Evaluating the response of δ ^{13}C in *Haloxylon ammodendron,* a dominant C_4 species in Asian desert ecosystem, to water and nitrogen addition as well as the availability of its δ^{13}C as the indicator of water use-efficiency

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Abstract

Variations in precipitation and atmospheric N deposition affect water and N availability in desert, and thus may have significant effects on desert ecosystems. *Haloxylon ammodendron* is a dominant plant in Asian desert, and addressing its physiological acclimatization to the changes in precipitation and N deposition can provide an insight into how desert plants adapt extreme environment by physiological adjustment. Carbon isotope ratio ($\delta^{13}C$) in plants has been suggested as a sensitive long-term indicator of physiological acclimatization. Therefore, this study evaluated the effect of precipitation change and increasing atmospheric N deposition on $\delta^{13}C$ of *H. ammodendron*. Furthermore, *Haloxylon ammodendron* is a C_4 plant, whether its $\delta^{13}C$ can indicate water use-efficiency (WUE) has not been addressed. In the present study, we designed a field experiment with a completely randomized factorial combination of N and water, and measured $\delta^{13}C$ and gas exchange of *H. ammodendron*, then we calculated the degree of bundle-sheath leakiness (φ) and WUE of the assimilating branches of *H. ammodendron*. $\delta^{13}C$ and φ remained stable under N and water supply, while N addition, water addition and their interaction affected gas exchange and WUE in *H. ammodendron*. In addition, $\delta^{13}C$ had no correlation with WUE. These results were associated with the irrelevance between $\delta^{13}C$ and the ratio of intercellular to ambient CO_2 concentration (c_i/c_a), which might be caused by a special value (0.37) of the degree of bundle-sheath leakiness (φ) or a lower activity of carbonic anhydrase (CA) of *H. ammodendron*. In conclusion, $\delta^{13}C$ of *H. ammodendron* is not sensitive to global change in precipitation and atmospheric N

deposition, and cannot be used for indicating its WUE.

1 Introduction

Recently, global precipitation pattern has changed significantly (Frank et al., 2015; Knapp et al., 2015), and atmospheric N deposition has continued to rise (Galloway et al., 2004; Liu et al., 2013; Song et al., 2017). Previous researchers have suggested that arid ecosystems are most sensitive to climate change (Reynolds et al., 2007; Huang et al., 2016), while global change in precipitation and atmospheric N deposition has an important impact on water and N availability in desert (Huang et al., 2018). Thus, these changes may have significant effects on desert ecosystems. *Haloxylon ammodendron* is a dominant species in desert regions, especially in Asia. Studying the physiological responses of *H. ammodendron* to global change can provide an insight into how desert plants adapt extreme environment by physiological adjustment. Carbon isotope ratio ($\delta^{13}C$) in plants depends on the ratio of intercellular to ambient CO_2 concentration (c_i/c_a), which reflects the balance between inward CO_2 diffusion rate, regulated by stomatal conductance (g_s), and CO_2 assimilating rate (A) (Farquhar and Richards, 1984), and has been suggested as a sensitive long-term indicator of physiological acclimatization (Battipaglia et al., 2013; Cernusak et al., 2013; Tranan and Schubertt, 2016; Wang and Feng, 2012). Therefore, investigating the variations in $\delta^{13}C$ of *H. ammodendron* under water and nitrogen addition can enhance understanding of physiological responses of desert plants to future changes in precipitation and atmospheric N deposition.

A large quantity of works has been devoted to the relationships between C_3 plant $\delta^{13}C$ and water availability or precipitation (e.g., Diefendorf et al., 2010; Kohn, 2010; Liu et al., 2005; Ma et al., 2012; Serret et al., 2018; Stewart et al., 1995; Wang et al., 2005, 2008) and nitrogen availability (e.g. Cernusak et al., 2007; Li et al., 2016; Sparks and Ehleringer, 1997; Yao et al., 2011; Zhang et al., 2015). However, a relatively small amount of research has focused on the responses of C_4 plant $\delta^{13}C$ to water availability or precipitation (Ellsworth et al., 2017; Liu et al., 2005; Rao et al., 2017; Wang et al., 2006) and nitrogen availability (Ma et al., 2016; Schmidt et al., 1993). For C_4 plants, $\delta^{13}C$ is controlled by both the c_i/c_a ratio and the degree of bundle-sheath leakiness (φ), the proportion of CO_2 produced within bundle sheath cells from C_4 acids that leaks back to mesophyll cells (Ellsworth and Cousins, 2016; Ellsworth et al., 2017; Farquhar, 1983). Thus, the responses of C_4 plant $\delta^{13}C$ to water and N availability are also affected by φ. Genetic factors control φ values, which causes the interspecific differences in $\delta^{13}C$, even the responses of plant $\delta^{13}C$ to water and N availability (Gresset et al., 2014). On the other hand, enzymatic activity of carbonic anhydrase (CA) may influence $\delta^{13}C$ in C_4 plants (Cousins et al., 2006). CA is an enzyme that catalyzes the hydration of CO_2 in mesophyll cells to form bicarbonate (HCO_3^-). Previous studies showed that CA activity in most C_4 plants is usually low, just sufficient to support photosynthesis (Cousins et al., 2006; Gillon and Yakir, 2000, 2001; Hatch and Burnell, 1990). *H. ammodendron* is a typical C_4 plant. How its $\delta^{13}C$ responds to water and N availability has never been addressed.

Foliar $\delta^{13}C$ in C_3 plants has been considered as a useful indicator of intrinsic water use-efficiency (WUE) (Farquhar, 1983). However, although some studies have suggested that $\delta^{13}C$ of C_4 plants could also indicate its WUE (Henderson et al., 1992; Wang et al., 2005; Cernusak et al., 2013; Ellsworth and Cousins, 2016), this statement is still controversial. The relationship between $\delta^{13}C$ and WUE is based on the links between c_i/c_a ratio and $\delta^{13}C$ and between c_i/c_a ratio and WUE (Ehleringer and Cerling, 1995). For C_3 plants, $\delta^{13}C$ always decreases with an increase in c_i/c_a ratio; but for C_4 plants, the correlation between $\delta^{13}C$ and c_i/c_a ratio depends on φ value (Cernusak et al., 2013) and CA activity (Cousins et al., 2006). As mentioned above, φ value is under genetic control, and the CA activity changes across species (Cousins et al., 2006; Gillon and Yakir, 2000, 2001; Hatch and Burnell, 1990), thus, the correlation between $\delta^{13}C$ and c_i/c_a ratio, as well as the relationship between WUE and $\delta^{13}C$, shows interspecific difference. Whether $\delta^{13}C$ of *H. ammodendron* indicates WUE has never been evaluated.

In this study, we designed an experiment with multiple water and nitrogen supply in the southern Gurbantunggut Desert in Xinjiang Uygur Autonomous Region, China. We measured the $\delta^{13}C$, gas exchange and WUE of the assimilating branches of *H. ammodendron*. We had two objectives. One objective was to evaluate the response of the dominant plant of Asian desert to future changes in precipitation and atmospheric N deposition by revealing the effects of water and N supply on $\delta^{13}C$ of *H. ammodendron*. The other was to explore the availability of $\delta^{13}C$ as the indicator of water use-efficiency in *H. ammodendron*.

2 Materials and methods

2.1 Definitions and Basic Equations

Stable carbon isotopic ratio ($\delta^{13}C$) of natural materials is expressed as:

$$\delta 13C(‰)=\left[\frac{(13C/12C)_{sample}}{(13C/12C)_{standard}}-1\right]\times 1000 \tag{1}$$

where $(^{13}C/^{12}C)_{sample}$ and $(^{13}C/^{12}C)_{standard}$ are the $^{13}C/^{12}C$ ratio of the sample and of Pee Dee Belemnite standard (PDB), respectively. . Farquhar (1983) proposed the pattern of carbon isotopic discrimination (Δ) in C_4 plant:

$$\Delta=\frac{\delta 13C_{air}-\delta 13C_{plant}}{1+\delta 13C_{plant}/1000}\approx \delta 13C_{air}-\delta 13C_{plant}=a+[b_4+\varphi\,(b-s)-a]\frac{c_i}{c_a} \tag{2}$$

where $\delta^{13}C_{plant}$ and $\delta^{13}C_{air}$ are the $\delta^{13}C$ values of plants and CO_2 in the ambient air，respectively. The parameter a (= 4.4‰, Craig, 1954) is the carbon isotopic fractionation in the diffusion of CO_2 into internal leaves; b_4 (= -5.9‰, O'Leary, 1984) is the combined carbon isotopic fractionations occurring in the processes of gaseous CO_2 dissolution, hydration/dehydration reactions of CO_2 and HCO_3^- in mesophyll cells, and HCO_3^-carboxylation by PEP (phosphoenolpyruvate) carboxylase; s (= 1.8‰, O'Leary, 1984) is the carbon isotopic fractionation during diffusion of CO_2 out of the bundle-sheath cells, and b (= 27‰, Farquhar and Richards, 1984) is the carbon isotopic fractionation of CO_2 carboxylation by RuBP (ribulose-1,5-bisphosphate) carboxylase. The variable φ is the proportion of CO_2 producing within bundle sheath cells from C_4 acids that leaks back to mesophyll cells, and c_i/c_a is the ratio of intercellular to ambient CO_2 concentration. Eq. (2) can be transformed into the following format:

$$\delta^{13}C_{plant}=-[b_4+\varphi(b-s)-a]\frac{c_i}{c_a}+\delta^{13}C_{air}-a \tag{3}$$

According to Eq. (3), if the coefficient $[b_4 + \varphi (b - s) - a]$ is greater than 0, $\delta^{13}C$ decreases with increasing c_i/c_a; if this coefficient is lower than 0, $\delta^{13}C$ increases with increasing c_i/c_a.

Water use-efficiency (WUE) is defined as the amount of assimilated carbon dioxide by plants under the consumption of per unit water. There are two characteristics of WUE, instantaneous WUE (ins-WUE) and intrinsic WUE (int-WUE), respectively. ins-WUE can be calculated by:

$$\text{ins-WUE} = A/E = (c_a - c_i)/1.6v = c_a(1-c_i/c_a)/1.6v \tag{4}$$

where A is photosynthetic rate, E is transpiration rate and v is calculated by:

$$v = (e_i-e_a)/p \tag{5}$$

where e_i and e_a are the water vapor pressure inside and outside the leaves, p is the atmospheric pressure.

The definition of int-WUE is:

$$\text{int-WUE} = A/g_s = (c_a - c_i)/1.6 = c_a(1-c_i/c_a)/1.6 \tag{6}$$

where g_s is stomatal conductance.

2.2 Study site and species

This experiment was conducted at the Fukang Station of Desert Ecology, Chinese Academy of Sciences, on the southern edge of the Gurbantunggut Desert (44°26′ N, 87°54′ E) in northwestern China. The altitude of the study site is 436.8 m above average sea level (a.s.l.). It is a typical continental arid, temperate climate, with a hot summer and cold winter in the area. The mean annual temperature is 7.1ºC and the mean annual precipitation is 215.6 mm, with a potential evaporation of about 2000 mm. The mean annual temperature and the annual rainfall amount in the sampling year are 10.23ºC and 122.7 mm (Cui, 2018). The soil type is grey desert soils (Chinese classification) with aeolian sands on the surface (0-100 cm). The percentages of clay (< 0.005 mm), silt (0.005-0.063 mm), fine sand (0.063-0.25 mm) and medium sand (0.25-0.5 mm) range from 1.63-1.76%, 13.79-14.15%, 55.91-56.21% and 20.65-23.23%, respectively (Chen et al., 2007). The soil is highly alkaline ($pH = 9.55 \pm 0.14$) with low fertility. The vegetation is dominated by *Haloxylon ammodendron* and *Haloxylon persicum* with about 30% coverage. Herbs include ephemerals, annuals and small perennials, with a cover of ca. 40% (Fan et al., 2013). Although the coverage of the two *Haloxylon* species is a little lower than that of herbs, the biomass of the former is much larger than that of the latter, because *Haloxylon* plants are shrubs with an average height of 1.5 m whereas the latter are very low herbaceous plants. Biological soil crusts are distributed widely on the soil between the herbs and *Haloxylon*, with almost 40% coverage (Zhang et al., 2007).

The present study focused on *Haloxylon ammodendron* because it is the dominant species in Asian desert. *Haloxylon ammodendron* is a species of Chenopodiaceae, which is a xerophytic and halophytic woody plant (Cui et al., 2017). The leaves of *H. ammodendron* have been completely degraded due to the extreme drought, and the assimilation branches, which are the glossy green branches (Fig. S1), perform the same functions as the leaves. Due to its drought tolerance, *H. ammodendron* is widely distributed in desert areas.

2.3 Experimental design

A field experiment with a completely randomized factorial combination of water and nitrogen has been conducted from 2014 to 2017. We designed two water addition levels (0, 60mm$\cdot$yr^{-1}; W0, W1) based on the prediction that precipitation will increase by 30% in northern China in the next 30 years (Liu et al., 2010), and three levels of N addition (0, 30, 60 kg N$\cdot$ha$^{-1}\cdot$yr^{-1}; N0, N1 and N2), because N deposition has reached 35.4 kg N$\cdot$ha$^{-1}\cdot$yr^{-1} in the nearby city, Urumqi (Cui et al., 2017) and will double by 2050 relative to the early 1990s (Galloway et al., 2008). Therefore, there were six treatments (W0N0, W0N1, W0N2, W1N0, W1N1, W1N2) in this experiment. Four replicates of each treatment were set, making a total of 24 plots with a size of 10 m × 10 m. A small sub-plot with a size of 1.5 m×1.5 m was set in each plot. A well-grown *H. ammodendron* was enclosed in the center of the sub-plot. The average height and coverage of an individual *H. ammodendron* were 1.5 m and 1.9 m^2, respectively, and did not vary significantly across the plots. The type of nitrogen used in the present study is NH_4NO_3. To simulate natural water and N inputs, the treatments were applied in equal amounts, twelve times, once a week in April, July and September, as 5 mm$\cdot$m^{-2} of water and 2.5 or 5 kg N$\cdot$ha^{-1} each week (Cui et al., 2017). Usually, water addition was with a sprinkler kettle, irrigating over the canopy of *H. ammodendron*.

2.4 Measurements of gas exchange and WUE

As mentioned above, the main assimilating organ of *H. ammodendron* is the assimilation branches. Thus, we conducted gas exchange measurements on the assimilation branches of the *H. ammodendron* grown in the sub-plots by LI-6400 portable photosynthesis system. The measurements were conducted on 27-29, June 2016, which is the main growing season of *H. ammodendron*. It may be the most appropriate to take measurements during this period, and the results of the measurements are therefore more representative. Previous studies have also usually conducted this measurement during the growing season (Nyongesah and Wang, 2013; Cui, 2018; Gong et al., 2019). The ins-WUE and the int-WUE were calculated based on these measured gas exchange traits by the Eq. (4), and int-WUE by Eq. (6). At each plot, the top assimilating branches of a mature individual was selected randomly for the measurement of gas exchange, which includes photosynthetic rate (A), stomatal conductance (g_s), transpiration rate (E), the ambient CO_2 concentration (c_a) and the intercellular CO_2 concentration (c_i). Before the measurement of gas exchange, it takes about 5s to stabilize after the assimilating branches was inserted in the cuvette. We repeated 10 times on the same assimilating branches for each measurement. Our measurements were carried out under the conditions of a standard 450 $mmol \cdot mol^{-1}$ CO_2 concentration at a flow rate of 500 $mmol \cdot s^{-1}$ above saturation in photo flux density of 1600 $mmol \cdot m^{-2} \cdot s^{-1}$. The temperature of the measuring assimilating branches varied from 29.5 °C to 30.5 °C during the entire period of gas exchange measurements.

2.5 Samples collection

Sample collection was conducted in 20, July, during the addition of water and nitrogen. Considering that there is a considerable difference in $\delta^{13}C$ between buds, young and matured leaves, so we collected the mature assimilating branches of *H. ammodendron* for the $\delta^{13}C$ measurements. All *H. ammodendron* individuals grown in plots (10 m × 10 m) were sampled. Eight pieces of the mature assimilating branches (15-20 cm long) were collected from each individual; two pieces of assimilating branches were collected at each of the four cardinal directions from the positions of full irradiance. All assimilating branches from the same plot were combined into one sample. After the samples were collected, they were immediately divided into two parts randomly and taken back to the laboratory at Fukang Station. The first part was used to determine the chlorophyll content. The second part was immediately inactivated in a 105 °C oven in the laboratory at Fukang Station, and then brought back to Beijing in a ziplock bag. The time interval between sample collection and inactivation is very short. After inactivation, the carbon exchange of the assimilating branches stop, so the isotope composition of the samples will not change anymore. All plant samples of the second part were air-dried immediately in the laboratory in Beijing. Then the samples were ground into a fine powder using a steel ball mixer mill MM200 (Retsch GmbH, Haan, Germany) for the measurements of $\delta^{13}C$ and N contents.

2.6 Measurements of plant $\delta^{13}C$, plant N and chlorophyll contents

The $\delta^{13}C$ and N measurements were performed on a Delta^{Plus} XP mass spectrometer (Thermo Scientific, Bremen, Germany) coupled with an automated elemental analyzer (Flash EA1112, CE Instruments, Wigan, UK) in a continuous flow mode, at the Stable Isotope Laboratory of the College of Resources and Environmental

Sciences, China Agricultural University. The carbon isotopic ratios were reported in the delta notation relative to the V-PDB standard. For this measurement, we obtained standard deviations low than 0.15‰ for $\delta^{13}C$ among replicate measurements of the same sample. And standard deviations for the N measurements were 0.1%.

The chlorophyll contents of all samples were determined immediately when the sample were taken back in the laboratory at Fukang Station. The samples were first extracted by 95% ethyl alcohol (0.5 g sample to 25 mL ethyl alcohol), and then the absorbancy was measured under the wave length of 665 and 649 mm by the spectrophotometer. The content of chlorophyll a, b was calculated by the follow equations:

$$\text{Chlorophyll a (mg/L)} = 13.95 \times \text{OD665} - 6.88 \times \text{OD649} \tag{7}$$

$$\text{Chlorophyll b (mg/L)} = 24.96 \times \text{OD649} - 7.32 \times \text{OD665} \tag{8}$$

where OD665 and OD649 are the absorbancy under the wave length of 665 and 649mm, respectively.

2.7 Calculation of the degree of bundle-sheath leakiness

The degree of bundle-sheath leakiness (φ) was calculated by the transformation of Eq. (2):

$$\varphi = \left(\frac{(\delta^{13}C_{air} - \delta^{13}C_{plant}) \big/ (1+\delta^{13}C_{plant}/1000) - a}{c_i/c_a} + a - b_4 \right) \Big/ (b - s) \tag{9}$$

In this equation, parameters a, b_4, b and s are constant, while $\delta^{13}C_{plant}$ and c_i/c_a are the measured values of our samples. We did not measure the $\delta^{13}C_{air}$ at our study site, so we had to use an approximation of the $\delta^{13}C_{air}$ to do this φ calculation. The approximated value we used is -9.77‰, which has been measured at Donglingshan

Mountain, Beijing, north China in September 2019. The two sites should have similar $\delta^{13}C_{air}$ because the two sites are located in countryside with less human activities and have a similar distance from the nearest city. The straight line distances between Donglingshan Mountain and the city center of Beijing, and between our study site and Urumqi city are about 90 km. In addition, since the $\delta^{13}C_{air}$ has large diurnal and seasonal variations, we used the published range of $\delta^{13}C_{air}$ from May to July in Shangdianzi, China (Data comes from Global Monitoring Laboratory, Earth System Research Laboratories, https://www.esrl.noaa.gov/gmd) to calculate the minimum and maximum $\delta^{13}C_{air}$ of the time period, which is the main growing season for *H. ammodendron*. Finally, the $\delta^{13}C_{air}$ used in the calculation ranged from -10.52‰ to -9.01‰ with an average of -9.77‰.

2.8 Statistical analysis

Statistical analyses were conducted using SPSS software (SPSS for Windows, Version 20.0, Chicago, IL, United States). One-way analysis of variance (ANOVA) and two-way analysis of variance (ANOVA) were used to compare the difference of $\delta^{13}C$ and other physiological traits between each treatment. Pearson analysis was used to determine the correlation among $\delta^{13}C$, WUE and c_i/c_a in *H. ammodendron*.

3 Results

3.1 Plant $\delta^{13}C$ under water and nitrogen addition

The $\delta^{13}C$ of the assimilating branches of *H. ammodendron* in the six treatments W0N0, W0N1, W0N2, W1N0, W1N1, W1N2 was -14.18 ± 0.19 ‰, -14.71 ± 0.35 ‰,

-14.45 ± 0.18 ‰, -14.67 ± 0.40 ‰, -14.65 ± 0.38 ‰, -14.34 ± 0.29 ‰, respectively. One-way ANOVA analyses showed no significant variation in $\delta^{13}C$ across treatments ($p = 0.79$, Fig. 1). Two-way ANOVA analyses suggested that $\delta^{13}C$ was not affected by water addition ($p = 0.68$), N addition ($p = 0.61$) and their interaction ($p = 0.56$, Table 1).

Fig.1

Table 1

3.2 Gas exchange and WUE under water and nitrogen addition

Photosynthetic rate (A), stomatal conductance (g_s), transpiration rate (E) and c_i/c_a ranged from 12.11 μmol CO_2 m^{-2} s^{-1} to 39.35 μmol CO_2 m^{-2} s^{-1}, from 0.09 mol H_2O m^{-2} s^{-1} to 0.31 mol H_2O m^{-2} s^{-1}, from 2.87 mmol H_2O m^{-2} s^{-1} to 8.49 mmol H_2O m^{-2} s^{-1} and 0.11 to 0.57, respectively. One-way ANOVA analyses showed significant changes in leaf gas exchange across the six treatments ($p = 0.01$for A, $p < 0.01$ for g_s, $p <= 0.01$ for E and c_i/c_a, Fig. 2). Two-way ANOVA analyses suggested that water addition had exerted effect on c_i/c_a ($p < 0.01$), that N additions influenced A ($p < 0.01$) and c_i/c_a ($p = 0.009$), and that the interaction between water and N supply played a role in g_s ($p < 0.001$), E ($p < 0.001$) and c_i/c_a ($p < 0.001$, Table 1).

Fig. 2

Instantaneous WUE (ins-WUE) and intrinsic WUE (int-WUE) ranged from 3.09 μmol CO_2 / mmol H_2O to 8.49μmol CO_2 / mmol H_2O and from 93.64μmol CO_2 / mol H_2O to 208.47μmol CO_2 / mmol H_2O, respectively. One-way ANOVA analyses showed significant changes in these two indexes across these treatments (both p <

0.001, Fig. 3). Two-way ANOVA analyses suggested that water addition, N addition and their interaction all have significant effect on these two indexes (all $p < 0.05$, Table 1).

Fig. 3

3.3 Correlations among $\delta^{13}C$, WUE and c_i/c_a ratio

In order to test whether $\delta^{13}C$ in *H. ammodendron* can indicate WUE, the relationships among $\delta^{13}C$, ins-WUE, int-WUE and c_i/c_a ratio were revealed in this study. Our results showed no correlation between $\delta^{13}C$ and ins-WUE ($p = 0.23$, Fig. 4a), between $\delta^{13}C$ and int-WUE ($p = 0.23$, Fig. 4c), and between $\delta^{13}C$ and c_i/c_a ratio ($p = 0.18$, Fig. 4e). However, there was a negative correlation between ins-WUE and c_i/c_a ratio ($p < 0.001$, Fig. 4b), and between int-WUE and c_i/c_a ratio ($p < 0.001$, Fig. 4d).

Fig. 4

3.4 The degree of bundle-sheath leakiness under water and nitrogen addition

The φ value calculated from the minimum $\delta^{13}C_{air}$ ranged from 0.16 to 0.50 with a mean value of 0.35; the φ value calculated from the maximum $\delta^{13}C_{air}$ ranged from 0.44 to 0.70 with a mean value of 0.55, and the φ value calculated from the average $\delta^{13}C_{air}$ ranged from 0.32 to 0.59 with a mean value of 0.45. One-way ANOVA analyses showed no significant variation in φ calculated from the minimum, average and maximum $\delta^{13}C_{air}$ across treatments ($p = 0.60$ for the φ calculated from the minimum $\delta^{13}C_{air}$, $p = 0.77$ for the φ calculated from the average $\delta^{13}C_{air}$, and $p = 0.90$ for the φ calculated from the maximum $\delta^{13}C_{air}$, Fig. 5). Two-way ANOVA analyses suggested that φ was not affected by water addition ($p = 0.46$ for the φ calculated from the minimum $\delta^{13}C_{air}$, p = 0.64 for the φ calculated from the average $\delta^{13}C_{air}$,, and p = 0.98 for the φ calculated from the maximum $\delta^{13}C_{air}$), N addition (p = 0.65 for the φ calculated from the minimum $\delta^{13}C_{air}$, p = 0.60 for the φ calculated from the average $\delta^{13}C_{air}$,, and p = 0.55 for the φ calculated from the maximum $\delta^{13}C_{air}$) and their interaction (p = 0.30 for the φ calculated from the minimum $\delta^{13}C_{air}$, p = 0.52 for the φ calculated from the average $\delta^{13}C_{air}$,, and p = 0.87 for the φ calculated from the maximum $\delta^{13}C_{air}$, Table 1).

Fig. 5

4 Discussion

The $\delta^{13}C$ of the assimilating branches in *H. ammodendron* did not change across treatments (Fig. 1, Table 1), suggesting that neither water addition nor nitrogen addition influenced the $\delta^{13}C$ of *H. ammodendron*. Previous studies also reported no significant relationship between $\delta^{13}C$ of C_4 plant and water availability (Swap et al., 2004; Wang et al., 2008), and between $\delta^{13}C$ of C_4 plant and nitrogen availability (Yao et al., 2011, Yang et al., 2017).

In general, the effects of water availability and nitrogen availability on $\delta^{13}C$ are dependent on c_i/c_a ratio, which reflects the balance between stomatal conductance (g_s) and photosynthetic rate (A) (Farquhar and Richards, 1984). Stomatal conductance (g_s) usually increases with increasing water availability under water addition. Although two-way ANOVA analyses suggested that water addition had no effect on both A and g_s (Table 1); one-way ANOVA analyses showed that g_s was higher in W1N0 than that in W0N0 (Fig. 2b), indicating that water addition had a positive effect on g_s under ambient N condition. Increasing g_s under water supply will lead to the rise of intercellular CO_2 because of the decrease of diffusional resistance to CO_2. As the results, c_i/c_a ratio was observed to increase with increasing moisture (Fig. 2d, Table 1). However, $\delta^{13}C$ remained stable under water addition (Fig. 1, Table 1). Thus, c_i/c_a ratio could not explain the observed response of $\delta^{13}C$ to water supply.

For most plants in natural ecosystems, nitrogen is the key factor limiting plant growth (Hall et al., 2011). Thus, nitrogen addition usually causes plants to absorb more N. However, extreme drought could prevent plants from absorbing N even under high N supply. In the present experiment, N supply was found to have an effect on N contents in *H. ammodendron*. Relative to the control treatment (W0N0), N contents increased with N supply under low N addition, but kept unchanged under high addition (Table S1, S2). Nitrogen is the main constituent of Rubisco (ribulose - 1,5 - bisphosphate carboxylase oxygenase) and chlorophyll in plants. Thus, chlorophyll a was found to have the similar pattern as N contents under water and N supply. Chlorophyll a was higher in W0N1 than W0N0, and there was no difference in chlorophyll a between W0N0 and W0N2 (Table S1). Increasing chlorophyll contents in W0N1 should lead to the increase in photosynthetic rate (A). However, different from our prediction, one-way ANOVA analyses suggested that A in W0N1 did not differ from that in W0N0, and that A in W0N2 was lower than that in W0N0 (Fig., 2a). Two-way ANOVA analyses showed that N addition had an influence on A (Table 1). Both the analyses suggested that N supply played a negative role in A. These results might be associated with the extremely high light intensity at the study site. Due to the high light intensity, photosynthetic rate might not be correlated with chlorophyll contents (Gabrielsen, 1948). The negative effect of N supply on A led to the decrease in the consumption of intercellular CO_2. Consequently, c_i/c_a ratio increased with N supply (Fig. 2d, Table 1). Therefore, the variations in c_i/c_a ratio with N addition could not account for the unchanged pattern in $\delta^{13}C$ under N supply (Fig. 1).

The co-application of water and nitrogen had a negative effect on A but no effect on g_s (W0N0 vs. W1N1, W1N2, Fig. 2a, b). The responses of A and g_s to the co-application of water and nitrogen resulted in an increase in c_i/c_a ratio (Fig., 2d). Since $\delta^{13}C$ remained unchanged under the co-application of water and nitrogen (Fig. 1), c_i/c_a ratio could not also explain the observed $\delta^{13}C$ response to the co-application of water and nitrogen.

In summary, the unchanged $\delta^{13}C$ across treatments was not dependent on the c_i/c_a ratio in *H. ammodendron* (Fig. 4e). The observed $\delta^{13}C$ stability across treatments might be associated with the φ value and carbonic anhydrase (CA) in *H. ammodendron*. For C_4 plants, the relationship between carbon isotope discrimination ($\Delta \approx \delta^{13}C_{air} - \delta^{13}C_{plant}$, see Eq. (2)) and c_i/c_a ratio is controlled by φ values (Ellsworth and Cousins, 2016; Ellsworth et al., 2017; Farquhar, 1983; Wang et al., 2008). Some studies suggested that φ value was stable for a given species under a wide range of environmental conditions (Henderson et al., 1992; Wang et al., 2008; Cernusak et al., 2013). However, other studies had different conclusions that φ value was influenced by irradiation (Bellasio and Griffiths, 2014; Kromdijk et al., 2010; Pengelly et al., 2010; Ubierna et al., 2013), temperature (von Caemmerer et al., 2014), water stress (Fravolini et al., 2002; Gong et al., 2017; Williams et al., 2001; Yang et al., 2017) and nitrogen supply (Fravolini et al., 2002; Meinzer and Zhu, 1998; Yang et al., 2017). In current study, the φ value of *H. ammodendron* remained unchanged across six treatments (Fig. 5), and two-way ANOVA analyses suggested that water supply and N supply had no effect on φ (Table 1). Therefore, the φ value of *H. ammodendron* was insensitive to water and N addition in this study. Even if the φ value remains stable, the relationship between Δ and c_i/c_a ratio is also associated with the magnitude of the φ value. Cernusak et al. (2013) predicted that when φ value is greater than 0.37, the correlation between Δ and c_i/c_a ratio is positive; conversely, when φ value is less than 0.37, the correlation is negative. In particular, when φ value is equal to 0.37, there will be no correlation between them, because the coefficient ($[b_4 + \varphi\,(b - s) – a]$ in Eq. (2)) of c_i/c_a ratio equals to 0 (Cernusak et al., 2013). The φ value calculated from the average $\delta^{13}C_{air}$ ranged from 0.32 to 0.59 with a mean value of 0.45 in present study. Thus, the correlation between Δ and c_i/c_a in *H. ammodendron* should be positive based on the prediction by Cernusak et al. (2013). Δ always changes in the opposite direction to $\delta^{13}C_{plant}$ changes according to Eq. (2), thus, a negative relationship between $\delta^{13}C_{plant}$ and c_i/c_a is expected. In fact, this study observed no correlation between $\delta^{13}C$ and c_i/c_ain *H. ammodendron* (Fig. 4e); this indicates that φ was not the driver of the observed $\delta^{13}C$ pattern in *H. ammodendron*. However, the measured $\delta^{13}C$ represents the fixed carbon isotope composition throughout the assimilation branch formation period, which usually spans at least several weeks. And the measured c_i/c_a

is an instant indicator. As a result, there were some uncertainties in the calculation of φ value using Eq. (2) based on the measured $\delta^{13}C$ and c_i/c_a. In addition, the mean φ value calculated from the minimum and maximum $\delta^{13}C_{air}$ were 0.35 and 0.55, respectively, suggesting that the φ value of *H. ammodendron* might be close to 0.37, which leaded to the observed insensitive response of $\delta^{13}C$ to water and N addition.

The enzymatic activity of CA may be another mechanism behind the unchanged $\delta^{13}C$ across treatments. Cousins et al. (2006) suggested that enzymatic activity of CA affects carbon isotope discrimination in most C_4 plants because CA can result in the parameter b_4 changes (see Eq. (2)). But in traditional view, the parameter b_4 was a constant. However, it is only true when the ratio of PEP carboxylation rate to the CO_2 hydration rate (V_p/V_h) is equal to zero, which is caused by a high CA activity. If V_p/V_h is not zero, b_4 will change and be controlled by V_p/V_h (Cousins et al., 2006). Previous studies reported that CA activity was low in most C_4 plants (Cousins et al., 2006; Gillon and Yakir, 2000, 2001; Hatch and Burnell, 1990). Thus, CA activity in *H. ammodendron* might also be low, leading to the change in b_4 with V_p/V_h, and thus $\delta^{13}C$. Cousins et al. (2006) added V_p/V_h into the discrimination pattern of C_4 plants and predicted that at a given φ value, when the V_p/V_h is 0 or 1, the correlation between Δ and c_i/c_a ratio is negative or positive, respectively. Since CA activity is low in most C_4 plants, and the V_p/V_h always ranges from 0 to 1, we speculate that no correlation between Δ and c_i/c_a ratio may also occur when the V_p/V_h is a certain value between 0 and 1. The irrelevance of Δ and c_i/c_a ratio also means that $\delta^{13}C_{plant}$ is not related to c_i/c_a ratio due to the negative correlation between Δ and $\delta^{13}C_{plant}$ according to Eq. (2). Thus, the uncorrelated pattern between $\delta^{13}C$ and c_i/c_a ratio in *H. ammodendron* might be related to this specific V_p/V_h value due to low CA activity.

In addition, the unchanged $\delta^{13}C$ across treatments may also be controlled by the water sources of *H. ammodendron*. Previous study has found that the root of *H. ammodendron* can be inserted into the soil layer deeper than 3 m (Sheng et al., 2004), which made it easy to uptake groundwater. Therefore, *H. ammodendron* may be less sensitive to water addition. However, a study conducted in the same region has found that the shallow soil water (0-40 cm) and groundwater are two important water sources for *H. ammodendron* (Dai et al., 2014), and another study has reported that water addition resulted in an increase of soil water contents in shallow soil layer (Cui, 2018). Moreover, gas exchange changed across treatments in the present study (Fig. 2). Thus, the utilization of groundwater by *H. ammodendro*n may be one of the reasons why its $\delta^{13}C$ was not sensitive to water and N addition, but it should not be the main reason.

Whether foliar $\delta^{13}C$ of C_4 plants can indicate their WUE is still controversial. Henderson et al. (1992) found that $\delta^{13}C$ of 10 C_4 species has negative correlation with their WUE. Although this result was just opposite to a positive relationship between $\delta^{13}C$ and WUE for C_3 plants (Farquhar, 1983; Duquesnay et al., 1998; Feng, 1998), it is a proof that $\delta^{13}C$ of C_4 plants can indicate their WUE. In the work of Henderson et al. (1992), they found that the φ values in 10 C_4 species was around 0.21 over a range of irradiance and leaf temperature. According to the suggestion by Cernusak et al. (2013) that Δ is negatively related to c_i/c_a ratio when φ value is less than 0.37, thus, the $\delta^{13}C$ of 10 C_4 species has a positive correlation with c_i/c_a ratio. In general, under fixed ambient CO_2 concentration, WUE is always negatively correlated with c_i/c_a ratio (see Eq. (4) and Eq. (6)). This is why a negative relationship between $\delta^{13}C$ and WUE was observed for the 10 C_4 species. The present study showed that ins-WUE and int-WUE both had no correlation with $\delta^{13}C$ in *H. ammodendron* (Fig. 4a, 4c), which was different from the results published by Henderson et al. (1992). In general, c_i/c_a ratio is the link between WUE and $\delta^{13}C$. As mentioned above, if the φ value equals to 0.37 and/or the activity of CA is very low, $\delta^{13}C$ would not correlate to c_i/c_a ratio, and thus leads to the uncorrelation between $\delta^{13}C$ and WUE. In addition, the different time scales of $\delta^{13}C$, ins-WUE and int-WUE may also result in this uncorrelation. As mentioned above, the measured $\delta^{13}C$ represents the long-term fixed carbon isotope composition (at least several weeks). And the value of ins-WUE and int-WUE were calculated from the gas exchange of a short-term measurement, which lasted only a few minutes. Therefore, this difference in time scale may also drive the uncorrelation between $\delta^{13}C$ and WUE. Although the defects in measurements could introduce some uncertainty in the observed relationship between $\delta^{13}C$ and WUE, $\delta^{13}C$ remained stable under water and nitrogen addition (Fig. 1, Table 1), while the measured ins-WUE and int-WUE was higher in the control treatment (W0N0) than other treatments (Fig. 3), suggesting and water and N supply had a significant effect on WUE (Table 1). These results indirectly confirmed that $\delta^{13}C$ of *H. ammodendron* could not indicate its WUE.

The present study has found that $\delta^{13}C$ of *H. ammodendron* could not be used as a indicator of its WUE. Although this conclusion cannot be analogous to all C_4 plants, the present study has important implications for the understanding of physiological responses of desert plants to future changes in precipitation and atmospheric N deposition. *H. ammodendron* is a dominant species in Asia desert, which has a great effect on the stabilization of sand dunes, the survival and development of understory plants and the structure and function of desert ecosystems (Sheng et al., 2005; Su et al., 2007; Cui et al., 2017). Thus, *H. ammodendron* is widely distributed in desert areas, and the prediction of its drought adaptation is crucial in desert ecosystem.

5 Conclusion

Global changes including precipitation and atmospheric N deposition have been proved to have an important influence on ecosystems, especially for the arid ecosystems. The present study showed that water and N addition had little effect on the $\delta^{13}C$ values and the degree of bundle-sheath leakiness (φ) of *H. ammodendron*, but played an important role in the change of its gas exchange and water use efficiency (WUE). In addition, different pattern of instantaneous WUE (ins-WUE), intrinsic WUE (int-WUE) and $\delta^{13}C$ across treatment and no correlation between instantaneous WUE (ins-WUE) and $\delta^{13}C$, and between intrinsic WUE (int-WUE) and $\delta^{13}C$ has been found in this study, suggesting that $\delta^{13}C$ of *H. ammodendron* could not indicate its WUE. This result was caused by the lack of the correlation between $\delta^{13}C$ and the ratio of intercellular to ambient CO_2 concentration (c_i/c_a), which might be associated with the degree of bundle-sheath leakiness (φ) or the low activity of carbonic anhydrase

(CA). Thus, the current experiment implies that the availability of $\delta^{13}C$ as the indicator of WUE could be not universal for C_4 species.

Conflict of interest

None declared.

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Authors' Contributions

G Wang and J Li designed the experiment and modified the manuscripts. Z Chen designed and executed the experiment and wrote the manuscripts. X Liu designed the experiment. X Cui executed the experiment. Y Han executed the experiment.

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Data availability

The datasets analyzed in this manuscript are not publicly available. Requests to access the datasets should be directed to gawang@cau.edu.cn.

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Table 1 The p values of all measured and calculated indexs in plants under two-way ANOVA analysis of water (W) and nitrogen (N) additions

	W	N	W*N
$\delta^{13}C$	0.678	0.607	0.563
Photosynthetic rate (A)	0.331	0.008**	0.183
Stomatal conductance (g_s)	0.533	0.871	<0.001***
Transpiration rate (E)	0.622	0.883	<0.001***
c_i/c_a	0.004**	0.009**	<0.001***
ins-WUE	0.002**	<0.001***	<0.001***
int-WUE	0.004**	0.018*	<0.001***
φ	0.644	0.600	0.521

Note. *, **, *** indicates a significant influence.

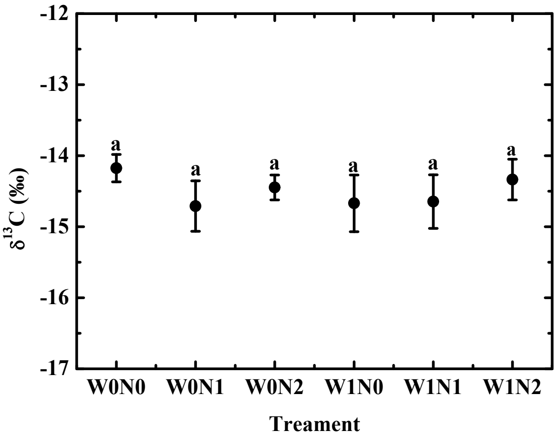

Fig. 1 The $\delta^{13}C$ of assimilating branches of *Haloxylon ammodendron* under water (W) and nitrogen (N) additions. The spot represents the mean value of four replicates with error bars denoting the standard error (SE).

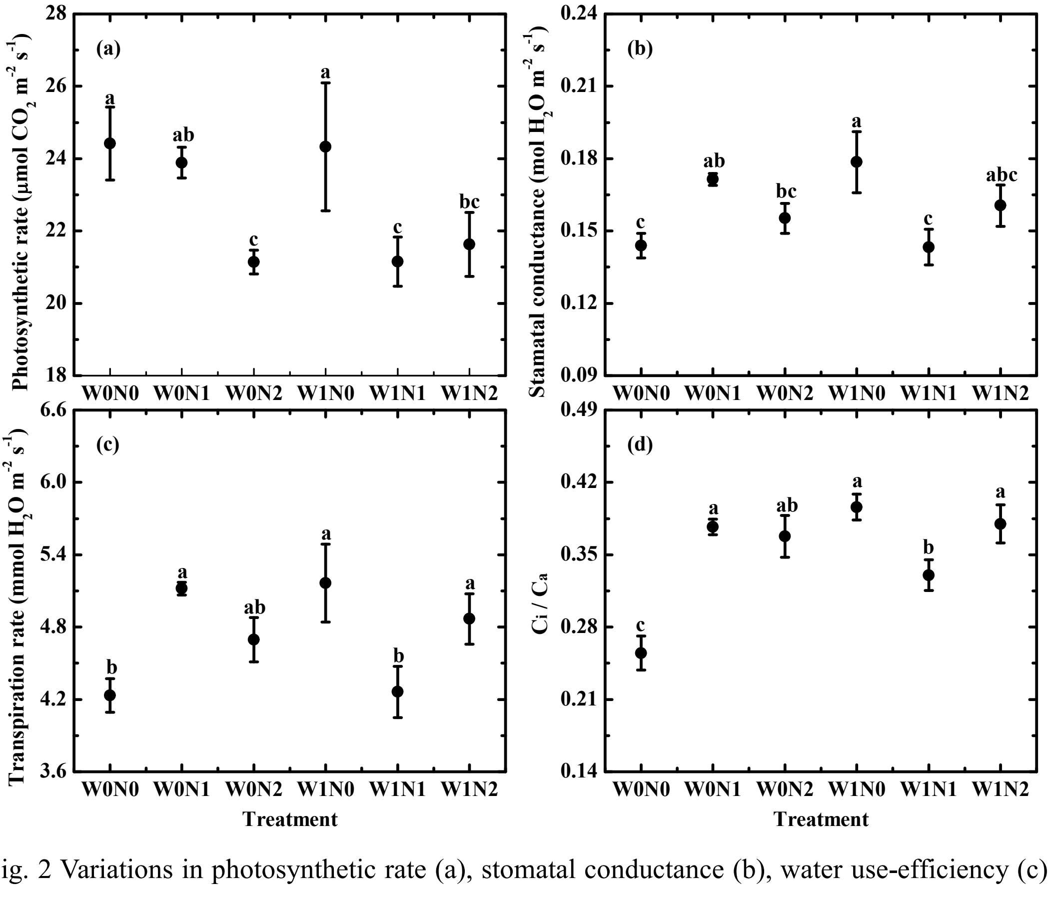

Fig. 2 Variations in photosynthetic rate (a), stomatal conductance (b), water use-efficiency (c) and

c_i/c_a (d) across water (W) and nitrogen (N) additions. The spot represents the mean value of four replicates with error bars denoting the standard error (SE).

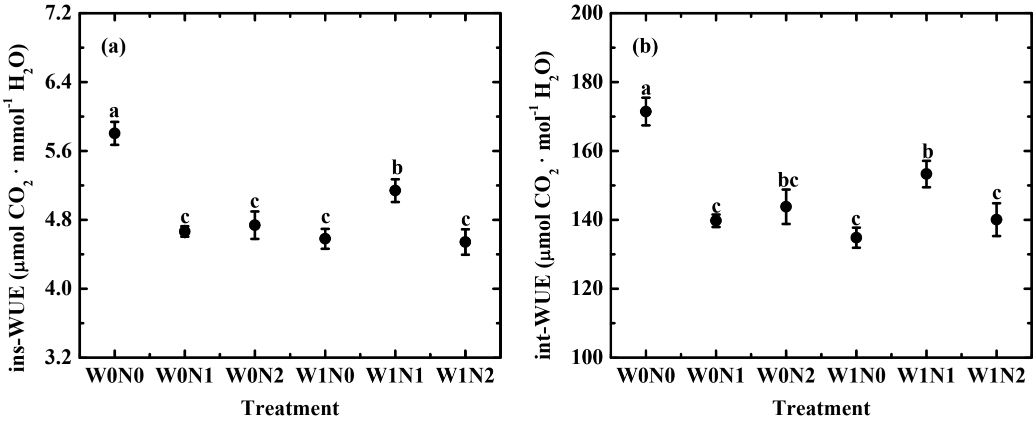

Fig. 3 Variations in ins-WUE (a) and int-WUE (b) across water (W) and nitrogen (N) additions. The spot represents the mean value of four replicates with error bars denoting the standard error (SE).

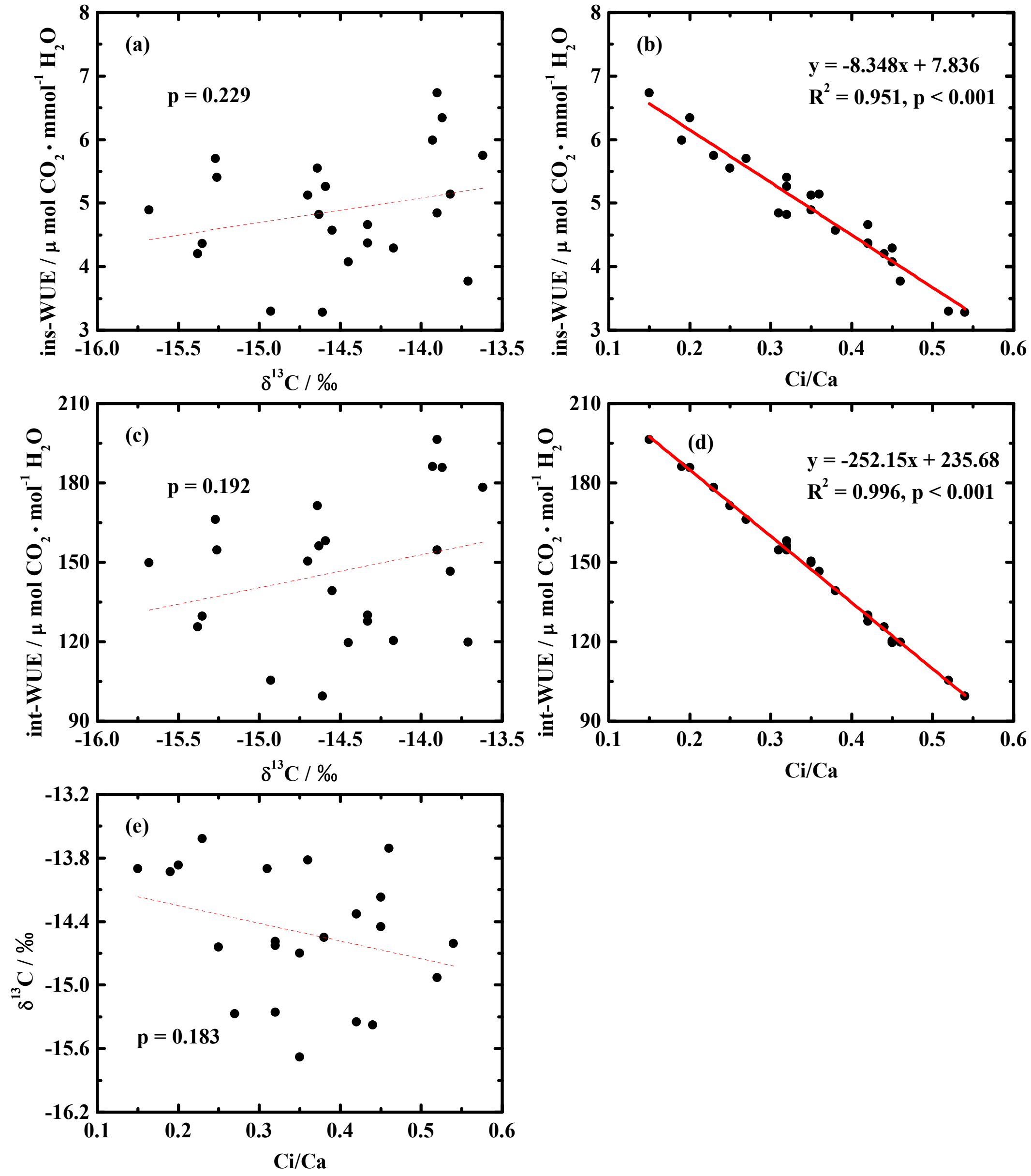

Fig. 4 Correlations of ins-WUE vs. $\delta^{13}C$ (a), ins-WUE vs. c_i/c_a (b), int-WUE vs. $\delta^{13}C$ (c), int-WUE vs. c_i/c_a (d), and $\delta^{13}C$ vs. c_i/c_a (e) of assimilating branches of *Haloxylon ammodendron*

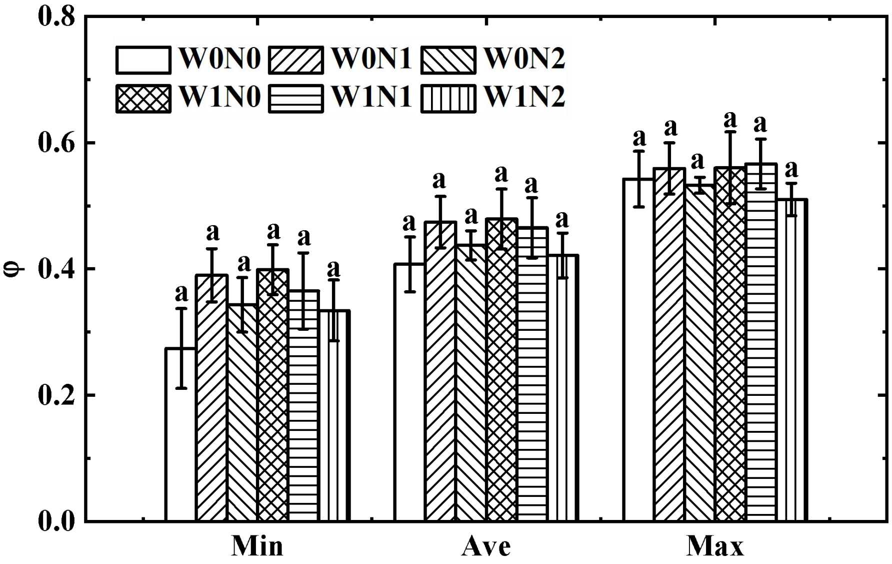

Fig. 5 Variations in φ across water (W) and nitrogen (N) additions. The spot represents the mean value of four replicates with error bars denoting the standard error (SE).