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# Effect of bicarbonate treatment on photosynthetic assimilation of inorganic carbon in two plant species of Moraceae

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## Abstract

Excessive levels of bicarbonate adversely affect the growth and metabolism of plants. *Broussonetia papyrifera* (L.) Vent. and *Morus alba* L., belonging to family Moraceae, possess the favorable characteristics of rapid growth and adaptability to adverse environments. We examined the response of these two plant species to bicarbonate stress in terms of photosynthetic assimilation of inorganic carbon. They were exposed to 10 mM sodium bicarbonate in the culture solution for 20 days. The photosynthetic response was determined by measuring the net photosynthetic rate of the leaf, water-use efficiency, and chlorophyll fluorescence on days 10 and 20. The bicarbonate-use capacity of the plants was studied by measuring the carbonic anhydrase activity and the compositions of the stable carbon and hydrogen isotopes. The photosynthetic response to high concentration of bicarbonate varied with plant species and treatment durations. High concentrations of bicarbonate decreased the photosynthetic assimilation of inorganic carbon in the two plant species to half that in the control plants on day 10. Bicarbonate treatment did not cause any damage to the reaction centers of photosystem II in *Morus alba*; it, however, caused a decline in the quantum efficiency of photosystem II in *B. papyrifera* on day 20. Moreover, *B. papyrifera* had a greater bicarbonate-use capacity than *M. alba* because carbonic anhydrase converted bicarbonate to $CO_2$ and $H_2O$ to a greater extent in *B. papyrifera*. This study showed that the effect of bicarbonate on photosynthetic carbon metabolism in plants was dual. Therefore, the concentration of bicarbonate in the soil should first be considered during afforestation and ecological restoration in karst areas.

*Additional key words*: bicarbonate-use capacity; carbonic anhydrase; chlorophyll fluorescence; photosynthesis; stable hydrogen isotope.

## Introduction

The bicarbonate ion, which is the major anion present in the soil solution of calcareous soils, is the major harmful factor for crops growing in karst regions, where the $HCO_3^-$ concentration in surface runoff water is about 5 mM (Yan *et al.* 2012). Reduced growth of plants on calcareous soils may often be attributed to the physiological effects of bicarbonate, excessive bicarbonate adversely affects protein synthesis and respiration, reduces absorption of nutrients, inhibits the growth of many plants, and increases chlorosis in many plants (Woolhouse 1966, Garg and Garg 1986, Lee and Woolhouse 1969, Alhendawi *et al*. 1997). The growth of beets (*Beta vulgaris L.*) is influenced to a lesser extent by the level of bicarbonate in the substrate compared to that of beans (Brown and Wadleigh 1955). Moreover, some studies indicated that the addition of bicarbonate can increase photosynthesis. Bicarbonate is considered an essential constituent of the water-oxidizing complex of photo-

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*Abbreviations*: BT − bicarbonate treatment; BUC − bicarbonate-use capacity; CA − carbonic anhydrase; Chl − chlorophyll; $E$ − transpiration rate; $F_m$ – maximum chlorophyll fluorescence; $F_m'$ – maximum fluorescence in the light-adapted state; $F_v$ – variable fluorescence; $F_v'$ – variable fluorescence in the light-adapted state; $F_s$ – fluorescence yield in the steady state; $F_0$ – minimum chlorophyll fluorescence; $F_v/F_m$ − maximum quantum yield of PSII; FM – fresh mass; $g_s$ − stomatal conductance; PDB − Pee Dee Belemnite; $P_N$ − net photosynthetic rate; $P_N'$ − corrected photosynthetic rate; PPFD – photosynthetic photon flux density; PSII − photosystem II; WA − Wilbur and Anderson; WUE − water-use efficiency; $\delta^{13}C$ − ratio of stable carbon isotopes; δD − ratio of stable hydrogen isotopes; $\Phi_p$ − photochemical efficiency of open PSII.
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system II, important for its functioning and stabilization; it could facilitate reassembly of the water-oxidizing complex; and this complex is stabilized by bicarbonate through its binding to other components of PSII (Van Rensen 2002, Klimov *et al*. 2003, Klimov and Baranov 2001). Thus, the response to bicarbonate exposure in terms of growth and physiological characteristics varies with plant species.

*B. papyrifera* (L.) Vent. and *M. alba* L., perennial tree species belonging to the family Moraceae, are characterized by higher growth rate and greater adaptability to adverse environments than other species in this family. Previous investigations have studied the physiological aspects of *B. papyrifera* (Wu *et al*. 2009). *M. alba* is one of the most important cash crops in south India, and the physiological changes in *M. alba* in response to water stress, high temperature, and salt have been previously studied (Barathi *et al*. 2001, Chaltanya *et al*. 2001, Kumar *et al*. 1999). Recently, we have also found that, compared with *M. alba, B. papyrifera* has a greater capacity for using bicarbonate (bicarbonate-use capacity, BUC) as the alternative source of inorganic carbon (Wu *et al*. 2011b). Nevertheless, the differences between *B. papyrifera* and *M. alba* in their responses to bicarbonate treatment in terms of the photosynthetic assimilation of inorganic carbon is still unknown.

Photosynthetic activities can be considered to represent the growth potential of a plant (Mooney 1972, Walters *et al*. 1993). Some plants can use not only the carbon dioxide present in air, but also the bicarbonate present in the substrate, as the source of inorganic carbon (Wu *et al*. 2010, Wu *et al*. 2011b). The utilization of bicarbonate by terrestrial plants involves the action of the enzyme carbonic anhydrase (CA, EC 4.2.1.1) (Wu *et al*. 2010, Wu *et al*. 2011b). CA is a zinc-containing metaloenzyme that catalyzes the reversible conversion of $CO_2$ to bicarbonate. It is widely distributed in animals, plants, archea, and eubacteria, where it is involved in diverse physiological processes, such as ion exchange, acid-base balance, carboxylation-decarboxylation reactions, and diffusion of inorganic carbon both (*1*) between the cell and its environment and (*2*) within the cell (Badger and Price 1994, Sasaki *et al*. 1998). CA regulates photosynthesis in response to water stress. Greater levels of CA activity enhance the conversion of bicarbonate to $H_2O$ and $CO_2$.

Chlorophyll (Chl) *a* in photosystem II (PSII) plays an important role in the photosynthetic response of the leaf to environmental stresses and its fluorescence is used to assess the integrity and efficiency of the photosynthetic apparatus, in addition to indicating the overall health of the plant tissues (Baker 1991, Roháček and Barták 1999). The photosynthetic apparatus, in particular PSII, is sensitive to different stresses. Changes in Chl fluorescence emission, arising mainly from PSII, provide information regarding almost all aspects of photosynthetic activity and therefore, reflect plant tolerance to environmental stresses, including drought (Panda *et al*. 2008). Fluorescence parameters have been applied to the identification of leaf injuries without any visible symptoms. Therefore, Chl fluorescence is also frequently used as a potential indicator of environmental stress and is used to screen for resistant plants (Guo *et al*. 2005).

The stable isotope technique is an important tool to identify the source of an element. The ratios of stable carbon and hydrogen isotopes ($\delta^{13}C$, $\delta D$) have been successfully used to study photosynthesis (Motomura *et al*. 2008, Schwender *et al*. 2004, Tcherkez *et al*. 2009) and water metabolism (Nichols *et al*. 2010). The ratios of stable carbon and hydrogen isotopes ($\delta^{13}C$, $\delta D$) in plants change when the carbon metabolic pathways and the sources of inorganic carbon consumed for photosynthesis are changed. The labeling of the stable carbon and hydrogen isotopes in exogenous bicarbonate can trace whether plants obtain $H_2O$ and $CO_2$ from the conversion of bicarbonate through the action of CA or not. Some studies reported that the bicarbonate was not the substrate in photosynthetic oxygen evolution but did not rule out the involvement of bicarbonate in water oxidation (Clausen *et al*. 2005). Therefore, $\delta^{13}C$ and $\delta D$ were measured to trace the metabolic route of the inorganic carbon source during photosynthesis (Motomura *et al*. 2008).

The present study examined photosynthesis, Chl fluorescence, and CA activity in these two plant species. The utilization of inorganic carbon sources was studied by comparing the differences between *B. papyrifera* and *M. alba* in terms of the foliar composition of the $\delta^{13}C$ and $\delta D$.

## Materials and methods

**Plant growth and bicarbonate ion treatment**: The experiments were conducted in a growth chamber at the Institute of Geochemistry, Chinese Academy of Sciences, Guizhou Province, China [26.35° N, 106.42° E]. The seedlings of *B. papyrifera* and *M. alba* were germinated and cultivated in 12 drain-hole-containing trays layered with quartz sand under a 12-h photoperiod, 300 μmol $m^{-2}$ $s^{-1}$ PPFD, a day/night temperature cycle of 28/20°C, and 60% relative air humidity. Plants were irrigated daily with 1/4-strength Hoagland solution (Hoagland and Arnon 1950). After two months of growth, the nutrient solution was replaced by a modified Hoagland solution containing 6 mM $KNO_3$, 4 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 2 mM Fe(Na)EDTA, 0.25 mM $NH_4H_2PO_4$, 0.75 mM $NH_4Cl$, 2 μM KCl, 50 μM $H_3BO_3$, 4 μM $MnSO_4$, 4 μM $ZnSO_4$, 0.2 μM $CuSO_4$, and 0.2 μM $(NH_4)_6MO_7O_{24}$ at pH 8.1 ± 0.5. The various treatments were as follows:

Treatment 1:10 mM $NaHCO_3$ was added into the modified Hoagland solution to culture the seedlings that germinated healthily and uniformly. The control group

had no exogenous $NaHCO_3$ added into the modified Hoagland solution. The $NaHCO_3$ was labeled with a $\delta^{13}C$ value of −17.4‰ PDB.

Treatment 2 was the same as Treatment 1, except that $NaHCO_3$ was replaced by a $\delta^{13}C$ value of –6.7‰ PDB.

The seedlings subjected to both the treatments were arranged in a completely randomized design, and 24 healthy and uniform seedlings from each species were studied. These treatments lasted for 20 d. During the experiments, the solution was changed every other day. Measurements were carried out in duplicate on days 10 and 20.

**Net photosynthetic rate ($P_N$), stomatal conductance ($g_s$), and transpiration rate ($E$)** were measured on the fourth youngest fully expanded leaf from the top between 09:30 and 11:00 h every 10 d from the onset of bicarbonate treatment. Three plants from each treatment group were used for the measurements. The photosynthetically active radiation, temperature, and $CO_2$ concentration during the measurements were 300 μmol $m^{-2}$ $s^{-1}$, 30°C, and 400 μmol $mol^{-1}$, respectively. A portable *LI-6400XT* photosynthesis measurement system (*LI-COR Inc.*, *Lincoln*, NE, USA) was used. Water-use efficiency (WUE) was calculated according to the following equation: $WUE = P_N/E$.

**Chl fluorescence** was measured with a portable *LI-6400XT* photosynthesis measurement systém (*LI-COR Inc.*, *Lincoln*, NE, USA). Leaves were dark-adapted for 30 min to ensure complete relaxation of all reaction centers before the measurements. As mentioned earlier, the fourth youngest fully expanded leaf from the top (three plants from each treatment group) was selected for measurement, which was conducted every 10 d after the onset of bicarbonate anion treatment. The minimum Chl fluorescence ($F_0$) was determined using a measuring beam, whereas the maximum Chl fluorescence ($F_m$) was recorded after a 0.8-s exposure to a saturating light pulse (6,000 μmol $m^{-2}$ $s^{-1}$). Then, actinic light (300 μmol $m^{-2}$ $s^{-1}$) was applied for 1 min on the adaxial side of the leaves to drive photosynthesis. Maximum fluorescence in the light-adapted state ($F_m'$), basic fluorescence after induction ($F_0'$), variable fluorescence ($F_v'$), and fluorescence yield in the steady state ($F_s$) were determined. Maximum quantum yield of PSII ($F_v/F_m$) was calculated as $(F_m - F_0)/F_m$, where $F_v = F_m - F_0$. The photochemical efficiency of open PSII ($\Phi_p$) was calculated as $F_v'/F_m'$.

**CA activity**: Every 10 d after the onset of bicarbonate treatment, the fourth and fifth youngest fully expanded leaves from the top (three plants from each treatment group) were chosen for measurement of the CA activity. Leaf tissues (0.1–0.2 g) were quickly frozen in liquid nitrogen and ground with 3 ml extraction buffer (0.01 M sodium barbitone with 0.05 M mercaptoethanol, pH 8.3). The homogenate was centrifuged at 10,000 × *g* at 0°C for 5 min and then placed on ice for 20 min. The supernatant was used to analyze the CA activity using the pH method described by Wilbur and Anderson (Wilbur and Anderson 1948) with slight modifications (Wu *et al*. 2011a). In brief, CA activity was assayed at 0–2°C in a mixture containing 4.5 ml of 0.02 M barbitone buffer (5, 5-diethylbarbituric acid; pH 8.3), 0.4 ml of a sample, and 3 ml of $CO_2$-saturated water. CA activity was expressed in Wilbur and Anderson as WA [WAU $g^{-1}$(FM)] = $(t_0/t) - 1$, where $t_0$ and $t$ are the time(s) recorded for the pH change from 8.2 to 7.2 with buffer alone ($t_0$) and with sample ($t$).

**Ratios of stable carbon ($\delta^{13}C$) and hydrogen (δD) isotopes** were determined for the first youngest fully expanded leaf from the top using gas-isotope-ratio mass spectrometry (*MAT-252*, *Finnigan MAT*, Bremen, Germany) and continuous-flow stable-isotope mass spectrometry (*Isoprime*, Manchester, United Kingdom). Four expanded leaves in each species of the Moraceae plants at each stress level were randomly detached from the 24 seedlings every 10 d after the onset of bicarbonate treatment.

**Calculations of bicarbonate utilization proportion and corrected photosynthetic rate**: According to the bivariate isotope-mixture model,

$$\delta_T = \delta_A - f_B \delta_A + f_B \delta_B \quad (1)$$

For the $NaHCO_3$ labeled with the $\delta^{13}C$ value of −17.4‰ PDB (Treatment 1), Eq. 1 can be rewritten as follows:

$$\delta_{T1} = \delta_{A1} - f_{B1} \delta_{A1} + f_{B1} \delta_{B1} \quad (2)$$

where $\delta_{T1}$ is the $\delta^{13}C$ value of the leaves of the plants that were cultivated with the $NaHCO_3$ labeled with the $\delta^{13}C$ value of −17.4‰ PDB, $\delta_{A1}$ is the $\delta^{13}C$ value of plant leaves when they used $CO_2$ as their unique carbon source, $\delta_{B1}$ is the $\delta^{13}C$ value of plant leaves when they used $NaHCO_3$ as their unique carbon source, and $f_{B1}$ is the bicarbonate utilization proportion of the plant.

For the $NaHCO_3$ labeled with the $\delta^{13}C$ value of −6.7‰ PDB (Treatment 2), Eq. 1 can be modified as follows:

$$\delta_{T2} = \delta_{A2} - f_{B2} \delta_{A2} + f_{B2} \delta_{B2} \quad (3)$$

where $\delta_{T2}$ is the $\delta^{13}C$ value of the leaves of the plants cultivated with the $NaHCO_3$ labeled with the $\delta^{13}C$ value of −6.7‰ PDB, $\delta_{A2}$ is the $\delta^{13}C$ value of plant leaves when they used $CO_2$ as their unique carbon source, $\delta_{B2}$ is the $\delta^{13}C$ value of plant leaves when they used $NaHCO_3$ as their unique carbon source, and $f_{B2}$ is the bicarbonate utilization proportion of the plant.

Comparing Eqs. 2 and 3, $\delta_{A1} = \delta_{A2}$, $f_B = f_{B1} = f_{B2}$; thus, Eqs. 2 and 3 can be used for calculations as follows:

$$f_B = \frac{\delta_{T1} - \delta_{T2}}{\delta_{B1} - \delta_{B2}} \quad (4)$$

$\delta_{B1} - \delta_{B2}$ in Eq. 4 can be replaced with $\delta_{C1} - \delta_{C2}$, where $\delta_{C1}$ is the $\delta^{13}C$ value of the first stable-isotope-labeled $NaHCO_3$, and $\delta_{C2}$ is the $\delta^{13}C$ value of the second stable-isotope-labeled $NaHCO_3$. Therefore, Eq. 4 can be rewritten as

$$f_B = \frac{\delta_{T1} - \delta_{T2}}{\delta_{C1} - \delta_{C2}} \quad (5)$$

Due to the influence of $CO_2$ present in the air on the nutrient solution containing low concentrations of bicarbonate, we did not calculate the bicarbonate utilization proportion of *B. papyrifera* in the control plants and only calculated, using Eq. 5, the value for plants grown under treatment with 10 mM sodium bicarbonate.

The carbon and hydrogen molar equivalence ratio is 1:1 when plants use $H_2O$ and $CO_2$ from the conversion of bicarbonate. The hydrogen in the photosynthate is derived from three sources: water, hydrogen (through the conversion of bicarbonate by CA), and cell metabolism. Therefore, the hydrogen isotopes of the plants used in the experiments are fit for investigation using the trivariate isotope-mixture model. According to that model,

$$\delta_S = f_P \delta_P + f_M \delta_M + f_N \delta_N \quad (6)$$
$$f_P + f_M + f_N = 1 \quad (7)$$

where $\delta_S$ is the δD value of leaves of the plants cultivated with $NaHCO_3$, $\delta_P$ is the δD value of plant leaves when they used $H_2O$ as their unique hydrogen source, $\delta_M$ is the δD value of plant leaves when they used $NaHCO_3$ as their unique hydrogen source, $\delta_N$ is the δD value of plant leaves when they used the metabolic hydrogen as their unique hydrogen source, $f_M$ is the bicarbonate-H utilization proportion of the plant, and $f_N$ is the metabolic hydrogen utilization proportion of the plant.

The bicarbonate-H and the metabolic hydrogen combine equivalently and produce water for plant photosynthesis. Therefore, $f_M$ is equal to $f_N$.

Thus, $f_M$ can also be calculated as follows:

$$f_M = \frac{\delta_S - \delta_P}{(\delta_M + \delta_N) - 2\,\delta_P} \quad (8)$$

where $\delta_S$ is the δD value of leaves of the plants cultivated with $NaHCO_3$, $\delta_P$ is the δD value of plant leaves when they used $H_2O$ as their unique hydrogen source, $\delta_M$ is the δD value of plant leaves when they used $NaHCO_3$ as their unique hydrogen source, $\delta_N$ is the δD value of plant leaves when they adopted metabolic hydrogen as their unique hydrogen source, and $f_M$ is the bicarbonate-H utilization proportion of the plant.

The value of $f_M$ in Eq. 8 is almost equal to the value of $f_B$ in Eq. 5.

The corrected photosynthetic rates, $P_N'$, were hence calculated as

$$P_N' = P_N + P_N \times f_B/(1 - f_B) \quad (9)$$

where $P_N$ is the net $CO_2$-assimilation rate of the plant leaves, and $f_B$ is the bicarbonate utilization proportion of the plant. Here, $P_N \times f_B/(1 - f_B)$ is defined as BUC.

**Statistical analysis**: All measurements were subjected to analysis of variance (*ANOVA*) to discriminate significant differences (defined as $p \leq 0.05$) between the group mean values. The data were shown as the means ± standard errors (SE). These mean data were analyzed statistically using a factorial design using the *SPSS* software (version *13.0, SPSS Inc*., CA, USA), and the mean results were compared using the least significant difference (LSD) post-hoc test at the 5% significance level ($p<0.05$).

## Results

***$P_N$, $g_s$,* and WUE**: Both *B. papyrifera* and *M. alba* showed a significant decrease in $P_N$, $g_s$, and WUE under bicarbonate treatment (Table 1). However, the responses of *B. papyrifera* and *M. alba* in terms of $P_N$, $g_s$, and WUE to bicarbonate treatment were different. *B. papyrifera* showed a greater decrease in $P_N$, $g_s$, and WUE values than *M. alba* under bicarbonate treatment. Moreover, the values of $P_N$ and WUE varied with the duration of bicarbonate treatment. The $P_N$ value of *B. papyrifera* under bicarbonate treatment was 42% that of the control group on day 10 and only 31% on day 20. Similarly, the $P_N$ value of *M. alba* under bicarbonate treatment was 45% that of the control group on day 10 and 52% on day 20. The $g_s$ values of *B. papyrifera* under bicarbonate treatment were all 50% that of the control on day 10 and 20, while the $g_s$ value of *M. alba* under bicarbonate treatment was 60% that of the control group on day 10 and 71% on day 20. The WUE values of *B. papyrifera* under bicarbonate treatment were only 12% and 43% that of the control group on days 10 and 20, respectively. Meanwhile, the WUE values of *M. alba* under bicarbonate treatment were 49% and 67% that of the control plants on days 10 and 20, respectively.

**Chl fluorescence**: Compared with the control plants, both *B. papyrifera and M. alba* did not show marked changes in the $F_v/F_m$ values under bicarbonate treatment, either on day 10 or day 20 (Table 2). Compared with the control plants, the $\Phi_p$ value of the two species increased a little due to the presence of bicarbonate; however, the difference is not significant on day 10. On day 20, the $\Phi_p$ values in *B. papyrifera* under bicarbonate treatment were significantly lower than those in the control plants, whereas the $\Phi_p$ values in *M. alba* under bicarbonate treatment were higher than those in the control plants (not significant).

Table 1. The net photosynthetic rate ($P_N$), stomatal conductance ($g_s$), and water-use efficiency (WUE) in the two species under different treatments. The means ± SE ($n$ = 5) followed by *different letters* in the same parameter of Moraceae plants differ significantly at $p \leq 0.05$, according to one-way *ANOVA* and *t*-test. BT – bicarbonate treatment. *This column stands for the percent value after BT with reference to that of the control plants.

| Parameter | Treatment | *B. papyrifera* | | | | *M. alba* | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Day 10 | | Day 20 | | Day 10 | | Day 20 | |
| | | Mean ± SE | [%]* | Mean ± SE | [%]* | Mean ± SE | [%]* | Mean ± SE | [%]* |
| $P_N$ [μmol($CO_2$) $m^{-2}$ $s^{-1}$] | Control | 5.91 ± 0.34$^{a}$ | 100 | 6.13 ± 0.02$^{a}$ | 100 | 4.08 (0.39)$^{c}$ | 100 | 4.89 ± 0.21$^{b}$ | 100 |
| | BT | 2.49 ± 0.14$^{de}$ | 42 | 1.91 ± 0.10$^{de}$ | 31 | 1.82 ± 0.32$^{e}$ | 45 | 2.55 ± 0.18$^{d}$ | 52 |
| $g_s$ [mol($H_2O$) $m^{-2}$ $s^{-1}$] | Control | 0.04 ± 0.005$^{bc}$ | 100 | 0.06 ± 0.005$^{ab}$ | 100 | 0.05 ± 0.004$^{b}$ | 100 | 0.07 ± 0.007$^{a}$ | 100 |
| | BT | 0.02 ± 0.002$^{c}$ | 50 | 0.03 ± 0.003$^{c}$ | 50 | 0.03 ± 0.002$^{c}$ | 60 | 0.05 ± 0.006$^{b}$ | 71 |
| WUE | Control | 13.14 ± 1.16$^{a}$ | 100 | 6.91 ± 0.42$^{b}$ | 100 | 5.70 ± 0.48$^{bc}$ | 100 | 4.02 ± 0.43$^{c}$ | 100 |
| [mmol ($CO_2$) $mol^{-1}$ ($H_2O$)] | BT | 1.56 ± 0.14$^{d}$ | 12 | 2.95 ± 0.39$^{cd}$ | 43 | 2.81 ± 0.60$^{cd}$ | 49 | 2.68 ± 0.45$^{cd}$ | 67 |

Table 2. The maximal PSII photochemical efficiency ($F_v/F_m$) and the photochemical efficiency of open PSII ($\Phi_p$) in the two species under different treatments. The means ± SE ($n$ = 9) followed by *different letters* in the same parameter of Moraceae plants differ significantly at $p \leq 0.05$, according to one-way *ANOVA* and *t*-test. BT – bicarbonate treatment.

| Parameter | Treatment | *B. papyrifera* | | *M. alba* | |
|---|---|---|---|---|---|
| | | Day 10 | Day 20 | Day 10 | Day 20 |
| $F_v/F_m$ | Control | 0.77 ± 0.02$^{ab}$ | 0.81 ± 0.00$^{a}$ | 0.76 ± 0.01$^{ab}$ | 0.74 ± 0.03$^{b}$ |
| | BT | 0.78 ± 0.01$^{ab}$ | 0.78 ± 0.01$^{ab}$ | 0.77 ± 0.01$^{ab}$ | 0.79 ± 0.01$^{a}$ |
| $\Phi_p$ | Control | 0.47 ± 0.04$^{bc}$ | 0.58 ± 0.02$^{a}$ | 0.40 ± 0.01$^{c}$ | 0.38 ± 0.03$^{c}$ |
| | BT | 0.53 ± 0.01$^{ab}$ | 0.50 ± 0.00$^{b}$ | 0.44 ± 0.02$^{c}$ | 0.44 ± 0.01$^{c}$ |

Table 3. Carbonic anhydrase (CA) activities in the two species under different treatments. The means ± SE ($n$ = 5) followed by *different letters* in the same parameter of Moraceae plants differ significantly at $p \leq 0.05$, according to one-way *ANOVA* and *t*-test. BT – bicarbonate treatment.

| Parameter | Treatment | *B. papyrifera* | | | | *M. alba* | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Day 10 | | Day 20 | | Day 10 | | Day 20 | |
| | | Mean | SE | Mean | SE | Mean | SE | Mean | SE |
| CA [WAU $g^{-1}$(FM)] | Control | 4,568 | 138$^{B}$ | 9,273 | 94$^{A}$ | 557 | 5$^{D}$ | 172 | 6$^{E}$ |
| | BT | 3,818 | 76$^{C}$ | 4,674 | 66$^{B}$ | 758 | 9$^{D}$ | 171 | 8$^{E}$ |

**CA activity** varied with plant species (Table 3). *B. papyrifera* had a significantly greater CA activity, at least five times greater than that in *M. alba*.

**Ratios of stable hydrogen isotope**: The δD value in *B. papyrifera* was higher than that in *M. alba* (Table 4). Furthermore, the δD value in *B. papyrifera* under bicarbonate treatment was higher than that under control conditions on both the 10$^{th}$ and 20$^{th}$ days. The δD values in bicarbonate-treated and control *M. alba* plants showed no significant difference on day 10. On day 20, the δD value in *M. alba* grown under bicarbonate treatment was lower than that in the control plants.

**BUC and corrected photosynthetic rates**: The seedlings of *B. papyrifera* were cultured with 10 mM of $NaHCO_3$, which had different $\delta^{13}C$ values, namely, –17.2‰ and –6.7‰ PDB. On day 20, the foliar $\delta^{13}C$ values in these seedlings, $\delta_{T1}$ and $\delta_{T2}$, were –32.3‰ and –29.3‰, respectively. According to Eq. 5, the $f_B$ was equal to 0.28.

The value of $f_B$ was almost equal to the value of the bicarbonate-H utilization proportion ($f_M$) of *B. papyrifera* when they were cultured with 10 mM of $NaHCO_3$ for 20 d.

On day 20, the δD value of *M. alba* was the lowest under bicarbonate treatment. Therefore, we hypothesize that no bicarbonate is utilized by *M. alba*. In this period, the δD value of *M. alba* was uniquely influenced by $H_2O$ from the culture solution. Therefore, the δD value of *M. alba* under bicarbonate treatment on day 20 was considered equal to $\delta_P$, *i.e.* $\delta_P$ = –107.6‰. According to Eq. 8, $\delta_M + \delta_N$ could be calculated as –134.6‰, that is, Eq. 8 can be rewritten as

$$f_M = \frac{\delta S + 107.6}{80.6} \quad (10)$$

Table 4. Values of δD in the two species under different treatments. The values are represented as means ± SE ($n$ = 5). BT – bicarbonate treatment.

| Parameter | Treatment | *B. papyrifera* | | *M. alba* | |
|---|---|---|---|---|---|
| | | Day 10 | Day 20 | Day 10 | Day 20 |
| δD [‰] | Control | –92.9 ± 1.6 | –92.4 ± 0.2 | –102.9 ± 1.9 | –94.5 ± 1.5 |
| | BT | –85.0 ± 0.8 | –85.0 ± 1.3 | –101.6 ± 2.7 | –107.6 ± 0.0 |

Table 5. Bicarbonate-use capacity (BUC) and corrected photosynthetic rate ($P_N$') of the two Moraceae plants under different treatments. BT – bicarbonate treatment. *This column stands for the percent value after bicarbonate treatment in relation to that of the control plants.

| Parameter | Treatment | *B. papyrifera* | | | | *M. alba* | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Day 10 | | Day 20 | | Day 10 | | Day 20 | |
| | | Mean | [%]* | Mean | [%]* | Mean | [%]* | Mean | [%]* |
| BUC [μmol($CO_2$) $m^{-2}$ $s^{-1}$] | Control | 2.54 | 100 | 2.64 | 100 | 0.73 | 100 | 0.88 | 100 |
| | BT | 0.97 | 38 | 0.74 | 28 | 0.15 | 21 | 0 | 0 |
| $P_N$' [μmol($CO_2$) $m^{-2}$ $s^{-1}$] | Control | 8.45 | 100 | 8.77 | 100 | 4.81 | 100 | 5.77 | 100 |
| | BT | 3.46 | 41 | 2.65 | 30 | 1.97 | 41 | 2.55 | 45 |

According to Eq. 10, the $f_M$ values of *B. papyrifera* under bicarbonate treatment on days 10 and 20 were both 0.28, whereas those of *M. alba* were 0.07 and 0.0, respectively. The $f_M$ values of *B. papyrifera* and *M. alba* under control conditions, in which the concentration of bicarbonate in the culture solution was 1.16 ± 0.08 (mean ± SE, $n$ = 5), can be considered to be 0.30 and 0.15, respectively (Wu *et al*. 2011b). The corrected photosynthetic rate ($P_N$') was calculated using Eq. 9.

*B. papyrifera* had at least three-fold BUC than *M. alba* during the same period and under the same treatment (Table 5). Both *B. papyrifera* and *M. alba* under control conditions had a greater BUC than under bicarbonate treatment. Under that treatment, *M. alba* had a weak BUC, the value being only 0.15 μmol $m^{-2}$ $s^{-1}$ on day 10; no BUC was observed on day 20. However, *B. papyrifera* had a large BUC even under bicarbonate treatment. The values of $P_N$' in both *B. papyrifera* and *M. alba* under control conditions were higher than the values under bicarbonate treatment. Under bicarbonate treatment, the $P_N$' value of *B. papyrifera* was 1.8 times that of *M. alba* on day 10; and slightly greater than that of *M. alba* on day 20.

## Discussion

Bicarbonate inhibits the $P_N$ and $P_N$' values of plants. The inhibition might have both long-term and short-term effects. The study highlights the differences between *B. papyrifera* and *M. alba* in terms of $P_N$ and $P_N$' values in response to 10 mM sodium bicarbonate on days 10 and 20. In *B. papyrifera*, the inhibition of $P_N$ and $P_N$' increased with increase in treatment duration; however, in *M. alba,* the inhibition decreased with increased treatment duration. *M. alba* showed a greater tolerance to bicarbonate than *B. papyrifera*. Both *B. papyrifera* and *M. alba* under bicarbonate treatment had $P_N$ and $P_N$' values lower than 50% of the control values on day 10, whereas *M. alba* had more than 50% $P_N$ on day 20. This result suggested that bicarbonate exerted its effect in a species-specific manner. The short-term effect, which decreased the photosynthetic assimilation of inorganic carbon in the two Moraceae plant species up to half the level in the control plants on day 10, was very obvious. The effect can be interpreted neither as bicarbonate requirement for the water-oxidizing complex of PSII (Klimov and Baranov 2001) nor as the probable inhibition of protein synthesis by bicarbonate (Nikolic *et al*. 2000, Srivastava *et al*. 1997, Stemler 2002). When bicarbonate inhibits photosynthetic assimilation of inorganic carbon, it inhibits WUE simultaneously. Therefore, the short-term effect of bicarbonate on photosynthetic assimilation of inorganic carbon might involve the decline of the activity of the enzymes involved in photosynthetic carbon metabolism (Kumar and Kumar 2001). However, the mechanism of inhibition of activity of the enzymes involved in photosynthetic carbon metabolism by bicarbonate was hitherto unknown.

The long-term effect of bicarbonate on photosynthetic assimilation of inorganic carbon might involve bicarbonate uptake and utilization. Under bicarbonate treatment, the values of $P_N$ and $P_N$' in *B. papyrifera* decreased with the increase of treatment duration, whereas those in *M. alba* increased. Considering the difference in bicarbonate use by the two species of Moraceae, we can conclude that the more the plant used bicarbonate, the greater is the

detrimental effect of bicarbonate. Greater bicarbonate uptake and utilization by plants may lead to a strong inhibition of saccharide metabolism and protein synthesis (Yang *et al*. 1993, Nikolic *et al*. 2000, Srivastava *et al*. 1997) and/or a gradual reduction of ribulose-1,5-bisphosphate carboxylase/oxygenase (Bertamini *et al*. 2001).

Bicarbonate has a stabilization effect on the oxygen-evolving complex of PSII (Klimov *et al*. 2003), which favors the enhancement of the quantum efficiency of open PSII. In this study, we found that the values of $F_v/F_m$ and $\Phi_p$ in *M. alba* slightly increased under bicarbonate treatment on days 10 and 20. It showed that the effect of bicarbonate treatment on the photosynthesis in *M. alba* did not involve any damage to the PSII reaction centers. However, bicarbonate decreased the $\Phi_p$ values in *B. papyrifera* on day 20. Therefore, the quantum efficiency of PSII in *B. papyrifera* was damaged on day 20.

The study showed that use of bicarbonate labeled with stable carbon isotopes and the determination of both stable hydrogen and carbon isotopes may yield the bicarbonate utilization proportion in *B. papyrifera* under treatment with high concentrations of bicarbonate. Thus, we obtained the BUC levels in both *B. papyrifera* and *M. alba*. From this study, we found that BUC directly reflects the conversion of bicarbonate to $CO_2$ and $H_2O$ by the action of CA. Greater CA activity in *B. papyrifera*, compared with that in *M.* alba, led to greater BUC. *B. papyrifera* had approximately 30% of its photosynthate derived from the $CO_2$ converted by CA, whereas *M. alba* had only 0–15%.Therefore, this study provides evidence that the $H_2O$ converted from bicarbonate is the substrate in photosynthetic oxygen evolution and not the bicarbonate itself. This result is consistent with the results of the study by Clausen *et al*. (2005).

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