Effects of Nitrogen Forms on Nitrogen Uptake, Distribution, and Lactone Component Accumulation in Andrographis paniculata (Postprint)

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Abstract

To clarify the utilization characteristics of different nitrogen forms in the medicinal plant *Andrographis paniculata* and their relationship with andrographolide component accumulation, this study employed 15N isotope tracing and physiological-biochemical analysis to investigate the effects of nitrogen forms on nitrogen absorption, distribution, and andrographolide component content in *A. paniculata* at different growth stages (rapid growth period, jointing stage, budding stage, and flowering stage), using nitrate nitrogen (NN), ammonium nitrogen (AN), amide nitrogen (UN), and amino acid nitrogen (GN) as single nitrogen sources. The results showed that: (1) Nitrogen content in leaves and roots gradually decreased with advancing growth stage, with NN treatment exhibiting lower nitrogen content. (2) The nitrogen absorption rate of *A. paniculata* was higher during the vegetative growth period and declined rapidly during the reproductive growth period, with higher absorption rates observed for AN, UN, and GN. (3) During the budding stage, the proportion of nitrogen allocated to leaves decreased while that to stems increased; compared with NN treatment, AN, UN, and GN treatments reduced leaf nitrogen allocation proportion while increasing stem and root nitrogen allocation proportions during this stage. (4) During the rapid growth period, NN treatment exhibited lower maximum carboxylation rate and maximum electron transport rate of photosynthesis, along with lower allocation proportions of leaf nitrogen in the carboxylation system and bioenergetic components; UN and AN treatments reduced leaf nitrogen allocation in the carboxylation system during the budding stage and flowering stage, respectively. (5) AN, UN, and GN increased the contents of andrographolide and dehydroandrographolide, decreased the content of 14-deoxyandrographolide during the budding and flowering stages, while different nitrogen forms had minimal effects on neoandrographolide. (6) The contents of andrographolide and neoandrographolide showed significant negative correlations with nitrogen content in leaves, stems, and roots, nitrogen

absorption rate, and nitrogen allocation proportions in leaves and roots, but significant positive correlation with nitrogen allocation proportion in stems, whereas 14-deoxyandrographolide showed the opposite pattern. In conclusion, the vegetative growth period represents the main stage for nitrogen absorption in *A. paniculata*; the plant can more effectively utilize ammonium nitrogen, amide nitrogen, and amino acid nitrogen, and promotes andrographolide component accumulation through optimized nitrogen allocation.

Full Text

Effects of Nitrogen Forms on Nitrogen Uptake, Allocation, and Andrographolide Component Accumulation in *Andrographis paniculata*

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Abstract: To elucidate the utilization characteristics of different nitrogen forms in the medicinal plant *Andrographis paniculata* and their relationship with andrographolide accumulation, we employed ^{15}N isotopic tracing combined with physiological and biochemical analyses to investigate the effects of nitrogen forms on nitrogen uptake, allocation, and andrographolide content across different growth stages (rapid growth, jointing, budding, and flowering). Nitrate nitrogen (NN), ammonium nitrogen (AN), amide nitrogen (UN), and amino acid nitrogen (GN) were supplied as sole nitrogen sources. The results showed: (1) Nitrogen content in leaves and roots decreased gradually with growth stage, with lower values under NN treatment. (2) Nitrogen uptake rate was high during the vegetative growth period but declined sharply during reproductive growth; *A. paniculata* exhibited higher uptake rates for AN, UN, and GN. (3) At the budding stage, the nitrogen allocation ratio decreased in leaves while increasing in stems. Compared with NN treatment, AN, UN, and GN treatments reduced leaf nitrogen allocation but enhanced stem and root allocation at this stage. (4) During the rapid growth stage, NN treatment showed lower maximum carboxylation rate and maximum electron transport rate of photosynthesis, along with reduced leaf nitrogen allocation to carboxylation and bioenergetic components; UN and AN treatments decreased leaf nitrogen allocation to the carboxylation system at budding and flowering stages, respectively. (5) AN, UN, and GN treatments increased andrographolide and dehydroandrographolide contents while decreasing 14-deoxyandrographolide content at budding and flowering stages, with minimal

effects on neoandrographolide. (6) Andrographolide and neoandrographolide contents were significantly negatively correlated with nitrogen content, uptake rate, and nitrogen allocation in leaves and roots, but significantly positively correlated with stem nitrogen allocation; the opposite pattern was observed for 14-deoxyandrographolide. In conclusion, the vegetative growth period represents the primary phase for nitrogen uptake in *A. paniculata*. The plant utilizes ammonium, amide, and amino acid nitrogen more efficiently, promoting andrographolide accumulation through optimized nitrogen allocation.

Keywords: nitrogen form, nitrogen uptake, nitrogen allocation, andrographolide, *Andrographis paniculata*

1.1 Experimental Materials and Treatments

Andrographis paniculata plants were used as experimental material. Original seeds were obtained from the seed bank of Guangxi Botanical Garden of Medicinal Plants and authenticated as *A. paniculata* (Burm. f.) Nees by Dr. Zhong Chu. A uniform and stable line was selected through screening and purification for subsequent experiments. Seeds were germinated in Petri dishes with moist filter paper under constant temperature (approximately 28 °C) in a growth chamber, maintaining complete filter paper saturation throughout. The photoperiod was 14 h light/10 h dark with photosynthetically active radiation (PAR) of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and relative humidity of 60%. After approximately 10 days, germinated seedlings were transplanted to seedling trays containing a mixture of vermiculite, perlite, and peat soil (volume ratio ~4:1:1) and grown with regular watering to prevent drought stress until reaching the 5-leaf-pair stage. Healthy, uniformly sized seedlings were then selected and transplanted into pots (one plant per pot) containing vermiculite and perlite (4:1) for nitrogen form treatments. Before transplanting, roots were gently shaken to remove adhering substrate. Each treatment comprised 48 pots (plants). Plants were maintained in a phytotron under the same light and temperature conditions described above.

Nitrogen sources were supplied as 2 $mmol \cdot L^{-1}$ KNO_3 and 2 $mmol \cdot L^{-1}$ $Ca(NO_3)_2$ (nitrate nitrogen, NN), 6 $mmol \cdot L^{-1}$ NH_4Cl (ammonium nitrogen, AN), 3 $mmol \cdot L^{-1}$ urea $[CO(NH_2)_2]$ (amide nitrogen, UN), and 6 $mmol \cdot L^{-1}$ glycine (amino acid nitrogen, GN). The nutrient solution contained additional mineral nutrients: 1 $mmol \cdot L^{-1}$ NaH_2PO_4, 2 $mmol \cdot L^{-1}$ KCl, 2 $mmol \cdot L^{-1}$ $CaCl_2$, 1 $mmol \cdot L^{-1}$ $MgSO_4$, 18 $\mu mol \cdot L^{-1}$ H_3BO_3, 0.15 $\mu mol \cdot L^{-1}$ $ZnSO_4$, 0.15 $\mu mol \cdot L^{-1}$ $CuSO_4$, 0.52 $\mu mol \cdot L^{-1}$ $(NH_4)_6Mo_7O_{24}$, 9.5 $\mu mol \cdot L^{-1}$ $MnSO_4$, and 36 $\mu mol \cdot L^{-1}$ Fe-EDTA. To maintain consistent K^+ and Ca^{2+} concentrations across treatments, KCl and $CaCl_2$ were omitted from the NN treatment, with K^+ and Ca^{2+} supplied by KNO_3 and $Ca(NO_3)_2$. Solution pH was adjusted to 5.8–6.0, and 10 $mg \cdot L^{-1}$ ampicillin was added to inhibit microbial growth. Nutrient solution (100 mL per pot) was applied twice weekly. Approximately 40 uniformly growing plants per treatment were available for sampling. Plants

were harvested for analysis after 30, 50, 70, and 85 days of treatment.

1.2.1 Photosynthetic CO_2 Response Curves

At 30 days (rapid growth stage), 50 days (jointing stage), 70 days (budding stage), and 85 days (flowering stage) after transplanting, three uniformly sized plants per treatment were selected. The CO_2 response characteristics of photosynthesis were measured on the 9th leaf from the main stem (leaf position varied with growth stage but was consistent across treatments within each stage) using a LI-6400XT portable photosynthesis system. Measurements employed the built-in LED light source with a red:blue ratio of 9:1, PAR of 1,500 $\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, leaf chamber temperature of 25 °C, relative humidity of 60–70%, and CO_2 concentration gradients of 50, 100, 150, 200, 300, 400, 600, 800, 1,000, 1,200, and 1,500 $\text{mol} \cdot \text{mol}^{-1}$.

Leaves were acclimated in the chamber at 1,500 $\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ PAR and 400 $\text{mol} \cdot \text{mol}^{-1}$ CO_2 for at least 20 minutes until parameters stabilized. Gas exchange parameters were then measured at each CO_2 concentration. The CO_2 response curves were fitted using the FvCB model (Farquhar et al., 1980) to calculate maximum carboxylation rate (Vcmax) and maximum electron transport rate (Jmax).

1.2.2 Chlorophyll Content, Specific Leaf Weight, and Specific Leaf Nitrogen

Following photosynthesis measurements, four leaf discs (6 mm diameter) were taken from both sides of the midrib in the middle portion of the leaf and placed in a 10 mL centrifuge tube with 5 mL of acetone:ethanol mixture (1:1, v/v). Samples were extracted in darkness at room temperature for 24 h until leaves became completely white. Absorbance of the extract was measured at 663 nm and 645 nm to calculate chlorophyll a, b, and total chlorophyll content (Zhang, 1986).

Additional leaf discs (five per side) were taken, oven-dried at 70 °C to constant weight, weighed, and then digested with H_2SO_4-H_2O_2 at 260 °C. Total nitrogen content was determined using the indophenol blue colorimetric method (Lü et al., 2004). Specific leaf weight (SLM) and specific leaf nitrogen (SLN) were calculated as leaf weight and nitrogen content per unit leaf area, respectively.

1.2.3 ^{15}N Isotopic Labeling and Determination

At each growth stage (rapid growth, jointing, budding, and flowering), four uniformly sized plants per treatment were selected. Six days before harvest, nitrogen sources in the nutrient solution were replaced with corresponding ^{15}N-labeled sources at 10% atom abundance. This treatment was applied once every three days. After treatment completion, intact root systems were removed, washed with tap water to remove adhering vermiculite and perlite, rinsed with 1 $\text{mmol} \cdot \text{L}^{-1}$ $CaCl_2$ for 1 minute, and then washed with deionized water. Plant

parts (roots, stems, leaves) were separated, placed in kraft paper bags, killed at 105 °C for 30 minutes, dried at 75 °C to constant weight, weighed, and ground into fine powder. ^{15}N atom abundance in each part was determined using a continuous-flow isotope ratio mass spectrometer coupled with a carbon-nitrogen elemental analyzer (ANCA-MS, PDZ-Europa), from which ^{15}N content and uptake rate were calculated.

1.2.4 Plant Biomass and Total Nitrogen Content

Biomass and total nitrogen content were determined using samples from section 1.2.3. Total nitrogen content was measured by the indophenol blue colorimetric method (Lü et al., 2004).

1.2.5 Andrographolide Component Content

Contents of andrographolide, dehydroandrographolide, 14-deoxyandrographolide, and neoandrographolide in dried leaf samples were determined using the method of Wang et al. (2022) on samples from section 1.2.3.

1.3 Data Processing and Statistical Analysis

Photosynthetic nitrogen allocation: The photosynthetic apparatus was divided into three components: carboxylation system (PNC), bioenergetics (PNB), and light-harvesting system (PNL) (Shi et al., 2015). The allocation proportion of nitrogen to each component was calculated using the following formulas:

c max 6.25 × cr × area 8.06 × mc × area B × area

Where: Vcmax is maximum carboxylation rate ($\mathrm{mol \cdot m^{-2} \cdot s^{-1}}$); Jmax is maximum electron transport rate ($\mathrm{mol \cdot m^{-2} \cdot s^{-1}}$), obtained by fitting CO_2 response curves using the FvCB model; CC is chlorophyll content ($\mathrm{mmol \cdot m^{-2}}$); Narea is nitrogen content per unit leaf area (SLN, $\mathrm{g \cdot m^{-2}}$); Vcr is Rubisco specific activity, valued at 20.78 $\mathrm{mol\ CO_2 \cdot g^{-1}}$ Rubisco $\cdot\, \mathrm{s^{-1}}$; Jmc is maximum electron transport rate per Cyt f, valued at 156 $\mathrm{mol\ e^- \cdot\ mol^{-1}}$ cyt f $\cdot\, \mathrm{s^{-1}}$; CB is the ratio of chlorophyll to nitrogen in the light-harvesting system, valued at 2.15 $\mathrm{mmol \cdot g^{-1}}$ N (Shi et al., 2015).

One-way ANOVA and LSD multiple comparisons were used to test for significant differences among treatments. Data are presented as means ± standard deviation (n = 3 or 4). Statistical analyses were performed using SPSS 19.0, and figures were prepared using Microsoft Excel 2023.

2.1 Effects of Different Nitrogen Sources on *A. paniculata* Growth

As shown in [Figure 1: see original paper], phenotypic differences among 5-leaf-pair *A. paniculata* plants treated with different nitrogen sources for 30 days (Fig. 1: A, S1), 50 days (Fig. 1: B, S2), 70 days (Fig. 1: C, S3), and 85

days (Fig. 1: D, S4) were minimal, though NN-treated plants entered the flowering stage slightly later. Plant height differences among treatments were minor during the first three stages, but at flowering, NN treatment resulted in significantly greater height than the other three treatments (Fig. 1: E). Plant biomass showed no significant differences among treatments at S1, but at S2, NN treatment was significantly higher than the other three treatments. At S3, AN and UN treatments showed lower biomass, while at S4, AN treatment had the lowest biomass, significantly lower than the other three treatments (Fig. 1: F). The proportion of leaf biomass to aboveground biomass decreased gradually with growth stage; it was lowest under UN treatment at S1, lowest under AN treatment at S2 and S3, and showed no significant differences among treatments at S4 (Fig. 1: G).

Scale bars represent 10 cm. Different letters on bars within the same stage indicate significant differences among treatments at $P < 0.05$. The same convention applies below.

2.2 Effects of Nitrogen Sources on Nitrogen Uptake, Accumulation, and Allocation in *A. paniculata*

Leaf nitrogen content decreased gradually with growth stage across all treatments. NN treatment consistently showed relatively low leaf nitrogen content, significantly lower than the other three treatments at S1 and S3 (Fig. 2 [Figure 2: see original paper]: A). Stem nitrogen content remained relatively stable throughout the growth period; at S1, NN and AN were relatively low, significantly different from UN; at S2, AN was lowest, significantly different from NN and GN; at S3, AN was lowest, significantly different from UN; and at S4, no significant differences were observed among treatments (Fig. 2: B). Root nitrogen content decreased with growth stage, with UN treatment showing the highest values at all stages and NN treatment the lowest at three stages (except S2). NN and AN treatments were significantly lower than UN and GN at S1, S2, and S4 (except AN at S4), while at S3, NN and GN were significantly lower than AN and UN (Fig. 2: C). Total plant nitrogen content decreased gradually with growth stage, with NN treatment generally showing lower values (Fig. 2: D).

Using ^{15}N stable isotope tracing, we measured nitrogen uptake rates under different nitrogen sources. As shown in [Figure 3: see original paper]A, nitrogen uptake rates were high during the first two growth stages, then declined sharply. Nitrate nitrogen uptake rate was consistently the lowest across all growth stages, significantly lower than AN at S1–S3, lower than UN at S3 and S4, and lower than GN at S1 and S4. These results demonstrate that *A. paniculata* can utilize ammonium, amide, and amino acid nitrogen more effectively.

Calculations of ^{15}N allocation proportions among organs revealed that during S1 and S2, absorbed ^{15}N was primarily distributed in leaves, while at S3 and S4, allocation to stems increased substantially, even exceeding that in leaves (Fig.

3: B). At S1, ^{15}N allocation patterns under AN and NN treatments were similar, as were those under UN and GN treatments. NN and AN treatments showed significantly higher ^{15}N allocation ratios in leaves but lower ratios in roots compared with UN and GN treatments. At S2, ^{15}N allocation proportions among organs under NN and AN treatments showed little change, whereas UN and GN treatments exhibited decreased ^{15}N allocation to roots and increased allocation to leaves and stems. At S3, AN, UN, and GN treatments showed significantly lower ^{15}N allocation ratios in leaves but higher ratios in roots compared with NN treatment. At S4, no significant differences in ^{15}N allocation among organs were observed across treatments.

2.3 Effects of Nitrogen Sources on Photosynthetic Nitrogen Allocation in *A. paniculata*

Different nitrogen sources significantly affected photosynthetic CO_2 response characteristics across growth stages. AN, UN, and GN treatments showed significantly higher Vcmax and Jmax than NN treatment at S1. At S2, AN treatment exhibited the highest Vcmax and Jmax, significantly greater than UN and NN treatments. At S3, AN treatment maintained significantly higher Vcmax and Jmax than the other three treatments, while no significant differences were observed among treatments at S4. Chlorophyll content was relatively low during early growth stages and higher in later stages, with no significant treatment differences at S1 and S4. At S2, GN treatment showed the highest chlorophyll content, significantly higher than NN and UN treatments. At S3, AN treatment had significantly higher chlorophyll content than other treatments. Specific leaf nitrogen (SLN) was relatively low at S1 and S2 but higher at S3 and S4. At S1, AN and UN treatments were significantly higher than GN treatment, while AN treatment was highest at S3 and S4.

[Table 1 about here]

Figure 4 [Figure 4: see original paper] illustrates nitrogen allocation within photosynthetic machinery. The proportion of leaf nitrogen allocated to photosynthetic apparatus ranged from 52.8% to 88.7%, showing an initial increase followed by a decrease with growth stage, peaking at S2—consistent with higher Vcmax and Jmax during this period. Among the three photosynthetic components, bioenergetics received the lowest nitrogen allocation (<10%). At S1 and S2, nitrogen allocation to light-harvesting and carboxylation systems was comparable among treatments, though NN treatment showed lower allocation to the carboxylation system at S1. At S3 and S4, leaf nitrogen was primarily allocated to the light-harvesting system, with minimal differences among treatments. The nitrogen allocation proportion to the carboxylation system was notably lower under UN treatment at S3 and AN treatment at S4 compared with other treatments.

2.4 Effects of Nitrogen Forms on Andrographolide Component Accumulation

As shown in [Figure 5: see original paper]A, andrographolide content was consistently higher under AN treatment across all stages, significantly exceeding the other three treatments at S1 and S2, where it was more than fourfold higher. At S3, andrographolide content increased rapidly under UN and GN treatments, with UN showing no significant difference from AN and GN significantly lower than both AN and UN but still significantly higher than NN. At this stage, AN and UN treatments produced approximately threefold higher andrographolide content than NN treatment. At S4, UN treatment maintained high andrographolide content, significantly higher than other treatments, while GN treatment content decreased to levels comparable with NN, both significantly lower than AN treatment.

Neoandrographolide content increased gradually with growth stage across all treatments. At S1, GN treatment showed the highest content, significantly higher than other treatments. At S2, UN treatment was lowest, significantly lower than NN treatment. No significant differences were observed among treatments at S3, while UN treatment was highest at S4, significantly higher than the other three treatments (Fig. 5: B). 14-Deoxyandrographolide content decreased gradually with growth stage, with NN treatment showing relatively high values throughout, significantly higher than other treatments at S3 and S4 (Fig. 5: C). Dehydroandrographolide content was consistently low under NN treatment, not detected at S1 and S3. AN treatment showed the highest content at all stages, significantly higher than other treatments. UN and GN treatments showed low dehydroandrographolide content during the first two stages but relatively higher levels in the latter two stages (Fig. 5: D). Total lactone content under AN treatment was significantly higher than other treatments at S1 and S2, with no significant differences among the other three treatments. At S3, AN, UN, and GN showed no significant differences among themselves but were all significantly higher than NN. At S4, UN treatment was highest, followed by AN, with NN and GN lowest (Fig. 5: E). Overall, ammonium, amide, and amino acid nitrogen enhanced andrographolide and total lactone contents.

2.5 Correlation Analysis Between Andrographolide Content and Nitrogen Uptake and Allocation

Correlation analysis results (Table 2) showed that andrographolide and neoandrographolide contents were negatively correlated with nitrogen content in roots, stems, and leaves, nitrogen uptake rate, and nitrogen allocation ratio in leaves and roots, but significantly positively correlated with nitrogen allocation ratio in stems. 14-Deoxyandrographolide content was extremely significantly negatively correlated with andrographolide and neoandrographolide contents, showing opposite correlations—significantly positively correlated with leaf and root nitrogen content and allocation and nitrogen uptake rate, but extremely signif-

icantly negatively correlated with stem nitrogen allocation ratio. Total lactone content showed patterns similar to andrographolide, being extremely significantly negatively correlated with nitrogen content in stems and roots and leaf nitrogen allocation ratio, but extremely significantly positively correlated with stem nitrogen allocation ratio. These results indicate that reducing plant nitrogen content while increasing nitrogen allocation to stems can effectively enhance andrographolide and total lactone contents.

Nitrogen contents among different organs were extremely significantly positively correlated, while stem nitrogen allocation ratio was extremely significantly negatively correlated with leaf and root nitrogen allocation ratios, particularly showing the strongest correlation with leaf nitrogen allocation ratio. Higher nitrogen content in leaves and roots increased their respective allocation ratios but decreased stem nitrogen allocation ratio.

[Table 2 about here]

3.1 Characteristics of *A. paniculata* Nitrogen Uptake from Different Sources

Plants can directly utilize both inorganic and organic nitrogen, though substantial differences exist in uptake and utilization efficiency among nitrogen forms. Most terrestrial plants primarily absorb nitrate nitrogen, with only a few such as rice, tea, and potato preferring ammonium nitrogen (Hao et al., 2020). Our ^{15}N tracing study revealed that *A. paniculata* exhibited significantly higher uptake rates for ammonium, amide, and amino acid nitrogen compared with nitrate nitrogen, with the highest rate observed for ammonium nitrogen, indicating a preference for this form. Additionally, plants treated with ammonium, amide, and amino acid nitrogen maintained relatively high leaf nitrogen content until the budding stage. These findings demonstrate that *A. paniculata* utilizes these three nitrogen forms more efficiently. Notably, although ammonium-treated plants had relatively smaller biomass, they did not exhibit typical ammonium toxicity symptoms such as leaf chlorosis, growth inhibition, or mortality (Jian et al., 2018; Hachiya et al., 2021). The mechanisms underlying ammonium-induced biomass reduction in *A. paniculata* require further investigation.

^{15}N isotopic tracing across growth stages revealed that the pre-jointing stage (S2) represents the primary period for nitrogen uptake in *A. paniculata*, with uptake rates declining substantially after entering the budding stage (S3). These results indicate that the vegetative growth period before jointing is a critical phase for nitrogen demand, which decreases sharply during reproductive growth. ^{15}N tracing also demonstrated that the budding stage is a key period for nitrogen reallocation at the whole-plant level. Due to reduced nitrogen uptake, leaf nitrogen allocation decreased, with nitrogen being translocated to stems and roots—particularly pronounced in plants receiving ammonium, amide, and amino acid nitrogen treatments. Furthermore, root nitrogen content under these three nitrogen sources was generally higher than under nitrate nitrogen, consistent with

^{15}N measurements. In summary, nitrogen fertilizer application for *A. paniculata* production should focus on the vegetative growth period, avoiding application during reproductive growth when nitrogen uptake declines and internal nitrogen redistribution predominates.

3.2 Effects of Nitrogen Sources on Andrographolide Components

This study demonstrates that nitrogen forms significantly influence andrographolide components in *A. paniculata*. While numerous reports have documented differential effects of nitrogen forms on active components in medicinal plants, the underlying mechanisms remain poorly understood. According to the carbon/nutrient balance hypothesis, higher nitrogen content typically reduces accumulation of carbon-rich secondary metabolites in medicinal plants (Luo & Fu, 2020). In our study, andrographolide, neoandrographolide, and total lactone contents were negatively correlated with nitrogen content and uptake rate in leaves, stems, and roots. However, ammonium, amide, and amino acid nitrogen treatments resulted in higher leaf nitrogen content and uptake rates compared with nitrate nitrogen treatment, yet also produced higher andrographolide content. The correlation analysis reflects relationships between nitrogen content changes across growth stages and andrographolide component variation, but differences in leaf nitrogen content and uptake rate among nitrogen form treatments were not the primary cause of andrographolide content differences.

Photosynthetic products serve as the initial material source for plant secondary metabolism, and maintaining high photosynthetic capacity can provide more assimilates for secondary metabolism. The higher photosynthetic capacity observed under ammonium, amide, and amino acid nitrogen treatments may contribute to elevated andrographolide content. Most leaf nitrogen exists in photosynthetic organs participating in photosynthesis (Makino et al., 2003). Therefore, leaf nitrogen content and its allocation among photosynthetic components significantly affect photosynthetic performance (Shi et al., 2015). The vegetative growth period is crucial for plant dry matter accumulation. In this study, *A. paniculata* plants under ammonium, amide, and amino acid nitrogen treatments maintained relatively high leaf nitrogen content throughout growth and exhibited higher photosynthetic capacity, Vcmax, and Jmax during vegetative stages (S1 and S2), indicating greater nitrogen investment in photosynthesis and reduced biochemical limitations. Analysis of nitrogen allocation within photosynthetic components revealed that at S1, nitrate nitrogen-treated plants allocated lower proportions of photosynthetic nitrogen to carboxylation and bioenergetic components than other treatments, likely contributing to their lower photosynthetic capacity.

The transition from vegetative to reproductive growth represents a critical period for nitrogen translocation and andrographolide component accumulation (Chen et al., 2014). In this study, andrographolide and neoandrographolide con-

tents began accumulating substantially at the budding stage (S3). During this stage, ammonium, amide, and amino acid nitrogen treatments showed significantly reduced leaf nitrogen allocation ratios compared with nitrate nitrogen treatment, with increased allocation to roots and stems. Correlation analysis also revealed that andrographolide and neoandrographolide contents were significantly negatively correlated with leaf nitrogen allocation but positively correlated with stem nitrogen allocation, suggesting that increased nitrogen translocation from leaves to stems at this stage promotes andrographolide accumulation. Leaves are the primary site for nitrogen assimilation, and excessive nitrogen accumulation during later growth stages increases carbohydrate consumption, which is unfavorable for secondary metabolite accumulation (Zhong et al., 2021). These findings partially support predictions of the carbon/nutrient balance hypothesis. Nitrogen allocation among photosynthetic components also affects nitrogen remobilization (Liu, 2018). Water-soluble proteins are more readily degraded and reused than other protein types. Nitrogen in the carboxylation system exists primarily as carboxylases, accounting for over half of water-soluble proteins (Carmo-Silva et al., 2015). Maintaining high nitrogen proportion in the carboxylation system not only reduces photosynthetic efficiency (Mu et al., 2016) but also decreases leaf nitrogen translocation and reuse. The reduced nitrogen proportion in the carboxylation system under ammonium and amide nitrogen treatments during later growth stages (S3 and S4) may facilitate nitrogen transfer from leaves.

In summary, ammonium, amide, and amino acid nitrogen affect plant and leaf nitrogen allocation in *A. paniculata*, enhance photosynthetic capacity during vegetative growth, and promote nitrogen translocation from leaves to stems during reproductive growth, providing the material basis for andrographolide synthesis and accumulation in leaves.

3.3 Potential Significance of This Study

Nitrogen forms influence active component accumulation in medicinal plants by regulating expression of enzyme genes in secondary metabolic pathways (Zhang et al., 2018). Andrographolide belongs to the labdane diterpenoid lactone family, and its biosynthetic pathway remains unelucidated. This study found that 14-deoxyandrographolide content was extremely significantly negatively correlated with andrographolide content, and that ammonium, amide, and amino acid nitrogen not only increased andrographolide content but also specifically enhanced dehydroandrographolide content, particularly under ammonium nitrogen. These results suggest that increased andrographolide content is closely associated with changes in 14-deoxyandrographolide and dehydroandrographolide contents. The findings support the recently proposed andrographolide biosynthetic pathway (Ren, 2023), in which andrographolide is formed from 14-deoxyandrographolide and dehydroandrographolide via monooxygenation reactions. Cytochrome P450 (CYP450) family enzymes catalyze monooxygenation reactions and play important roles in modifying

chemical components in medicinal plants (Xu et al., 2015). We hypothesize that ammonium, amide, and amino acid nitrogen may induce upregulation of certain CYP450 genes, promoting conversion of 14-deoxyandrographolide and dehydroandrographolide to andrographolide. Although numerous studies have investigated CYP450 genes in *A. paniculata* (Garg et al., 2015; Liang et al., 2020; Sun et al., 2022), key CYP450 genes catalyzing andrographolide synthesis have not been reported. This study provides a foundation for mining CYP450 genes involved in andrographolide biosynthesis.

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