Variation in carbon isotope discrimination in *Cleistogenes squarrosa* (Trin.) Keng: patterns and drivers at tiller, local, catchment, and regional scales

Hao Yang¹,³, Karl Auerswald¹, Yongfei Bai²*, Maximilian H. O. M. Wittmer¹ and Hans Schnyder¹

¹ Lehrstuhl für Grünlandlehre, Technische Universität München, Alte Akademie 12, 85350 Freising, Germany
² State Key Laboratory of Vegetation and Environmental Change, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China
³ Synthesis Research Center of Chinese Ecosystem Research Network, Key Laboratory of Ecosystem Network Observation and Modeling, Institute of Geographic Sciences and Natural Resources Research, Chinese Academy of Sciences, Beijing 100101, China

* To whom correspondence should be addressed. E-mail: yfbai@ibcas.ac.cn

Received 15 November 2010; Revised 3 March 2011; Accepted 14 March 2011

Abstract

Understanding the patterns and drivers of carbon isotope discrimination in C₄ species is critical for predicting the effects of global change on C₃/C₄ ratio of plant community and consequently on ecosystem functioning and services. *Cleistogenes squarrosa* (Trin.) Keng is a dominant C₄ perennial bunchgrass of arid and semi-arid ecosystems across the Mongolian plateau of the Eurasian steppe. Its carbon isotope discrimination (δ¹³C) during photosynthesis is relatively large among C₄ species and it is variable. Here the δ¹³C of *C. squarrosa* and its potential drivers at a nested set of scales were examined. Within cohorts of tillers, δ¹³C of leaves increased from 5.1‰ to 8.1‰ from old to young leaves. At the local scale, δ¹³C of mature leaves varied from 5.8‰ to 8.4‰ increasing with decreasing grazing intensity. At the catchment scale, δ¹³C of mature leaves varied from 6.2‰ to 8.5‰ and increased with topsoil silt content. At the regional scale, δ¹³C of mature leaves varied from 5.5‰ to 8.9‰ increasing with growing-season precipitation. At all scales, δ¹³C decreased with increasing leaf nitrogen content (Nleaf). Nleaf was positively correlated with grazing intensity and leaf position along tillers, but negatively correlated with precipitation. The presence of the correlations across a range of different environmental contexts strongly implicates Nleaf as a major driver of δ¹³C in *C. squarrosa* and, possibly, other C₄ species.

Key words: C₄ species, ¹³C discrimination, grazing, Inner Mongolian steppe, leaf nitrogen content, precipitation.

Introduction

Grasslands have been experiencing rapid shifts in botanical composition, structure, and functioning driven primarily by global climate change and human disturbances (Alward et al., 1999; Grime et al., 2000; Sage and Kubien, 2003). Such changes may also shift the dominance of the two distinct photosynthetic pathways, C₃ and C₄, which differ in the mode of initial CO₂ fixation and have profound effects on ecosystem processes (Farquhar et al., 1989; Hunt et al., 1996; Sage and Kubien, 2003; Kuzyakov et al., 2006). Most of the world’s C₄ species are grasses (Teeri and Stowe, 1976; Ehleringer et al., 1997). The ratio of C₃ to C₄ photosynthetic types is an important component in understanding ecosystem processes because it can affect biomass production, carbon sequestration, and water cycling (Tieszen et al., 1997; Pyankov et al., 2000; Sage and Kubien, 2003). It can also influence the terrestrial carbon isotope signal, which is used for the land–ocean partitioning of CO₂ fluxes (Still et al., 2003; Suits et al., 2005).

The actual C₃/C₄ ratio is often obtained from the stable carbon isotope compositions of C₃ and C₄ plants and bulk...
samples in a two-member mixing model (Murphy and Bowman, 2007), where bulk samples may comprise soil organic matter (Wittmer et al., 2010), n-alkanes (Zhang et al., 2003), wool (Auerswald et al., 2009), or other materials. This approach is based on the difference in discrimination against the heavy carbon stable isotope (\(^{13}\Delta\)) during photosynthesis between C\(_3\) and C\(_4\) plants (Farquhar et al., 1989). A single mean C\(_3\) end-member value is usually assumed in the two-member mixing model (Murphy and Bowman, 2007; Wittmer et al., 2010), whereas variable C\(_3\) end-member values are estimated, based on the well-known correlations between the \(^{13}\Delta\) of C\(_3\) plants and environmental conditions (Mole et al., 1994; Männel et al., 2007; Wittmer et al., 2008). The reason for this is that \(^{13}\Delta\) of C\(_4\) plants is reported to be less responsive to environmental variables than that of C\(_3\) plants (Van de Water et al., 2002; Murphy and Bowman, 2009). However, this view has been challenged by a number of studies that have examined variations in \(^{13}\Delta\) of C\(_4\) plants, and which suggest that the \(^{13}\Delta\) of C\(_4\) species can vary with environmental variables (Henderson et al., 1992; Buchmann et al., 1996; Wang et al., 2005) and that this variability is related to ecophysiological responses and resource efficiencies (Henderson et al., 1992; Ghannoum et al., 2002; Wang et al., 2005). For instance, the \(^{13}\Delta\) of *Amaranthus cruentus* was \(-6^{\circ}\) higher in low-light leaves than in high-light leaves (Tazoe et al., 2006). The \(^{13}\Delta\) of *Bothriochloa ischaemum* increased \(2^{\circ}\) with an increase in annual precipitation from 350 mm year\(^{-1}\) to 700 mm year\(^{-1}\) (Liu et al., 2005). In *Saccharum* spp., \(^{13}\Delta\) declined by \(2^{\circ}\) with increasing leaf nitrogen content (N\(_{leaf}\)) in a nitrogen-stress experiment (Meinzer and Zhu, 1998).

The \(^{13}\Delta\) of a plant can be obtained from the carbon isotope composition of a plant (\(\delta^{13}C_{plant}\)) and that of the air (\(\delta^{13}C_{air}\)) in which it grows:

\[
^{13}\Delta = \frac{\delta^{13}C_{air} - \delta^{13}C_{plant}}{1 + \delta^{13}C_{plant}}
\]  

\(^{13}\Delta\) of C\(_4\) plant biomass is influenced by many factors, including isotope effects during diffusion of CO\(_2\) through the boundary layer, stomatal pore, cell walls, membranes, and in the liquid phase, dissolution, hydration by carbonic anhydrase, fixation of bicarbonate by phosphoenolpyruvate carboxylase (PEPc) in mesophyll cells, fixation of CO\(_2\) by Rubisco in bundle sheath cells, leakage of CO\(_2\) from bundle sheath cells to mesophyll cells, and carbon allocation and partitioning, including photorespiration and dark respiration (Farquhar, 1983; von Caemmerer and Furbank, 2003). This complexity has not been analysed in full detail, mainly due to lack of instrumentation and experimental protocols for the measurements of all the necessary variables. To solve the problem, Farquhar (1983) proposed a simplified, but testable version of the model, which was subsequently used widely to investigate the influence of the supposedly strongest drivers of variation in \(^{13}\Delta\). This model states that \(^{13}\Delta\) of C\(_4\) plants depends on bundle sheath leakiness (\(\Phi\), the proportion of C fixed by PEP carboxylation, which subsequently leaks out of the bundle sheath) and the ratio of internal and ambient CO\(_2\) concentrations (C\(_i\)/C\(_a\)):

\[
^{13}\Delta = a + (b_4 + b_3 \times \Phi - a) \times \frac{C_i}{C_a}
\]  

where \(a\) is the discrimination of \(^{13}\)C during diffusion of CO\(_2\) through stomata (4.4\(^{\circ}\)), \(b_3\) is the discrimination during fixation by Rubisco [27\(^{\circ}\) for C\(_4\) plants; von Caemmerer et al. (2008)] and \(b_4\) is the discrimination during hydration of CO\(_2\) to HCO\(_3^-\) and fixation by PEPc.

Eqn (2), predicts that \(^{13}\Delta\) variation during photosynthesis of C\(_4\) plants depends on \(b_4\), \(\Phi\), and on C\(_i\)/C\(_a\). These variables in turn depend on a host of environmental conditions. Temperature is the biggest influence on \(b_4\), inducing variation from \(-4.8^{\circ}\) at 33.8 \(^{\circ}\)C to \(-6.2^{\circ}\) at 21.0 \(^{\circ}\)C (Henderson et al., 1992). \(\Phi\) is an important ecophysiological trait that is directly associated with photosynthetic quantum yield and nitrogen use efficiency of C\(_4\) plants (Henderson et al., 1992; Ghannoum et al., 2002; Wang et al., 2005). Any CO\(_2\) that leaks from bundle sheath cells decreases the energetic efficiency of C\(_4\) photosynthesis, through futile costs of ATP for the regeneration of PEP (Furbank et al., 1990). \(\Phi\) depends on the balance of the biochemical capacities of the C\(_4\) cycle in the mesophyll cells and the C\(_3\) cycle in bundle sheath cells, and the CO\(_2\) conductance of the bundle sheath exterior wall (von Caemmerer and Furbank, 2003). Factors that could affect \(\Phi\) include the activity ratio of Rubisco to PEPc (Farquhar, 1983), such as N\(_{leaf}\) (Sage et al., 1987), temperature (Pittermann and Sage, 2001), and light (Tazoe et al., 2008). C\(_i\)/C\(_a\) depends on the ratio of stomatal conductance to photosynthetic activity and, hence, influences intrinsic water use efficiency (Ghannoum et al., 2002). Because there is an interaction between the influences of \(\Phi\) and C\(_i\)/C\(_a\) on \(^{13}\Delta\), \(^{13}\Delta\) can increase or decrease with C\(_i\)/C\(_a\) depending on the term \((b_4 + b_3 \times \Phi - a)\) (Farquhar, 1983). For \(\Phi>0.37\), this term is greater than zero with \(^{13}\Delta>4.4^{\circ}\), and it increases with C\(_i\)/C\(_a\). For \(\Phi<0.37\), this term is smaller than zero with \(^{13}\Delta<4.4^{\circ}\), and it decreases with increasing C\(_i\)/C\(_a\). For \(^{13}\Delta\) differing from 4.4\(^{\circ}\), \(^{13}\Delta\) will thus vary with C\(_i\)/C\(_a\) if \(\Phi\) is constant.

Studies of the \(^{13}\Delta\) of C\(_4\) plants and its response to environmental variables have been conducted primarily in grasslands in North America, Europe, and Australia (Schulze et al., 1996; Murphy and Bowman, 2009; Ghannoum et al., 2011) or in experiments under controlled conditions (Ranjith et al., 1995; Tazoe et al., 2006). Few studies of this type have been reported from the mixed C\(_3\)/C\(_4\) grassland of the Mongolian plateau, where the C\(_4\) community of the semi-arid steppe is dominated by *Cleistogenes squarrosa* (Trin.) Keng, a C\(_4\) (NAD-ATP) perennial bunchgrass. *C. squarrosa* has a wide range occurring at meadow steppe, typical steppe, desert steppe, and sand dune ecosystems across the Mongolian plateau. The \(^{13}\Delta\) in *C. squarrosa* is well above 4.4\(^{\circ}\) (Pyankov et al., 2000; Chen et al., 2002; Gong et al., 2008) and it is known to vary with soil moisture (Chen et al., 2002).

In this study the \(^{13}\Delta\) variability in *C. squarrosa* and its controlling factors were investigated across a range of ecological scales on the Mongolian plateau. It was hypothesized that \(^{13}\Delta\) variability in *C. squarrosa* is driven by factors
controlling $N_{\text{leaf}}$, which influence $C_{\text{i}}/C_{\text{a}}$ and $\Phi$. Factors influencing $N_{\text{leaf}}$ may include leaf age, grazing pressure, soil properties, or weather conditions, all of which vary at different scales. Hence the $\delta^{13}D$ of *C. squarrosa* was studied with a nested set of scales (i.e. tiller, local, catchment, and regional) to identify and quantify controls of $N_{\text{leaf}}$ and, in turn, $\delta^{13}D$.

**Material and methods**

**Study area**

The study area was located between 106.15 °E and 117.13 °E longitude and 40.62 °N and 45.57 °N latitude in the Inner Mongolia Autonomous Region, China (Fig. 1). This area of ~200 000 km$^2$ constitutes the regional scale. Samples were taken at altitudes ranging from 1010 to 1635 m elevation.

The temperate, semi-arid climate is characterized by warm summers and cold, dry winters (Chen, 1988) with mean annual temperature (MAT) between 0.3 °C and 5.6 °C and mean July temperature (MJulT) between 18.7 °C and 22.7 °C. Mean annual precipitation (MAP) increases from 160 mm year$^{-1}$ in the western part to 350 mm year$^{-1}$ in the eastern part, and 60–80% of the annual total occurs in the May–August growing season (Bai et al., 2007). Regional sampling took place in five different years (2005–09) to take account of the large inter-annual variation in precipitation in the region.

The Xilin river basin covers an area of 10 786 km$^2$ in the northeast of the region. Within this catchment, MAP increases from 275 to 346 mm year$^{-1}$ along a northwest–southeast precipitation gradient, with elevation ranging from 1019 to 1482 m. MAT varies from 0.3 °C in the southeast to 2.5 °C in the northwest of the basin, with MJulT ranging from 18.7 °C to 20.9 °C. The soils in the Xilin river basin include phaeozems, shallow and stony soils at rocky outcrops, and sandy soils of low water-holding capacity in sand dune areas (Wiesmeier et al., 2011).

A grazing experiment (116.67 °E, 43.56 °N) near the Inner Mongolia Grassland Ecosystem Research Station (IMGERS) of the Chinese Academy of Sciences at the middle reach of the Xilin river represents the local scale. MAT, MJulT, and MAP at IMGERS are 0.4 °C, 19.0 °C, and 337 mm year$^{-1}$, respectively (Zheng et al., 2011).

**Meteorological data and $\delta^{13}C$ of atmospheric CO$_2$**

Growing season precipitation was geostatistically determined according to Wittmer et al. (2008) for each sampling location and time of sampling. This was done by interpolating between meteorological stations and taking into account 1.5×1.5 km$^2$ resolution maps of MAP, mean monthly precipitation, MJulT, and MAT (Climate Source, Inc., Corvallis, OR, USA). The mean daily precipitation (mm d$^{-1}$) between 1 May, which is the date that usually corresponds with the onset of growth of *C. squarrosa* (Liang et al., 2002), and the day of sampling was calculated. This measure of precipitation takes account of the fact that samples were collected on different days and in different years.

$\delta^{13}C_{\text{air}}$ was obtained from a long-term monitoring station in Ulan Uul (Climate data online; NOAA NNDC, 2009), Mongolia, ~460 km northwest of IMGERS.

**Soil analyses**

On the catchment scale sampling locations were selected to cover the full range of soils as known from soil mapping campaigns. Soil pits were dug to 1 m at locations representing the mapping units and the soils were sampled horizontally. A comprehensive set of soil properties was measured (for details see Wiesmeier et al., 2011), including bulk density, texture, pH, and nitrogen content. Either topsoil properties (e.g. silt content) because water uptake by *C. squarrosa* is restricted to the topsoil (Yang et al., 2011), or whole-soil properties (e.g. N stocks) were used.
Grazing experiment

The grazing experiment had 28 annually grazed plots (Fig. 1, grazing experiment) within two experimental blocks differing in topography (flat and gently sloping). The plots had been grazed between June and September each year since 2005 at seven stocking rates: 0, 37.5, 75.0, 112.5, 150.0, 187.5, and 225.0 sheep km\(^{-2}\) with three times higher stocking densities during the 4 months of grazing. Further information about the grazing experiment can be found elsewhere (Schönbach et al., 2009).

C. squarrosa sampling

The tiller scale was sampled in an ungrazed plot, where no leaves were lost by grazing. Leaves from several individuals were sampled and pooled according to their phytomer position (Fig. 1). Leaves were collected in mid-July, end of July, and mid-August 2008. Resampling over time allowed us to distinguish between new leaves and old leaves grown between sampling dates. Old leaves at low phytomer positions were then compared between consecutive sampling dates to identify changes over time.

At the local, catchment, and regional scales, mature, fully expanded, and sun-exposed leaves from several non-flowering individuals were sampled. At the local scale, leaves were collected from the plots of the grazing experiment in June, July, and August of 2007, and in May, June, and July of 2008. These two years differed in precipitation: 2007 was dry with 240 mm year\(^{-1}\), and 2008 was an average year with 362 mm year\(^{-1}\). At the catchment and regional scales, samples were collected in each of the five years 2005–09. The precise locations and elevations of all sampling positions were recorded with a mobile GPS. In total, 51, 144, 64, and 47 samples at the tiller, local, catchment, and regional scales, respectively, were collected.

Isotope and N\(_{\text{leaf}}\) analysis

All samples were dried at 60 °C for 48 h in a forced-draught oven and ground to homogeneity with a ball mill. Carbon isotope composition and nitrogen content were then measured using an elemental analyser (NA 1110; Carlo Erba, Milan, Italy) interfaced (ConFlo III; Finnigan MAT, Bremen, Germany) to an isotope ratio mass spectrometer (Delta Plus; Finnigan MAT) following the protocol by Wittmer et al. (2008). Carbon isotope data were specified as \(\delta^{13}\text{C}\) relative to the Vienna Pee Dee Belemnite standard:

\[
\delta^{13}\text{C} = \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1
\]

where \(R_{\text{sample}}\) and \(R_{\text{standard}}\) are the ratios of \(^{13}\text{C}/^{12}\text{C}\) in the sample and standard.

The precision for sample repeats was better than 0.15% for \(\delta^{13}\text{C}\) and 0.04% for nitrogen content in dry matter.

Statistical analysis

Linear regressions were used to evaluate relationships between \(\Delta\), \(N_{\text{leaf}}\), and the environmental variables of interest at a certain scale: leaf position, grazing intensity, soil properties, and growing season precipitation. The coefficient of determination was tested with a two-sided test for significance of the regression. Confidence intervals (95%) were calculated for the relationship between \(\Delta\) and \(N_{\text{leaf}}\) and for the population. All procedures were carried out in SPSS Version 16.0 (SPSS, Inc., Chicago, IL, USA).

Results

Relationship between \(\Delta\) and leaf position at the tiller scale

The \(\Delta\) of C. squarrosa decreased and \(N_{\text{leaf}}\) increased from the lowermost leaf to the uppermost leaf (Fig. 2). Thus, young leaves had a low \(\Delta\) but high \(N_{\text{leaf}}\). The \(\Delta\) mainly changed from leaf 5 to younger leaves, while \(N_{\text{leaf}}\) mainly changed among leaves older than leaf 7. Hence in a bulk sample of undefoliated plants, the variation in \(\Delta\) can mainly be attributed to younger leaves whereas the variation in \(N_{\text{leaf}}\) can mainly be attributed to older leaves.

The \(N_{\text{leaf}}\) of a leaf produced at a given time remained unchanged between mid-July (average=1.6%, SD=0.2%) and August (average=1.6%, SD=0.3%), which suggested no nitrogen redistribution over time. \(N_{\text{leaf}}\) of the three uppermost (newly grown) leaves (average=2.4%, SD=0.1%) was significantly higher (\(P<0.01\)) in August than those grown in July (average=1.7%, SD=0.2%).

\(\Delta\), stocking rate, and topography at local scale

At the local scale, \(\Delta\) varied between 5.8%\(_{\text{oo}}\) and 8.4%\(_{\text{oo}}\) with an SD of 0.5%\(_{\text{oo}}\), \(N_{\text{leaf}}\) varied between 1.8% and 3.8% with an SD of 0.4%. \(\Delta\) decreased (\(R^2=0.04, N=144, P<0.05\)) while the \(N_{\text{leaf}}\) increased (\(R^2=0.11, N=144, P<0.001\)) with stocking rate (Fig. 3). \(\Delta\) and \(N_{\text{leaf}}\) also varied with year (\(P<0.001\)) and month (\(P<0.001\)), but not with topographic position (\(P>0.05\)).

\(\Delta\) and soil variables at catchment scale

Across the Xilin river catchment, \(\Delta\) of C. squarrosa varied between 6.2%\(_{\text{oo}}\) and 8.5%\(_{\text{oo}}\) (SD=0.5%\(_{\text{oo}}\)), \(N_{\text{leaf}}\) varied between 1.5% and 3.7% (SD=0.3%), and topsoil silt content varied from 2% to 40% (SD=10%). \(\Delta\) increased with topsoil silt content (\(R^2=0.42, N=24, P<0.001\)) despite there being differences in slope between the two years (\(P<0.05\)), but \(N_{\text{leaf}}\) was not correlated with silt content (\(P>0.05\)) (Fig. 4). Similar patterns were found with soil nitrogen stocks. The \(\Delta\) increased significantly with soil nitrogen stocks (\(R^2=0.40, N=25, P<0.01\)), but there was no relationship between soil nitrogen stocks and \(N_{\text{leaf}}\) (\(P>0.05\)). The influence of soil nitrogen stocks and silt content on
$^{13}$D could not be separated due to their correlation with each other; this also applied to other soil properties.

$^{13}$D and precipitation at regional scale

As expected, the range of $^{13}$D of *C. squarrosa* (3.4‰) was considerably larger at the regional scale than at the local (2.6‰) and catchment scales (2.3‰). Precipitation during the growing season had a significant, positive effect on $^{13}$D ($R^2=0.31$, $N=47$, $P<0.001$) and a negative effect on $N_{\text{leaf}}$ ($R^2=0.56$, $N=47$, $P<0.001$) (Fig. 5). The $^{13}$D of *C. squarrosa* increased ($P<0.05$) whereas $N_{\text{leaf}}$ decreased ($P<0.01$) from the desert steppe to the typical steppe, and to the meadow steppe.

$^{13}$D and $N_{\text{leaf}}$

At tiller, local, and regional scales, $^{13}$D was negatively correlated with $N_{\text{leaf}}$ (Fig. 6) although the range of values and factors that affected the variation in $N_{\text{leaf}}$ differed between scales (Figs 2, 3, 5). In spite of no significant relationship at the catchment scale ($P>0.05$), all except four of the total of 47 data points were included in the 95% confidence intervals for all pooled samples. $N_{\text{leaf}}$ explained 23% of the variation in $^{13}$D when all samples were pooled. When regressed separately, the slope of the regression was significantly steeper at the tiller scale than other scales ($P<0.01$), but remained within the 95% confidence interval of the common regression slope for the other scales (Fig. 6).
Fig. 6. Correlations between $\delta^{13}$C and Nleaf of mature leaves of C. squarrosa at the regional (steppe area of Inner Mongolia), catchment (Xilin river catchment), local (grazing experiment), and tiller scales (an ungrazed plot). The central line in each panel denotes the regression for the pooled samples, the inner envelopes (solid lines) are the 95% confidence intervals for the regression, and the outer envelopes (dashed lines) are the 95% confidence intervals for individual samples.

Discussion

This study represents the first comprehensive examination of the $\delta^{13}$C variability of a C₄ (NAD-ME) perennial bunchgrass, C. squarrosa, and its controlling factors across a range of scales (i.e. tiller, local, catchment, and regional scale) on the Mongolian plateau. The results reported here have shown that $\delta^{13}$C of C. squarrosa was large (on average: 7.2‰) and, at the region scale, it varied over a 4‰ range (5–9‰) across the Inner Mongolian steppe. This range is larger than that for other C₄ species in the study area. For instance, Salsola collina, a dicot sampled together with C. squarrosa in the grazing experiment, varied by 3‰ under identical conditions (data not shown). A previous study has reported that B. ischaemum, a dominant C₄ perennial bunchgrass on the Chinese loess plateau, varied by only 2‰ across its range (Liu et al., 2005). This suggested that C. squarrosa could be a model species for studying the effect of environmental conditions on the $\delta^{13}$C of C₄ plants, and that $\delta^{13}$C of the C₄ community is likely to vary, largely due to the change in relative abundance of C. squarrosa.

Many studies have demonstrated that foliar δ¹³C of plants is negatively correlated with water availability (e.g. soil moisture and annual precipitation) across an environmental gradient (Farquhar et al., 1989; Stewart et al., 1995; Liu et al., 2005). The mechanisms underpinning these patterns, however, have yet to be explored. The results reported here indicate that Nleaf has a strong and consistent effect on $\delta^{13}$C of C. squarrosa at all scales and explained 23% of the variation in $\delta^{13}$C, even though the factors influencing Nleaf varied at different scales. Therefore confidence can be placed in this relationship, and it may be expected that other environmental variables that affect Nleaf would also affect $\delta^{13}$C in a similar way.

Factors influencing Nleaf

Factors that affect Nleaf include factors that control N availability, such as fertilization (Bai et al., 2010), factors that influence N uptake, such as root density and root activity (Farooq et al., 2009), and factors that relate to ontogenesis and organogenesis, such as the availability of water and nutrients, which determine the velocity of leaf expansion and leaf dimensions where grazing could contribute (Anten et al., 1998).

The influence of leaf position on Nleaf may have been influenced by soil temperature, which was ~12.6 °C in late May and increased to 21.1 °C in mid-August (average of 10 d at 10 cm depth, data from IMGERS). N uptake of C₄ is especially sensitive to low temperature (Farooq et al., 2009). Remarkably, neither Nleaf nor $\delta^{13}$C of newly formed leaves changed between mid-July and August, indicating that the influence of leaf position was not caused by redistribution of N from old to young leaves. The increase in leaf nitrogen with grazing intensity is well known and relates to the faster cycling of N and decrease in mean leaf age (Mattson, 1980). Decreasing Nleaf with increasing precipitation is likely to be related to the dilution effect caused by additional plant growth. Temperature-related N uptake may also add to the effect of precipitation, as regional temperatures and precipitation are closely negatively correlated ($R^2=0.93$, Bai et al., 2007) and hence their effects cannot be separated. Finally, the Birch effect of enhanced mineralization after a pronounced dry spell (Birch, 1964; Austin et al., 2004) could also contribute to improved N availability in drier areas where rainfall occurs infrequently.

At the catchment scale, the influences on Nleaf are less clear. $\delta^{13}$C increased with silt content whereas Nleaf did not, although the relationship between $\delta^{13}$C and Nleaf was similar for this scale as for the other scales. At this scale the variation of soil is expected to have a strong influence but, in contrast to leaf position, grazing intensity, or precipitation acting on the other scales, the soil cannot be arranged along only one single variable like silt content. Soil is an
aggregation of several soil properties, which may exert influence on Nleaf and in turn on $^{13}\Delta$.

The effect of Nleaf on $^{13}\Delta$

In principle, the variability in $^{13}\Delta$ could be derived from: (i) discrimination associated with photosynthesis; (ii) post-photosynthetic fractionation, which includes CO$_2$ release in dark respiration, or (iii) any other simplifications included in Eqn (2). Although the latter processes have not been studied in detail in C$_4$ plants (but see Kromdijk et al., 2008; Pengelly et al., 2010), evidence for the contribution of post-photosynthetic fractionation comes from the fact that $^{13}\Delta$ obtained by online measurement of photosynthetic $^{13}$CO$_2$/12CO$_2$ exchange is often less than $^{13}\Delta$ estimated from the carbon isotope composition of leaf dry mass (Evans et al., 1986; Kubásek et al., 2007). For instance, $^{13}\Delta$ obtained from online measurement was $\sim 0.75–1.5\%$ lower than that from leaf dry mass (Kubásek et al., 2007). Respiration and carbon allocation are responsive to environmental conditions including temperature and water stress (Ryan, 1991; Flexas et al., 2005) and thus can contribute to the observed variability. However, changing chemical composition seems unlikely to explain the correlation of $^{13}\Delta$ with Nleaf. Proteins differ isotopically from cellulose only by $4\%$ (Benner et al., 1987). A difference in Nleaf of $2\%$, corresponding to a difference in protein content of $12\%$, is insufficient to explain a difference in $^{13}\Delta$ of $3\%$. Also, secondary compounds, which increase with leaf age and may thus relate to Nleaf, cannot explain the variability of $^{13}\Delta$ at the tiller scale, because the young leaves present in mid-July did not change in isotopic composition until mid-August. Hence variation in leaf $^{13}\Delta$ was most likely caused by the variation in discrimination during photosynthesis.

In Eqn (2), $^{13}\Delta$ mainly depends on $\Phi$ and C$_{C_{\alpha}}$ as $b_{\alpha}$ changes little within the 4°C range in air temperature in the study area. C$_{C_{\alpha}}$ should increase with stomatal openness (Turner et al., 1984), but decrease with the activity of carboxylating enzymes (Sage et al., 1987; Sage and Pearcy, 1987), which should increase with Nleaf (Ranjith et al., 1995; Sage et al., 1987). $\Phi$ depends on the activity ratio of Rubisco and PEPc, which is influenced by the allotment of nitrogen to them (Sage et al., 1987). At the local scale, C$_{C_{\alpha}}$ was measured for young mature leaves close to the top of tillers by Zheng et al. (2011) simultaneously with our sampling. C$_{C_{\alpha}}$ varied between 0.41 and 0.66 (Table 1), similar to the range of 0.34–0.59 found by Chen et al. (2005) (Table 2). Using these C$_{C_{\alpha}}$ data and the $^{13}\Delta$ data reported here, $\Phi$ was estimated using Eqn (2). This indicated that $^{13}\Delta$ ranged between 0.56 and 0.58 (Table 1). Nleaf from this study correlated with both C$_{C_{\alpha}}$ and $\Phi$ although variation of $\Phi$ was small. Therefore, $^{13}\Delta$ seemed to be influenced mostly by C$_{C_{\alpha}}$, which is related to water use efficiency.

Conclusions

The findings reported herein demonstrate that Nleaf is a key factor driving leaf $^{13}\Delta$ variation of C. squarrosa. Increasing Nleaf was associated with decreased $^{13}\Delta$, which seemed to be related to improved water use efficiency. The leaf-level response of $^{13}\Delta$ to Nleaf was found at all scales from tiller to local, catchment, and regional although the reasons for Nleaf variation differed. Within cohorts of tillers, $^{13}\Delta$ decreased with leaf position. Within the field experiment site, $^{13}D$ decreased with grazing intensity. Within the Xilin river catchment, $^{13}D$ decreased with decreasing topsoil silt content. Across the regional study area, $^{13}D$ decreased with decreasing growing season precipitation. Accordingly, it is proposed that water use efficiency increased for leaves

Table 1. Variation in the ratio of internal to ambient CO$_2$ concentration (C$_{C_{\alpha}}$) and leakiness ($\Phi$) for the local scale and correlations with Nleaf

| Variable | Minimum–maximum | Correlation with Nleaf |
|----------|-----------------|------------------------|
| C$_{C_{\alpha}}$ | 0.41–0.66 | -0.866 |
| $\Phi$ | 0.56–0.58 | +0.832 |

Gas exchange measurements were taken from Zheng et al. (2011).

Table 2. Leakiness ($\Phi$) and ratio of internal to ambient CO$_2$ concentrations (C$_{C_{\alpha}}$) of NAD-ME plants from published sources

| Species | $\Phi$ | C$_{C_{\alpha}}$ | References |
|---------|--------|----------------|------------|
| Amaranthus caudatus | 0.37 | 0.43–0.46 | Kubásek et al. (2007) |
| Amaranthus edulis | 0.32 | 0.43 | Henderson et al. (1992) |
| Amaranthus retroflexus | 0.40 | 0.40–0.90 | Sage and Pearcy (1987) |
| Atriplex lentiformis | 0.54–0.62 | – | Zhu et al. (1999) |
| Atriplex rosea | 0.45 | 0.47 | Henderson et al. (1992) |
| C. squarrosa | – | 0.34–0.59 | Chen et al. (2005) |
| Cynodon dactylon | – | 0.12–0.28 | Mantilana et al. (2008) |
| Eleusine coracana | 0.40 | 0.38 | Henderson et al. (1992) |
| Eleusine indica | 0.37, 0.27 | 0.48, 0.58 | Farquhar (1983) |
| Eragrostis lehmanniana | 0.45–0.57 | 0.08–0.20 | Frevolini et al. (2002), Mantilana et al. (2008) |
| Panicum schinzi | 0.32 | 0.38 | Henderson et al. (1992) |
| Total range | 0.27–0.62 | 0.08–0.90 | |
| Mean | 0.42 | 0.44 | |
produced later in the growing season, and that it also increased with grazing intensity. But it declined with increasing silt content and water-holding capacity of the soil and precipitation during the growing period.

Acknowledgements

The authors thank Monika Michler and Anja Schmidt for assistance with sample preparation, group III and IV of MAGIM for setting up the grazing experiment, and group VII and Martin Wiesmeier for soil data. They are grateful to Joanna Hsu and Rudi Schäufele for helpful discussions. This research was funded by the DFG within the research group 536 MAGIM, the State Key Basic Research Development Program of China (2009CB421102), and the Natural Science Foundation of China (30825008).

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