

Article

Differential Responses of Nitrate/Ammonium Use to Bicarbonate Supply in Two *Brassicaceae* Species under Simulated Karst Habitat

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Abstract: In the karst habitats with nitrate-abundant and ammonium-rare soil, the bicarbonate supply plays a crucial role in both inorganic carbon and nitrogen assimilation in various plant species. Consequently, two carbon sources, carbon dioxide (CO₂) and bicarbonate (HCO₃⁻), and two nitrogen sources, namely nitrate (NO₃⁻) and ammonium (NH₄⁺) are available for plants. However, variations in the absorption and utilization of nitrate, ammonium, and inorganic carbon during bicarbonate supply in different plants are not well-depicted. In this study, we evaluated the nitrate/ammonium use efficiency and their contributions to the total nitrogen assimilation/utilization capacity at different bicarbonate levels using a bidirectional stable nitrogen isotope tracer approach. The inorganic carbon assimilation, such as the photosynthesis, carbon/nitrogen enzymatic activities, carbon/nitrogen content, nitrogen assimilation/utilization capacity, and nitrate/ammonium contributions to plant growth, were also evaluated to decipher the responses of both carbon and nitrogen metabolism to bicarbonate supply in karst habitats. With the increasing bicarbonate level, *Orychophragmus violaceus* (*Ov*) was found to be more available for nitrate to total nitrogen assimilation and utilization than *Bn* (*Brassica napus*). Further, it enhanced the contributions of nitrate and nitrogen accumulation/utilization capacity and inorganic carbon assimilation, increasing photosynthesis, carbon/nitrogen enzymatic activities, and carbon accumulation, and promoted the growth in *Ov*. Though the highest bicarbonate level was conducive to ammonium utilization and water use efficiency in both *Ov* and *Bn*, it inhibited total inorganic carbon and nitrogen assimilation, leading to growth suppression in *Bn* compared to *Ov*. Moreover, considering the optimistic responses of both carbon and nitrogen assimilation to the high bicarbonate supply in nitrate-abundant, as well as ammonium-rare, environments, we conclude that *Ov* was more adaptable to the karst habitats. This study provides a novel approach to elucidate the responses of nitrate/ammonium utilization and inorganic carbon assimilation to bicarbonate. Furthermore, the current study reveals the complex interactions among different carbon–nitrogen metabolism pathways in various plants and their adaptations to karst habitats.



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1. Introduction

High bicarbonate (high pH), abundant nitrite, bare ammonium, and drought are typical characteristics of karst habitats [1]. The widely exposed carbonatite corroded by long-term karstification leads to loose soil structures and poor water storage capacity, which is the major reason causing drought in the karst habitat [2]. Moreover, abundant bicarbonate derived from the dissolved carbonatite promotes soil alkalinity, resulting in high bicarbonate and pH [3,4]. Previously, it has been shown that the bicarbonate content in the karst wet soils of Guizhou, China, is about 5–10 mM or even higher [5]. Thus, the hydrogen ion is rapidly consumed, and ammonium is volatilized in the drought and high bicarbonate

(high pH) habitats, leading to a nitrate-abundant and ammonium-rare environment [1,6]. Keeping this in mind, the important role of bicarbonate in karst habitats has been reported in various studies; based on which, the perception of the bicarbonate's role in plant growth has changed universally. Earlier, it was believed that the bicarbonate exerts negative effects, such as the inhibition of photosynthesis, and nitrogen assimilation, leading to the decline in plant growth [7,8]. Although studies have widely reported the variation in inorganic carbon or nitrogen utilization during bicarbonate supplementation among different plant species [4,9], there are few reports on the response of nitrate/ammonium utilization to the bicarbonate supply in plants under karst habitats.

In plants, the inorganic carbon and nitrogen utilization strategy reflect their karst adaptation mechanisms, dominated by both inorganic carbon and nitrogen sources in the environment [10,11]. In the karst habitats, plant growth is affected by two inorganic carbon sources, carbon dioxide and bicarbonate, and two inorganic nitrogen sources, nitrate and ammonium [12]. The positive response to high bicarbonate, which reflected the bicarbonate use capacity (BUC), is one of plant's most essential inorganic carbon metabolism strategies employed for adaptation to karst habitats [3,13]. Compared to the nonadaptable plants, the adaptable species are more efficient in bicarbonate utilization, which not only protects the roots from high bicarbonate and pH environment but also maintains the photosynthesis level to support the normal growth of plants [14,15]. In the karst adaptable plant species, the BUC is attributed to affluent carbonic anhydrase (CA), which facilitates the conversion of bicarbonate into carbon dioxide and water [16]. This process protects plants from insufficient carbon and water conditions and restores photosynthesis in the karst habitats. Wu et al. [3] and Rao et al. [9] confirmed that bicarbonate, which was absorbed by roots, participated in plants' total inorganic carbon assimilation in *Camptotheca acuminata*. Moreover, karst-adaptable plants are more efficient in BUC than karst-nonadaptable plants to escape their growth inhibitions due to high bicarbonate-containing soil [4]. In this view, it is found that *Broussonetia papyrifera* (Bp., a typical species of karst-adaptable plant) could utilize more bicarbonate for total carbonate assimilation by enhancing both the glycolytic pathway and pentose phosphate pathway [17] and improving the photosystem electron transfer reaction [18,19] to increase karst drought resistance. Overall, the bicarbonate promotes roots to secrete organic acids, which help in maintaining cellular homeostasis.

Evidence suggests that the efficient utilization of nitrate and ammonium is an important mechanism in plants for karst adaptation [20,21]. It has also been established that karst-adaptable plants exhibit outstanding nitrate utilization efficiency [1], which might attribute to better BUC and total nitrogen assimilation capability, resulting in efficient nitrate reduction, which is promoted by more electronic supplies from excessive bicarbonate in the karst habitats [6]. Altogether these reports provide partial scientific evidence highlighting the role of inorganic carbon or nitrogen metabolisms in plant species adaptable to karst habitats. Interestingly, under high bicarbonate, pH, and drought conditions, intricate interactions between the inorganic carbon and nitrogen metabolism have been observed in plants. However, alteration in the nitrate reduction and ammonium assimilation in response to the bicarbonate supply is not clearly understood to date.

The nitrogen isotope value is performed for the quantification of inorganic nitrogen assimilation in plants [22]. The $\delta^{15}\text{N}$ value, ranging between -10% and 22% , represents the inorganic nitrogen assimilation, and the $\delta^{15}\text{N}$ value of the leaves reflects the total nitrogen utilization [23]. In complex karst adversities, it is difficult to quantify the responses of nitrate and ammonium to the bicarbonate supply using a single isotope tracer at near-natural abundance levels [24,25]. Therefore, the bidirectional stable nitrogen isotope tracer approach was employed to study the differential contributions of nitrate and ammonium in plants [1]. To solve this difficult problem, two stable nitrogen isotope treatments labeled L and H with a difference in $\delta^{15}\text{N}$ values higher than 10% were used to calculate the responses of nitrate/ammonium utilization to bicarbonate in two plant species. Further, the plants were transferred to habitats with different bicarbonate levels under the same drought and total inorganic nitrogen supply [21]. Under karst habitats, the bidirectional

stable nitrogen isotope treatments were used to quantify the contributions of nitrate and ammonium at differential bicarbonate levels [26]. We observed that the energy and reducing power mechanisms, combined with inorganic carbon assimilation and nitrogen utilization, were dominated by photosynthesis, carbon/nitrogen metabolism enzymes, and growth [27]. Here, we highlighted intricate interactions among different pathways, such as the contributions of nitrate/ammonium, photosynthesis, carbon/nitrogen metabolism enzymes, and plant growth, further revealing the response of nitrate/ammonium utilization to bicarbonate supply in karst habitat-adaptable plants.

Compared with other cruciferous plants, *Orychophragmus violaceus* (*Ov*) has great advantages in adapting well to karst desertification. Moreover, *Ov* is available for widespread cultivation with its abundant fatty acids and fuel properties [13]. In addition, *Brassica napus* (*Bn*) is a typical economic crop, which is widely grown for oil production [16]. In this study, *Ov* and *Bn* were selected as experimental plants. In karst regions, the nutrient content is bare, and the nitrate is dominant compared to ammonium [28]. Under the same nitrogen supply, bicarbonate levels triggered differential nitrate/ammonium utilization and inorganic carbon assimilation efficiencies in *Ov* and *Bn* [12]. We aimed to answer the following questions: (1) Evaluation of the nitrate/ammonium utilization and their contributions to the total inorganic nitrogen assimilation in *Ov* and *Bn* at different bicarbonate levels. (2) Exploring the differential responses of inorganic carbon assimilation to bicarbonate supply between two plant species. (3) Revealing the adaptation mechanisms of karst plants by the interactions among nitrate reduction, ammonium assimilation, and inorganic carbon assimilation at different bicarbonate levels.

2. Materials and Methods

2.1. Plant Materials

The seeds of *Ov* and *Bn* (Zayou NO.59, a variety of *Brassica napus*), selected as experimental material for the study, were obtained, respectively, from the Guizhou Vocational College of Agriculture, Guizhou and Shanxi Agricultural Reclamation Scientific Research Center, Shanxi, China. The experiments were conducted in an artificial greenhouse with a length, width, and height of $10 \times 5 \times 4$ m at the Institute of Geochemistry, Chinese Academy of Sciences (Guiyang, China). A metal halide lamp (HPI-T400 W/645, Philips, The Netherlands) was used as a light source, and the temperature was controlled by air conditioning. The greenhouse environment was maintained as follows: light $500 \pm 23 \mu\text{mol m}^{-2} \text{s}^{-1}$, temperature (day/night): $25/19$ °C, constant light time of 12 h per day, and relative humidity range: 55–60%. *Ov* and *Bn* seeds were stirred uniformly with 70% ethanol, sterilized for 1 min, repeatedly rinsed 3–5 times, and soaked for 6–8 h. The cavity trays (twelve-hole size, $19 \times 15 \times 9.5$ cm), the substrate (perlite: vermiculite = 1:3), and modified Hoagland solution were used to grow *Ov* and *Bn* seedlings. In addition, it was reported that karst drought had the properties of drought: high pH and high bicarbonate. Therefore, we provided PEG6000 with 10 g/L solution to simulate a drought habitat, and the pH is kept at 8.30 ± 0.02 . The nutrient solution was changed every 3 days, and the seedlings were transplanted for 28 days for the subsequent experiment. The *Ov* and *Bn* seedlings with uniform growth were categorized as 3 plants/pot, 3 pots/group, and 3 groups/treatment for differential bicarbonate treatments for 10 days.

2.2. Bicarbonate Treatments

In this study, the drought treatment induced with $10 \text{ g}\cdot\text{L}^{-1}$ PEG6000 and the total nitrogen sources (nitrate: ammonium = 14 mM:1 mM) were kept to mimic the karst habitats for *Ov* and *Bn*, along with the supplementation of 1 mM, 5 mM and 15 mM NaHCO_3 (Table 1) with the same capacity at 9:00 a.m daily. The soil water content was maintained between 20 and 25% constantly. Two labeled stable nitrogen isotopes were used as the nitrogen source, which were divided into high (H) and low (L) natural ^{15}N abundance in NaNO_3 with $\delta^{15}\text{N}$ values of 22.67‰ and 8.08‰, respectively. NH_4Cl with a $\delta^{15}\text{N}$ value of -2.64 ‰ was supplied for the ammonium during the plants' growth. The pH of the

Hoagland nutrient solution was adjusted to 8.30 ± 0.05 with $1 \text{ mol}\cdot\text{L}^{-1}$ KOH to maintain the bicarbonate concentration. The *Ov* and *Bn* under various groups were planted in a growth chamber at $25 \text{ }^\circ\text{C}$ with a 12-h photo period.

Table 1. Different bicarbonate treatments to *Ov* and *Bn* in simulated karst habitats.

Treatment	Reagent	Substance Content
B1	NaHCO ₃	1 mM
B3	NaHCO ₃	5 mM
B5	NaHCO ₃	15 mM
NO ₃ ⁻ :NH ₄ ⁺	NaNO ₃ ($\delta^{15}\text{N}_\text{H}$:22.72‰):NH ₄ Cl	14 mM:1 mM
NO ₃ ⁻ :NH ₄ ⁺	NaNO ₃ ($\delta^{15}\text{N}_\text{L}$:12.7‰): NH ₄ Cl	14 mM:1 mM
pH	KOH	$1 \text{ mol}\cdot\text{L}^{-1}$, 8.30 ± 0.05
Drought	PEG6000	$10 \text{ g}\cdot\text{L}^{-1}$

2.3. Sample Collection for Biomass Estimation

The *Ov* and *Bn* under various treatments were collected in 3 parts: root, stem, and leaf; heated at $108 \text{ }^\circ\text{C}$ for 30 min; and dried at $70 \text{ }^\circ\text{C}$ to obtain the organic biomass and total biomass of *Ov* and *Bn*.

2.4. Measurement of Photosynthesis in Plants

The 2nd to 3rd expanded leaves of *Ov* and *Bn* were used for the measurement of photosynthesis from 9:00 to 11:00 a.m. The Li-6400 photosynthesizer (LI-COR, Lincoln, NE, USA) was used to measure the photosynthesis, including the net photosynthetic rate (P_n , $\mu\text{mol}/\text{m}^2\cdot\text{s}^{-1}$), stomatal conductance (Cond , $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$), transpiration rate (Tr , $\text{mmol H}_2\text{O m}^{-2}\cdot\text{s}^{-1}$), and intercellular CO₂ concentration (C_i , $\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ air}^{-1}$) in *Ov* and *Bn*. The water use efficiency (WUE) was analyzed according to Formula (1). The other parameters were set as temperature $25 \text{ }^\circ\text{C}$, CO₂ concentration $400 \mu\text{mol}/\text{mol}$ in the buffered glass bottles, and photosynthetically active radiation intensity $500 \mu\text{mol}/\text{m}^2\cdot\text{s}^{-1}$.

$$\text{WUE (\%)} = P_n/\text{Tr} \quad (1)$$

2.5. Measurement of Carbon and Nitrogen Enzymes

The fresh leaves of *Ov* and *Bn* were collected, frozen in liquid nitrogen, and stored at $-20 \text{ }^\circ\text{C}$. Enzyme reagent kits (Sangon, Shanghai, China) were used for the quantification of Rubisco (Ribulose biphosphate carboxylase oxygenase), SS (Sucrose synthetase), NR (nitrate reductase), and glutamate synthase (GOGAT) activities in the leaves.

2.6. Analysis of Leaf Carbon and Nitrogen Content

Dried leaves of *Ov* and *Bn* were used to determine the carbon and nitrogen contents using an elemental analyzer (Vario MACRO cube, Langensfeld, Germany).

2.7. The Total Nitrogen Assimilation/Utilization Capacity

The nitrogen accumulation capacity (NAC) was determined by the quantity of total inorganic nitrogen assimilated in plants. The nitrogen utilization capacity (NUC) according to Formulas (2) and (3) were referred to Wu (2019), who depicted the amount of total inorganic nitrogen used in plants. The atomic mass of N was 14, as well as the relative molecular mass was 62.

$$\text{NAC} = \text{DW} \times N_{\text{content}}; (\text{DW: drought weight of plants}) \quad (2)$$

$$\text{NUC (\%)} = 100 \times (\text{NAC}/M_{\text{N}})/M(\text{NO}_3^-) [M_{\text{N}}:14; M(\text{NO}_3^-):62] \quad (3)$$

2.8. Bidirectional Stable Nitrogen Isotope Tracer Method

2.8.1. The Nitrogen Isotope Ratio in Plants

The collected, dried leaves of *Ov* and *Bn* were ground to a fine powder for nitrogen isotope measurement. $\delta^{15}\text{N}$ was determined by gas isotope ratio mass spectrometer (MAT 253, Thermo Fisher Scientific, Langensfeld, Germany) according to Formula (4). In the formula, R_{sample} refers to the sample's nitrogen isotope ratio, and R_{standard} stands for the nitrogen isotope of N_2 in the air. The reference materials, including IAEA N1, IAEA N2, and IAEA NO_3 , were used to calibrate the instrument with a standard deviation of 0.2 % [29]. $\Delta^{15}\text{N}$ values refers to the total inorganic nitrogen assimilate capacity of plants. According to Formula (5), $\delta^{15}\text{N}_{\text{substrate}}$ stands for the nitrogen isotope of samples, and $\delta^{15}\text{N}_{\text{product}}$ was the reference nitrogen isotope ratio at 8.08‰ under the natural background.

$$\delta^{15}\text{N} (\text{‰}) = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000 \quad (4)$$

$$\delta^{15}\text{N} = \delta^{15}\text{N}_{\text{product}} - \delta^{15}\text{N}_{\text{substrate}} \quad (\delta^{15}\text{N}_{\text{product}} = 8.08\text{‰}) \quad (5)$$

2.8.2. Evaluation of Nitrate and Ammonium Contributions in Plants

The mixed nitrogen sources, including nitrate and ammonium, have been used to determine the nitrogen isotope values in plant species. In this study, two nitrate sources with different nitrogen isotope values (more than 10‰) were supplied for nitrate utilization. The $\delta^{15}\text{N}$ value of ammonium was less than those of the nitrate sources. Therefore, the nitrogen isotope value reflected the mixed results of assimilated nitrate and ammonium [30]. Consequently, Formula (6) showed the foliar $\delta^{15}\text{N}$ value (δ_{T}), obtained by measuring nitrogen isotope composition. After that, the bidirectional nitrogen isotope method was calibrated to qualify the contributions of nitrate and ammonium. δ_{A} and δ_{B} were the $\delta^{15}\text{N}$ value derived from nitrate/ammonium assimilation, and the labels of f_{A} and f_{B} were the contributions of nitrate/ammonium assimilation.

$$\delta_{\text{T}} = f_{\text{A}}\delta_{\text{A}} + f_{\text{B}}\delta_{\text{B}} = f_{\text{A}}\delta_{\text{A}} + 1 - f_{\text{A}}\delta_{\text{B}} \quad (6)$$

In this study, two kinds of labeled nitrogen isotope ratios, which differed by 10% ($\delta^{15}\text{N}$ values, L:12.7–H:22.72‰) were used to qualify the nitrate contribution (f_{A}) and ammonium contribution (f_{B}). The two-terminal meta-model was expressed as Formula (7).

$$\delta_{\text{TH}} = f_{\text{AH}}\delta_{\text{AH}} + f_{\text{B}}\delta_{\text{B}} = f_{\text{AH}}\delta_{\text{AH}} + 1 - f_{\text{AH}}\delta_{\text{B}} \quad (7)$$

In contrast, only the $\delta^{15}\text{N}$ value of leaves changed when the f_{A} and f_{B} were adjusted to the treatment with high-labeled (H) nitrogen isotope groups [8]. According to the above statements, the low-labeled group was expressed in Formula (8).

$$\delta_{\text{TL}} = f_{\text{AL}}\delta_{\text{AL}} + f_{\text{B}}\delta_{\text{B}} = f_{\text{AL}}\delta_{\text{AL}} + 1 - f_{\text{AL}}\delta_{\text{B}} \quad (8)$$

In this experiment, the same culture conditions were maintained for both plant species. Therefore, the contributions of nitrate and ammonium were the same, except for the nitrogen isotope value in the high (H) and light (L) treatments [30]. However, the physiological processes, metabolism, and growth were considered consistent under the same total nitrogen level [1]. Consequently, there was a specific formula given in (9) and (10).

$$f_{\text{A}} = f_{\text{AH}} = f_{\text{A}} \quad (9)$$

$$1 - f_{\text{AH}} = 1 - f_{\text{AL}} \quad (10)$$

Based on Formulas (9) and (10), we designed subsequent Formulas (11) and (12).

$$f_{\text{A}} = (\delta_{\text{TH}} - \delta_{\text{TL}})/(\delta_{\text{AH}} - \delta_{\text{AL}}) \quad (11)$$

$$f_B = 1 - f_A \quad (12)$$

However, previous studies merely depict the contributions of nitrate and ammonium to total inorganic nitrogen assimilate capacity (NAC) and nitrogen utilization capacity (NUC). The f_A and f_B of plants can be confirmed by bidirectional nitrogen isotope methods with different nitrogen isotope sources. It is also feasible to calculate the contributions of nitrate/ammonium to NAC by Formula (13), (NACA) and Formula (14), (NACB) and the contributions of nitrate/ammonium to NUC by Formula (15), (NUCA) and Formula (16), (NUCB).

$$\text{NACA} = \text{NAC} \times f_A \quad (13)$$

$$\text{NACB} = \text{NAC} \times f_B \quad (14)$$

$$\text{NUCA} = \text{NUC} \times f_A \quad (15)$$

$$\text{NUCB} = \text{NUC} \times f_B \quad (16)$$

2.9. Statistical Analysis

The experimental data were measured by an analysis of variance (ANOVA), and Tukey's test ($p < 0.05$) was performed for the comparison among various experimental treatments. The results were shown as the mean \pm standard deviation (SD), and the figures were designed using Origin 2019.

3. Results

3.1. Growth-Related Features of Different Plant Species

The influence of bicarbonate levels on growth was different between *Ov* and *Bn* (Table 2). The *Ov* exhibited less increase in the biomass than the *Bn* at various bicarbonate supplies ranging from 1 to 15 mM. With the increasing concentration of the bicarbonate, the root, stem, leaves, and total biomass of *Ov* increased significantly, specifically at 5 mM NaHCO_3 . However, the growth of *Ov* declined at the highest concentration of 15 mM NaHCO_3 in the study, especially the stem reduced by 24.86% compared to the lowest bicarbonate level (1 mM NaHCO_3). However, the increasing bicarbonate levels persistently inhibited the growth of *Bn*, resulting in a significant decrease in total biomass at the highest bicarbonate concentration (15 mM NaHCO_3) used in our study.

Table 2. The growth of *Ov* and *Bn* at various bicarbonate concentrations.

NaHCO_3^- , mM	1	5	15	1	5	15
Plant species	<i>Ov</i>			<i>Bn</i>		
root/mg	96.19 \pm 3.09 d	131.97 \pm 5.11 b	112.11 \pm 2.38 c	315.3 \pm 7.98 a	158.99 \pm 4.05 b	95.8 \pm 3.35 d
stem/mg	125.11 \pm 4.5 d	260.48 \pm 6.46 c	94.01 \pm 4.07 e	464.6 \pm 12.69 a	356.47 \pm 7.04 b	132.95 \pm 4.7 d
leaves/mg	128.11 \pm 4.48 e	288.15 \pm 4.25 c	144.64 \pm 6.27 e	521.85 \pm 10.54 a	402.85 \pm 9.86 b	218.39 \pm 6.98 d
biomass/mg	395.86 \pm 15.74 e	742.46 \pm 17.75 c	388.59 \pm 14.23 e	1443.28 \pm 35.21 a	1076.92 \pm 26.85 b	525.06 \pm 18.69 d

Ov *Orychophragmus violaceus* and *Bn* *Brassica napus*. The ratio of nitrate/ammonium within consistent with the total nitrogen concentration was 14 mM:1 mM under various bicarbonate treatment. Each value represents the mean \pm SD ($n = 3$), and diverse letters in each value are significantly different by ANOVA ($p > 0.05$).

3.2. The Photosynthetic Efficiency of Plants

The photosynthetic efficiency of *Bn* and *Ov* was affected by different bicarbonate concentrations during various treatments, as depicted in Figure 1. The increase in photosynthesis is attributed to the increasing bicarbonate level in *Ov* as compared to *Bn*. The increasing bicarbonate concentration promoted the photosynthesis in *Ov* with the maximum effect at 5 mM NaHCO_3 . *Ov* showed the highest Pn during all the bicarbonate concentrations, attributing to the maximum Cond, Ci, and WUE. Therefore, the photosynthesis of *Ov* was decreased at the maximum level of 15 mM NaHCO_3 , resulting in the decline of Pn, Cond, Tr, and Ci with no significant difference from the lowest level

(1 mM NaHCO₃). On the other hand, less photosynthetic efficiency was observed in *Bn* as compared to *Ov* with the increasing concentration of bicarbonate, leading to the maximum decline of Pn, Cond, Ci, and Tr at the highest level (15 mM NaHCO₃).

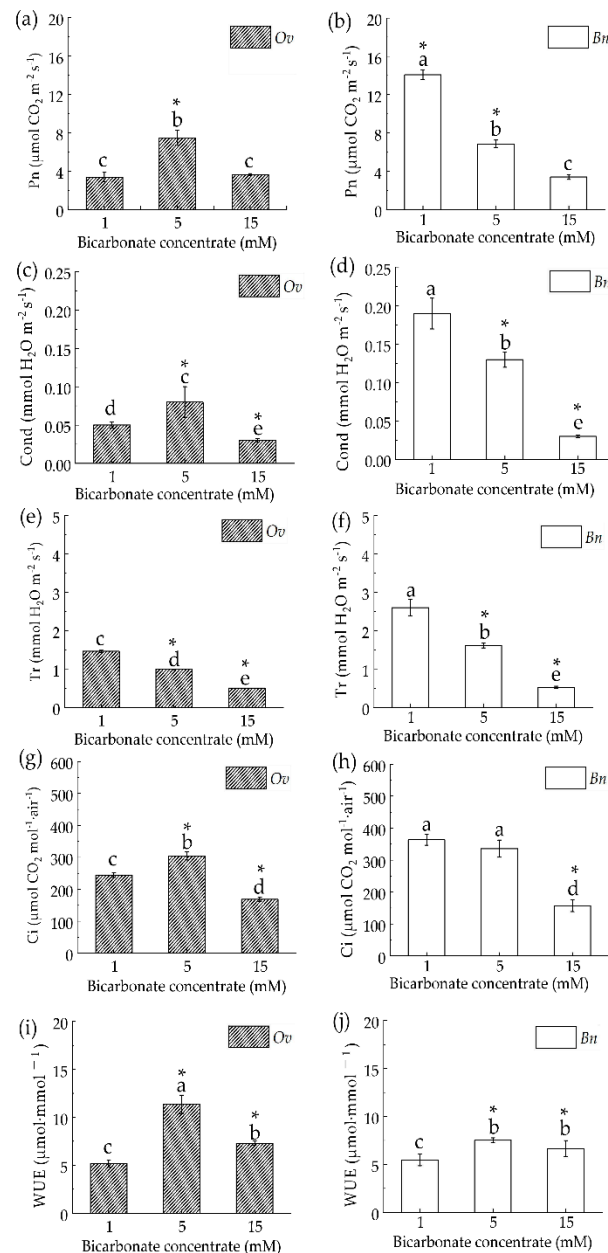


Figure 1. The photosynthesis of *Ov* and *Bn* under different bicarbonate supplies. *Ov*—*Orychophragmus violaceus*, *Bn*—*Brassica napus*, Pn-net—photosynthetic rate, Cond—stomatal conductivity, Tr—transpiration, Ci—intercellular carbon dioxide concentration, and WUE—water use efficiency. (a,b)—The Pn of *Ov*/*Bn*, (c,d)—The Cond of *Ov*/*Bn*, (e,f)—The Tr of *Ov*/*Bn*, (g,h)—The Ci of *Ov*/*Bn*, (i,j)—The WUE of *Ov*/*Bn*. The ratio of nitrate/ammonium within consistent total nitrogen concentration was 14 mM:1 mM under various bicarbonate treatments. Each value represents the mean \pm SD ($n = 3$), and diverse letters with “*” in each value are significantly different by ANOVA ($p > 0.05$).

3.3. Responses of Carbon and Nitrogen Metabolizing Enzymes of Two Plant Species

The responses of carbon and nitrogen metabolizing enzymes to different bicarbonate levels were measured between *Ov* and *Bn* (Figure 2). The increasing concentration of bicarbonate (from 1 to 15 mM NaHCO₃) promoted the activity of carbon and nitrogen metabolizing enzymes in *Ov* more significantly than *Bn*. At the middle level

(5 mM NaHCO₃), the maximum activities of Rubisco, SS, NR, and GOGAT were measured in *Ov*, whereas the inhibited activities of these enzymes were observed in *Bn*. Furthermore, reduction in the activities of Rubisco, SS, NR, and GOGAT was observed less significantly reduced in *Ov* as compared to *Bn* at the highest bicarbonate level (15 mM NaHCO₃).

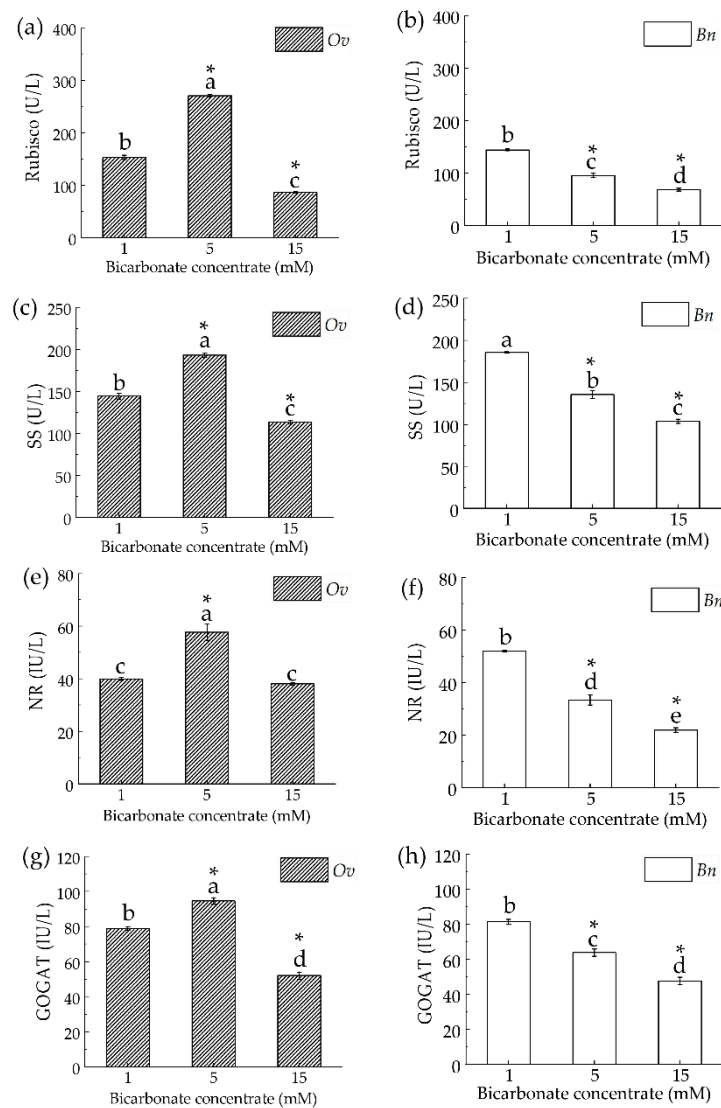


Figure 2. The responses of enzymes involved in carbon and nitrogen metabolism to bicarbonate in *Ov* and *Bn*. *Ov*—*Orychophragmus violaceus* and *Bn*—*Brassica napus*, Rubisco—ribulose biphosphate carboxylase oxygenase, SS—sucrose synthetase, NR—nitrate reductase, and GOGAT—glutamate synthase. (a,b)—The Rubisco of *Ov/Bn*, (c,d)—The SS of *Ov/Bn*, (e,f)—The NR of *Ov/Bn*, (g,h)—The GOGAT of *Ov/Bn*. The ratio of nitrate/ammonium within consistent total nitrogen concentration was 14 mM:1 mM under various bicarbonate treatments. Each value represents the mean \pm SD ($n = 3$), and diverse letters with “*” in each value are significantly different by ANOVA ($p > 0.05$).

3.4. The Inorganic Carbon and Nitrogen Contents of Leaves in *Ov* and *Bn*

With the increasing bicarbonate concentration, the carbon and nitrogen contents in leaves were found to be different between *Ov* and *Bn* (Figure 3). The carbon content of leaves in *Ov* first increased and then declined constantly during treatments from 1 to 15 mM; however, the pattern in *Bn* was subject to *Ov*. Moreover, the carbon content was the maximum in *Ov* at the middle bicarbonate level (5 mM) and significantly higher than that in *Bn*. In addition, the nitrogen content of leaves in *Ov* increased at 5 mM bicarbonate, whereas

it declined consonantly in *Bn* and obtained the minimum level at 15 mM. Further, *Ov* exhibited more significantly enhanced NAC and NUC than *Bn* with increasing bicarbonate levels. Consequently, the C/n ratio of leaves in *Ov* declined extremely than that in *Bn*.

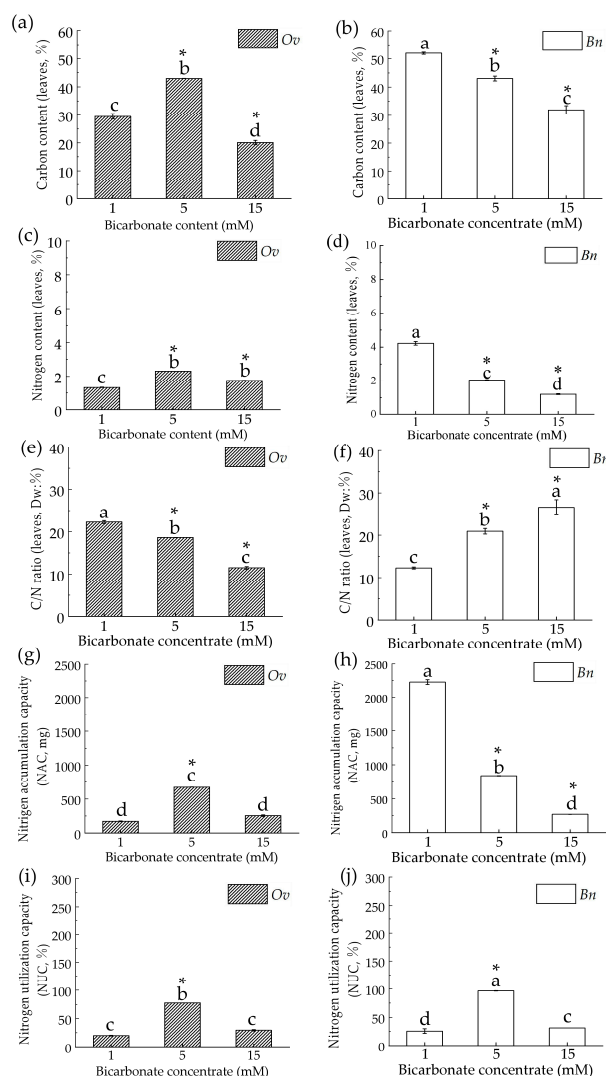


Figure 3. The content of the carbon/nitrogen component in leaves between *Ov* and *Bn* at different bicarbonate levels. *Ov*—*Orychophragmus violaceus* and *Bn*—*Brassica napus*. (a,b)—The carbon content of *Ov*/*Bn*, (c,d)—The nitrogen content of *Ov*/*Bn*, (e,f)—The C/N ratio of *Ov*/*Bn*, (g,h)—The NAC of *Ov*/*Bn*, (i,j)—The NUC of *Ov*/*Bn*. The ratio of nitrate/ammonium within consistent total nitrogen concentration was 14 mM:1 mM under various bicarbonate treatments. Each value represents the mean \pm SD ($n = 3$), and diverse letters with “*” in each value are significantly different by ANOVA ($p > 0.05$).

3.5. The Contributions of Nitrate/Ammonium in Two-Plant Species

The contributions of nitrate/ammonium in *Ov* and *Bn* labeled with the high (H) and low (L) treatments were opposite during the treatment with all the concentrations of bicarbonate (Figure 4). The $\delta^{15}\text{N}$ values of *Ov* and *Bn* leaves were different during all the bicarbonate supplies (Figure 4a,b). In both the H and L groups, the $\delta^{15}\text{N}$ value of *Ov* first declined and then enhanced with increasing the bicarbonate levels. The minimum and maximum $\delta^{15}\text{N}$ values of *Ov* were obtained at 5 mM and 15 mM NaHCO_3 , respectively. However, the $\delta^{15}\text{N}$ values of *Bn* were increased consistently in all the groups. The differences in the $\delta^{15}\text{N}$ values between *Ov* and *Bn* were attributed to the distinctive utilization of nitrate (f_A) and ammonium (f_B) in two-plant species (Figure 4c,d), which were deter-

mined by different bicarbonate levels. In both *Ov* and *Bn*, the nitrate utilization (f_A) was subjected to the results of $\delta^{15}\text{N}$, while the ammonium utilization (f_B) was adjudicative with the increasing bicarbonate supplies. Predictably, the increasing bicarbonate concentration showed more enhancement in *Ov* at the middle bicarbonate level (5 mM NaHCO_3), where nitrate was majorly available for inorganic nitrogen assimilation in *Ov*, contributing to the higher nitrate utilization (f_A) as compared to *Bn*. However, when the bicarbonate enhanced consistently exceeded 5 mM NaHCO_3 , the nitrate utilization was decreased in both *Ov* and *Bn*, leading to lower f_A and higher f_B . At the highest bicarbonate level (15 mM NaHCO_3), the ammonium utilization increased more obviously than the nitrate utilization in both *Ov* and *Bn*. Moreover, ammonium could be considered the major source of inorganic nitrate supply in *Bn* at 15 mM NaHCO_3 , where f_B increased by more than half percent of the lowest bicarbonate level (1 mM NaHCO_3) and had the maximum ammonium utilization. Overall, the contribution of ammonium was enhanced to the inorganic nitrogen assimilation in both *Ov* and *Bn* during increasing bicarbonate levels from 1 to 15 mM NaHCO_3 .

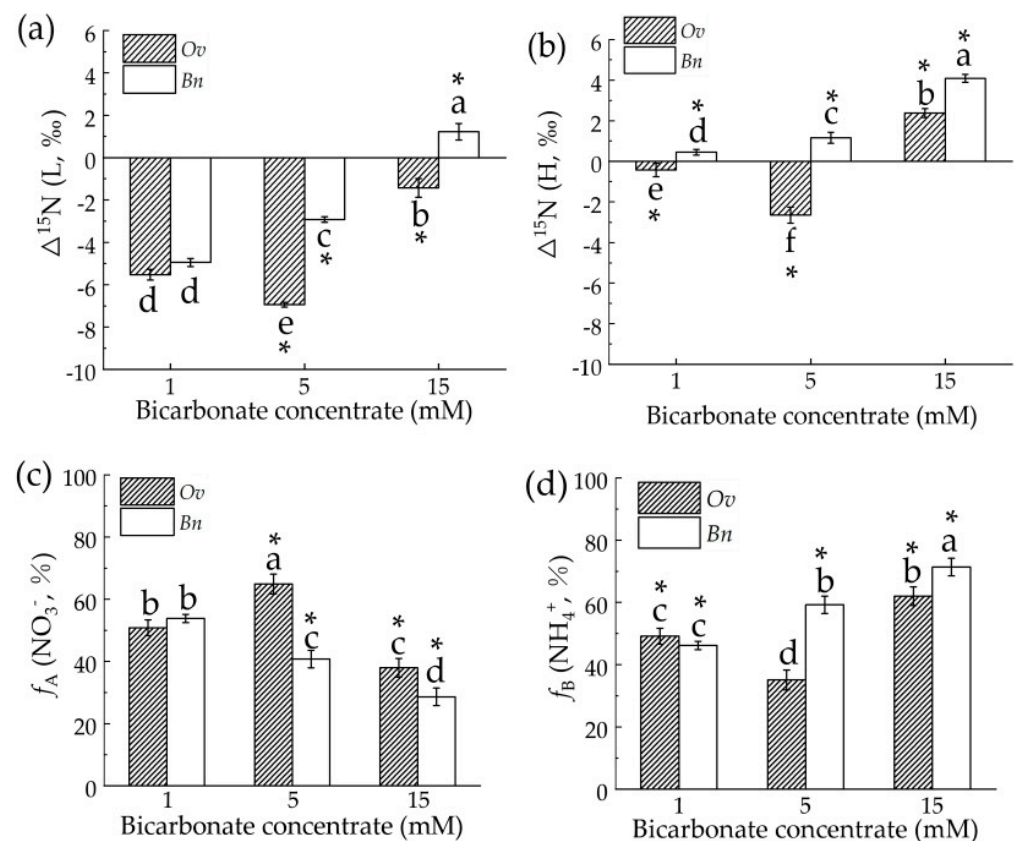


Figure 4. The contributions of nitrate/ammonium of leaves in *Ov* and *Bn* at different bicarbonate levels. *Ov*—*Orychophragmus violaceus*, *Bn*—*Brassica napus*, $\Delta^{15}\text{N}$ —the $\delta^{15}\text{N}$ value, f_A —the contribution of nitrate, and f_B —the contribution of ammonium. (a,b)—the $\delta^{15}\text{N}$ value of *Ov* and *Bn*, (c,d)—the contribution of nitrate/ammonium of *Ov* and *Bn*. The ratio of nitrate/ammonium within consistent total nitrogen concentration was 14 mM:1 mM under various bicarbonate treatments. Each value represents the mean \pm SD ($n = 3$), and diverse letters with “*” in each value are significantly different by ANOVA ($p > 0.05$).

3.6. The Contribution of Nitrate/Ammonium to Nitrogen Assimilation and Utilization Capacity

In this study, NACA/NACB and NUCA/NUCB separately represented the contribution of nitrate/ammonium to nitrogen assimilation capacity (NAC) and nitrogen utilization capacity (NUC). As depicted in Figure 5, the increasing bicarbonate levels triggered NAC and NUC in *Ov* more than *Bn*. Compared to the lowest bicarbonate level (1 mM NaHCO_3), *Ov* showed enhanced NAC and NUC at 5 mM NaHCO_3 , utilizing nitrate as the major con-

tributor, leading to the maximum NACA, NACB, NUCA, and NUCB. We observed that the results in *Bn* were different from *Ov*. The increasing bicarbonate level constantly inhibited NAC and NUC in *Bn*, leading to an excessive decline in NACA, NACB, NUCA, and NUCB. The lowest assimilation/utilization in *Bn* was observed at the highest bicarbonate level (15 mM NaHCO₃). Altogether, our results suggested that the increasing bicarbonate enhanced more f_A to NAC and NUC in *Ov* than *Bn*.

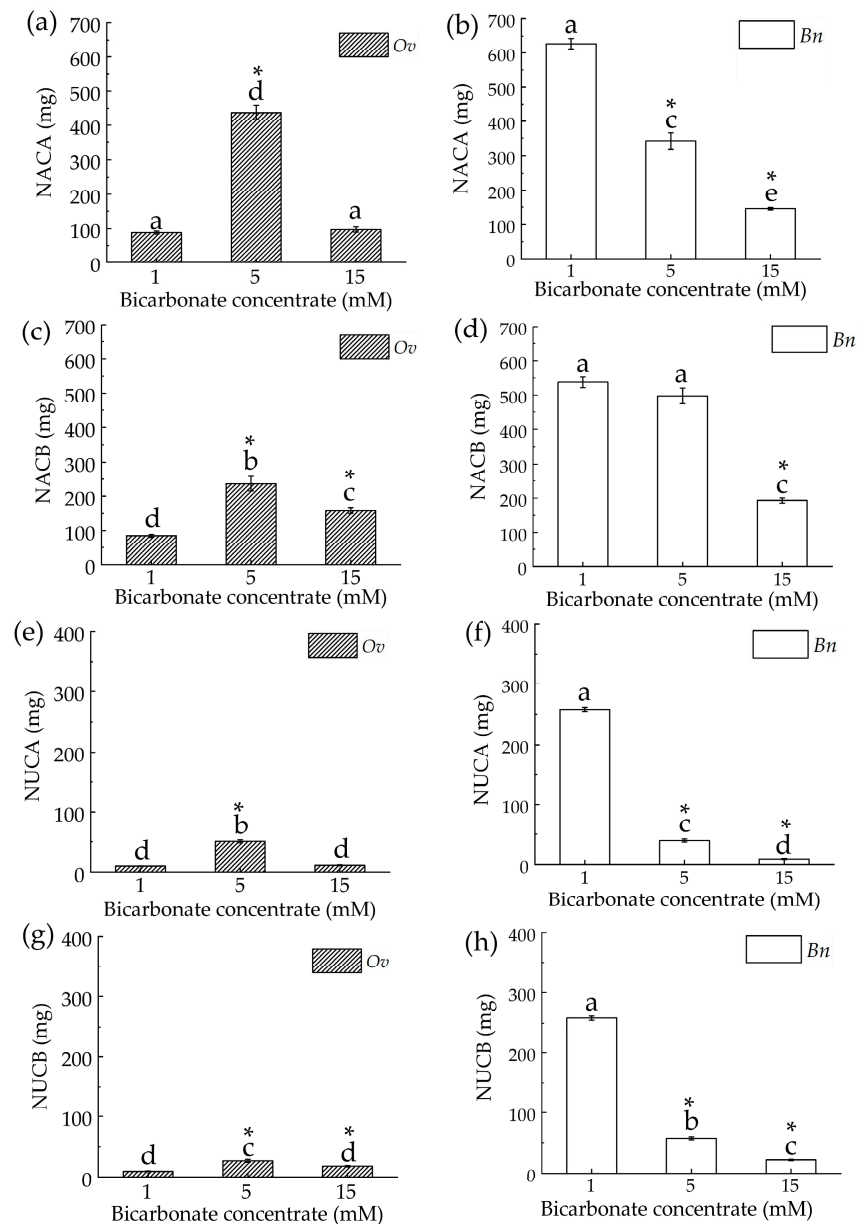


Figure 5. The contribution of nitrate/ammonium to NAC and NUC in *Ov* and *Bn* at different bicarbonate levels. *Ov*—*Orychophragmus violaceus*, *Bn*—*Brassica napus*. NACA—the contribution of nitrate to total nitrogen accumulation capacity, NACB—the contribution of ammonium to total nitrogen accumulation capacity, NUCA—the contribution of nitrate to total nitrogen utilization capacity, and NUCB—the contribution of ammonium to total nitrogen utilization capacity. (a,b)—the NACA of *Ov/Bn*, (c,d)—the NACB of *Ov* and *Bn*. (e,f)—the NUCA of *Ov* / *Bn*, (g,h)—the NUCB of *Ov/Bn*. The ratio of nitrate/ammonium within consistent total nitrogen concentration was 14 mM:1 mM under various bicarbonate treatments. Each value represents the mean \pm SD ($n = 3$), and diverse letters with “*” in each value are significantly different by ANOVA ($p > 0.05$).

4. Discussion

4.1. Differential Regulation of Inorganic Carbon Assimilation during Bicarbonate Supplementation in *Ov* and *Bn*

In this study, we found that the growth of *Ov* and *Bn* exhibited different patterns in response to the changing bicarbonate supply. With an increase in the bicarbonate concentration, the growth of root, stem, and leaf and total biomass increased at the middle bicarbonate level (5 mM HCO_3^-) in *Ov*, while consistently decreased in *Bn*. Previously, it has been reported that karst-adaptable plants have stronger BUC, which can utilize more bicarbonates for inorganic carbon assimilation [1]. For instance, *Camptotheca acuminata* could incorporate more bicarbonate into nonstructural carbohydrates (NSC) to alleviate the extreme conditions of karst habitats such as drought, high bicarbonate, and high pH [9]. In addition, at different bicarbonate levels, photosynthesis was promoted in *Ov* compared to *Bn*. Precisely, the photosynthesis in *Ov* obtained the maximum rate at the middle bicarbonate level (5 mM) but consistently decreased in *Bn* with the minimum photosynthetic rate at 15 mM HCO_3^- . Wu et al. [1] reported that bicarbonate was more readily available for karst-adaptable plants than non-karst plants, which is attributed to the activity of active carbonic anhydrases (CA). CA converts bicarbonate into carbon dioxide and water to alleviate water and carbon source deficits and restore photosynthesis in plants. Wang et al. [13] showed that *Brassica juncea* L (*Bj*), a non-karst-adaptable plant, had less bicarbonate use capacity, resulting in a decrease in photosynthesis. Though the Pn, Ci, Cond, and Tr decreased at the highest bicarbonate level more than the middle level, we found that the WUE significantly increased in both *Ov* and *Bn*, indicating that these two plant species have enhanced water use efficiency to adapt to increasing bicarbonate, which is consistent with the results shown by Hang et al. [4] and Gimenez et al. [31].

In addition, we found that change in the bicarbonate concentration was more significantly associated with the activities of carbon metabolizing enzyme in *Ov* compared to *Bn*. With increasing bicarbonate, the Rubisco and SS activities in *Ov* reached to a maximum at the middle bicarbonate level (5 mM) but consistently decreased in *Bn* with the minimum at the highest bicarbonate level (15 mM). It could be due to higher BUC in *Ov* [3,16], which restored the photosynthesis, increasing the carbon metabolizing enzyme activities. Xia et al. [12] also confirmed that *Ov* had more stable carbon metabolic enzyme activities than *Bn* in simulated karst habitats. However, high bicarbonate inhibited the carbon metabolizing enzyme activities in plants [32,33]. In this study, at the highest bicarbonate level (15 mM), Rubisco and SS activities decreased in both *Ov* and *Bn*. However, the growth, photosynthetic capacity, and carbon metabolic enzyme activities in *Ov* decreased less than *Bn*, indicating that bicarbonate imposed slight inhibitions on *Ov*. Further, it highlighted the outstanding adaptability of inorganic carbon assimilation to high bicarbonate supply.

4.2. The Differential Characteristics of Nitrate/Ammonium Utilization at Various Bicarbonate Levels in Two Plant Species

With nitrate-abundant and ammonium-rare soil in karst habitats, the bicarbonate had a pronounced effect on nitrate/ammonium contributions in plants, resulting in the nitrogen isotope value during inorganic nitrogen utilization [34]. It was reported previously that the nitrogen isotope value results from both nitrate and ammonium contributions [35]. In this study, we estimated the contributions of nitrate/ammonium utilization in *Ov* and *Bn* using the bidirectional nitrogen isotope tracer method [21]. The results showed that *Ov* and *Bn* exhibited differential nitrate/ammonium utilization at different bicarbonate levels. Compared to *Bn*, the bicarbonate promoted significant nitrogen utilization in *Ov*, leading to the maximum enhancement of f_A , NAC, and NUC at the middle bicarbonate level (5 mM), which attributed to the higher photosynthetic capacity and growth (Table 1 and Figure 1). It has been shown that increasing photosynthesis promotes the inorganic nitrogen assimilation in plants, increasing both inorganic nitrogen accumulation and utilization capacity [34]. Moreover, the $\Delta^{15}\text{N}$ value, which is opposite to the inorganic nitrogen assimilation capacity [35], was reduced to the minimum at 5 mM HCO_3^- (Figure 4). It was

shown that karst-induced drought inhibits the stomatal movement of leaves, resulting in a decrease in the carbon dioxide level and water use efficiency [1]. Precisely, it reduced the carbon dioxide assimilation, mediating a decline in the photosynthetic capacity [4]. Conversely, it might inhibit the photo electron transfer by decreasing the water photolysis, which is not conducive to inorganic nitrogen metabolism in plants [3]. Nevertheless, bicarbonate could be used to provide electrons for nitrate reduction, maintaining normal inorganic nitrogen metabolism in plants [6,18]. In this study, 5 mM HCO_3^- enhanced nitrate utilization and NR activity in *Ov*, which was conducive to ammonium assimilation and enzyme formation [27], leading to the enhancement of ammonium utilization, GOGAT activity, and total inorganic nitrogen assimilation. In addition, at the highest level of bicarbonate (15 mM), the nitrate utilization was reduced, but the ammonium utilization was increased in *Ov*, which helped in attaining the NAC and NUC values consistent with those at the minimum bicarbonate level (1 mM). It was observed that ammonium utilization was increased in *Ov* to maintain the nitrogen assimilation capacity at a high bicarbonate level, which conformed to the findings by Lu et al. [6] and Wu et al. [1].

However, with the increasing bicarbonate, we found that the nitrogen assimilation was consistently inhibited in *Bn*, resulting in the lowest f_A , NACA, and NACB at the maximum bicarbonate level (15 mM). These results were attributed to its weak BUC, since bicarbonate was rarely used, leading to the occurrence of stress on cellular osmotic pressure [36]. Increased osmotic pressure inhibited the absorption of nitrate and ammonium, as well as photosynthesis and growth [6], which is not conducive to inorganic nitrogen assimilation of *Bn*. In addition, we found that, though the utilization of ammonium was increased in *Bn*, NAC and NUC were reduced to a minimum at 15 mM HCO_3^- . It was due to the bare ammonium environment, which was only 1 mM in our study. The abundant nitrate and bare ammonium in karst soil results in the minimal absorption of ammonium by plants [34,35,37]. Additionally, plants rarely receive ammonium for direct assimilation. Thus, nitrate reduction restoring nitrate to ammonium majorly participates in the total inorganic nitrogen assimilation [30]. Consequently, in this experiment, the bare ammonium might be sufficient to enhance total inorganic nitrogen metabolism in *Bn*. Though the bicarbonate supply increased, the ammonium utilization in *Bn* counteracted the negative effects on plant growth, leading to a decline in the total inorganic nitrogen utilization.

4.3. The Differential Responses of Inorganic Carbon and Nitrogen Metabolisms to Bicarbonate Supply in *Ov* and *Bn* under Karst Habitats

In the simulated karst habitats with drought, high bicarbonate, abundant nitrate, and bare ammonium soil, we found that the responses of nitrate/ammonium utilization to bicarbonate were contrasting in *Ov* and *Bn* (Figure 6). In this study, the bicarbonate was found to be more conducive to the growth of *Ov* than *Bn*, resulting in the promotion of photosynthesis, enzymatic activities, carbon concentrations, and nitrogen reduction. Bicarbonate concentrations not only affected the contributions of nitrate and ammonium but also impacted the total nitrogen assimilation and utilization capacity in plants [1,6]. However, due to weak adaptability to high bicarbonate and abundant nitrate habitats, *Bn* exhibited more decline in carbon assimilation and nitrogen reduction with increasing bicarbonate, leading to excessive growth inhibitions but an obvious promotion in water use efficiency, as well as ammonium utilization efficiency. Hence, *Ov* exhibited more exceptional responses related to both carbon and nitrogen metabolism to high bicarbonate and nitrate soil than *Bn*, which was primarily attributed to its preferable adaptations to karst habitats.

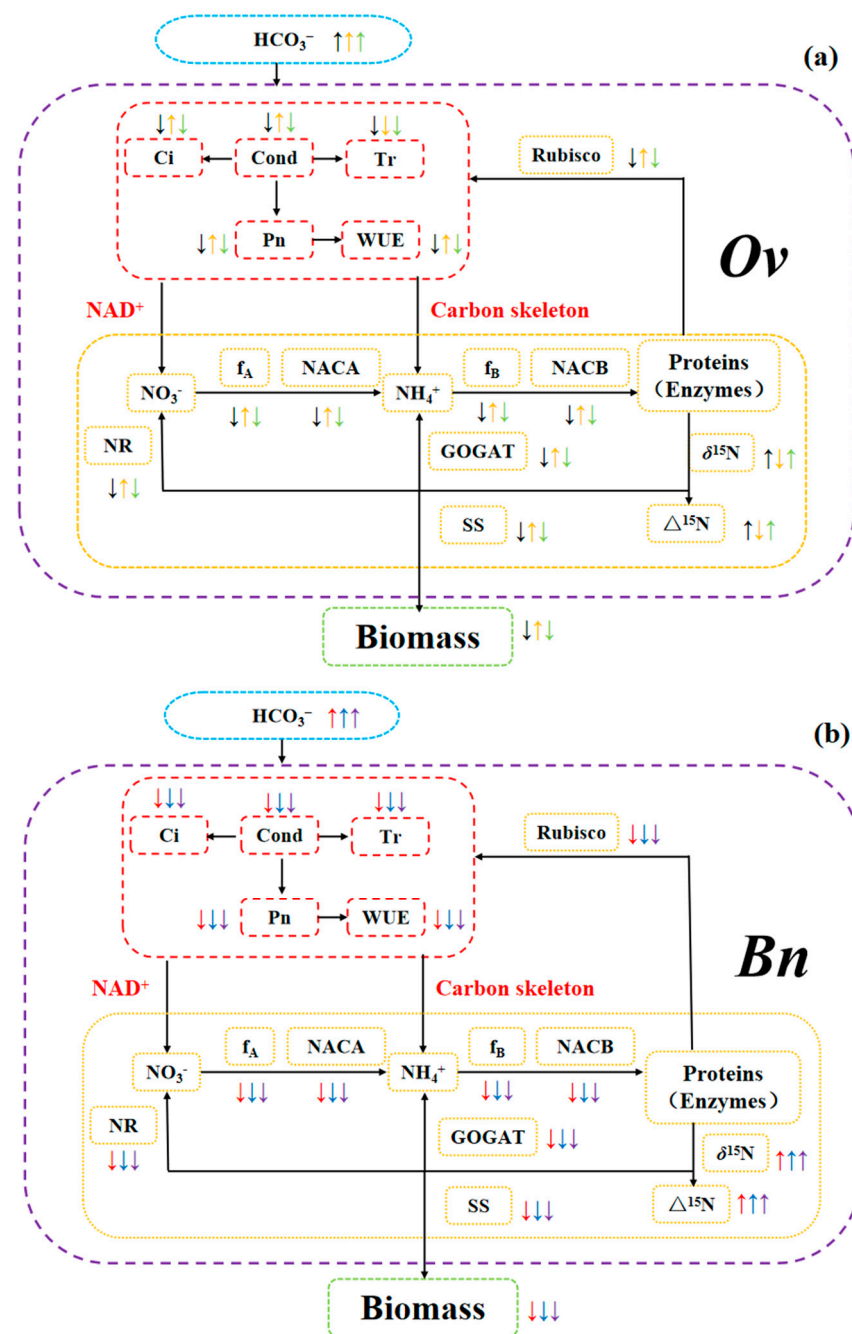


Figure 6. Differential responses of carbon and nitrogen metabolism to bicarbonate in *Ov* and *Bn* under simulated karst habitats. ↑: Increase, ↓: Decrease. (a,b) represented differential responses of carbon and nitrogen metabolism to bicarbonate in *Ov*/*Bn* under simulated karst habitats. The black, yellow and green/the red, blue, and purple arrows represent different bicarbonate treatments at 1 mM, 5 mM, and 15 mM in *Ov*/*Bn*.

5. Conclusions

Under simulated karst habitats, the bidirectional stable nitrogen isotope tracing method accurately estimated nitrate and ammonium utilization in plants at different bicarbonate levels. Compared to a non-karst-adaptable plant species (*Bn*), the bicarbonate significantly promoted inorganic carbon assimilation and nitrogen utilization in the karst-adaptable plant (*Ov*), resulting in an increase in the photosynthesis, carbon metabolizing enzyme activities, and biomass. Additionally, the nitrogen enzyme activities, nitrate utilization, and total inorganic nitrogen metabolism were enhanced in *Ov*. Moreover, high

bicarbonate promoted ammonium utilization to counteract the decline in the inorganic nitrogen assimilation capacity in *Ov*. Therefore, *Ov* can be considered a widely grown pioneer plant species in karst areas and a valuable resource for oil production and fuel properties, etc.

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Abbreviation:

Abbreviation	Meaning
<i>Bj</i>	<i>Brassica juncea</i> L.
<i>Bn</i>	<i>Brassica napus</i>
BUC	bicarbonate use capacity
CA	carbonic anhydrase
Ci	intercellular carbon dioxide concentration
Cond	stomatal conductivity
f_A	the contribution of nitrate
f_B	the contribution of ammonium
GOGAT	glutamate synthase
HCO_3^-	bicarbonate
NH_4^+	ammonium
NO_3^-	nitrate
NR	nitrate reductase
NAC	nitrogen accumulation capacity
NUC	nitrogen utilization capacity
NACA	the contribution of nitrate to total nitrogen accumulation capacity
NACB	the contribution of ammonium to total nitrogen accumulation capacity
NUCA	the contribution of nitrate to total nitrogen utilization capacity
NUCB	the contribution of ammonium to total nitrogen utilization capacity
NSC	nonstructural carbohydrate
<i>Ov</i>	<i>Orychophragmus violaceus</i>
Pn	photosynthetic rate
Rubisco	ribulose biphosphate carboxylase oxygenase
SS	sucrose synthetase
Tr	transpiration
WUE	water use efficiency

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