Evaluation of Inorganic Nitrogen Supply-Demand Relationship in Mulberry Seedlings under Different Nitrate Nitrogen Concentrations Using Δ15N Values (Postprint)

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Abstract

Nitrate nitrogen dominates in soils of karst regions, but exhibits temporal and spatial heterogeneity. Consequently, mulberry seedlings cultivated in karst regions may experience low nitrogen stress. To provide scientific inorganic nitrogen management for mulberry seedlings in karst regions, this study employed a hydroponic experiment using mulberry seedlings as experimental material, modified Hoagland nutrient solution as the culture medium, and sodium nitrate with a δ^{15}N value of 22.35‰ as the sole nitrogen source. Three nitrate nitrogen concentration gradients were established (0.5, 2, and 8 $\mathrm{mmol \cdot L^{-1}}$), and photosynthetic characteristics along with dry weight, carbon content, nitrogen content, and δ^{15}N values of leaves, stems, and roots were measured. Physiological responses under different nitrogen supply levels were analyzed, the relationship between nitrogen demand and supply in mulberry seedlings was evaluated through stable nitrogen isotope fractionation at the whole-plant scale, and the carbon-nitrogen coupling relationship was investigated through plant nitrogen and carbon accumulation. The results showed: (1) When nitrate concentration ranged from 0.5 to 2 $\mathrm{mmol \cdot L^{-1}}$, increasing concentration significantly enhanced chlorophyll content and net photosynthetic rate, thereby promoting biomass accumulation. However, when nitrate concentration exceeded 2 $\mathrm{mmol \cdot L^{-1}}$, additional supply (8 $\mathrm{mmol \cdot L^{-1}}$) did not significantly increase chlorophyll content, net photosynthetic rate, or biomass. (2) Increased nitrate supply promoted nitrogen assimilation, with nitrogen accumulation gradually increasing alongside nitrate nitrogen supply; however, carbon accumulation showed no significant change at nitrate concentrations of 2 and 8 $\mathrm{mmol \cdot L^{-1}}$. (3) The stable nitrogen isotope fractionation value of nitrate nitrogen assimilation products reached a minimum at 2 $\mathrm{mmol \cdot L^{-1}}$. Thus, inorganic nitrogen supply at 2 $\mathrm{mmol \cdot L^{-1}}$ approximated the demand of mulberry seedlings, and proximity to equilibrium

between external supply and plant demand implies effective coordination of carbon and nitrogen metabolism, thereby achieving synchronized growth of carbon and nitrogen assimilation products.

Full Text

Use of Δ15N Value to Estimate the Relationship Between Nitrogen Supply and Demand in Mulberry Seedlings Under Different Nitrate Concentrations

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Abstract: Nitrate predominates in karst soils, yet its content exhibits significant temporal and spatial heterogeneity. Consequently, mulberry seedlings cultivated in karst regions may experience low nitrogen stress. To provide scientific guidance for inorganic nitrogen management of mulberry seedlings in these areas, we conducted a hydroponic experiment using mulberry seedlings as experimental material. A modified Hoagland nutrient solution served as the culture medium, with sodium nitrate ($ 15N = 22.35‰)asthesolenitrogensourceatthreeconcentrationgradients(0.5, 2, and8mmol· L^{-1}$). We measured photosynthetic characteristics, dry weight, carbon content, nitrogen content, and $ 15Nvaluesofleaves, stems, androotstoanalyzephysiologicalresponsesunderdiffe
plantstablenitrogenisotopefractionation, whilecarbon−nitrogencouplingwasinvestigatedviaplantnitrogenan
(1)Whennitrateconcentrationsincreasedfrom0.5to2mmol·L{-1}, chlorophyllcontentandnetphotosyntheticrateincrease
1}, additionalnitratesupply(8mmol·L{-1})didnotproducesignificantincreasesinchlorophyllcontent, netphotosyntheticrate, or
1}$ nitrate concentrations. (3) The stable nitrogen isotope fractionation value of nitrate assimilation products reached its minimum at 2 $\mathrm{mmol} \cdot \mathrm{L}^{-1}$ nitrate concentration. This indicates that an inorganic nitrogen supply at 2 $\mathrm{mmol} \cdot \mathrm{L}^{-1}$ approximates the inorganic nitrogen demand of mulberry seedlings. A close balance between external nitrogen supply and plant nitrogen demand signifies effective coordination of carbon and nitrogen metabolism, thereby achieving synchronized growth of carbon and nitrogen assimilates.

Keywords: nitrate nitrogen, *Morus alba*, carbon and nitrogen metabolism, stable nitrogen isotope fractionation, nitrogen demand

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Introduction

Inorganic nitrogen absorbed and utilized by plants consists primarily of nitrate and ammonium nitrogen. However, in well-aerated or alkaline soils, ammonium is rapidly converted to nitrate through nitrification by aerobic bacteria. Karst bedrock is rich in calcium (Huang et al., 2015), resulting in calcareous soils with high pH (Wan et al., 2009). Consequently, ammonium nitrogen in karst soils oxidizes to nitrate at high rates, exacerbating the characteristic low-ammonium, high-nitrate profile of these regions. Since nitrate dominates karst soils, investigating plant physiological responses to varying nitrate concentrations holds significant research importance.

Nitrate plays multiple roles in plant growth, with 99% of organic nitrogen in the biosphere derived from plant-assimilated nitrate (Wang et al., 1993). As an essential nutrient, nitrate also functions as a signaling molecule that regulates ethylene and abscisic acid synthesis and interacts with cytokinins to modulate plant senescence (Wen et al., 2020). Plants have evolved high-affinity and low-affinity transport systems to adapt to the heterogeneous distribution of soil nitrate. Previous research indicates that soil nitrate accumulation is the primary factor determining nitrogen fertilizer efficiency and the effectiveness of different nitrogen forms (Miao et al., 2014). Therefore, investigating the effects of varying nitrate concentrations under alkaline conditions is crucial for scientific management of inorganic nitrogen supply to plants in karst environments.

Plant photosynthetic carbon assimilation and nitrogen assimilation are tightly linked, exhibiting both interdependence and competition. Photosynthetic carbon assimilation utilizes ATP and NADPH generated during light reactions to reduce CO_2 into carbohydrates and other organic compounds. Inorganic nitrogen assimilation involves plant uptake of environmental NO_3^-, utilizing reductive power and energy from both light and dark reactions while employing carbon assimilation products as amino acceptors for amino acid synthesis. This amino acid synthesis intimately connects carbon and nitrogen assimilation pathways (Wang et al., 2014; Busch et al., 2018). Appropriate nitrogen application promotes both nitrogen and carbon metabolism (Zhang et al., 2022), while nitrogen assimilation consumes substantial photosynthetic products and reductive power. Photosynthetic carbon assimilation provides energy and carbon skeletons for nitrogen assimilation, creating competition for photosynthetically derived reductive power and intermediates (Geng et al., 2010). Consequently, determining optimal nitrogen application rates is essential for improving plant yield and quality.

Following absorption from the culture medium, inorganic nitrogen is partially assimilated in roots, with the remainder transported to leaves for assimilation or returned to the medium (Hu et al., 2022). During inorganic nitrogen assimilation, plants preferentially assimilate lighter nitrogen isotopes (^{14}N), while unassimilated nitrogen exported from roots becomes enriched in heavier isotopes (^{15}N). Consequently, whole-plant $ $15N values become relatively depleted compared to the nitrogen source (Hu & Guy, 2020). The difference between whole-plant and source $ $15N values results from nitrogen isotope fractionation (Kalcsits & Guy, 2013). Under nitrate as the sole nitrogen source, whole-plant stable nitrogen isotope fractionation depends on nitrate reductase activity and reductive power supply. High nitrate reductase activity with adequate reductive power maximizes assimilation of incoming nitrate, reducing the amount of unassimilated nitrate exported from roots and consequently minimizing whole-plant stable nitrogen isotope fractionation. Conversely, limitations in nitrate reductase activity or reductive power increase unassimilated nitrate export from roots, leading to greater whole-plant nitrogen isotope fractionation (Mariotti et al., 1982). Thus, whole-plant stable nitrogen isotope fractionation values correlate closely with plant nitrate supply-demand dynamics. Based on isotope mass balance equations (Hayes, 2004), we can calculate whole-plant stable nitrogen isotope fractionation values across different nitrate concentrations, enabling temporal assessment of plant nitrate supply-demand status and overcoming limitations of traditional methods.

Mulberry (*Morus alba*), a perennial deciduous woody plant, is widely distributed throughout China, which leads the world in mulberry cultivation area and produces 80% of global silkworm cocoons. Combining high ecological and economic value, mulberry represents an excellent species for diversified development and ecological poverty alleviation. Sericulture involves low costs and technical barriers. Southwestern China comprises karst regions characterized by mountainous terrain, limited arable land, and fragile substrates, where mulberry cultivation yields significantly higher benefits than sugarcane, corn, or soybean crops. With its extensive root system, mulberry provides water conservation and windbreak functions (Zhao, 2019), offering important value for restoration and management of southwestern China's karst areas with shallow soils, slow pedogenesis, and severe water erosion. However, insufficient nitrogen supply in mulberry plantations results in slow branch and leaf growth, soft and thin shoots, small leaf size, poor leaf quality, and low yield (Yuan, 2018). Moreover, appropriate nitrate application is key to maintaining high yield and quality under stress conditions (Pang et al., 2014). Current mulberry cultivation remains at a extensive management stage, with farmers commonly applying nitrogen fertilizer blindly to increase yield. Excessive nitrogen application causes serious waste and environmental problems. Previous studies indicate mulberry is nitrate-preferring (Xu et al., 2012). Therefore, quantifying the supply-demand relationship of mulberry seedlings under different nitrate concentrations can prevent both nitrate deficiency and excess, enabling scientific nitrate management.

Based on these objectives, we cultivated mulberry seedlings in an artificial climate greenhouse using solution culture. By measuring growth, carbon-nitrogen content, and stable nitrogen isotope values under different nitrate concentrations, we investigated: (1) physiological responses of mulberry seedlings to varying nitrate concentrations; (2) the relationship between nitrate supply and inorganic nitrogen demand; and (3) carbon-nitrogen coupling under different nitrate concentrations. Through this analysis, we aim to provide theoretical foundations for scientific nitrogen application and mulberry cultivation management in southwestern China's karst regions.

Materials and Methods

Plant Cultivation and Treatments

The experiment was conducted in a greenhouse. Healthy, plump, and uniformly sized mulberry seeds (Qiangsang No. 1, purchased from a seed company) were soaked and sown in 12-cell seedling trays, which were kept moist with water. After germination, seedlings were cultured with 1/8 Hoagland nutrient solution. Sixty days after sowing, 16 germinated and vigorously growing seedlings were selected and transplanted into nursery pots for hydroponic culture using 1/4 Hoagland nutrient solution. After 23 days, nine uniformly growing mulberry seedlings were selected for formal experimentation using modified 1/2 Hoagland nutrient solution, with three pots per group and three groups total. The modified 1/2 Hoagland nutrient solution contained: 1 $\mathrm{mmol\cdot L^{-1}}$ $MgSO_4\cdot 7H_2O$, 0.125 $\mathrm{mmol\cdot L^{-1}}$ KH_2PO_4, 2.5 $\mathrm{mmol\cdot L^{-1}}$ KCl, 4 $\mathrm{mmol\cdot L^{-1}}$ $CaCl_2$, 0.1875 $\mathrm{mmol\cdot L^{-1}}$ K_2SO_4, 50 $\mathrm{mol\cdot L^{-1}}$ Fe(Na)EDTA, 25 $\mathrm{mol\cdot L^{-1}}$ H_3BO_3, 2 $\mathrm{mol\cdot L^{-1}}$ $MnSO_4\cdot H_2O$, 2 $\mathrm{mol\cdot L^{-1}}$ $ZnSO_4\cdot 7H_2O$, 0.1 $\mathrm{mol\cdot L^{-1}}$ $CuSO_4\cdot 5H_2O$, 0.04 $\mathrm{mol\cdot L^{-1}}$ $CoCl_2\cdot 6H_2O$, and 0.1 $\mathrm{mol\cdot L^{-1}}$ $Na_2MoO_4\cdot 2H_2O$. Sodium nitrate with \$ \$15N = 22.35‰ served as the sole nitrogen source. The entire treatment lasted 20 days, with solution changes every 2 days (500 mL per plant) to maintain a relatively stable nitrogen environment.

Based on the actual condition that nitrate content in karst soils is far below 10 $\mathrm{mmol\cdot L^{-1}}$ and exhibits heterogeneous distribution, we established three nitrate concentration gradients: 0.5, 2, and 8 $\mathrm{mmol\cdot L^{-1}}$. The 0.5 $\mathrm{mmol\cdot L^{-1}}$ treatment simulated low nitrogen levels in karst regions, 2 $\mathrm{mmol\cdot L^{-1}}$ simulated medium nitrogen levels, and 8 $\mathrm{mmol\cdot L^{-1}}$ simulated high nitrogen levels. The photoperiod was 12 h with light intensity of (500 ± 25) $\mathrm{mol\cdot m^{-2}\cdot s^{-1}}$, daytime temperature of (25 ± 2)°C, nighttime temperature of (19 ± 2)°C, relative humidity of 55–60%, and culture solution pH of 7.5 ± 0.1.

Measurement of Plant Growth Parameters

Before treatment initiation, three uniformly growing mulberry seedlings were selected to determine root, stem, and leaf dry weights. The average values served as initial dry weights for roots, stems, and leaves throughout the experiment. Correspondingly, average carbon and nitrogen contents of these three seedlings approximated initial carbon and nitrogen contents, while average stable nitrogen isotope values approximated initial stable nitrogen isotope values for roots, stems, and leaves.

On the final day of treatment, plants were separated into roots, stems, and leaves. Fresh weights were measured using an electronic balance, then samples were placed in a forced-air oven at 108°C for 40 minutes, dried at 80°C to constant weight, and dry weights were recorded. Roots, stems, and leaves were then ground into powder for subsequent carbon-nitrogen content and stable nitrogen isotope measurements.

Chlorophyll Content and Photosynthetic Parameter Measurements

On the final treatment day, SPAD values of the second fully expanded leaf from the top were measured using a SPAD-502Plus chlorophyll meter. Net photosynthetic rate (Pn), stomatal conductance (Gs), transpiration rate (Tr), and intercellular CO_2 concentration (Ci) were measured using a portable photosynthesis system (Li-6800, LI-COR, Lincoln, NE, USA). A 6800-01A fluorescence leaf chamber was used, maintaining CO_2 concentration at 400 mol · mol^{-1} with a small CO_2 cylinder, gas flow rate at 500 mmol · s^{-1}, photosynthetic photon flux density at 500 mol · m^{-2} · s^{-1}, and leaf temperature at 27°C.

Photosynthetic Nitrogen Use Efficiency

Photosynthetic nitrogen use efficiency (PNUE) is defined as the ratio of net photosynthetic rate to leaf nitrogen content (Poorter & Evans, 1998), calculated as:

$$PNUE = \frac{Pn}{C_N}$$

where Pn is leaf net photosynthetic rate and C_N is leaf nitrogen content.

Carbon and Nitrogen Content Determination and Accumulation Calculation

Total carbon and nitrogen contents of mulberry seedling roots, stems, and leaves were determined using an elemental analyzer (vario MACRO cube, Germany), expressed as mass percentages of dry weight.

Nitrogen accumulation amount (NAA) was calculated as:

$$NAA = (DW_{leaf1} \times N_{leaf1} + DW_{stem1} \times N_{stem1} + DW_{root1} \times N_{root1}) - (DW_{leaf0} \times N_{leaf0} + DW_{stem0} \times N_{stem0} + DW$$

where DW_{leaf1}, DW_{stem1}, and DW_{root1} are leaf, stem, and root dry weights after treatment; N_{leaf1}, N_{stem1}, and N_{root1} are nitrogen contents after treatment; DW_{leaf0}, DW_{stem0}, and DW_{root0} are initial dry weights; and N_{leaf0}, N_{stem0}, and N_{root0} are initial nitrogen contents. Standard errors for NAA were calculated using error propagation formulas.

Carbon accumulation amount (CAA) was calculated as:

$$CAA = (DW_{leaf1} \times C_{leaf1} + DW_{stem1} \times C_{stem1} + DW_{root1} \times C_{root1}) - (DW_{leaf0} \times C_{leaf0} + DW_{stem0} \times C_{stem0} + DW$$

where C_{leaf1}, C_{stem1}, and C_{root1} are carbon contents after treatment, and C_{leaf0}, C_{stem0}, and C_{root0} are initial carbon contents. Standard errors for CAA were calculated using error propagation formulas.

Stable Nitrogen Isotope Determination

Plant sample \$ $15 N values were measured using a gas isotope ratio mass spectrometer (MAT-$
$253, Germany) with a precision of 0.2‰. IAEA N1, IAEA N2, and IAEA NO\_\{3\}$\$

standards were used for instrument calibration. Stable nitrogen isotope values were calculated as:

$$\delta^{15}N = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \times 1000‰$$

where R_{sample} is the $^{15}N/^{14}N$ isotope ratio of mulberry seedlings and R_{standard} is the $^{15}N/^{14}N$ ratio of the standard material (atmospheric N_2).

After determining $ $15N values of leaves, stems, and roots, whole-plant $ 15*Nvalues*($15N_{whole}-plant) were calculated (Robinson et al., 2000; Wang et al., 2016) as:

$$\delta^{15}N_{whole-plant} = \frac{m_{leaf} \times \delta^{15}N_{leaf} + m_{stem} \times \delta^{15}N_{stem} + m_{root} \times \delta^{15}N_{root}}{m_{leaf} + m_{stem} + m_{root}}$$

where m_{leaf}, m_{stem}, and m_{root} are total nitrogen amounts (g) in leaves, stems, and roots (calculated as dry weight × nitrogen content), and $ $15N_{leaf}, $ $15N_{stem}, and $ $15N_{root} are corresponding stable nitrogen isotope values.

After calculating whole-plant $ 15*Nvaluesbeforeandaftertreatment, thestablenitrogenisotopevalueofnitroge* during the experimental period was determined based on isotope mass balance equations (Hayes, 2004):

$$\delta^{15}N_{assimilates} = \frac{m_1 \times \delta^{15}N_{whole-plant1} - m_0 \times \delta^{15}N_{whole-plant0}}{m_1 - m_0}$$

where $ $15N_{whole}-plant1 and $ $15N_{whole}-plant0 are whole-plant $ $15N values after and before treatment; m_1 and m_0 are total nitrogen amounts after and before treatment (sum of leaf, stem, and root nitrogen). Standard errors for $ $15N_{assimilates} were calculated using error propagation formulas.

The stable nitrogen isotope fractionation value of nitrate assimilation products (Δ15N_{assimilates}) was calculated as (Evans et al., 1996):

$$\Delta^{15}N_{assimilates} = \delta^{15}N_{substrate} - \delta^{15}N_{assimilates}$$

where $ $15N_{substrate} = 22.35‰. Standard errors for Δ15N_{assimilates} were calculated using error propagation formulas.

Data Processing and Analysis

All data are presented as mean ± standard error (Mean ± SE). Single-factor significance analysis was performed using DPS statistical software (Tukey's test, $P < 0.05$). Figures were generated using Origin software (version 2019b).

Results

Effects of Different Nitrate Concentrations on Mulberry Seedling Growth

Nitrate supply significantly affected mulberry seedling growth (Table 1). Increased nitrate supply improved seedling growth. Plant dry weight at 2 $\mathrm{mmol \cdot L^{-1}}$ nitrate was significantly higher than at 0.5 $\mathrm{mmol \cdot L^{-1}}$. At 8 $\mathrm{mmol \cdot L^{-1}}$, plant dry weight was also significantly higher than at 0.5 $\mathrm{mmol \cdot L^{-1}}$ but showed no significant difference compared to 2 $\mathrm{mmol \cdot L^{-1}}$. These results indicate that moderate nitrate concentration increases promote seedling growth, but the growth-promoting effect becomes non-significant beyond a certain threshold.

Table 1 Effects of different nitrate concentrations on the growth of *Morus alba* seedlings

Parameters	Nitrate concentration ($\mathrm{mmol \cdot L^{-1}}$)	
	0.5	2
Dry weight of leaf (g)	0.68\$±0.04*b*|0.87±0.08*ab*|0.91±0.02*a*||*Dryweightofstem*(*g*)|0.46±0.01*a*|0.56±0.04*a*|0.60± *plant*^{-1})|1.54±0.04*b*|1.91±0.12*a*|1.95±\$0.04a	

Note: Values represent mean ± SE (n = 3). Different letters within each row indicate significant differences by Tukey's test ($P < 0.05$). The same notation applies below.

Effects of Different Nitrate Concentrations on Photosynthetic Parameters and Chlorophyll Content

Photosynthesis is crucial for plant growth. As shown in Table 2 , net photosynthetic rate at 2 and 8 $\mathrm{mmol \cdot L^{-1}}$ nitrate was significantly higher than at 0.5 $\mathrm{mmol \cdot L^{-1}}$, but showed a slight decline when nitrate concentration increased from 2 to 8 $\mathrm{mmol \cdot L^{-1}}$. This suggests that the photosynthetic enhancement effect saturates beyond a certain nitrate supply level. Increased nitrate concentration facilitated chlorophyll biosynthesis, with chlorophyll content at 2 and 8 $\mathrm{mmol \cdot L^{-1}}$ significantly exceeding that at 0.5 $\mathrm{mmol \cdot L^{-1}}$, though no significant difference existed between 2 and 8 $\mathrm{mmol \cdot L^{-1}}$ treatments.

Table 2 Effects of different nitrate concentrations on photosynthesis and chlorophyll content of *Morus alba* seedlings

Parameters	Nitrate concentration ($\mathrm{mmol \cdot L^{-1}}$)	
	0.5	2

Parameters	Nitrate concentration (mmol · L^{-1})	
Net photosynthetic rate (mol · m^{-2} · s^{-1})	10.14$±0.35$b|13.28±0.72a|13.00±0.68a||Intercellular CO_2 concentration (mol · mol^{-1})	287.73$±2.9$15a|289.80±19.93a|252.91 m{-2}·s{- 1})|2.83±0.32a|3.92±0.84a|2.75±0.20 m{-2}·s{- 1})|0.17±0.02a|0.24±0.06a|0.16±0.01

Effects of Different Nitrate Concentrations on Carbon-Nitrogen Content and Accumulation

Compared to carbon content, different nitrate concentrations exerted more pronounced effects on nitrogen content (Figure 1 [Figure 1: see original paper]). Across treatments, stem and root carbon contents showed no significant differences among the three nitrate concentrations, except that leaf carbon content at 0.5 mmol · L^{-1} was significantly lower than at 2 and 8 mmol · L^{-1}. However, nitrogen application significantly increased nitrogen content in all three plant parts, which rose progressively with increasing nitrate concentration. Overall, carbon was relatively evenly distributed among leaves, stems, and roots, while nitrogen concentrated primarily in leaves.

Values represent mean ± SE (n = 3). Bars with different letters indicate significant differences (Tukey's test, $P < 0.05$). The same notation applies below.

Figure 1 Effects of different nitrate concentrations on carbon contents (A) and nitrogen contents (B) of leaf, stem, and root of *Morus alba* seedlings [Figure 1: see original paper]

Carbon and nitrogen accumulation calculations after treatment are shown in Figure 2 [Figure 2: see original paper]. Carbon accumulation increased initially then plateaued with rising nitrate concentration, showing significant improvement from 0.5 to 2 mmol · L^{-1} but no significant change between 2 and 8 mmol · L^{-1}. In contrast, nitrogen accumulation increased continuously with nitrate concentration. These findings demonstrate that nitrogen fertilization promotes carbon assimilation within a certain range, but the carbon assimilation enhancement does not persist indefinitely with increasing nitrogen application, indicating limited promotional effects on biomass.

Error bars were calculated using error propagation formulas.

Figure 2 Effects of different nitrate concentrations on carbon accumulation amount (A) and nitrogen accumulation amount (B) of *Morus alba* seedlings [Figure 2: see original paper]

Effects of Different Nitrate Concentrations on Photosynthetic Nitrogen Use Efficiency

As shown in Figure 3 [Figure 3: see original paper], mulberry seedlings achieved higher photosynthetic nitrogen use efficiency at 0.5 and 2 mmol · L^{-1} nitrate, while excessive nitrate supply (8 mmol $\cdot$ L^{-1}) reduced efficiency. This indicates that increasing inorganic nitrogen application within a certain range does not reduce photosynthetic nitrogen use efficiency, but excessive application causes nitrogen waste.

Figure 3 Effects of different nitrate concentrations on photosynthetic nitrogen use efficiency of *Morus alba* seedlings [Figure 3: see original paper]

Effects of Different Nitrate Concentrations on $ $15N and Δ15N Values of Plant Nitrogen Assimilates

As shown in Figure 4A [Figure 4: see original paper], $ $15N values of nitrogen assimilation products were lower than the nitrogen source (sodium nitrate) across all treatments, indicating stable nitrogen isotope fractionation occurred during nitrate assimilation. $ *15Nvaluesofplantnitrogenassimilationproductsfirstincreasedthendecreased,r* *L*^{-1}$ and declining at 8 mmol $\cdot$ L^{-1}. Correspondingly, Δ15N values showed the opposite trend, reaching minimum at 2 mmol $\cdot$ L^{-1} and increasing when nitrate concentration rose to 8 mmol $\cdot$ L^{-1}.

Error bars were calculated using error propagation formulas.

Figure 4 Effects of different nitrate concentrations on $ $15N values (A) and Δ15N values (B) of N assimilates in whole *Morus alba* seedlings [Figure 4: see original paper]

Discussion and Conclusion

Nitrogen management is a crucial measure for regulating crop growth, improving photosynthetic characteristics, and increasing yield. Research demonstrates that crop growth and yield are closely related to nitrogen supply (Cui & Lee, 2002). Appropriate nitrogen application significantly promotes mulberry growth (Xu et al., 2012). As the primary inorganic nitrogen source for plants and a nitrate-preferring species (Xu et al., 2012), mulberry responses to nitrate concentration warrant investigation. Our results show that moderate nitrate concentration increases significantly promoted mulberry seedling growth, likely because 2 mmol $\cdot$ L^{-1} inorganic nitrogen supply substantially enhanced chlorophyll biosynthesis and consequently increased net photosynthetic rate. However, higher nitrate supply (8 mmol $\cdot$ L^{-1}) did not produce linear growth increases, suggesting nitrate supply exceeded seedling inorganic nitrogen demand. Chlorophyll content and net photosynthetic rate showed no significant differences between 2 and 8 mmol $\cdot$ L^{-1} treatments, indicating a saturating concentration for photosynthetic enhancement. Furthermore, higher nitrate supply intensifies nitrogen assimilation, consuming substantial photosynthetic products (Geng et al., 2010) and potentially limiting biomass accumulation.

Typically, nitrogen content accounts for 1.5–5% of dry weight in higher plants (Novoa & Loomis, 1981), and increased inorganic nitrogen supply raises plant nitrogen content (Gulmon & Chu, 1981). Our experiment confirmed that increased nitrate concentration significantly elevated nitrogen content in mulberry leaves, stems, and roots, with nitrogen accumulation increasing accordingly. Although maximum nitrogen accumulation occurred at 8 mmol · L^{-1}, the 4-fold increase in nitrate supply from 2 to 8 mmol · L^{-1} only increased nitrogen accumulation by 1.54-fold, whereas the same 4-fold increase from 0.5 to 2 mmol · L^{-1} increased nitrogen accumulation by 2-fold. This suggests stronger nitrogen acquisition capacity at 2 mmol · L^{-1} nitrate concentration.

Nitrate reductase is an inducible enzyme whose activity increases with nitrate supply, promoting nitrate absorption and assimilation (Kaiser & Huber, 2001; Black et al., 2002). However, nitrate assimilation is an active, energy-consuming process (Tsay et al., 1993) that utilizes substantial carbon and energy reserves (Huppe & Turpin, 1994). Plants consume approximately 20 ATP molecules per nitrate molecule assimilated (Salsac et al., 1987), meaning greater nitrogen accumulation entails higher energy consumption. Our net photosynthetic rate data showed no significant difference between 2 and 8 mmol · L^{-1} treatments, indicating that 8 mmol · L^{-1} nitrate supply did not further enhance photosynthesis. Since photosynthesis provides materials and energy for growth (Walters et al., 1993) and nitrate assimilation depends on photosynthetically derived energy and reductive power (Larsson et al., 1985), maximum nitrogen accumulation at 8 mmol · L^{-1} entailed substantial energy consumption, resulting in minimal carbon accumulation increase compared to 2 mmol · L^{-1}. In contrast, increasing nitrate supply from 0.5 to 2 mmol · L^{-1} achieved synchronized increases in both carbon and nitrogen accumulation. The significant carbon accumulation increase at 2 mmol · L^{-1} likely resulted from enhanced photosynthesis, as net photosynthetic rate was significantly higher than at 0.5 mmol · L^{-1}. Overall, appropriate inorganic nitrogen supply enhances both carbon and nitrogen metabolism, while excessive supply primarily enhances nitrogen metabolism with limited promotion of carbon metabolism. Therefore, increasing yield through excessive nitrogen application is not scientifically sound.

Increasing nitrate supply enhances leaf nitrate reductase activity (Kaiser & Huber, 2001; Black et al., 2002), leading to greater nitrogen accumulation. While high nitrate reductase activity typically corresponds to high \$ \$15N values in assimilation products (Pate et al., 1993), \$ \$15N values of mulberry seedling nitrate assimilation products did not increase linearly with nitrate concentration. The \$ $15Nvaluepeakedat2mmol \cdot L$^{-1}\$ and declined at 8 mmol · L^{-1}, suggesting factors beyond nitrate reductase activity influence assimilation product \$ $15Nvalues.Thedeclineat8mmol \cdot L$^{-1}\$ may relate to insufficient reductive power supply (Mariotti et al., 1982), as photosynthetic nitrogen use efficiency decreased by 26.40% when nitrate concentration increased from 2 to 8 mmol · L^{-1}, indirectly indicating reductive power limitation.

Based on \$ $15Nvaluesofnitrogenassimilationproducts, Equation(7)enabledcalculationofstablenitrogenisoto$
$plantnitrateassimilationunderdifferentnitrateconcentrations.Stablenitrogenisotopefractionationindicate$
$demand), whilelargervaluessuggestsupplyisbeloworexceedsdemand.Ourresultsshowminimum\Delta 15Nat2mmo$
L^{-1}\$ nitrate, with significantly higher values at 8 mmol · L^{-1}. This indicates that 8 mmol · L^{-1} nitrate supply substantially exceeded seedling inorganic

nitrogen demand, while 2 mmol • L^{-1} represented the optimal balance among the three concentrations.

In summary, stable nitrogen isotope technology enables quantification of nitrogen isotope fractionation in assimilation products under different nitrate concentrations, allowing assessment of inorganic nitrogen supply-demand balance. Ensuring this balance prevents nitrogen waste and deficiency. At 2 mmol • L^{-1} nitrate, mulberry seedlings approached supply-demand equilibrium, enabling effective coordination of carbon and nitrogen metabolism and achieving synchronized growth of carbon and nitrogen assimilates.

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Note: Figure translations are in progress. See original paper for figures.