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ARTICLE

Effects of Arbuscular Mycorrhizal Fungi on the Physiology and Saponin Synthesis of *Paris polyphylla* var. *yunnanensis* at Different Nitrogen Levels

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ABSTRACT

Arbuscular mycorrhizal fungi (AMF) are important members of the plant microbiome and affect the uptake and transfer of mineral elements by forming a symbiotic relationship with plant roots. Nitrogen (N), as an important mineral element, can directly affect plant growth and development at different N levels. It has been confirmed that inoculation with AMF can improve the efficiency of N utilization by plants. However, there are still fewer reports on the dynamic relationship between arbuscular mycorrhizal and plant secondary metabolites at different nitrogen levels. In this experiment, the physiological indexes and genes related to saponin synthesis were determined by applying different concentration gradients of nitrogen to the medicinal plant *P. polyphylla* var. *yunnanensis* infested by AMF as the test material. It was found that nitrogen addition increased the biomass, chlorophyll content, and nutrient content of above- and below-ground plant parts and increased the content of saponin content of *P. polyphylla* var. *yunnanensis* to some extent, but AMF inoculation increased the saponin content of *P. polyphylla* var. *yunnanensis* more significantly. AMF inoculation also promoted the expression of genes related to the saponin synthesis pathway, including 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMGS), squalene epoxidase 1 (SE1), and cycloartenol synthase (CAS), which is in according with the accumulation of saponin in plants. It also may increase the saponin content of AMF plants by altering the expression of P450s and UGTs related to saponin synthesis.

Keywords: Nitrogen; Arbuscular mycorrhizal fungi; Saponin; *P. polyphylla* var. *yunnanensis*

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1. Introduction

Nitrogen (N) fertilizer application has long been one of the main tools for agricultural yield improvement [1]. But along with the increased use of nitrogen levels comes an increasingly high economic and environmental cost. However, excessive N can cause dramatic changes in the plant growth environment and can lead to soil acidification and salinization, which in turn can lead to significant loss of soil carbonate [2]. Excessive N application can also reduce soil microbial activity, decreasing the bacterial community composition [3] and enhancing the spread of soil diseases [4]. In recent years, many studies have shown that soil symbiotic fungi with plants, especially arbuscular mycorrhizal fungi, play an important role in helping plants to absorb nitrogen and phosphorus nutrients [5,6]. How to apply nitrogen fertilizer to plants more scientifically, rationally and efficiently can more effectively reduce nitrogen deposition in natural ecosystems, enhance the synergistic effect of nutrient transport between plants and microorganisms, and achieve longer-term sustainable development, are important issues that continue to be explored.

Arbuscular mycorrhizal fungi (AMF) are widely symbiotic in terrestrial plants, and when AMF are symbiotic with plants, AMF can help plants to absorb more water and mineral elements through mycelial transport, which can account for the majority of the total nutrients absorbed by plants [7]. As an important nutrient required for plant growth, the main N in soil is ammonium nitrogen (NO^{3-}) and nitrate nitrogen (NH^{4+}). Many studies have found that AMF can take up ammonium and nitrate nitrogen from the soil, as well as other forms of organic nitrogen. N is taken up by AMF and enters the AMF mycelium and is assimilated into arginine (arg), which is transported through the mycelium to the periplasmic cavity (PAM), where it is hydrolyzed to form NH^{4+}, which enters the plant cell through the PAM and is then transported by cellular NH^{4+} transporters (AMTs) transport [8-11]. In addition, AMF inoculation can also promote plant growth and increase the secondary metabolite content of plants. For example, AMF inoculation significantly increased the yield as well as vitamin content of strawberry at low phosphorus levels [12]. The relationship between microorganisms and secondary metabolites is also complex; for example, certain microorganisms are capable of producing some active ingredients themselves, including secondary metabolites of plants [13]. In medicinal plants, secondary metabolites mainly help plants to resist ecological conditions. The common ones are triterpene saponins, steroid saponins, alkaloids, phenolics, etc. AMF plants are able to alter the accumulation of phenolics in the plant by increasing the uptake of P [14]. In response to biotic stresses and to improve plant adaptations, mycorrhizalization can increase the content of plant alkaloids such as camptothecin [15], in studies on terpenoids, it was found that mycorrhizal symbiosis facilitates the expression of genes on the terpenoid synthesis pathway in medicinal plants [16,17]. In medicinal plants, AMF enhances the accumulation of medicinal components in licorice by inducing the genes SQS1, β-AS, CYP88D6 and CYP72A154 on the glycyrrhetinic acid and glycyrrhizin synthesis pathways [18]. Field trials also demonstrated that AMF inoculation increased artemisinin content in *Artemisia annua* leaves while improving the nutritional status of the plant, and that different AMFs differed significantly in enhancing artemisinin accumulation [19]. Although numerous studies have shown that AMF can effectively increase the content of active chemical components in medicinal plants [20], and regulates the accumulation of secondary plant metabolites by changing root morphology [21], however, the main studies have usually focused on the relationship between P and AMF, the effects of inoculation with mycorrhizal fungi of Artemisia bushes at different nitrogen levels on plant growth and secondary metabolites have not been reported. In addition, there were differences in the effects of different types of AMF on plants, and the analysis of the metabolome revealed significant differences in the effects of different types of AMF on the metabolite accumulation patterns of plants [22]. For example, Glomus caledonium inoculation increased rosmarinic and caffeic acid production in Ocimum basilicum,

while F. mosseae only increased caffeic acid production [23]. In the case of medicinal plants, the promotion effect of AMF symbiosis is also closely related to the growth environment of the plant, as different nutrient conditions, light time, harvesting time and cultivation methods can have significant effects on AMF-plant symbiosis [24]. Through previous studies in the laboratory, it was found that among Glomus moseae, Glomus etunicatum, Glomus eburneum, and Paraglomus occultum, the symbiosis of *P. polyphylla* var. *yunnanensis* rebus with Glomus eburneum and Cryptococcus occultum was better, and the symbiotic colonization rate of the remaining two AMF fungi with *P. polyphylla* var. *yunnanensis* rebus was lower.

P. polyphylla var. *yunnanensis* is a valuable Chinese herb with steroidal saponins as its main active constituents, which have various effects such as antitumor, hemostatic, antibacterial, and immunomodulatory [25]. Steroidal saponins are synthesized mainly through the cytoplasmic mevalonate (MVA) pathway and the plastidic 2-C-methyl-d-erythritol-4-phosphate (MEP) pathway for the biosynthesis of saponin elements, which are then modified by glycosyltransferases and P450 to eventually form different saponins [26,27]. In order to investigate the effect of AMF inoculation on the nitrogen uptake and secondary metabolism synthesis of the plant, the present study was conducted to investigate the effects on the growth physiology and active components of *P. polyphylla* var. *yunnanensis* after symbiosis with different species of AMF under different nitrogen levels and the molecular mechanism behind it.

2. Materials and methods

2.1 Biomaterials and culture

The seedlings were purchased from Yunnan Baotian Agricultural Technology Co. Ltd, and two-year-old seedlings were selected as the test material. The AMF fungi used were purchased from the Germplasm Repository of Congenital Mycorrhizal Fungi (BGC), the Institute of Plant Nutrition and Resources, Beijing Academy of Agricultural and Forestry Sciences (BAAFS), Beijing, China. The purchased fungi were *Glomus eburneum* (Ge) and *Paraglomus occultum* (Po). Mycorrhizal substrates containing approximately 100 spores per 10 g were purchased for subsequent expansion inoculation. The soil substrates used in this experiment were humus, loess, and sand. Humus was purchased from Dounan Flower and Bird Market in Kunming, Yunnan Province, and loess was selected from the test site at Yunnan Agricultural University. The three soils were mixed in a volume ratio of 3:1 (humus: loess) and 2:1 (humus: sand), then sterilized in an autoclave at 121 °C for 1 h, bagged and sealed, and set aside in a cool place. The cooled soil was packed into pots of about 1.5 kg each, with a diameter of 21 cm, bottom diameter of 20 cm and height of 20 cm. 10% sodium hypochlorite was sprayed on the surface for 1 h before use, and then rinsed with sterile water to dry. Two layers of 8-needle shade net were covered in the rain-proof greenhouse, and trays were placed at the bottom of the planting pots to prevent rapid water loss and to ensure no cross-contamination between treatments. Before the test treatment, the *P. polyphylla* var. *yunnanensis* seedlings were transplanted into pots, 2/3 of the substrate soil was poured in first, then the roots of *P. polyphylla* var. *yunnanensis* were put into the soil, and finally the soil was filled, and 3 or 4 *P. polyphylla* var. *yunnanensis* plants were transplanted into each pot. The transplanting was completed in June 2020. Until the second year of seedling emergence regularly watered with sterile water, and to ensure the rate of shade of the greenhouse, control of pests and diseases, keep the greenhouse clean and good air permeability, weed at the right time.

2.2 AMF propagation

AMF was colonized using sandy soil with humus as substrate. Maize seeds were selected in July 2020 after surface disinfection by 10% sodium hypochlorite spraying for 1 h and rinsing and drying with sterile water, buried 5 cm below the surface soil, and 2 g of AMF fungicide was evenly applied around them, 3-4 maize seeds were planted into each pot, a total of 10 pots were cultivated, 5 pots for each type of AMF, and Hoagland nutrient solution was applied

regularly (once every 2 weeks) during the rest of the time, sterile water was poured to ensure that the soil moisture content was maintained between 20% and 30% (measured using a moisture meter) in order to increase the AMF colonization rate.

2.3 AMF inoculation

After 4 months of expansion culture, the aboveground part of the successfully expanded maize was cut, and the roots were cut into root segments of about 1 cm and mixed evenly with the root soil as a follow-up AMF fungicide. AMF was inoculated at the rhizomes of *P. polyphylla* var. *yunnanensis*, the soil at the rhizomes of *P. polyphylla* var. *yunnanensis* prepared for inoculation was removed, and then the spare fungicide (about 50 g) weighed in advance was spread evenly around the rhizomes of *P. polyphylla* var. *yunnanensis* again, and finally, the soil was filled, finish the transplanting and inoculation process. AMF inoculation of *P. polyphylla* var. *yunnanensis* was completed in March 2021.

2.4 Different nitrogen concentration treatment

The nitrogen test was divided into 3 nitrogen addition gradients: N1 (application of 1 mM NH_4NO_3), N2 (application of 2.5 mM NH_4NO_3), and N3 (application of 5 mM NH_4NO_3). Each nitrogen gradient consisted of three inoculation treatments: no AMF (AM^-), inoculation with *Glomus eburneum* (Ge), and inoculation with *Paraglomus occultum* (Po), for a total of nine treatments: N1CK; N1Ge; N1Po; N2CK; N2Ge; N2Po; N3CK; N3Ge; N3Po. Nitrogen was added every 2 weeks by accurately weighing 16.00 g into a 1000 mL volumetric flask, using sterilized pure water to fix the volume to 1000 mL to obtain 16 g/L NH_4NO_3 solution. The NH_4NO_3 solutions of 1 mM/250 mL, 2.5 mM/250 mL and 5 mM/250 mL were obtained by dividing 5 mL, 12.5 mL and 25 mL into 250 mL volumetric flasks and adding sterilized pure water to 250 mL, respectively. After 90 days of nitrogen addition, the corresponding indexes of *P. polyphylla* var. *yunnanensis* were measured. The nitrogen addition was started in June 2021.

2.5 AMF colonization rate

AMF was determined with reference to the Taipan Blue staining method, determination by reference to the method [28]: (1) Fixation: 1 cm root segments (lateral roots) of *P. polyphylla* var. *yunnanensis* rebus were taken and fixed in FAA solution (130 mL formaldehyde, 50 mL glacial acetic acid, 2000 mL 5% ethanol) for 24 h. (2) Transparency: Root segments were cleaned with distilled water, immersed in 10% KOH solution, heated in a water bath at 90 °C for 60 min, and heated until the root color was slightly white and transparent. (3) Acidification: The residual KOH on the roots was cleaned with distilled water, immersed in 2% HCL solution, acidified for 10 min, and then cleaned with distilled water. (4) Staining: Put the washed root segments into 0.05% Taipan blue staining solution, heated in a 90 °C water bath for 30 min, removed and cooled, then put into lactic acid glycerol solution for decolorization for more than 60 min. (5) Filming observation: According to the amount of mycorrhizal structure of each root segment, the infestation rate was graded by 0%, < 5%, < 10%, < 30%, > 50%, > 70%, > 90%.

2.6 Determination of elemental content

Soil nitrogen: The determination was performed by the alkaline solution method. Soil phosphorus: The determination was carried out by atomic absorption spectrometry using ammonium acetate leaching. Soil phosphorus: Molybdenum antimony anti-colorimetric method was used for determination.

Organic matter: The oxidation capacity method of potassium dichromate was used for the determination. Plant nitrogen: Measured with reference to the agricultural industry standard NY/T297-1995 of the People's Republic of China. Plant phosphorus: Measured with reference to NY/T2421-2013, the agricultural industry standard of the People's Republic of China. Plant potassium: Measured with reference to NY/T2420-2013, the agricultural industry standard of the People's Republic of China. Plant organic

matter: Measured with reference to NY/T1121.6-2006, the agricultural industry standard of the People's Republic of China.

2.7 Determination of plant physiological indicators

Determination of chlorophyll content

To determine the photosynthetic pigment content of *P. polyphylla* var. *yunnanensis* plants [29], fresh plant leaves of uniform length were selected, the midvein was removed, cut, and weighed to 0.1 g. The samples were placed in a mortar, and 2-3 mL of 95% ethanol and a small amount of calcium carbonate ($CaCO_3$) was added to make a homogenous slurry, 95% ethanol was added dropwise until the tissue turned white, and left to stand; the filter paper was placed on a funnel, moistened with ethanol, and filtered into a 25 mL volumetric flask. The filter paper was placed on a funnel, moistened with ethanol, and filtered into a 25 mL volumetric flask. Rinse the chlorophyll on the filter paper with 95% ethanol into a volumetric flask, fix the volume to 25 mL, shake well, and set aside. The above samples were taken into the cuvette, and the values of 665 nm, 649 nm and 470 nm were measured with 95% ethanol reagent as blank.

Calculation formula:

$$Ca = 13.95\ A665 - 6.88\ A649$$

$$Cb = 24.96\ A649 - 7.32\ A665$$

$$Cx = (1000\ A470 - 2.05\ Ca - 114.8\ Cb)/245$$

$$\text{Content (mg/g)} = [\text{C(mg/L)} \times \text{total extract (25 mL)}]/[\text{weight of leaf sample (0.1 g)} \times 1000]$$

2.8 Determination of Abscisic Acid (ABA), 3-Indoleacetic acid (IAA) and Proline (Pro) in plants

Determination of ABA and IAA

Both ABA and IAA were determined using enzyme-linked immunoassay kits, and ABA was determined using plant ABA enzyme-linked immunosorbent assay kits [30]. Extraction of primary enzyme solution: Cut the leaf tissue into the grinding bowl, then liquid nitrogen grinding into powder, 1 g of plant tissue plus 9 mL of homogenate (PBS), 4000-5000 rpm/min centrifugation for 15 minutes to take the supernatant. Dilution of standards: The kit provides one original multiple standard, which can be diluted in small test tubes according to the following chart: No. 5 standard: 150 μL of original multiple standard added to 150 μL standard dilution; No. 4 standard: 150 μL of No. 5 standard added to 150 μL standard dilution; No. 3 standard: 150 μL of No. 4 standard added to 150 μL standard dilution; No. 2 standard: 150 μL of No. 4 standard added to 150 μL standard dilution; No. 3 standard: 150 μL of No. 4 standard added to 150 μL standard dilution. Dilution; No. 2 standard: 150 μL of No. 3 standard added to 150 μL standard dilution; No. 1 standard 150 μL of No. 2 standard added to 150 μL standard dilution, the concentrations are 48 pmol/L, 24 pmol/L, 12 pmol/L, 6 pmol/L, 3 pmol/L. Adding samples: Set up blank wells respectively (blank control). The wells were set up as blank control wells (no sample and enzyme reagent were added to the wells, the rest of the steps were the same), standard wells and wells for the samples to be tested. Add 50 μL of standard to the enzyme plate, 40 μL of sample dilution to the sample wells, and then 10 μL of sample to be tested (the final dilution of the sample is 5 times). Add the sample to the bottom of the well of the enzyme plate without touching the wall of the well as much as possible, and gently shake and mix. Warming: Seal the plate with sealing film and incubate at 37 °C for 30 minutes. Configurate liquid: 30-fold washing solution diluted 30-fold with distilled water backup washing: Uncover closure plate membrane, discard liquid, dry by swing, add full washing solution to each well, rest for 30 seconds, repeat 5 times, dry by pat. Add enzyme: Add hrP-conjugate reagent 50 μL to each well, except the blank well. Incubation: Operation with 3. Washing: Operation with 5. Color-developing: Add color developing agent A50 μL and color-developing agent B50 μL to each well, mix gently, and avoid light developing for 10 minutes at 37 °C. Termination: Add 50 μL termination solution to each well to terminate the reaction (at this

time, the blue color turns to yellow). Determination: Set the blank hole to zero, and measure the absorbance (OD value) of each hole in sequence at 450 nm wavelength. The determination should be carried out within 15 minutes after adding termination solution.

Proline determination

Proline was extracted and determined by referring to the method of Bates et al. [31]. The proline content in *P. polyphylla* var. *yunnanensis* was determined by using UV spectrophotometer. About 0.5 g of *P. polyphylla* var. *yunnanensis* leaves were weighed and added to 4.5 mL of homogenization medium, and mechanical homogenization was carried out in an ice-water bath, followed by centrifugation at 3500 rpm for 10 min, and the supernatant was taken afterwards. The absorbance was measured in a 1-cm cuvette at 520 nm by adding corresponding reagents in a boiling water bath for 30 min according to the proline test kit.

2.9 Determination of saponin content

The content of steroidal saponins in the rhizomes of *P. polyphylla* var. *yunnanensis* seedlings was determined by high performance liquid chromatography (HPLC) on an Agilent 4.6 × 250 mm column with the following chromatographic conditions: mobile phase acetonitrile (A): water (B) (gradient elution, 0→40 min (A:B = 30:70→60:40), 40→41 min (A:B = 60:40→80:20), 41→44 min (A:B = 80:20→80:20). The rhizomes were collected from the treated rhizomes, washed, dried to constant weight at 45 °C, and crushed. The sample was weighed precisely according to the method of Chinese Pharmacopoeia and placed in a 50 mL volumetric flask with 0.5 g of crushed sample (passed through No. 3 sieve, 50 mesh), added methanol to 20 mL, weighed at this point, soaked for 30 min, extracted by ultrasonication for 30 min, cooled at room temperature, then methanol was used to make up the lost weight, shaken well, and the extract was filtered through a 0.45 μm microporous membrane. The extract was filtered through a 0.45 μm microporous membrane. 700 uL was taken into a brown injection bottle and injected into the machine.

2.10 Determination of genes and transcriptome

Seven genes related to the steroidal saponin synthesis pathway of *P. polyphylla* var. *yunnanensis* were selected for fluorescent quantitative PCR analysis based on previous experiments. The seven related genes were: 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMGS), vanilloid diphosphate synthase (GPPS), farnesyl pyrophosphate synthase (FPPS), squalene synthase (SS), squalene cyclooxygenase 1 (SE1), cycloazidyl synthase (CAS), and squalene cyclooxygenase 2 (SE2). The transcriptome assays were performed on selected rhizome fractions of *P. polyphylla* var. *yunnanensis* and sent to Shanghai Meiji Biological Company for determination. The transcripts were assembled de novo using Trinity software with default parameters. The overlapping groups were assembled into unigenes based on paired-end information and subsequently annotated against the National Center for Biotechnology Information (NCBI) database using the BLAST program, and homology searches were performed using BLAST against the following protein databases: NCBI non-redundant (nr), Kyoto Encyclopedia of Genes and Genomes (KEGG) and GO (Gene Ontology) [32]. The best comparison results were selected for single gene annotation. Sequences of unigenes belonging to cytochrome P450 and UDP-glycosyltransferase (UGT) were identified from the transcriptome, and unigenes > 1000 bp in length were screened for phylogenetic analysis. Phylogenetic tree mapping was performed using MEGA7, and some of the UGT and P450 from other plants were selected, and then some of the unigenes sequences of UGT and P450 whose functions had been identified and those of UGT and P450 screened from the transcriptome of *P. polyphylla* var. *yunnanensis* were selected for phylogenetic analysis.

2.11 Data analysis

The obtained experimental data were statistically analyzed by Excel 2013 software; SPSS 20.0 (SPSS, Chicago, USA) analysis software was used for statis-

tical analysis, and one-way ANOVA (one-way analysis of variance) was used for the analysis of significance of differences; comparison of means between treatments was performed using Duncan's multiple comparison method for analysis of significance of differences; and Prism software and Origin2019b software were used for graphing.

3. Results

3.1 AMF colonization rate

Under different N conditions, the roots of *P. polyphylla* var. *yunnanensis* inoculated with AMF treatment were all infiltrated by mycelium, and all with mycelium or spore structures, while the treatments without inoculation had no mycorrhizal structures (**Figure 1**). As seen in the colonization rate, the colonization rate of Ge decreased significantly under the highest concentration of N treatment, but the difference was not significant at low and medium concentrations, while the colonization rate of Po remained consistent with the N concentration gradient (**Figure 2**) ($p > 0.01$).

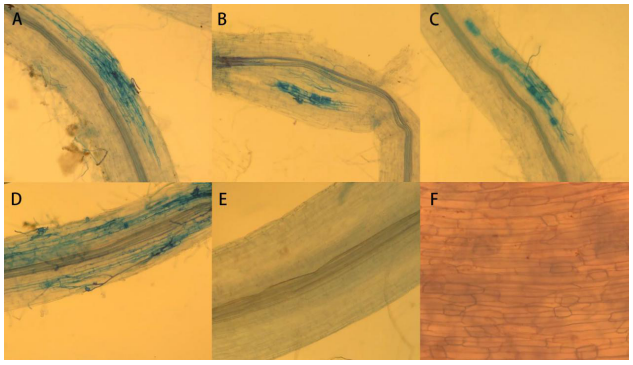

Figure 1. Mycorrhizal structure of the root system of *Pp*.

Note:

A: Mycorrhizal structure of mycorrhizal infestation by Ge under N3 treatment,

B: Mycorrhizal structure of mycorrhizal infestation by Po under N1 treatment,

C: Mycorrhizal structure of mycorrhizal infestation by Ge under N2 treatment,

D: Mycorrhizal structure of mycorrhizal infestation by Ge under N1 treatment,

E: Non-mycorrhizal structure of mycorrhizal infestation under N1 treatment,

F: Non-mycorrhizal structure of mycorrhizal infestation under N2 treatment.

3.2 Agronomic traits of *P. polyphylla* var. *yunnanensis*

After 90 days of nitrogen treatment, the agronomic trait indexes of *P. polyphylla* var. *yunnanensis* were measured. The differences in leaf length and leaf width of plants under most treatments were not significant, with leaf length under the N1Ge treatment being higher than the other treatments, while leaf length under N3Po was lower. Plant height under the N1 treatment without AMF inoculation showed lower values, while plant height under the other treatments did not differ significantly, and plant stem and root weights also showed this trend (**Table 1**).

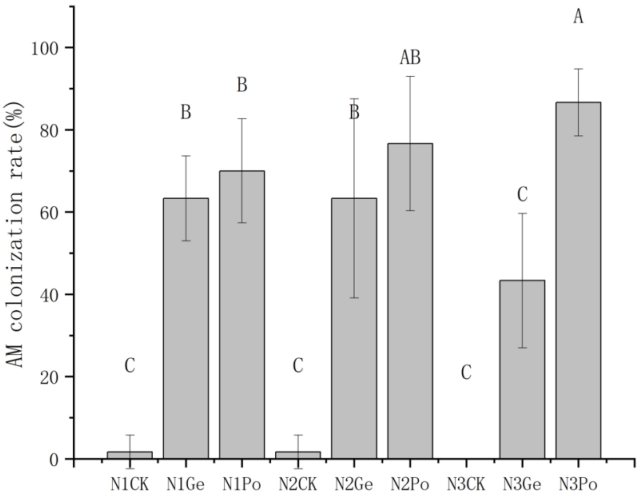

Figure 2. Root colonization rate of *Pp.* under different nitrogen treatments ($p > 0.01$).

3.3 Chlorophyll content

By applying different nitrogen to *P. polyphylla* var. *yunnanensis*, it was found that inoculation with Ge favored the accumulation of chlorophyll a, b, and carotenoids at low nitrogen concentrations, while inoculation with Po increased chlorophyll in the plants less than Ge (**Figure 3**). In the medium N treatment, both AMFs significantly increased chlorophyll a, b and carotenoid contents of the plants (**Figures 3-6**). In the high N treatment, inoculation with Po reduced the chlorophyll a and carotenoid content of the plants, with no significant difference for chlorophyll b, whereas inoculation with Ge reduced only the carotenoid content, but the total chlorophyll content was the same as in plants without AMF (**Figure 3D**).

Table 1. Effects of different nitrogen treatments on the morphological indexes of *P. polyphylla* var. *Yunnanensis.*

Treatment	Leaf length (cm)	Leaf width (cm)	Plant height (cm)	Stem weight (g)	Root weight (g)	Root length (cm)
N1CK	5.275 ± 0.57AB	3.16 ± 0.30 ns	14.65 ± 0.88B	1.0796 ± 0.05B	0.96205 ± 0.16B	9.53 ± 1.42 ns
N1Ge	5.545 ± 0.64A	3.1 ± 0.33 ns	15.66 ± 1.26A	1.1162 ± 0.04AB	1.1329 ± 0.17A	10.055 ± 0.83 ns
N1Po	5.47 ± 0.55AB	3.085 ± 0.26 ns	16.215 ± 0.67A	1.1499 ± 0.05A	1.06785 ± 0.18AB	9.175 ± 1.47 ns
N2CK	5.38 ± 0.47AB	3.045 ± 0.27 ns	16.03 ± 1.15A	1.11125 ± 0.08AB	1.03375 ± 0.17AB	9.66 ± 1.72 ns
N2Ge	5.475 ± 0.61AB	3.005 ± 0.30 ns	16.315 ± 1.08A	1.1467 ± 0.07A	1.0854 ± 0.12A	10.01 ± 1.79 ns
N2Po	5.535 ± 0.46A	3.005 ± 0.32 ns	16.205 ± 0.82A	1.12865 ± 0.06AB	1.10405 ± 0.21A	9.43 ± 1.79 ns
N3CK	5.535 ± 0.46A	3.035 ± 0.33ns	16.05 ± 0.74A	1.1392 ± 0.06AB	1.058 ± 0.18AB	9.775 ± 1.76 ns
N3Ge	5.415 ± 0.53AB	3.17 ± 0.33 ns	15.93 ± 0.90A	1.1293 ± 0.08AB	1.02415 ± 0.11AB	9.64 ± 1.78 ns
N3Po	5.12 ± 0.71B	2.96 ± 0.33 ns	15.81 ± 0.95A	1.11885 ± 0.08AB	1.07005 ± 0.16AB	10.1 ± 1.67ns

The data in the table are the mean ± standard error (n = 3). Different lowercase letters after the data in the same column indicate significant differences in the level of variation under different treatments ($p < 0.01$).

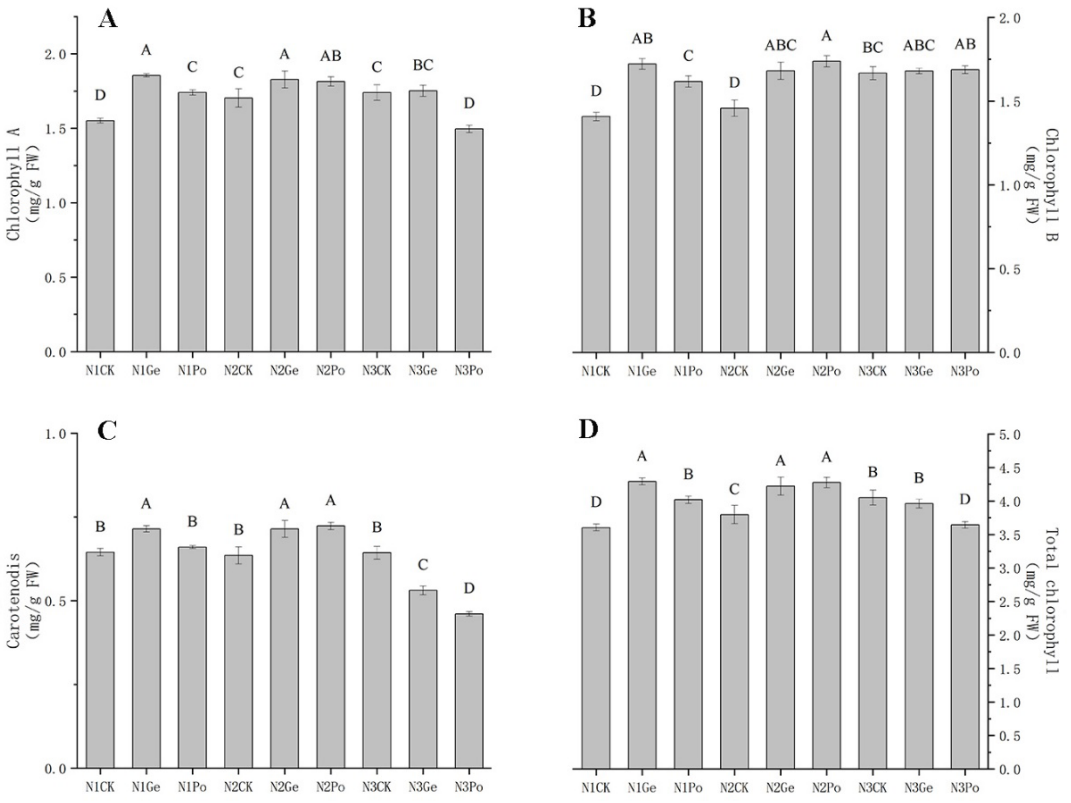

Figure 3. Effect of different nitrogen treatments on the chlorophyll content of *Pp*.

Note: A: chlorophyll a, B: chlorophyll b, C: carotenoids, D: total chlorophyll.

3.4 The contents of ABA, IAA and Pro in *P. polyphylla* var. *yunnanensis*

In this experiment, the IAA content of the roots of *P. polyphylla* var. *yunnanensis* showed an increasing trend with the increase of N concentration (**Figure 4**). In both low and medium N treatments, both AMFs increased the IAA content of plant roots up to 36.82 ng/g in N2Ge treatment, while in high N concentration, the IAA content of N3Ge was 37.39 ng/g, which was significantly higher than that of N3CK and N3Po. The accumulation trends of proline content and IAA content of *P. polyphylla var. yunnanensis* graveolens in different N treatments were similar (**Figure 5B**), with low and medium N levels, both AMF increased the Pro content of plants, but under high N treatment, the Pro content of N3CK and N3Po was significantly higher than that of N3Ge, and the highest Pro content was 20.87 ng/g and 21.5433 ng/g for N3CK and N3Po, respectively and relative to the difference between high and medium N, the AMF on plant IAA and Pro The effect of AMF on plant IAA and Pro varied most significantly from low N to medium N. In contrast, the trend of ABA accumulation in *P. polyphylla* var. *yunnanensis* was opposite to that of IAA and Pro. Under low and medium N treatments, AMF plants reduced the ABA content of *P. polyphylla* var. *yunnanensis* roots (**Figure 5A**), and the lowest ABA content of 29.54 ng/g was found in N3Ge under high N treatment, which was significantly lower than that of N3CK and N3Po.

3.5 Elemental content of the plant and soil

By applying different concentrations of nitrogen, it was found that the quick-acting N content of the soil showed a significant increase, showing a similar trend to the nitrogen gradient (**Table 2**). And under the N3 treatment, the concentration of fast-acting N was found to be higher in the AMF-inoculated soil than in the non-AMF-inoculated soil, but the increase was less than that of the different N treatments. The results of effective phosphorus showed that AMF inoculation promoted the accumulation of effective phosphorus in the soil under low N concentration. And the content of fast-acting potassium in the soil was also higher than that in the soil before nitrogen application, but the difference was not significant between different N treatments.

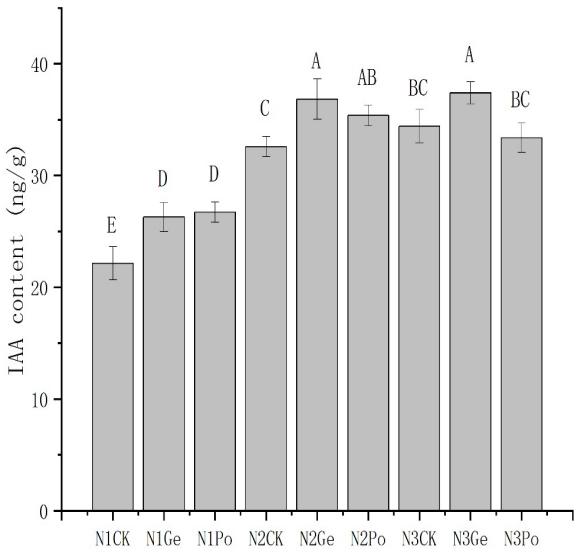

Figure 4. Effect of different nitrogen treatments on the IAA of *P. polyphylla* var. *yunnanensis*.

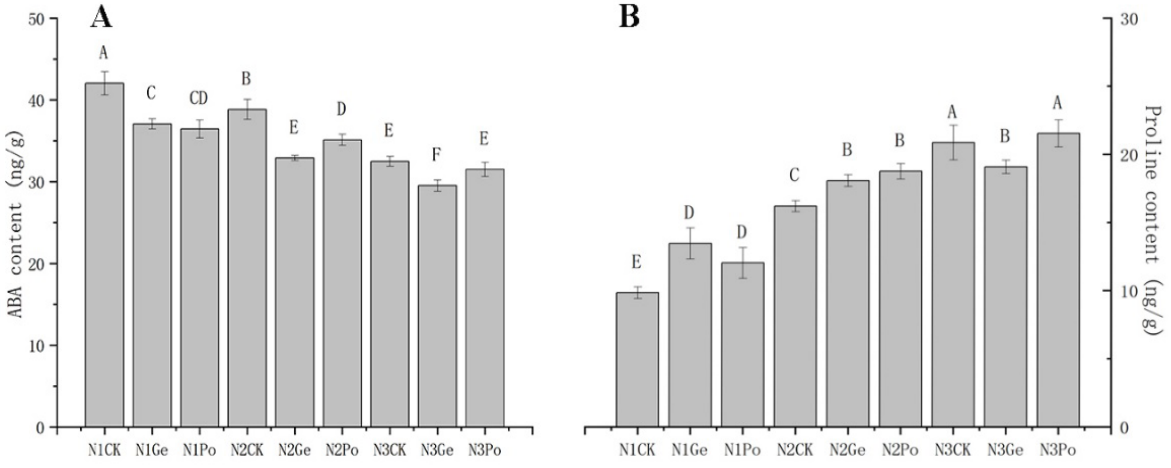

Figure 5. Effect of different nitrogen treatments on ABA and Pro of *P. polyphylla* var. *yunnanensis*.

Note: A: ABA, B: Pro.

Table 2. Effect of different nitrogen treatments on soil NPK content.

Treatment	Organic matter g/kg	Effective phosphorus mg/kg	Fast-acting potassium mg/kg	Quick-acting nitrogen mg/kg
N0(before applying N)	535.26 ± 60.92 ns	67.25 ± 1.34B	551.19 ± 34.35C	252.26 ± 4.89E
N1CK	549.13 ± 27.62 ns	66.62 ± 1.64B	630.89 ± 12.38A	298.69 ± 3.87D
N1Ge	544.46 ± 15.11 ns	71.51 ± 1.12A	632.81 ± 14.38A	296.82 ± 2.91D
N1Po	533.09 ± 11.52 ns	72.79 ± 3.15A	613.47 ± 11.74AB	300.50 ± 3.14D
N2CK	566.09 ± 9.38 ns	66.14 ± 1.38B	581.71 ± 15.97BC	359.93 ± 4.07C
N2Ge	541.25 ± 11.56 ns	64.97 ± 1.07B	602.25 ± 14.71AB	354.11 ± 6.54C
N2Po	555.80 ± 7.99 ns	64.85 ± 2.11B	611.98 ± 13.50AB	359.70 ± 5.45C
N3CK	545.93 ± 2.56 ns	65.89 ± 1.07B	600.63 ± 19.34AB	414.73 ± 1.57B
N3Ge	547.17 ± 12.82 ns	66.69 ± 0.73B	606.03 ± 11.69AB	426.51 ± 3.24A
N3Po	566.36 ± 6.82 ns	65.10 ± 0.64B	594.14 ± 5.21AB	427.01 ± 2.31A
N	F = 0.322574	F = 30.244988**	F = 3.709461*	F = 169.771011**
AMF	F = 2.675380	F = 2.950613ns	F = 0.991322 ns	F = 0.166143 ns
N*AMF	F = 1.192012	F = 4.982080**	F = 0.753217 ns	F = 1.177272 ns

The data in the table are the mean ± standard error (n = 3). Different lowercase letters after the data in the same column indicate significant differences in the level of variation under different treatments ($p < 0.01$).

CNPK measurements on the above-ground parts of nitrogen-treated *P. polyphylla* var. *yunnanensis* revealed that the C content of plants inoculated with AMF was significantly increased under low N treatment (**Table 3**), and the C% of N1Ge and N2Po increased by 3% and 2%, respectively, compared to N1CK, while this trend was reflected in the P, K, and N contents, with the greatest increase being in the P content under low N conditions. Under medium N treatment, both AMFs increased the C content of the plants, but the difference in P content was not significant and the increase in their K and N content was found only in plants inoculated with Ge. Po inoculation had no significant effect on the NPK content of the above-ground parts of the plants under medium N concentration. The C, N, P and K contents of the plants inoculated with AMF did not improve under high N treatment, and especially the C, K and N contents of N3Po showed a decreasing trend. the highest C, K and N contents were found in the N3CK treatment, while the highest P contents were found in the N1Ge-treated plants.

Measurements of C, N, P, and K in the nitrogen-treated subterranean parts of *P. polyphylla* var. *yunnanensis* revealed that both AMFs increased the C, N, P, and K contents of the subterranean parts of *P. polyphylla* var. *yunnanensis* in the low-nitrogen treatment (**Table 4**). In the medium nitrogen treatment, inoculation with Ge still ensured an increase in C, N, P, and K of the plants, but Po symbiosis with the plants only increased the C and N content of the plants. Under the high N treatment, the C and N contents of N3CK, N3Ge and N3Po were not significantly different, while the P contents of N3Ge and N3Po were significantly lower than those of N3CK, and those of N3Po were higher than those of N3CK and N3Ge. In the underground part of the assay, the P contents of AMF plants were all higher than those of other treatments at N1 concentration, while N1Po had the highest K content and N1Ge as well as N2Po had the highest N content.

3.6 Saponin content of *P. polyphylla* var. *yunnanensis*

In this experiment, the content of saponins I, II, VI, VII, D, and H was examined in *P. polyphylla* var. *yunnanensis* after 90 d of treatment with different concentrations of nitrogen. And saponin VI was not detected in any of the treatments. In the detection of saponin I (**Figure 6A**), the highest saponin I content was found in N1Ge and N1Po, which reached 0.18% and 0.167%, respectively, while the saponin I content of N1CK was only 0.047%. In the medium nitrogen treatment, Po significantly increased the saponin I content of the plants,

Table 3. Effects of different nitrogen treatments on the NPK content of above-ground parts of *P. polyphylla* var. *yunnanensis*.

Stem				
Treatment	**C%**	**P%**	**K%**	**N%**
N1CK	32.35 ± 0.49E	0.0653 ± 0.00054C	4.1036 ± 0.18029E	0.9547 ± 0.01341E
N1Ge	35.23 ± 0.63BC	0.0790 ± 0.00152A	5.3951 ± 0.0934C	1.1274 ± 0.00976C
N1Po	34.20 ± 0.61CD	0.0716 ± 0.00384B	5.0317 ± 0.13248CD	1.1099 ± 0.00878C
N2CK	33.03 ± 0.80DE	0.0721 ± 0.00295B	4.0904 ± 0.09526E	1.0755 ± 0.00891D
N2Ge	36.39 ± 0.50AB	0.0678 ± 0.00366BC	4.8664 ± 0.24111D	1.1158 ± 0.01523C
N2Po	36.40 ± 0.67AB	0.0680 ± 0.00158BC	4.1506 ± 0.21127E	1.0680 ± 0.01003D
N3CK	37.36 ± 0.37A	0.0668 ± 0.00071BC	6.8121 ± 0.21127A	1.2251 ± 0.01077A
N3Ge	36.22 ± 0.47AB	0.0682 ± 0.0019BC	5.9878 ± 0.26285B	1.2056 ± 0.02079AB
N3Po	34.14 ± 0.16CD	0.0689 ± 0.00079BC	4.2568 ± 0.09027E	1.1902 ± 0.01283B
N	F = 23.928812**	F = 7.348639**	F = 128.242518**	F = 73.197138**
AMF	F = 19.056139**	F = 2.989605ns	F = 67.923281**	F = 9.360673 **
N*AMF	F = 27.547554**	F = 5.783699**	F = 89.785862 **	F = 12.432372 **

The data in the table are the mean ± standard error (n = 3). Different lowercase letters after the data in the same column indicate significant differences in the level of variation under different treatments ($p < 0.01$).

but Ge decreased the saponin I content, while the plants not inoculated with AMF were significantly higher than the low nitrogen treatment. Under high N treatment, the saponin I content of plants not inoculated with AMF was significantly higher than that of medium N treatment, but no saponin I was detected in N3Ge, while the content of N3Po was not significantly different from that of N3CK. The results of saponin II assay showed that AMF significantly increased the accumulation of saponin II in *P. polyphylla* var. *yunnanensis* under low N treatment (**Figure 6B**), and in the medium N treatment, the saponin II content of N2CK was significantly higher than that of N1CK, but still significantly lower than that of N2Ge and N2Po, where N2Ge had the highest content of 0.713%, which was significantly higher than that of N2Po. In the high N treatment, the saponin II content of N3CK was also higher than N2CK, but not significantly different from N3Po. The lowest saponin II content of 0.481% was detected in N3Ge at high N concentration, which was significantly lower than the other treatments of N3. Only four groups of treatments in this experiment detected saponin D content (**Figure 6D**), which were N2CK > N3Po > N1Po > N1CK in descending order, 0.0641%, 0.0411%, 0.0172% and 0.0110%, respectively. The saponin H content of P. polyphylla var. yunnanensis without AMF inoculation increased with increasing N (**Figure 6E**), and under low N treatment, Ge and Po significantly increased the accumulation of saponin H. In the medium N treatment, the accumulation of saponin H was in the order of N2Ge > N2Po > N2CK with 0.13%, 0.08% and 0.04%, respectively, with significant differences among the three, and under high N treatment, N3CK and N3Po showed no significant difference in saponin H content, while no saponin H was detected in N3Ge under high N treatment. The content of non-AMF *P. polyphylla* var. *yunnanensis* saponin VII was significantly lower under the low N treatment than the medium and high N treatments (**Figure 6E**), Ge increased the saponin VII content of *P. polyphylla* var. *yunnanensis* under all three N treatments, especially under the medium N concentration, where saponin VII reached 0.433%, Po was able to increase the saponin VII content under the medium and low N treatments, but was not significantly different from non-AMF inoculated plants under the high N treatment. Overall, in the low and medium nitrogen treatments, inoculation with AMF helped to increase the total saponin content of *P. polyphylla* var. *yunnanensis*, and Ge was significantly more helpful than Po for total saponin accumulation, while when *P. polyphylla* var. *yunnanensis* was under high nitrogen treatment (**Figure 6F**), Po did not significantly improve the accumulation of total saponin in *P. polyphylla* var. *yunnanensis*, and the symbiosis of Ge with *P. polyphylla* var. *yunnanensis* reduced the total saponin content of the plants instead.

Table 4. Effects of different nitrogen treatments on the NPK content of the underground fraction of *P. polyphylla* var. *yunnanensis.*

Roots				
Treatment	**C%**	**P%**	**K%**	**N%**
N1CK	30.9716 ± 0.14946D	0.0632 ± 0.00095D	3.7397 ± 0.09179D	1.0071 ± 0.03796E
N1Ge	38.3554 ± 0.56021A	0.0795 ± 0.00168A	4.0259 ± 0.05849B	1.5314 ± 0.02330A
N1Po	36.6945 ± 0.33912B	0.0787 ± 0.00206A	4.1838 ± 0.02211A	1.4936 ± 0.01543AB
N2CK	33.11927 ± 0.53777C	0.0688 ± 0.00077C	3.7828 ± 0.06047CD	1.3230 ± 0.02394D
N2Ge	38.2088 ± 0.74297A	0.0747 ± 0.00101B	4.0909 ± 0.02555AB	1.4962 ± 0.00959AB
N2Po	37.7540 ± 0.81660AB	0.0708 ± 0.00117C	3.6792 ± 0.03545D	1.5508 ± 0.03056A
N3CK	37.3892 ± 0.45074AB	0.0685 ± 0.00112C	3.8742 ± 0.03398C	1.4205 ± 0.01857C
N3Ge	37.3140 ± 0.67672AB	0.0643 ± 0.00095D	3.8813 ± 0.06786C	1.4636 ± 0.02790BC
N3Po	37.6074 ± 0.35413AB	0.0644 ± 0.00054D	4.0177 ± 0.03687B	1.4576 ± 0.01555BC
N	F = 14.664061**	F = 68.609142**	F = 12.645827**	F = 3.511144 ns
AMF	F = 67.804434**	F = 27.104054**	F = 35.510770**	F = 26.893784 **
N*AMF	F = 19.015849**	F = 39.669369**	F = 44.406771**	F = 7.810122*

The data in the table are the mean ± standard error (n = 3). Different lowercase letters after the data in the same column indicate significant differences in the level of variation under different treatments ($p < 0.01$).

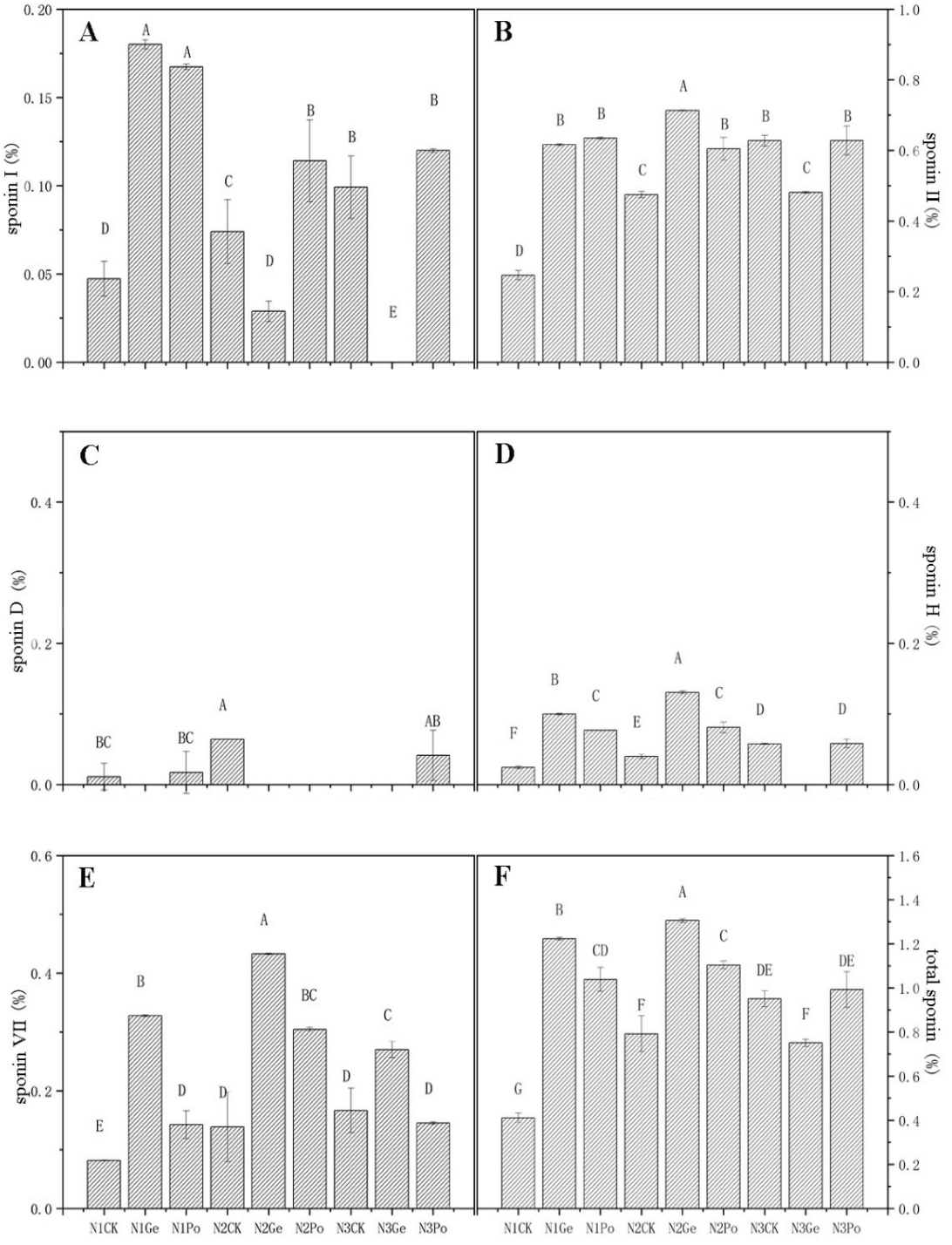

Figure 6. Effect of different nitrogen treatments on saponins of *Pp.*

Note: A: saponin I, B: saponin II, C: saponin D, D: saponin H, E: saponin VII, F: total saponin.

3.7 Saponin synthesis-related genes

Real-time fluorescence quantification of internal reference genes (β-actin) and target genes (HMGS, GPPS, FPPS, SS, SE1, CAS, SE2) was performed for the relevant genes on the steroid saponin synthesis pathway. The expression results were calculated and the specific results are shown in **Figure 7**. In this experiment, AMF increased the expression of HMGS in plants at low N concentration, but its expression was lower than that of N3CK plants at high N concentration, and in plants not inoculated with AMF, the elevated N content promoted the expression of HMGS. The expression of SE1 was significantly higher in AMF plants than in non-AM plants at low N concentration, but lower than in non-AM plants at medium N concentration, and at high N concentration, an increase in SE1 was observed only under Po treatment. AMF also increased the expression of CAS in the plants at low and medium N concentrations, but this trend did not differ significantly, and CAS expression was higher in the Po and CK treatments than in the Ge treatment at high N. There was no significant difference in SE2 expression among treatments at low and medium nitrogen concentrations, but SE2 expression was higher in each treatment at medium nitrogen concentration than at low nitrogen concentration, while high nitrogen decreased SE2 expression. An increase in SS expression was observed only in AM plants at low N concentration and decreased with increasing N. The treatments with higher GGPS expression were N1Ge, N2CK and N2Po, while the differences between the other treatments were not significant.

3.8 Transcriptome analysis

The transcriptome analysis of *P. polyphylla* var. *yunnanensis* with different nitrogen treatments revealed that the Q30 (%) of each treatment was more than 80%, and no significant deviation was found in the base content distribution. 242520 unigene were obtained by the assembly, of which 46.28% were annotated. Through the functional query analysis of the transcriptome of *P. polyphylla* var. *yunnanensis* between different nitrogen treatments, it was found from GO annotation that among the biological process categories, the unigenes annotated by metabolic

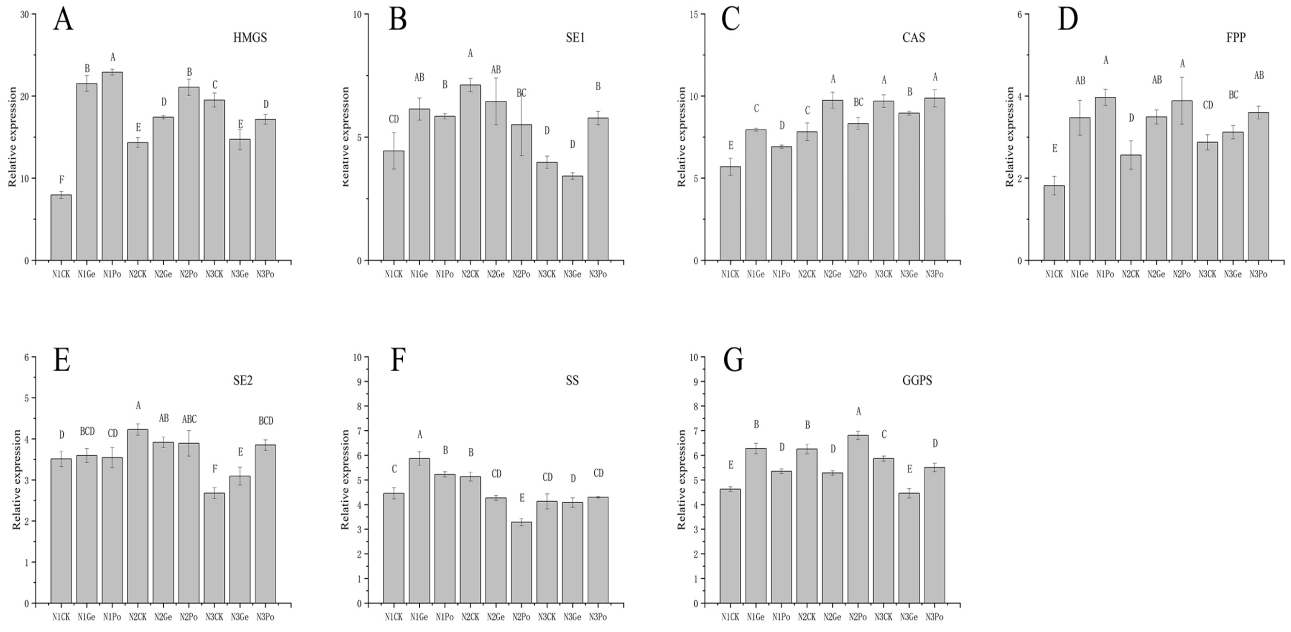

Figure 7. Effect of different nitrogen treatments on saponins of *P. polyphylla* var. *yunnanensis.*

Note: A: *HMGS*, B: *SE1*, C: *CAS*, D: *FPP*, E: *SE2*, F: *SS*, G: *GGPS.*

processes were second only to cellular processes and higher than bioregulation, among the cellular component categories, the cellular and organelle parts were annotated with the most unigenes, and among the molecular function categories, they were mainly focused on catalytic and binding functions (**Figure 8**). The metabolic pathways were found to be the most enriched in unigenes by KEGG analysis, with the most being the metabolism of carbohydrates (5509 unigenes), while the secondary metabolite pathways were enriched with 1484 unigenes **(Figure 9**). The identification of P450 and UGT-related genes was performed by KEGG, GO and NR annotation, and 30 P450 and 297 UGT genes were identified. Cds greater than 1000 bp were screened for subsequent analysis, and 16 eligible P450 unigenes and 75 UGT unigenes were obtained.

Phylogenetic trees were constructed by comparing P450s genes with genes from *Arabidopsis thaliana* (L.) Heynh., *Panax ginseng* C. A. Meyer, *Lycopersicon esculentum* Miller and *Isatis tinctoria* Linnaeus using ML method, where DN14416_c0_g2, DN29717_c0_g1 and DN820_c0_g1 were mainly concentrated in Ath CYP86 clade (Ath CYP94D2), while DN7630_c0_g1 was close to Ath CYP72 clade, DN66132_c0_g1 was close to PpCYP90G4 (**Figure 10A**), and nine unigenes were enriched in Ath CYP51G clade, namely DN342541_c0_g1, DN32483_c0_g1, DN473834_c0_g1, DN47915_c0_g1, DN36083_c0_g1, DN317186_c1_g1, DN119469_c1_g1, DN119469_c1_g3, and DN484778_c0_g1 **(Figure 10A**).

A phylogenetic tree was constructed by constructing a phylogenetic tree of UGTs genes and selected some genes known to be related to steroid, flavonoid and triterpenoid synthesis and UGTs of *Arabidopsis thaliana* (L.) Heynh. Some of the genes were clustered around the triterpene synthesis-related genes (OAGT, UGT73), and seven unigenes tended to be enriched with the Ath UGT73 family and steroid synthesis-related UGTs (**Figure 10B**), namely DN58096_c1_g3, DN43198_c1_g1, DN452515_c0_g1, DN428098_c0_g1, DN217552_c0_g1, DN7369_c0_g3, and DN49172_c1_g1.

CYP450 proteins are the largest family of plant proteins that catalyze most of the oxidative processes in plant secondary metabolism. Glycosyltransferases (UGTs) are another large multigene family in plants. Usually, glycosylation is the final step in the biosynthesis of secondary metabolites, and glycoconjugation enhances stability and water solubility. Eleven P450s and seven UGTs were analyzed by constructing a phylogeny, which was clustered. Under low nitrogen conditions, Ge increased the expression of plants DN32483, DN66132 and DN473834, while Po increased the expression of DN119469, DN484778, DN7630 and DN436083, and under medium nitrogen conditions, non-AM plants showed an increase in DN7630 but a decrease in DN484778 expression, while Po increased the expression of DN111387 and DN287723, and Ge did not increase the expression of P450 under medium nitrogen conditions. Under high nitrogen conditions, non-AM plants increased the expression of genes DN47915, DN119469, and DN342541, while Ge significantly decreased the expression of DN7630 and Po increased the expression of DN317186 (**Figure 11A**). Analysis of UGTs revealed that the screened UGTs were down-regulated in two AM plants under high nitrogen conditions, while three UGTs, DN217552, DN49172, and DN58096, were up-regulated in non-AM plants. N1CK increased the expression of DN43198 and N1Po increased the expression of DN7369 and DN42515 under low nitrogen conditions. Under medium nitrogen conditions, only N2Ge up-regulated DN428098 (**Figure 11B**).

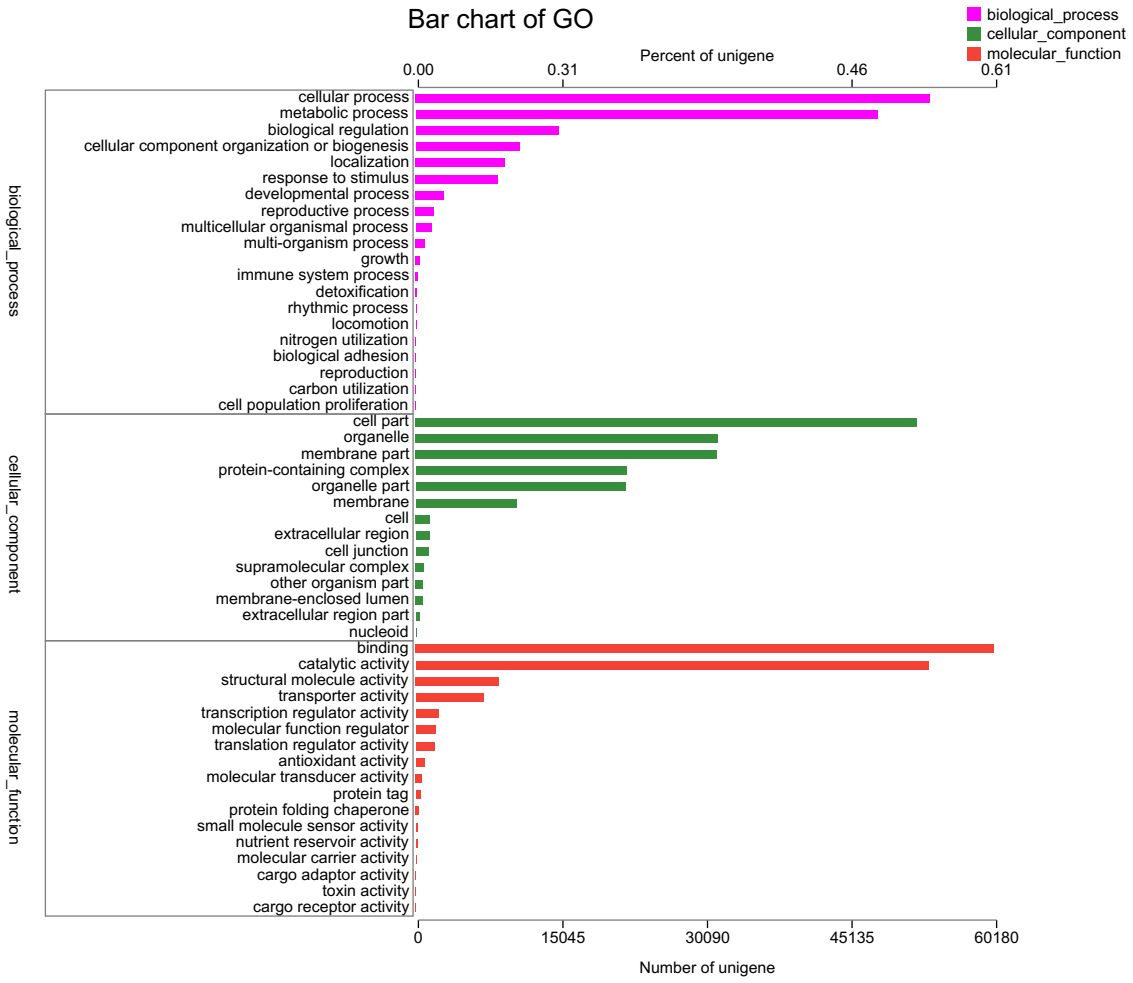

Figure 8. GO annotation of the transcriptome of *P. polyphylla* var. *yunnanensis*.

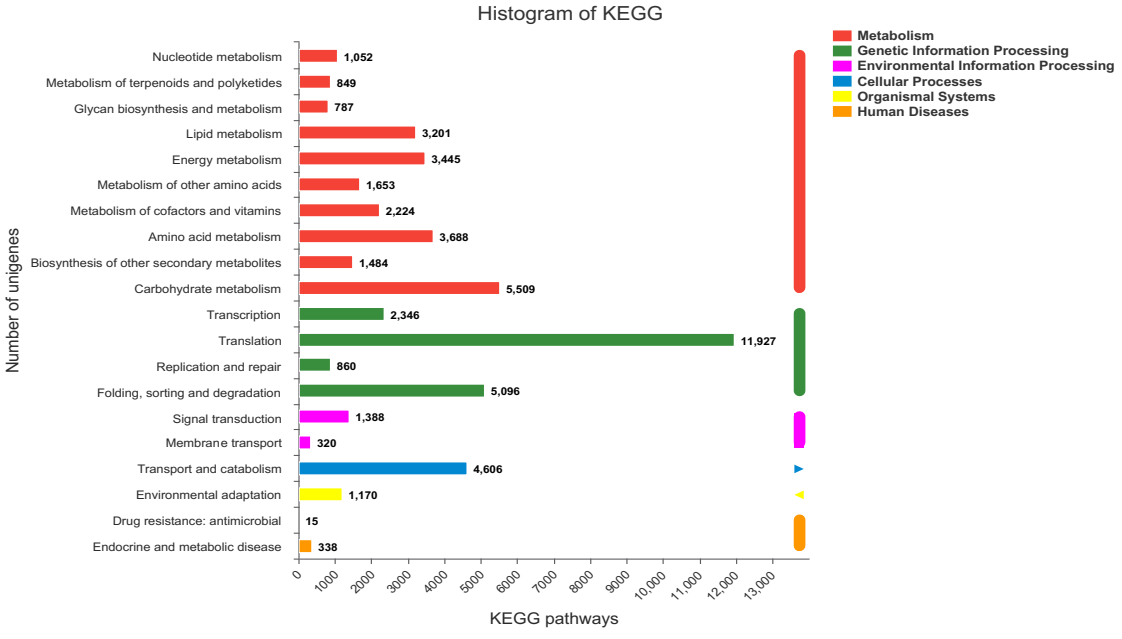

Figure 9. KEGG annotation of the transcriptome of *P. polyphylla* var. *yunnanensis*.

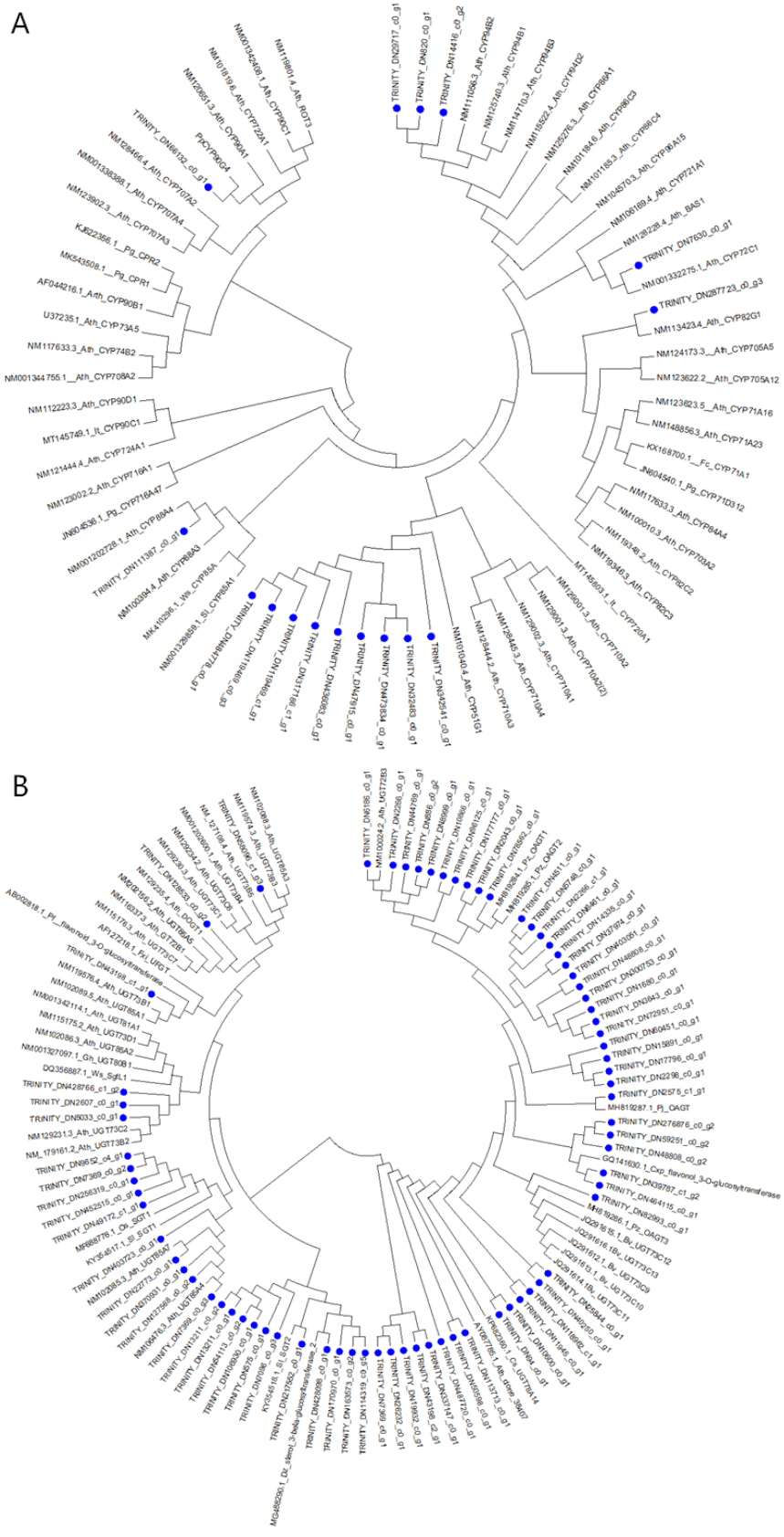

Figure 10. Phylogenetic analysis of unigenes (blue) screened from the transcriptome of *P. polyphylla* var. *yunnanensis*.

Note: A: Phylogenetic analysis of P450s, B: Phylogenetic analysis of UGT.

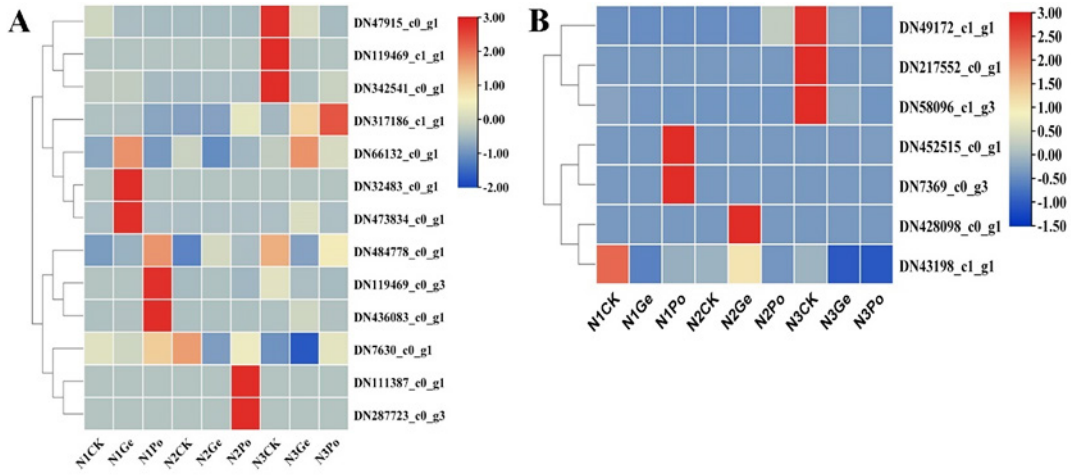

Figure 11. Clustering of transcriptome gene expression profiles under different nitrogen treatments of *P. polyphylla* var. *yunnanensis*.

Note: A: Clustering of UGT gene expression profiles, B: Clustering of P450 gene expression profiles.

4. Discussion

4.1 High nitrogen reduces the colonization rate of AMF

The symbiosis between terrestrial plants and AMF is common, and the two achieve mutual benefits by exchanging nutrients, and usually, plants prefer to form this symbiosis in an adverse environment. In nitrogen experiments, it was found that Ge and Po had very different symbiotic strategies, and with increasing N concentration, Ge showed a decreasing trend in colonization rate at high N concentration, while Po showed an increasing trend in colonization rate. Nitrogen nutrients are the second only to phosphorus nutrients as a determinant of AMF symbiosis [33], and many studies have shown that too much N may lead to a decrease in AMF symbiosis rate [34], which is due to the fact that plants in high. This is consistent with Ge's strategy in this experiment, while the increase in Po colonization rate indicates that plants are more inclined to symbiosis with *P. polyphylla* var. *yunnanensis* regardless of whether they are under environmental stresses such as drought or nutrient-rich environments, and some studies have shown that tomato plants are more inclined to symbiosis with AMF in high nutrient environments [35], which is consistent with Po's symbiosis strategy is consistent. AMFs of different populations may be able to adjust their symbiotic strategies with plants through their own unique growth and developmental patterns to obtain more suitable survival conditions.

4.2 AMF promotes the growth of plants

Most studies have demonstrated that by helping plants to obtain more nutrients AMF can help plants to increase their biomass and promote plant growth and development [36]. In the present, AMF increased plant root weight, stem weight, and plant height, but had less effect on the increase in root length, perhaps because plants prioritize nutrient supply to aboveground parts after inoculation with AMF, which was found to increase plant biomass in a previous study [37], which is similar to the results of the present experiment. In addition, AMF increased the accumulation of IAA in plants, especially under medium to high N treatment, the IAA content of AM plants was significantly higher than that of non-AM plants, indicating that AMF may help more in plant growth under N sufficiency conditions, which is contrary to the trend of AMF colonization rate, which may be due to the fact that the optimal strategy of plant-AMF symbiosis does not follow the range of symbiosis, but the efficiency of symbiosis [38]. With the increase of nitrogen concentration, the ABA content of *P. polyphylla* var. *yunnanensis* repens decreased significantly, and the hormone ABA has

a significant effect on AMF infestation [39], which may be one of the reasons for the decrease of AMF colonization rate. Usually, when plants are subjected to drought or salt stress, the ABA content in the plant will be significantly increased and ABA, as a signaling molecule, can regulate the stomatal conductance of the plant to cope with the water changes occurring in the plant, and the inoculation of AMF led to a significant decrease in the ABA content in *P. polyphylla* var. *yunnanensis*, which can indicate that AMF prevents the change of osmotic pressure brought about by the increase of soil species N content by changing the ABA content in the plant. It can be suggested that AMF can prevent water imbalance in plants by changing the ABA content in plants in response to the increase of soil N content. The study showed that the relationship between Pro and AMF is diverse, and in this experiment, the Pro content of plants inoculated with AMF showed an increasing trend with the increase of N concentration, and Pro is an important indicator of osmotic stress in plants, which can help plants regulate osmotic balance. The change of Pro content can be regarded as a protective measure taken by plants to regulate the osmotic balance. The increase of Pro content by AMF was mainly concentrated in plants treated with medium N concentration, while there was no significant difference in the change of Pro content by AMF under high N treatment, which proved that inoculation of AMF can help plants change the metabolic changes in their bodies and cope with different natural environments.

The determination of soil physicochemical properties showed that the application of N did increase the content of fast-acting N in the soil. In the detection of NPKC in plants, it was found that compared to non-AM plants, the C and N contents of the root parts of AMF plants were significantly increased, especially at N2 concentrations where their enhancement was most obvious, which is also consistent with the trend of its colonization rate, and with the increase of soil N concentration, the uptake of nutrients by *P. polyphylla* var. *yunnanensis* was also significantly increased, but the change of its content by AMF was relatively small. AMF can help plants to absorb various N elements as well as nutrients [40,41], AMF has also been shown to increase the expression of plant phosphate transporter proteins and nitrogen transporter proteins in previous mycorrhizal experiments and this result is consistent with the present experiment [42,43], but when N application was increased, the improvement of plant nutrients by AMF was significantly reduced, and in the N3 treatment, the C, P, and N contents of non-AM plants were higher than those of AM plants, indicating that excessive nutrient addition reduces the help of AMF to plants. In addition, the trend of AMF inoculation on the nutrient accumulation in the above- and below-ground parts of the plants differed. In the above-ground part of the *P. polyphylla* var. *yunnanensis* assay, it was found that the treatments with the highest plant nutrient contents were basically in N3, while in the below-ground part of the assay, the highest C contents were N1Ge and N2Ge, the highest P contents were N1Ge and N1Po, the highest K content was N1Po, and the highest N content was It can be seen that although N application increased the overall nutrient content of the above-ground part of the plant, for the below-ground part, AMF plants were still more dominant than non-AM plants, and the plant roots are the main nutrient uptake site of the plant [40] and can influence the nutrient transport and transfer in the plant, and at the same N concentration, the nutrient content of the below-ground part of AM plants was significantly higher than that of non-AM plants, while at the same N concentration, the nutrient content of the below-ground part of AM plants was significantly higher than that of non-AM plants, while the above-ground part was not significantly different from that of non-AM plants, indicating that AM plants have higher nutrient uptake than non-AM plants and their nutrient regulation is more flexible among different tissues.

4.3 AMF promotes the accumulation of saponins

AMF symbiosis with plants can promote the growth and development of plants, while the addi-

tion of N-substance is also the main way to increase the yield of crops. Usually, the most important indicator of a crop is its yield, but the most important substance of a medicinal plant is its medicinal component. Studies have shown that AMF is effective in increasing the accumulation of secondary metabolites in plants [44] and also in increasing the synthesis of terpenoids in plants [45]. In this experiment, the content of *P. polyphylla* var. *yunnanensis* I appeared significantly elevated after inoculation with AMF, with N1Ge and N1Po being the treatments with the greatest elevation, while in the N2 and N3 treatments, Ge inoculation instead reduced the content of *P. polyphylla* var. *yunnanensis* I, with a trend in the opposite direction to the trend of N vegetation concentration, while Po, although it also showed a reduction, still had a higher saponin I content than non-AM plants. In saponin II, Ge and Po increased the content of saponin II under N1 and N2, but Ge decreased the content of saponin II under N3 treatment. The content of saponin D was detected only in N1CK, N1Po, N2CK and N3Po, with lower levels under N1 treatment and relatively higher levels under N2 and N3. The increase in N content also increased the content of saponin H, and Ge and Po increased it the most, but there was no difference in the effect of the two AMFs on saponin content at high N concentrations, a trend similar to that of *P. polyphylla* var. *yunnanensis* saponin VII changes were similar. From this experiment, it is clear that the symbiosis of *P. polyphylla* var. *yunnanensis* with AMF at low N concentration can promote the accumulation of its saponin content, mainly in saponin I, II, VII and H. It shows that the promotion of secondary metabolite accumulation by AMF is selective [46]. Under low and medium nitrogen conditions, plants relied more on the AMF pathway for nutrient uptake, and through increased nutrient accumulation, the content of precursors for saponin synthesis was increased, thus improving the synthesis of total saponins. With the increase of N concentration, AM *P. polyphylla* var. *yunnanensis* saponin also possessed higher saponin content at medium N concentration, but when the soil N concentration increased to high N, the advantage brought by AMF inoculation was not significant and even decreased, and the accumulation trend of saponin in *P. polyphylla* var. *yunnanensis* saponin was not uniform for both AMFs when they were in high N soil. It can be shown that AMF under low and medium nitrogen on *P. polyphylla* var. *yunnanensis* saponin content accumulation helps more, in the nitrogen test, the nutrient content of the underground part of AM *P. polyphylla* var. *yunnanensis* is significantly higher than non-AM plants, and the highest content of the same in low and medium nitrogen concentration treatment, this trend is similar to the saponin accumulation trend of *P. polyphylla* var. *yunnanensis* saponin, which may be due to AMF can not only help the plant to absorb more mineral nutrients but also help plants to better regulate nutrient transfer transport and in this way increased the synthesis of saponins in *P. polyphylla* var. *yunnanensis*, which is similar to some studies [47]. At high N concentrations, the root nutrient and saponin contents of the plants were much less enhanced than those of non-AM plants at low N concentrations, although they were higher than those of AM plants. For medicinal plants, their yield is far more important than their biomass increase, and further experiments are needed to determine which N content is more appropriate for AM *P. polyphylla* var. *yunnanensis* to accumulate saponin components in symbiosis between AMF and *P. polyphylla* var. *yunnanensis*. In the present study, we also found that saponin VI was not detected in both AMF plants and under all three nitrogen level treatments, suggesting that AMF is specific for the alteration of secondary metabolites, which may be related to AMF itself. During plant growth, plants preferentially increase their biomass [48].In contrast, secondary metabolites are usually not directly involved in plant growth and development and only help plants to improve ecological adaptations, which may be one of the reasons why sustained increases in N do not always improve saponins. AMF alters the nutrient balance in plants by increasing nutrient uptake capacity, and AM plants have higher C:N compared to non-AMF plants, which allows more nutrients to be used for the synthesis of secondary

metabolites [18].

4.4 AMF promotes the expression of genes related to saponin synthesis

Studies have shown that overexpression of key genes on the steroid saponin synthesis pathway, HMGS, GGPS, FPPS, SS, CAS and SE2, promotes the accumulation of bioactive components and improves saponins in host plants [47], and in this experiment, it can be found that AMF increased the expression of HMGS, a key enzyme on the steroid saponin synthesis pathway, in the plants at low and medium N concentrations, indicating that AMF plants promoted the synthesis of saponin in the heavy floor by absorbing more nutrient elements, but its expression was lower than that of CK plants at high N concentrations, which may be related to the decrease in AM colonization rate, and although the expression of HMGS also appeared elevated at this N concentration, its increase was less than that of AM plants, which is also consistent with the trend of saponin accumulation in the plants in the experiment. Secondly, AMF also increased the expression of SE1 and CAS under low nitrogen concentration, indicating that AMF played a role in promoting saponin synthesis to some extent. To some extent, AMF is able to achieve changes in plant saponin content by regulating relevant genes on the steroid saponin synthesis pathway. In non-AMF plants, increased nitrogen concentration also promoted the expression of HMGS, CAS, and FPP. HMGS acts as the main rate-limiting enzyme gene and the resulting cascade reaction is able to increase the saponin content of the plant [49].

4.5 Transcriptome analysis

Transcriptome sequencing is a cost-effective method that is currently used extensively to uncover genomic information from different non-model medicinal plants that do not have a reference genome [50]. In this study, we used different nitrogen and AMF treatments of *P. polyphylla* var. *yunnanensis* and then analyzed its transcriptome data to uncover the P450 and UGT genes involved in the *P. polyphylla* var. *yunnanensis* saponin biosynthesis pathway. Steroid saponins are mainly synthesized from cholesterol via oxidation at the C-16, C-22 and C-26 positions [51], where P450 enzymes play an important role in the catalysis of these transformations. CYP90G4 and CYP51G have been shown to aid in the synthesis of steroid saponins in Dendrobium plants [52,53], and DN66132_c0_g1 is close to PpCYP90G4 and has nine unigenes enriched in the Ath CYP51G family, suggesting their possible involvement in steroid saponin synthesis. In addition to P450s, UGTs are also involved in the final modification of saponins, where the glycosylation of C-26 is catalyzed by UGTs, and it was shown that genes in the UGT73 family are able to glycosylate the C-26 position of saponin elements [26]. It suggests that seven unigenes concentrated in the UGT73 family may play a catalytic role in the glycosylation of *P. polyphylla* var. *yunnanensis* saponin. Cluster analysis showed that both Ge and Po promoted the expression of different P450s and UGTs in low and medium nitrogen treatments, whereas, in non-AM plants, significant up-regulation of P450s and UGTs was found only in N3 treatment, a pattern also similar to that of saponin accumulation. Both AMFs had no up-regulation on plant P450s and UGTs at high N concentrations, which may also be one of the reasons for the low total saponins in AM plants in high N treatments. HMGS, CAS, and FPP increased their expression with nitrogen addition, while the expression of SE1, SE2, and SS showed down-regulation under high nitrogen treatment, but in non-AM plants, the saponin content reached its maximum under high nitrogen treatment, indicating that oxidative glycosylation by the upstream rate-limiting gene HMGS and the downstream P450 and UGT are key factors in saponin synthesis pathway, and the up-regulation of P450 and UGT could offset the negative effects of the decreased expression of SE1, SE2, and SS to some extent. In addition, the P450 gene 43198 appeared to be down-regulated in AM plants under high nitrogen treatment (**Figure 11B**), which may be one of the reasons for the decrease in saponin content, and the synthesis of steroidal saponins generally oc-

curs through the MVA pathway during plant growth metabolism [54], in turn, free sterols are also a major component in controlling cell membrane fluidity and permeability [55]. In high nitrogen environments, plants do not need to rely too much on AMF for nutrient uptake, which may lead to the need for more sterol substances for membrane permeability regulation in response to excessive nitrogen in the environment, and the precursor substances for steroid saponin synthesis are then decreased, which may also be one of the reasons for the down-regulation of P450.

5. Conclusions

In order to investigate the effect of AMF on saponin synthesis of *P. polyphylla* var. *yunnanensis* under nitrogen addition, this experiment used *P. polyphylla* var. *yunnanensis* as the study material and initially investigated the effect of AMF on saponin synthesis of *P. polyphylla* var. *yunnanensis* under nitrogen addition by measuring the growth, chlorophyll, hormones, nutrients, saponin content and gene expression on the saponin synthesis pathway of *P. polyphylla* var. *yunnanensis*. Nitrogen addition was able to increase the saponin content of *P. polyphylla* var. *yunnanensis* to some extent, but AMF plants increased the saponin content of *P. polyphylla* var. *yunnanensis* more significantly, and inoculation with AMF also promoted the expression of genes related to the saponin synthesis pathway, HMGS, SE1 and CAS. Thus, the accumulation of saponin in the plants was increased. And probably by altering the expression of saponin synthesis-related P450 and UGT, thus increasing the saponin content of AMF plants.

Author Contributions

Shubiao Qian, was responsible for the whole experiment, raw data collection, collation and analysis, and original manuscript writing. Huang Can assisted in the experiment. Xiaoxian Li, Xiahong He, Shuhui Zi, Congfang Xi, Rui Shi, and Tao Liu and others are responsible for guiding the experiment and article revision.

Conflict of Interest

The authors declare that they have no conflict of interests concerning the current research publication.

Data Availability Statement

The original read registration numbers are PRJNA864763, located in the NCBI SRA database, https://www.ncbi.nlm.nih.gov/bioproject/PRJNA864763

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References

[1] Guo, J.H., Liu, X.J., Zhang, Y., et al., 2010. Significant acidification in major Chinese croplands. Science. 327(5968), 1008-1010. DOI: https://doi.org/10.1126/science.1182570

[2] Raza, S., Miao, N., Wang, P., et al., 2020. Dramatic loss of inorganic carbon by nitrogen-induced soil acidification in Chinese croplands. Global Change Biology. 26(6), 3738-3751. DOI: https://doi.org/10.1111/gcb.15101

[3] Ramirez, K.S., Craine, J.M., Fierer, N., 2012. Consistent effects of nitrogen amendments on soil microbial communities and processes across

biomes. Global Change Biology. 18(6), 1918-1927.
DOI: https://doi.org/10.1111/j.1365-2486.2012.02639.x

[4] Walters, D.R., Bingham, I.J., 2007. Influence of nutrition on disease development caused by fungal pathogens: Implications for plant disease control. Annals of Applied Biology. 151(3), 307-324.
DOI: https://doi.org/10.1111/j.1744-7348.2007.00176.x

[5] Hestrin, R., Hammer, E.C., Mueller, C.W., et al., 2019. Synergies between mycorrhizal fungi and soil microbial communities increase plant nitrogen acquisition. Communications Biology. 2(1), 233.
DOI: https://doi.org/10.1038/s42003-019-0481-8

[6] Müller, L.M., Harrison, M.J., 2019. Phytohormones, miRNAs, and peptide signals integrate plant phosphorus status with arbuscular mycorrhizal symbiosis. Current Opinion in Plant Biology. 50, 132-139.
DOI: https://doi.org/10.1016/j.pbi.2019.05.004

[7] Smith, S.E., Smith, F.A., Jakobsen, I., 2004. Functional diversity in arbuscular mycorrhizal (AM) symbioses: The contribution of the mycorrhizal P uptake pathway is not correlated with mycorrhizal responses in growth or total P uptake. New Phytologist. 162(2), 511-524.
DOI: https://doi.org/10.1111/j.1469-8137.2004.01039.x

[8] Johansen, A., Jakobsen, I., Jensen, E.S., 1993. Hyphal transport by a vesicular-arbuscular mycorrhizal fungus of N applied to the soil as ammonium or nitrate. Biology and Fertility of Soils. 16, 66-70.
DOI: https://doi.org/10.1007/bf00336518

[9] Hodge, A., Campbell, C.D., Fitter, A.H., 2001. An arbuscular mycorrhizal fungus accelerates decomposition and acquires nitrogen directly from organic material. Nature. 413(6853), 297-299.
DOI: https://doi.org/10.1038/35095041

[10] Koegel, S., Ait Lahmidi, N., Arnould, C., et al., 2013. The family of ammonium transporters (AMT) in s orghum bicolor: Two AMT members are induced locally, but not systemically in roots colonized by arbuscular mycorrhizal fungi. New Phytologist. 198(3), 853-865.
DOI: https://doi.org/10.1111/nph.12199

[11] Chen, A., Gu, M., Wang, S., et al., 2018. Transport properties and regulatory roles of nitrogen in arbuscular mycorrhizal symbiosis. Seminars in Cell & Developmental Biology. 74, 80-88.
DOI: https://doi.org/10.1016/j.semcdb.2017.06.015

[12] Bona, E., Lingua, G., Manassero, P., et al., 2015. AM fungi and PGP pseudomonads increase flowering, fruit production, and vitamin content in strawberry grown at low nitrogen and phosphorus levels. Mycorrhiza. 25, 181-193.
DOI: https://doi.org/10.1007/s00572-014-0599-y

[13] Bills, G.F., Gloer, J.B., 2017. Biologically active secondary metabolites from the fungi. The Fungal Kingdom. 4(6), 1087-1119.
DOI: https://doi.org/10.1128/microbiolspec.FUNK-0009-2016

[14] Boby, V.U., Bagyaraj, D.J., 2003. Biological control of root-rot of Coleus forskohlii Briq. using microbial inoculants. World Journal of Microbiology and Biotechnology. 19(2), 175-180.
DOI: https://doi.org/10.1023/A:1023238908028

[15] Yu, Y., Yu, T., Wang, Y., et al., 2010. Effect of inoculation time on camptothecin content in arbuscular mycorrhizal Camptotheca acuminata seedlings. Chinese Journal of Plant Ecology. 34(6), 687.
DOI: https://doi.org/10.3724/SP.J.1142.2010.40521

[16] Mandal, A., Mandal, S., Park, M.H., 2014. Genome-wide analyses and functional classification of proline repeat-rich proteins: Potential role of eIF5A in eukaryotic evolution. PloS One. 9(11), e111800.
DOI: https://doi.org/10.1371/journal.pone.0111800

[17] Mandal, S., Upadhyay, S., Singh, V.P., et al., 2015. Enhanced production of steviol glycosides in mycorrhizal plants: A concerted effect of arbuscular mycorrhizal symbiosis on transcription of biosynthetic genes. Plant Physiology and

Biochemistry. 89, 100-106.
DOI: https://doi.org/10.1016/j.plaphy.2015.02.010

[18] Xie, W., Hao, Z., Zhou, X., et al., 2018. Arbuscular mycorrhiza facilitates the accumulation of glycyrrhizin and liquiritin in Glycyrrhiza uralensis under drought stress. Mycorrhiza. 28, 285-300.
DOI: https://doi.org/10.1007/s00572-018-0827-y

[19] Chaudhary, V., Kapoor, R., Bhatnagar, A.K., 2008. Effectiveness of two arbuscular mycorrhizal fungi on concentrations of essential oil and artemisinin in three accessions of Artemisia annua L. Applied Soil Ecology. 40(1), 174-181.
DOI: https://doi.org/10.1016/j.apsoil.2008.04.003

[20] Zeng, Y., Guo, L.P., Chen, B.D., et al., 2013. Arbuscular mycorrhizal symbiosis and active ingredients of medicinal plants: Current research status and prospectives. Mycorrhiza. 23, 253-265.
DOI: https://doi.org/10.1007/s00572-013-0484-0

[21] Weng, W., Yan, J., Zhou, M., et al., 2022. Roles of arbuscular mycorrhizal fungi as a biocontrol agent in the control of plant diseases. Microorganisms. 10(7), 1266.
DOI: https://doi.org/10.3390/microorganisms10071266

[22] Tsiokanos, E., Cartabia, A., Tsafantakis, N., et al., 2022. The metabolic profile of Anchusa officinalis L. differs according to its associated arbuscular mycorrhizal fungi. Metabolites. 12(7), 573.
DOI: https://doi.org/10.3390/metabo12070573

[23] Toussaint, J.P., Smith, F.A., Smith, S.E., 2007. Arbuscular mycorrhizal fungi can induce the production of phytochemicals in sweet basil irrespective of phosphorus nutrition. Mycorrhiza. 17(4), 291-297.
DOI: https://doi.org/10.1007/s00572-006-0104-3

[24] Zhao, Y., Cartabia, A., Lalaymia, I., et al., 2022. Arbuscular mycorrhizal fungi and production of secondary metabolites in medicinal plants. Mycorrhiza. 32(3-4), 221-256.
DOI: https://doi.org/10.1007/s00572-022-01079-0

[25] Wu, X., Wang, L., Wang, H., et al., 2012. Steroidal saponins from *Paris polyphylla* var. *yunnanensis*. Phytochemistry. 81, 133-143.
DOI: https://doi.org/10.1016/j.phytochem.2012.05.034

[26] Upadhyay, S., Jeena, G.S., Shukla, R.K., 2018. Recent advances in steroidal saponins biosynthesis and in vitro production. Planta. 248(3), 519-544.
DOI: https://doi.org/10.1007/s00425-018-2911-0

[27] Gao, X., Zhang, X., Chen, W., et al., 2020. Transcriptome analysis of *Paris polyphylla* var. *yunnanensis* illuminates the biosynthesis and accumulation of steroidal saponins in rhizomes and leaves. Phytochemistry. 178, 112460.
DOI: https://doi.org/10.1016/j.phytochem.2020.112460

[28] Trouvelot, A., Kough, J.L., Gianinazzi-Pearson, V. (editors), 1985. Mesure du taux de mycorhization VA d'un système radiculaire. Recherche de méthode d'estimation ayant une signification fonctionnelle. Physiological and genetical aspects of mycorrhizae: Proceedings of the 1st european symposium on mycorrhizae (French) [Measurement of the VA mycorrhization rate of a root system. Physiological and genetical aspects of mycorrhizae: Proceedings of the 1st european symposium on mycorrhizae]. European symposium on mycorrhizae (1; Dijon 1985-07-01); 1985 1-5 July; Dijon. Paris:INRA, Paris. p. 217-221.

[29] Sartory, D.P., Grobbelaar, J.U., 1984. Extraction of chlorophyll a from freshwater phytoplankton for spectrophotometric analysis. Hydrobiologia. 114, 177-187.
DOI: https://doi.org/10.1007/BF00031869

[30] Li, T., Sun, Y., Ruan, Y., et al., 2016. Potential role of D-myo-inositol-3-phosphate synthase and 14-3-3 genes in the crosstalk between Zea mays and Rhizophagus intraradices under drought stress. Mycorrhiza. 26, 879-893.
DOI: https://doi.org/10.1007/s00572-016-0723-2

[31] Bates, L.S., Waldren, R.A., Teare, I.D., 1973. Rapid determination of free proline for water-stress studies. Plant and Soil. 39, 205-207.
DOI: https://doi.org/10.1007/bf00018060

[32] Conesa, A., Götz, S., García-Gómez, J.M., et al., 2005. Blast2GO: A universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics. 21(18), 3674-3676. DOI: https://doi.org/10.1093/bioinformatics/bti610

[33] Lanfranco, L., Fiorilli, V., Gutjahr, C., 2018. Partner communication and role of nutrients in the arbuscular mycorrhizal symbiosis. New Phytologist. 220(4), 1031-1046. DOI: https://doi.org/10.1111/nph.15230

[34] Zhou, Z., Wang, C., Zheng, M., et al., 2017. Patterns and mechanisms of responses by soil microbial communities to nitrogen addition. Soil Biology and Biochemistry. 115, 433-441. DOI: https://doi.org/10.1016/j.soilbio.2017.09.015

[35] Wang, L., Chen, X., Du, Y., et al., 2022. Nutrients regulate the effects of arbuscular mycorrhizal fungi on the growth and reproduction of cherry tomato. Frontiers in Microbiology. 13, 843010. DOI: https://doi.org/10.3389/fmicb.2022.843010

[36] Zhang, S., Lehmann, A., Zheng, W., et al., 2019. Arbuscular mycorrhizal fungi increase grain yields: A meta-analysis. New Phytologist. 222(1), 543-555. DOI: https://doi.org/10.1111/nph.15570

[37] Ma, N., Yokoyama, K., Marumoto, T., 2007. Effect of peat on mycorrhizal colonization and effectiveness of the arbuscular mycorrhizal fungus Gigaspora margarita. Soil Science and Plant Nutrition. 53(6), 744-752. DOI: https://doi.org/10.1111/j.1747-0765.2007.00204.x

[38] Clark, R.Á., Zeto, S.K., 2000. Mineral acquisition by arbuscular mycorrhizal plants. Journal of Plant Nutrition. 23(7), 867-902. DOI: https://doi.org/10.1080/01904160009382068

[39] Foo, E., Ross, J.J., Jones, W.T., et al., 2013. Plant hormones in arbuscular mycorrhizal symbioses: An emerging role for gibberellins. Annals of Botany. 111(5), 769-779. DOI: https://doi.org/10.1093/aob/mct041

[40] Averill, C., Bhatnagar, J.M., Dietze, M.C., et al., 2019. Global imprint of mycorrhizal fungi on whole-plant nutrient economics. Proceedings of the National Academy of Sciences. 116(46), 23163-23168. DOI: https://doi.org/10.1073/pnas.1906655116

[41] Wang, W., Shi, J., Xie, Q., et al., 2017. Nutrient exchange and regulation in arbuscular mycorrhizal symbiosis. Molecular Plant. 10(9), 1147-1158. DOI: https://doi.org/10.1016/j.molp.2017.07.012

[42] Kobae, Y., Tamura, Y., Takai, S., et al., 2010. Localized expression of arbuscular mycorrhiza-inducible ammonium transporters in soybean. Plant and Cell Physiology. 51(9), 1411-1415. DOI: https://doi.org/10.1093/pcp/pcq099

[43] Wang, S., Chen, A., Xie, K., et al., 2020. Functional analysis of the OsNPF4. 5 nitrate transporter reveals a conserved mycorrhizal pathway of nitrogen acquisition in plants. Proceedings of the National Academy of Sciences. 117(28), 16649-16659. DOI: https://doi.org/10.1073/pnas.2000926117

[44] Dang, H., Zhang, T., Wang, Z., et al., 2021. Succession of endophytic fungi and arbuscular mycorrhizal fungi associated with the growth of plant and their correlation with secondary metabolites in the roots of plants. BMC Plant Biology. 21, 1-16. DOI: https://doi.org/10.1186/s12870-021-02942-6

[45] Sharma, E., Anand, G., Kapoor, R., 2017. Terpenoids in plant and arbuscular mycorrhiza-reinforced defence against herbivorous insects. Annals of Botany. 119(5), 791-801. DOI: https://doi.org/10.1093/aob/mcw263

[46] Cartabia, A., Tsiokanos, E., Tsafantakis, N., et al., 2021. The arbuscular mycorrhizal fungus Rhizophagus irregularis MUCL 41833 modulates metabolites production of Anchusa officinalis L. under semi-hydroponic cultivation. Frontiers in Plant Science. 12, 724352. DOI: https://doi.org/10.3389/fpls.2021.724352

[47] Pedone-Bonfim, M.V.L., da Silva, D.K.A., da Silva-Batista, A.R., et al., 2018. Mycorrhizal inoculation as an alternative for the sustainable production of Mimosa tenuiflora seedlings with improved growth and secondary compounds

content. Fungal Biology. 122(9), 918-927.
DOI: https://doi.org/10.1016/j.funbio.2018.05.009

[48] McCarthy, M.C., Enquist, B.J., 2007. Consistency between an allometric approach and optimal partitioning theory in global patterns of plant biomass allocation. Functional Ecology. 21(4), 713-720.
DOI: https://doi.org/10.1111/j.1365-2435.2007.01276.x

[49] Enfissi, E.M., Fraser, P.D., Lois, L.M., et al., 2005. Metabolic engineering of the mevalonate and non-mevalonate isopentenyl diphosphate-forming pathways for the production of health-promoting isoprenoids in tomato. Plant Biotechnology Journal. 3(1), 17-27.
DOI: https://doi.org/10.1111/j.1467-7652.2004.00091.x

[50] Gupta, P., Goel, R., Agarwal, A.V., et al., 2015. Comparative transcriptome analysis of different chemotypes elucidates withanolide biosynthesis pathway from medicinal plant Withania somnifera. Scientific Reports. 5(1), 18611.
DOI: https://doi.org/10.1038/srep18611

[51] Thimmappa, R., Geisler, K., Louveau, T., et al., 2014. Triterpene biosynthesis in plants. Annual Review of Plant Biology. 65, 225-257.
DOI: https://doi.org/10.1146/annurev-arplant-050312-120229

[52] Christ, B., Xu, C., Xu, M., et al., 2019. Repeated evolution of cytochrome P450-mediated spiroketal steroid biosynthesis in plants. Nature Communications. 10(1), 3206.
DOI: https://doi.org/10.1038/s41467-019-11286-7

[53] Bak, S., Kahn, R.A., Olsen, C.E., et al., 1997. Cloning and expression in Escherichia coli of the obtusifoliol 14α-demethylase of Sorghum bicolor (L.) Moench, a cytochrome P450 orthologous to the sterol 14α-demethylases (CYP51) from fungi and mammals. The Plant Journal. 11(2), 191-201.
DOI: https://doi.org/10.1046/j.1365-313x.1997.11020191.x

[54] Liao, P., Chen, X.J., Wang, M.F., et al., 2018. Improved fruit α-tocopherol, carotenoid, squalene and phytosterol contents through manipulation of *Brassica juncea* 3-HYDROXY-3-METHYLGLUTARYL-COA SYNTHASE1 in transgenic tomato. Plant Biotechnology Journal. 16(3), 784-796.
DOI: https://doi.org/10.1111/pbi.12828

[55] Piironen, V., Lindsay, D.G., Miettinen, T.A., et al., 2000. Plant sterols: Biosynthesis, biological function and their importance to human nutrition. Journal of the Science of Food and Agriculture. 80(7), 939-966.
DOI: https://doi.org/10.1002/(SICI)1097-0010(20000515)80:7<939::AID-JSFA644>3.0.CO;2-C