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Effects of vapor pressure deficit, nitrogen fertilizer and leaf turnover on the oxygen isotope composition of cellulose in leaves of a perennial C$_4$ grass (*Cleistogenes squarrosa*)

Haitao Liu

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ABSTRACT

Aims: The oxygen isotope composition of cellulose in organic archives is widely used to reconstruct temperature, relative humidity in paleoclimate and reflect stomatal conductance in plant physiology. However, the mechanism of incorporation of oxygen isotope from leaf water to cellulose and environmental effects on this processes are still not well understood. The general aim of the present thesis was to understand the relationship between oxygen isotope composition of leaf water and cellulose, and effects of external factors on that relationship in *Cleistogenes squarrosa*, a perennial C_4 grass, and to explore the possibility of oxygen isotope in cellulose of successive leaf formed along the tiller as a chronometer. A fundamental unknown of ^{18}O incorporation in cellulose synthesis of leaf blades, the real proportion of unenriched (source) water at the site of synthesis, was analyzed with a range of C_3 and C_4 grass species.

Material & Methods: All experiments were conducted in controlled-environmental chambers, which allowed monitoring air temperature, relative humidity, CO_2 concentration and light intensity. *C. squarrosa*, a perennial C_4 grass endemic to the Central Asia steppe, was grown with different combinations of oxygen isotope composition of CO_2 ($\delta^{18}O_{CO2}$), nitrogen (N) fertilizer supply and vapor pressure deficit (VPD) to investigate the direct and interactive effects of these treatments on the oxygen isotope composition of leaf water ($\delta^{18}O_{LW}$) and cellulose ($\delta^{18}O_{Cel}$), oxygen isotope enrichment of leaf water ($\Delta^{18}O_{LW}$) and cellulose ($\Delta^{18}O_{Cel}$) above source water, and $p_{ex}p_x$, the proportion of oxygen in cellulose that exchanged with unenriched water at the site of cellulose synthesis. The incorporation of oxygen isotope in leaf cellulose during leaf development was assessed in the same experiment by swapping *C. squarrosa* between VPD environments and exposure to the new environment for 7 d with simultaneous $^{13}CO_2$ labeling. In addition, to determine oxygen and hydrogen isotope composition of water in the leaf growth and differentiation zone (LGDZ) of grasses, two C_3 (*Triticum aestivum*, *Lolium multiflorum*) and three C_4 grass species (*C. squarrosa*, *Pennisetum americanum*, *Panicum maximum*) were grown in low and high VPD conditions.

Results & Discussion: $\delta^{18}O_{CO2}$, N supply and their interaction with VPD did not affect $\delta^{18}O_{Cel}$, $\Delta^{18}O_{LW}$, $\Delta^{18}O_{Cel}$ and $p_{ex}p_x$ in *C. squarrosa*. High VPD increased $\Delta^{18}O_{LW}$ and $\Delta^{18}O_{Cel}$, but decreased $p_{ex}p_x$. The proportion of unenriched water in LGDZ (p_x) in five grass species showed no significant difference between low and high VPD with an average value of p_x 0.95 for oxygen and 1.06 for hydrogen. Remarkably, VPD had no effect on growth or morphology

in *C. squarrosa*. As p_x was close to source water in contrasting scenarios of VPD, the observed VPD effect on $p_{ex}p_x$ must be explained by variation of p_{ex} or another condition of the Barbour & Farquhar (2000) model such as variation of equilibrium fractionation factor between carbonyl oxygen and water (ε_o), or deviation of the oxygen isotope composition of sucrose from bulk leaf water in the steady state. For the incorporation of new carbon or oxygen into leaf cellulose, the direction of change, from low to high or high to low VPD, had no differential effect on new oxygen and carbon incorporation in cellulose. Thus, successive leaves produced by tillers of *C. squarrosa* provided a $\delta^{18}O_{Cel}$ record useful for reconstructions of short-term VPD dynamics.

Conclusions: The results of this study indicated that water in the LGDZ is close to source water without influence of VPD in grass species. Variation of ε_o or disequilibrium between sucrose and bulk leaf water might cause the observed effect of VPD on $p_{ex}p_x$. Analysis of oxygen incorporation in cellulose of successively formed leaves along tillers demonstrated that the $\delta^{18}O_{Cel}$ of successive leaves in grass species can be interpreted as a short-term isotopic chronometer.

ZUSAMMENFASSUNG

Zielsetzung: Die Sauerstoffisotopenzusammensetzung der Zellulose ist ein wichtiger Proxy für die Rekonstruktion der Temperatur und relativen Luftfeuchtigkeit im Paläoklima. Darüber hinaus wird vermutet, dass die Sauerstoffisotopenzusammensetzung der Zellulose auch ein Signal der stomatären Leitfähigkeit, eine wesentliche physiologische Komponente der Wassernutzungseffizienz, enthält. Allerdings, ist der Mechanismus des Transfers von Sauerstoff aus dem Blattwasser in die Zellulose, und deren Abhängigkeit von Umweltbedingungen noch nicht hinlänglich gut bekannt. Ziel der vorliegenden Dissertation war es, die Beziehung zwischen der Sauerstoffisotopenzusammensetzung der Blattzellulose und des Blattwassers in *Cleistogenes squarrosa* (ein ausdauerndes C_4 Gras welches in der zentralasiatischen Steppe beheimatet und verbreitet ist), und der Wirkung von Umweltfaktoren, insbesondere des atmosphärischen Wasserdampfsättigungsdefizits und der Stickstoffernährung, auf diese Beziehungen zu untersuchen und besser zu verstehen. Darüber hinaus sollte eruiert werden, inwiefern die Sauerstoffisotopenzusammensetzung der sukzessive gebildeten Blätter entlang von Trieben als zeitlich hochauflösender 'Chronometer' des Wasserdampfsättigungsdefizits (VPD) genutzt werden kann. Eine grundsätzliche Unbekannte im Beziehungsgefüge zwischen Sauerstoffisotopensignatur des Blattwassers und der Zellulose, der effektive Anteil des nicht-^{18}O-angereicherten Wassers am Ort der Zellulosesynthese, wurde mit verschiedenen C_4 und C_3 Grasarten untersucht.

Material und Methoden: Die Experimente wurden in Klimakammern durchgeführt. Diese gestatteten eine sorgfältige und reproduzierbare Steuerung der Lufttemperatur, Luftfeuchtigkeit, CO_2-Konzentration und Lichtintensität. *C. squarrosa* wurde mit differenzierter Stickstoffernährung bei hohem und niedrigem Wasserdampfsättigungsdefizit in Gegenwart von CO_2 mit unterschiedlicher Sauerstoffisotopenzusammensetzung ($\delta^{18}O_{CO2}$) kultiviert. An diesen Pflanzen wurden dann die direkten und interaktiven Wirkungen der genannten Behandlungen auf die Sauerstoffisotopenzusammmensetzung des Blattwassers ($\delta^{18}O_{LW}$) und der Blattzellulose ($\delta^{18}O_{Cel}$), sowie die Sauerstoffisotopenanreicherung im Blattwasser ($\Delta^{18}O_{LW}$) und in der Blattzellulose ($\Delta^{18}O_{Cel}$) gegenüber dem aufgenommenen Wasser, und $p_{ex}p_x$, der Anteil des aufgenommenen und nicht evaporativ ^{18}O-angereicherten Wassers in der Blattzellulose untersucht. Im selben Experiment wurde der Einbau von Sauerstoff und Kohlenstoff in die Blattzellulose während des Blattwachstums durch Transfer von Pflanzen zwischen Klimakammern mit kontrastierendem VPD und unterschiedlicher Kohlenstoffisotopenzusammensetzung des CO_2 untersucht. In diesem Markierungsexperiment verblieben die Pflanzen während 7 Tagen im neuen Milieu; die Pflanzen wurden dann nach

Blattaltersklassen beerntet und die ^{13}C und ^{18}O-Isotopensignatur der Blattzellulose bestimmt. In einem weiteren Experiment untersuchte ich die Sauerstoff- und Wasserstoffisotopensignatur des Wassers am Ort der Zellulosesynthese in der Blattwachstums- und Differenzierungszone von zwei C$_3$ Gräsern (*Triticum aestivum*, *Lolium multiflorum*) and drei C$_4$ Gräseren (*C. squarrosa*, *Pennisetum americanum*, *Panicum maximum*) welche bei hohem und niedrigem VPD heranwuchsen.

Ergebnisse und Diskussion: Sowohl $\delta^{18}O_{CO2}$, als auch die differenzierte Stickstoffernährung und deren Interaktion mit den kontrastierenden VPDs hatten keinen Einfluss auf $\delta^{18}O_{Cel}$, $\Delta^{18}O_{LW}$, $\Delta^{18}O_{Cel}$ und $p_{ex}p_x$ in *C. squarrosa*. $\Delta^{18}O_{LW}$ und $\Delta^{18}O_{Cel}$ wurden bei hohem VPD erhöht, während $p_{ex}p_x$ erniedrigt wurde. Der Anteil des nicht evaporativ ^{18}O- und ^{2}H-angereicherten Wassers in der Wachstums- und Differenzierungszone der Blätter (p_x) betrug in den verschiedenen Gräsern durchschnittlich 0.95 bei Sauerstoff und 1.06 bei Wasserstoff und wurde durch VPD nicht beeinflusst. Bemerkenswerterweise hatte VPD keinen Einfluss auf das Wachstum und morphologische Parameter von *C. squarrosa*. Da die Sauerstoffisotopensignatur des Wassers in der Wachstums- und Differenzierungszone sehr nahe an der Sauerstoffisotopensignatur des Source- bzw. von den Wurzeln aufgenommenen Wassers lag, muss die beobachtete VPD-Wirkung auf $p_{ex}p_x$ mit Variation von p_{ex} oder anderen Bedingungen des Modells von Barbour & Farquhar (2000) erklärt werden. Infrage kommt hierbei Variation im biosynthetischen Fraktionierungsfaktor (ε_o), oder in der Relation der Sauerstoffisotopensignatur der Sacharose zur steady-state Sauerstoffisotopensignatur des gesamten Blattwassers im Licht. Der Wechsel des VPD – von niedrigem VPD zu hohem VPD, oder von hohem zu niedrigem VPD – hatte keinen differenziellen Einfluss auf die Einbaukinetik von aktuell assimiliertem Sauerstoff in die Blattzellulose wachsender Blätter. Es zeigte sich also, dass wechselnde VPD-Bedingungen durch $\delta^{18}O_{Cel}$ der sukzessive gebildeten Blätter zuverlässig und mit relativ hoher zeitlicher Auflösung abgebildet werden.

Schlussfolgerungen: Die vorliegende Studie belegt, dass die Sauerstoff- und Wasserstoffisotopensignatur des Wassers am Ort des Blattwachstums und seiner Differenzierung – unabhängig von der Grasart – sehr nahe an derjenigen des von den Wurzeln aufgenommenen Wassers liegt und von VPD nicht beeinflusst wird. Diese Tatsache bedeutet, dass die beobachtete VPD-abhängige Variation in der Beziehung zwischen der Sauerstoffisotopensignatur des Blattwassers und der Blattzellulose wahrscheinlich auf Variation des biosynthetischen Fraktionierungsfaktors oder der Relation der Sauerstoffisotopensignatur der Sacharose zur Sauerstoffisotopensignatur des Blattwassers

gründet. Untersuchungen des Sauerstoffeinbaus in die Zellulose sukzessive gebildeter Blätter deuten darauf hin, dass $\delta^{18}O_{Cel}$ der Blätter entlang eines Triebes von *C. squarrosa* als isotopischer Chronometer wöchentlicher VPD Schwankungen interpretiert werden kann.

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experimental run (high N), respectively. Each data point and error bar represents the mean ± standard error of three to six individual plants ($n = 3$ to 6)....................................32

Fig. 4.1 Schematic illustration of a vegetative grass tiller (right hand side) composed of a growing leaf and three fully expanded leaves of increasing age (1-3). The basal part of the tiller is composed of a whorl of encircling leaf sheaths (sometimes termed 'pseudostem') that enclose the leaf growth and differentiation zone (LGDZ) of the growing leaf (shown on the left hand side). The LGDZ includes the successive zones of cell division, expansion and differentiation (also see Fig. 5.14 in MacAdam, 2009). The exposed part of the growing leaf is fully differentiated and photosynthetically competent. Leaf growth begins with the tip of the blade (lamina) and terminates with the basal portion of the sheath. Throughout the growth of a leaf, the LGDZ remains at the base of the leaf and, hence, is always fully enclosed by the sheath of the next older leaf (Schnyder *et al.*, 1990). The blade-sheath interface, also termed 'joint' (see arrow) or 'collar', often contains a thin outgrowth (termed 'ligule') that surrounds the basal exposed part of the growing leaf (not shown). Leaf elongation stops at the time when the leaf blade bends away from the vertical as a result of differential growth in the joint

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Chapter 1

General introduction

Knowledge of reconstruction of paleoclimate, plant physiology and cellulose

Analysis of stable isotopes in fossil and modern plant cellulose is widely applied in many fields, such as reconstruction of paleoclimate, plant ecophysiology, and ecology of carbon and water balance (Barbour, 2007; Sternberg, 2009; Gessler *et al.*, 2014). Comprehensive understanding of isotopic signal in cellulose requires better knowledge of environmental effects on stable isotope ratio in cellulose, such as precipitation, temperature, relative humidity (RH), and vapor pressure deficit (VPD), which can all affect the incorporation of oxygen isotopes into cellulose. For various reasons, cellulose is a material that has attracted the attention of scientists. Firstly, cellulose is an important structural component of the primary cell wall of plants, which are widely distributed over the globe and can be sampled easily for reconstruction of precipitation and temperature at a finer temporal and spatial scale, than for instance ice core sites which are distributed more sparsely and remotely (Marino & McElroy, 1991; Marino *et al.*, 1992; Brienen *et al.*, 2012). Second, cellulose is a polysaccharide polymer. The structure of cellulose consists of long polymer chains of glucose units connected by a beta acetal linkage. All of the monomer units are beta-D-glucose; once synthesized, cellulose is preserved without any D-glucose addition to the chain (O'Sullivan, 1997; Klemm *et al.*, 2005), and no carbon or oxygen exchanges between cellulose and the environment occurs after cellulose synthesis (Farquhar *et al.*, 1998; Sternberg *et al.*, 2003). Therefore, the oxygen isotope in cellulose has the potential to record environmental variations at the time of cellulose formation. Consequently, a deeper understanding the effects of environmental factors, and morphological and physiological parameters of plants on the oxygen isotope composition of cellulose ($\delta^{18}O_{Cel}$) will greatly enhance the potential application of the oxygen isotope signal in cellulose for reconstruction of VPD in paleoclimate and stomatal conductance in plant physiology.

Knowledge of oxygen isotope

The stable isotopes of a chemical element share the same number of protons, but differ in the number of neutrons, giving rise to different atomic masses. The isotopes of an element have

the same chemical but (slightly) different physical properties. The abundance of oxygen with atomic mass 16 (^{16}O) and 18 (^{18}O) are 99.76% and 0.21%, respectively.

By convention, the relative abundance of an isotope is expressed by its δ-value. The δ^{18}O of a sample ($\delta^{18}O_{sample}$) gives the per mil deviation of the molar abundance ratio (*R*) of the rare relative to the abundant isotope (^{18}O/^{16}O) in a sample relative to that of an international standard. Thus, $\delta^{18}O_{sample}$ is expressed in per mil (‰) as

$$\delta^{18}O_{sample} = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \times 1000,$$ Eqn 1.1

with $R_{standard}$ the ^{18}O/^{16}O ratio in the Vienna Standard Mean Ocean Water (V-SMOW) standard.

Factors affecting oxygen isotopic signal of cellulose

The $\delta^{18}O_{Cel}$ is mainly controlled by the δ^{18}O of soil water ($\delta^{18}O_{SW}$) at the depth of root water uptake, which occurs without isotopic fractionation (White *et al.*, 1985; Flanagan & Ehleringer, 1991a; Ehleringer & Dawson, 1992; Dawson & Ehleringer, 1993) and the δ^{18}O of leaf water ($\delta^{18}O_{LW}$) which is enriched relative to the soil water due to isotope effects in transpiration (Barbour & Farquhar, 2000; Farquhar *et al.*, 2007; Cernusak *et al.*, 2016). $\delta^{18}O_{SW}$ depends on the δ^{18}O of precipitation, which varies as a function of temperature (and hence altitude and latitude), amount of precipitation (Dansgaard, 1964; Rozanski *et al.*, 1992; Rozanski *et al.*, 1993; Bowen, 2010), residence time of water in the soil and evaporation of soil surface water (Allison *et al.*, 1983; Barnes & Allison, 1983; Barnes & Allison, 1988; Yakir & Sternberg, 2000). Soil water reaches leaf blades *via* the xylem and becomes enriched in ^{18}O due to evaporative enrichment at the water layer that lines the stomatal cavity. ^{18}O enrichment at the site of evaporation ($\Delta^{18}O_e$) in the stomatal cavity can be predicted from the modified model of ^{18}O enrichment of a water body (Craig & Gordon, 1965; Dongmann *et al.*, 1974; Flanagan *et al.*, 1991a; Farquhar & Lloyd, 1993), as

$$\Delta^{18}O_e = \varepsilon^+ + \varepsilon_k + (\Delta^{18}O_v - \varepsilon_k)\frac{e_a}{e_i} \,,$$ Eqn 1.2

with ε^+ the equilibrium fractionation between liquid and vapor at the air-water interface, ε_k the kinetic fractionation that occurs during diffusion from the leaf intercellular air space to the atmosphere, $\Delta^{18}O_v$ the ^{18}O enrichment of atmospheric vapor relative to source water, and e_a/e_i the ratio of ambient to intercellular vapor pressures. ε^+ is temperature dependent (Bottinga & Craig, 1969; Majoube, 1971; Horita & Wesolowski, 1994), and ε_k is determined by stomatal

conductance and boundary layer conductance (Farquhar *et al.*, 1989). The Craig & Gordon model is an isotopic steady state model which assumes that the oxygen isotope of composition of transpired vapor equals that of source water. Numerous researches have reported that ^{18}O enrichment of leaf water predicted by $\Delta^{18}O_e$ according to the Craig & Gordon model overestimates the measured ^{18}O enrichment of leaf water, $\Delta^{18}O_{LW}$ (Allison *et al.*, 1985; Bariac *et al.*, 1989; Walker *et al.*, 1989; Yakir *et al.*, 1990; Flanagan *et al.*, 1991a, b; Wang *et al.*, 1998). At present, one of the most accepted explanations of that discrepancy between the observed and modeled ^{18}O of leaf water enrichment is a Péclet effect (proposed by Farquhar & Lloyd, 1993), that results from the diffusion of enriched water away from the sites of evaporation and opposed by convection of unenriched water towards evaporative sites driven by transpiration. The Péclet number ($\wp$) describes the ratio of convection to diffusion

$$\wp = \frac{EL}{CD},$$

Eqn 1.3

where E is transpiration rate (mol m^{-2} s^{-1}), L is the effective path length (m), C is the density of water (5.55×10^4 mol m^{-3}), and D is the diffusivity of $H_2{}^{18}O$ in water (2.66×10^{-9} m^{-2} s^{-1}).

Accordingly, average ^{18}O enrichment of leaf water (relative to source water) at steady state is given by (Farquhar & Lloyd, 1993):

$$\Delta^{18}O_{LW} = \frac{\Delta^{18}O_e \left(1 - e^{-\wp}\right)}{\wp},$$

Eqn 1.4

where $\Delta^{18}O_e$ is from Eqn 1.2. Barbour *et al.* (2004) found that the Péclet effect may affect ^{18}O enrichment of leaf cellulose ($\Delta^{18}O_{Cel}$) *via* the variation of $\Delta^{18}O_{LW}$.

Effects of environmental, physiological and biochemical factors on the relationship between $\Delta^{18}O_{LW}$ and $\Delta^{18}O_{Cel}$

The $\delta^{18}O_{Cel}$ of cellulose is an integrator of environmental effects on leaf water enrichment, especially vapor pressure deficit (VPD). Thus, it has been suggested that the oxygen isotope enrichment of cellulose could be used to infer long-term, integrated characteristics of VPD when $\delta^{18}O$ of source water is constant (Farquhar *et al.*, 1989; Sternberg *et al.*, 1989; Kahmen *et al.*, 2011). Moreover, the $\delta^{18}O$ of tree rings has been widely used to reconstruct past precipitation and temperature (Burk & Stuiver, 1981; Anderson *et al.*, 2002; Treydte *et al.*, 2006; Brienen *et al.*, 2012), because some oxygen atoms at specific position in cellulose derived from source water directly (Sternberg *et al.*, 2003; Waterhouse *et al.*, 2013). It is well known that the $\delta^{18}O_{LW}$ is the main factor which controls the oxygen isotopic signature of leaf

cellulose. However, the $\delta^{18}O_{Cel}$ does not completely reflect that of the leaf water due to attenuation effects (see below). Therefore, qualitative and quantitative descriptions of the relationship between $\Delta^{18}O_{LW}$ and $\Delta^{18}O_{Cel}$, and understanding of the mechanisms underlying the relationships are necessary for precise reconstruction of paleoclimate parameters from cellulose archives. Barbour & Farquhar (2000) developed an empirical and biochemical model to predict ^{18}O enrichment of cellulose from ^{18}O-enrichment of leaf water:

$$\Delta^{18}O_{Cel} = (1 - p_{ex}\,p_x)\,\Delta^{18}O_{LW} + \varepsilon_o. \qquad\qquad \text{Eqn. 1.5}$$

In that model, $p_{ex}p_x$ represents an 'attenuation factor', which has two components: 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 unenriched water at the site. ε_o is the equilibrium fractionation factor between carbonyl oxygen and water. Up to now, it has been commonly accepted that p_{ex} is close to 0.4, and p_x is close to unity in tree ring cellulose (Sternberg *et al.*, 1986; Yakir & DeNiro, 1990; Roden *et al.*, 2000; Sternberg *et al.*, 2003). However, more and more studies indicated that p_{ex} may not be a constant value (Barbour & Farquhar, 2000; Gessler *et al.*, 2009; Offermann *et al.*, 2011; Ellsworth & Sternberg, 2014; Song *et al.*, 2014a). Cernusak *et al.* (2005) reviewed published values of p_{ex} with different plant species and indicated that p_{ex} may vary from 0.31 to 0.77 when substrates for cellulose synthesis differ between species. Furthermore, p_{ex} was suggested to correlate with growth rate (Barbour & Farquhar, 2000) and turnover rate of carbohydrates (Song *et al.*, 2014a). By applying the combination of Barbour & Farquhar model and tree ring model for grass species, Helliker & Ehleringer (2002a, b), partitioned p_{ex} and p_x, and indicated that p_x ranged in the order of 0.50 − 0.62 in several grass species. That estimation assumed that p_{ex} ranged between 0.40 and 0.50, and found tentative support for estimates of p_x from sampling tissue water at the base of leaf blades of *Lolium multiflorum*. However, that tissue is not the site of cellulose synthesis in leaf blades (see below and chapter 4). Thus, understanding the environmental response of $p_{ex}p_x$ is of great importance for reconstructions of VPD, temperature and precipitation in paleoclimate using fossil archives. For instance, under- or overestimations of environmental variation from data of $\delta^{18}O$ of cellulose may occur if $p_{ex}p_x$ is erroneously treated as a fixed value. Given the variation of morphological parameters and biochemical processes in plants under variable environmental conditions, a non-constant value of $p_{ex}p_x$ is a likely scenario.

Current Challenges

An effect of the oxygen isotope composition of CO$_2$ on cellulose

So far, only one study has investigated the effect of the δ^{18}O of CO$_2$ on cellulose. That work was performed with wheat, a C$_3$ species, and found no effect (Deniro & Epstein, 1979). As yet, it is unknown if the δ^{18}O of CO$_2$ affects the δ^{18}O$_{Cel}$ in C$_4$ species, which have much lower activity of carbonic anhydrase (Gillon & Yakir, 2001a) which could limit oxygen exchange between water and CO$_2$.

Nitrogen supply and VPD effects on ^{18}O-enrichment of leaf water and cellulose

Nitrogen fertilizer affects growth rate, turnover of carbohydrate pools, and morphological parameters like leaf length, interveinal distance, and leaf area index, which may affect p_{ex} and δ^{18}O$_{LW}$. Brooks & Coulombe (2009) observed that δ^{18}O$_{Cel}$ increased with nitrogen fertilization (in association with leaf area), decrease of stomatal conductance, compared with controlled experiments when the source water and RH were the same between treatments. Brooks & Mitchell (2011) found that after fertilization and thinning treatments, δ^{18}O of tree ring cellulose was higher only under thinning. They thought that the thinning effect on δ^{18}O of tree ring cellulose was related to the thinning effect on canopy microclimate (decrease in RH and increase in leaf temperature). Both those two experiments were conducted in field conditions, where many unpredictable factors may give rise to variation of δ^{18}O of leaf water and cellulose. So far, no study investigated the effects of nitrogen supply on δ^{18}O of leaf water, cellulose and $p_{ex}p_x$ in controlled environmental conditions.

VPD is the main environmental factor that controls ^{18}O-enrichment of leaf water; also VPD is recognized as a major factor controlling ^{18}O-enrichment of leaf cellulose (Barbour, 2007; Kahmen *et al.*, 2011; Gessler *et al.*, 2014; Cernusak *et al.*, 2016). However, the oxygen isotopic information of leaf water is not fully translated into that of cellulose due to the isotopic exchange (affecting p_{ex}, see Eqn 1.5) with less enriched water (influencing p_x) at the site of cellulose synthesis. In grass leaves, cellulose synthesis occurs in the leaf-growth-and-differentiation-zone (LGDZ) that is located at the base of the growing leaf (fully enclosed within the whorl of mature leaf sheaths) and includes zones of cell division, cell elongation and maturation (Schnyder *et al.*, 1990; MacAdam & Nelson, 2002; MacAdam, 2009). Primary cell walls and secondary cell walls, which include cellulose, are synthesized in the LGDZ (Taylor, 2008; Vogel, 2008). In addition, leaf wax of grasses is synthesized largely inside the LGDZ (Richardson *et al.*, 2005, 2007). Moreover, the hydrogen isotope

composition of leaf wax of grasses is influenced by the water at the site of wax synthesis (Sachse *et al.*, 2012; Zhou *et al.*, 2016). Therefore, it is imperative to know the oxygen and hydrogen isotope composition of water inside the LGDZ ($\delta^{18}O_{LGDZ}$, δ^2H_{LGDZ}) as it represents the medium and synthesis water for cellulose and leaf wax formation that affect the respective values of p_x ($p_{x\ 18O}$ and $p_{x\ 2H}$). Thus far, however, there has been no report of the values of $\delta^{18}O_{LGDZ}$ and δ^2H_{LGDZ} in grasses.

Incorporation of oxygen isotope in leaf blade cellulose

For many decades, researchers have investigated several materials that have potential as indicators of environmental changes at different temporal scales, such as ice cores, tree rings, lake sediments and peat cores (Grootes *et al.,* 1993; Von Grafenstein *et al.,* 1998; Hong *et al.,* 2000; Roden *et al.,* 2002). In the last two decades, scientists also started to explore the usefulness of new materials, such as grass leaf blades, to reconstruct atmospheric CO_2 at a finer temporal scale (Marino & McElroy, 1991; Marino *et al.*, 1992; Lichtfouse *et al.*, 2003; Kèlomé *et al.*, 2006). In addition, it was shown that changes of relative humidity during blade development of grasses are recorded as corresponding changes of $\delta^{18}O_{Cel}$ (Helliker & Ehleringer, 2002a). However, isotopic signals in cellulose in response to environmental variation may also be influenced by the use of stored materials in cellulose synthesis (Gessler *et al*, 2009, 2013, 2014). Such a potential complication has not been considered in grasses.

Objective of this dissertation

The objective of this work is to improve understanding of the effects of environmental factors on the relationship between ^{18}O-enrichment of leaf water and leaf cellulose in a C_4 perennial grass, *Cleistogenes squarrosa*, addressing the above-mentioned uncertainties. Specifically, the work asks the following questions: a) Do the oxygen atoms in CO_2 affect the oxygen isotope composition of cellulose, as carbonic anhydrase activity is low in C_4 grasses (Chapter 2)? b) How do vapor pressure deficit, nitrogen fertilizer supply, and their interaction influence morphological parameters of *C. squarrosa*, and ^{18}O-enrichment of leaf water and cellulose, and the relationship between ^{18}O-enrichment of leaf water and cellulose? (Chapter 2) c) Can cellulose in the successively formed leaves of *C. squarrosa* provide us with a high temporal resolution oxygen isotope record resulting from changes of VPD in its growth environment? (Chapter 3) d) What is the proportion of unenriched water ($p_{x\ 18O}$ and $p_{x\ 2H}$) at the site of cellulose synthesis in the LGDZ and how is it affected by VPD? (Chapter 4) To ascertain, the generality of that work included additional (C_3 and C_4) grass species. Finally, with the new knowledge, we attempt a new interpretation of the conditions/hypotheses of the Barbour &

Farquhar (2000) model and its implications for p_{ex}, the proportion of oxygen in metabolites of cellulose synthesis that exchange with medium water in the LGDZ (Chapter 5).

Chapter 2

Oxygen isotope composition of leaf water and cellulose in a C$_4$ grass: effects of vapor pressure deficit, nitrogen nutrition, δ^{18}O of CO$_2$, canopy position and leaf age [1]

ABSTRACT

The oxygen composition of cellulose follows that of leaf water, although in a variably attenuated fashion which is under environmental and morpho-physiological control. Here we explore putative direct and interactive effects of nitrogen fertilizer supply, vapor pressure deficit (VPD), leaf age, canopy position and δ^{18}O of CO$_2$ (δ^{18}O$_{CO2}$) on the relation between leaf water- and cellulose-^{18}O in blades and sheaths of a C$_4$ grass. Stands of *Cleistogenes squarrosa* were grown at low (0.63 kPa) or high (1.58 kPa) VPD with low or high nitrogen supply and different δ^{18}O$_{CO2}$ in controlled-environment mesocosms. Nitrogen supply altered growth and morphology, but had no effect on ^{18}O-enrichment of leaf water (Δ^{18}O$_{LW}$) or cellulose (Δ^{18}O$_{Cel}$). Conversely, VPD did not influence growth or morphology, but strongly affected Δ^{18}O$_{LW}$, Δ^{18}O$_{Cel}$ and $p_{ex}p_x$ (attenuation factor in relationship between Δ^{18}O$_{LW}$ and Δ^{18}O$_{Cel}$). Also, at high, but not low VPD, Δ^{18}O$_{Cel}$ of blades was 2‰ higher than that of sheaths. δ^{18}O$_{CO2}$, leaf age and canopy position had no effect on Δ^{18}O$_{Cel}$. VPD was the sole driver of variation in Δ^{18}O$_{LW}$, Δ^{18}O$_{Cel}$ and $p_{ex}p_x$. Variation of $p_{ex}p_x$ was possibly related to the p_x component (the proportion of unenriched water at site of cellulose synthesis), resulting from ^{18}O-enriched phloem water.

INTRODUCTION

The oxygen isotope composition of cellulose ($\delta^{18}O_{Cel}$) is a function of environmental conditions and morpho-physiological plant traits which influence the $\delta^{18}O$ of water in chloroplasts, cellular compartments involved in the synthesis of transport sugars, and sink tissues where sugars are metabolized and used in cellulose synthesis (Barbour, 2007). As it contains environmental and physiological information, the $\delta^{18}O$ of cellulose is of great interest to a range of scientific disciplines, including palaeoecology, global change science, plant physiology and plant breeding (Barbour, 2007; Farquhar *et al.*, 2007; Kahmen *et al.*, 2011; Xiao *et al.*, 2012). Current knowledege on the relationship between $\delta^{18}O$ in cellulose and $\delta^{18}O$ in source and leaf water has been summarized in a quantitative model by Barbour & Farquhar (2000) (see Chapter 1, Eqn 1.5). In that model, the value of ε_o has been estimated near 27‰ (Sternberg & DeNiro, 1983; Sternberg *et al.*, 1984; Yakir & DeNiro, 1990), with a- possibly temperature-related (Sternberg & Ellsworth, 2011), uncertainty of about 3‰ (DeNiro & Epstein, 1981; Waterhouse *et al.*, 2002; Ellsworth & Sternberg, 2014). That model also assumes that oxygen in CO_2 exchanges completely with leaf water (Farquhar *et al.*, 1993; Flanagan *et al.*, 1994) – a process that is greatly enhanced by carbonic anhydrase (Gillon & Yakir, 2001a, b; Cousins *et al.*, 2006a, b). Futhermore, metabolic intermediates in the synthesis of leaf sucrose – a primary photosynthetic product, transport sugar and substrate for cellulose synthesis – lead to a full isotopic equilibrium of sucrose with average leaf water (Cernusak *et al.* 2003, Barbour 2007), but see also Gessler *et al.* (2013). However, direct empirical evidence for the absence of an effect of the $\delta^{18}O$ of CO_2 ($\delta^{18}O_{CO2}$) on the $\delta^{18}O$ of cellulose is presently limited to a single, non-replicated experiment which used seedlings of 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 evaporative sites in stomatal cavities (Dongmann *et al.*, 1974; Flanagan *et al.*, 1991a), and this effect propagates through leaves by diffusion of enriched water away from the evaporative sites, against the convective flux of unenriched water, a Péclet effect (Farquhar & Lloyd, 1993; Farquhar & Gan, 2003). Leaf water evaporative enrichment thus elevates ^{18}O in leaf water above that of source water ($\Delta^{18}O_{LW} > 0$).

Of all environmental factors, vapor pressure deficit (VPD) has the strongest effect on $\Delta^{18}O_{Cel}$ (Burk & Stuiver, 1981; Kahmen *et al.*, 2011). This (stimulating) effect derives from

the VPD effect on $\Delta^{18}O_{LW}$ (Flanagan *et al.*, 1991b; Sheshshayee *et al.*, 2005; Farquhar *et al.*, 2007), and is particularly clear from experiments in controlled conditions (Barbour & Farquhar, 2000; Helliker & Ehleringer, 2000, 2002a, b), where the effects of different climatic parameters can be disentangled. Temperature and relative humidity cause changes in $\Delta^{18}O_{LW}$ (Flanagan & Ehleringer, 1991b; Barbour & Farquhar, 2000; Barbour *et al.*, 2004; Song *et al.*, 2014a), which are related to their relationship with VPD (Dongmann *et al.*, 1974; Barbour, 2007; Ripullone *et al.*, 2008). Whether or not other climatic factors have an effect on $\Delta^{18}O_{LW}$ independently of their association with VPD is less certain. For instance, different light levels did not lead to differences in $\Delta^{18}O_{LW}$ of *Ricinus communis* when VPD was controlled (Song *et al.*, 2014a), and contrasts of $\Delta^{18}O_{LW}$ between well-lit (upper) and shaded (lower) canopy positions of wheat and corn were attributed (at least in part) to within-canopy gradients of VPD (Xiao *et al.*, 2012). Also, it is presently unknown, if nitrogen nutrition – a major limiting factor for plant growth which affects leaf expansion rate, leaf dimensions, leaf area index and canopy microclimate (Novoa & Loomis, 1981; Simon & Lemaire, 1987) – has a direct effect on $\Delta^{18}O_{LW}$ which would feed forward to $\Delta^{18}O_{Cel}$.

Understanding of the controls of $p_{ex}p_x$ is even more limited, partly due to methodological difficulties in the assessment of its components p_{ex} and p_x (Song *et al.*, 2014a). In reality, the p_{ex} component of $p_{ex}p_x$ must be > 0, as carbohydrate metabolism during cellulose synthesis invariably leads to the formation of carbonyl groups in a certain proportion of the carbon atoms in metabolic intermediates (Barbour & Farquhar, 2000). In particular, futile cycling of hexose through triose phosphates enhances p_{ex} to a great extent (Hill *et al.*, 1995; Barbour & Farquhar, 2000). In this context, it has been proposed that turnover time of non-structural carbohydrate pools should affect $p_{ex}p_x$ *via* an effect on p_{ex} (Song *et al.*, 2014a). Nitrogen (N) nutrition generally increases or decreases the turnover time of carbohydrate pools (White, 1973; Novoa & Loomis, 1981), but it is currently unknow if it also influences $p_{ex}p_x$.

The term p_x can be < 1, due to the presence of enriched phloem water or evaporative enrichment of the water in the cellulose-forming tissue (Barbour, 2007). In general, it is thought that $p_x \approx 1$ in non-transpiring tissue that is remote of photosynthesizing tissue, such as stems of trees (Roden *et al.*, 2000), while p_x should be much smaller for cellulose synthesis inside concurrently expanding and transpiring dicot leaves, such as in *R. communis* (Cernusak *et al.*, 2003; Gessler *et al.*, 2007). The situation is probably intermediate for grass leaves, in which the meristem/growth zone of the growing leaf is non-transpiring as it is enclosed by the

sheath of the next-older leaf (Helliker & Ehleringer, 2002a). One might expect that VPD effects on leaf water enrichment may translate to differences in p_x in association with (differentially) enriched phloem water. However, experiments with different VPDs have not reported effects on $p_{ex}p_x$ in leaves of cotton (Barbour & Farquhar, 2000), castor bean (Song *et al.*, 2014a), or a range of C_3 and C_4 grasses (Helliker & Ehleringer, 2002b).

Given the incompleteness of our current mechanistic understanding of the controls of $p_{ex}p_x$ and, hence, of the ^{18}O enrichment in cellulose, this work aimed at a comprehensive assessment of the direct and interactive effects of a diverse set of factors, including leaf age/developmental stage and canopy effects, and contrasts of VPD, N nutrition and $\delta^{18}O_{CO2}$, on the relationship between $\Delta^{18}O_{LW}$, $\Delta^{18}O_{Cel}$ and hence $p_{ex}p_x$ of leaf blades and leaf sheaths in *Cleistogenes squarrosa* (Trin.) Keng, in controlled environments. *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), and a co-dominent member of the 'typical steppe' of Inner Mongolia (Kang *et al.*, 2007). That species has exhibited remarkably high and variable ^{13}C discrimination among different leaves (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.

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 (-14.2 ± 0.04 ‰ in the 1st and 2nd run, -0.8 ± 0.26 ‰ in the 3rd and 4th run), while the other two received CO_2 that was relatively depleted in ^{18}O (-36.5 ± 0.08 ‰ in the 1st and 2nd run, -15.6 ± 0.26 ‰ in the 3rd and 4th run), see **Table 2.1**. The CO_2 gases were obtained from CARBO Kohlensäurewerke (Bad Hönningen, Germany) and Linde AG (Unterschleissheim, Germany).

Table 2.1 Experimental plan that gives the assignment of treatments to the different growth chambers (no. 1 – 4) in the successive experimental runs (1st – 4th). Treatments consisted of combinations of low or high VPD (V1 or V2) with low or high N fertilizer supply (N1 or N2). Each treatment combination was replicated four times, two times with ^{18}O-enriched CO_2 (denoted by 'E') and two times with ^{18}O-depleted CO_2 (denoted by 'D').

	Treatment								
Exp.	N1 V1		N2 V1			N1 V2		N2 V2	
run	E	D	E	D		E	D	E	D
	--------------------------------------- Chamber no. -------------------------------------								
1st	-	-	4	1		-	-	2	3
2nd	3	4	-	-		1	2	-	-
3rd	1	2	-	-		3	4	-	-
4th	-	-	2	1		-	-	4	3

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 2.1**), 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.

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 pots (4.5 cm diameter, 35 cm deep) filled with quartz sand (0.3-0.8 mm diameter). Pots were placed in free-draining plastic containers (length: 77 cm, width: 57 cm, depth: 30 cm) with 164 pots in each container. Two containers 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 pot, 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. 2.1**).
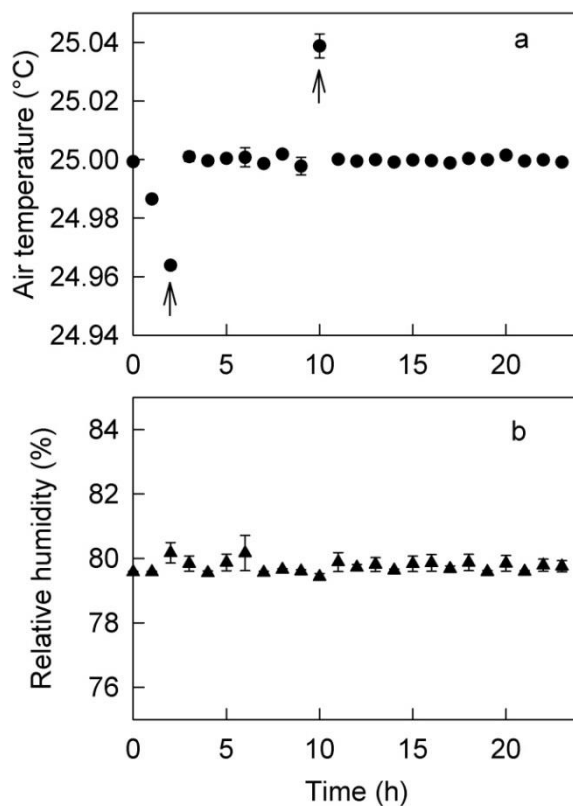
Fig. 2.1 Typical diurnal time course for (a) air temperature and (b) relative humidity. Data were taken from chamber 1 in the 3rd run (see **Table 2.1**), and give hourly averages of data recorded on the 32nd, 33rd, 37th, 38th, 39th, 41th, 49th and 50th day after imbibition. Shown are the means ± standard errors (n = 8). The dark period lasted from 2 to 10 hours. The arrows in (a) indicate the start of dark and light periods.

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 vapor,

and distributed between the four chambers described by Schnyder *et al.* (2003). CO_2 and water vapor concentrations in chamber air were measured by an infrared gas analyzer (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 vapor 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 relative humidity (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. 2.1**).

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. 2.2**). 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 hours 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 two days of sampling which were closest to the days of sampling for leaf water and cellulose.

Fig. 2.2 A photograph of a major tiller of *Cleistogenes squarrosa*. Letters/numbers refer to: a, the youngest visible, growing leaf blade; 1 – 10, fully expanded leaf blades numbered from the tip (youngest part) to the base (oldest part) of the tiller; DT, a daughter tiller which has emerged from the axillary bud of leaf 9.

Leaf water and cellulose in upper and lower canopy

Samples for leaf blade water and cellulose 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 from all chambers at approx. two hours after the beginning of the light period and sampled quickly using a scalpel to minimize changes of the content and δ^{18}O of

leaf water. For each plant, mature (fully expanded) blades in the upper part of the canopy (from the top to the center of tillers) and lower canopy (from the center to the base of tillers) were sampled separately. All in all, for each sample, 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 and sheaths of different age categories

Additional samples were collected to assess developmental changes/age gradients in ^{18}O of cellulose in leaf blades and sheaths along tillers. These samplings occurred in two experimental runs, on the 53rd day after imbibition in the 2nd experimental run (N1 V1, N1 V2) and the 44th day in the 4th run (N2 V1, N2 V2) (cf. **Table 2.1**). 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. Phytomer age categories were numbered from the top of the tiller (youngest phytomer) to the bottom (oldest) (cf. **Fig. 2.2**).

Blades and sheaths of successive phytomers were then clipped off from the major tillers. To obtain sufficient material for cellulose extraction, 16 to 20 blades (or sheaths) per phytomer age category were pooled in one sample. This procedure provided three samples for each plant fraction (individual age categories of leaf blades or sheaths) for each growth chamber.

Leaf water extraction

Leaf water was extracted for 3 h using a custom-built cryogenic vacuum distillation apparatus. Water extraction was virtually complete, as was confirmed by no 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.

Cellulose extraction

50 mg or 25 mg of dry sample material were used to extract α-cellulose using the procedure of Brendel *et al.* (2000) as modified by Gaudinski *et al.* (2005).

Measurement of δ^{18}O in CO$_2$ and vapor

The δ^{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 δ^{18}O of vapor in the growth chambers (δ^{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 two hours after the beginning of the light period. Values of measured δ^{18}O$_v$ were stored when the reading of the vapor concentration measured by the CRDS became stable. In the 2nd experimental run, water vapor 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 two hours using a pump. Vapor collection started two hours after the beginning of the light period. δ^{18}O$_v$ did not differ significantly between growth chambers and treatments, and averaged −13.0 ‰ (± 0.6 ‰ standard error, *n* = 16).

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

δ^{18}O of water samples (leaf water, collected vapor or nutrient solution) was analyzed on 300 μL aliquots using the CRDS analyzer 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^{TM} 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 (δ^{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 δ^{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 (2-5mm, 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:

$$\Delta^{18}O_{LW} = \frac{\delta^{18}O_{LW} - \delta^{18}O_{SW}}{1 + \delta^{18}O_{SW}/1000},$$
Eqn 2.1

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 $\delta^{18}O_{LW}$ 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):

$$\Delta^{18}O_e = (1 + \varepsilon^+) [(1 + \varepsilon_k) (1 - w_a/w_i) + w_a/w_i (1 + \Delta^{18}O_v)] - 1$$
Eqn 2.2

where w_a/w_i is the ratio of water vapor mole fraction in the air to that in the intercellular air space, ε^+ is the equilibrium ^{18}O fractionation between liquid water and vapor, ε_k is the kinetic ^{18}O fractionation for combined diffusion through the stomata and the boundary layer, $\Delta^{18}O_v$ is the ^{18}O enrichment of vapor (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 relative humidity 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 ($n = 2$). Due to 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 $\times$ 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 1.5, 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. The effects of N supply and VPD on the difference between $\delta^{18}O$ of blade and sheath were also tested by using confidence intervals from the mean values of each individual plant. 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 $\pm$ 1.3‰ around the measured value. In the latter sensitivity tests, ε_o was fixed at 27‰.

RESULTS

The effect of ^{18}O in CO_2 on ^{18}O in 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 2.1**). That difference in $\delta^{18}O_{CO2}$ amounted to approx. 20‰ in the 1st and 2nd experimental run and 13 ‰ in the 3rd and 4th experimental run, when comparing measurements taken at the outlet of the rapidly ventilated chambers (**Table 2.2**). When measured at the inlet of the chamber, those differences were even larger (**Table 2.2**, 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 2.3**). 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. 2.3**). 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 ± 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 vapor in leaves. So, as $\delta^{18}O_{CO2}$ had no effect on any of the relationships analyzed 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.
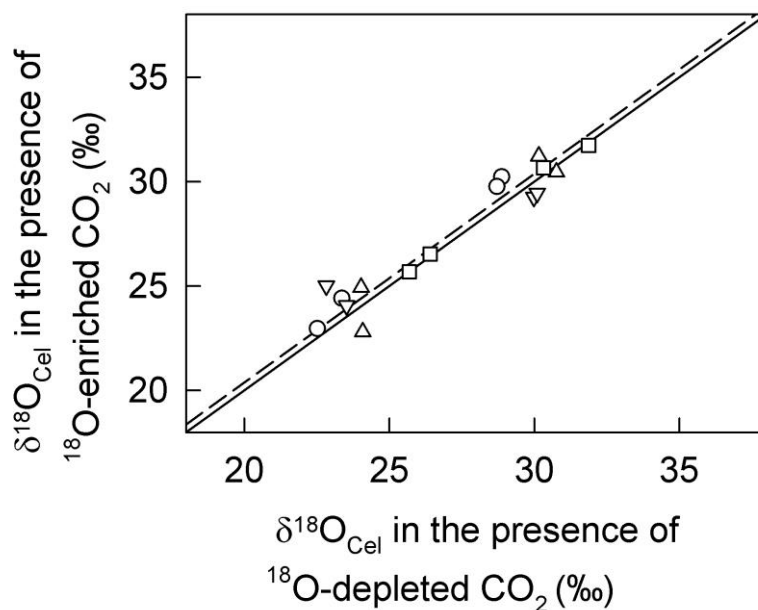
Fig. 2.3 $\delta^{18}O_{Cel}$ of leaf blades grown in the presence of ^{18}O-enriched CO_2 (y-axis) *versus* ^{18}O-depleted CO_2 (x-axis). The $\delta^{18}O$-difference between the CO_2 sources was approx. 20‰ in the 1st and 2nd experimental run, and 13‰ in the 3rd and 4th run (cf. **Table 2.2**). Variation of $\delta^{18}O_{Cel}$ was associated with different VPD treatments. The two CO_2 sources were used in parallel that is in two growth chambers of the same treatment in the same experimental run. $\delta^{18}O$ of CO_2 was measured at the outlet of the growth chambers, and reflected the $\delta^{18}O$ of CO_2 in the well-mixed atmosphere of the growth chamber. 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

δ^{18}O$_{Cel\_E}$ and δ^{18}O$_{Cel\_D}$ was 0.76‰ (±0.15‰ standard error). Cellulose was extracted from leaf blades sampled from the upper and lower parts of canopies. Three samples were collected in each canopy section in each chamber. Samples from the different runs are designated as: 1st run, up-pointing triangles; 2nd run, circles; 3rd run, down-pointing triangles, and 4th run, squares. For assignment of treatments to runs, see **Table 2.1**.

Table 2.2 Oxygen isotope composition of CO$_2$ measured at the inlet (δ^{18}O$_{CO2\ inlet}$) and outlet of the growth chambers (δ^{18}O$_{CO2\ outlet}$) during light periods. δ^{18}O$_{CO2\ outlet}$ reflects the δ^{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 2.1**, and Materials and Methods). Data are shown as averages of 10 d-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	δ^{18}O$_{CO2\ inlet}$ (‰)		δ^{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

Table 2.3 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 and morphology

In all treatments, plant dry mass increased near-linearly with time after canopy closure at around 35 days after imbibition (**Fig. 2.4**). 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 ($\pm$ 0.02 confidence interval) g d^{-1} plant^{-1} at low N and 0.16 $\pm$ 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 $\pm$ 0.04 than that of plants grown at low N (0.80 $\pm$ 0.02, $n = 8$, averaged over VPD levels). High N stimulated plant growth by 45% (relative to low N), increased shoot weight per plant by 42% and enhanced LAI by 53% (**Table 2.4**). 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. Also, VPD (and its interaction with N supply) had no significant effect on any of the morphological variables in **Table 2.4**.
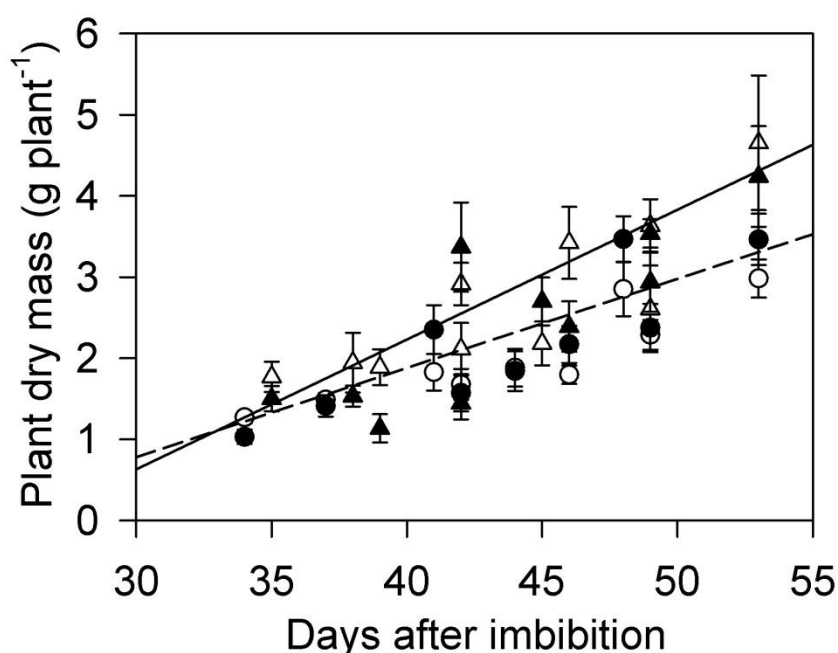
Fig. 2.4 Growth of *C. squarrosa* plants in stands at low or high N fertilizer supply (N1 or N2) combined with low or high VPD (V1 or V2) in growth chambers: N1 V1 (open circle), N1 V2 (closed circle), N2 V1 (open triangle) and N2 V2 (closed triangle). Plant growth rate was determined as the slope of a linear regression. Regression parameters for plant growth rate under low and high VPD did not differ significantly. The dashed and solid line represent the linear regression lines for the low and high N treatment. Each data point and error bar represent the mean and standard error of eight plants harvested on the same day.

The effects of VPD and nitrogen nutrition on $\Delta^{18}O$ of leaf blade water and cellulose in the lower and upper half of the canopy, $\Delta^{18}O_e$ and $p_{ex}p_x$

Across all treatments, $\Delta^{18}O_{LW}$ in the upper canopy was $1.3 \pm 0.1‰$ (mean ± standard error, $n = 16$) higher than in the lower canopy (**Fig. 2.5a**). This effect was similar for the different

VPD and N levels; thus, there was no interaction between VPD and N for this effect ($P >$ 0.05). In contrast, $\Delta^{18}O_{Cel}$ did not differ between lower canopy and upper canopy (**Fig. 2.5b**). High VPD increased $\Delta^{18}O_{LW}$ by approx. 6.4‰ in comparison to low VPD in both the lower and upper canopy sections (average of both N treatments) (**Fig. 2.5a**). In the same way, high VPD increased $\Delta^{18}O_{Cel}$ by approx. 5.7‰ in both canopy sections. N supply had no significant effect on $\Delta^{18}O_{LW}$ in both canopy sections, and $\Delta^{18}O_{Cel}$ in the upper canopy. However, N did appear to affect $\Delta^{18}O_{Cel}$ in the lower canopy, although that effect was relatively small (average +1.7‰ of both VPD treatments). There was no significant interaction effect of VPD and N supply on $\Delta^{18}O_{LW}$ or $\Delta^{18}O_{Cel}$ in both the upper and lower canopy sections.

In the following results, only ^{18}O enrichment of leaf water $\Delta^{18}O_{LW}$ and cellulose $\Delta^{18}O_{Cel}$ in the upper canopy were involved in the calculation of $p_{ex}p_x$ due to the much greater photosynthetic activities of upper canopy relative to lower canopy leaves (Yang Fang, unpublished).

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. 2.6**). 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. 2.7**). 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. 2.6**). Also, 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. 2.7**). 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. 2.7**).

The $p_{ex}p_x$, calculated with Eqn 1.5 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. 2.6**). Also, 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. 2.8**), 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 $\pm$ 1.3‰ (that is $\pm$ 20% of the measured difference between $\Delta^{18}O_{LW}$ at high and low VPD, see **Fig. 2.5a**) also generally supported the significance of the VPD effect on $p_{ex}p_x$ (**Fig. 2.9**). 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. 2.10**).

Table 2.4 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 2.1**). 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 1st (N2 V1 and N2 V2) and 2nd run (N1 V1 and N1 V2), day 44 and 48 after imbibition in the 3rd run (N1 V1 and N1 V2), and day 45 and 49 after imbibition in the 4th 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).

Parameter	Treatment				Significance		
	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
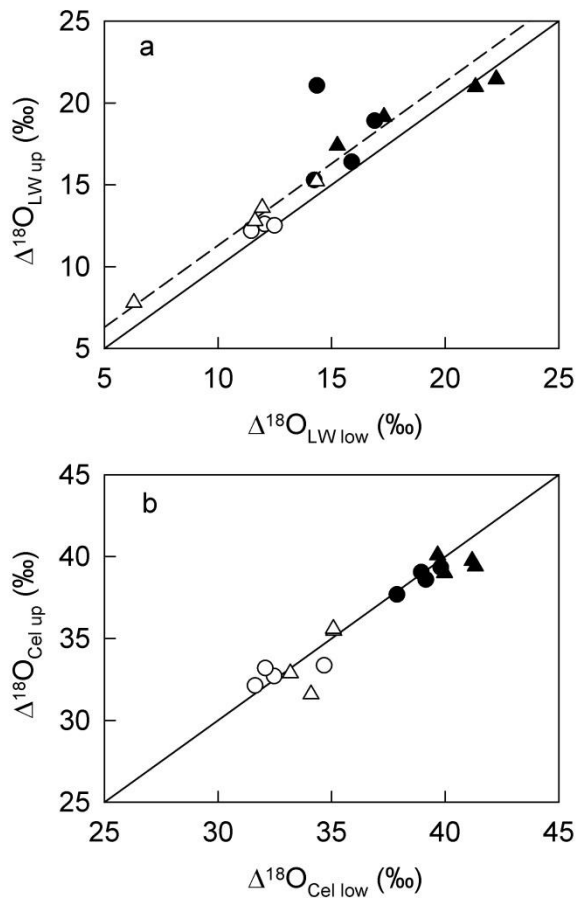
Fig. 2.5 Relationships between ^{18}O enrichment in the upper canopy and lower canopy for (a) leaf water ($\Delta^{18}O_{LW\ up}$ *versus* $\Delta^{18}O_{LW\ low}$, ‰) and (b) leaf blade cellulose ($\Delta^{18}O_{Cel\ low}$ *versus* $\Delta^{18}O_{Cel\ up}$, ‰) in *C. squarrosa* stands grown at low or high N fertilizer supply (N1 or N2) combined with low or high VPD (V1 or V2) in growth chambers: N1 V1 (open circle), N1 V2 (closed circle), N2 V1 (open triangle) and N2 V2 (closed triangle). All treatments (N × V combinations) had four true replications, arranged in four growth chambers in four experimental runs (cf. **Table 2.1**). The solid line gives the 1:1 relationship; the dashed line in (a) is a linear regression: $\Delta^{18}O_{LW\ up} = \Delta^{18}O_{LW\ low} + 1.30$. The linear relationship between $\Delta^{18}O_{Cel\ low}$ and $\Delta^{18}O_{Cel\ up}$ did not differ significantly from the 1:1 relationship. Samples were collected on the 54th, 55th, 37th and 46th day following imbibition of seeds in the 1st, 2nd, 3rd and 4th experimental run, respectively.
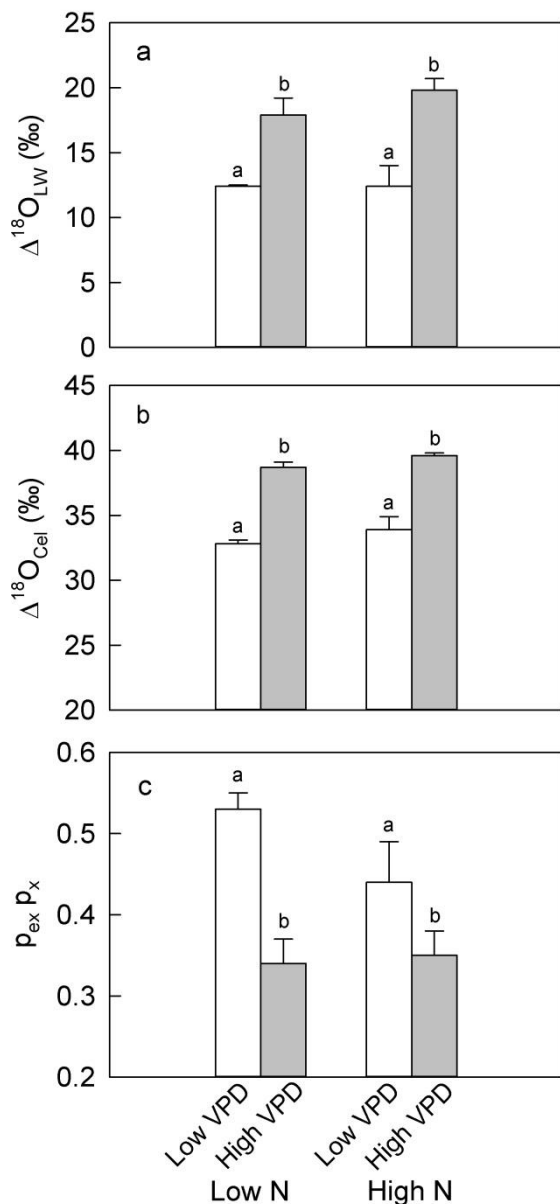
Fig. 2.6 The effects of VPD and nitrogen fertilizer supply (N) on (a) ^{18}O-enrichment of leaf water ($\Delta^{18}O_{LW}$, ‰), (b) ^{18}O-enrichment of leaf blade cellulose ($\Delta^{18}O_{Cel}$, ‰) and (c) $p_{ex}p_x$, the attenuation factor of the relationship between $\Delta^{18}O$ of leaf water and cellulose, estimated using $\varepsilon_o = 27$‰ (cf. Eqn 1.5) in *C. squarrosa* stands under low VPD (white bars) and high VPD (gray bars) with low and high N supply levels. All treatments (N × VPD combinations) had four true replications, arranged in four growth chambers in four experimental runs (see **Table 2.1**). Samples were collected on the 54th, 55th, 37th and 46th day following imbibition of seeds in the 1st, 2nd, 3rd and 4th 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 ($P > 0.05$).
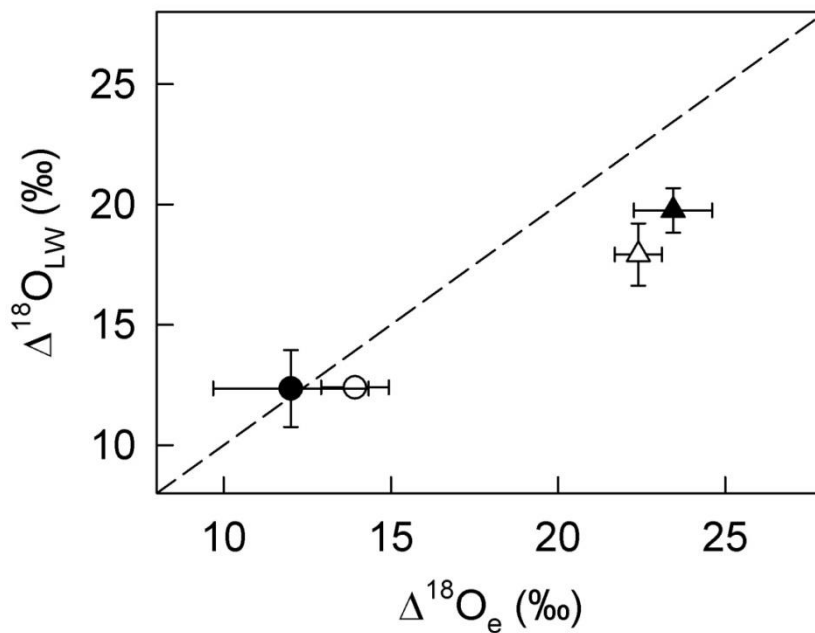
Fig. 2.7 Relationship between observed values of $\Delta^{18}O_{LW}$, measured for bulk leaf blade water, and Craig-Gordon modeled evaporative site ^{18}O-enrichment, $\Delta^{18}O_e$, under low VPD with low nitrogen (empty circle) and high nitrogen supply (filled circle), and under high VPD with low 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 ($P > 0.05$).
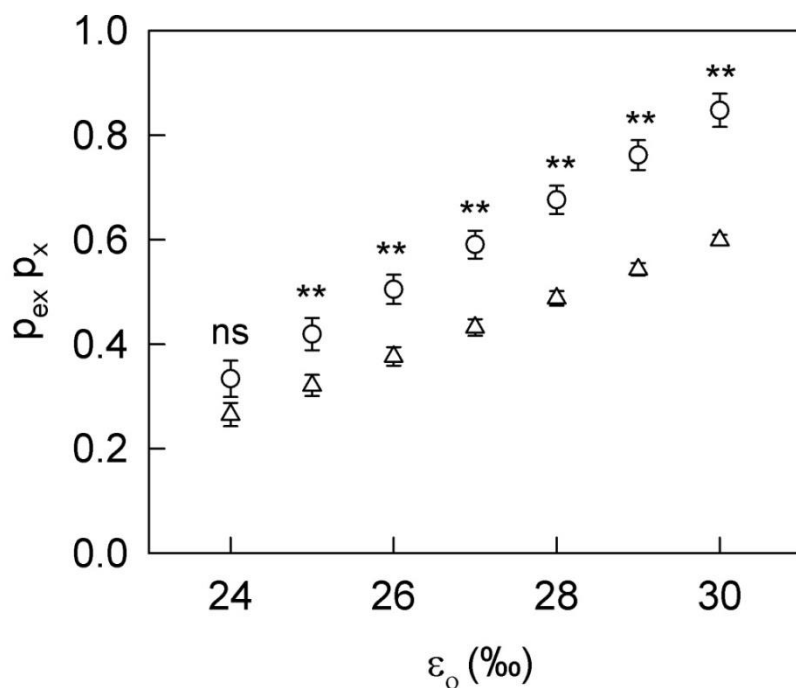
Fig. 2.8 Sensitivity analysis showing the effect of uncertainty in ε_o on calculated $p_{ex}p_x$ for leaf blade cellulose of upper leaves at low (circles) and high VPD (triangles). Data were taken from **Fig. 2.6**. Data from the two N fertilizer levels were pooled for the two VPD levels, as N fertilizer levels and interactions with VPD were not significant for ε_o ranging within $\pm$ 3‰. Values are means $\pm$ standard error ($n = 8$). Significance levels according to two-way ANOVA: ns, not significant; *, $P < 0.05$; **, $P < 0.01$.
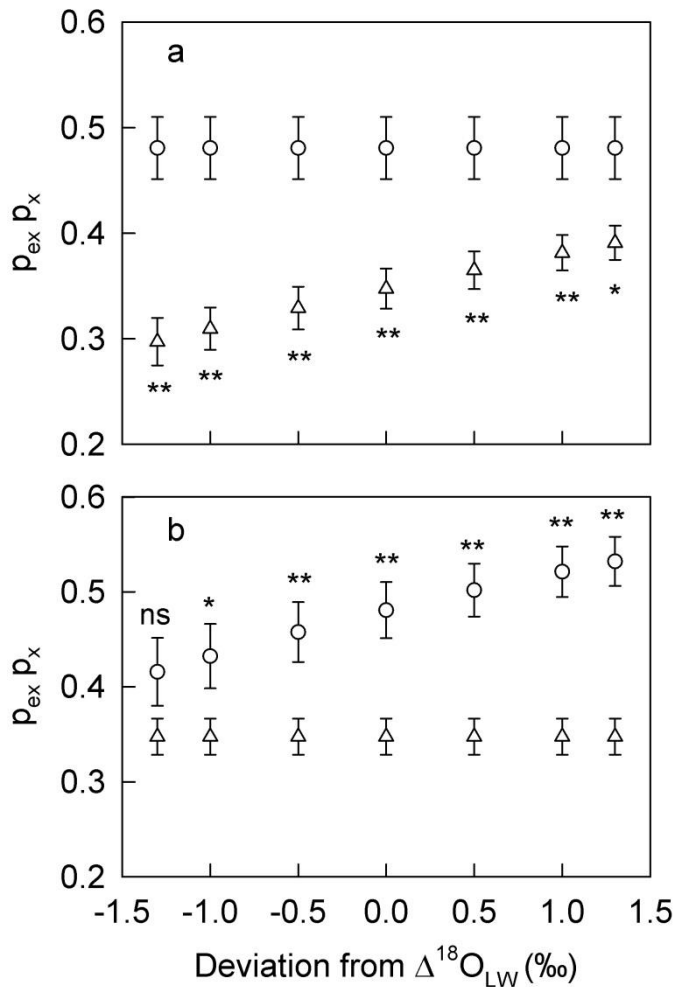
Fig. 2.9 Sensitivity analysis testing the effect of varying oxygen isotope enrichment of leaf water ($\Delta^{18}O_{LW}$) on the significance of the VPD effect on $p_{ex}p_x$ of upper canopy leaves. Data were taken from **Fig. 2.6**. Data from the two N fertilizer levels were pooled for the two VPD levels, as N fertilizer levels and interactions with VPD were not significant (cf. **Fig. 2.6**). In (a) $\Delta^{18}O_{LW}$ at high VPD was varied by ± 1.3‰ in steps of 0.5‰, 1‰ and 1.3‰ while $\Delta^{18}O_{LW}$ at low VPD was kept constant; in (b) $\Delta^{18}O_{LW}$ at low VPD was varied by ± 1.3‰ in steps of 0.5‰, 1‰ and 1.3‰ while $\Delta^{18}O_{LW}$ at high VPD was kept constant. The significance of a VPD effect on $p_{ex}p_x$ was tested by two-way ANOVA with a constant ε_o of 27‰. Values are means ± standard error (n = 8). Significance levels: ns, not significant; *, $P < 0.05$; **, $P < 0.01$.

$\Delta^{18}O_{Cel}$ and leaf age

Oxygen isotope enrichment of leaf blade cellulose ($\Delta^{18}O_{Cel\ blade}$) along a developmental gradient, from the youngest leaf blades at the top to the oldest leaf blade at the bottom of a

tiller, were studied in two of the four replicates and were very similar within a treatment; thus, the strong VPD effect on $\Delta^{18}O_{Cel\ blade}$ was found in all age categories of leaf blades, and, again, no N effect was apparent (**Fig. 2.10**).

According to our monitoring of phytomer development ((Yang *et al.*, 2016), the leaf blade of a given phytomer and the sheath of the next older phytomer grow simultaneously. Thus, the difference between $\delta^{18}O$ of cellulose in the leaf blade ($\delta^{18}O_{Cel\ blade}$) of a given phytomer and the sheath ($\delta^{18}O_{Cel\ sheath}$) of the next older phytomer, provides a measure of leaf tissue-effects on $\delta^{18}O_{Cel}$. This demonstrated a significant difference between $\delta^{18}O_{Cel}$ in the blade relative to that in the sheath in the high VPD, but not in the low VPD treatment (**Fig. 2.11**). The mean $\delta^{18}O_{Cel}$-differences between leaf blades and sheaths ($\pm$ confidence interval; n = 6) in the different treatments were: N1 V1, -0.13 ± 1.09‰; N1 V2, 1.62 ± 1.61‰; N2 V1, 0.57 ± 0.66‰; and N2 V2, 2.19 ± 1.23‰ (**Fig. 2.11**). The $\delta^{18}O_{Cel}$-difference between leaf blades and sheaths translated to different $p_{ex}p_x$ for blades and sheaths at high VPD, with a 8% higher $p_{ex}p_x$ in the leaf blade than in the sheath (**Fig. 2.12**). No such effect was seen at low VPD (not shown), as $\delta^{18}O_{Cel}$ of blades and sheaths did not differ (**Fig. 2.11**). These relationships were not affected by nitrogen supply.
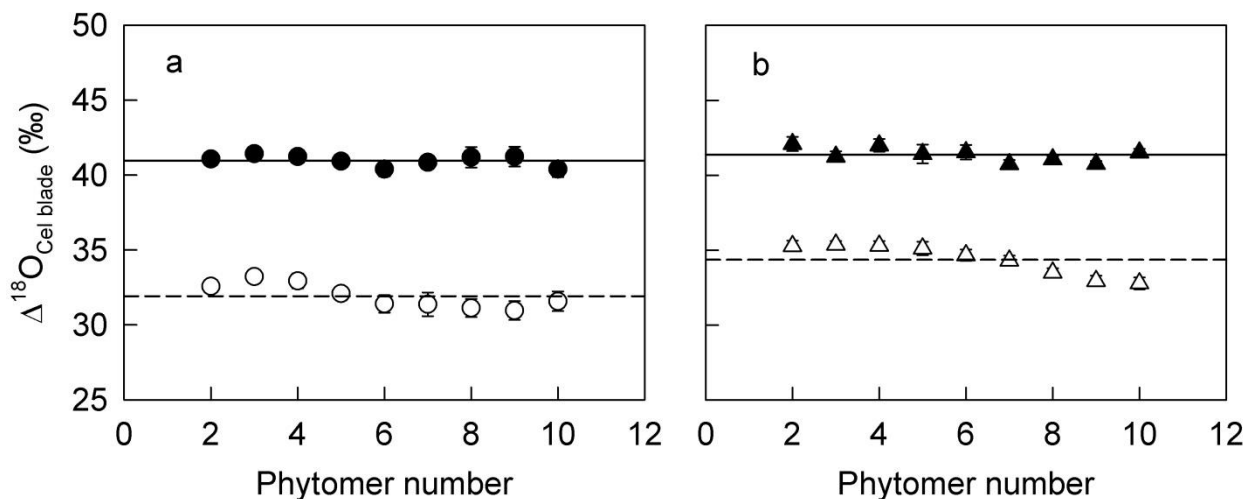
Fig. 2.10 Developmental changes in ^{18}O enrichment of leaf blade cellulose ($\Delta^{18}O_{Cel\ blade}$, ‰). Developmental age was defined by phytomer number: 1 refers to the youngest fully expanded leaf blade near the tip of the tiller, 10 to the oldest leaf blade at the base of the tiller (cf. **Fig. 2.2**). (a) low VPD (open circle) and high VPD (closed circle) at low N fertilizer supply (2nd experimental run); (b) low VPD (open triangle) and high VPD (closed triangle) at high N fertilizer supply (4th experimental run). In (a) and (b), the dashed and solid lines give the mean

$\Delta^{18}O_{Cel\ blade}$ of all leaves at low and high VPD, respectively. The samples were collected on the 53rd and 44th day after imbibition of seed in the 2nd and 4th experimental run (developmental changes were not recorded in the 1st and 3rd experimental run). Each data point and error bar represents the mean ± standard error of six individual plants ($n = 6$).
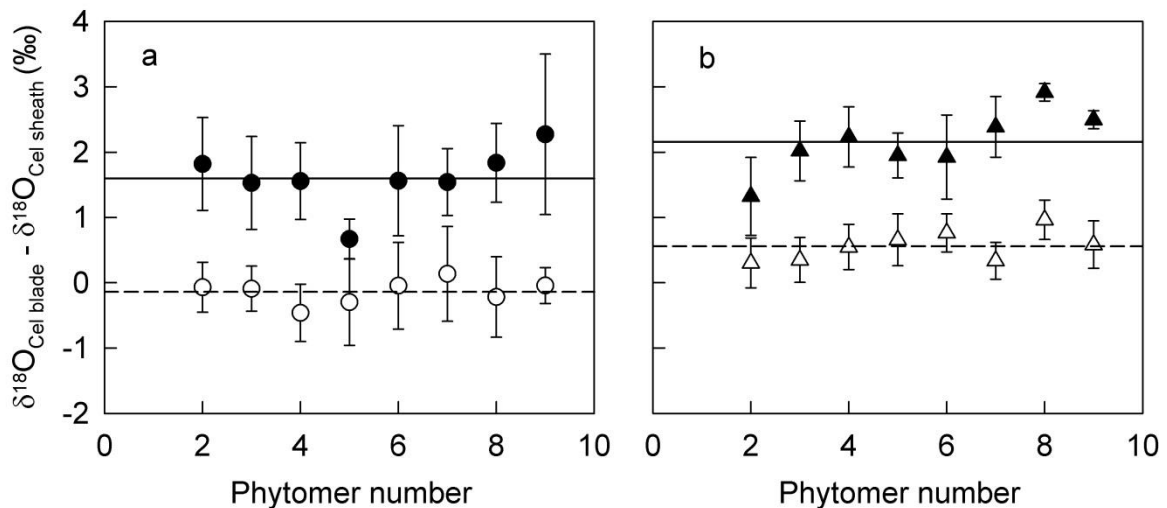
Fig. 2.11 $\delta^{18}O$-offset between cellulose in leaf blades and sheaths, $\delta^{18}O_{Cel\ blade} - \delta^{18}O_{Cel\ sheath}$, as a function of developmental age (see **Fig. 2.10**) of *C. squarrosa* growing at (a) low VPD (open circle) and high VPD (closed circle) at low N fertilizer supply (2nd experimental run) and (b) low VPD (open triangle) and high VPD (closed triangle) at high N fertilizer supply (4th experimental run). Tissue expansion and associated cellulose synthesis occur near-simultaneously in the leaf blades of a give phytomer and the leaf sheath of the next older phytomer (Yang *et al.*, 2016). Hence the ^{18}O-offset was estimated as the difference between the $\delta^{18}O_{Cel\ blade}$ of a given phytomer and the $\delta^{18}O_{Cel\ sheath}$ of the next older phytomer. Samples were harvested on day 53 and 44 after imbibition of seeds in the 2nd (low N) and 4th experimental run (high N), respectively. Each data point and error bar represents the mean ± standard error of three to six individual plants ($n = 3$ to 6).
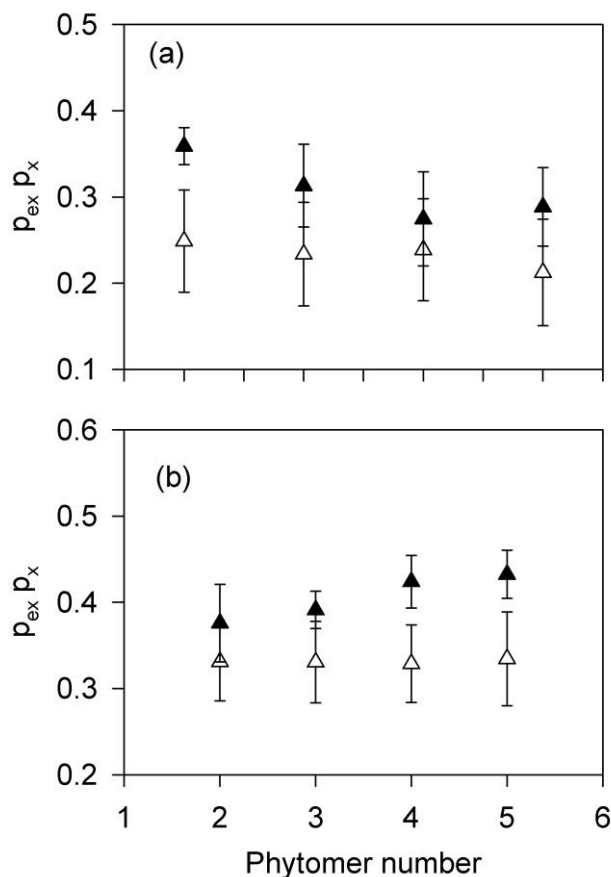
Fig. 2.12 $p_{ex}p_x$ for leaf blade (open symbols) and leaf sheath cellulose (closed symbols) of *C. squarrosa* grown under high VPD (triangles) at low N fertilizer supply (a) and high N supply (b). The difference of oxygen isotope composition in cellulose between blade and sheath under low VPD was not significant (see **Fig. 2.2**), so here only $p_{ex}p_x$ under high VPD treatment is shown. For explanation of the developmental scale, see **Fig. 2.2** and **Fig. 2.11**. $p_{ex}p_x$, the attenuation factor of the relationship between the ^{18}O enrichment of cellulose and leaf water was calculated by assuming $\varepsilon_o = 27$‰. For all leaf blades and sheaths, the ^{18}O enrichment of leaf water used in Eqn 1.5 was taken as the mixture of leaf blade water in the upper canopy sampled at 55 and 46 days after imbibition of seeds in the 2nd and 4th experimental run. Samples for blade and sheath cellulose extraction were collected at 53 and 44 days after imbibition in the 2nd (a, low N supply) and 4th (b, high N supply) experimental run. Each data point and error bar represent the mean and standard error of 3 to 6 samples from two chambers with the same VPD.

DISCUSSION

This work found no significant direct or interactive effects of the ^{18}O content of CO_2 or N fertilizer supply on the ^{18}O content of leaf cellulose in *C. squarrosa*. Also, ^{18}O enrichment of leaf water and, $p_{ex}p_x$, the attenuation factor in the relationship between ^{18}O enrichment of cellulose and leaf water, were not influenced by nitrogen nutrition or ^{18}O content of CO_2. This was all true, except for a small (and possibly spurious) effect of N nutrition on ^{18}O enrichment of cellulose in the lower part of the canopy. These findings were based on four runs of controlled experiments, with four growth chambers, and independent replications of the different growth environments.

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, a C_3 grass, and providing support for Eqn 1.5 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 catalyzing 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 and LMA) or non-existent (leaf thickness).

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-existant) effects on leaf morphological parameters, and lack of a N effect on stomatal conductance and transpiration rates (Gong *et al.*, 2016). Importantly as well, $\Delta^{18}O_e$, estimated by the Craig-Gordon model (Craig & Gordon, 1965; Dongmann *et al.*, 1974; Flanagan *et al.*, 1991a; 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. 2.7**). A similar overprediction of $\Delta^{18}O_{LW}$ by $\Delta^{18}O_e$ in C$_4$ grasses was noted by Webb and 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 due to 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.

Canopy position affects ^{18}O enrichment of leaf water but not cellulose

^{18}O enrichment of cellulose was independent of canopy position (as was the δ^{18}O of cellulose in leaves of different age, which correlates with positions in the canopy), while ^{18}O-enrichment of leaf water was greater (average +1.3‰) in the upper than in the lower canopy. The near-invariance with age and canopy position of δ^{18}O of cellulose must be related (at least partly) to (i) the constancy of environmental conditions in the growth chambers, (ii) the fact that successive leaf growth and associated cellulose synthesis occur in the youngest phytomers at tip of the grass tillers, where environmental conditions (e.g. irradiance) are unaltered by canopy effects, and (iii) the substrate for cellulose synthesis is mainly provided by the young (and mostly) unshaded leaves at the top of the canopy (Ryle & Powell, 1974;

Dale, 1985, 1988). Short-term ^{13}C labeling experiments confirmed that most carbon assimilation occurred in the upper part of the canopy (Yang *et al.*, unpublished data).

The greater ^{18}O enrichment of leaf water in the upper as compared to the lower canopy agrees with findings in corn and wheat sampled in the field near midday, when leaf water ^{18}O enrichment had attained a near-steady state (Xiao *et al.*, 2012). Such a canopy gradient in ^{18}O enrichment may be connected with a VPD gradient between the top and the bottom of the canopy. The humidity sensitivity of ^{18}O enrichment has been found to range between −0.4 and −0.2‰ per % relative humidity (RH) (Farquhar *et al.*, 2007; WELP *et al.*, 2008; Kim & Lee, 2011; Wen *et al.*, 2012; Xiao *et al.*, 2012). In this work, the sensitivity was −0.22‰ per % RH, in close agreement with that observed by Xiao *et al.* (2012) in wheat and corn fields (−0.2‰ per % RH) and Wen *et al.* (2012) in short steppe grassland (−0.25‰ per % RH). If the the canopy effect was entirely related to VPD, then RH humidity in the lower canopy must have been about 6% greater than in the upper canopy.

VPD affects $p_{ex}p_x$ in leaf blades and leaf sheaths

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 (2002b) with a range of C$_3$ and C$_4$ grasses. Also, 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 affect 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 (Gong *et al.*, 2016) 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, b; 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 (2002b) – 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 $p_{ex}p_x$ for the range of RH explored in our work. In their work, the calculated $p_{ex}p_x$ 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 $p_{ex}p_x$ at the high VPD level, similar to our work.

In this study, variation of $p_{ex}p_x$ for leaf blade cellulose and the divergence of $p_{ex}p_x$ between leaf blades and sheaths were entirely dependent on VPD. As plant growth rate and associated morphological parameters had no effect on $p_{ex}p_x$. we suggest that all variation of $p_{ex}p_x$ observed in this work was due (at least in part) to variation of p_x. This interpretation is supported by Helliker & Ehleringer (2002a), who observed a VPD-dependent ^{18}O enrichment of leaf meristem/growth zone water in *Lolium multiflorum*, a C$_3$ grass. Empirical and theoretical studies indicate that p_{ex} ranges between 0.3 and 0.64 (Barbour & Farquhar, 2000; Ellsworth & Sternberg, 2014), with ~0.4 (Cernusak *et al.*, 2005) the most-commonly used (default) value in the absence of direct measurements. Using a $p_{ex} = 0.4$ and $\varepsilon_o = 27‰$ in Eqn 2. 1, we obtain a range of p_x for leaf blade cellulose from 0.85 (low N high VPD) to 1.3 (low N low VPD). These estimates of p_x are much higher than the range of 0.50 to 0.62 in C$_3$ and C$_4$ grasses reported by (Helliker & Ehleringer, 2002a, b). Evidently, the $p_x = 1.3$ must be an overestimation, indicating that either ε_o was < 27‰ and/or that $p_{ex} > 0.4$. If we set ε_o at 26‰ and p_{ex} at 0.5, we obtain a range of p_x from 0.56 to 0.90, showing that estimates of p_x are highly sensitive to relatively modest adjustments/modifications of ε_o and p_{ex}. Still, provided that ε_o and p_{ex} were both constant in a reasonable range, VPD always had a marked effect on p_x. The mechanism underlying such a putative VPD effect on p_x has not been explored, but may be related to variation in the proportion or ^{18}O enrichment of phloem-derived water at the sites of cell wall synthesis.

Using the same assumptions as above, high VPD also caused a 8% greater proportion of unenriched water at the site of cellulose synthesis in sheaths than in blades. This result was unexpected, as grass leaf blades and sheaths are formed successively by the same (heterotrophic) intercalary meristem/growth zone (Sharman, 1942; Schnyder *et al.*, 1990) and ^{18}O enrichment in leaf blade cellulose of *L. multiflorum* grown at a high constant VPD did not present a longitudinal (Helliker & Ehleringer, 2002a), which might extrapolate to a changed ^{18}O enrichment in leaf sheaths. It is presently unknown which factors may have caused a

greater p_x in sheaths than in leaves at high but not low VPD. But again, variation in the proportional contribution of phloem water at the site of cellulose synthesis or of its ^{18}O enrichment may have played a role.

CONCLUSION AND OPEN QUESTIONS

This study provided a systematic analysis of some unexplored putative controls of the relation between ^{18}O in leaf water and cellulose in leaves of a perennial C$_4$ grass. Only VPD had significant effects, whereas N nutrition (and its associated effect on plant growth rate and morphological parameters), $\delta^{18}O_{CO2}$, the position of leaves in a canopy and leaf age had no significant effect on $\Delta^{18}O_{Cel}$.

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 Δ^{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 (MacAdam & Nelson, 1987; Schnyder *et al.*, 1990; MacAdam & Nelson, 2002), also see 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 throught 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 δ^{18}O in leaf water on δ^{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, b; Cernusak *et al.*, 2005; Barbour, 2007; Gessler *et al.*, 2014).

Chapter 3

An oxygen isotope chronometer of cellulose: the successive leaves formed by tillers of a C$_4$ grass

ABSTRACT

Multiannual time series of (palaeo)hydrological information can be reconstructed from the oxygen isotope composition of cellulose (δ^{18}O$_{Cel}$) in biological archives, e.g. tree-rings, but our ability to temporally resolve information at subannual scale is limited. We capitalized on the short and predictable leaf appearance interval (2.4 d) of a perennial C$_4$ grass (*Cleistogenes squarrosa*), to assess its potential for providing highly time-resolved δ^{18}O$_{Cel}$ records of vapor pressure deficit (VPD). Plants grown at low (0.63 kPa) or high (1.58 kPa) VPD, were swapped between VPD environments, and exposed to the new environment for 7 d with simultaneous ^{13}CO$_2$ labeling. Then, leaves were sampled by age/position along individual tillers. Five leaves at different developmental stages were growing simultaneously. The period of most-active leaf elongation, from 10% to 90% of final length, lasted 6.6 d and ~80% of all carbon and oxygen incorporation in whole-leaf cellulose occurred within 7 d. Cellulose synthesis stopped at (or shortly after) full leaf expansion. The direction of change, from low to high or high to low VPD, had no differential effect on new oxygen and carbon incorporation in cellulose. Successive leaves produced by tillers of *C. squarrosa* provide a δ^{18}O$_{Cel}$ record useful for reconstructions of short-term hydrological dynamics.

INTRODUCTION

The oxygen isotope composition of cellulose ($\delta^{18}O_{Cel}$) is determined by environmental conditions, mainly temperature and relative humidity – which determine evaporative demand – and morpho-physiological plant traits which affect the evaporative enrichment of ^{18}O in leaf water, and the $\delta^{18}O$ of water in plant compartments that are involved in the metabolism of sugars leading up to cellulose synthesis (Barbour, 2007; Gessler *et al.*, 2009; Sternberg, 2009). As they store environmental information, chronologies of $\delta^{18}O_{Cel}$ are powerful tools for (paleo-)climate reconstructions (Libby *et al.*, 1976; Hong *et al.*, 2000; Roden *et al.*, 2000; Anderson *et al.*, 2002; Treydte *et al.*, 2007; Sternberg, 2009; Kahmen *et al.*, 2011). In addition, as stomatal conductance is thought to modify the $\delta^{18}O$ of leaf water and hence that of cellulose (Farquhar *et al.*, 1998; Scheidegger *et al.*, 2000), it is also expected that retrospective analyses of $\delta^{18}O_{Cel}$ archives can shed light on physiological responses of plants' gas exchange to past climate changes. Annually resolved tree-ring series have spanned periods of centuries up to more than 10 000 years in some regions (Pilcher *et al.*, 1984; Briffa *et al.*, 1990; Esper *et al.*, 2002; Treydte *et al.*, 2006), but tree-rings have also unique potential for analyses at the subannual time scale (Barbour *et al.*, 2002; Helle & Schleser, 2004; Roden *et al.*, 2009). Some state-of-the-art methodologies attain a sampling-width resolution as fine as 10-100 μm (Helle & Schleser, 2004; Schulze *et al.*, 2004; Schollaen *et al.*, 2014), equivalent to average tree-ring increments of <1 d to a few weeks in some cases. Yet, exact dating of subannual tree-ring sections remains a challenge (Schleser *et al.*, 1999; Kagawa *et al.*, 2005), as growth of individual subannual sections cannot be observed directly. Hence, various indirect approximations have been used for (tentative) dating of subannual sections in tree-rings, such as coincidence of drought periods and maxima of $\delta^{13}C$ within tree-rings (Schleser *et al.*, 1999) or extrapolations from dendrometer measurements (Barbour *et al.*, 2002; Gessler *et al.*, 2009).

Although they can not replace tree-rings as long-term records, other morphological units of plants offer a specific potential for resolving subannual records with precisely dated chronologies of tissue formation. For instance, Helle & Schleser (2004) monitored leaf and bud sprouting, and dated tree-ring sections by comparison of the carbon isotope composition of cellulose ($\delta^{13}C_{Cel}$) in the sprouting leaves and in highly spatially-resolved tree-ring sections. Helliker & Ehleringer (2002a) demonstrated that segments of grass leaves provide a longitudinal (spatial) record of $\delta^{18}O_{Cel}$ variation that results from effects of changed atmospheric vapor pressure deficit (VPD) on the ^{18}O composition of leaf water and assimilates that were produced during the respective periods of leaf segement growth. Grass-

tissue-based isotopic indicators are of great importance for grassland ecosytems, e.g. semiarid steppes in North China where water stress is the major limiting factor for plant growth (Gong *et al.*, 2016). Vegetative tillers of grasses – and some other taxa (Schleip *et al.*, 2013) – exhibit a repetitive pattern of leaf production throughout the vegetation period. C_4 grasses may be particularly suitable for dynamic isotope time series analyses, as they exhibit short and predictable leaf appearance intervals (phyllochrons) and have several leaves at different developmental stages of the leaf growth process during vegetative development (Yang *et al.*, 2016). Furthermore, $\delta^{13}C_{Cel}$ of C_4 plants is not directly applicable for indicating evaporative demind due to the involvement of the variance in bundle sheath leakiness (Gong *et al.*, 2016). Thus, in this context, $\delta^{18}O_{Cel}$ of C_4 plants might be an alternative. But that potential has not been explored to date.

A specific challenge in the evaluation of highly spatially-resolved ^{18}O records is their unknown temporal integration (Kagawa *et al.*, 2005). In other words, it is generally not known what time period of assimilation is actually reflected in the $\delta^{18}O_{Cel}$ of a given sample. The integration is primarily a function of the turnover time of substrate pools in source and sink tissue (including storage/mobilization processes of sugars that may participate in cellulose synthesis), and the diversity of cell types, developmental stages and characteristic cellulose deposition dynamics of the different cell types in the sample (Hemming *et al.*, 2001; Damesin & Lelarge, 2003; Gessler *et al.*, 2009; Gessler *et al.*, 2014). To our best knowledge, there have been no explicit, quantitative investigations of the temporal integration in short-term ^{18}O records. However, in 1 d-long $^{13}CO_2$ pulse labelling experiments with *Cryptomeria japonica* (Japanese cedar) performed in spring and fall, Kagawa *et al.* (2005) observed a half-width of the labeling peak – which comprizes ~80% of the label within the peak (given a Gaussian distribution) – equivalent to 9 to 28 d of earlywood and 33 to 42 d of latewood formation. However, whether or not carbohydrate stores contributed to cellulose synthesis was not analyzed. In principle, one may expect a similar or higher temporal resolution of the ^{18}O record of successively formed leaves in grasses as rates of successive leaf appearance and elongation are rapid in grasses (Skinner & Nelson, 1995) and growth substrate pools for leaf growth have a high turnover (Lattanzi *et al.*, 2005).

The objective of this work was to characterize the temporal resolution of the ^{18}O record that is generated by the successive leaves formed by tillers of *C. squarrosa* following a change of VPD, and hence to evaluate its potential as a quantitative high-resolution biological chronometer of ^{18}O signals. In the same experiment, we performed a detailed analysis of the development of successive phytomers, and of their blade, sheath and internode parts, for

plants grown in contrasting VPDs (Yang *et al.*, 2016). Plants were grown at high and low VPD, swapped between VPD environments and exposed to the new environment for 7 d while simultaneously labeling them with $^{13}CO_2/^{12}CO_2$ mixtures of known constant composition at near-ambient CO_2 concentration. After 7 d, the incorporation of new oxygen (derived from assimilation in the new VPD environment) and labelled carbon were evaluated in leaves of different developmental stages.

MATERIALS AND METHODS

Plant material and growth conditions

This experiment used the same plant material and growth conditions as the studies of Liu *et al.* (2016) and Yang *et al.* (2016). The work was performed in growth chambers that formed part of a new version of the controlled environment $^{13}CO_2/^{12}CO_2$ mesocosm system described by Schnyder *et al.* (2003). That system permitted continuous measurements and control of air temperature, relative humidity, and concentration and ^{13}C composition of CO_2 ($\delta^{13}C_{CO2}$) in each chamber.

Plants were established from seed. Four seeds of *C. squarrosa*, were sown in individual tubes (4.5 cm diameter, 35 cm deep) filled with quartz sand. Tubes were arranged in free-draining plastic containers (length: 77 cm, width: 57 cm, depth: 30 cm) with 164 tubes per container. Two containers were placed in each growth chamber. Before germination, the conditions in all chambers were kept the same with a relative humidity (RH) of 80% at a constant growth temperature of 25 ^{o}C, thus yielding a vapor pressure deficit (VPD) of 0.63 kPa. The time of first watering is referred to as day 0 of the experiment. One week after imbibition, plants were thinned to one per tube, and RH adjusted to 50% (providing a VPD of 1.58 kPa) in two chambers, while maintaining the VPD of 0.63 kPa in the other chambers. The two chambers of a given VPD regime were supplied with CO_2 of different $\delta^{13}C$: a ^{13}C enriched CO_2 with a $\delta^{13}C_{CO2}$ of -6.2 ± 0.1‰ (mean $\pm$ SD, $n = 20$) was supplied to one chamber and a ^{13}C depleted CO_2 with a $\delta^{13}C_{CO2}$ of -48.8 ± 0.1‰ (mean $\pm$ SD, $n = 20$) to the other (cf. **Fig. 3.1**). Due to the carbon isotope discrimination by the plants, a $\delta^{13}C_{CO2}$ inside the growth chambers with the ^{13}C enriched CO_2 was -5.3 ± 0.2‰ (mean $\pm$ SD) and was -47.7 ± 0.1‰ inside the growth chambers with the ^{13}C depleted CO_2 (cf. Gong *et al.,* 2016). Except for the VPD and CO_2 sources, all other environmental parameters were kept the same.

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. Irradiance was kept constant by periodic adjustments of the distance between the fluorescent tubes and

the top of the canopy following measurements with a quantum sensor (LI-190R, LI-COR, Lincoln, Nebraska, USA). Air temperature was kept constant at 25 °C during dark and light periods. CO_2 concentration was controlled at 390 μmol mol^{-1} during the light period. CO_2 and water vapor concentration in chamber air were measured by an infrared gas analyzer (LI-6262, LI-COR Inc. Lincoln, USA). A modified Hoagland nutrient solution, with 7.5 mM nitrate, was supplied three times per day by an automatic irrigation system as described by Liu *et al.* (2016).
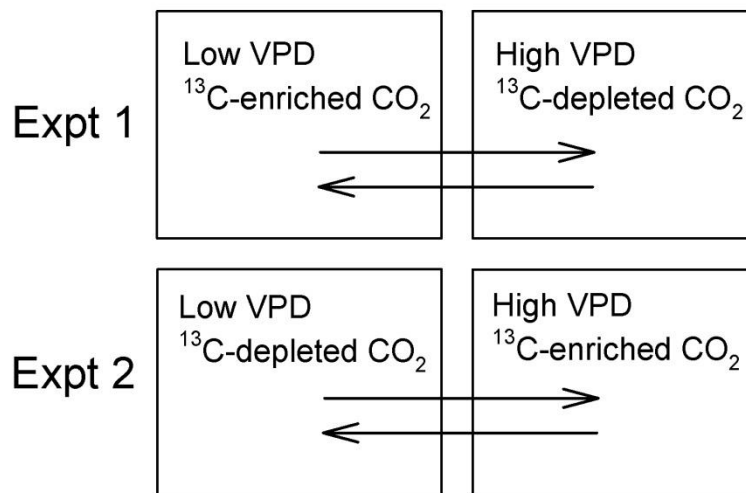
Fig. 3.1 Experimental scheme. Two independent experiments were performed with plants of *C. squarrosa* exposed to four treatments in growth chambers: constant low VPD, constant high VPD, transfer from low to high VPD, and transfer from high to low VPD. In both experiments, the low and high VPD environments received CO_2 with contrasting $\delta^{13}C_{CO2}$ (^{13}C-depleted CO_2, −48.8‰; and ^{13}C-enriched CO_2, −6.2‰). Transferred plants were kept in the new environment (high or low VPD) for 7 d and then sampled in parallel with plants kept constantly at low and high VPD. Three samples were collected in each treatment in each experiment. Except for VPD and $\delta^{13}C_{CO2}$ all conditions were kept the same in all growth chambers.

Treatments and sampling

The experiment was run twice. Each experimental run had two transfer treatments (high-to-low VPD, and low-to-high VPD) and constant VPD 'controls' with plants grown

continuously at low or high VPD. Some of the $\delta^{18}O_{Cel}$ data from the constant VPD treatments were already reported by Liu *et al.* (2016). In transfer treatments, eight plants each were swapped with plants growing in contrasting conditions of VPD and $\delta^{13}C_{CO2}$. For example, plants grown at low VPD in the presence of the ^{13}C enriched CO_2 ($\delta^{13}C_{CO2}$ = −6.2‰) were exchanged with plants grown at high VPD and the ^{13}C depleted CO_2 ($\delta^{13}C_{CO2}$ = −48.8‰).

Transfers occurred at 1 h before the start of the light period on the 44th day (see **Fig. 3.1**). Plants were harvested 7 d later at the same daytime. Harvests also included plants that had remained in the same environment throughout the experiment (controls, see above). Leaves on major tillers (i.e. tillers that had >10 fully developed leaves) were sampled by position, i.e. age category (**Fig. 3.2**). Except for the youngest exposed leaf, all other leaf blades were sampled integrally, by cutting at the ligule. Daughter tillers that developed in the leaf axils of older leaves (DT in **Fig. 3.2**) were discarded. Same age leaves from 15 to 20 tillers were combined in one sample, and three separate samples of each age category collected in every treatment in every chamber. The youngest sampled leaf (no. 1, **Fig. 3.2**) was cut at the point of emergence from the surrounding older leaf and often included an additional tiny leaf tip that had emerged and was enrolled by leaf no. 1. All samples were oven-dried for 48 hours at 60 ˚C, and then stored in an exsiccator until milling and cellulose extraction.

Estimation of leaf blade developmental and growth stage

Leaf blade samples collected along tillers were numbered consecutively from tip, starting with the youngest sampled leaf (see above). For every leaf age category (1 to *n*), the stage of blade development and growth (as a fraction of final blade length, f_{FBL}) at the times of transfer and harvest was estimated from knowledge of the leaf appearance interval, development of individual phytomers, and the contribution of the leaf blade component to phytomer development in the same study (Yang *et al.*, 2016). **Fig. 3.3** illustrates the time course of leaf blade elongation, as calculated from phytomer elongation and the fractional contribution of leaf blades to phytomer elongation for the constant low and high VPD treatments in plants grown at 'low N' (cf Fig. 3a and 5 in Yang *et al.* 2016), the 'control' treatments presented here. The tip of the leaf blade emerged from the enclosing sheath of the next older leaf when the blade had reached 31% of its final length and the period of maximum leaf elongation rate − taken as the time for expansion from 10% to 90% of final length − was 6.6 d, and lasted from 2.1 d before tip emergence to 4.5 d after tip emergence (**Fig. 3.3**). Also, the leaf appearance interval (2.4 d) and final leaf blade length were the same in plants grown constantly in the same low or high VPD environment (Yang *et al.,* 2016). These relationships

were not affected by VPD. Also, transfer of plants from one VPD environment to the other did not affect the leaf appearance interval (data not shown).
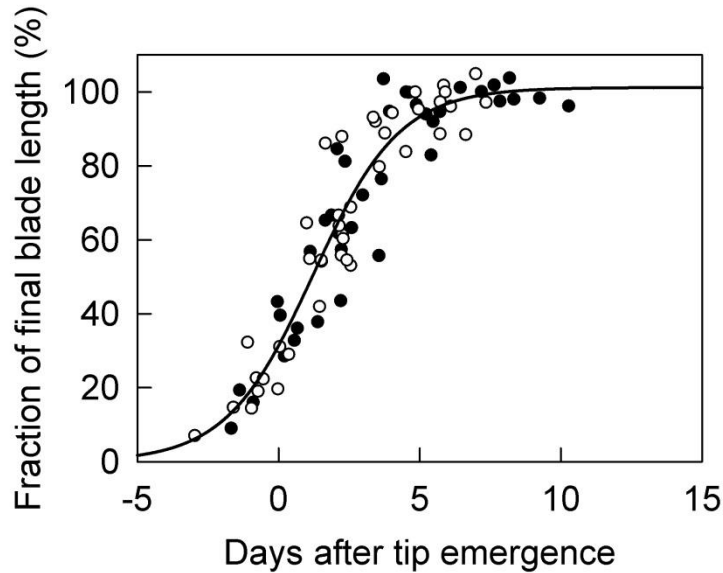
Fig. 3.3 Time course of leaf blade elongation of *C. squarrosa* in relation to the time of leaf tip emergence. Plants were grown at low VPD (closed circles) and high VPD (open circles). Leaf blade length is expressed as a fraction of final length (f_{FBL}). Emergence is defined as the time when the leaf tip passes through the surrounding sheath of the next-older leaf. The solid curve denotes a 3-parameter sigmoidal function for all data, y = 101.19/{1 + exp[(1.22 − x)/1.53]}, $R^2 = 0.89$. VPD had no effect on the parameters of the function ($P > 0.05$).

Cellulose extraction

Aliquots of 50 mg or 25 mg of ball-milled, dry sample material were used to extract α-cellulose using the procedure of Brendel *et al.* (2000) as modified by Gaudinski *et al.* (2005).

Measurements of carbon and oxygen isotope composition

Carbon or oxygen isotope composition was expressed in per mil (‰) as

$$\delta^{13}C \text{ or } \delta^{18}O = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \times 1000 \qquad \text{Eqn 3.1}$$

where, R_{sample} is the $^{13}C/^{12}C$ or $^{18}O/^{16}O$ ratios of the sample, and $R_{standard}$ is $^{13}C/^{12}C$ ratio in the international V-PDB standard or $^{18}O/^{16}O$ ratios in the Vienna Standard Mean Ocean Water standard (V-SMOW).

Carbon isotope discrimination as expressed in leaf blade cellulose ($\Delta^{13}C_{Cel}$) of plants kept continuously in a given growth chamber was calculated as:

$$\Delta^{13}C_{Cel} = \frac{\delta^{13}C_{chamber\ CO2} - \delta^{13}C_{Cel}}{1 + \delta^{13}C_{chamber\ CO2}/1000} \qquad \text{Eqn 3.2}$$

with $\delta^{13}C_{chamber\ CO2}$ referring to the $\delta^{13}C$ of CO_2 in the atmosphere of the respective growth chamber.

Oxygen isotope enrichment of leaf blade cellulose relative to source water ($\Delta^{18}O_{Cel}$) was obtained as:

$$\Delta^{18}O_{Cel} = \frac{\delta^{18}O_{Cel} - \delta^{18}O_{SW}}{1 + \delta^{18}O_{SW}/1000}, \qquad \text{Eqn 3.3}$$

with $\delta^{18}O_{SW}$ the oxygen isotope composition of the nutrient solution (Liu *et al.*, 2016).

Cellulose samples were redried at 40 °C for 24 h, and 700 μg aliquots were packed in tin cups for carbon and silver cups for oxygen isotope (size: 3.3 × 5 mm, LüdiSwiss, Flawil, Switzerland) and stored above Silica Gel orange (2-5mm, ThoMar OHG, Lütau, Germany) in exsiccator vessels. For measurements of $\delta^{18}O_{Cel}$, 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 lab 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). For $\delta^{13}C_{Cel}$ measurements, each sample was measured against a laboratory working standard gas, which was previously calibrated against a secondary isotope standard (IAEA-CH6 for $\delta^{13}C$, accuracy of calibration ± 0.06% SD). A laboratory standard (a fine ground wheat flour) was run after every tenth samples. The long-term precision for the SILS was <0.2‰ for $\delta^{13}C$ and <0.3‰ for $\delta^{18}O$.

Fraction of new carbon or oxygen in leaf blade cellulose

The fraction of new carbon or oxygen (f_{new}) in cellulose of a given leaf age category of transferred plants was calculated as

$$f_{new} = (\delta_{sample} - \delta_{old})/(\delta_{new} - \delta_{old}), \qquad \text{Eqn 3.4}$$

with δ_{sample}, δ_{old} and δ_{new} designating the $\delta^{13}C_{Cel}$ or $\delta^{18}O_{Cel}$ (as appropriate) of that same leaf age category in transferred plants, plant kept in the chamber of origin (old) or in the new chamber throughout the experiment, following the rationale in Schnyder (1992). For the fraction of new oxygen ($f_{new\ O}$) in plants that were transferred from low to high VPD in the

2nd experiment, the mean value of repeated measurements from blade 7 to 10 of labeled/transferred plants was used as δ_{old}, see the explanation in discussion.

Statistical analysis

The differences of $\delta^{13}C$ or $\delta^{18}O$ of blade cellulose within the successive leaf blades of controlled plants and also between old blades from the 6 to 11 of controlled plants and transferred plants were compared by the 95% of confidence interval. The confidence intervals of slopes and intercept of linear relationship between fraction of new carbon ($f_{new\ C}$) or fraction of new oxygen ($f_{new\ O}$) in each leaf blade cellulose of plants transferred from low to high VPD and from high to low VPP were also used to test the coincidence between the linear relationship and 1:1 line. One-way ANOVA was used to test the difference of $f_{new\ C}$ and also $f_{new\ O}$ in each leaf blade cellulose with the two transfer treatments as random factors.

RESULTS

Leaf development and growth stages

The weighted mean time appearance of tiny blades enrolled by leaf no. 1 was assumed at 1.2 d, because 15-20 leaf blades with different time appearances ranged between 0 and 2.4 d were combined for one sample. Based on the time course of leaf blade elongation in relation to the time of leaf tip emergence (**Fig. 3.3**) and the observations at sampling we estimated that the tip of leaf no. 1 had emerged 3.6 d (1.2 + leaf appearance interval of 2.4 d) before sampling (cf. also **Fig. 3.2**) on average of all sampled leaves in this age category in all treatments. Also based on **Fig. 3.3**, leaf no. 2 had reached 97% of its final length at sampling (**Table 3.1**, **Fig. 3.2**). At 7 d before sampling, the time of transfer from one VPD environment to the other, this leaf (no. 1) had reached only 5% of its final length. As the leaf appearance interval (2.4 d) was constant and the same for the different treatments, times of leaf tip emergence of successively older leaf numbers were obtained as $((n-1) \times 2.4\ d) + 3.6\ d$ by assuming that the average time when the tip of the tiny blade enrolled by leaf no. 1 passing through the whorl of older sheath was 1.2 d. Leaf blade was thought as its final blade length when it reached 98% of its final length at 6.5 days after tip emergence, see **Fig. 3.3**. Thus, for instance, in leaf no. 3 the leaf tip had emerged at 8.4 d before sampling, meaning that this leaf had already reached 54% of its final length at the time of transfer, had continued to elongate for 5.1 d after the transfer, and – hence – stopped growing 1.9 d before sampling. These observations indicated that only leaf no. 1 and 2 elongated throughout the 7 d long period following transfer, while successively older leaves were at increasingly advanced developmental stages at transfer, and – accordingly – stopped elongating earlier. The oldest still expanding leaf age category (no. 5)

stopped to expand very shortly after the transfer and leaf blade no. 6 and older were fully expand at the time of transfer.
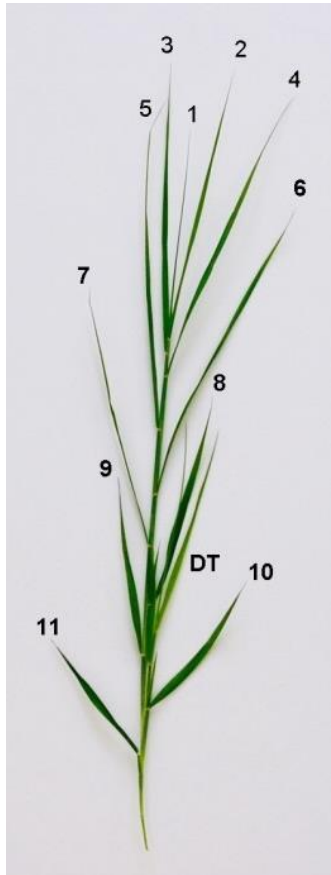
Leaf number	(f_{FBL}) at times of		Growth duration after transfer
	Transfer	Harvest	transfer
	%	%	d
1	5	84	7.0
2	19	97	7.0
3	54	100	5.1
4	85	100	2.7
5	97	100	0.3
$\geq$6	100	100	0

Fig. 3.2 Photograph of a tiller of *C. squarrosa*. Leaf blades are numbered consecutively from the tip to the base of the tiller, with no. 1 the youngest (exposed) leaf and no. 11 the oldest. DT refers to a daughter tiller which has emerged from the axillary bud of leaf no. 10 (photo taken from **Fig. 2.2**).

Table 3.1 Developmental stages of leaf blades at times of transfer to a new VPD environment and harvest/sampling 7 d later, and growth duration in the new environment following the transfer (for details, see Materials and Methods and Results). Developmental stage is given as leaf blade length expressed as a fraction of final length (f_{FBL}), and was inferred from the leaf appearance interval (2.4 d at both low and high VPD) and the time course of leaf blade elongation shown in **Fig. 3.3**. Growth duration after the transfer was calculated as the time needed to reach 98% of final blade length.
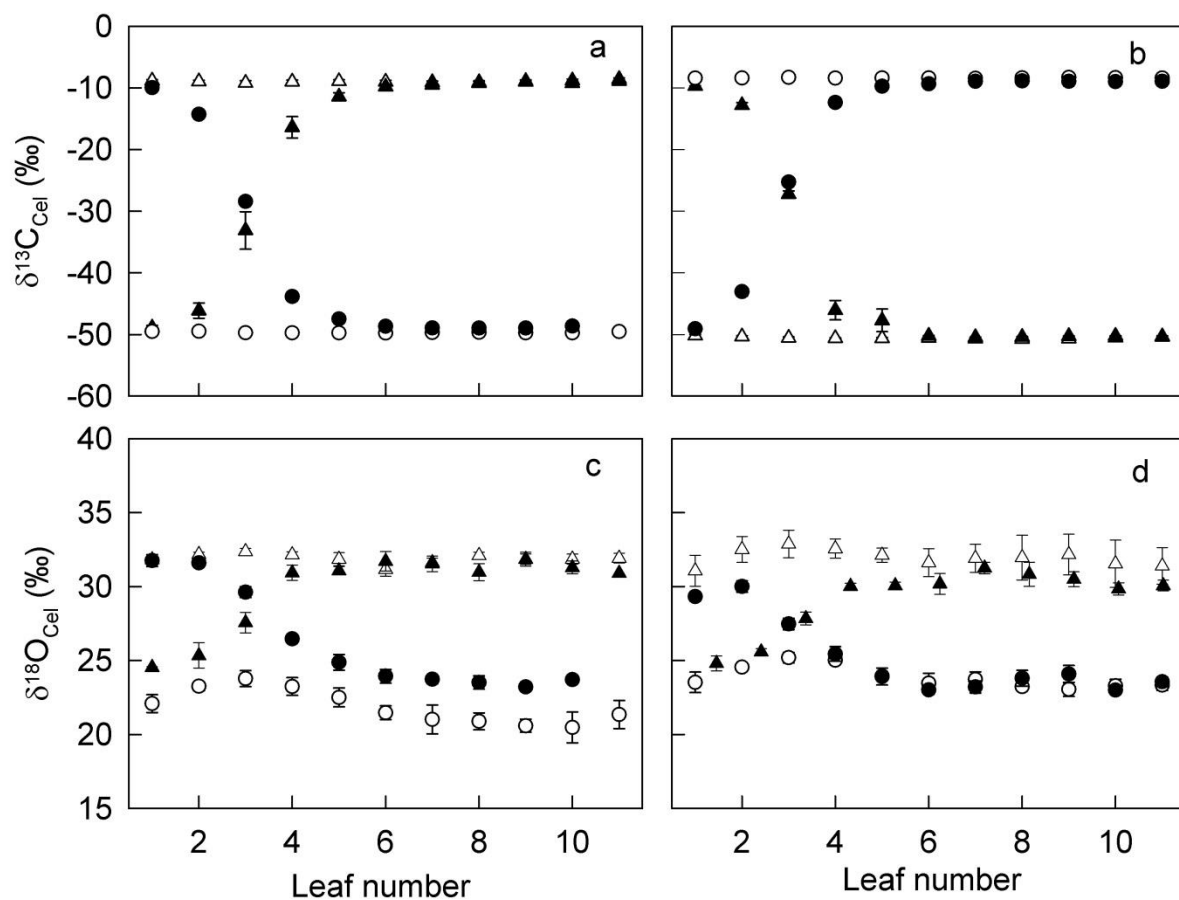
Fig. 3.4 Carbon isotope composition, $\delta^{13}C_{Cel}$ (a, b), and oxygen isotope composition (c, d) of cellulose, $\delta^{18}O_{Cel}$, in the different leaves formed along a tiller of *C. squarrosa*. Leaves were numbered from youngest to oldest (cf. **Fig. 3.2**). Plants were either kept constantly in the same environment (open symbols) or transferred from one VPD environment to the other (low to high VPD or high to low VPD) and kept in the new environment for 7 d before sampling (closed symbols). In all cases, the transfer of plants was associated with exposure to an altered $\delta^{13}C_{CO2}$ (see Materials and Methods). The experiment had two replications: 1st experiment (a, c), 2nd experiment (b, d). Symbols: constant low VPD (open circles); constant high VPD (open triangles); low to high VPD transfer (closed circles); high-to-low VPD transfer (closed triangles). In the 1st (2nd) experiment the low VPD chamber received ^{13}C-depleted CO_2 (^{13}C-enriched CO_2) and the high VPD chamber ^{13}C-enriched CO_2 (^{13}C-depleted CO_2). For details of treatments see **Fig. 3.1** and Materials and Methods. Values are means ± standard error ($n = 3$).

Carbon isotope composition

When plants were kept in constant conditions of VPD, the $\delta^{13}C_{Cel}$ of leaf blades did not exhibit any significant variation with leaf age ($P > 0.05$). That is the $\delta^{13}C_{Cel}$ of the

successively produced leaves was virtually constant (**Fig. 3.4a, b**). As expected, $\delta^{13}C_{Cel}$ was strongly influenced by $\delta^{13}C_{CO2}$. The contrast in $\delta^{13}C_{Cel}$ of plants kept in the presence of the ^{13}C-enriched and ^{13}C-depleted CO_2 source was 41.0‰ ($\pm$ 0.1‰ SD) in the 1st experiment (**Fig. 3.4a**) and 42.2‰ ($\pm$ 0.2‰ SD) in the 2nd (**Fig. 3.4b**). This contrast was largely independent of VPD, as $\Delta^{13}C_{Cel}$ differed little between VPD treatments (being 0.6 $\pm$ 0.1‰ SD higher at low relative to high VPD).

Where plants were transferred between environments with contrasting $\delta^{13}C_{CO2}$ (and VPD), and kept in the new environment for 7 d, the $\delta^{13}C_{Cel}$ depended strongly on developmental stage/age of the leaf. When leaves had stopped to expand prior to the transfer (mature leaves; no. 6 to 11; **Fig. 3.2** and **Table 3.1**), their $\delta^{13}C_{Cel}$ was virtually the same as that in plants kept in the old/original environment (**Fig. 3.4**). But, within the class of still growing (immature) leaves, the younger the leaf at time of the transfer the nearer was its $\delta^{13}C_{Cel}$ to that of the plants grown continuously in the new environment. For the youngest leaf of transferred plants, the $\delta^{13}C_{Cel}$ was almost the same as that of the corresponding leaves of plants kept constantly in the respective environment.

Oxygen isotope composition

As expected, continuous growth of plants at low and high VPD led to a different $\delta^{18}O_{Cel}$ of leaf blades. On average of all age classes, the $\delta^{18}O_{Cel}$ was 22.9‰ ($\pm$ 1.2‰ SD) at low VPD and 31.9‰ ($\pm$ 1.1‰ SD) at high VPD (**Fig. 3.4c** and **d**). At high VPD, the $\delta^{18}O_{Cel}$ was very similar in the two experiments. However, at low VPD, the $\delta^{18}O_{Cel}$ was lower in the 1st (**Fig. 3c**) than in the 2nd experiment (**Fig. 3.4d**), mainly due to the lower $\delta^{18}O_{Cel}$ of the older leaf blades (blades no. 6 to 11). In addition, $\delta^{18}O_{Cel}$ exhibited some apparently systematic age-related variation among the younger leaf age classes. Again, that effect was particularly evident at low VPD where the $\delta^{18}O_{Cel}$ of leaf blade no. 3 was 1.9‰ higher than that of the other leaves. That variation was important as it corresponded to 21% of the (average) $\delta^{18}O_{Cel}$-difference between low and high VPD.

Similar to $\delta^{13}C_{Cel}$, the $\delta^{18}O_{Cel}$ in leaf blades of transferred plants depended on developmental stage at transfer. Thus, within the class of still growing leaves, the younger the leaf the closer was its $\delta^{18}O_{Cel}$ to that of plants grown continuously in the respective environment. With older leaves that had stopped to expand before the transfer (that is leaves no. 6 through 11), $\delta^{18}O_{Cel}$ did not vary systematically as a function of age class. Also, the $\delta^{18}O_{Cel}$ of these leaves did not differ significantly from that of plants kept in the original environment throughout the experiment. This was true except for the $\delta^{18}O_{Cel}$ of the older leaves (no. 6 and older) in the low to high VPD transfer treatment in the 1st experiment, which

was 2.7‰ higher than that of same age leaves in the constant low VPD treatment in the same experimental run. We had already noted the particularly low $\delta^{18}O_{Cel}$ of leaves no. 6 to 11 in the constant low VPD treatment in the 1st experiment.
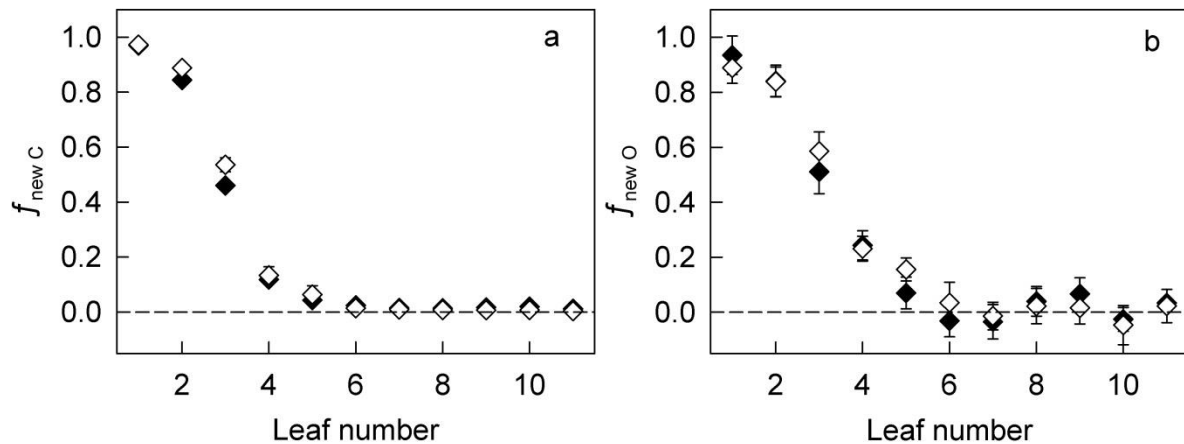
Fig. 3.5 Fractions of new carbon, $f_{new\ C}$ (a) and new oxygen, $f_{new\ O}$ (b) in cellulose of leaf blades of different ages in plants transferred from low to high VPD (closed diamonds) and from high to low VPD (open diamonds). Leaves are numbered from the youngest to the oldest (cf **Fig. 3.2**). Transferred plants were exposed to the new VPD environment for a period of 7 d before sampling. In all treatments, transferred plants were exposed to an altered $\delta^{13}C_{CO2}$ that provided for a differential labeling of all carbon assimilation in the new VPD environment (see Materials and Methods and **Fig. 3.1**). Values are presented as mean ± standard error (*n* = 6).

The fractions of new carbon and oxygen in cellulose of transferred plants

The developmental stage-dependent variations of $\delta^{13}C_{Cel}$ and $\delta^{18}O_{Cel}$ observed in plants transferred to a different (new) VPD and altered $\delta^{13}C_{CO2}$ were evaluated in terms of 'labeling kinetics', that is fractions of carbon ($f_{new\ C}$) and oxygen ($f_{new\ O}$) assimilated in the new environments and incorporated into leaf blade cellulose (**Fig. 3.5**).
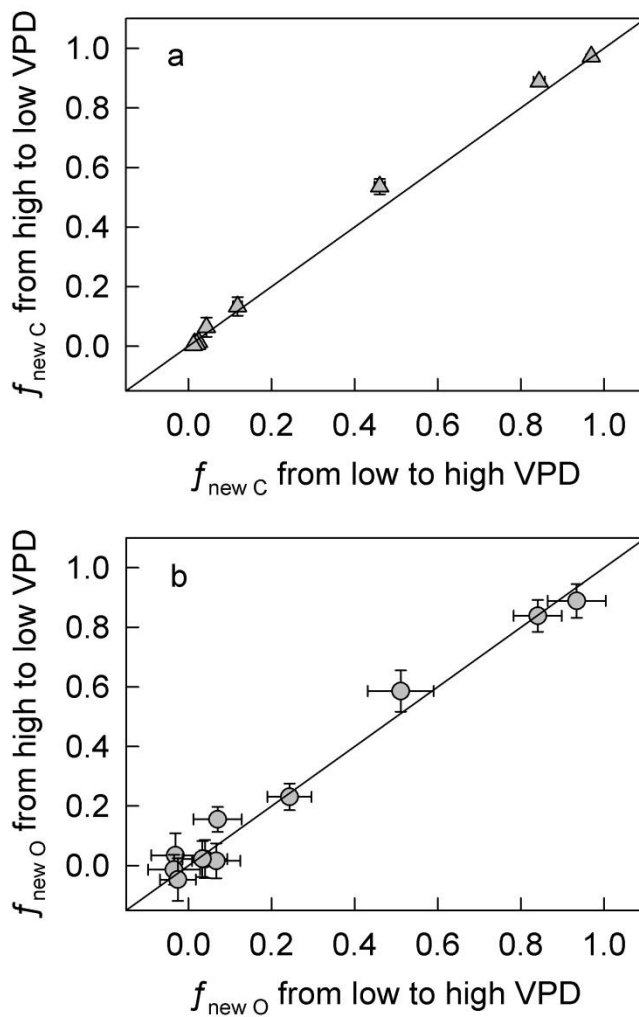
Fig. 3.6 (a) Fraction of new carbon ($f_{new\,C}$) in leaf blade cellulose of plants transferred from high to low VPD (y-axis) *versus* $f_{new\,C}$ in plants transferred from low to high VPD (x-axis). (b) Fraction of new oxygen ($f_{new\,O}$) in plants transferred from low to high VPD (x-axis) *versus* $f_{new\,O}$ in plants transferred from high to low VPD (y-axis). Data points compare $f_{new\,C}$ (or $f_{new\,O}$) for same age leaves. The solid lines give the 1:1 relationship. Data points show the mean ± standard error ($n = 6$). The 95% confidence intervals of slope and intercept in the linear relationship between $f_{new\,C}$ of high to low or low to high VPD both overlapped with 1 and 0, respectively. The same was true for $f_{new\,O}$.

The fractions of new carbon and oxygen revealed near-identical relationships with leaf age in the low to high and the high to low VPD transfer treatments (**Fig. 3.5** and **Fig. 3.6**). In leaves no. 6 and older, both $f_{new\,C}$ and $f_{new\,O}$ were not significantly different from 0. With leaf no. 5 and younger, both the $f_{new\,C}$ and $f_{new\,O}$ increased in a sigmoidal fashion with decreasing leaf age. In leaf no. 1, the leaf blade was virtually fully labeled with new carbon (i.e. $f_{new\,C}$ =

0.97 ±0.01 SE), while the estimated $f_{\text{new O}}$ averaged 0.91 (±0.02 SE). These relationships corresponded with the fraction of final leaf blade length that was already present at the time of the transfer (**Fig. 3.7**) and, hence, time since emergence of the leaf. Thus, the $f_{\text{new C}}$ and $f_{\text{new O}}$ decreased near-exponentially with the fraction of final blade length that was already present at the time of the transfer.

Although the general relationships of $f_{\text{new C}}$ and $f_{\text{new O}}$ with leaf age/developmental stage were similar, the $f_{\text{new O}}$ was 88% higher than $f_{\text{new C}}$ ($P < 0.05$) for leaf no. 4 (**Fig. 3.8** and **Fig. 3.9**).
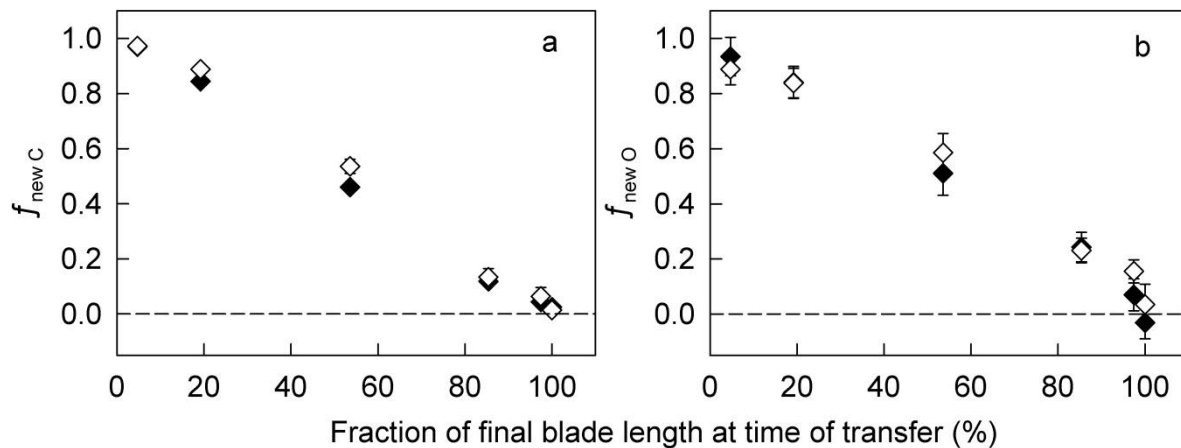
Fig. 3.7 Fractions of new carbon, $f_{\text{new C}}$ (a) and new oxygen, $f_{\text{new O}}$ (b) in cellulose of leaf blades of different ages in plants transferred from low to high VPD (closed diamonds) and from high to low VPD (open diamonds) as a function of the fraction of leaf blade length that was already attained at the time of transfer. Leaves are numbered from the youngest to the 6th old blade which wass already fully expanded at the time of transfer (cf. **Fig. 3.2** and **Table 3.1**). Transferred plants were exposed to the new VPD environment for a period of 7 d before sampling. Values are presented as mean ± standard error ($n = 6$). For further details see Materials and Methods, **Fig. 3.1** and **Fig. 3.4**.
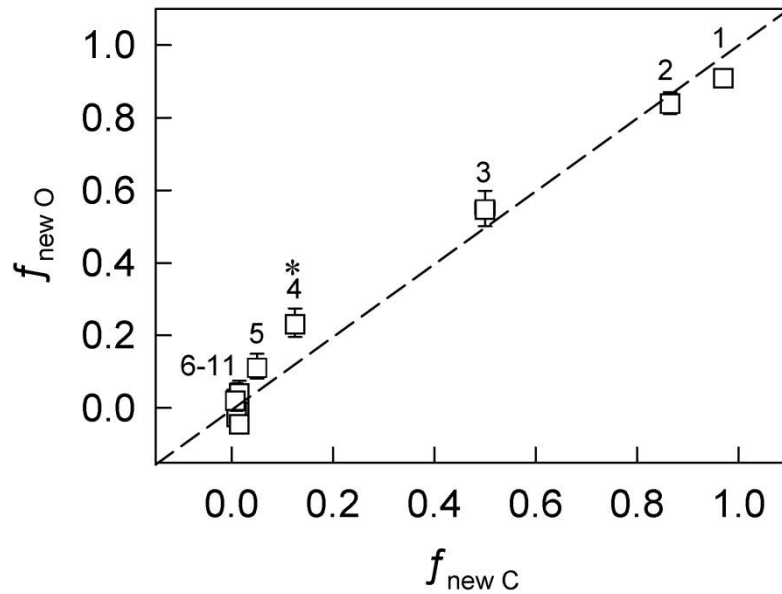
Fig. 3.8. Relationship between the fractions of new carbon ($f_{new\,C}$) and oxygen ($f_{new\,O}$) in cellulose of leaf blades of different age in plants. Data of $f_{new\,C}$ and $f_{new\,O}$ transferred from low to high VPD and from high to low VPD was combined together due to the similar general relationship between $f_{new\,C}$ and $f_{new\,O}$ with leaf age/developmental stage. Numbers refer to leaf age as indicated in **Fig. 3.2**, **Table 3.1**. * above data points indicates a significant difference between $f_{new\,C}$ and $f_{new\,O}$ of leaf no. 4 ($P < 0.05$), while there is no significant difference between $f_{new\,C}$ and $f_{new\,O}$ of other leaf categories. The dashed lines are 1:1 lines, and values are presented as means ± standard error ($n = 6$).
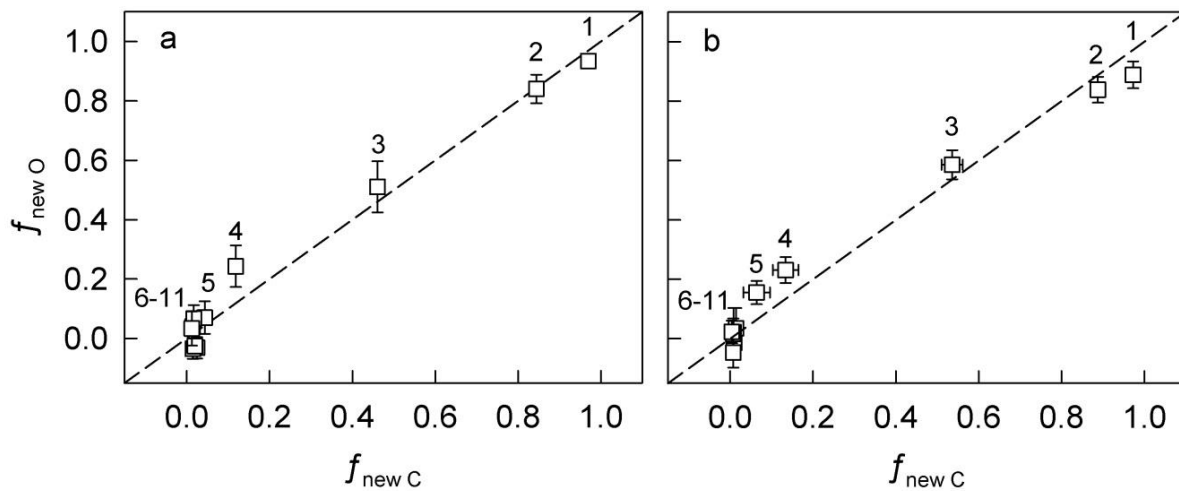
Fig. 3.9 Relationship between the fractions of new carbon ($f_{\text{new C}}$) and oxygen ($f_{\text{new O}}$) in cellulose of leaf blades of different age in plants transferred from low to high VPD (a) and from high to low VPD (b). Numbers refer to leaf age as indicated in **Fig. 3.2**, **Table 3.1**. The dashed lines are 1:1 lines, and values are presented as means ± standard error ($n = 6$).

DISCUSSION

The short-term VPD effect on cellulose-^{18}O of growing leaves is independent of VPD in the original growth environment

The transfer treatments – low to high and high to low VPD – did not differ in their effects on $f_{\text{new O}}$ (or $f_{\text{new C}}$) of the different age classes of growing leaves. This indicates that the $\delta^{18}O_{\text{Cel}}$ of cellulose synthesized after the transfer was determined entirely by the VPD in the new environment and, hence, was independent of any morpho-physiological traits acquired in the original growth environment. Thus the original growth environment seemed to have no significant after- or memory effects on ^{18}O-enrichment of leaf water and (consequently) carbohydrates in the new VPD environment and the propagation of the ^{18}O signal of carbohydrates to that of the newly synthesized cellulose. Principally, a memory effect may be expected if the original growth environment has some constitutive effect on stomatal conductance that would influence the posterior ^{18}O-enrichment of leaf water and hence carbohydrates when plant are exposed to a new VPD regime. However, in modeling the separate effects of VPD and stomatal conductance on $\delta^{18}O_{\text{Cel}}$, Barbour *et al.* (2002) found only a very minor effect of stomatal conductance when compared with that of VPD. Stomatal

conductance was 20% less in plants of *C. squarrosa* when grown (and measured) with a low VPD in the same experiment (Gong *et al.*, 2016), but that effect probably resulted mostly from the direct effect of VPD during measurement on stomatal conductance (Schulze, 1986; Monteith, 1995). Except for transpiration (+27% at high relative to low VPD), measurements of other morpho-physiological parameters indicated no or little effect of VPD (Gong *et al.*, 2016; Liu *et al.*, 2016): leaf area index of the canopies, plant growth rate and mass, leaf mass and dimensions (individual leaf area and thickness), and nitrogen per unit leaf area or mass were not affected by VPD in the original growth environment, while net photosynthesis was increased by 11% at low relative to high VPD. Additionally, the absence of aftereffects of prior growth conditions on the ^{13}C-labeling of cellulose ($f_{\text{new C}}$) in growing leaves supports the view that the two VPD transfer treatments had no differential effect on allocation of newly synthesized carbohydrates to leaf growth, the turnover rate and size of the relevant carbohydrate pools and carbohydrate metabolism associated with cellulose synthesis.

The temporal resolution of short-term VPD effects on whole-leaf cellulose-^{18}O

The temporal resolution of the oxygen isotope record represented by whole-leaf cellulose of successively formed leaf blades is influenced (i) by the participation of stores (which dampen and broaden the isotopic signal in the carbohydrate supply to cellulose synthesis), (ii) the leaf appearance interval (which dictates the rate of formation of the individual morphological units that resolve the temporal record), (iii) the duration of leaf expansion (that delimits the integration time of the isotopic signal of cellulose in primary cell walls at the whole leaf level) and (iv) the duration of post-expansion cell wall synthesis (that is related to post-expansion, continued secondary cell wall synthesis).

In general, one should expect that carbohydrate stores can supply substrate to cellulose synthesis during leaf growth. In the present work, a contribution of carbohydrate stores to cellulose synthesis is potentially detectable in transferred plants based on an increased ratio of $f_{\text{new O}}$ to $f_{\text{new C}}$. In the theoretical situation, that metabolism of storage-derived substrate involves no carbonyl oxygen formation, which is unlikely (Hill *et al.*, 1995; Barbour, 2007; Gessler *et al.*, 2014; Song *et al.*, 2014a), or that such formation occurs only in unenriched medium water, see chapter 4, the oxygen isotope composition of storage-derived metabolites is unaffected by a VPD change. However, if storage-derived metabolites exchange carbonyl oxygen with evaporatively enriched water (e.g. in source leaves), such exchange would generate an 'oxygen tag' or label in the new VPD environment, while the carbon isotope composition of the metabolite would remain unchanged, elevating the ratio of $f_{\text{new O}}$ to $f_{\text{new C}}$. In particular, one should expect to see such an effect in leaves that terminate cellulose

synthesis shortly after the VPD transition, when stored carbon still reflects the carbon isotope composition of the original growth environment. In agreement with such an expectation, we observed a significant divergence of the $f_{\text{new O}}$ to $f_{\text{new C}}$ ratio, supporting some role of stores in cellulose synthesis of leaf no. 4. In leaf no. 5, that effect was not statistically significant. Notably, however, such an effect was not evident for the younger leaves (the $f_{\text{new O}}$ to $f_{\text{new C}}$ ratio for leaves no. 1 to 3 was 1.01 ± 0.04 SE), indicating that stores supplying leaf growth must be turned over very rapidly by assimilates produced in the new environment. This conclusion is supported by findings with *Paspalum dilatatum*, where stored carbon contributed only 7% to the total carbon incorporation in leaf growth zones during undisturbed growth (Lattanzi *et al.*, 2005).

Cellulose synthesis ceased at or very shortly after termination of leaf blade expansion. That finding is consistent with studies of MacAdam & Nelson (2002) in *Festuca arundinacea* (Schreb.) that indicated a termination of secondary cell wall synthesis in leaf blade tissue at around the time of emergence of that part of blade tissue from the surrounding sheath of the next-older leaf. Thus virtually all cellulose synthesis in leaf tissue occurs while that part of tissue is fully enclosed within the surrounding sheath of the older leaf and non-transpiring, supporting the claim of Liu *et al*. (unpublished) that water in the leaf-growth-and-differentiation zone indeed represents the medium water for cellulose synthesis, also see chapter 4.

The dominant factor determining the temporal integration of the ^{18}O-signal in whole-leaf cellulose ($\delta^{18}O_{\text{Cel}}$) was the duration of leaf expansion, as we have shown (above) that stores likely contributed little to cellulose synthesis and cellulose synthesis was mainly associated with the expansion phase. This interpretation is in line with that of Helliker & Ehleringer (2002a) and is also consistent with the observation that the $\delta^{18}O_{\text{Cel}}$ of leaf no. 1 was essentially determined by assimilate produced concurrently with its growth during the 7 d-long exposure to an altered VPD. Leaves at more advanced growth stages at the time of transfer experienced divergent VPD conditions during the different phases of leaf growth, which must have led to divergent $\delta^{18}O_{\text{Cel}}$ in the leaf sections formed in the different phases as in Helliker & Ehleringer (2002a).

The phase of most-active leaf blade elongation lasted 6.6 d, with − on average − 2.8 leaves at different developmental stages present in that phase simultaneously. At the same time, our data indicate that $f_{\text{new O}}$ increased from 0.1 to 0.9 over a leaf number-interval of 3.4 (growing) leaves, which corresponds to a 8.2 d-long period (obtained by multiplying the leaf

number-interval by the 2.4 d-long leaf appearance interval). The same calculation indicated a period of 5.8 d for $f_{new\ C}$. These relationships all support the idea that similar to 80% of all oxygen and carbon incorporation into a leaf blade derived from current assimilation over a period of approx. 7 d, again supporting the view that leaf expansion duration was the single most important parameter determining the temporal resolution of the whole-leaf blade based $\delta^{18}O_{Cel}$ record presented by the sequential leaves produced by a tiller of *C. squarrosa*. That interpretation coincides with that of Wright & Leavitt (2006) who noted a fair correspondence between the duration of needle growth and the duration of cellulose synthesis in needles of *Pinus aizonica*.

Remarkably, the leaf appearance interval and leaf growth duration of grass species (and species of other taxa with continuous or 'succeeding' leaf production; e.g. (Kikuzawa, 1984; Schleip *et al*. 2013) can be predicted/modeled relatively well, as they are determined by growth-effective temperature, expressed in degree days above a certain base temperature GDD (the sum of daily average temperature above a certain base temperature), or other, more complex, functional relationships with (soil or air) temperature and other environemntal factors such as day length (Cao & Moss, 1994; Fournier & Andrieu, 1998; Fournier *et al.*, 2005). Also, these parameters can be verified by simple means including visual inspection (e.g. Yang *et al*., 2016). These findings all suggest that the ^{18}O record presented by the successive leaves formed along tillers of *C. squarrosa* (and probably other C_3 and C_4 grasses and dicotyledonous taxa) can be exploited as short-term isotopic chronologies. Such chronologies could be combined with analyses of ^{18}O in phytoliths (Webb & Longstaffe, 2006) and ^{13}C in biomass or cellulose of the same tissue (Köhler *et al.*, 2016) for comprehensive reconstructions of environmental conditions and physiological adaptations (Barbour 2007, Franks *et al.,* 2013) using plant tissue from e.g. herbaria (e.g. Peñuelas & Azcón-Bieto 1992, Bonal *et al.* 2011) or other artificial (e.g. Silvertown *et al.,* 2006) and natural archives such as bogs (Ménot-Combes *et al.,* 2002).

Chapter 4

$\delta^{18}O$ and δ^2H of water in the leaf growth-and-differentiation zone of grasses is close to source water in both humid and dry atmospheres

ABSTRACT

The oxygen and hydrogen isotope composition of water in the leaf growth and differentiation zone, LGDZ, ($\delta^{18}O_{LGDZ}$, δ^2H_{LGDZ}) of grasses influences the isotopic composition of leaf cellulose (oxygen) and wax (hydrogen) –important for understanding (paleo)environmental and physiological information contained in these biological archives–but is presently unknown. This work determined $\delta^{18}O_{LGDZ}$ and δ^2H_{LGDZ}, ^{18}O- and ^2H-enrichment of LGDZ ($\Delta^{18}O_{LGDZ}$ and Δ^2H_{LGDZ}), and the ^{18}O- and ^2H-enrichment of leaf blade water ($\Delta^{18}O_{LW}$, Δ^2H_{LW}) in two C_3 (*Triticum aestivum*, *Lolium multiflorum*) and three C_4 grasses (*Cleistogenes squarrosa*, *Pennisetum americanum*, *Panicum maximum*) grown at high and low vapor pressure deficit (VPD). The proportion of unenriched water (p_x) in the LGDZ ranged from 0.9 to 1.0 for ^{18}O and 1.0 to 1.2 for ^2H. VPD had no effect on the proportion of ^{18}O- and ^2H-enriched water in the LGDZ, and species effects were small or non-significant. Deuterium discrimination caused depletion of ^2H in LGDZ water, increasing (apparent) p_x-values >1.0 in some cases. Results explain why δ^2H of leaf cuticular wax in grasses has shown relatively little (or no) evaporative enrichment and indicate that the VPD effect on ^{18}O-enrichment of grass leaf cellulose may be more similar to tree rings than leaves of dicots.

INTRODUCTION

To date, the oxygen and hydrogen isotope composition of water in the leaf growth and differentiation zone (LGDZ) of grasses ($\delta^{18}O_{LGDZ}$ and $\delta^{2}H_{LGDZ}$) is unknown (Liu *et al.*, 2016). Knowledge of $\delta^{18}O_{LGDZ}$ and $\delta^{2}H_{LGDZ}$ is, however, necessary for understanding the biophysical and biochemical mechanisms determining the oxygen isotope composition of leaf cellulose ($\delta^{18}O_{Cellulose}$) and the hydrogen isotope composition of leaf cuticular wax ($\delta^{2}H_{Wax}$), which are thought to store important (paleo)climatological and environmental, geographic, and physiological information (Barbour & Farquhar, 2000; Helliker & Ehleringer, 2002a,b; Kahmen *et al.*, 2013). The effect of $\delta^{18}O_{LGDZ}$ on $\delta^{18}O_{Cellulose}$ and of $\delta^{2}H_{LGDZ}$ on $\delta^{2}H_{Wax}$, respectively, derives from the fact that they represent the medium or biosynthetic waters at the sites of cellulose and wax synthesis in the LGDZ, providing oxygen and hydrogen atoms for metabolic intermediates of cellulose and wax synthesis (Barbour, 2007; Sachse *et al.*, 2012; Zhou *et al.*, 2016).

The LGDZ of grass leaves (**Fig. 4.1**) is enclosed within a whorl of encircling sheaths of older leaves (Kemp, 1980; Volenec & Nelson, 1981). It contains zones of cell production/division, expansion and differentiation/maturation that are organized along a spatial gradient from the base of the growing leaf, near the point of attachment of the leaf to the stem, to the point where leaf tissue emerges from the enclosing sheath (Schnyder *et al.*, 1990; Skinner & Nelson, 1995; Tardieu *et al.*, 2000; Rademacher & Nelson, 2001; Kavanová *et al.*, 2006). The LGDZ is entirely heterotrophic, that is fully dependent on assimilate supply from other parts of the plant, including the exposed part of the growing leaf itself (Allard & Nelson, 1991). Tissue within the growth zone is displaced away from the leaf base as a result of production and expansion of younger cells at more basal positions in the leaf growth zone (Schnyder *et al.*, 1990). When it emerges into light, the leaf tissue is virtually fully differentiated and photosynthetically competent (Wilhelm & Nelson, 1978; Allard & Nelson, 1991; MacAdam & Nelson, 2002). Cell wall material, which includes cellulose, is synthesized throughout the LGDZ during processes of primary and secondary cell wall formation (MacAdam & Nelson, 1987, 2002). Cuticular wax is deposited in the distal half to two-thirds of the LGDZ (that is the distal part of the cell elongation zone and the differentiation zone), but may continue beyond the point where tissue emerges into light (Richardson *et al.,* 2005, 2007).

The principal unsolved question with regard to the water isotope composition of the LGDZ is whether it is evaporatively enriched above source water for ^{18}O and ^{2}H ($\Delta^{18}O_{LGDZ}$ and $\Delta^{2}H_{LGDZ}$). In general, evaporative ^{18}O- and ^{2}H-enrichment occurs at the evaporative site

in stomatal cavities (Dongmann *et al.*, 1974; Flanagan *et al.*, 1991a) of light exposed leaf tissue and propagates through leaves by diffusion of the enriched water away from the evaporative sites, against the convective flux of water to the stomata (Farquhar & Lloyd, 1993; Farquhar & Gan, 2003). Although it has not been analyzed directly, there have been notions on the $\delta^{18}O$ and δ^2H of medium and synthesis waters for grass leaf cellulose and cuticular wax synthesis. These ideas root primarily in the seminal works of Helliker & Ehleringer (2000) and Helliker & Ehleringer (2002a,b), which analyzed $\delta^{18}O$ of cellulose and distinct water pools in C_3 and C_4 grass leaf blades. From the $\delta^{18}O$ of cellulose in leaf blades, source water and leaf water, and assumptions on the proportion of oxygen exchange of metabolic intermediates of cellulose synthesis with medium water at the site of cellulose synthesis (p_{ex}) of different C_3 and C_4 grasses, along with knowledge of the $\delta^{18}O$ of water in the most basal portion of leaf blades of *Lolium multiflorum*, they concluded that the proportion of unenriched water (that is source water) at the site of leaf blade cellulose synthesis ($p_{x\,18O}$) ranged between 50 and 62% in a number of C_3 and C_4 grass species exposed to different atmospheric humidities. That work also influenced views on the δ^2H of medium or synthesis waters for grass leaf cuticular wax formation (McInerney *et al.*, 2011; Sachse *et al.*, 2012; Kahmen *et al.*, 2013; Gamarra & Kahmen, 2016; Gamarra *et al.*, 2016). However, recent work suggested that p_{ex} for oxygen in cellulose synthesis may vary in a greater range than previously assumed (Song *et al.*, 2014a). Also, the leaf base tissue water sampled by Helliker & Ehleringer (2002a) was perhaps not reflective of the water isotope composition of the LGDZ (**Fig. 4.1**; see also Fig. 1 in Helliker & Ehleringer, 2002b). As the LGDZ is fully enclosed, it was thought that it is non-transpiring and that evaporatively enriched water in the LGDZ stems from diffusion from adjacent transpiring tissue or import of water *via* the phloem (Gamarra *et al.*, 2016; Liu *et al.*, 2016).

To shed light on this knowledge gap, this study investigated the oxygen and hydrogen isotope composition of tissue water in the LGDZ ($\delta^{18}O_{LGDZ}$ and δ^2H_{LGDZ}), and the ^{18}O- and ^2H-enrichment of LGDZ water above source water ($\Delta^{18}O_{LGDZ}$ and Δ^2H_{LGDZ}) along with the ^{18}O- and ^2H-enrichment of leaf blade water ($\Delta^{18}O_{LW}$, Δ^2H_{LW}) in grasses. Experiments were performed with C_3 and C_4 grasses grown at constant low (0.63 kPa) and high (1.58 kPa) vapor pressure deficit (VPD). We show that water isotope composition of the LGDZ is much closer to that of source water than has been assumed previously, and is unaffected by VPD.

Fig. 4.1 Schematic illustration of a vegetative grass tiller (right hand side), composed of a growing leaf and three fully expanded leaves of increasing age (1-3). The basal part of the tiller is composed of a whorl of encircling leaf sheaths (sometimes termed 'pseudostem') that enclose the leaf growth and differentiation zone (LGDZ) of the growing leaf (shown on the left hand side). The LGDZ includes the successive zones of cell division, expansion and differentiation (also see Fig. 5.14 in MacAdam, 2009). The exposed part of the growing leaf is fully differentiated and photosynthetically competent. Leaf growth begins with the tip of the blade (lamina) and terminates with the basal portion of the sheath. Throughout the growth of a leaf, the LGDZ remains at the base of the leaf and, hence, is always fully enclosed by the sheath of the next older leaf (Schnyder *et al.*, 1990). The blade-sheath interface, also termed 'joint' (see arrow) or 'collar', often contains a thin outgrowth (termed 'ligule') that surrounds the basal exposed part of the growing leaf (not shown). Leaf elongation stops at the time when the leaf blade bends away from the vertical as a result of differential growth in the joint region. Helliker & Ehleringer (2002a) sampled water from the basal part of the leaf blade (see ellipse in **Fig. 4.1**, and Fig. 1 of Helliker & Ehleringer, 2002b), while Barnard *et al.* (2006) collected tissue water from the pseudostem of *Zea mays*, *Dactylis glomerata* and *Lolium perenne*, after removal of the outermost leaf sheath (cf. dash-lined box). From Schnyder (unpublished).

MATERIALS AND METHODS

Plant material and growth conditions

The experiment was performed with two C_3 grasses (*Triticum aestivum* and *Lolium multiflorum*) and three C_4 grasses (*Cleistogenes squarrosa*, *Pennisetum americanum* and *Panicum maximum*). All species were grown at low and high VPD (see below) in two growth chambers (Conviron PGR15, Conviron, Winnipeg, Canada). Plants were established from seed, with four to six seeds of a given species sown in individual pots (4.5 cm diameter, 35 cm deep) filled with quartz sand (0.3-0.8 mm diameter). Pots were placed in four free-draining plastic containers (length: 77 cm, width: 57 cm, depth: 30 cm) and two containers placed in each growth chamber. In each chamber, one container held the pots with *C. squarrosa* (n = 164) while the other species were arranged in the other container (*P. americanum*, 15; *P. maximum*, 15; *T. aestivum*, 20; *L. multiflorum*, 25). The time of first watering is referred to as imbibition of seeds and denotes the start of the experiment. Before germination, the conditions in both chambers were kept the same with a relative humidity of 80% at a temperature of 25 $^{\circ}$C (providing for a VPD of 0.63 kPa). After one week, seedlings were thinned to one plant per pot. At the same date, a VPD of 1.58 kPa (high VPD) was set in one chamber by lowering relative humidity to 50%. Meanwhile a VPD of 0.63 kPa (low VPD) was maintained in the other. All other growth conditions were identical in the two chambers: a photosynthetic photon flux density (PPFD) of 800 μmol m^{-2} s^{-1} at the height of the container was supplied by cool white fluorescent tubes during 16 h-long light periods. A modified Hoagland nutrient solution, that corresponded to the high N solution described by Liu *et al*. (2016), was supplied to each container, and maintained approx. 7 cm deep in each container by adding new nutrient solution every two days until day 30; thereafter nutrient solution was added once daily.

Source water sampling

The nutrient solution in the different containers was sampled at 8 h into the light period, at intervals of one or two days, just before addition of new nutrient solution. In addition, we sampled water from individual pots at the time of sampling plants from one of the containers in each chamber. Samples were collected with a pipette, placed in *12 mL Exetainer* vials (*Labco* Ltd, High Wycombe, UK), vials were sealed with Parafilm (PM996, Bemis Company, Wisconsin, USA) and stored in a freezer at −20 °C.

Sampling and extraction of LGDZ and leaf water

Samples for water extraction from the LGDZ and leaf blades were collected between 32 and 35 days after the start of the experiment for *T. aestivum*, *L. multiflorum*, *P. americanum* and *P. maximum*. *C. squarrosa* was growing more slowly, mainly because of the small seed, and was sampled between 42 and 44 d. Sampling started at 5 h after the beginning of light periods. At the time of sampling, the number of major tillers (with at least two fully expanded leaf blades per tiller) per plant was: *C. squarrosa*, 5-8 tillers; *P. americanum*, 1; *P. maximum*, 5-7, *T. aestivum*, 3-5; and *L. multiflorum*, 4-5. Six replicates were sampled for each species by VPD combination.

For leaf water samples, one replicate consisted of one plant, from which the youngest fully expanded leaves (from the first to the third or fourth blade) from mature tillers were combined in one sample. As in the comparative analysis of C_3 and C_4 grass species of Helliker & Ehleringer (2002b), the mid vein was not removed in this study. The total number of leaf blades in a sample depended on leaf size: *C. squarrosa*, ~15; *P. maximum*, 8; *T. aestivum* 3-5; *L. multiflorum*, 3-5; *P. americanum*, 1. Sampling was done swiftly and sampled leaves were immediately sealed in a *12 mL Exetainer* vial, capped and then wrapped with Parafilm.

For LGDZ water, we combined LGDZ tissue from several plants in one sample (*C. squarrosa*, 4 plants; *P. americanum*, 3; *P. maximum*, 2; *T. aestivum*, 2; *L. multiflorum*, 4). The total number of LGDZ combined in on sample was: *C. squarrosa*, 36; *P. americanum*, 3; *P. maximum*, 14; *T. aestivum*, 10; *L. multiflorum*, 16. Growing leaves were identified as the youngest leaf emerging from the surrounding sheath of the youngest fully-expanded leaf (**Fig. 4.1**). The LGDZ was excised quickly following removal of the enclosing sheath of the next-older leaf, by cutting at the base (the point of attachment to the tiller axis) and ~3-5 mm below the point of emergence from the surrounding sheath of the next-older leaf (**Fig. 4.1**). Excised LGDZ tissue was immediately sealed in a 12 mL Exetainer vial, as above. In a small proportion of cases the LGDZ contained growing sheath tissue. In those instances the LGDZ was cut at the ligule, recognizable as a faint whitish band around the LGDZ tissue (cf. Schnyder *et al.,* 1990).

All vials were weighed before and after filling with samples. Samples were stored in a freezer at −20 °C until cryogenic extraction of water. Water was extracted as in Liu *et al.* (2016).

Isotope analysis

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

$$\delta^{18}O \text{ or } \delta^2H = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \times 1000, \hspace{2cm} \text{Eqn 4.1}$$

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

The detailed method of liquid water isotope measurements was described by Liu *et al.* (2016). In brief, $\delta^{18}O$ and δ^2H of water samples from LGDZ, leaf blades, nutrient solution, and pot and tap water were analyzed on 300 μL aliquots using a Cavity Ring-Down Spectroscopy Analyzer (L2110-i), coupled to an A0211 high precision vaporizer set at 110 °C (both: Picarro Inc., Sunnyvale, Ca, USA). After every 20-25 samples, two laboratory water standards were analyzed, spanning the range of the isotopic compositions of samples ($\delta^{18}O$ +14.2‰ and −23.0‰, δ^2H +121.1‰ and −164.0‰, respectively) for possible drift correction and normalizing results to the SMOW-scale. Standards were previously calibrated against V-SMOW, V-GISP and V-SLAP using the same analytical procedure. Analytical uncertainty (the SD for repeated measurements) for $\delta^{18}O$ and δ^2H was ± 0.1 ‰ and 1.0‰, respectively. In a preliminary study, we compared the spectroscopy-based measurements with pyrolysis-based measurements performed by use of a TC/EA HTC coupled to a Delta^{Plus} XL isotope ratio mass spectrometer (both from Finnigan MAT, Bremen, Germany). The samples included in that comparison spanned the entire range of sample types collected in the present work, and provided virtually identical data (within analytical error) for $\delta^{18}O$ and δ^2H.

^{18}O or ^{2}H enrichment of samples above source water and the fraction of unenriched water in the LGDZ

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

$$\Delta S = (\delta S - \delta^{18}O_{SW})/(1 + \delta^{18}O_{SW}/1000), \hspace{2cm} \text{Eqn 4.2}$$

where *S* denotes LGDZ or leaf water, as appropriate, and *SW* refers to the oxygen isotope composition of source water, that is the nutrient solution sampled from the containers in which the plants were grown. ^2H-enrichment was calculated accordingly.

The fraction of ^{18}O-unenriched water (that is source water) in the LGDZ, $p_{x\ 18O}$, was calculated as:

$$p_{x\ 18O} = (\delta^{18}O_{LGDZ} - \delta^{18}O_{LW})/(\delta^{18}O_{SW} - \delta^{18}O_{LW}), \hspace{1cm} \text{Eqn 4.3a}$$

which is equivalent to

$$p_{x\ 18O} = 1 - \Delta^{18}O_{LGDZ}/\Delta^{18}O_{LW}. \hspace{2cm} \text{Eqn 4.3b}$$

The fraction of ^2H-unenriched water in the LGDZ, $p_{x\ 2H}$, was obtained accordingly.

Statistical analysis

The values of ^{18}O and ^2H enrichment of LGDZ and leaf water, and $p_{x\ 18O}$ and $p_{x\ 2H}$ are presented as mean ± 95% confidence intervals. For these parameters, the standard deviation was estimated by error propagation that considered the standard deviation of each parameter on the right side of Eqns 4.3 and 4. 4a according to the method of Phillips & Gregg (2001). The significance of differences between any two means of given VPD by species combinations was tested by comparing the means of one member with the 95% confidence interval of the other. If the mean of one member was included in the 95% confidence intervals of the other, the difference was considered non-significant ($P > 0.05$).

RESULTS AND DISCUSSION

Source water

The $\delta^{18}O$ and δ^2H of the nutrient solution ($\delta^{18}O_{SW}$ and δ^2H_{SW}) sampled from containers holding pots with plants was constant with time ($P > 0.05$) and did not differ significantly from that of the tap water used to generate the nutrient solution or the water collected from pots at times of plant sampling (**Table 4.1**). This was true for both VPD treatments, except for the $\delta^{18}O_{SW}$ and δ^2H_{SW} in the container holding pots with *P. americanum, P. maximum, T. aestivum, L. multiflorum* in the high VPD treatment, which revealed some evaporative enrichment of both the nutrient solution and the water collected from the pots (+2.4‰ for ^{18}O and +7.7‰ for ^2H, relative to the container that held plants of *C. squarrosa*). This effect was related to the fact that this container was not filled completely with pots, so that approx. 40% of the container base area was directly exposed to light.

Leaf water

Leaf water was always enriched in ^{18}O and ^2H relative to source water (**Fig. 4.2a**, **d**). The enrichment was enhanced by VPD and differed between some of the species for both $\Delta^{18}O_{LW}$ and Δ^2H_{LW} (**Fig. 4.2a**, **d**). Thus, *C. squarrosa* presented a greater ^{18}O and ^2H enrichment than the other species. Conversely, *P. americanum* showed a lower ^{18}O and ^2H enrichment than most of the other species. The latter was likely related to the (much more) prominent mid vein, and associated greater proportion of unenriched water in bulk leaf water, in *P. americanum*.

At low VPD, $\Delta^{18}O_{LW}$ ranged between 7.2‰ and 11.5‰, while Δ^2H_{LW} varied between 16.8‰ and 30.2‰ in the different species. In all species, the enrichment was greater at high VPD, with $\Delta^{18}O_{LW}$ varying between 11.3‰ and 18.3‰, and Δ^2H_{LW} between 25.5‰ and 36.0‰. As the number of species in the C_3 and C_4 groups was small, we did not test for respective group effects. Nevertheless, there were no obvious differences between groups, and

observed effects of species and VPD on $\Delta^{18}O_{LW}$ and Δ^2H_{LW} were similar to those observed and discussed by others (Helliker & Ehleringer, 2002b; McInerney *et al.*, 2011; Kahmen *et al.*, 2013), supporting the idea that the experimental setting was representative of the experimental scenarios used by others.

Is LGDZ water evaporatively enriched in ^{18}O and ^2H?

The $\Delta^{18}O_{LGDZ}$ and Δ^2H_{LGDZ} was not influenced by VPD or species (**Fig. 4.2b** and **e**). This was true, except for $\Delta^{18}O_{LGDZ}$ of *C. squarrosa* at high VPD, which was slightly greater than that of the other species. Notably, $\Delta^{18}O_{LGDZ}$ and Δ^2H_{LGDZ} was very small and often not significantly different from 0, particularly for Δ^2H_{LGDZ}. On average of all species and both VPD levels, $\Delta^{18}O_{LGDZ}$ was 0.6‰ and Δ^2H_{LGDZ} was −1.6‰. In *C. squarrosa* and *P. maximum*, $\Delta^{18}O_{LGDZ}$ was significantly higher than zero at both VPD levels, while such a significant difference occurred only at the high VPD level in the other species. Meanwhile, Δ^2H_{LGDZ} was not significantly different from zero, except in *L. multiflorum* (at both low and high VPD) and *T. aestivum* (low VPD).

The fractional contribution of unenriched water to total LGDZ water ($p_{x\,18O}$ and $p_{x\,2H}$) ranged between 0.9 and 1.0 for ^{18}O, and between 1.0 and 1.2 for ^2H (**Fig. 4.2 c** and **f**). In none of the species did we observe a significant VPD effect on $p_{x\,18O}$ or $p_{x\,2H}$. Also, species did generally not differ systematically in $p_{x\,18O}$ or $p_{x\,2H}$. As an exception, $p_{x\,18O}$ of *C. squarrosa* was slightly lower than that of *P. maximum*, *T. aestivum* and *L. multiflorum* (for both VPD levels) and *P. americanum* for high VPD. For $p_{x\,18O}$, the 95% confidence interval of *L. multiflorum* was not significantly different from 1 under both low and high VPD. Similarly, the $p_{x\,18O}$ of *T. aestivum* did not differ from 1 at low VPD. In all other cases, including the C_4 species at both VPD levels, $p_{x\,18O}$ was significantly smaller than 1, although none of the values were smaller than 0.9. Meanwhile, $p_{x\,2H}$ differed between none of the species at low VPD. Also, it did not differ between *P. americanum*, *T. aestivum* and *L. multiflorum* at high VPD. As an exception, the $p_{x\,2H}$ of *L. multiflorum* at low VPD was slightly higher than that of *C. squarrosa* and *P. maximum* at high VPD. In *L. multiflorum* and *T. aestivum*, $p_{x\,2H}$ was greater than 1 at both VPD levels, while $p_{x\,2H}$ did not differ from 1 in the three C_4 species.

Table 4.1 $\delta^{18}O$ and δ^2H of tap water, nutrient solution water, water collected from pots in containers, and atmospheric water vapor inside plant growth chambers at low and high VPD. Each chamber held two containers: *C. squarrosa* was placed in container 1 (low VPD) and 3 (high VPD); *T. aestivum*, *L. multiflorum*, *P. americanum* and *P. maximum* were held in container 2 (low VPD) and 4 (high VPD). Nutrient solution in the containers was sampled at 8 hours after the beginning of light periods every one or two days before adding new nutrient solution ($n = 7\text{-}16$ in the different containers). Water inside pots was collected when sampling plants from containers 1 and 4 ($n = 4$). The isotopic composition of vapor did not change over time ($P > 0.05$). Values are presented as mean ± standard deviation; n.d. not determined.

	Low VPD		High VPD	
	Container 1	Container 2	Container 3	Container 4
Tap water				
$\delta^{18}O$		−10.4 ± 0.2		
δ^2H		−70.1 ± 0.6		
Nutrient solution				
$\delta^{18}O$	−10.2 ± 0.1	−9.9 ± 0.3	−10.1 ± 0.2	−7.9 ± 0.2
δ^2H	−71.9 ± 0.9	−71.7 ± 1.0	−71.5 ± 0.9	−65.6 ± 1.2
Water inside pots				
$\delta^{18}O$	−10.0 ± 0.1	n.d.	n.d.	−7.7 ± 0.4
δ^2H	−71.0 ± 0.9	n.d.	n.d.	−63.8 ± 1.2
Vapor				
$\delta^{18}O$	−18.1 ± 0.2		−18.2 ± 0.2	
δ^2H	−144.5 ± 0.6		−144.6 ± 0.7	
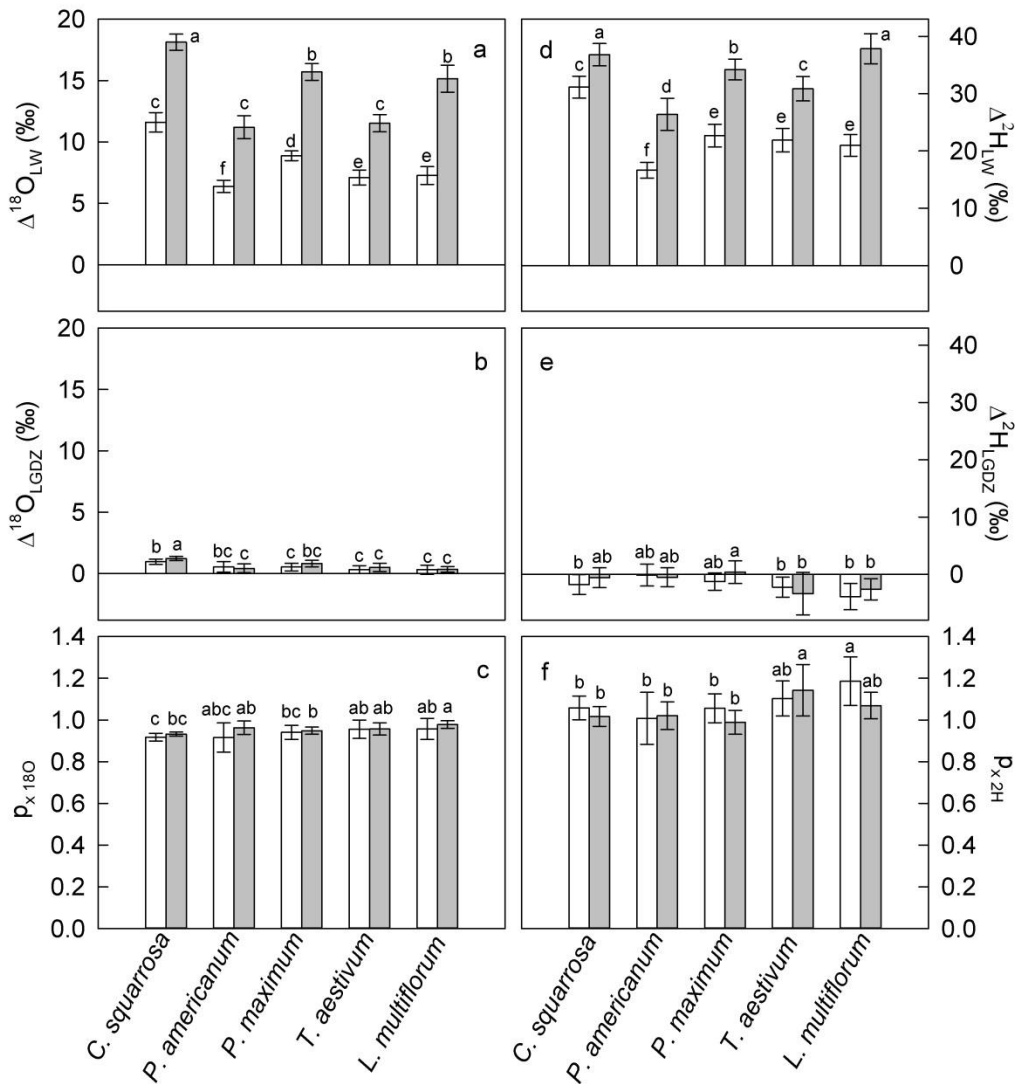
Fig. 4.2 (a) ^{18}O-enrichment of bulk leaf blade water ($\Delta^{18}O_{LW}$), (b) ^{18}O-enrichment of water in the leaf growth and differentiation zone, LGDZ, ($\Delta^{18}O_{LGDZ}$), (c) proportion of ^{18}O-unenriched water in the LGDZ ($p_{x\ 18O}$), (d) ^2H-enrichment of bulk leaf blade water (Δ^2H_{LW}), (e) ^2H-enrichment of water in the LGDZ (Δ^2H_{LGDZ}), and (f) proportion of ^2H-unenriched water in the LGDZ ($p_{x\ 2H}$) in three C_4 (*C. squarrosa*, *P. americanum*, *P. maximum*) and two C_3 grass species (*T. aestivum*, *L. multiflorum*) grown at low VPD (empty bar) or high VPD (grey bar). Values represent means ± 95% confidence interval ($n = 6$).

The observation of a very small and often non-significant evaporative ^{18}O- and ^2H-enrichment of LGDZ water in several C_3 and C_4 grasses grown at low and high VPD modifies previous notions on the ^{18}O and ^2H composition of medium water at the sites of cellulose and

surface wax synthesis in the LGDZ of grasses (e.g. Barbour, 2007; Kahmen *et al.,* 2013). The data also indicate that the evaporative enrichment of tissue water in the basal 5 mm of leaf blades of *L. multiflorum* observed by Helliker & Ehleringer (2002a) is not representative for water in the LGDZ. That difference was likely related to the fact that the leaf base tissue (cf. the 'intercalary meristem' in Fig. 1 of Helliker & Ehleringer, 2002b) was sampled when the respective tissue was already exposed to the atmospheric environment. Clearly, the results of the present work (average $p_{x\ 18O}$ 0.95) contrast markedly with the $p_{x\ 18O}$ of 0.50 to 0.62 reported by Helliker & Ehleringer (2002a, b). Interestingly and significantly, our observed $\Delta^{18}O_{LGDZ}$ was very close to the $\Delta^{18}O$ of pseudostem water reported by Barnard *et al.* (2006), who analyzed water extracted from the pseudostem of one C_4 (*Zea mays*) and two C_3 grasses (*Dactylis glomerata* and *Lolium perenne*), following removal of the outermost leaf sheath (cf. **Fig. 4.1**). Being fully enclosed by the sheath of the outermost mature leaf, the physical environment of the enclosed sheaths is very similar to that of the LGDZ (which was actually sampled as a part of the pseudostem by Barnard *et al.,* 2006). In the present investigations, each LGDZ was enclosed in a whorl of 2-5 sheaths, depending on species. Yet, the number of sheaths did not show a relationship with $\Delta^{18}O_{LGDZ}$ or $p_{x\ 18O}$, suggesting that a small number of sheaths provided an effective protection against evaporative enrichment of LGDZ water.

The close similarity of the isotopic composition of source water and LGDZ indicates that LGDZ water was very similar to xylem water. However, the supply of growth zones with assimilates must involve a considerable influx of phloem sap, which – coming from source leaves – is originally enriched relative to xylem water (Cernusak *et al.*, 2002). However, the close vicinity of xylem and phloem elements leads to an exchange of water along the transport path (McCully & Mallett, 1993; Sevanto, 2014) effectively quenching the original phloem sap ^{18}O signal by that of xylem water (Cernusak *et al.*, 2005).

Remarkably, $p_{x\ 2H}$ was significantly greater than 1 in *L. multiflorum* and *T. aestivum*. Alternative definitions of source water, either by designating the tap water or water sampled from pots as source water, did not change the statistical significance of this result for these C_3 grasses. Also, $p_{x\ 2H}$ tended to be greater than 1 in the C_4 grasses, although that difference was not significant. That effect cannot be explained by a simple mixing of non-enriched and enriched water pools, and must have resulted from discrimination of ^2H in LGDZ water. If we assumed that the real $p_{x\ 2H}$ was identical with $p_{x\ 18O}$, the discrepancy between estimated/modelled and observed Δ^2H_{LGDZ} suggested an average ^2H discrimination of 2.9‰ (independent of VPD). Depletion of ^2H in root tissue water of halophitic plants was previously observed by Lin *et al.* (1993). Recently, Zhao *et al.* (2016) found a significant

depletion of ^{2}H in tissue water relative to xylem or source water of *Populus eupratica*, and discussed that effect in relation to ^{2}H discrimination during water transport through aquaporins (Mamonov *et al.*, 2007) and in metabolism (e.g. Yakir, 1992; Roden *et al.,* 2000). Further, Chen *et al.* (2016) reported a strong ^{2}H depletion (and a minor ^{18}O depletion) of the surface water layer in a wide variety of hydrophilic organic materials. That effect was not related to transport or metabolic phenomena (as it was observed in metabolically inert materials), or effects of solutes, equilibration time, incomplete extraction, or exchange processes with the materials, and appeared to be a genuine effect of the (hydrophilic) surface on the isotopic composition of the adsorbing surface water layer. Although we are presently unable to identify the specific biophysical mechanism(s) underlying the ^{2}H depletion of LGDZ water, it is notable that the LGDZ is a highly metabolically active tissue. Thus, during active leaf elongation, total LGDZ biomass is turned over in a matter of a few days, and the relative growth rate of tissue at the position of most active cell expansion is in the order of 6 to 8% h^{-1} (e.g. Schnyder & Nelson, 1987; Schnyder *et al.,* 1990). Also, there is a marked difference between C_3 and C_4 grasses in LGDZ metabolism: expanding leaf cells of C_3 grasses store large concentrations of fructan that is subsequently used for structural biomass synthesis during the differentiation phase of leaf tissue (Schnyder & Nelson 1987, 1989; Schnyder *et al.,* 1988); conversely, fructan metabolism is absent in C_4 grasses (Pollock & Cairns, 1991). It remains to be investigated whether differences in carbohydrate metabolism in LGDZ of C_3 and C_4 grasses could contribute to a difference in ^{2}H depletion of LGDZ water between the two metabolic groups.

CONCLUSION AND IMPLICATIONS

As they provide the first direct determinations of the isotopic composition of water in the LGDZ of grasses, the present results are immediately relevant to the interpretation of reports of the δ^2H of cuticular wax or the δ^{18}O of cellulose in grass leaves, as these compounds are predominantly (wax, see below) or virtually exclusively (cellulose) synthesized inside the LGDZ.

If we use a $p_{x\,18O}$ of 0.94 for *C. squarrosa* (this work), the mean $p_{ex\,18O}$ (proportion of oxygen in cellulose that has exchanged with LGDZ water) was 0.44 in the work of Liu *et al.* (2016), closely matching the most commonly accepted plausible range of 0.4 to 0.5 (Cernusak *et al.*, 2005; Barbour, 2007). Also, the data explain why Hou *et al.* (2008) and McInerney *et al.* (2011) found no effect of atmospheric humidity on ^{2}H enrichment of leaf wax *n*-alkanes of grasses, and why the enrichment is much greater in dicot leaves (Kahmen *et al.*, 2013), which expand while being directly exposed to the atmospheric environment. Observations of a

comparatively small ^{2}H enrichment of leaf wax *n*-alkanes in grasses relative to dicots, and the much smaller effect of atmospheric humidity on that enrichment in grasses (Kahmen *et al.*, 2013; Gamarra *et al.*, 2016), may be related to the fact that some *n*-alkane synthesis occurs outside of the LGDZ (Richardson *et al.*, 2005), or that leaf surface waxes are turned over post-emergence, when leaf tissue is directly exposed to the atmospheric environment (Gamarra & Kahmen, 2016).

Given that $p_{x\ 18O}$ varied so little among C_3 and C_4 grass species (and leaf cellulose synthesis occurs virtually exclusively inside the LGDZ), that parameters appears to represent a particularly well constrained parameters for models of the oxygen isotope composition of cellulose. Thus, the absence of a VPD effect on $p_{x\ 18O}$ suggests that the previously observed variation in the relationship between ^{18}O enrichment of cellulose ($\Delta^{18}O_{Cel}$) and $\Delta^{18}O_{LW}$ in the work of Liu *et al.* (2016) and Helliker & Ehleringer (2002b; see discussion in Liu *et al.* 2016) must be explained in a large part by variation of p_{ex}, or the equilibrium fractionation factor between carbonyl oxygen and water, or another condition of the Barbour & Farquhar (2000) model of the oxygen isotope composition of cellulose, such as a deviation of the oxygen isotope composition of sucrose from average (bulk) leaf water in the steady state.

As $p_{x\ 18O}$ in the LGDZ of grass leaves as observed here was very similar to that discussed for growing xylem cells in stems of trees (cf. Cernusak *et al.,* 2005) and higher than has been assumed for leaves of dicots (Helliker & Ehleringer, 2002b) one may wonder if the VPD response of $\Delta^{18}O_{Cellulose}$ of grass leaves is more similar to that of tree rings than of dicot leaves. However, direct measurements of $p_{x\ 18O}$ in leaves of dicot species have not been reported (but see discussion in Song *et al.,* 2014a), to the best of our knowledge.

Chapter 5

GENERAL AND SUMMARIZING DISCUSSION

This study explored in detail the relationship between $\delta^{18}O$ of leaf water and cellulose in *C. squarrosa*, the effects of VPD and nitrogen nutrition on that relationship. Further, it analyzed the incorporation of the oxygen istopic signal in growing leaf blades of the same species. Lastly, the study addressed the oxygen and hydrogen isotopic signals in LGDZ water in several C_3 and C_4 grass species, including *C. squarrosa*. It is well known that VPD is the primary environmental control of the oxygen isotopic signal of leaf water (Barbour, 2007; Cernusak *et al.,* 2016) and cellulose (Kahmen *et al.,* 2011), and that fertilizer supply can accelerate the growth rate and turnover rate of carbohydrates pools in plants. The latter was thought to modify $p_{ex}p_x$ (Barbour & Farquhar, 2002; Song *et al.,* 2014a), subsequently affecting the $\delta^{18}O$ of cellulose. However, previous studies had not explored if VPD and nitrogen fertilizer supply influence the relationship between ^{18}O-enrichment of leaf water and cellulose in controlled environmental conditions. To exclude an effect of $\delta^{18}O$ of CO_2 on the $\delta^{18}O$ of cellulose, this work used CO_2 with contrasting $\delta^{18}O$ to assess the direct and interactive effects of VPD and nitrogen supply on the ^{18}O-enrichment of leaf water and cellulose (Chapter 2). In chapter 3, the incorporation of the oxygen isotope signal of changed VPD in cellulose of growing grass leaves was investigated to test its potential as a high-resolution temporal recorder of environmental variation. Chapter 4 explored the effects of contrasting VPDs on the $\delta^{18}O$ and δ^2H of water in the leaf growth and differentiation zone (LGDZ) of two C_3 and three C_4 grass species. The proportion of unenriched water in the LGDZ ($p_{x\ 18O}$ and $p_{x\ 2H}$) was estimated from the $\delta^{18}O$ and δ^2H of source and leaf water. As p_x appears well constrained (near unity), it should now also be possible to interpret or discuss the Barbour & Farquhar model (2000) in terms of p_{ex} (the proportion of oxygen atoms exchanging with medium water in the LGDZ during cellulose synthesis) or other conditions/hypotheses of that model (see below).

Exclusion of CO_2 sources effect on oxygen isotope in cellulose

Although it is known that C_4 grasses have a low activity of carbonic anhydrase (Gillon & Yakir, 2001a, b; Cousins *et al.,* 2006a, b), this work established that the $\delta^{18}O$ of CO_2 has no influence on the $\delta^{18}O$ of leaf cellulose in *C. squarrosa*. This is the first observation of that fact in a C_4 species. The result is consistent with a previous with wheat that reported no effect of

$\delta^{18}O$ of CO_2 on that of cellulose in wheat (Deniro & Epstein, 1979), a species that has a much higher activity of carbonic anhydrase. Although the present finding with *C. squarrosa* does not necessarily prove that the oxygen of CO_2 does exchange completely with leaf water, it does indicate strongly that if such an exchange is incomplete, then posterior exchange between metabolites leading up to sucrose will do the rest. Clearly, the result implies that any significant effect of the $\delta^{18}O$ of CO_2 on the $\delta^{18}O$ of cellulose can be safely disregarded.

The value of p_x in grass species

Helliker & Ehleringer (2002a) found a $\Delta^{18}O$ of water in the basal tissue of fully-expanded leaf blades (termed 'intercalary meristem') of *L. multiflorum* of 3.2 to 6.7‰. In parallel, the $\Delta^{18}O$ of leaf water ranged from -10.5 to 5.6‰ as a result of varied relative humidity (from 93% to 35%). Based on the assumption that the 'intercalary meristem' water actually formed the medium water for leaf cellulose synthesis, they concluded that the medium water was actually a mixture of source water and evaporatively enriched leaf blade water. Similarly, the values of p_x for five C_3 and five C_4 grass species exposed to low and high VPD conditions were estimated at 0.62 to 0.5, when the value of p_{ex} was constrained between 0.4 and 0.5 (Helliker & Ehleringer, 2002b). However, according to that assumption, Gamarra *et al.* (2016) hypothesized that grass species which had a high transpiration rate will reduce effect of leaf water ^2H-enrichment on leaf wax *n*-alkane δ^2H values relative to the low transpiration, because the dilution of enriched water at the site of wax synthesis could be enhanced by the unenriched source water *via* high transpiration rate. However, their results showed that transpiration rate did actually not correlate strongly with the ^2H enrichment of leaf wax, in agreement with a previous observation by McInerney *et al.* (2011). In our studies, the mean value of $p_{x\ 18O}$ and $p_{x\ 2H}$ at the LGDZ of five grass species under both low and high VPD was 0.95 and 1.1, respectively, showing that the oxygen isotope composition of water at the LGDZ was very close to that of source water in all grass species. To our knowledge, this is the first report that actually shows the $\delta^{18}O$ and δ^2H of water in the LGDZ of grasses, a finding that is key to understanding the mechanisms generating the oxygen isotope signal in leaf cellulose and the hydrogen isotope signal in surface leaf waxes of grasses.

The relationship between ^{18}O-enrichment of leaf water and cellulose

As shown by the Barbour & Farquhar (2000) model, the relationship between ^{18}O-enrichment of leaf water and cellulose is determined by the $1 - p_{ex}p_x$. It has been accepted that p_{ex} is a roughly constant value, close to 0.42, suggested by a number of experimental studies with a range of species and growth conditions reviewed by Cernusak *et al.* (2005). However,

variation of p_{ex} from 0.2 to 0.42 during the growing season in tree rings of Scots pine (*Pinus sylvestris*) was reported by Gessler *et al.* (2009), while Offermann *et al.*, (2011) found a variation from 0 to 0.76 in European beech (*Fagus sylvatica* L.). Furthermore, it was sugegsted that p_{ex} depends on the growth rate (Barbour & Farquhar, 2000) and turnover rate of carbohydrates (Song *et al.*, 2014a). In addition, Ellsworth & Sternberg (2014) demonstrated that salinity can increase p_{ex} during leaf cellulose synthesis in *Arabidopsis thaliana*, and suggested this effect was related to an excess of soluble carbohydrates. In the present study, VPD had a significant effect on $p_{ex}p_x$ in *C. squarrosa* in a tightly controlled environment. Remarkably, increased VPD led to decreased $p_{ex}p_x$, although growth rate was not affected by VPD. As p_x was constant, near unity under both low and high VPD for a range of grasses, it seemed reasonable to speculate that the relationship between ^{18}O-enrichment of leaf water and cellulose under VPD treatments was actually related to variation of p_{ex} (provided that ε_o was constant at 27‰). That variation was supported by the analysis of the Δ^{18}O of leaf water (**Fig. 3.2a**) and cellulose **Fig. 5.1a**) of the five grass species studied in Chapter 4. Using the values of $p_{ex}p_x$, predicted from the Barbour & Farquhar model in assuming a ε_o of 27‰, and measured p_x for each species (**Fig. 3.2c**), estimates of p_{ex} were obtained for each species × VPD combination. Results demonstrated that high VPD significantly increased ^{18}O enrichment of cellulose, increasing $p_{ex}p_x$ for all species with the exception of *L. multiflorum,* but decreased p_{ex} for all species (**Fig. 5.1c**). In general, the values of p_{ex} in that study were much larger than the commonly accepted value of 0.42, especially under low VPD conditions. However, considering the uncertainties of ε_o and the fundamental assumption of Barbour & Farquhar model that oxygen atoms in sucrose equilibrate completely with average leaf water in the steady state, the effect of VPD on estimated p_{ex} may be not real. However, leaf temperature varied only between 26 to 27°C at low and high VPD in this study, and ε_o was found constant in this range by Sternberg & Ellsworth (2011). Therefore, one could expect that errors in estimation of p_{ex} could come from VPD- and species-dependent variation in the oxygen isotopic disequilibrium between sucrose and bulk leaf water. Sucrose synthesis occurs in the cytosol of mesophyll cells in C_3 species and bundle sheath cells in C_4 species (Lunn & Furbank, 1999). Isotopic signatures of water in mesophyll and bundle sheath cells could differ as a function of distance to the stomata and veins (Yakir *et al.*, 1989, 1990, 1994; Yakir, 1992b; Song *et al.*, 2015; Song & Barbour, 2016). One should expect that the isotopic composition of water in mesophyll cells should be closer to that of water at the site of evaporation, than that of the bundle sheath cells. For the latter one would expect an isotopic composition that is closer to that of source water (Smith *et al.*, 1991; Zhou

et al., 2016). Clearly, the empirical support for the full equilibration of sucrose oxygen isotope composition with that of average leaf water (in the steady state) is relatively small and merits to be expanded to a greater range of species, including C_3 and C_4 grasses, and environmental conditions. In addition, cellulose synthesis and deposition presumably occur during both day and night (Schnyder & Nelson, 1988; Schnyder *et al.*, 1988), and the sources of sucrose used to form cellulose may differ between day and night periods. During daytime, sucrose is synthesized in mature leaf blades and translocated to the LGDZ where cellulose synthesis occurs. Simultaneously, some carbohydrates are used to build up stores of starch in C_4 grasses and fructan in C_3 grasses. At night time, much of the stored carbohydrates are mobilized and used to synthesize sucrose for export, which may also support cellulose synthesis cellulose. The size and turnover rate of relevant carbohydrate pools, including that of sucrose, may thus differ between day and night, increasing the complexity of processes underlying variation of p_{ex}. Clearly, more work is required to elucidate potential spatial and temporal variation of p_{ex}.

The incorporation of oxygen isotope in the growing leaf blade

This study proved the potential of the oxygen isotope record of cellulose in the successively produced leaves along the tillers of *C. squarrosa* as a chronometer of VPD variation at short, i.e. weekly, timescale, that complement tree ring and ice cores which can be used to reconstruct inter-annual variation of hydrological signals over millenia. As they are highly predictable, the unique leaf production characteristics of grasses (and in particular C_4 grasses) provide a near-ideal model to study environmental signals imprinted in cellulose or other biomass components, such as leaf surface waxes. In this study, VPD signals were incorporated into leaf blade cellulose at the time of cellulose synthesis, supporting the observation by Helliker & Ehleringer (2002a). This also meant that stored carbohydrates must have contributed very little to oxygen isotope incorporation in leaf cellulose in *C. squarrosa*. This result has practical implications for (paleo)climatic reconstructions of VPD in grassland using grass leaves as recorder of seasonal changes. Certainly, analysis of [18]O in grass leaf cellulose coupled with ^{13}C analysis could be used to analyze grassland ecosystem carbon and water relations at a fine temporal scale.

Conclusion and outlook

Understanding the effects of plant physiology and biochemistry on the relationship between ^{18}O-enrichment of leaf water and cellulose is imperative for the application of oxygen isotope in the climate reconstruction, plant breeding and plant physiology. Incorporation of oxygen

isotope in cellulose – an integrator of environmental signals in the successive leaves formed along the grass tillers – provided a new plant material to reconstruct short-term hydrological changes. In addition, this work proved that the water in the LGDZ of grasses is very close to source water that is hardly influenced by evaporative enrichment. This finding has important implications for the understanding of the $\delta^{18}O$ of leaf cellulose and δ^2H in leaf waxes of grasses. More studies and multidisciplinary efforts are needed to understand the mechanisms controlling variation of p_{ex} in different species under contrasting environmental conditions.
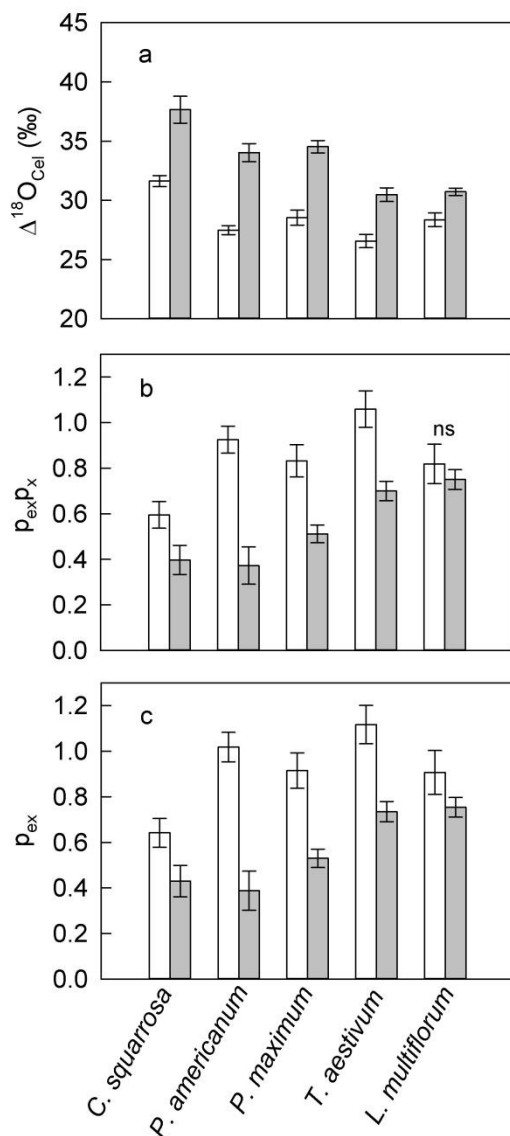
Fig. 5.1 (a) ^{18}O enrichment of leaf cellulose ($\Delta^{18}O_{Cel}$, ‰), (b) $p_{ex}p_x$ calculated from the ^{18}O enrichment of leaf water from the data of **Fig. 4.2a** and cellulose by application of Barbour & Farquhar model (2000) with ε_o as 27‰, (c) p_{ex} calculated from $p_{ex}p_x$ with the known values of p_x for each of five grass species (**Fig. 4.2c**) grown under low (empty bar) and high (grey bar)

VPD. The values are presented as mean $\pm$ 95% confidence interval, $n = 6$. The differences of $\Delta^{18}O_{Cel}$, $p_{ex}p_x$, p_{ex} between low and high VPD were all significant except $p_{ex}p_x$ of *L. multiflorum*. The 'ns' above a bar means no significant difference between VPD treatments.

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CURRICULUM VITAE
<u>Persönliche Angaben</u>

Name   Haitao Liu

Geburtstag und −ort   30. November 1984 in He Nan, China

Nationalität   China

Familienstand   Ledig

<u>Ausbildung</u>

09/2005-07/2009   Studium Biological Science, College of Life Science, Luo Yang Normal University, Luo Yang, He Nan, China

Abschluss: B.Sc

09/2009-07/2012   Studium Grassland Science, College of Animal Science and Technology, Northwest A&F University, Yangling, Shaanxi, China

Abschluss: M.Sc

09/2012-03/2016   Doktorand, Lehrstuhl für Grünlandlehre, Technische Universität München

<u>Berufliche Tätigkeit</u>

09/2012-03/2017   Wissenschaftlicher Mitarbeiter, Lehrstuhl für Grünlandlehre, Technische Universität München

<u>Publikationen</u>

Liu HT, Gong XY, Schäufele R, Yang F, Hirl RT, Schmidt A, Schnyder H. 2016. Nitrogen fertilization and δ^{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? *Plant, Cell & Environment* **39**: 2701-2712.

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