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Stable Nitrogen Isotopes as an Effective Tool for Estimating the Nitrogen Demand of *Broussonetia papyrifera* (L.) Vent Seedlings under Variable Nitrate Concentrations

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Abstract: Poor growth is often observed in artificial young forests due to insufficient inorganic nitrogen in karst soils. However, little is known about the assimilatory demand of the whole plant for nitrate and the partitioning of nitrate assimilation in roots and leaves in woody plants grown in karst habitats. In this study, *Broussonetia papyrifera* (L.) Vent (*B. papyrifera*) seedlings were grown under nearly hydroponic conditions. The isotope mass balance approach was employed to quantify the $\delta^{15}N$ values of the N assimilates in plant organs and in whole plants for *B. papyrifera* seedlings grown at different nitrate concentrations. The $\delta^{15}N$ values of the N assimilates in the whole *B. papyrifera* seedlings showed a rising trend with increasing nitrate concentration. Increasing the supply of nitrate decreased the leaf–root difference in the $\delta^{15}N$ values of the N assimilates for *B. papyrifera* seedlings. Quantifying the $\delta^{15}N$ values of N assimilates in the whole *B. papyrifera* seedlings grown under different nitrate concentrations contributes to estimating the assimilatory demand of the *B. papyrifera* seedlings for nitrate. The leaf–root difference in the $\delta^{15}N$ values of the N assimilates can be used to estimate the partitioning of nitrate assimilation in the roots and leaves.

Keywords: karst; artificial forest; nitrate; isotope mass balance approach; assimilatory demand

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1. Introduction

Vegetation restoration is the key to controlling karst rocky desertification. However, poor growth is often observed in artificial young forests due to insufficient inorganic nitrogen in karst soils [1,2]. Hence, it is necessary to effectively manage the supply of inorganic nitrogen in artificial forests. The major sources of inorganic nitrogen utilized by plants have been suggested to be nitrate and ammonium [3–5]. However, weakly alkaline soils (pH 7.8–8.4) are often observed in karst rocky desertification areas [6], and high pH values in soils usually lead to ammonia volatilization [7,8]. Hence, a high-nitrate and low-ammonium environment occurs in karst rocky desertification areas [8,9]. As a result, the supply of nitrate may be suitable for artificial young forest growth in such habitats. The uptake and assimilation of nitrate within plants depend on the internal demand and external supply [10,11]. Generally, a balance between internal nitrogen demand and external nitrogen supply is an ideal status for plants and contributes to preventing excess or limited nitrogen supply. However, little is known about the assimilatory demand of the whole plant for nitrate and the partitioning of nitrate assimilation in roots and leaves in woody plants grown in karst rocky desertification areas.

After uptake, nitrate may be assimilated in roots and/or leaves, and partially unassimilated nitrate returns to the medium [10,12]. The lighter N isotope (^{14}N) is favored due to the kinetic process, and as a result, the heavier isotope (^{15}N) is depleted in the product [10,13,14]. Accordingly, the effluxed unassimilated nitrate is enriched in ^{15}N, and the whole-plant δ^{15}N values are negative relative to the source [15]. The deviations in whole-plant δ^{15}N values relative to source nitrogen δ^{15}N values are referred to as the nitrogen isotope fractionation value [16]. Under the condition that nitrate is the sole nitrogen source, the nitrogen isotope discrimination of whole plants depends on nitrate reductase activity and the supply of reductants [17]. Generally, the influxed nitrate will be assimilated to as great a degree as possible with strong nitrate reductase activity and an adequate supply of reductant. Consequently, the amount of effluxed unassimilated nitrate will decrease, which will minimize the observed isotope fractionation for nitrate assimilation by plants. However, when plants experience nitrate reductase activity restriction and/or reductant restriction, the amount of effluxed unassimilated nitrate increases. Then, greater nitrogen isotope fractionation is observed. Hence, the nitrogen isotope fractionation value of whole plants is closely related to the assimilatory demand for nitrate.

Generally, there is preexisting nitrogen in young woody plants [18]. It is difficult to quantify the nitrogen isotope fractionation value of a whole woody plant grown at different nitrate concentrations owing to interference from the δ^{15}N values of preexisting nitrogen. A simpler, more convenient approach is to quantify the nitrogen isotope fractionation value of N assimilates in whole woody plants grown at different nitrate concentrations to determine the assimilatory demand for nitrate over a greater time scale. Based on the isotope mass balance approach [19], the δ^{15}N value of N assimilates in whole woody plants can be quantified for woody plants grown at different nitrate concentrations. Moreover, the δ^{15}N values of N assimilates in plant organs can also be quantified using the isotope mass balance approach [19]. The δ^{15}N values of newly acquired N assimilates in stems are derived from the mix of δ^{15}N values of N assimilates in the roots and leaves. Consequently, the proportion of stem nitrogen obtained from the leaves (i.e., $f_{\text{leaf stem}}$) can be estimated using a two end-member isotope mixing model [20,21]. Furthermore, the leaf–root difference in the δ^{15}N values of N assimilates is closely linked with the partitioning of assimilatory activity between roots and leaves. Hence, quantifying the δ^{15}N values of N assimilates in plant organs contributes to the estimation of the partitioning of nitrate assimilation in the roots and leaves.

There is increasing interest in using *Broussonetia papyrifera* (L.) Vent (*B. papyrifera*) for ecological reclamation [22]. *B. papyrifera* trees can rapidly colonize abandoned factories and are employed for mining rehabilitation. In addition, this species presents a wide array of potential uses, such as the utilization of its bark for paper production, leaves as a source of forage, roots and fruits for traditional Chinese medicinal purposes, and the entire plant as a bioethanol source. Its rapid growth, strong adaptability to adverse environments, and high economic value make *B. papyrifera* suitable for karst rocky desertification control [23–26]. In the present study, *B. papyrifera* seedlings were subjected to different nitrate regimes. The effects of different nitrate concentrations on the growth, photosynthesis, chlorophyll fluorescence, nitrate reductase activity in leaves and roots, nitrogen content of plant organs, and δ^{15}N values of plant organs of *B. papyrifera* seedlings were investigated. The following were our main aims: (1) to estimate the assimilatory demand of whole *B. papyrifera* seedlings for nitrate over a greater time scale and (2) to estimate the partitioning of nitrate assimilation in the roots and leaves for *B. papyrifera* seedlings grown at different nitrate concentrations.

2. Materials and Methods

2.1. Plant Material and Experimental Treatments

Seeds of *B. papyrifera* were germinated in 12 drainage-hole-containing double-layer basin with a mixture of perlite and vermiculite (1:1 *v*/*v*) for 2 weeks at a temperature of 26/20 °C in the light/dark and 50–55% relative humidity. The lower basin contained a certain amount of water to keep the mixture moist. Seedlings of *B. papyrifera* were then

incubated under a 12-h photoperiod, with 500 ± 20 μmol m^{-2} s^{-1} of photosynthetic photon flux density (PPFD). The lower basin contained adequate 1/8 strength Hoagland nutrient solution [27], and the solution was completely replaced every 3 days. After 6 weeks, vigorous seedlings were transplanted to pots (height of 8.5 cm, bottom diameter of 9 cm, and 12 holes at the bottom, which were 0.9 cm in diameter). Two layers of nylon mesh were placed inside the pot, and then a mixture of perlite and vermiculite (1:1 v/v) was added to the pot to fix the roots of the seedling. Each pot contained only one seedling. Six pots were placed in a tray that contained adequate 1/4 strength Hoagland nutrient solution [27]. The seedlings in the pots grew well without extra aeration. The tray (not including the pot) was covered with aluminum foil to prevent algal growth from light infiltration into the solution. The solution in the tray was completely replaced every 3 days, and the surface of the pot and the tray were cleaned to avoid algal contamination. After 3 weeks of growth, the nutrient solution was replaced by a modified Hoagland solution containing 1 mM $MgSO_4 \cdot 7H_2O$, 0.125 mM KH_2PO4, 2.5 mM KCl, 4 mM $CaCl_2$, 0.1875 mM K_2SO_4, 50 μM Fe(Na)EDTA, 25 μM H_3BO_3, 2 μM $MnSO_4 \cdot 1H_2O$, 2 μM $ZnSO_4 \cdot 7H_2O$, 0.1 μM $CuSO_4$, 0.04 μM $CoCl_2 \cdot 6H_2O$, and 0.1 μM $Na_2MoO_4 \cdot 2H_2O$ at a pH of 7.3 ± 0.1. $NaNO_3$, with a $\delta^{15}N$ of 22.35‰, was employed as the sole nitrogen source. The nitrate concentrations in the three treatments were set at 0.5 mM, 2 mM, and 8 mM. Each treatment contained three replicates, and each seedling was treated as a replicate. The seedlings in all treatments achieved the same growth status. Each pot was placed in a tray that contained 500 mL modified Hoagland solution. The small tray (not including the pot) was covered with aluminum foil to prevent algal growth from light infiltration into the solution. The modified Hoagland solution in the small tray was completely replaced every other day, and the surface of the pot and the tray were cleaned to avoid algal contamination. The treatments lasted for 20 days.

2.2. Measurements of Growth

After 20 days of culture, all *B. papyrifera* seedlings were harvested and divided into leaves, stems, and roots. The dry weights of the leaves, stems, and roots were determined after oven drying to constant mass at 80 °C. To obtain the dry weight of the leaves, stems, and roots of the *B. papyrifera* seedlings at the start of the experiment, three *B. papyrifera* seedlings with the same growth status were selected, and the corresponding dry weights were measured. The average dry weight of the leaves, stems, and roots of the three *B. papyrifera* seedlings at the start of the experiment was approximately equal to the initial dry weight of the leaves, stems, and roots of the *B. papyrifera* seedlings in this study [see Table A1]. Accordingly, the average nitrogen contents of the leaves, stems, and roots of the three *B. papyrifera* seedlings at the start of the experiment were approximately equal to the initial nitrogen contents of the leaves, stems, and roots of the *B. papyrifera* seedlings in this study [see Table A1]. In addition, the average $\delta^{15}N$ values of the leaves, stems, and roots of the three *B. papyrifera* seedlings at the start of the experiment were approximately equal to the initial $\delta^{15}N$ values of the leaves, stems, and roots of the *B. papyrifera* seedlings in this study [see Table A1].

2.3. Measurement of Chlorophyll Content and Gas Exchange

At the final harvest, the chlorophyll (Chl) content in the second fully expanded leaf was determined using the chlorophyll meter SPAD-502Plus (Konica Minolta, Tokyo, Japan). The gas exchange measurements were performed with a portable photosynthesis system LI-6800 (LI-COR, Lincoln, NE, USA). The photosynthetically available radiation, leaf temperature, relative humidity, and CO_2 concentration during the measurements were 500 μmol m^{-2} s^{-1}, 27.0 °C, 55%, and 400 μmol m^{-2} s^{-1}, respectively. The net photosynthetic rate (Pn), stomatal conductance (Gs), transpiration rate (Tr), and intercellular CO_2 concentration (Ci) of the second fully expanded leaves were measured in the fluorescence leaf chamber using 6800-01A from 09:00 to 11:00.

2.4. Chlorophyll Fluorescence Measurements

Leaves were dark-adapted for 30 min to ensure complete relaxation of all reaction centers before the measurements. As mentioned earlier, the second fully expanded leaves were selected for Chl fluorescence measurements with a portable photosynthesis system LI-6800 (LI-COR, Lincoln, NE, USA). The initial (F_o) and maximum (F_m) Chl fluorescence were measured, and then, the maximum photochemical efficiency (F_v/F_m) was calculated. The maximum fluorescence (F_m') in the light-adapted state, basic fluorescence after induction (F_o'), and fluorescence yield in the steady state (F_s) were simultaneously determined while determining the Pn. The F_v/F_m, the actual photochemical efficiency of PSII (Φ_p), the photochemical quenching coefficient (q_P), the nonphotochemical quenching coefficient (q_N), and the electron transport rate (ETR) were calculated according to the following formulae:

$$F_v/F_m = (F_m - F_o)/F_m \quad (1)$$

$$\Phi_p = (F_m\prime - F_s)/F_m\prime \quad (2)$$

$$q_p = (F_m\prime - F_s)/(F_m\prime - F_o\prime) \quad (3)$$

$$q_N = (F_m - F_m\prime)/(F_m - F_o\prime) \quad (4)$$

$$ETR = PPFD \times \Phi_p \times 0.85 \times 0.5 \quad (5)$$

2.5. Analysis of Elements and Determination of $\delta^{15}N$ in Plants

The nitrogen contents of the dried leaves, stems, and roots were determined using an elemental analyzer (vario MACRO cube, Langenselbold, Germany). The $\delta^{15}N$ values of the leaves, stems, and roots were measured using a gas isotope ratio mass spectrometer (MAT-253, Thermo Fisher Scientific, Langenselbold, Germany). The $\delta^{15}N$ values were calculated according to the following equation:

$$\delta^{15}N(‰) = (R_{sample}/R_{standard} - 1) \times 1000 \quad (6)$$

where R_{sample} refers to the nitrogen isotope ratio of the plant material and $R_{standard}$ refers to the isotope ratio of a known standard (N_2 in air). IAEA N_1, IAEA N_2, and IAEA NO_3 reference materials were used to calibrate the instrument to reach a precision of 0.2‰ [28].

2.6. Photosynthetic Nitrogen Use Efficiency

After determining the nitrogen contents and net photosynthetic rate of leaves, the photosynthetic nitrogen use efficiency (PNUE) was obtained by dividing the value of Pn by the N content of the leaf [29,30].

2.7. Nitrate Reductase Activity Determination

The nitrate reductase activity (NRA) in the leaves and roots was tested using a Micro Nitrate Reductase Assay Kit (Solarbio, Beijing, China). The NRA was expressed as U/g FW.

2.8. The $\delta^{15}N$ of the Whole Plant

Using an isotope mass balance approach [19], the integrated $\delta^{15}N$ values of the whole plant consisting of leaves, stems, and roots were calculated as follows:

$$\delta^{15}N_{whole\text{-}plant}(‰) = (m_{leaf} \times \delta^{15}N_{leaf} + m_{stem} \times \delta^{15}N_{stem} + m_{root} \times \delta^{15}N_{root})/(m_{leaf} + m_{stem} + m_{root}) \quad (7)$$

where m_{leaf}, m_{stem}, and m_{root} are the total N accumulation (g) of the leaves, stems, and roots, respectively. $\delta^{15}N_{leaf}$, $\delta^{15}N_{stem}$, and $\delta^{15}N_{root}$ represent the $\delta^{15}N$ values of the leaves, stems, and roots, respectively.

2.9. The $\delta^{15}N$ of N Assimilates in the Whole Plant

Based on the isotope mass balance approach [19], we were able to calculate the $\delta^{15}N$ of the N assimilates in the whole plant when the $\delta^{15}N$ values of the initial and final whole plant (i.e., integrated $\delta^{15}N$ values of the whole plant at the beginning and harvest stages) were calculated. The $\delta^{15}N$ of the N assimilates in the whole plant ($\delta^{15}N_{assimilates}$) was calculated using the following equations:

$$\begin{aligned}\delta^{15}N_{assimilates}(‰) &= (m \times \delta^{15}N_{whole\text{-}plant1} - m_0 \times \delta^{15}N_{whole\text{-}plant0})/(m - m_0)\\ &= (m_l \times \delta^{15}N_l + m_s \times \delta^{15}N_s + m_r \times \delta^{15}N_r - m_{l0} \times \delta^{15}N_{l0} - m_{s0} \times \delta^{15}N_{s0} - m_{r0} \times \delta^{15}N_{r0})/(m - m_0)\end{aligned} \quad (8)$$

where $\delta^{15}N_{whole\text{-}plant1}$ and $\delta^{15}N_{whole\text{-}plant0}$ represent the $\delta^{15}N$ values of the final and initial whole plant, respectively. m and m_0 are the total N accumulation (g) of the final and initial whole plant, respectively. $\delta^{15}N_l$, $\delta^{15}N_s$, and $\delta^{15}N_r$ represent the $\delta^{15}N$ values of the leaves, stems, and roots at the final harvest, respectively. m_l, m_s, and m_r are the total N accumulation (g) of the leaves, stems, and roots at the final harvest, respectively. $\delta^{15}N_{l0}$, $\delta^{15}N_{s0}$, and $\delta^{15}N_{r0}$ represent the $\delta^{15}N$ values of the leaves, stems, and roots at the start of the experiment, respectively. m_{l0}, m_{s0}, and m_{r0} are the total N accumulation (g) of the leaves, stems, and roots at the start of the experiment, respectively. The standard error (SE) of the $\delta^{15}N_{assimilates}$ was achieved using the error propagation formula [31].

2.10. The $\delta^{15}N$ of N Assimilates in Plant Organs

For woody plant species, we treat the plant as having three major organs. We assume that the major sites of nitrogen assimilation are only leaves and roots. The $\delta^{15}N$ of the N assimilates in the stem can be predicted using a two end-member isotope mixing model using the $\delta^{15}N$ of the N assimilates in the leaves and roots as end members depending on the source for that stem nitrogen [20,21]:

$$\delta^{15}N_{stem\text{-}assimilates}(‰) = (\delta^{15}N_{leaf\text{-}assimilates} \times f_{leaf\ stem}) + (\delta^{15}N_{root\text{-}assimilates} \times f_{root\ stem}) \quad (9)$$

Equation (9) was rearranged to yield the fraction of stem N that is from the roots ($f_{root\ stem}$) or the leaves ($f_{leaf\ stem}$). For the fraction from the leaves, note that $f_{root\ stem} = 1 - f_{leaf\ stem}$:

$$f_{leaf\ stem} = \frac{(\delta^{15}N_{stem\text{-}assimilates} - \delta^{15}N_{root\text{-}assimilates})}{(\delta^{15}N_{leaf\text{-}assimilates} - \delta^{15}N_{root\text{-}assimilates})} \quad (10)$$

where $\delta^{15}N_{leaf\text{-}assimilates}$, $\delta^{15}N_{stem\text{-}assimilates}$, and $\delta^{15}N_{root\text{-}assimilates}$ can be calculated using an isotope mass balance approach. The mass balance equations for $\delta^{15}N_{leaf\text{-}assimilates}$, $\delta^{15}N_{stem\text{-}assimilates}$, and $\delta^{15}N_{root\text{-}assimilates}$ are as follows:

$$\delta^{15}N_{leaf\text{-}assimilates}(‰) = (m_l \times \delta^{15}N_l - m_{l0} \times \delta^{15}N_{l0})/(m_l - m_{l0}) \quad (11)$$

$$\delta^{15}N_{stem\text{-}assimilates}(‰) = (m_s \times \delta^{15}N_s - m_{s0} \times \delta^{15}N_{s0})/(m_s - m_{s0}) \quad (12)$$

$$\delta^{15}N_{root\text{-}assimilates}(‰) = (m_r \times \delta^{15}N_r - m_{r0} \times \delta^{15}N_{r0})/(m_r - m_{r0}) \quad (13)$$

where $\delta^{15}N_{leaf\text{-}assimilates}$ and $\delta^{15}N_{root\text{-}assimilates}$ represent the $\delta^{15}N$ values of N assimilates in leaves and roots, respectively. $\delta^{15}N_{stem\text{-}assimilates}$ represents the $\delta^{15}N$ values of newly acquired N assimilates in stems. The standard error (SE) of the $f_{leaf\ stem}$, $\delta^{15}N_{leaf\text{-}assimilates}$, $\delta^{15}N_{stem\text{-}assimilates}$, and $\delta^{15}N_{root\text{-}assimilates}$ was determined using the error propagation formula [31].

2.11. Statistical Analysis

The data were subjected to analysis of variance (ANOVA). The means of the different groups were compared via Tukey's test ($p < 0.05$). The data are shown as the mean ± standard

deviation (SE). All analyses were conducted using Data Processing System (DPS) software 7.05 (Hangzhou Ruifeng Information Technology Co., Ltd., Hangzhou, China).

3. Results

3.1. Growth

The nitrate concentration had a significant effect on the growth of *B. papyrifera* seedlings. As shown in Table 1, the dry weight of the *B. papyrifera* seedlings increased significantly with increasing nitrate supply. However, compared to the dry weight of the leaves, stems, and roots at a 2 mM nitrate concentration, the excessive nitrate supply (8 mM) did not lead to a significant increase in dry biomass accumulation. In addition, the shoot length of *B. papyrifera* seedlings did not show a significant increase when the nitrate concentration increased from 2 to 8 mM. These results indicated that excessive nitrate supply did not significantly promote the growth of *B. papyrifera* seedlings.

Table 1. The growth parameters of *Broussonetia papyrifera* (L.) Vent seedlings under the three nitrate regimes.

Parameters	Nitrate Concentration (mM)		
	0.5	**2**	**8**
Dry weight (g/plant)	1.917 ± 0.078 b	2.814 ± 0.172 a	3.035 ± 0.112 a
Leaf DW (g)	0.771 ± 0.070 b	1.224 ± 0.111 ab	1.460 ± 0.132 a
Stem DW (g)	0.464 ± 0.037 b	0.622 ± 0.017 ab	0.653 ± 0.057 a
Root DW (g)	0.683 ± 0.071 a	0.967 ± 0.102 a	0.921 ± 0.075 a
Shoot length (cm)	12.8 ± 0.6 b	16.1 ± 0.9 ab	17.8 ± 1.2 a

Each value represents the mean ± SE (n = 3). Values with the same letter in each line are not significantly different based on Tukey's test ($p > 0.05$).

3.2. Photosynthesis, SPAD, and Chl Fluorescence

We monitored the SPAD values and gas exchange parameters to determine the effects of different nitrate levels. With increasing nitrate supply, SPAD, Pn, Gs, and Tr showed a significant increasing trend (Table 2). The SPAD values and gas exchange parameters of *B. papyrifera* seedlings showed a positive response to nitrate concentration.

Table 2. The photosynthetic parameters, chlorophyll fluorescence, and SPAD of *Broussonetia papyrifera* (L.) Vent seedlings under the three nitrate regimes.

Parameters	Nitrate Concentration (mM)		
	0.5	**2**	**8**
Pn (μmol m^{-2} s^{-1})	4.714 ± 0.244 c	11.005 ± 0.189 b	14.083 ± 0.815 a
Gs (mol m^{-2} s^{-1})	0.059 ± 0.012 b	0.115 ± 0.002 ab	0.169 ± 0.022 a
Tr (mmol m^{-2} s^{-1})	1.067 ± 0.199 b	2.037 ± 0.034 ab	2.862 ± 0.334 a
Fv/Fm	0.740 ± 0.010 b	0.801 ± 0.005 a	0.819 ± 0.003 a
Φp	0.287 ± 0.009 c	0.509 ± 0.011 b	0.623 ± 0.007 a
qP	0.641 ± 0.012 c	0.802 ± 0.001 b	0.866 ± 0.005 a
qN	0.788 ± 0.015 a	0.586 ± 0.024 b	0.379 ± 0.025 c
ETR	60.477 ± 1.939 c	107.343 ± 2.305 b	131.276 ± 1.549 a
SPAD	40.533 ± 1.408 c	47.367 ± 1.049 b	57.200 ± 0.436 a

Each value represents the mean ± SE (n = 3). Values with the same letter in each line are not significantly different based on Tukey's test ($p > 0.05$).

Chl fluorescence was further investigated to understand the internal causes of the effects of different nitrate levels on photosynthesis. The *B. papyrifera* seedlings grown at the lowest nitrate concentration had a significantly lower F_v/F_m, Φ_p, qP, and ETR, while the qN was the highest (Table 2). These results suggested that the activity of PSII was significantly affected by the supply of nitrate. Because of the N deficiency (0.5 mM nitrate), the *B. papyrifera* seedlings seemed to suffer from photoinhibition.

3.3. Nitrogen Content in the Leaves, Stems, and Roots of *B. papyrifera* Seedlings

Increasing the nitrate supply significantly promoted nitrogen assimilation in the *B. papyrifera* seedlings. As shown in Figure 1, the nitrogen content in the leaves, stems, and roots showed a significant rising trend with increasing nitrate concentration. In addition, the nitrogen content of leaves was markedly higher than that of roots and stems in all treatments.

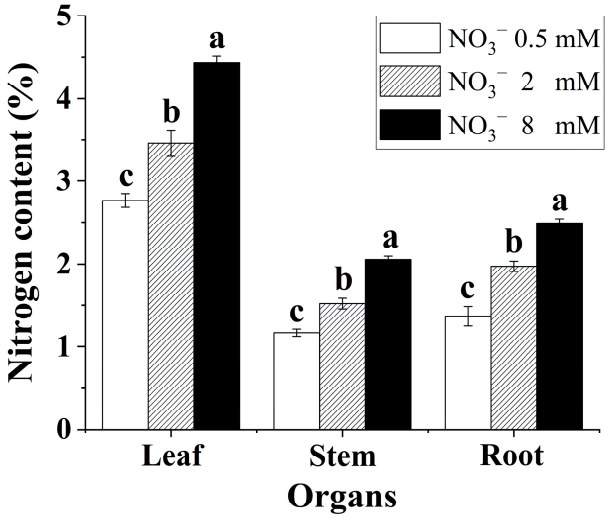

Figure 1. Nitrogen content of the *Broussonetia papyrifera* (L.) Vent seedlings under the three nitrate regimes. The mean ± SE (n = 3) followed by different letters in the same legend differ significantly (Tukey's test, $p < 0.05$).

3.4. Photosynthetic Nitrogen-Use Efficiency (PNUE)

The PNUE of the *B. papyrifera* seedlings only showed significant differences at the lowest nitrate concentration. As shown in Figure 2, increasing the nitrate supply did not significantly affect the PNUE of the *B. papyrifera* seedlings when the nitrate concentration was in the range of 2 to 8 mM.

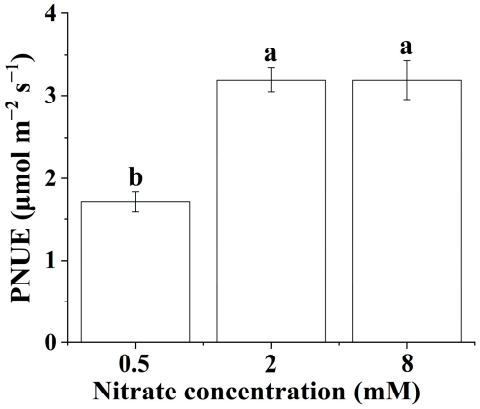

Figure 2. Photosynthetic nitrogen-use efficiency of *Broussonetia papyrifera* (L.) Vent seedlings under the three nitrate regimes. The mean ± SE (n = 3) followed by different letters in the same legend differ significantly (Tukey's test, $p < 0.05$).

3.5. NRA in the Leaves and Roots

Nitrate supply had a significant effect on the NRA in the leaves and roots (Figure 3). Increasing the nitrate concentration contributed to enhancing the NRA in the leaves and roots. Generally, the NRA in the leaves was markedly higher than that in the roots for the *B. papyrifera* seedlings.

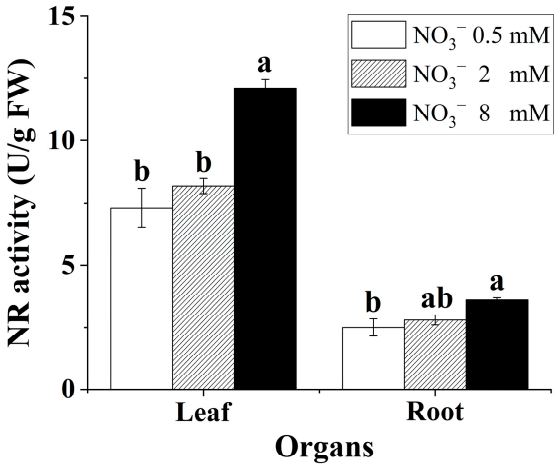

Figure 3. NRA in the leaves and roots of *Broussonetia papyrifera* (L.) Vent seedlings under the three nitrate regimes. The mean ± SE (n = 3) followed by different letters in the same legend differ significantly (Tukey's test, $p < 0.05$).

3.6. Nitrogen Isotope Composition in the Leaves, Stems, and Roots of the B. papyrifera Seedlings

There were differences in organ-level $\delta^{15}N$ for the *B. papyrifera* seedlings grown under the three nitrate regimes (Figure 4). Generally, the leaves were consistently enriched in ^{15}N relative to the roots. In addition, the $\delta^{15}N$ in the leaves, stems, and roots showed a significant rising trend with increasing nitrate concentration. Increasing the supply of nitrate contributed to enriching ^{15}N in the *B. papyrifera* seedlings.

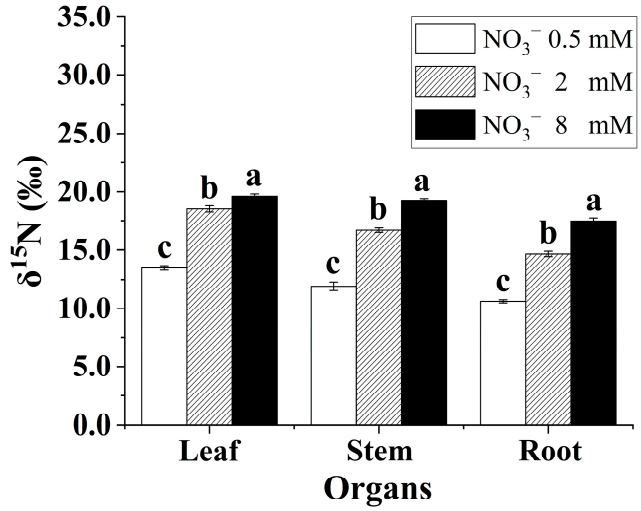

Figure 4. The $\delta^{15}N$ in leaves, stems, and roots of the *Broussonetia papyrifera* (L.) Vent seedlings under the three nitrate regimes. The mean ± SE (n = 3) followed by different letters in the same legend differ significantly (Tukey's test, $p < 0.05$).

3.7. The $\delta^{15}N$ Values of N Assimilates in the Whole Plant

The $\delta^{15}N$ values of the N assimilates in the whole *B. papyrifera* seedlings were negative compared to the initial source $\delta^{15}N$ under the three nitrate regimes (Figure 5), which suggested that isotope discrimination occurred during the process of nitrate assimilation. Increasing the nitrate concentration contributed to enriching ^{15}N in the N assimilates in the whole *B. papyrifera* seedlings. The $\delta^{15}N$ values of the N assimilates in the whole *B. papyrifera* seedlings at the lowest nitrate concentration were distinctly lower than that at other nitrate concentrations.

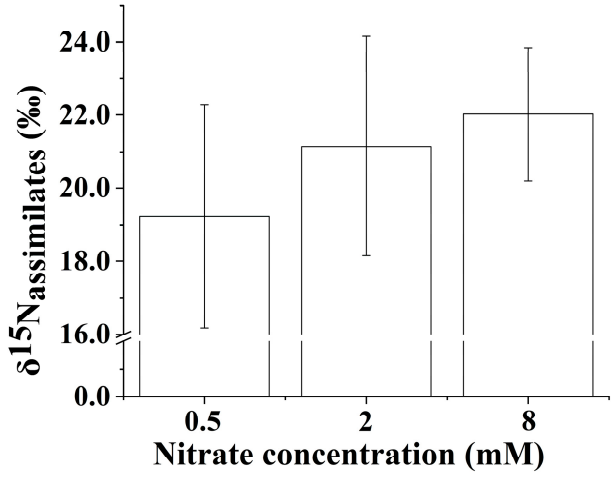

Figure 5. The $\delta^{15}N$ values of the N assimilates in the whole *Broussonetia papyrifera* (L.) Vent seedlings under the three nitrate regimes. The error bars were calculated using the error propagation formula.

3.8. Proportion of Stem N Derived from the Leaves

The proportion of stem N derived from the leaves showed a linear increase with nitrate concentration for the *B. papyrifera* seedlings (Figure 6). As shown in Figure 6, increasing the supply of nitrate contributed to the translocation of the N assimilates from the leaves to the stems. We observed that approximately 60% of stem N was derived from the translocation of N assimilates from the leaves when the nitrate concentration reached 8 mM.

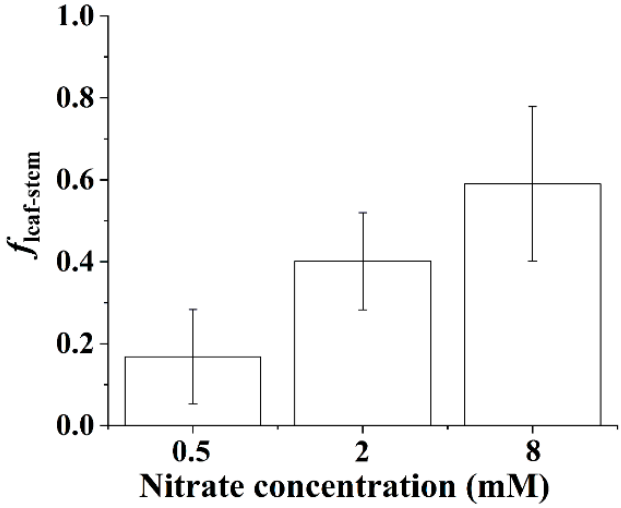

Figure 6. Proportion of stem N derived from the leaves of the *Broussonetia papyrifera* (L.) Vent seedlings under the three nitrate regimes. The error bars were calculated using the error propagation formula.

3.9. The $\delta^{15}N$ of N Assimilates in Plant Organs

The $\delta^{15}N$ values of the N assimilates in the leaves, stems, and roots of the *B. papyrifera* seedlings depended on the nitrate supply. As shown in Figure 7, the $\delta^{15}N$ values of the N assimilates in the leaves showed a decreasing trend with increasing nitrate concentration. However, increasing the supply contributed to enriching ^{15}N in the N assimilates of the stems and roots of the *B. papyrifera* seedlings. In addition, the difference between the $\delta^{15}N$ values of the N assimilates in the leaves and roots decreased gradually with increasing nitrate concentration.

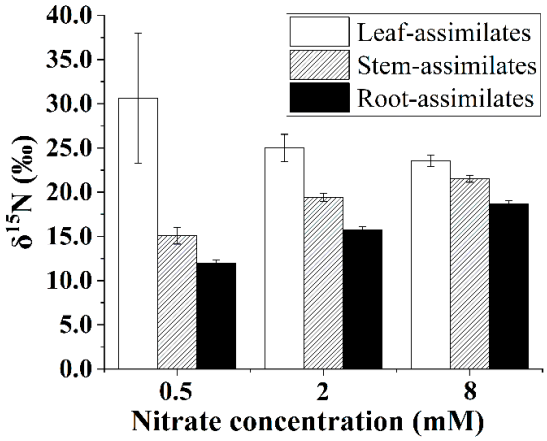

Figure 7. The $\delta^{15}N$ values of the N assimilates in the *Broussonetia papyrifera* (L.) Vent organs under the three nitrate regimes. The error bars were calculated using the error propagation formula.

4. Discussion

Plant $\delta^{15}N$ is a physiological indicator of N demand and fractionation that reflects changes in metabolic N fluxes and/or environmental effects [10,11]. Generally, variation in N supply can affect organ-level nitrogen isotope composition [13,32]. As shown in Figure 4, there was variation in organ-level $\delta^{15}N$ for the *B. papyrifera* seedlings grown under the three nitrate regimes. Enrichment of leaf $\delta^{15}N$ relative to root $\delta^{15}N$ for *B. papyrifera* grown at all nitrate concentrations indicated that some unassimilated inorganic nitrogen in the roots translocated to the shoot through the xylem [33]. Increasing the supply of nitrate enhanced the enrichment of ^{15}N in the leaves, stems, and roots of the *B. papyrifera* seedlings, which might be attributed to an increased capacity to assimilate nitrate in the leaves and/or roots [33]. Increasing the supply of nitrate also led to a significant increase in the nitrogen content of the leaves, stems, and roots (Figure 1), which suggested that increasing the nitrate concentration contributed to improving the nitrogen assimilation ability of *B. papyrifera* seedlings. Hence, there might be a positive response between the enrichment of ^{15}N and the nitrogen assimilation ability of *B. papyrifera* seedlings.

In the present study, the roots of *B. papyrifera* seedlings were always grown in the solution, which contributed to minimizing localized ^{15}N enrichment of the solution around the roots. The uptake of nitrogen by *B. papyrifera* seedlings caused the nitrate concentration in the tray to decrease, which resulted in an ^{15}N enrichment of the residual nitrate over time. Generally, the degree of ^{15}N enrichment in the residual nitrate depended on the change in nitrate concentration in the solution; namely, the more the nitrate concentration in the solution decreased, the greater the residual nitrate enriched ^{15}N [34]. After 20 days of culture, the *B. papyrifera* seedlings grown in three nitrate regimes (0.5 mM, 2 mM, and 8 mM) accumulated 0.0157 ± 0.0021 g N (n = 3, SE), 0.0510 ± 0.0056 g N (n = 3, SE), and 0.0807 ± 0.0048 g N (n = 3, SE), respectively. The total nitrogen supply of the three nitrate regimes (0.5 mM, 2 mM, and 8 mM) was 0.035 g N, 0.140 g N, and 0.560 g N, respectively. Hence, the $\delta^{15}N$ value of the residual nitrate at low nitrate concentrations would be greater than that at high nitrate concentrations. Based on the isotope mass balance approach [19], the $\delta^{15}N$ values of the N assimilates in the whole *B. papyrifera* seedlings grown at three nitrate regimes could be quantified. Because the actual $\delta^{15}N$ value of the source (i.e., the residual nitrate in the solution) could not be known, it was very difficult to precisely calculate the nitrogen isotope discrimination for the N assimilates in the whole *B. papyrifera* seedlings. However, the $\delta^{15}N$ values of the N assimilates in the whole *B. papyrifera* seedlings could still be used to indirectly indicate the degree of nitrogen isotope discrimination. The $\delta^{15}N$ values of the N assimilates in the whole *B. papyrifera* seedlings gradually increased with increasing nitrate concentration (Figure 5). The actual $\delta^{15}N$ value of the source (i.e., the residual nitrate in the solution) at the three nitrate concentrations was above 22.35‰ (the $\delta^{15}N$ value of the initial source). Meanwhile, the actual $\delta^{15}N$ value of the source (i.e., the residual nitrate in the

solution) gradually decreased with increasing nitrate concentration. Hence, we concluded that increasing the nitrate concentration reduced the nitrogen isotope discrimination for *B. papyrifera* seedlings when the nitrate concentration was in the range of 0.5 to 8 mM [16].

In general, nitrogen isotope discrimination is dependent on the relationship between nitrogen supply and nitrogen demand [11]. Increased nitrogen isotope discrimination usually corresponds to a reduced assimilatory demand for nitrogen [35], while the enhanced assimilatory demand for nitrogen will reduce the efflux and result in decreased nitrogen isotope discrimination [34]. As a result, we speculated that the decrease in nitrogen isotope discrimination value (i.e., the increase in the $\delta^{15}N$ value of the N assimilates in the whole *B. papyrifera* seedlings) was caused by enhanced assimilatory demand for nitrate in this study. Generally, increasing the supply of inorganic nitrogen leads to an increase in the nitrogen isotope discrimination value [13]. However, nitrogen isotope discrimination is not only dependent on the external inorganic nitrogen concentration. Nitrogen isotope discrimination was observed only when the demand for nitrogen was lower than the nitrogen supply [13]. Buschhaus [34] also found that increasing the supply of inorganic nitrogen resulted in a decrease in nitrogen isotope discrimination, which was attributed to the increased nitrogen demand resulting from stimulating growth. Hence, the assimilatory demand for nitrogen could be estimated using the $\delta^{15}N$ value of the N assimilates in the whole plant. In this study, increasing the supply of nitrate increased the $\delta^{15}N$ values of the N assimilates in the whole *B. papyrifera* seedlings when the nitrate concentration was in the range of 0.5 to 8 mM, which suggested that a relatively high external nitrate supply (8 mM) did not exceed the demand for nitrogen. In addition, the growth potential of the *B. papyrifera* seedlings also reflected that increasing the supply of nitrate contributed to enhancing the assimilatory demand for nitrate. As shown in Table 2, the Fv/Fm values of *B. papyrifera* seedlings were below 0.75 when the nitrate concentration was only 0.5 mM, which suggested that low N stress caused photoinhibition [36,37]. Photoinhibition usually damages the photosynthetic structure, thus significantly reducing the electron transport rate [37]. As a result, poor photosynthetic capacity was observed for *B. papyrifera* seedlings grown at 0.5 mM nitrate. Accordingly, the assimilatory demand for nitrate would be very low for *B. papyrifera* seedlings grown at 0.5 mM nitrate. As a whole, increasing the supply of nitrate could significantly enhance the photosynthetic capacity of *B. papyrifera* seedlings (Table 2). The enhanced photosynthetic capacity promoted the growth of *B. papyrifera* seedlings, which suggested that the demand for nitrate must have increased. Furthermore, increasing the nitrate concentration significantly elevated the nitrogen content of leaves, stems, and roots of *B. papyrifera* seedlings, which suggested that increasing the supply of nitrate enhanced the assimilatory demand for nitrate for *B. papyrifera* seedlings. Hence, quantifying the $\delta^{15}N$ value of the N assimilates in the whole plant could estimate the assimilatory demand of the whole plant for nitrate over a greater time scale, which contributed to preventing the waste and insufficiency of the nitrate supply.

Given that effluxed nitrogen is enriched in ^{15}N [15], the increased $\delta^{15}N$ values of the N assimilates in the whole *B. papyrifera* seedlings indicated that the net efflux of nitrate in the roots decreased. Correspondingly, increasing the supply of nitrate enhanced the assimilation of nitrate in the *B. papyrifera* seedlings. Leaves and roots are thought to be the major sites of nitrogen assimilation [11]. Hence, the efflux of nitrate in the roots was closely related to the assimilation of nitrate in the leaves and roots. Generally, nitrate reductase is required for the assimilation of nitrate, NRA is inducible with available nitrate [38,39], and increasing the supply of nitrate contributes to enhancing the NRA [39,40]. In this study, the NRA in the leaves and roots showed a positive response to the nitrate concentration (Figure 3). However, the NRA was not the only factor that affected nitrogen isotopic fractionation. As shown in Figure 3, when the nitrate concentration was in the range of 0.5 to 2 mM, no significant difference was observed in the NRA in the roots, and the NRA in the leaves did not show a significant change. The $\delta^{15}N$ values of the N assimilates in the whole *B. papyrifera* seedlings showed a marked increase when the nitrate concentration

increased from 0.5 to 2 mM, which suggested that the increased assimilation of nitrate might benefit from an adequate supply of reductant [17]. As shown in Figure 2, the PNUE level was significantly higher in the 2 mM nitrate concentration than in the 0.5 mM nitrate concentration. The high PNUE level was accompanied by an adequate supply of reductant. The PNUE level did not show a significant increase when the nitrate concentration increased from 2 to 8 mM. Hence, the increased $\delta^{15}N$ values of the N assimilates in the whole *B. papyrifera* seedlings might be attributed to the significantly enhanced NRA in the leaves when the nitrate concentration increased from 2 to 8 mM (Figure 3). Overall, the degree of nitrogen isotopic fractionation observed for nitrate assimilation depended on the NRA and the supply of reductant for the young, rapidly growing *B. papyrifera* seedlings.

Generally, the leaves had higher $\delta^{15}N$ values than the roots [15,18,33], which was the expected pattern because residual unassimilated nitrate (enriched in ^{15}N) is transported from roots to shoots. Accordingly, the $\delta^{15}N$ values of the N assimilates in the leaves were higher than those in the roots for the *B. papyrifera* seedlings (Figure 7). As shown in Figure 7, increasing the nitrate concentration decreased the leaf–root difference in the $\delta^{15}N$ values of the N assimilates, which might have been related to the partitioning of assimilatory activity between the roots and leaves. Although the NRA in the leaves was considerably greater than that in the roots (Figure 3), the location of nitrate assimilation appeared to depend on the internal demand of the *B. papyrifera* seedlings as well as the external nitrate concentration [33]. Under nonsaturating nitrate conditions, substantial nitrate will be assimilated by root nitrate reductase, and less nitrate may be assimilated in leaves [10]. A previous study also showed that assimilation is weighted more toward roots, particularly at concentrations below 1 mM nitrate [41]. Low assimilatory demand for nitrate was evidenced by the $\delta^{15}N$ value of the N assimilates in the whole *B. papyrifera* seedlings grown at 0.5 mM nitrate. Hence, only a smaller proportion of nitrate was assimilated in the leaves of the *B. papyrifera* seedlings grown at 0.5 mM nitrate. As a result, distinct leaf–root differences in the $\delta^{15}N$ values of the N assimilates were observed for the *B. papyrifera* seedlings grown at 0.5 mM nitrate (Figure 7). With increasing nitrate concentration, the assimilatory demand for nitrate in the roots reached saturation, and then, substantial nitrate was assimilated in the leaves, which led to a decrease in the $\delta^{15}N$ values of the N assimilates in the leaves. Consequently, a gradually decreased leaf–root difference in the $\delta^{15}N$ values of the N assimilates was observed for *B. papyrifera* seedlings when the nitrate concentration increased from 2 to 8 mM (Figure 7). Hence, the leaf–root difference in the $\delta^{15}N$ values of the N assimilates could be used to estimate the partitioning of nitrate assimilation in the roots and leaves. The newly acquired organic nitrogen in the stems is translocated from the leaves and roots [11,15]. Hence, when the $\delta^{15}N$ values of the N assimilates in the leaves, stems, and roots are quantified using the isotope mass balance approach [19], the proportion of stem N derived from the leaves (i.e., $f_{\text{leaf stem}}$) can be calculated using Equation (10). As shown in Figure 6, the proportion of stem nitrogen obtained from the leaves showed an obviously rising trend with increasing nitrate concentration, which suggested that increasing the supply of nitrate promoted the translocation of the N assimilates from the leaves to the stems. The increased translocation of the N assimilates from the leaves to the stems might imply that the nitrate assimilation was weighted more toward leaves with increasing nitrate concentration. Hence, quantifying the $\delta^{15}N$ values of the N assimilates in the plant organs not only contributes to estimating the partitioning of nitrate assimilation in roots and leaves but also provides an alternate way to calculate the proportion of stem N derived from the leaves.

5. Conclusions

Based on the isotope mass balance approach, the $\delta^{15}N$ values of N assimilates in plant organs and in whole plants can be quantified for *B. papyrifera* seedlings grown at different nitrate concentrations. The $\delta^{15}N$ values of N assimilates in whole *B. papyrifera* seedlings can be used to estimate the assimilatory demand of whole *B. papyrifera* seedlings for nitrate over a greater time scale. Increasing the supply of nitrate contributes to enhancing the assimila-

tory demand of the whole *B. papyrifera* seedlings for nitrate when the nitrate concentration was in the range of 0.5 to 8 mM. However, our results suggested that a concentration of 0.5 mM nitrate was insufficient to maintain the health of *B. papyrifera* seedlings. The optimal nitrate supply for *B. papyrifera* seedlings varies depending on the intended objective. To achieve ecological restoration, a 2 mM nitrate supply is recommended, whereas a nitrate supply of 8 mM is optimal for achieving a leaf forage with high protein content. Hence, quantifying the $\delta^{15}N$ values of N assimilates in whole *B. papyrifera* seedlings grown under different nitrate concentrations contributes to preventing the waste and insufficiency of the nitrate supply, which provides a theoretical basis for effective inorganic nitrogen management in *B. papyrifera* seedlings grown in karst regions. The partitioning of nitrate assimilation in the roots and leaves can be estimated using the leaf–root difference in the $\delta^{15}N$ values of the N assimilates for *B. papyrifera* seedlings grown under different nitrate concentrations. Increasing the nitrate supply contributes to increasing the partitioning of nitrate assimilation in leaves relative to roots.

Author Contributions: K.Z. and Y.W. conceived and designed the experiment. F.Z. performed most of the experiment. H.L. performed some of the experiment. K.Z. and F.Z. analyzed the data. K.Z. wrote the manuscript. Y.W. and Y.S. reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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Appendix A

Table A1. The initial biomass, nitrogen content and $\delta^{15}N$ of the *Broussonetia papyrifera* (L.) Vent seedlings at the start of the experiment.

Parameters	Plant Organs		
	Leaves	Stems	Roots
Dry weight (g)	0.348 ± 0.031	0.075 ± 0.006	0.070 ± 0.011
Nitrogen content (%)	4.53 ± 0.02	2.81 ± 0.01	3.15 ± 0.01
$\delta^{15}N$ (‰)	7.51 ± 0.09	6.97 ± 0.04	6.46 ± 0.02

Note: Each value represents the mean ± SE (n = 3).

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