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ORIGINAL ARTICLE

Root-derived bicarbonate assimilation in response to variable water deficit in *Camptotheca acuminate* seedlings

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Abstract Water deficit is one of the key factors that limits the carbon (C) assimilation and productivity of plants. The effect of variable water deficit on recently root-derived bicarbonate assimilation in *Camptotheca acuminate* seedlings was investigated. Three-month-old seedlings were subjected to three water regimes, well-watered (*WW*), moderate stress (*MS*), and severe stress (*SS*) induced by polyethyleneglycol, in conjunction with relatively high (*H*) and low (*L*) natural ^{13}C-abundance of $NaHCO_3$-labeled treatments in hydroponics for 14 days. The $\delta^{13}C$ of the newly expanded leaves in *H* were generally more enriched in heavy isotopes than were those in *L*, indicative of the involvement of bicarbonate in aboveground tissues. The C isotope fractionation of newly expanded leaves relative to air ($\Delta^{13}C_{air\text{-}leaves}$) ranged from 17.78 to 21.78‰ among the treatments. The $\Delta^{13}C_{air\text{-}leaves}$ under the *MS* and *SS* treatments in *H* were both more negative than was that in *L*. A linear regression between *Ci/Ca* and $\Delta^{13}C_{air\text{-}leaves}$ in both *L* and *H* were different from the theoretical regression. On the basis of the two end-member mixing model, the proportion of fixed CO_2 supplied from bicarbonate contributing to the total photosynthetically inorganic C assimilation were 10.34, 20.05 and 16.60% under the *WW, MS*, and *SS* treatments, respectively. These results indicated that the increase in water deficit decreased the atmospheric CO_2 gain but triggered a compensatory use of bicarbonate in *C. acuminate* seedlings.

Keywords Water deficit · Bicarbonate utilization · Carbon assimilation · Carbon isotope fractionation

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Introduction

Water deficit is one of the key factors that limits the carbon (C) assimilation and productivity of plants (Chaves and Oliveira. 2004; Ramírez et al. 2012; Sapeta et al. 2013). These have become important issues worldwide since the global climate has undergone much change in recent decades. The shortage of water was believed to be increased in many areas around the earth due to frequently extreme precipitation events (IPCC 2014). The effects of water deficit on plants include the routine modulation of morpho-anatomical characteristics (Pang et al. 2011; De Micco and Aronne 2012; Jung et al. 2014), biochemical (Ancillotti et al. 2015; Kumar et al. 2015; Wang et al. 2015) and photochemical (Zivcak et al. 2013; Lauteri et al. 2014) processes, proteomics and gene expression (Krasensky and Jonak 2012) at different regulatory levels. To diminish water loss via transpiration, leaf gas exchange is regulated by stomatal closure, which often leads to the remarkable reduction of atmospheric C gain. Overall, a critical trade-off between water retention and C acquisition is mediated in response to water deficit.

Higher plants can acquire various C sources for survival. The majority of inorganic C is sequestered from atmospheric CO_2 by terrestrial plants via photosynthesis. As has been stated already, this process is almost always

hindered by environmental stress, such as drought, heat, chilling, salinity and other biotic factors and their combinations (Suzuki et al. 2014; Martínez-Lüscher et al. 2015). In addition, plants can also fix a minor amount of C internally from respiration of stem tissues, roots and microbes (Stringer and Kimmerer 1993; Ford et al. 2007; Grossiord et al. 2012). Early studies revealed that dissolved inorganic carbon (DIC) in root xylem contributed to aboveground C gain (Teskey and McGuire 2007; Aubrey and Teskey 2009). Bloemen et al. (2016) reviewed that a large fraction of root-respired CO_2 could be delivered to aboveground tissues by the transpiration stream. To our knowledge, the form of DIC (CO_2, HCO_3^-, and CO_3^{2-}) was pH-dependent. Previous studies have shown that the pH of xylem sap ranges from 4.2 to 6.8 and varies with the species (Levy et al. 1999; Wegner and Zimmermann 2004). This may be one reason that many studies have focused on CO_2 in the xylem rather than HCO_3^-. Nevertheless, there is a trend that an increasing number of studies have seemed to shed light on the utilization of HCO_3^- by higher plants. The formation of HCO_3^- usually results from the hydrolyzation of carbonate. A low level of bicarbonate results in an increase in biomass or had no effect on it (Bialczyk and Lechowsk 1995; Covarrubias and Rombolà 2013), while a high concentration of bicarbonate inhibited the growth, net photosynthetic rate, and chlorophyll content (Yang et al. 2009; Wu and Xing 2012). Wegner and Zimmermann (2004) accurately detected bicarbonate-induced alkalization of the xylem sap pH using a novel xylem pH probe, which seemed to be beneficial to the transport of HCO_3^-. Stringer and Kimerer (1993) observed that ^{14}C-labeled bicarbonate was delivered to veins and then mostly incorporated into sugar, starch, and protein in the Populus leaves. Rombolà et al. (2005) used high ^{13}C-abundance $Ca^{13}CO_3$ (20 atom % enriched ^{13}C) to trace the fate of bicarbonate assimilation and found 3.81 and 1.09% of ^{13}C abundance in the fine roots and leaf blades of sugar beet plants, respectively. All of these results demonstrated that plants could utilize bicarbonate as a way of C sequestration in response to bicarbonate. However, the relationship between atmospheric CO_2 and root-derived bicarbonate with respect to photosynthetically inorganic C assimilation is still poorly understood.

Camptotheca acuminate is a perennial woody plant of the Nyssaceae family that is used for timber and camptothecin extraction (Yu et al. 2012; Ying et al. 2014). In addition, *C. acuminate* is native to China and adapted to the calcareous soil. The lime soil is characterized by high pH and high concentration of bicarbonate. The bicarbonate concentration was reported to increase with increasing soil water content and $CaCO_3$ content (Zuo et al. 2007). In well-aerated soil, HCO_3^- concentration was about 5 mM while more than 10 mM was observed in wet soil (Boxma 1972; Bloom and Inskeep 1986). During the growing season, plants usually exposed to variable water deficit. *C. acuminate* was described as drought tolerant by improving water retention, antioxidation, and membrane integrity (Ying et al. 2014). However, the research did not fully explain how this species adapted to calcareous soil with co-occurring water deficit and bicarbonate, which is very common under natural conditions (Deng et al. 2012; Hu et al. 2013). In this regard, we hypothesized that water deficit would enhance the development of the root system, helping to promote water uptake and bicarbonate utilization. We also hypothesized that bicarbonate might be a potentially important C source for photosynthesis under the circumstance of drought-induced stomatal closure. This paper is the first to explore the proportion of bicarbonate utilization under variable water deficit in *C. acuminate*.

Stable isotope techniques are used as a nonradioactive tracer to reveal the uptake and accumulation of isotope in plants (Dawson et al. 2002; Tang et al. 2016). To trace the fate of bicarbonate, we used the natural abundance ^{13}C of $NaHCO_3$ for labeling and examined the variation of the ^{13}C signature in plant organs. $H^{13}CO_3^-$ ions are passively absorbed, translocated from root to leaves, and catalyzed into CO_2 by carbonic anhydrase (Moroney et al. 2001). Thus, atmospheric CO_2 and the fixed CO_2 supplied from root-derived bicarbonate are both assimilated in expanded leaves via photosynthesis. The new photosynthate is exported from the expanded leaves to the developing leaves and roots (Durand et al. 2016) as a result of the remobilization of storage material for growth (Masyagina et al. 2016). When plants are subjected to variable environmental stress, the response is usually reflected in the $\delta^{13}C$ of newly assimilated carbohydrate. The rapid turnover C can significantly influence the overall $\delta^{13}C$ of leaves (Cranswick et al. 1987; Brendel 2001). In addition, the $\delta^{13}C$ of carbohydrate closely reflects the gas exchange parameters (Brugnoli et al. 1988; Göttlicher et al. 2006). The stable C isotope techniques, in combination with gas exchange measurement, might provide precise evidence to investigate bicarbonate assimilation under water deficit treatments.

In the present study, *C. acuminate* seedlings were exposed to different water regimes and $NaH^{13}CO_3$-labeled treatments. The following are our aims: (1) to study how *C. acuminate* responded to the labeling, bicarbonate, water deficit, and their interaction morphologically and physiologically; (2) to examine the C isotope composition and fractionation of newly expanded leaves; and (3) to quantify the proportions of fixed CO_2 supplied from bicarbonate contribution to the total photosynthetically inorganic C assimilation under different water regimes in *C. acuminate* seedlings.

Methods

Plant materials

This work was conducted in the phytotron of the Institute of Geochemistry, Chinese Academy of Sciences (CAS), Guiyang, China. The phytotron was $10 \times 5 \times 4$ m in length, width, and height, respectively. The light was provided by the metal halide lamps (HPI-T400W/645, Philips, the Netherlands) while the temperature was controlled by air-condition. Two small ventilation fans were installed to control the gas exchange at regular intervals. Seeds of *C. acuminate* were obtained from the nearby mother tree at the mature stage. Uniform seeds were selected and disinfected with 75% ethanol for 1 min and were then washed with distilled water three times. The seeds were germinated in trays with a moist bed covered with perlite for 2 weeks at a temperature of 25/19 °C in the light/dark and 55–60% relative humidity. Seedlings were then incubated in a photoperiod of 16/8 h of light/dark conditions, along with 500 ± 23 µmol m^{-2} s^{-1} of photosynthetic photon flux density. The seedlings were irrigated with 1/2 strength Hoagland nutrient solution every 3 days. After 76 days of growth, uniform seedlings were chosen and randomly transplanted to pots. The plastic pots were $19.5 \times 14.5 \times 5.6$ cm in length, width, and height, respectively. With a tray beneath, each pot had several apertures for drainage. A soil mixture of perlite and vermiculite (1:3 v/v) was added to fix the plants in the pots.

Experimental design

We carried out a two-way factorial design with six treatments, which contained two labeled treatments in conjunction with three water regimes. Each treatment consisted of three replicates, and each seedling was treated as a replicate. The labeled treatments were separated into relatively high (*H*) and low (*L*) natural ^{13}C-abundance of NaHCO$_3$, with a δ^{13}C of $-9.76‰$ in *H* and $-26.78‰$ in *L*. The *H*-labeled treatment was consistent with many studies which suggested that the ^{13}C values of bicarbonate ranged from -7 to $-14‰$ in calcareous soil (Salomons and Mook 1986; Pan et al. 2002; Rovira and Vallejo 2008). Both of the bicarbonate labeled treatments contained three water regime sub-treatments: well-water (*WW*), moderate stress (*MS*), and severe stress (*SS*) treatments. Polyethylene glycol (PEG 6000) was used to induce water deficit (van der Weele et al. 2000; Ancillotti et al. 2015). The addition of PEG for *WW, MS*, and *SS* stress was 0, 100, and 200 g per liter in the solution, respectively. The values of the water potential (ψ_w) in the solution approximately equaled -0.01 Mpa (*WW*), -0.2 Mpa (*MS*), and -0.6 Mpa (*SS*), respectively. After the start of the experiment, the pots were irrigated with 250 ml modified solution, and the residual solution was collected and renewed every other day. The concentration of HCO$_3^-$ was determined by Aqua-merck alkalinity test (MColortest, Merck, Germany). The modified 1/2 strength Hoagland nutrient solution contained 10 mM NaHCO$_3$, 2 mM Ca(NO$_3$)$_2$, 2.5 mM KNO$_3$, 0.5 mM NH$_4$NO$_3$, 0.125 mM KH$_2$PO$_4$, 1 mM MgSO$_4$, and micronutrients. The solution pH was adjusted to 8.3 ± 0.2 at which the HCO$_3^-$ is the most abundant species among DIC (Millero 2003). The treatments lasted for 14 days.

Measurements of growth and gas exchange

Growth characteristics were monitored at the end of treatments by measuring the shoot length and leaf length (the second expanded leaf). All seedlings were harvested at the end of the experiment. The fresh weight of the whole plant and root length were determined. The dry weight of shoot and root were determined after drying the plant material at 70 °C for 3 days (until a constant weight was achieved). In the second fully expanded leaves, gas exchange measurements were performed with a portable leaf photosynthesis and fluorescence system Li-6400 (Li-Cor Inc, Lincoln, NE, USA). The air flow rate was set at 500 µmol s^{-1} and the CO$_2$ concentration at 380 µmol m^{-2} s^{-1}. The net photosynthetic rate (*Pn*), stomatal conductance (g_s), and the ratio of intercellular to ambient CO$_2$ partial pressures (*Ci/Ca*) were measured in the leaf chamber by LI-6400-02B from 09:00 to 11:00 in the morning.

Measurement of carbon isotope signature

At the final harvest, newly expanded leaves were collected and soaked in 1 M HCl for 1 h to remove exogenous inorganic C. Samples were then dried at 70 °C for 3 days and ground for C isotope testing. Measurement of the δ^{13}C value was performed by isotope ratio mass spectrometer (MAT252, Finnigan, Germany) at the Institute of Geochemistry, CAS. The δ^{13}C was expressed as: δ^{13}C $(‰) = [(R_{Sample}/R_{Standard}) - 1] \times 1000$, where R_{Sample} and $R_{Standard}$ are the ^{13}C/^{12}C ratio of the samples and the PDB, respectively. A small amount of atmospheric CO$_2$ dissolved in the solution and mixed with the initially added NaH^{13}CO$_3$, resulting in the mixed bicarbonate solution. To determine the δ^{13}C of bicarbonate, 15 ml of the solution was sampled at equal time intervals of 2 days. CO$_2$ was generated in a reaction of the bicarbonate solution with phosphoric acid in a sealed and vacuumed bottle. CO$_2$ was then purified and transferred to MAT252 for measurement of the δ^{13}C values. The ^{13}C composition of air (δ_a) was collected at the regular time points of different days during the treatments in this phytotron. The δ_a

was determined by a trace gas analyzer (Isoprime, GV. Instruments, UK), with the value of $-15.11 \pm 0.37\%o$.

Estimation of the contribution of bicarbonate utilization to the total photosynthetically inorganic carbon assimilation

A simplified model as described by Farquhar et al. (1989) was used as follows to predict the instantaneous discrimination against ^{13}C:

$$\Delta^{13}C_i = a + (b - a)C_i/C_a, \tag{1}$$

where a is the discrimination of diffusion (4.4‰) and b is the discrimination by RubisCO (27‰). To compare with $\Delta^{13}C_i$, the fractionation between air (source) and leaves (product) was determined by the following equation (Brugnoli et al. 1988):

$$\Delta^{13}C_{air-leaves} = \frac{\delta_{air} - \delta_{leaves}}{1 + \delta_{leaves}/1000} \tag{2}$$

In addition, a two end-member mixing model was designed to investigate the proportion of fixed CO_2 supplied from bicarbonate contributing to the total photosynthetically inorganic C assimilation. In L treatment, the two end-member model was written as:

$$(\delta_{NL} + \varepsilon) = (\delta_{SL} - \Delta_{1L})f_L + (\delta_a - \Delta_{2L})(1 - f_L), \tag{3}$$

where δ_{NL} is the $\delta^{13}C$ of newly expanded leaves, ε is the fractionation between source and sink during carbohydrate export, δ_{SL} is the mean $\delta^{13}C$ of the bicarbonate in the solution, Δ_{1L} is the fractionation from bicarbonate to carbohydrate, f_L is the proportion of the fixed CO_2 supplied from bicarbonate contributing to the total photosynthetically inorganic C assimilation, and Δ_{2L} is the fractionation from atmospheric CO_2 to carbohydrate and equals $\Delta^{13}C_i$. In contrast, the two end-member mixing model in H treatment was expressed as:

$$(\delta_{NH} + \varepsilon) = (\delta_{SH} - \Delta_{1H})f_H + (\delta_a - \Delta_{2H})(1 - f_H) \tag{4}$$

As suggested by Wu and Xing (2012), when the gas exchange and the solution absorption parameters were approximately equal under the same treatment in L and H labeling treatments, there were specific equations: $\Delta_{1L} = \Delta_{1H}, \Delta_{2L} = \Delta_{2H}, f = f_L = f_H$, which could be well demonstrated in the following text. Then Eqs. 3 and 4 were obtained:

$$f = \frac{\delta_{NL} - \delta_{NH}}{\delta_{SL} - \delta_{SH}} \tag{5}$$

Statistical analysis

A generalized linear model (GLM) was used for analysis of variance (ANOVA) of the treatments and replications, as well as the interactions. Significant differences between the mean values were calculated with Duncan's Multiple Range Tests. Tests were considered significant at the $p < 0.05$ level and extremely significant at a level of $p < 0.01$. Mean values were expressed with the standard error (SE). All analyses were performed using the SAS 9.4 statistical package (SAS Institute Inc, NC, US).

Results

Solution absorption

Some alterations of the solution pH, the concentration, and ^{13}C composition of bicarbonate were observed due to the continuing dissolution of atmospheric CO_2 into the solution (Table 1). However, the pH values and the HCO_3^- concentration showed no difference in all treatments ($p > 0.05$). The mean $\delta^{13}C$ values of the bicarbonate were $-23.35\%o$ in L and $-15.31\%o$ in H. In L, the uptake rate of the solution was on average 103.6, 58.5 and 34.1 ml/day for WW_L, MS_L and SS_L, respectively ($p < 0.01$). Similar results were observed in H. Furthermore, the evaporation was estimated to be approximately 5–7.5 ml/day in every pot (data not shown).

Table 1 The characteristics of the solution under the three water regimes in two labeled treatments

Treatment	pH	$c(HCO_3^-)$ (mmol/L)	$\delta^{13}C$ of the bicarbonate (‰)	Uptake of solution (ml/day)
WW_L	8.17 ± 0.20	12.37 ± 1.86	-23.71 ± 1.71 b	103.59 ± 6.35 a
MS_L	7.99 ± 0.27	13.44 ± 2.59	-23.49 ± 1.69 b	58.51 ± 5.01 b
SS_L	8.09 ± 0.11	11.98 ± 0.95	-22.83 ± 2.01 ab	34.09 ± 2.91 c
WW_H	8.21 ± 0.06	12.42 ± 1.67	-15.78 ± 3.18 ab	98.39 ± 7.14 a
MS_H	7.90 ± 0.23	13.55 ± 2.79	-15.31 ± 2.95 a	60.11 ± 3.82 b
SS_H	8.03 ± 0.14	11.90 ± 0.94	-14.82 ± 2.59 a	32.60 ± 2.96 c

WW_L, MS_L, SS_L: three water deficits in L. WW_H, MS_H, SS_H: three water deficits in H. Date was shown with mean ± SE, $n = 3$. Values in the same column were followed by a different letter which were significantly different at $p < 0.05$

Growth and morphology

The growth and morphological traits of *C. acuminate* under all treatments are shown in Table 2. The results indicated that the biomass and shoot length were not affected by any treatments. The leaf length was nearly twice that of *pre-trt* under WW_L and WW_H treatments ($p < 0.05$). Approximately 62.14 and 65.05% of the leaf length increment was observed under MS_L and MS_H treatments in comparison to *pre-trt*, respectively ($p < 0.01$). Furthermore, the leaf length was totally inhibited when the SS_L and SS_H treatments were imposed. The bicarbonate, as a stress rather than labeling factor, had extremely significant influence on leaf length as compared to *pre-trt* (Tables 2, 3). In addition, water deficit and the interaction of bicarbonate and water deficit significantly affected the leaf length, while the labeling factor had no effect on it. Of all the growth indicators, the root length was the only one that increased under all treatments ($p < 0.01$). A general increase in the root length was observed with the increase in the water deficit. Bicarbonate, rather than water deficit or labeling factor, had an extremely significant effect on root length (Table 3).

Leaf gas exchange

Overall, *Pn, g_s*, and *Ci/Ca* exhibited dramatic variation (Fig. 1). The tendency of *Pn* in *L* was consistent with that in *H* when the same water deficit treatment was imposed (Fig. 1a). Under *WW* treatment, the *Pn* gradually increased and reached the maximal values of 5.15 µmol m^{-2} s^{-1} for WW_L and 5.34 µmol m^{-2} s^{-1} for WW_H at the end of the experiment. When the *MS* treatment was imposed, the *Pn* was kept steady at approximately 4 µmol m^{-2} s^{-1} for both *L* and *H* during the first week and then declined in the second week, with the final values of 3.03 µmol m^{-2} s^{-1} for MS_L and 2.26 µmol m^{-2} s^{-1} for MS_H. Under *SS* treatment, the *Pn* declined sharply by 70.45 and 67.11% for SS_L and SS_H in the first 4 days, respectively. The effect of the water deficit on the *Pn* was extremely significant in the two labeled treatments (Table 3).

The g_s increased at first 4–6 days and then declined for WW_L and WW_H (Fig. 1b). *MS* treatments induced 60.72 to 64.29% reduction of g_s in *L* and *H* during the 14 days. In addition, the g_s decreased quickly to the level of 0.01 after *SS* treatment was imposed in *L* and *H*. The effect of bicarbonate, water deficit, and their interaction were extremely notable on g_s (Table 3).

The *Ci/Ca* showed the same tendency in *L* and *H* (Fig. 1c). A slight decrease in *Ci/Ca* was observed under WW_L and WW_H treatments. Under the *MS* treatments, the *Ci/Ca* decreased dramatically within the first 2 days and then decreased slowly, with the final mean values of 0.27 and 0.31 for the MS_L and MS_H treatments, respectively. Similar results were found in the SS_L and SS_H treatments, in comparison with those in the MS_L and MS_H treatments. During the treatments, the mean values of *Ci/Ca* were 0.73, 0.36, and 0.32 in *L* under the *WW*, *MS*, and

Table 3 Effect of bicarbonate, water deficit, labeling and their interaction on growth, gas exchange, and C isotope signature

Index	Source of variation*				R^2
	Bicarbonate (b)	Water deficit (w)	Labeling	$b \times w$	
Leaf length	0.001	0.001	0.913	0.030	0.931
Root length	0.008	0.161	0.413	0.387	0.632
P_n	0.806	0.001	0.707	0.433	0.910
g_s	0.001	0.001	0.158	0.001	0.973
Ci/Ca	0.001	0.001	0.538	0.001	0.951
$\delta^{13}C$ of leaves	0.213	0.001	0.002	0.103	0.829
$\delta^{13}C$ of roots	0.082	0.001	0.001	0.160	0.908
$\Delta^{13}C_{air-leaves}$	0.213	0.001	0.002	0.103	0.829
$\Delta^{13}C_{leaves-roots}$	0.017	0.035	0.041	0.236	0.598

Statistical differences were tested with three-way ANOVA

*p values for the *F*-test

Table 2 Growth and morphology indicators of seedlings under the three water regimes in two labeled treatments

Treatments	Fresh weight (g/plant)	DW of shoot (g)	DW of root (g)	Shoot length (cm)	Leaf length (cm)	Root length (cm)
Pre-trt	43.68 ± 1.91	7.73 ± 0.13	3.33 ± 0.10	35.69 ± 0.57	3.09 ± 0.10 c	13.85 ± 0.53 c
WW_L	45.83 ± 4.22	8.99 ± 0.60	3.73 ± 0.24	36.97 ± 1.40	6.44 ± 0.42 a	16.17 ± 1.56 bc
MS_L	38.74 ± 2.26	7.65 ± 1.24	4.05 ± 0.41	36.90 ± 0.80	5.01 ± 0.37 b	18.94 ± 1.17 ab
SS_L	36.06 ± 2.62	6.13 ± 0.56	3.12 ± 0.36	35.35 ± 0.56	3.18 ± 0.16 c	20.38 ± 1.56 a
WW_H	44.78 ± 4.76	8.55 ± 0.82	3.61 ± 0.41	36.58 ± 0.38	6.37 ± 0.20 a	17.43 ± 0.68 abc
MS_H	42.46 ± 6.24	6.81 ± 0.62	3.08 ± 0.30	36.50 ± 0.78	5.10 ± 0.30 b	20.59 ± 1.22 a
SS_H	34.60 ± 2.67	6.49 ± 0.19	2.83 ± 0.15	35.31 ± 0.34	3.12 ± 0.09 c	19.37 ± 0.81 ab

DW Dry weight, *Pre-trt* pretreatment, WW_L, MS_L, SS_L: three water deficits in *L*. WW_H, MS_H, SS_H: three water deficits in *H*. Date was shown with mean ± SE, $n = 3$. Values in the same column were followed by a different letter which were significantly different at $p < 0.05$

Fig. 1 Parameters of leaf photosynthetic capacity under the three water deficits (*WW, MS,* and *SS*) in two labeled treatments (*L, H*). *WW_L*: open rectangle; *MS_L*: open circle; *SS_L*: open triangle; *WW_H*: closed rectangle; *MS_H*: closed circle; *SS_H*: closed triangle. **a** *Pn* in *L* and *H*; **b** *g_s* in *L* and *H*; **c** *Ci/Ca* in *L* and *H*. Date was shown with mean ± SE, *n* = 3
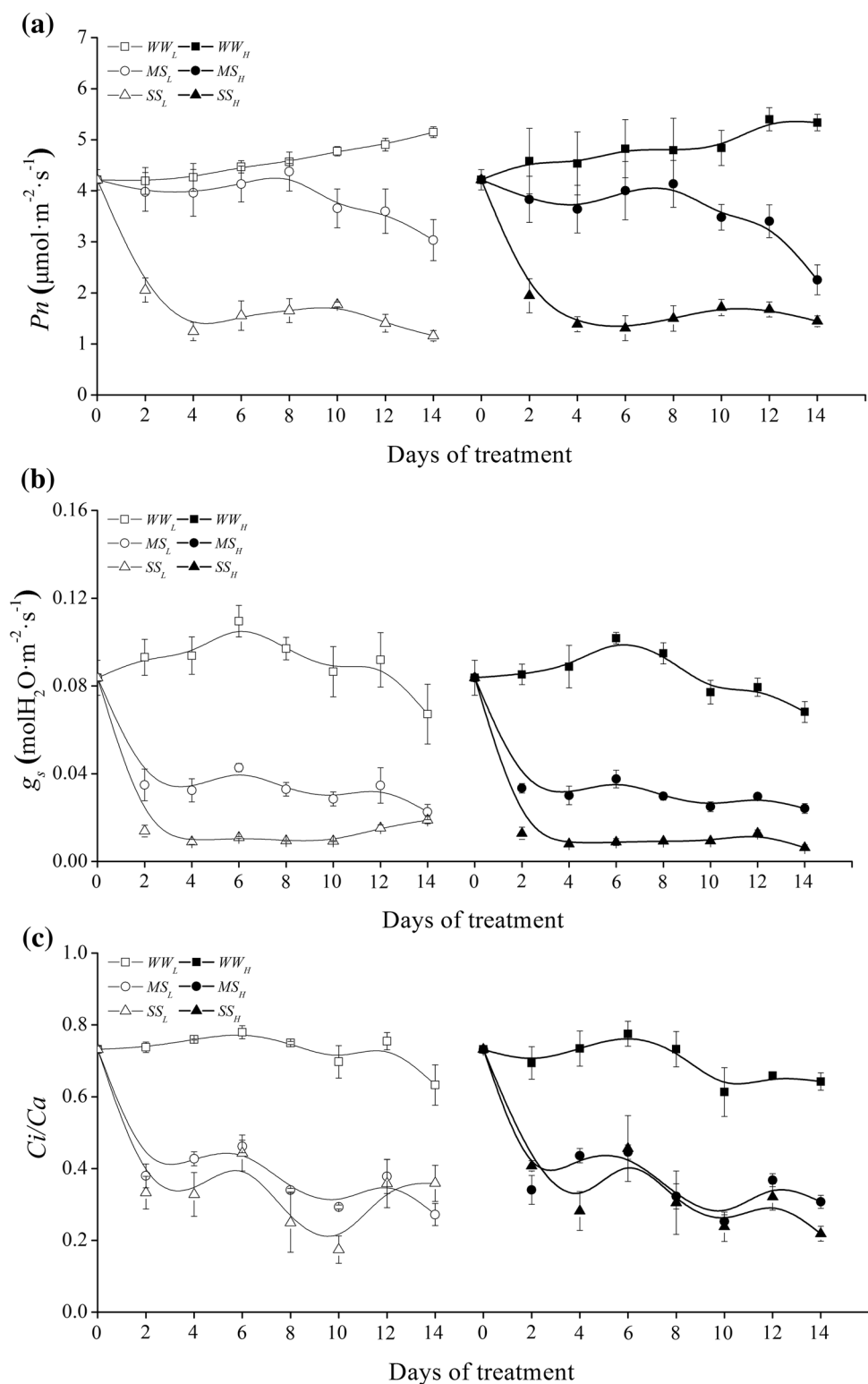
SS treatments, respectively, and those in the *H* were 0.69, 0.35, and 0.32, respectively. In addition, the effect of bicarbonate, water deficit, and their interaction were extremely notable on *Ci/Ca* (Table 3).

C isotope composition of newly expanded leaves and roots

The treatments led to a large range of $\delta^{13}C$ of the newly expanded leaves, as compared to *pre-trt* (Fig. 2a). In
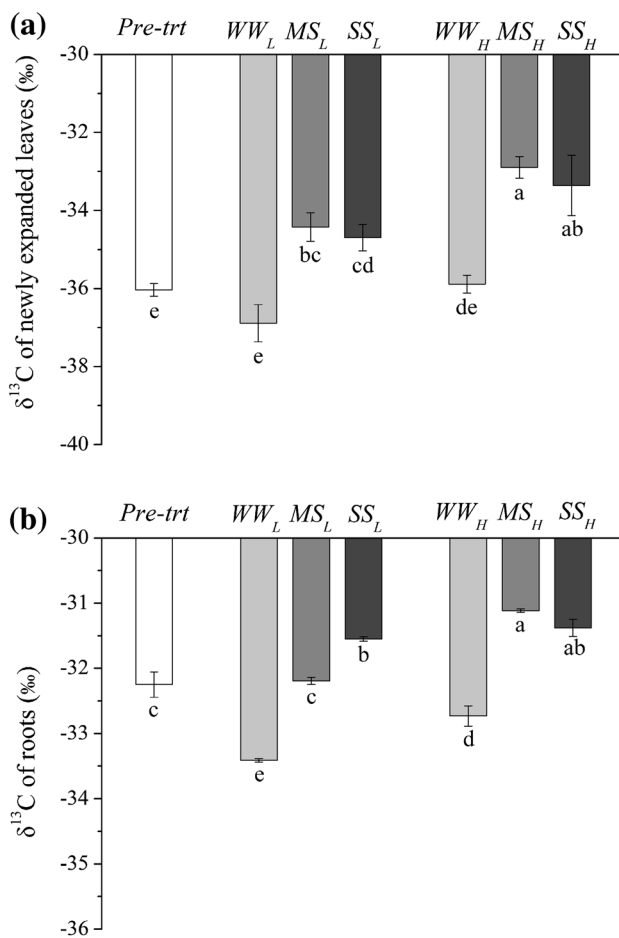
Fig. 2 C isotope composition ($\delta^{13}C$, ‰) of different organs under three water regimes(*WW, MS,* and *SS*) in two labeled treatments (*L*, *H*). **a** $\delta^{13}C$ of newly expanded leaves; **b** $\delta^{13}C$ of roots. The *bar chart* was noted by *different letters* which were significantly different at $p < 0.05$. Date was shown with mean $\pm$ SE, $n = 3$

L, the mean values of newly expanded leaves under the WW_L, MS_L, and SS_L treatments were −36.89, −34.42, and −34.69‰, respectively. In contrast, the $\delta^{13}C$ under the three water deficits in *L* were approximately 1–1.5‰ ^{13}C-depleted compared to *H* ($p < 0.01$). Overall, the $\delta^{13}C$ increased significantly from the *WW* to *MS* treatments and then decreased slightly from the *MS* to *SS* treatments in both *L* and *H*. An interaction analysis revealed that water

deficit and labeling had an extremely significant effect on the $\delta^{13}C$ of leaves (Table 3). Among the treatments, the $\delta^{13}C$ of roots throughout the study ranged from −31.12 to −33.41‰ (Fig. 2b). A significant difference was observed as the water deficit increased from the *MS* to *SS* treatment, but it differed in the change pattern among *L* and *H*. Furthermore, the $\delta^{13}C$ under the three water deficits in *L* were approximately 0.2–0.7‰ negative compared to *H* ($p < 0.01$). The water deficit and labeling had extremely significant effects on the $\delta^{13}C$ values of roots (Table 3).

C isotope fractionation

Great C isotope fractionation was observed from air to leaves, mainly due to CO_2 diffusion and Rubisco catalysis (Table 4). In all cases, the $\Delta^{13}C_{air\text{-}leaves}$ ranged from 17.78 to 21.78‰. The $\Delta^{13}C_{air\text{-}leaves}$ of the WW_L and WW_H treatments were not affected by the water deficit and bicarbonate as compared to the *pre-trt*. The *MS* treatment led to the lowest $\Delta^{13}C_{air\text{-}leaves}$ among the three treatments in both *L* and *H* ($p < 0.01$). Moreover, the $\Delta^{13}C_{air\text{-}leaves}$ of the MS_H and SS_H treatments were both more negative than were those of the MS_L and SS_L treatments, respectively ($p < 0.01$). The data showed that the water deficit and labeling had extremely significant effects on the $\Delta^{13}C_{air\text{-}leaves}$ (Table 3). The $\Delta^{13}C_{solution\text{-}leaves}$ showed pronounced differences between *L* and *H* ($p < 0.01$). The range of the $\Delta^{13}C_{solution\text{-}leaves}$ observed (Table 4), 10.93–13.18‰ in *L* and 17.58–20.11‰ in *H*, was correlated with the mean $\delta^{13}C$ of bicarbonate in the solution (Table 1). In comparison with the $\Delta^{13}C_{solution\text{-}leaves}$, the $\Delta^{13}C_{leaves\text{-}roots}$ in *H* tended to be close to that of *L*. MS_L, MS_H, and SS_H led to significant differences in comparison with *pre-trt*. Bicarbonate, water deficit, and labeling had significant effects on the $\Delta^{13}C_{solution\text{-}leaves}$ values (Table 3).

There was a linear regression between Ci/Ca and $\Delta^{13}C_{air\text{-}leaves}$ in both *L* and *H* (Fig. 3). The correlation coefficients revealed that 61% and 76% of the variation of $\Delta^{13}C_{air\text{-}leaves}$ in *L* and *H* could be explained by Ci/Ca, respectively. In addition, The fitted equation in *L* ($y = 5.43x + 17.64$, $R^2 = 0.61$) was similar with that in *H* ($y = 7.57x + 15.49$, $R^2 = 0.76$). This result showed that the $\Delta^{13}C_{air\text{-}leaves}$ in *L* were larger than that in *H* when

Table 4 C isotope fractionation ($\Delta^{13}C$) between air and leaves, between solution and leaves and between leaves and roots

C isotope fractionation	Pre-trt	WW_L	MS_L	SS_L	WW_H	MS_H	SS_H
$\Delta^{13}C_{air\text{-}leaves}$	20.93 ± 0.16a	21.78 ± 0.48a	19.31 ± 0.37 cd	19.58 ± 0.34bc	20.78 ± 0.23ab	17.78 ± 0.28e	18.25 ± 0.77de
$\Delta^{13}C_{solution\text{-}leaves}$	–	13.18 ± 0.48c	10.93 ± 0.37d	11.86 ± 0.34 cd	20.11 ± 0.23a	17.58 ± 0.28b	18.54 ± 0.77b
$\Delta^{13}C_{leaves\text{-}roots}$	−3.79 ± 0.13d	−3.48 ± 0.45 cd	−2.23 ± 0.37abc	−3.14 ± 0.33bcd	−3.16 ± 0.35bcd	−1.78 ± 0.29a	−1.98 ± 0.66ab

Pre-trt pretreatment. WW_L, MS_L, SS_L: three water deficits in *L*. WW_H, MS_H, SS_H: three water deficits in *H*. Date was shown with mean $\pm$ SE, $n = 3$. Values in the same row were followed by different letters which were significantly different at $p < 0.05$. - no data
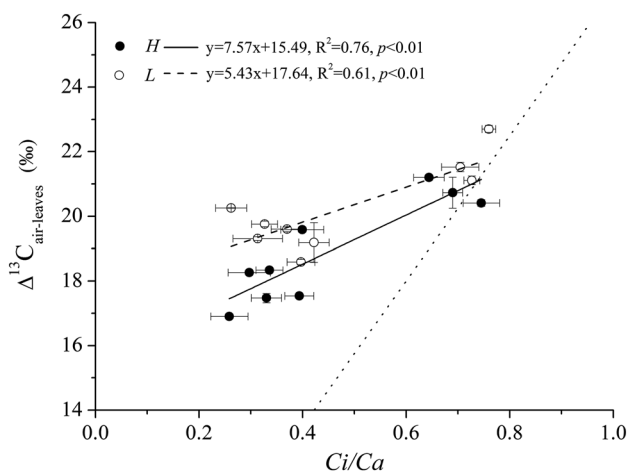
Fig. 3 Relationship between $\Delta^{13}C_{air\text{-}leaves}$ and Ci/Ca in L (*open dots* and *dashed line*) and H (*black dots* and *solid line*). *Dot line*, theoretical regression equation: $y = 4.4 + 22.6x$. $N = 27$
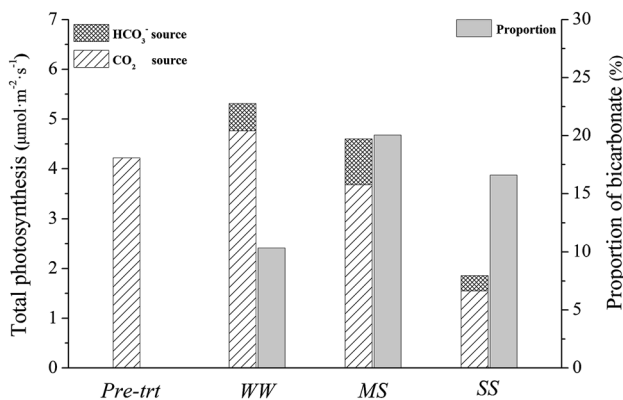

Fig. 4 Total photosynthetically inorganic C assimilation and proportions of fixed CO_2 supplied from bicarbonate under the three water deficits. *Pre-trt*: pretreatment. *WW, MS, SS*: three water deficits in both L and H

the Ci/Ca was equal. However, these two equations were different from the theoretical regression equation ($y = 22.6x + 4.4$).

Quantification of bicarbonate utilization contributing to the total photosynthetically inorganic C assimilation

Under the *WW, MS*, and *SS* treatments, the proportions of fixed CO_2 supplied from bicarbonate contributing to total photosynthesis were 10.34, 20.05, and 16.60% in both L and H, respectively, representing 0.55, 0.92, and 0.31 $\mu mol\ m^{-2}\ s^{-1}$ of Pn, respectively (Fig. 4).

Discussion

Contribution of bicarbonate utilization to the total photosynthetically inorganic C assimilation

It was observed that 10.36 to 20.05% of fixed CO_2 supplied from bicarbonate contribution to total photosynthesis. Under the water stress, there was a higher proportion of transported bicarbonate used by photosynthesis than when well-watered. It was clear that root-derived bicarbonate could enter the plants. The highest enrichment was always observed in the stem, veins, and petiole (Stringer and Kimerer 1993; Bloemen et al. 2015), whereas the portion in the leaves ranged from 0.8 to 8% (Enoch and Olesen 1993; Ford et al. 2007; Aubrey and Teskey 2009). Nevertheless, most studies did not quantify the proportion of fixed CO_2 supplied from DIC via photosynthesis. It is known that about 40% of a plant's dry mass was C which was fixed by photosynthesis (Lambers et al. 2008). The acquisition of atmospheric CO_2 and the bicarbonate utilization both contributed to the plant's C gain. In the present study, the bicarbonate contributed substantial proportion of the total C assimilation under *WW* treatment. The greater proportion (28%) of bicarbonate utilization was observed in paper mulberry (*Broussonetia papyrifera*) (Wu and Xing 2012). Hang and Wu (2016) suggested that the bicarbonate accounted for approximately 3.10 and 13.28% of the total C assimilation in two Brassicaceae plants, respectively. These results were, most likely, species-specific.

Water stress decreased the atmospheric CO_2 gain but triggered a compensatory effect that maximized the bicarbonate utilization in *C. acuminate* seedlings. The water deficit induced stomatal closure was unfavorable to meet the continued demand for maintaining metabolism, leading eventually to C starvation (McDowell et al. 2008). Thus, it promoted the proportion of fixed CO_2 supplied from bicarbonate utilization. Under the *MS* treatment, near 100% of increase in the proportion of bicarbonate utilization was observed over the *WW* treatment. Apparently, it was likely due to stomata closure limiting CO_2 diffusion from atmosphere more than the constrain of transpiration (delivery of bicarbonate). For instance, as the water deficit increased from *WW* to *MS*, the mean g_s values and uptake rate of solution decreased by approximately 64 and 40%, respectively. However, the results also showed that the decrease of Pn values were not proportionate to the decline of g_s values. The mean Pn values only decreased about 17% in L and 28% in H when the water stress increased from *WW* to *MS*. It seemed that the stomatal conductance did not correlate with photosynthesis capacity under the water stress. Previous study showed that the activity of intercellular Carbonic anhydrase increased and catalyzed bicarbonate into CO_2 quickly under the water stress (Pandey et al. 2000).

Thus, we speculated that the carbonic anhydrase might have played an important role in promoting the proportion of bicarbonate utilization. Furthermore, *MS* treatment might be the turning point of bicarbonate utilization. The decline of the proportion revealed that the *SS* treatment exceeded the capacity of the maximum bicarbonate utilization in *C. acuminate* seedlings. The dramatic decline of g_s and the total inhibition of growth, revealed that the plants suffered from great disadvantage. This adverse condition could not be totally compensated by the utilization of bicarbonate.

In this paper, the mixing model, which took bicarbonate and atmospheric CO_2 as the two end-members, along with *L* and *H* labeling treatments, was used to calculate the relative contribution of root-derived bicarbonate utilization to the total photosynthesis. This simple model had ignored discrimination resulted from mesophyll conductance, respiration or photorespiration (O'Leary 1981; Gillon and Griffiths 1997), which might have caused the predicted discrimination differing from observed. Nevertheless, the specially designed *L* and *H* labeling played a vital role in interpreting the source and composition of isotope. The labeling factor only affected the isotope composition of newly expanded leaves and roots, and had no effect on the physiological process, like growth, gas exchange, and systematic discrimination. This phenomenon had been extensively observed among many plant materials in this and our previous work (Wu and Xing 2012; Hang and Wu 2016). We assumed that the discrimination resulted from mesophyll conductance, respiration, or photorespiration in L equaled to that in H under the same treatment. This ignored discrimination could be also eliminated by comparing Eq 3 and Eq 4. In addition, this approach differed from the traditional labeling (^{14}C or high abundance of ^{13}C), which might be underestimate due to respiration of ^{13}C tracer from newly fixed carbohydrate. It was also distinguished from those methods calculating the transpiration ratio or the transport of CO_2 in the xylem sap (Stringer and Kimerer 1993; Levy et al. 1999). Moreover, we had no idea about the measurement of bicarbonate transport in the xylem or leaves. The amount of fixed CO_2 supplied from bicarbonate might be less than the amount of bicarbonate uptake. Some studies showed that a part of the bicarbonate was catalyzed by PEPC and produced malic acid in the roots and xylem sap or reversibly converted it into CO_2 and then fixed it in the bark via corticular photosynthesis (Msilini et al. 2009; Cernusak and Hutley 2011; Covarrubias and Rombolà 2013). Altogether, the enhanced water deficit triggered a compensatory effect that maximized bicarbonate utilization. The water deficit highlighted the critical role of bicarbonate as a part of the global C budget and soil C sequestration. Field experiments must be conducted for the purpose of investigating the proportion of bicarbonate utilization under natural conditions.

Indication of C isotope signature

The influence of *L*- and *H*-labeled treatments on seedlings was faithfully recorded in the C isotope composition of newly expanded leaves and roots. The range of $\delta^{13}C$ in newly expanded leaves reflected the different degree of mixing between atmospheric CO_2 and bicarbonate assimilation under various water deficits. In the bicarbonate endmember, the different $\delta^{13}C$ of photosynthate between *L* and *H* was attributed to the mean $\delta^{13}C$ of the solution (Table 1). The proportion of bicarbonate assimilation was correlated with the solution uptake rate, which was approximately equal in *L* and *H*. In the atmospheric CO_2 end-member, the *Pn* and $\Delta^{13}C_i$ in *L* were both equal to that in *H* under the same water regimes. Thus, it could explain the phenomenon that the $\delta^{13}C$ of newly expanded leaves in *L* showed ^{13}C-depletion in heavy isotopes as compared to *H*. Furthermore, the $\Delta^{13}C_{leaves-roots}$ ranged from −1.92 to −3.30‰, which was in good agreement with some studies (Badeck et al. 2005; Lamade et al. 2016). It suggested that the $\delta^{13}C$ of roots was mostly influenced by the new photosynthate rather than the labeled solution remaining in the roots. Additionally, water deficit also enhances the export of C to the roots (Durand et al. 2016). All of the above observations indicated the involvement of bicarbonate assimilation in the short time scale.

The bicarbonate, water deficit, and their combination could impose osmotic stress on the plants (Ahmed et al. 2013), which results in a rapid gas exchange response like g_s and *Ci*/*Ca* alteration. *Ci*/*Ca* has a close correlation with the yield and $\delta^{13}C$ and $\Delta^{13}C$ (Yasir et al. 2013; Lauteri et al. 2014). The alteration of *Ci* has an effect on the relative accumulation of the $^{13}CO_2$ reaction with Rubisco and has therefore influenced the isotope composition (Farquhar et al. 1989). However, the plot of *Ci*/*Ca* versus $\Delta^{13}C_{air-leaves}$ in Fig. 3 showed a different pattern from the predictive model (Brugnoli et al. 1988). Obviously, *Ci*/*Ca* affected discrimination values in the simple predictive model. However, there was still a large difference under *MS* and *SS* treatments where the most root-derived bicarbonate was assimilated. The different isotopic composition of this internal source was not included in the simple predictive model. It was not in line with previous study which observed a significant linear regression between $\Delta^{13}C_i$ and $\Delta^{13}C_{air-leaves}$ (Brendel 2001). Initially, the *Ci* in the predictive model was under the circumstance without considering the exogenous DIC addition. The root-derived bicarbonate could contribute to the amount of *Ci* due to its reversible conversion to CO_2 (Moroney et al. 2001). The fixed CO_2 supplied from bicarbonate involved in photosynthesis and subsequently affected C isotope discrimination. The underestimated amount of *Ci* explained that $\Delta^{13}C_i$ was less than $\Delta^{13}C_{air-leaves}$ (Fig. 3). In addition, $\Delta^{13}C_i$ only represented

the atmospheric CO_2 end-member assimilation. In our experiment, the proportion of fixed CO_2 supplied from bicarbonate also influenced the overall $\delta^{13}C$. As a indirect substrate for photosynthesis, the mean $\delta^{13}C$ of bicarbonate was negative than that of atmospheric CO_2 in both L and H. The ^{13}C-depleted photosynthate in the bicarbonate end-member was mixed with a relatively ^{13}C-enriched photosynthate in the atmospheric CO_2 end-member at different proportions, resulting in a $\Delta^{13}C_i$ that was less than $\Delta^{13}C_{air\text{-}leaves}$. Moreover, the Pn and Ci/Ca were maintained at a relatively high level in the first week but a low level in the second week under MS and SS treatments (Fig. 1). This process therefore led to both the yield of photosynthesis and $\Delta^{13}C_i$ in the first week that was larger than that in the second week. The time-integrated Ci/Ca probably made it the third reason that $\Delta^{13}C_i$ was less than $\Delta^{13}C_{air\text{-}leaves}$.

Conclusion

Water deficit has always led to a reduction in leaf gas exchange while constraining the atmospheric CO_2 gain in *C. acuminate* seedlings. Nevertheless, bicarbonate stimulates increases in the root length and the formation of fine roots, helping with the uptake of water as well as with HCO_3^- utilization. The bicarbonate was transported to leaves and then reversibly converted to CO_2 as a substrate for photosynthesis. The different mixing proportions of CO_2 and bicarbonate led to the differentiation of $\delta^{13}C$ in newly expanded leaves and its fractionation relative to air and solution in two labeled treatments. Evidence from the natural ^{13}C-abundance of $NaHCO_3$-labeled treatments supported the hypothesis that with the enhancement of water deficit, the proportion of fixed CO_2 supplied from bicarbonate contributing to the total photosynthesis increased to some extent. The role of bicarbonate was so important that it contributed greatly to the total C assimilation, which had been ignored or underestimated in past decades.

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