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Determination of leaf carbon isotope discrimination in C4 plants under variable N and water supply

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Understanding the mechanisms underlying variations in carbon isotope discrimination (Δ) in C4 plants is critical for predicting the C3/C4 ratio in C3/C4 mixed grassland. The value of Δ is determined by bundle sheath leakiness (Φ) and the ratio of intercellular to ambient CO₂ concentration (C_i/C_a). Leaf nitrogen concentration (N_{leaf}) is considered a driver of Δ in C4 plants. However, little is known about how N_{leaf} affects Φ and C_i/C_a and subsequently Δ . Here leaf carbon isotope composition, N_{leaf} , Φ , and leaf gas exchange were measured in *Cleistogenes squarrosa*, a dominant C4 species in the Inner Mongolia grassland. Δ remained relatively stable under variable N and water supply. Higher N supply and lower water supply increased N_{leaf} stimulated photosynthesis and further decreased C_i/C_a . High N supply increased Φ , which responded weakly to water supply. N_{leaf} exerted similar effects on C_i/C_a and on Φ in the field and pot experiments. Pooling all the data, N_{leaf} explained 73% of the variation in C_i/C_a . Overall, both Φ and C_i/C_a determined Δ ; however, the contribution of Φ was stronger. N_{leaf} influenced Δ primarily though C_i/C_a rather than Φ . Φ should be considered in estimating Δ of C4 endmember.

The carbon isotope discrimination (Δ) of C4 plants, C3 plants, and bulk samples (e. g., bulk vegetation, soil organic matter, wool, and horn) are widely used to calculate the C3/C4 ratio based on a two-member mixed model^{1–3}. A single mean value of C4 end-member is usually used because it is weak responsive to environmental variables. However, this view should be changed based on a number of studies. The Δ of C4 plants is closely related to the environmental variables of precipitation^{4,5}, atmospheric CO₂ concentration^{6,7} and human disturbance, such as grazing⁸, as these factors can affect the ecophysiological responses of C4 plants. Further, leaf nitrogen concentration (N_{leaf}) is considered a possible physiological driver of the variation in Δ ⁸. A better understanding of the mechanisms underlying the influence of N_{leaf} on Δ is fundamental to predict the variation in Δ in C4 plants, especially in the light of the doubled availability of reactive global nitrogen over the last 50 years⁹.

The Δ in C4 plants is influenced by many factors, such as isotope effects during diffusion of CO₂ through stomatal pore and cell walls, fixation of bicarbonate by phosphoenolpyruvate carboxylase (PEPC) in mesophyll cells, fixation of CO₂ by Rubisco in bundle sheath cells, and leakage of CO₂ from bundle sheath cells to mesophyll cells^{10,11}. Farquhar *et al.*¹¹ proposed a simplified model, which was widely used to analyze the relationships between environment factors and Δ . The Δ in C4 plants depends on the ratio of intercellular to ambient CO₂ concentration (C_i/C_a) and bundle sheath leakiness (Φ , the proportion of C fixed by PEP carboxylation, which subsequently leaks out of the bundle sheath):

$$\Delta = a + (b_4 + b_3 \cdot \phi - a) \cdot C_i/C_a \quad (1)$$

where a is the discrimination of ¹³C during diffusion of CO₂ through stomata (4.4‰), b_3 is the fixation by Rubisco 27‰ for C4 plants¹², and b_4 is the hydration of CO₂ to HCO₃[−] and fixation by PEP carboxylase (PEPC) 5.7‰ depending on the temperature¹³.

In equation (1), Δ varies with Φ and C_i/C_a . Φ is an important C4 photosynthesis parameter and is mostly affected by the CO₂ concentration gradient between bundle sheath and mesophyll cells, and thus by factors influencing the activity ratio of Rubisco to PEPC¹¹. A high value of Φ represents inefficiency in the CO₂ concentration processes and increases the quantum requirement in C4 photosynthesis^{11,14}. Variation in N_{leaf} can influence the

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	Source of variation ^a					
	A	g_s	N_{leaf}	C_i/C_a	Δ	Φ
N supply	*	ns	**	**	ns	**
W supply	**	**	*	*	ns	ns
N supply × W supply	**	*	ns	**	ns	*

Table 1. Statistical significance of photosynthetic rate (A), stomatal conductance (g_s), leaf nitrogen content (N_{leaf}), the ratio of intercellular to ambient CO₂ concentration (C_i/C_a), carbon isotope discrimination (Δ), and bundle sheath leakiness (Φ) in *Cleistogenes squarrosa* responses to N and water (W) supply in the pot experiment. **, *, and ns for $P < 0.05$, $P < 0.01$, and not significant, respectively.

allotment of nitrogen to Rubisco and PEPC. Under nitrogen-rich conditions, N_{leaf} is high and PEPC activity can increase to a greater extent than Rubisco, resulting in a high CO₂ concentration gradient between bundle sheath and mesophyll cells and then a high Φ value^{15, 16}. However, different results were also reported in several studies^{17–19} and thus the relationship between N_{leaf} and Φ might be species-specific. In terms of C_i/C_a , increasing N_{leaf} improves the allotment of nitrogen to photosynthetic enzymes and further decreases the C_i/C_a through stimulating the photosynthetic capacity of the plant^{20, 21}. C_i/C_a also depends on stomatal conductance, which is influenced by the vapor pressure deficit and leaf water potential affected by available water in the soil²². Low soil water availability could decrease C_i/C_a by closing stomata and influence the nitrogen uptake by roots. Hence, the effects of N_{leaf} and soil water on C_i/C_a might interact and be difficult to distinguish. Thus, the mechanism of how N_{leaf} influence both Φ and C_i/C_a , and in turn Δ is still poorly understood.

Cleistogenes squarrosa (Trin. ex Ledeb.) Keng is a dominant C₄ plants that occurs across a wide range of habitats, such as meadow steppe, typical steppe, desert steppe, and sand dune ecosystems in the semi-arid Inner Mongolian grassland. As a NAD-ME subtype lacking a suberized lamella²³, *C. squarrosa* is more sensitive to N and water supply. In this study, we measured Δ and photosynthetic gas exchange and obtained Φ under increased N supply and limited soil water (W). The effects of N_{leaf} on Φ and C_i/C_a and the determination of Φ and C_i/C_a on Δ were assessed. Specifically, the following three questions were addressed: Firstly, how do different N and W supply affect Δ , N_{leaf} , and related gas exchange? Secondly, how does N_{leaf} affect Φ and C_i/C_a ? Thirdly, which is the major factor affecting $\Delta - \Phi$ or C_i/C_a ?

Results

Effects of N and water supply on Δ and related parameters in the pot experiment. In the pot experiment, the values of Δ varied from 5.14‰ to 6.85‰ (SD = 0.37‰). N and W supply had no significant effect on Δ , although $P = 0.06$ for N supply effect (Table 1). High N supply and low W supply enhanced N_{leaf} and decreased C_i/C_a , and the N × W interaction was significant for C_i/C_a . C_i/C_a was lower, in spite of higher g_s , under low W supply. Φ increased with N supply and showed no response to water supply while the N × W interaction was significant. Φ and C_i/C_a varied between 0.43 and 0.77 (average = 0.51, SD = 0.07) and between 0.18 and 0.67 (average = 0.43, SD = 0.17), respectively.

The effects of N supply on A, g_s , C_i/C_a , and N_{leaf} were different under different W supply (Fig. 1). Under high W supply, higher N supply enhanced N_{leaf} and stimulated A. C_i/C_a decreased rapidly with more N supply although g_s increased. Compared to no N supply, A was three times higher and C_i/C_a was three times lower at the N supply of 56.0 g N m⁻². Under low W supply, N supply had no significant effect on A, g_s , or C_i/C_a , except for increasing N_{leaf} . The effects of W supply on A, g_s , C_i/C_a , and N_{leaf} were also different under different N supply (Fig. 1). At no or low N supplies (e.g., 10.5 g N m⁻²), low W supply enhanced N_{leaf} and g_s and then stimulated A, and decreased C_i/C_a . However, at the N supply of 56.0 g N m⁻², low W supply decreased A and C_i/C_a in spite of a slight increase in N_{leaf} .

The responses of Φ and Δ to N supply were dependent on W supply (Fig. 1). Under high W supply, N supply increased Φ and had no effect on Δ . Φ was highest at the N supply of 56.0 g N m⁻² and lowest at no N supply. Under low W supply, Φ had no response to N supply, while Δ was slightly higher at the N supply of 56.0 g N m⁻² compared to no N supply. In terms of W supply, higher W supply increased Φ at the N supply of 56.0 g N m⁻².

Effects of N supply on Δ and related parameters in the field experiment. In the field experiment, N supply had no significant effect on Δ and the related parameters of A, g_s , C_i/C_a , N_{leaf} , and Φ . However, N_{leaf} and Φ showed trends of increasing, while C_i/C_a showed a decreasing trend, which was similar to the results of the pot experiment. Φ and C_i/C_a varied between 0.52 and 0.77 (average = 0.59, SD = 0.06) and between 0.30 and 0.70 (average = 0.54, SD = 0.11) respectively. The averages of C_i/C_a were higher in the field experiment than in the pot experiment, which was probably due to high rainfall (35 mm higher in 2011 than in 2011 during June–July). The values of Δ were higher in the field experiment (average = 7.57‰, SD = 0.18‰) than in the pot experiment (average = 5.84‰, SD = 0.37‰) partly due to higher C_i/C_a .

Effect of N_{leaf} on Φ and C_i/C_a . N_{leaf} exerted similar effects on C_i/C_a and on Φ in the field and pot experiments (Fig. 2). N_{leaf} was negatively related with C_i/C_a ($R^2 = 0.67$, $P = 0.046$ in the field experiment; $R^2 = 0.70$, $P = 0.009$ in the pot experiment) and was positively related with Φ ($R^2 = 0.53$, $P = 0.103$ in the field experiment; $R^2 = 0.52$, $P = 0.045$ in the pot experiment). SEM models showed that N_{leaf} had a strong effect on C_i/C_a but a slight effect on Φ (Fig. 3). Pooling all data, N_{leaf} explained 73% of the variation in C_i/C_a ($R^2 = 0.73$, $N = 14$, $P < 0.001$). C_i/C_a decreased by approximately 71% with increasing N_{leaf} from 2.2% to 4.1%, and g_s had no significant effect on C_i/C_a .

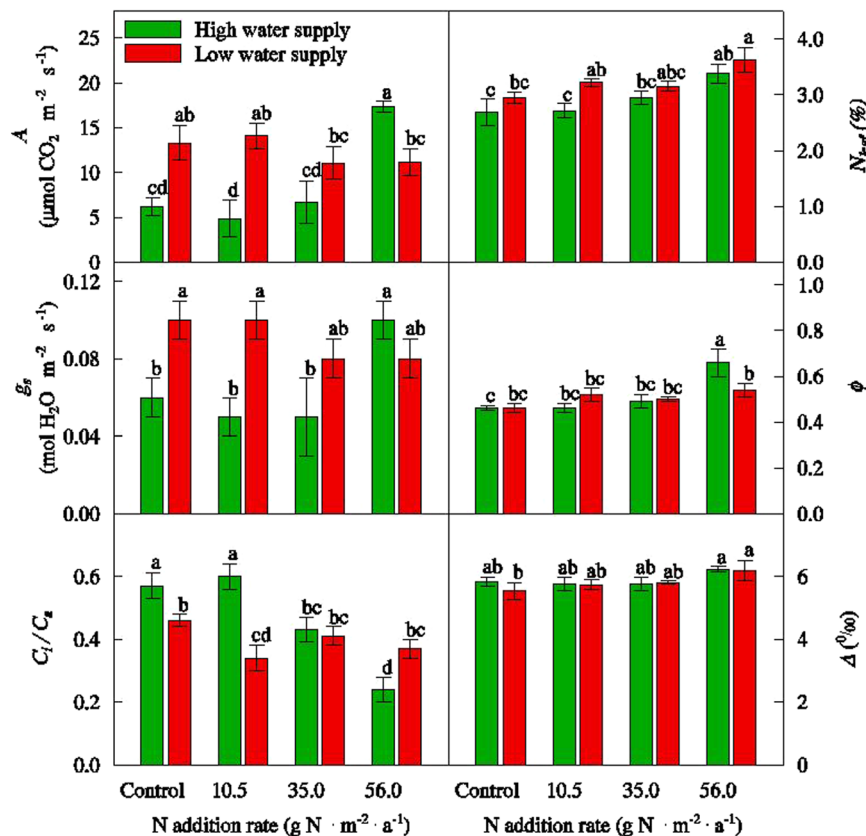


Figure 1. Photosynthetic rate (A), stomatal conductance (g_s), ratio of internal to ambient CO_2 partial pressure (C_i/C_a), leaf nitrogen concentration (N_{leaf}), bundle sheath leakiness (Φ), and carbon isotope discrimination (Δ) in *Cleistogenes squarrosa* grown under variable N and water supply in the pot experiment. Error bars indicate standard error ($N = 4$). Different letters indicate significant differences between treatments at $P = 0.05$.

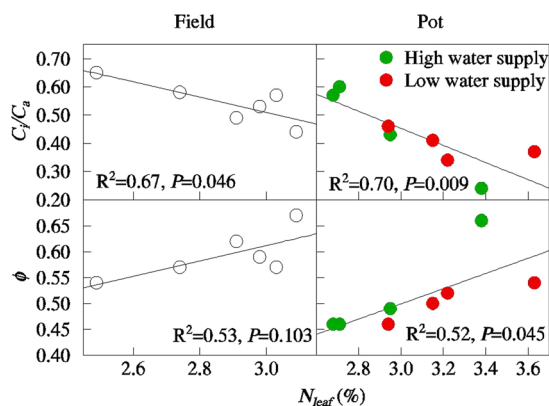


Figure 2. Relationships among leaf nitrogen concentration (N_{leaf}), bundle sheath leakiness (Φ), and the ratio of internal and ambient CO_2 concentrations (C_i/C_a). Each data point shows the mean of samples taken from the pot experiment ($N = 4$) or the field experiment ($N = 3$).

Effect of Φ and C_i/C_a on Δ . C_i/C_a was negatively related with Δ ($R^2 = 0.86$, $P = 0.008$ in the field experiment; $R^2 = 0.31$, $P = 0.152$ in the pot experiment) while Φ was positively related with Δ ($R^2 = 0.71$, $P = 0.034$ in the field experiment; $R^2 = 0.65$, $P = 0.015$ in the pot experiment) (Fig. 4). SEM models further showed that both Φ and C_i/C_a were important to variations in Δ and the contribution of Φ was higher than C_i/C_a (Fig. 3). N_{leaf} influenced Δ primarily through C_i/C_a . The effect of g_s on Δ was weak.

Discussion

Our results showed N_{leaf} had more effect on C_i/C_a than Φ (Fig. 3). Firstly, the increase in N_{leaf} strongly decreased C_i/C_a . N_{leaf} might stimulate photosynthesis and decrease C_i/C_a by increasing the amount of Rubisco and PEPC,

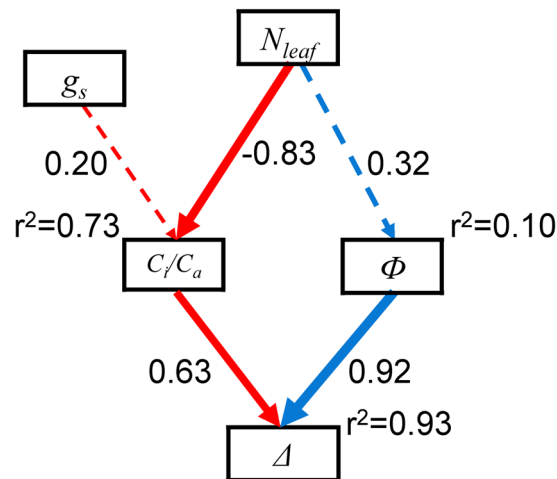


Figure 3. Structural equation modeling (SEM) analysis examining the effects of leaf nitrogen concentration (N_{leaf}) and stomatal conductance (g_s) on the ratio of internal and ambient CO₂ concentrations (C_i/C_a) and leakiness (Φ), and stable carbon isotope discrimination (Δ). Square boxes indicate variables included in the model. Results of model fitting: $\chi^2 = 6.838$, $P = 0.233$, d.f. = 5, $N = 14$ (Note that high P -values associated with χ^2 tests indicate good model fit to data, i.e., no significant discrepancies). Solid arrows connecting the boxes indicate significant positive and negative effects ($P < 0.05$), respectively; the pathways without significant effects are indicated by broken lines ($P > 0.05$). r^2 values associated with response variables indicate the proportion of variation explained by relationships with other variables. Values associated with solid arrows represent standardized path coefficients.

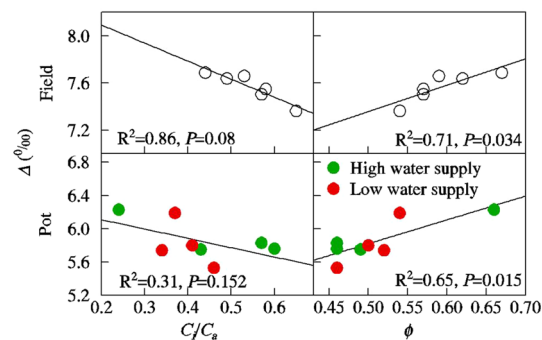


Figure 4. Relationships among stable carbon isotope discrimination (Δ), bundle sheath leakiness (Φ), and the ratio of internal and ambient CO₂ concentrations (C_i/C_a). Each data point shows the mean of samples taken from the pot experiment ($N = 4$) or the field experiment ($N = 3$).

or by increasing allocation to photosynthetic organs, such as chloroplasts²⁴. Secondly, N_{leaf} had a slight effect on Φ . This species might be able to co-ordinate the activities of Rubisco and PEPC, maintaining a stable Rubisco/PEPC ratio and the difference between dissolved CO₂ in the bundle sheath and in the mesophyll cells, similar to other NAD-ME subtypes⁶. The stability in Φ indicates stable photosynthetic efficiency, which could be one of the physiological reasons why *C. squarrosa* occurs widely in different habitats across the Mongolia grassland.

Our previous survey of N_{leaf} and Δ in *C. squarrosa* showed that N_{leaf} was negatively correlated with Δ across precipitation at the regional scale, the stocking rate in the grazing experiment, and the leaf position within a tiller on the Mongolia Plateau⁸. This study further demonstrated that N_{leaf} influenced Δ , mostly by C_i/C_a rather than Φ (Fig. 3). However, Φ was also important to the variation in Δ and should be considered, although it was not influenced by N_{leaf} . Previous studies on C4 plant physiology reported that the Δ values in dry leaf were higher in shade conditions due to relatively more leakage of CO₂ produced during bundle sheath respiration^{25–27}. The intensity of shade could be expected to be higher in regions with more precipitation and higher plant biomass²⁸, or in areas with higher stocking rate and lower plant cover within the concept of grazing experiment²⁹, or for the lowermost leaf within a tiller. Hence, higher Φ produced by shade in these places could also contribute to higher Δ , which could make the negative relationship between N_{leaf} and Δ stronger. This effect of shade on Δ could also have occurred in the field plots with N addition in this study as dominant C3 plants (above 30 cm) were significant taller than *C. squarrosa* (approximately 10 cm).

Two factors can influence the bundle sheath leakiness of C4 plants. One is the difference between dissolved CO₂ in the bundle sheath and in the mesophyll cells determined by the Rubisco/PEPC ratio, and another is the conductance to leakage determined by a bundle sheath cell wall containing a suberized lamella¹¹. The conductance to leakage in *C. squarrosa* was high due to the lack of suberized lamella, which is typical of NAD-ME subtypes^{30,31}. Hence, the value of leakiness should be strongly influenced by the ratio of Rubisco and PEPC activity. Under higher N addition, soil available N could be rich, resulting in higher N_{leaf} (Fig. 1). Higher N_{leaf} could contribute to higher Φ (Fig. 1) by decreasing the Rubisco/PEPC ratio, which was indirectly confirmed³² and supported by previous studies on the negative relationship between leakiness and Rubisco/PEPC ratio^{15,16}. High W supply improved the effect of N_{leaf} on Φ though increasing soil moisture and then plant nitrogen uptake. Meanwhile, Φ had no response to W supply, which was not consistent with the result from previous studies showing an increase in Φ during drought^{7,33} or high vapour pressure deficit³². Possible reason could be different species or methods. The underlying mechanisms of low W supply on Φ though decreasing C_i/C_a was still unclear. In terms of the survival of *C. squarrosa* stable Φ under low W supply highlights the physiological tolerance.

The extent to which C_i/C_a and Φ affect Δ is a subject or debate. Previous studies reported that Δ was mostly influenced by C_i/C_a due to variations in g_s ^{34,35}. Conversely, C_i/C_a was primarily controlled by N_{leaf} not g_s , and Φ accounted for most of the variations observed in Δ in *C. squarrosa* under changing N and W conditions. This phenomenon can also appear in some NADP-ME and PCK subtypes^{6,33,36}. For instance, *Aristida* spp. deviates from the classical NADP-ME bundle sheath anatomy as it lacks suberized lamellae³⁷. Schulze *et al.*³⁸ reported a wide range of Δ in *Aristida*. The value of Δ was substantially mirrored by changes in Φ . Hence, we think that influences of C_i/C_a and Φ on Δ may be species-dependent and vary with different morphological and anatomical characteristics of bundle sheath cells. Also, our results shed light on the effect of Φ on Δ because g_s were normally regarded as a major cause. More and more studies in recent years reported that Φ can be changed by environmental conditions using online measurement or model methods. For instance, high vapour pressure deficit increased Φ ³²; shade reduced Φ ¹⁹. It was demonstrated that the balance of C3 cycle and C4 cycle could be changed by those environmental conditions. Hence, how to predict Δ became a challenge.

Implications. The variation in C4 end-member value in the C3/C4 mixed equation contributes to the uncertainty of estimated C4 percent. Approximately 20 C4 species occur frequently in the Inner Mongolia grassland³⁹. More than 70% of them are annual and their growth is opportunistic depending on the frequency and amount of rainfall. The Δ value of *C. squarrosa* can represent the majority of vegetation as it is a dominant perennial species in the C4 community across the Inner Mongolia grassland. Pooling the measured values in our study with those from the same region⁸, *C. squarrosa* had a large range in Δ from 5.14‰ to 8.90‰. Although the value of Δ is relatively stable and independent of soil available N and W or air temperature³, the large range of 3.76‰ can cause a bias in the estimated C4 percent. For instance, given that the mean of C3 plants and bulk vegetation is 17‰ and 15‰ respectively³, the resulting C4 is 17% when 5.14‰ is used and 25% when 8.90‰ is used. Hence, we suggest that it is better to use a well modeled Δ of C4 plants (e.g., geographic distribution based on more sampling) in the C3/C4 mixed equation.

Φ is a key parameter which can affect the photosynthetic efficiency of C4 plants and it is extremely difficult to measure it directly. Previous studies reported dry matter Δ yield values of 0.31–0.45^{10,11,13}. The average of Φ in *C. squarrosa* was 0.55 although scattered and higher than that reported above, which indicates its lower photosynthetic efficiency in C4 plants. It appears that the photosynthetic performance of this species is closer to C3 plants than other C4 plants such as *Setaria viridis* and *Amaranthus retroflexus*. Relatively low photosynthetic efficiency, together with low height and shallow root depth, could be the major reason for weak competitiveness capacity compared to coexistent C3 plants.

The new technology development, such as tunable diode laser absorption spectroscopy, provides new opportunities for rapidly and concurrently measuring Δ and CO₂ assimilation, which facilitate measurement of photosynthetic Δ and calculation of Φ ⁴⁰. The advantage of this method compared to dry matter Δ is that it is not influenced by post-photosynthesis discrimination⁴¹. In order to better understand the effect of Φ on dry matter Δ , more measurements are needed to obtain photosynthetic Δ .

Conclusions

Leaf nitrogen influenced the Δ of C4 plants primarily though C_i/C_a , rather than Φ . Both Φ and C_i/C_a determined Δ together and the contribution of Φ was stronger. Our study highlights that Φ should be well considered in predicting the Δ of C4 plants.

Methods

Study site. The study was carried out at the Inner Mongolia Grassland Ecosystem Research Station (IMGERS: 43°13'N, 116°14'E), which is located in the Xilin River Basin, Inner Mongolia Autonomous Region of China⁴². The climate in the area is continental temperate semi-arid climate, which is characterized by a cold and dry winter with a warm and moist summer⁴³. Long-term (1980–2013) mean annual temperature was 0.9°C. Long-term mean annual precipitation was 351.4 mm, with 72.8% falling during the growing season (May–August). The soil is classified as Calcic-Orthic Aridisol by the U.S. soil classification system. *Stipa grandis* and *Leymus chinensis*, which are domain C3 species, together accounted for >60% of aboveground biomass in the community. *C. squarrosa* is a domain C4 species and normally starts growing at the beginning of June. The annual ambient atmosphere N deposition was <1.0 g N m⁻²⁹.

Pot experiment. To address the first scientific question, we used controlled pot experiment. The pot experiment was carried out at the IMGERS in 2013. To examine the response of *C. squarrosa* to N addition and drought, we used a two-way factorial experimental design. Four N addition treatments were used: control, 10.5, 35.0, and

56.0 g N m⁻² (as urea) and two W supply treatments: high W supply (ambient rainfall, 160 mm from the beginning of June to the end of July) and low W supply (65% ambient rainfall, 104 mm). Four replicate pots were set for each treatment. We collected seeds from a grassland population near the IMGERS and sowed them in the pots at the beginning of May. Approximately 20 seedlings were sown in one pot (30 cm diameter × 30 cm deep) and received natural rainfall until the end of May. N fertilizers were dissolved in 1 L water, equal to 3.5 mm rainfall, and were then applied to each pot at the beginning of June. The same amount of water was also applied to no N addition plots. A rain shelter was built to prevent ambient rainfall. The amounts of water representing precipitation were 104 mm in June and July and equal amounts of water were applied every 5 days. The water came from a nearby well, with N, phosphorous, and potassium concentrations below detectable levels.

The rain shelter was made of steel pipes, covering 90 m² (6 m × 15 m), and had a slanted roof made of waterproof cloth that could be wound up with a steel roller. The heights of the frame were 50 cm at the south side and 90 cm at the north side respectively. On dry days, the waterproof cloth was rolled to the top of the north side to withdraw the roof from the shelter. When rain was coming, the cloth was rolled back to form the roof of the shelter, and rolled back after rain stopped. Because the shelter was only used during rain events, no significant difference occurred in air moisture and temperature, or light between the inside and outside the shelter. We placed the pots in the soil with a 3-cm edge above the soil surface and the pots at least 1 m away from the edge of each side which followed the suggestions of Heisler-White *et al.*⁴⁴ and Liu *et al.*⁴⁵ that a buffer zone of 0.5 m is effective to avoid receiving rainfall. The aisles between treatments pots were 0.4 m.

Field experiment. To address the second and third questions, we used a long-term field N addition experiment⁴⁶. The field N addition experiment was conducted in a *Leymus chinensis* grassland which had been fenced since 1999 to prevent grazing by large animals. Seven treatments included: control, 0, 5.6, 11.2, 22.4, 39.2, 56.0 g N m⁻² (added as urea). Each treatment had six replicates. Each plot, except for the control, also received 1.6 g P m⁻² (as KH₂PO₄). The fertilizer mixed with sand applied to the plot surfaces in May during 2006–2011.

Gas exchange measurement, sample collection and laboratory analysis. The three most mature, fully expanded, and sun-exposed leaves were chosen from each treatment to measure photosynthetic gas exchange parameters at the end of July 2011 (in the field experiment) and at the beginning of August 2013 (in the pot experiment). Gas exchange parameters including photosynthetic rate (*A*), stomatal conductance (*g_s*), and *C_i* were measured during 08:00–11:30 with an open gas-exchange system (LI-6400, Li-Cor, Lincoln, NE, USA). A 2 × 3 cm² broad leaf chamber with a light source (LI-6400-02B, Li-Cor, Lincoln, NE, USA) was used with the CO₂ concentration at 400 μmol mol⁻¹ and a saturation irradiance of 1500 μmol m⁻² s⁻¹ for the light source. After measuring gas exchange we collected 30 individual leaves from each treatment in both experiments. All samples were dried at 60 °C for 24 hours in a forced-draught oven and homogenized with a ball mill. The carbon isotope composition and N content were then measured using an elemental analyzer (NA 1110; Carlo Erba, Milan) interfaced (ConFlo III; Finnigan MAT, Bremen) with an isotope ratio mass spectrometer (Finnigan MAT253). Carbon isotope data were specified as δ¹³C relative to the Vienna Pee Dee Belemnite standard:

$$\delta^{13}\text{C} = \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \quad (2)$$

where *R_{sample}* and *R_{standard}* are the ratios of ¹³C/¹²C in the sample and standard, respectively.

The precision for sample repeats was better than 0.15‰ for δ¹³C and 0.04‰ for N content in dry matter.

To calculate discrimination, the δ¹³C_{air} values for 2011 and 2013 were obtained from the US National Oceanic and Atmospheric Administration using data from the Ulaan Uul station, which is the closest one located approximately 460 km northwest of IMGERS. Discrimination was calculated as:

$$\Delta = \frac{\delta^{13}\text{C}_{\text{air}} - \delta^{13}\text{C}_{\text{leaf}}}{1 + \delta^{13}\text{C}_{\text{leaf}}} \quad (3)$$

The values of Φ were calculated based on Δ and *C_i/C_a* using equation (1).

Statistical analysis. Linear regressions were used to evaluate relationships between *N_{leaf}*, Φ, and *C_i/C_a* and between *C_i/C_a*, Φ, and Δ. Two-way analysis of variance (ANOVA) was used to assess effects of N and W supply on *A*, *g_s*, *C_i/C_a*, *N_{leaf}*, Φ, and Δ. One-way ANOVA followed by the LSD multiple range tests was used to evaluate the effects of N addition on these response variables under the treatments of ambient precipitation and drought. Structural equation modeling (SEM) was performed to analyze different hypothetical pathways that may explain the effect of *N_{leaf}* and *g_s* on *C_i/C_a* and Φ and determine the extent to which Δ was influenced by Φ and *C_i/C_a*. All procedures were carried out in SPSS Version 18.0 (SPSS Inc., Chicago, USA).

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Author Contributions

Hao Yang, the first and corresponding author of the paper, main responsibility for experimental design, data collection, analysis and writing; Qiang Yu, Wen-ping Sheng, Sheng-gong Li, Jing Tian, significant contribution to the interpretation of data. All authors reviewed the manuscript.

Additional Information

Competing Interests: The authors declare that they have no competing interests.

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