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# Effects of $NO_3^-/NH_4^+$ ratios on growth, enzyme activity and nitrogen assimilation-related gene expression in *Toona sinensis* seedlings

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## Abstract

Nitrogen is an essential nutrient for plants. Different nitrate ($NO_3^-$)/ammonium ($NH_4^+$) ratios have different effects on plant growth. However, the underlying mechanism in *Toona sinensis* remains unclear. Thus, we determined the effects of five different $NO_3^-/NH_4^+$ ratios (16/0, 12/4, 8/8, 4/12, and 0/16, denoted T1, T2, T3, T4, and T5, respectively) in nutrient media on *T. sinensis* seedling growth. When the nitrogen source was $NH_4^+$ alone (T5) or $NO_3^-$ alone (T1), the soluble protein content in the leaves was the lowest. Additionally, the activities of key nitrogen assimilation-related enzymes, such as nitrate reductase (NR), glutamate synthase (GOGAT), and glutamine synthetase (GS), were altered by the $NO_3^-/NH_4^+$ ratio. Principal component analysis (PCA) revealed that the T2 treatment was optimal for *T. sinensis* seedling growth. The $NO_3^-/NH_4^+$ ratio regulates nitrogen assimilation at the transcription level, as under high $NO_3^-$ conditions, the expressions of *NR*, *GS*, and *NADH-GOGAT* were high, and nitrate transporter (NRT) family members *NRT1*, *NRT1.1*, and *NRT1.7* played leading roles in nitrogen transport. However, under low $NO_3^-$ conditions, the level of *NRT2.7* increased to ensure nutrient absorption. Our results provide a theoretical basis for understanding how different $NO_3^-/NH_4^+$ ratios affect *T. sinensis* growth.

*Keywords*: ammonium, ammonium transporter, Chinese toon, nitrate, nitrate transporter, nitrogen assimilation-related enzymes.

## Introduction

Nitrogen is typically applied as a chemical fertilizer to support food production worldwide (Oldroyd and Leyser, 2020). There are various forms of nitrogen in soil, including organic soluble protein and amino acids, inorganic nitrate-nitrogen, ammonia-nitrogen, and urea. Plants absorb and utilize inorganic nitrogen from the soil, which exists in two main types: nitrate ($NO_3^-$) and ammonium ($NH_4^+$). The ability of plants to absorb $NO_3^-$ or $NH_4^+$ depends on their traits and the soil status. Under anaerobic conditions, nitrification is inhibited and denitrification occurs easily (Lin et al., 2009). Therefore, it is difficult to utilize $NO_3^-$-nitrogen under anaerobic conditions. In most aerobic soils, the major form of nitrogen is $NO_3^-$, whereas $NH_4^+$ is dominant in acidic and/or anaerobic soils (Maathuis, 2009;

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*Abbreviations*: AMT - ammonium transporter; CLC - chloride channel; Fd - ferredoxin; GOGAT - glutamate synthase; GS - glutamine synthetase; HAT - high affinity transport; LAT - low affinity transport; NADH - nicotinamide adenine dinucleotide; NiR - nitrite reductase; NLP - nodule inception (NIN)-like protein; NPF - nitrate transporter 1(NRT1)/peptide transporter (PTR) family; NR - nitrate reductase; NRT - nitrate transporter; PCA - principal component analysis; PTR - peptide transporter; RT-qPCR - reverse transcription real-time PCR; SLAC/SLAH - slowly activating anion channel.

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Zhu et al., 2011). Generally, plants adapted to acidic soil tend to take up $NH_4^+$, whereas plants adapted to alkaline soils and more aerobic conditions preferentially take up $NO_3^-$ (Maathuis, 2009). The preference for $NO_3^-$ and $NH_4^+$ varies among different plant species (Cruz et al., 2011; Hachiya and Sakakibara, 2017). The concentration of $NH_4^+$ in anaerobic paddy field soils used for rice cultivation is much higher than that of $NO_3^-$ (Pratiwi et al., 2016; Gao et al., 2019). Therefore, rice is generally considered an $NH_4^+$-tolerant crop. Studies have uncovered the mechanism of $NH_4^+$ tolerance in rice. A combined transcriptome and metabolomics analysis revealed that $NH_4^+$ tolerance in rice is related to rhizosphere pH, and that rice absorbs $NO_3^-$ and releases $OH^-$, which alkalizes the rhizosphere and induces the accumulation of large amounts of insoluble iron on the root surface, resulting in iron deficiency and the inhibition of rice growth (Chen et al., 2018). $NO_3^-$ inhibits the expression of $NH_4^+$ transporter (AMT) 1 through the $NO_3^-$ transporter (NRT) 1.1B - nodule inception (NIN)-like protein 3 (NLP3) signal transduction pathway, reduces the absorption rate of $NH_4^+$, and promotes the glutamine synthetase (GS)/glutamate synthase (GOGAT) cycle to increase the rate of $NH_4^+$ assimilation, thereby reducing the accumulation of $NH_4^+$ and enhancing the tolerance of rice to $NH_4^+$ (Yan et al., 2023). However, an increasing number of studies have shown that a combination of $NO_3^-$ and $NH_4^+$ is more beneficial for plant growth than a single nitrogen source (Tabatabaei et al., 2008; Hu et al., 2015; Zhu et al., 2018). A 5/1 $NH_4^+$/$NO_3^-$ ratio promotes the growth and improves the quality of blueberries (Zhang et al., 2021). Additionally, the content of various nutrients was significantly greater in pepper fruits treated with an $NH_4^+$/$NO_3^-$ ratio of 25/75 than in the group treated with a 0/100 ratio (Zhang et al., 2019). Even in rice plants, which preferentially absorb $NH_4^+$, the application of a small amount of $NO_3^-$ can improve nitrogen utilization efficiency and increase yield (Guo et al., 2007). Therefore, the combination of $NO_3^-$ and $NH_4^+$ and the $NO_3^-$/$NH_4^+$ ratio strongly affect plant growth.

$NO_3^-$ is absorbed by plant roots through active transport; a small portion is stored in the roots while most is transported to the shoots (Xuan et al., 2017). First, $NO_3^-$ is reduced and converted into nitrite ($NO_2^-$) by NR in the cytosol; then, $NO_2^-$ is reduced and converted into $NH_4^+$ by nitrite reductase (NiR) in the plastids or chloroplasts; and finally, $NH_4^+$ can be assimilated (Wang et al., 2018). The GS/GOGAT cycle is the main pathway of $NH_4^+$ assimilation in plants and includes the GS and GOGAT, which are key enzymes in the catalytic generation of $NH_4^+$. GS binds $NH_4^+$ to one molecule of glutamate to synthesize glutamine. GOGAT then catalyzes the reaction of glutamine and 2-oxyglutarate to form glutamate (Miflin and Habash 2002; Masclaux-Daubresse et al., 2010; Foyer et al., 2011). The glutamate and glutamine formed by $NH_4^+$ assimilation can be rapidly used to synthesize other compounds, such as amides, amino acids, urea compounds, amines, and peptides, which can be further incorporated into proteins, nucleic acids, and other macromolecular organic compounds.

Plants have evolved two types of GOGATs: ferredoxin (Fd)-GOGAT, which functions mainly in the leaves, and nicotinamide adenine dinucleotide (NADH)-GOGAT, which functions mainly in the roots and many other nonphotosynthetic tissues (Vanoni et al., 2005; Masclaux-Daubresse et al., 2010). Similar to GOGAT, cytosolic GS and chloroplastic GS are two forms of GS in different plant tissues (Bernard and Habash, 2009).

NRTs and AMTs are located in plant cells, and are protein carriers that specifically transport $NO_3^-$ and $NH_4^+$, respectively (Nacry et al., 2013). NRT protein families include the NRT1/peptide transporter (PTR) family (NPF), NRT2 family, chloride channel (CLC) family, and slowly activating anion channel (SLAC/SLAH) family (Krapp et al., 2014). Plants have two pathways for transporting $NO_3^-$ that is absorbed from the soil by the roots: a low-affinity transport (LAT) system and a high-affinity transport (HAT) system. Members of the NRT1 family, except NRT1.1, are generally considered to belong to the LAT system, whereas NRT1.1 is considered a dual affinity $NO_3^-$ transporter (Xing et al., 2023). The HAT system relies mainly on NRT2 (Williams and Miller, 2001). Moreover, AMTs can be divided into two classes: AMT1, which has been relatively extensively studied, and AMT2 (Zhu et al., 2021). Both NRTs and AMTs have distinct spatial distributions. *AtNRT1.1*, an extensively studied gene in *Arabidopsis*, that is predominantly localized in the root epidermis, and *AtNRT1.2* exhibits a certain expression pattern only in the root epidermis (Huang et al., 1999). *AtAMT1.1* and *AtAMT1.2* are also expressed mainly in *Arabidopsis* roots (Yuan et al., 2007).

*Toona sinensis*, also known as the Chinese toon, is a perennial deciduous tree of the *Meliaceae* family that is planted mostly in mountainous forests. Chinese toon buds are a widely popular vegetable because of their unique flavor. The Chinese toon requires large amounts of nutrients during growth, among which nitrogen plays crucial roles in its response to external environmental changes and development (Liu et al., 2022). In our previous study, appropriate nitrogen levels promoted the growth of Chinese toon (Zhao et al., 2024), but the effects of different nitrogen ratios on certain biological processes were unclear.

In this study, the effects of five different $NO_3^-$/$NH_4^+$ ratios on growth-related biological and physiological processes in the Chinese toon, such as seedling morphology, biomass, and activities of key enzymes and expression of key genes related to nitrogen assimilation, were investigated to reveal the physiological mechanism by which different $NO_3^-$/$NH_4^+$ ratios regulate Chinese toon growth. This study provides a theoretical reference for improving nitrogen fertilizer application and increasing nitrogen utilization efficiency during *T. sinensis* cultivation.

## Materials and methods

**Plant material and treatments:** After germination, *Toona sinensis* Heiyouchun plants with a pair of cotyledons were selected and transplanted into pots containing nonnutritive soil. The nutrient solution composition was calculated according to the Hoagland nutrient solution, except for the nitrogen supply, which differed. The experiment was conducted in pots, and potassium nitrate ($KNO_3$) and ammonium sulfate [$(NH_4)_2SO_4$] were used as the $NO_3^-$ and $NH_4^+$ sources, respectively. Three total nitrogen concentrations were applied (0.32, 0.16, and 0.08 mM), and at each concentration, five $NO_3^-/NH_4^+$ ratios were used: T1 ($NO_3^-/NH_4^+=16/0$), T2 ($NO_3^-/NH_4^+=12/4$), T3 ($NO_3^-/NH_4^+=8/8$), T4 ($NO_3^-/NH_4^+=4/12$), and T5 ($NO_3^-/NH_4^+=0/16$) (Table 1). Each $NO_3^-/NH_4^+$ ratio treatment included three biological replicates, and each biological replicate consisted of five plants. To evaluate seedling morphology and measure seedling biomass, experiments were conducted under three total nitrogen concentrations (0.32, 0.16, and 0.08 mM) and five $NO_3^-/NH_4^+$ ratios (T1, T2, T3, T4, and T5). To measure chlorophyll accumulation, enzyme activities, and gene expression, experiments were conducted with 0.16 mM total nitrogen at five $NO_3^-/NH_4^+$ ratios (T1, T2, T3, T4, and T5). Ninety-day-old seedlings were collected, flash frozen in liquid nitrogen and then stored at -80°C.

**Biomass:** In accordance with the methods of Wang et al. (2022), biomass was determined in this study by measuring the dry mass. The roots, stems, and leaves were harvested and washed with deionized water, and the surface moisture was removed with filter paper. For dry mass measurements, the samples were placed in an oven and dried at 80°C for 48 h, until the sample weight was stable.

**Chlorophyll content:** The chlorophyll *a*, chlorophyll *b*, and total chlorophyll content was measured according to the methods of Witham et al. (1971). For these assessments, the leaves from five plants were cut and mixed to constitute a biological replicate. A total of 0.1 g of each leaf sample was extracted with 80% acetone using a mortar and pestle. The chlorophyll content was expressed as mg per g (mg $g^{-1}$) of fresh mass (FM). All the chlorophyll content measurements included three biological replicates.

**Soluble protein and amino acids:** The total soluble protein content was quantified by the Bradford (1976) method. For each biological replicate, a total of 1.0 g of tissue was homogenized with 10 mL of cold 0.1 M sodium phosphate buffer containing 2% (m/v) polyvinylpolypyrrolidone (PVPP) in a cold bath and centrifuged at 16 000 × *g* for 30 min at 4°C. The supernatant was collected and used for the soluble protein assessment. The free amino acid content was determined using the ninhydrin chromogenic method, as described by Praxedes et al. (2006). Briefly, for each biological replicate, a total of 1.0 g of tissue was homogenized with 5 mL of 80% (v/v) aqueous ethanol. The sample was shaken and incubated at 80°C for 20 min and then centrifuged at 16 000 × *g* for 10 min; the pellets were extracted two more times with aqueous ethanol. All the free amino acid measurements included three biological replicates, and with each biological replicate consisting of five plants.

**Enzyme activities:** A total of 0.1 g of leaf or root sample was used to determine the activity of each enzyme. The activities of NR (EC 1.7.99.4), GOGAT (EC 1.4.7.1), and GS (EC 6.3.1.2) were determined according to the instructions of the corresponding kit (BC0080, BC0070 and BC0915, respectively; *Solarbio*, Beijing, China). Briefly, the samples were weighed and the corresponding kit solutions were added to extract the target enzymes, after which the samples were homogenized and centrifuged. The supernatant of each sample was collected for testing. All the enzyme activity measurements included three biological replicates, with each biological replicate consisting of five plants.

**Reverse transcription real-time PCR (RT-qPCR) analysis:** Total RNA was extracted from 100 mg of root or leaf sample using an *RNAprep Pure Plant Plus* kit (*Tiangen*, Beijing, China). Single cDNA was synthesized with *FastKing gDNA Dispelling RT SuperMix* (*Tiangen*) following the manufacturer's protocols. RT-qPCR analysis was performed with a *Bio-Rad CFX96* instrument (Hercules, USA) using *Super Real PreMix Plus* (*SYBR Green*) (*Tiangen*). The expressions of key genes involved in nitrogen assimilation, *NR*, *GS*, *Fd-GOGAT*, *NADH-GOGAT*, and those encoding NRT family members (*NRT1*, *NRT1.1*, *NRT1.7*, and *NRT2.7*) and AMT family members (*AMT1.1* and *AMT1.2*), were analyzed. These genes were selected on the basis of our transcriptome data. Detailed information about these genes and the primer sequences used are included in Table 1 Suppl. The housekeeping gene *Actin* was used as the reference for calculating the relative gene expressions according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) and are presented as the means ± standard errors (M ± SE) obtained from three biological replicates.

Table 1. Total nitrogen contents and $NO_3^-/NH_4^+$ ratios.

| Total nitrogen [mM] | $NO_3^-/NH_4^+$ | T1<br>16/0 | T2<br>12/4 | T3<br>8/8 | T4<br>4/12 | T5<br>0/16 |
|---|---|---|---|---|---|---|
| 0.32 | $KNO_3$ | 0.32 | 0.24 | 0.16 | 0.02 | 0 |
| | $(NH_4)_2SO_4$ | 0 | 0.02 | 0.08 | 0.16 | 0.16 |
| 0.16 | $KNO_3$ | 0.16 | 0.12 | 0.08 | 0.02 | 0 |
| | $(NH_4)_2SO_4$ | 0 | 0.02 | 0.04 | 0.06 | 0.08 |
| 0.08 | $KNO_3$ | 0.08 | 0.06 | 0.04 | 0.02 | 0 |
| | $(NH_4)_2SO_4$ | 0 | 0.01 | 0.02 | 0.03 | 0.04 |

**Statistical analysis:** Statistical analyses were performed using *SPSS 19.0* software, and the means were examined by analysis of variance (*ANOVA*) using Duncan's test at a 95% confidence threshold ($P < 0.05$). A total of six (roots) or seven (leaves) variables, including biomass, soluble protein content, amino acid content, NR activity, GOGAT activity, GS activity, and total chlorophyll content (leaves only) were used for principal component analysis (PCA). *Origin 2021* software was used for PCA and to create the bar charts.

## Results

As the total nitrogen content varied (Fig. 1), so did the growth status of the Chinese toon seedlings (Fig. 1). Additionally, at the same total nitrogen content the growth parameters of the Chinese toon seedlings treated with different $NO_3^-/NH_4^+$ ratios (T1, T2, T3, T4, and T5) also differed (Fig. 1). Fig. 1 shows that when $NO_3^-$ or $NH_4^+$ was the only nitrogen source, the Chinese toon exhibited relatively rapid growth. As shown in Table 2 Suppl., at the same $NO_3^-/NH_4^+$ ratio, the biomass of root, stem, and leaf biomass was the greatest at a total nitrogen content of 0.16 mM, followed by 0.08 and 0.32 mM total nitrogen. These results indicate that the growth of Chinese toon seedlings is affected not only by the total nitrogen content but also by the $NO_3^-/NH_4^+$ ratio. As a total nitrogen concentration of 0.16 mM was more suitable for Chinese toon seedling growth, this concentration was selected to study the effects of the $NO_3^-/NH_4^+$ ratio.

As shown in Fig. 2, the lowest chlorophyll *a* and total chlorophyll contents were observed in the T3 ($NO_3^-/NH_4^+$=8/8) treatment, but no obvious difference in chlorophyll *b* was detected among the treatments. These findings indicate that equal contents of $NO_3^-$ and $NH_4^+$ are not conducive to chlorophyll accumulation but applying different amounts of $NO_3^-$ and $NH_4^+$.

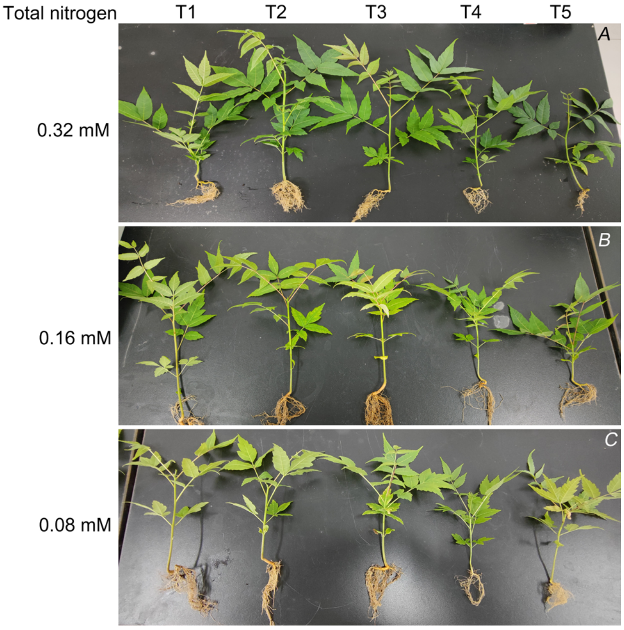

Fig. 1. Phenotypes of Chinese toon seedlings grown with 0.32 mM (*A*), 0.16 mM (*B*), and 0.08 mM (*C*) total nitrogen with different ratios of $NO_3^-/NH_4^+$ [16/0 (T1), 12/4 (T2), 8/8 (T3), 4/12 (T4), and 0/16 (T5)].

The total soluble protein content in Chinese toon plants varied along with the $NO_3^-/NH_4^+$ ratio. In the roots, the soluble protein content decreased gradually with decreasing concentrations of $NO_3^-$, and more soluble protein accumulated in the roots when only $NO_3^-$ was present (T1, $NO_3^-/NH_4^+$=16/0) or when the $NO_3^-/NH_4^+$ ratio was 12/4 (Fig. 3*A*). The soluble protein content in the leaves increased with increasing $NH_4^+$ proportion, and the highest soluble protein content was detected in the T2 ($NO_3^-/NH_4^+$=12/4) treatment group (Fig. 3*A*).

The content of free amino acids increased with decreasing $NO_3^-$ proportion in the roots, and the highest content was detected in the T2 ($NO_3^-/NH_4^+$=12/4) treatment group (Fig. 3*B*). This result suggests that under T2 ($NO_3^-/NH_4^+$=12/4), the roots have a strong ability to assimilate nitrogen into amino acids for further protein synthesis. The free amino acid content in the leaves was greatest when $NH_4^+$ was the only nitrogen source (T5, $NO_3^-/NH_4^+$=0/16).

$NO_3^-$ absorbed by seedlings can be reduced to $NO_2^-$ by NR, which catalyzes the rate-limiting step in nitrogen assimilation. The activity of NR in the roots of Chinese toon plants differed at various $NO_3^-/NH_4^+$ ratios, and the highest NR activity was observed in the T4 ($NO_3^-/NH_4^+$=4/12) treatment group. The T2 ($NO_3^-/NH_4^+$= 2/4) treatment group presented the highest NR enzyme activity in the leaves, with was 2 - 3 times greater than those in the other treatment groups (Fig. 4*A*).

GOGAT and GS are involved in regulating ammonia assimilation in plants. The GOGAT activity in the roots was greatest in the T4 ($NO_3^-/NH_4^+$=4/12) treatment group, whereas the GOGAT activity in the leaves first increased but then decreased with increasing $NH_4^+$ proportion, peaking in the T3 ($NO_3^-/NH_4^+$=8/8) treatment group (Fig. 4*B*).

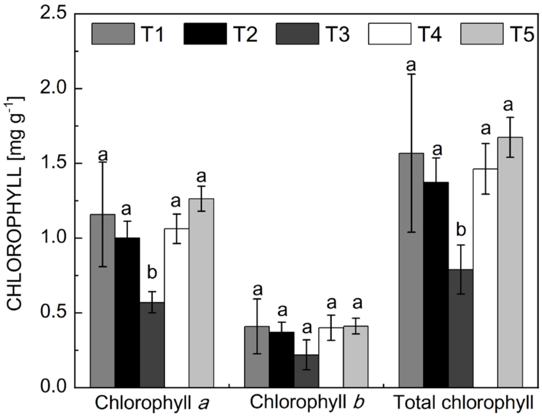

Fig. 2. Effects of different $NO_3^-/NH_4^+$ ratios [16/0 (T1), 12/4 (T2), 8/8 (T3), 4/12 (T4), and 0/16 (T5)] on chlorophyll content. Means ± SEs of three biological replicates. *Different letters* indicate significant differences according to Duncan's test, $P < 0.05$.

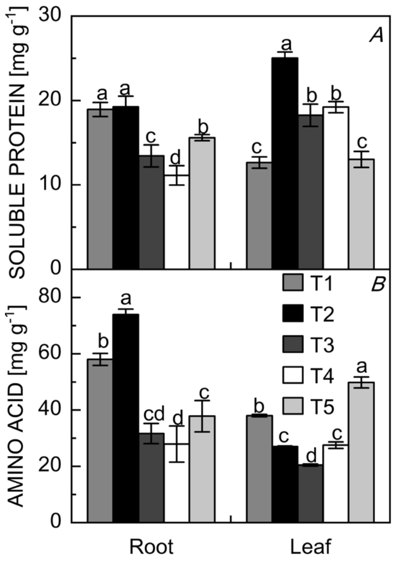

Fig. 3. Effects of different ratios of $NO_3^-/NH_4^+$ [16/0 (T1), 12/4 (T2), 8/8 (T3), 4/12 (T4), and 0/16 (T5)] on the total soluble protein content (*A*) and amino acid content (*B*). Means ± SEs of three biological replicates. *Different letters* indicate significant differences according to Duncan's test, $P < 0.05$.

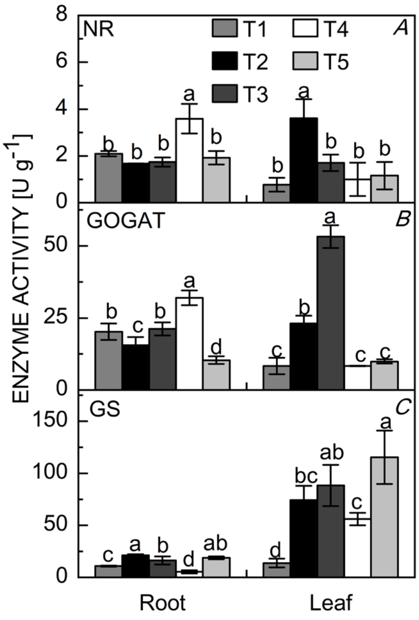

Fig. 4. Effects of different ratios of $NO_3^-/NH_4^+$ [16/0 (T1), 12/4 (T2), 8/8 (T3), 4/12 (T4), and 0/16 (T5)] on the enzyme activities of NR (*A*), GOGAT (*B*), and GS (*C*). Means ± SEs of three biological replicates. *Different letters* indicate significant differences according to Duncan's test, $P < 0.05$.

The activity of GS in the roots was weaker than that in the leaves (Fig. 4*C*). The T5 ($NO_3^-/NH_4^+$=0/16) treatment group presented the highest GS enzyme activity in the leaves, and the GS activity in the leaves of *T. sinensis* seedlings was greater in the T2 and T3 groups than in the T4 group (Fig. 4*C*).

Two principal components (PCs) were extracted from a total of six (roots) or seven (leaves) variables, including biomass, soluble protein content, amino acid content, NR activity, GOGAT activity, GS activity, and total chlorophyll content (leaves only) *via* PCA. The first two PCs captured 87.1% and 73.4% of the total observed variance in the roots and leaves, respectively (Fig. 5). In the roots, biomass, soluble protein content, and amino acid content contributed significantly to PC1 (Fig. 5*A*). In the leaves, biomass, soluble protein content, amino acid content, and NR activity contributed significantly to PC1 (Fig. 5*B*). For the both roots and the leaves, T2 treatment gave larger values of PC1, indicating that this treatment had the greatest positive effect on the growth of Chinese toon seedlings.

The effect of the $NO_3^-/NH_4^+$ ratio on Chinese toon seedlings was further studied at the gene expression level. In both the roots and leaves, the highest expressions of NR and GS were detected in the T1 ($NO_3^-/NH_4^+$=16/0) treatment group (Fig. 6*A,B*), indicating that $NO_3^-$ can induce the expression of both *NR* and *GS*. *NADH-GOGAT* plays a major role in roots, and its expression gradually decreased with increasing $NH_4^+$ concentration (Fig. 6*C*). *Fd-GOGAT* functions mainly in leaves, and its expression was greatest in the T4 ($NO_3^-/NH_4^+$=4/12) treatment group and lowest in the T3 ($NO_3^-/NH_4^+$=8/8) treatment group (Fig. 6*C*).

The expressions of several *NRT*s and *AMT*s, including *NRT1*, *NRT1.1*, *NRT1.7*, *NRT2.7*, *AMT1.1*, and *AMT1.2* were also analyzed (Fig. 7). In roots, the expressions of *NRT1*, *NRT1.1*, and *NRT1.7* were the highest in the T1 ($NO_3^-/NH_4^+$=16/0) treatment group (Fig. 7*A-C*). In contrast, the expression of *NRT2.7* showed the opposite trend, as its expression was the lowest in the T1 ($NO_3^-/NH_4^+$=16/0) treatment group and the highest level occurring in the T3 ($NO_3^-/NH_4^+$=8/8) treatment group (Fig. 7*D*). With increasing $NH_4^+$ concentration, the *AMT1.1* expression level first decreased but then increased (Fig. 7*E*). Furthermore, the expression level of *AMT1.2* was much greater in T1 ($NO_3^-/NH_4^+$=16/0) than in the other treatment groups (Fig. 7*F*).

In the leaves, the expression levels of *NRT1*, *NRT1.1*, and *NRT1.7* were the highest in the T3, T2, and T2 treatment groups, respectively (Fig. 7*G-I*). The lowest level of *NRT2.7* was detected in the T3 treatment group (Fig. 7*J*). *AMT1.1* and *AMT1.2* expression increased with increasing $NH_4^+$ concentration, peaking in the T3 and T4 treatment groups (Fig. 7*K,L*).

## Discussion

Biomass is the most intuitive index of plant growth. Nitrogen and the ratio of its different forms ($NO_3^-$ and $NH_4^+$) directly affect plant life activities and are ultimately reflected in the morphology and biomass of the plant (Sun et al., 2020; Wang et al., 2022). Mini Chinese cabbage seedlings fertilized with $NO_3^-$ nitrogen alone presented longer primary roots, but a lower shoot and root biomass than did plants fertilized with both $NH_4^+$ and $NO_3^-$ (Hu et al., 2015). The photosynthetic pigment content, net photosynthetic rate, and stomatal conductance

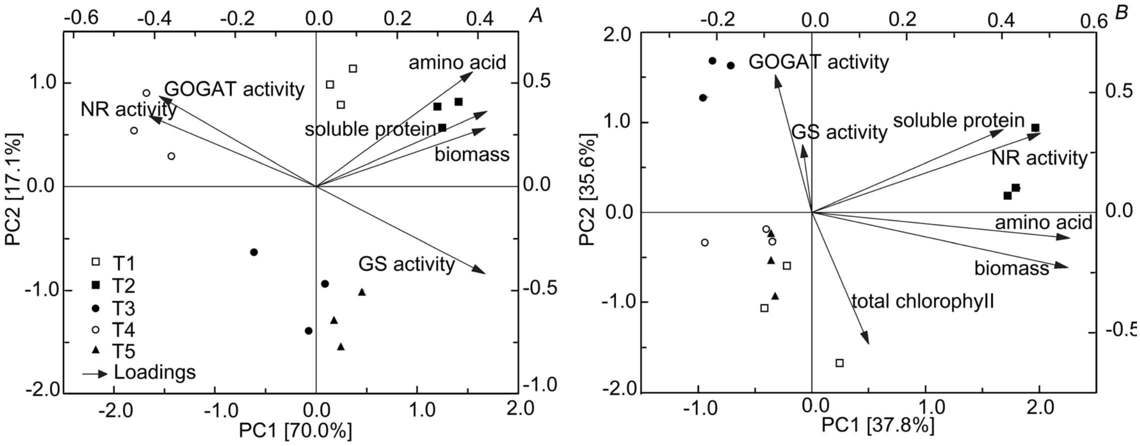

Fig. 5. Principal component analysis (PCA) of the total biomass, soluble protein content, amino acid content, NR activity, GOGAT activity, and GS activity in roots (*A*) and total biomass, soluble protein content, amino acid content, NR activity, GOGAT activity, GS activity, and total chlorophyll content in leaves (*B*).

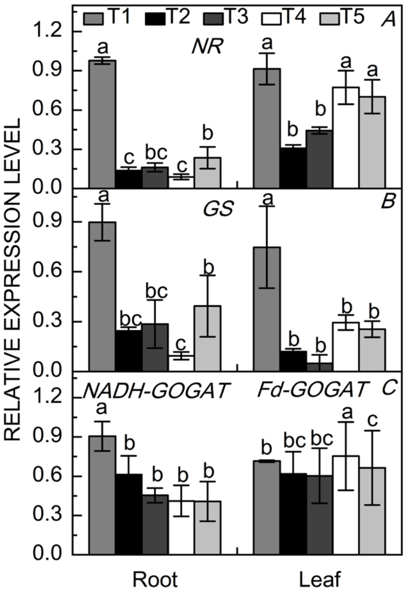

Fig. 6. Effects of different ratios of $NO_3^-/NH_4^+$ [16/0 (T1), 12/4 (T2), 8/8 (T3), 4/12 (T4), and 0/16 (T5)] on the expressions of *NR* (*A*), *GS* (*B*), and *GOGAT* (*C*). *NADH-GOGAT* and *Fd-GOGAT* are different forms of *GOGAT* found in the roots and leaves, respectively. Means ± SEs of three biological replicates. *Different letters* indicate significant differences according to Duncan's test, $P < 0.05$.

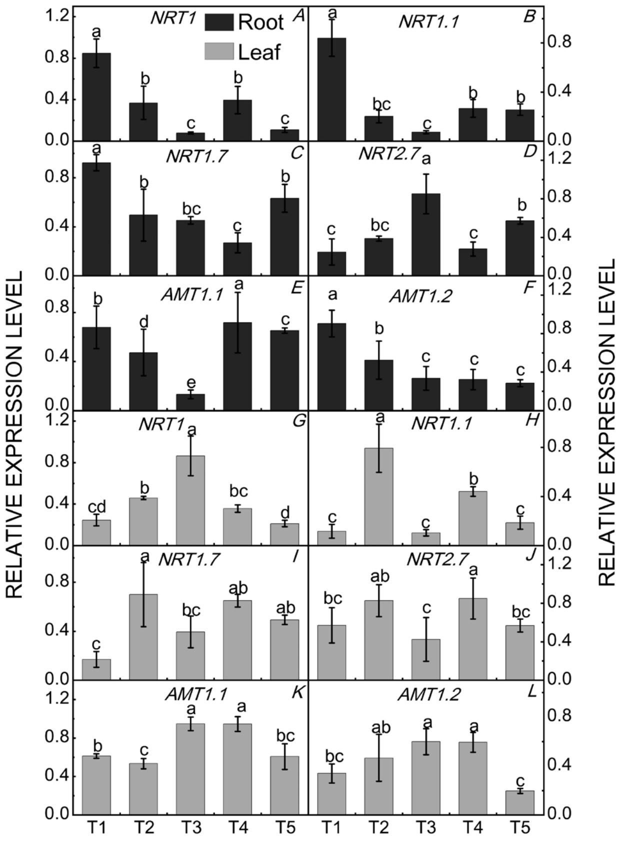

Fig. 7. Effects of different ratios of $NO_3^-/NH_4^+$ [16/0 (T1), 12/4 (T2), 8/8 (T3), 4/12 (T4), and 0/16 (T5)] on the expressions of key genes related to nitrogen transport in roots (*A-F*) and leaves (*G-L*). Means ± SEs of three biological replicates. *Different letters* indicate significant differences according to Duncan's test, $P < 0.05$.

also decrease after fertilization with $NO_3^-$ alone (Hu et al., 2015). Furthermore, the application of $NO_3^-$ only to Chinese cabbage resulted in the lowest soluble protein and flavonoid content but the highest nitrate content (Zhu et al., 2018). These studies indicated that the combined application of $NH_4^+$ and $NO_3^-$ was more beneficial to plant growth than the addition of $NH_4^+$ or $NO_3^-$ alone.

In this study, when the total nitrogen application was constant, the $NO_3^-/NH_4^+$ ratio had different effects on the roots, stems and leaves of Chinese toon seedlings (Table 2 Suppl.). With increasing $NH_4^+$ content in the nutrient mixture, the biomass of the Chinese toon seedlings tended firstly to increase but then to decrease. In general, the growth phenotype and biomass were not optimal upon fertilization with $NH_4^+$ alone (T5) or $NO_3^-$ alone (T1).

Consistent with previous work, our research has provided further evidence that an appropriate $NO_3^-/NH_4^+$ ratio can improve plant growth (Zhu et al., 2018, 2021).

PCA was used to visualize how the $NO_3^-/NH_4^+$ ratio affected the Chinese toon seedlings (Fig. 5). The samples from the different treatment groups clustered in different areas, indicating that Chinese toon seedling growth was affected by differences in the $NO_3^-/NH_4^+$ ratio. For the roots, the T3 samples were located mainly in the third quadrant, indicating that this treatment was not suitable for the growth of Chinese toon roots. For both the roots and leaves, biomass, soluble protein content, and amino acid content, contributed significantly to PC1 (Fig. 5). Moreover, the growth parameters (Fig. 1, Table 2 Suppl.) and indexes determined by a series of physiological measurements (Figs. 2, 3, 4) were better in the T2 group than other treatment group, and these results were supported by PCA, indicating that T2 was the optimal treatment for the growth of Chinese toon seedlings.

The main pathway of nitrogen assimilation in plants is generally believed to involve the formation of $NO_2^-$ from $NO_3^-$ through NR and the subsequent formation of $NH_4^+$ through NiR (Meyer and Stitt, 2001). $NH_4^+$ is incorporated into amino acids and then into various proteins, mainly through the GS/GOGAT circular reaction, in which GS is a key enzyme in $NH_4^+$ assimilation (Unno et al., 2006). In higher plants, NR is a nitrogen assimilation rate-limiting enzyme and a substrate-inducing enzyme exists in the cytoplasm of the roots and leaves of higher plants (Meyer and Stitt, 2001). NR activity is regulated and induced by $NO_3^-$ in cells, wherein $NO_3^-$ reduction occurs mainly in the cytoplasm (Wang et al., 2004; Meyer and Stitt, 2001; Masclaux-Daubresse et al., 2010). The trends in NR and GOGAT activity in the roots were consistent but opposite to the trends in GS activity (Fig. 4). On the basis of these results, we hypothesize that when the content of $NO_3^-$ is not lower than that of $NH_4^+$ (*i.e.*, T1, T2, and T3), NR can maintain the $NO_3^-$ reduction rate, and GOGAT can assimilate the $NH_4^+$ that is reduced by $NO_3^-$, and directly absorbed by the roots; moreover, the concentration of $NH_4^+$ is maintained within an appropriate range. When the proportion of $NH_4^+$ was high, the activities of NR and GOGAT increased, and the reduction in $NO_2^-$ and glutamate production were faster. However, the contents of soluble protein and free amino acids decreased, indicating that nitrogen assimilation efficiency was low at this time, which may be related to the low GS activity and the limitations of the GS/GOGAT cycle.

The activity of GS in the leaves, was greater than that in the roots (Fig. 4*C*), indicating that $NH_4^+$ assimilation was more active in Chinese toon leaves. When the $NO_3^-$ proportion was not greater than the $NH_4^+$ proportion (*i.e.*, T3, T4, and T5), GS activity was greater, which may be related to the increased NR activity that promoted $NO_3^-$ conversion and $NH_4^+$ formation. When $NH_4^+$ was the only nitrogen source (T5, $NO_3^-/NH_4^+$=0/16), the activity of GS peaked, indicating that GS converts $NH_4^+$ to glutamine to protect seedlings from the toxic effects of excessive $NH_4^+$. In addition, in the process of nitrogen assimilation, NR and GOGAT require the reducing power of NADH or Fd, whereas GS requires energy (Masclaux-Daubresse et al., 2010). At the expense of energy, plants must reduce nitrate before nitrogen can be used to synthesize amino acids (Andrews et al., 2013; Kuppe et al., 2024). As $NH_4^+$ is energetically cheaper than $NO_3^-$, the high activity of GS under T5 treatment ($NO_3^-/NH_4^+$=0/16) may be related to the fact that less energy is consumed by assimilating $NH_4^+$.

Flavin adenine dinucleotide, heme, and molybdenum cofactors are linked to the NR monomer when functional and complete, NR is a homodimer (Meyer and Stitt, 2001). Moreover, GS is not a protein encoded by a single gene and has a polymer structure (Unno et al., 2006). In plants, NADH-GOGAT and Fd-GOGAT are located in both nonphotosynthetic plastids and chloroplasts. Fd-GOGAT is encoded by *GLU1* and *GLU2* in *Arabidopsis*, and NADH-GOGAT is encoded by *GLT* (Suzuki and Knaff, 2005). Cytosolic GS and NADH-GOGAT may be involved in the primary assimilation of ammonia in roots (Tabuchi et al., 2007; Fortunato et al., 2023), but chloroplastic GS and Fd-GOGAT participate in ammonia assimilation in leaves, a step that is highly important under light conditions (Tabuchi et al., 2007; Ferreira et al., 2019; Fortunato et al., 2023). The changes in the expression of genes encoding NR, GS, and GOGAT were not completely consistent with the changes in enzyme activity, suggesting that nitrogen assimilation and utilization in *T. sinensis* are a complicated life processes rather than controlled by the expression of a single gene; furthermore, the mechanism of nitrogen assimilation and utilization at the protein level may be even more complex. Thus, the underlying mechanism needs to be further explored.

$NO_3^-$ has been suggested to act as a regulatory factor of gene expression to directly regulate plant nitrogen metabolism at the gene transcription level (Wang et al., 2004), which was confirmed in the present study. When only $NO_3^-$ was applied (T1), the expressions of *NR*, *GS*, *Fd-GOGAT*, and *NADH-GOGAT* were significantly greater than those in the other treatments (Fig. 6*C*). Furthermore, *NRT* and *AMT* expressions were also notably affected by the $NO_3^-/NH_4^+$ ratio. The difference in trends of the expression of *NRT1*s (*NRT1*, *NRT1.1*, and *NRT1.7*) and *NRT2.7* may be related to the two nitrogen transport systems. Under relatively high $NO_3^-$ conditions, the LAT system, which includes *NRT1*, *NRT1.1* (a dual-affinity $NO_3^-$ transporter), and *NRT1.7*, plays vital roles in the uptake of nitrogen and its transport from the roots to the shoots. The upregulation of *NRT2.7* with decreasing $NO_3^-$ concentration indicated that the HAT system functioned to ensure nutrient absorption and utilization. In terms of $NH_4^+$ transport, the expression of *AMT1.2* was inhibited by $NH_4^+$, while the expression of *AMT1.1* in the roots reached its highest level under T4 ($NO_3^-/NH_4^+$=4/12). The interaction between $NH_4^+$ and $NO_3^-$ and the control mechanism regulated by *AMT1.2* have been previously reported in *Brassica campestris* L. (Zhu et al., 2020). However, the functions of *AMT* vary in different plant species (Loqué and von Wirén 2004; Zhu et al., 2020). The expression patterns of *AMT1.1* and *AMT1.2* in response to different $NO_3^-/NH_4^+$ ratios may reflect the different nitrogen transport strategies in *T. sinensis* seedlings. The expression patterns of *AMT1.1* and *AMT1.2* in the leaves were not similar to those in roots; however, more data is needed to explain these mechanisms.

## Conclusions

In addition to the total nitrogen content, the $NO_3^-/NH_4^+$ ratio also affects the growth of Chinese toon seedlings. The soluble protein content, free amino acid content, and activities of key metabolic enzymes involved in nitrogen assimilation were affected by the $NO_3^-/NH_4^+$ ratio. A total nitrogen content of 0.16 mM with $NO_3^-/NH_4^+=12/4$ was optimal for *T. sinensis* seedling growth. $NO_3^-$ can directly regulate nitrogen assimilation and utilization in *T. sinensis* seedlings at the transcription level, and the expression levels of *NR*, *GS*, and *NADH-GOGAT* were greater in the T1 treatment group than in the other groups. Under high $NO_3^-$ application, the LAT system, which includes *NRT1*, *NRT1.1*, and *NRT1.7*, plays a leading role in nitrogen transport. As the concentration of $NO_3^-$ decreased, the expression of *NRT2.7* increased, and the HAT system functioned to ensure nutrient absorption and utilization.

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