

Article

Nitrogen Forms Alter Triterpenoid Accumulation and Related Gene Expression in *Cyclocarya paliurus* (Batalin) Iljinsk. Seedlings

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Abstract: *Cyclocarya paliurus* (Batalin) Iljinsk. is a multiple function tree species distributed in subtropical areas, and its leaves have been used in medicine and nutraceutical foods in China. However, little information on the effects of nitrogen (N) forms and ratios on growth and secondary metabolite accumulation is available for *C. paliurus*. The impact of five $\text{NO}_3^-/\text{NH}_4^+$ ratios on biomass production, triterpenoid accumulation and related gene expression in *C. paliurus* seedlings was evaluated at the middle N nutrition supply. Significant differences in seedling growth, triterpenoid accumulation and relative gene expression were observed among the different $\text{NO}_3^-/\text{NH}_4^+$ ratio treatments. The highest triterpenoid content was achieved in a sole NO_3^- or NH_4^+ nutrition, while the mixed N nutrition with equal ratio of NO_3^- to NH_4^+ produced the highest biomass production in the seedlings. However, the highest triterpenoid accumulation was achieved at the treatment with the ratio of $\text{NO}_3^-/\text{NH}_4^+ = 2.33$. Therefore, the mixed N nutrition of NO_3^- and NH_4^+ was beneficial to the triterpenoid accumulation per plant. The relative expression of seven genes that are involved in triterpenoid biosynthesis were all up-regulated under the sole NH_4^+ or NO_3^- nutrition conditions, and significantly positive correlations between triterpenoid content and relative gene expression of key enzymes were detected in the leaves. Our results indicated that NO_3^- is the N nutrition preferred by *C. paliurus*, but the mixture of NO_3^- and NH_4^+ at an appropriate ratio would improve the leaf triterpenoid yield per area.

Keywords: *Cyclocarya paliurus*; biomass; nitrogen nutrition; secondary metabolite; trade-off

1. Introduction

As one of the important secondary metabolites in plants, triterpenoids allow plant to better adapt to the environment, such as their defence against natural enemies and plant communication [1], but also have been confirmed to contain a variety of health-promoting effects in human beings, such as anti-hyperglycemic, anti-hyperlipidemic and antioxidant effects [2–4]. In plants, triterpenoids are synthesized by the isoprenoid pathway, which is composed of three synthetic stages: (1) 3-isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) are formed by mevalonate acid (MVA) or the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway [5]; (2) 2,3-oxidosqualene is formed by geranyl diphosphate synthase (GPS), farnesyl diphosphate synthase (FPPS), squalene synthase (SQS) and squalene epoxidase (SE), which is subsequently cyclized to triterpenoid skeletons by 2,3-oxidosqualene cyclases (OSCs). Then, cytochrome P450 monooxygenase (PDMO) catalyzes oxidation of triterpenoid skeletons to produce aglycones; (3) the aglycones are glycosylated to triterpenoid saponins by glycosyltransferase (GT) [6] (Figure 1).

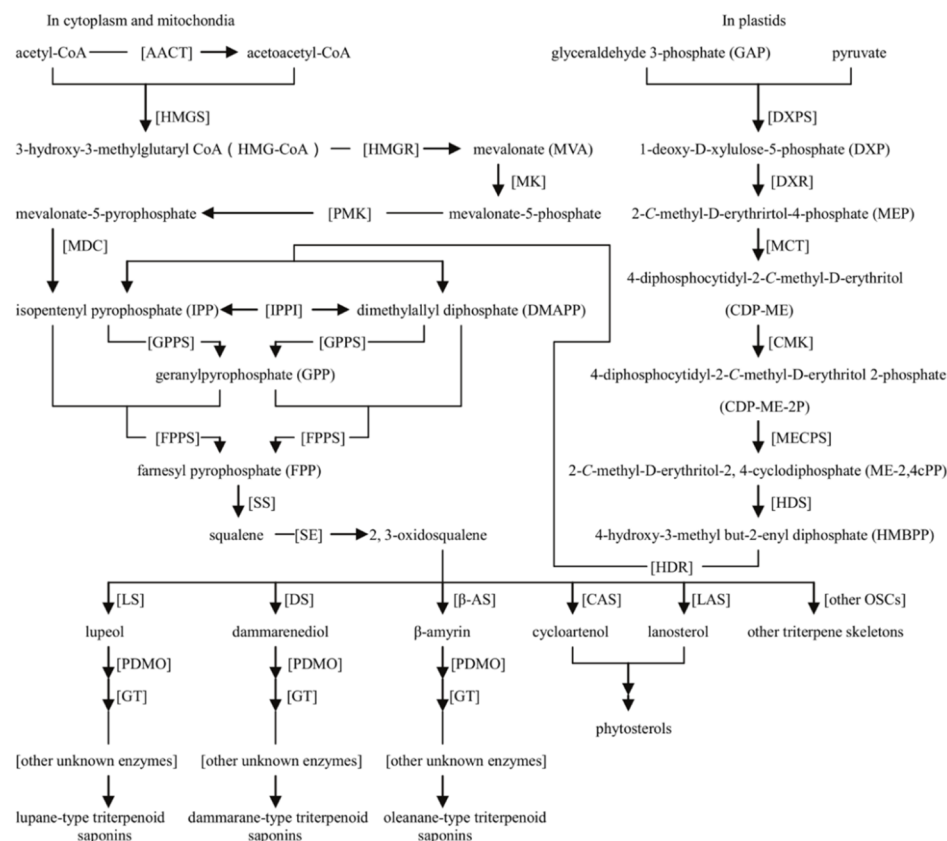


Figure 1. Biosynthetic pathway of triterpenoid in higher plants. Abbreviations: AACT: acetyl CoA: acetyl CoA C-acetyltransferase or acetoacetyl-CoA thiolase; CAS: cycloartenol synthase; CMK: 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase; DS: dammaradiol synthase; DXPS, i.e., DXS: 1-deoxy-D-xylulose-5-phosphate synthase; DXR1-deoxy-D-xylulose-5-phosphate reductoisomerase; FPPS: farnesyl diphosphate synthase; GPPS: geranyl diphosphate synthase; GT: glycosyltransferases; HDR: 4-hydroxy-3-methyl but-2-(E)-enyl diphosphate reductase; HDS: 4-hydroxy-3-methyl but-2-(E)-enyl diphosphate synthase; HMGR: 3-hydroxy-3-methylglutaryl CoA reductase; HMGS: 3-hydroxy-3-methylglutaryl CoA synthase; IPPI: isopentenyl diphosphate isomerase; LAS: lanosterol synthase; LS, i.e., LUS: lupeol synthase; MCT: 2-C-methyl-D-erythritol 4-phosphate cytidyl transferase or 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol synthase; MDC: mevalonate-5-pyrophosphate decarboxylase; MECPS: 2-C-methyl-D-erythritol-2, 4-cyclodiphosphate synthase; MK: mevalonate kinase; OSCs: 2, 3-oxidosqualene cyclases; PDMO: cytochrome P 450-dependent monooxygenases; PMK: phosphomevalonate kinase; SE: squalene epoxidase; SS: squalene synthase; β-AS: β-amyrin synthase (quoted from Zhao et al. [7]).

Cyclocarya paliurus (Batalin) Iljinsk., a member of Juglandaceae, is mainly scattered in the mountainous across sub-tropical regions of China [8]. Leaves of *C. paliurus* have been made into nutraceutical tea for a long time in China, while its leaf, bark and root are used in traditional Chinese medicine [9,10]. Moreover, the leaves of *C. paliurus* have been listed as new food raw material by the National Health and Family Planning Commission of China since 2013 [11]. Previous studies have found that *C. paliurus* leaves contain various bioactive constituents, including polyphenolics, flavonoids, triterpenoids, polysaccharides [4,11,12]. However, triterpenoids in *C. paliurus* play a very important role in the health-promoting effect, such as enhancing antihyperlipidemic activity and ameliorating diabetes [13,14]. Moreover, there are several specific triterpenoids that have been identified and isolated from *C. paliurus* leaves [15,16]. Due to its various health-promoting effects, a huge production of *C. paliurus* leaves is required for tea production and for medical use. However, *C. paliurus* is mainly distributed in natural forests and there are not enough *C. paliurus* plantations to produce the leaves.

Therefore, there is an urgent need to develop *C. paliurus* plantations for meeting the strong market demand for high-quality raw material.

Environmental factors can affect growth, secondary metabolite biosynthesis and accumulation in plants. It was reported that the concentration of CO₂ in the Earth's atmospheric would increase to between 600 and 1000 µmol/mol by the end of 21st century [17]. This will affect the ratio of carboxylation to oxygenation, and consequently decrease photorespiration. Nitrogen (N), as an important environmental factor for plant growth, is primarily absorbed by plants in the form of ammonium (NH₄⁺) and nitrate (NO₃⁻) [18]. Many studies have indicated that the response of higher plants to elevated CO₂ is determined by nitrogen availability, and is primarily related to the different forms of nitrogen nutrition [17,19–21]. Modification of the NO₃⁻/NH₄⁺ ratio can modulate the relative uptake of other anion and cations, consequently modifying primary and secondary metabolism and influencing plant growth and quality. Therefore the N forms and the most effective ratio of NO₃⁻/NH₄⁺ have been received considerable attentions in the research of N nutrition of plants [22]. However, most of the studies were focused on effects of N forms on the growth and quality of crops, vegetables and some herbal medicinal plants [17,22–24]. For example, previous studies showed that nitrogen form and ratio affect not only plant growth but also the secondary metabolite of herbal medicinal plants, such as *Artemisia annua* [25], *Eleutherococcus koreanum* [26], *Centella asiatica* [23], and *Prunella vulgaris* [27]. However, the responses of different plants to the nitrogen form and ratio vary with species [17,22,27,28], which could be linked to alterations in gene expression and readjustments of metabolic processes [29]. Alterations in secondary metabolite profiles of medicinal plants may improve their pharmacological properties, and can be potentially used to stimulate the synthesis of phytochemicals with beneficial properties in medicinal plants. However, biomass production is another key parameter for the cultivation of medicinal plants [30,31]. Hence, the trade-off between biomass production and pharmaceutically active metabolite content is a key parameter for culturing medicinal plants. To date, the effects of nitrogen forms and ratios on secondary metabolites were most reported in agricultural crops, horticultural and herbal medicinal plants, and little information is available for *C. paliurus*. Furthermore, the response of triterpenoid biosynthesis genes to nitrogen forms and ratios in *C. paliurus* is poorly understood. Therefore, it is critical to systematically assess the impact of fertilization on the growth, development, and secondary metabolism of woody medicinal plants. The objective of this study was to detect the effect of nitrogen forms and ratios on triterpenoid accumulation, and to elucidate the response of triterpenoid biosynthesis genes to nitrogen forms and ratios in *C. paliurus*. The result aims to provide optimal cropping strategies for harvesting the higher yield of triterpenoid of *C. paliurus*.

2. Material and Methods

2.1. Plant Material and Experimental Design

The experiment was carried out at Nanjing Forestry University (31°59' N, 119°18' E) in 2018. Seeds of *C. paliurus* were collected from Wufeng county (30°41' N, 119°41' E), Hubei province, China in late October 2016. The seeds were subjected to exogenous gibberellin A3 (GA3) and stratification treatments to break seed dormancy, according to the methods of Fang et al. [8]. The germinated seedlings were planted in non-woven containers filled with turfy soil/perlite/composted poultry manure/soil (4/2/2/2, v/v/v/v) in April 2018. The substrate was a loam with pH 6.4, organic matter content of 86.3 g/kg, total N content of 63.5 g/kg, total P content of 3.0 g/kg, and total K content of 10.1 g/kg. After 3 months of cultivation, *C. paliurus* seedlings with base diameter around 3.5 mm and height around 21.0 cm were moved into a greenhouse and transferred to the 35 L polypropylene containers with full-strength Hoagland nutrient solution. The full-strength nutrient solution contained the following macronutrients (mg/L): NO₃⁻-N (224), NH₄⁺-N (14.0), PO₄³⁻-P (15.5), K (298.0), Mg (48.1), Ca (210) and micronutrients (mg/L): B (0.5), Mn (0.5), Zn (0.5), Cu (0.5), Mo (0.5) and Fe (5.6).

After cultivated in the nutrient solution for 1 week, *C. paliurus* seedlings were carried out for N form and ratio treatments. Twenty-one seedlings for each treatment were used, which were arranged in a completely randomized design. Treatments consisted of N at 238 mg/L supplied as (T1) 100% NO_3^- -N: 0% NH_4^+ -N, (T2) 70% NO_3^- -N: 30% NH_4^+ -N, (T3) 50% NO_3^- -N: 50% NH_4^+ -N, (T4) 30% NO_3^- -N: 70% NH_4^+ -N, and (T5) 0% NO_3^- -N: 100% NH_4^+ -N. NH_4^+ -N was supplied as $(\text{NH}_4)_2\text{SO}_4$, while NO_3^- -N was supplied as $\text{Ca}(\text{NO}_3)_2$ and KNO_3 . Throughout the experiment, the pH of each nutrient solution was adjusted to 5.5 ± 0.2 with 0.1 mol/L NaOH or 0.1 mol/L HCl. To prevent nitrification, the nitrification inhibitor dicyandiamide (DCD, 7% of total nitrogen content) was added to the nutrient solution. The solution was replaced on a weekly basis and aerated with an electric pump.

Environmental factors were monitored using a hand-held Agricultural Weather Station (TNHY series model, Zhejiang Top Instrument Co. Ltd., Hangzhou, China). The air temperature varied between 13 and 27 °C. The relative humidity of air varied between 55 and 80%. The photosynthetic photon flux density (PPFD) varied between 256.4 and 487.6 $\mu\text{mol}/(\text{m}^2 \cdot \text{s})$.

2.2. Plant Material Sampling and Biomass Measurements

Plant materials (leaf, stem and root) were harvested and sampled at 30, 60 and 90 days after the N form treatments respectively, based on the mean base diameter and height of seedlings for each treatment. Three replicates of each treatment were sampled and harvested for biomass measurement, then washed and separated according to the components (leaf, stem and root). All of the sampling components were dried to constant weight at 70 °C. Total biomass of each seedling was calculated as the sum of root, stem and leaf biomass.

2.3. Measurement of Triterpenoid Contents

Triterpenoids were extracted as described by Cao et al. [32]. The total triterpenoid content was measured following the method of Fan and He [33], and the oleanolic acid was used as standard curve for calculating triterpenoid content.

The individual triterpenoid content was detected using high-performance liquid chromatography [32]. The mobile phases were composed of water/acetic acid (10,000/1, v/v) (A) and acetonitrile (B). The column temperature was 45 °C. The gradient program was as follows: 8–19% B at 0–13 min; 19–21% B at 13–28 min; 21–50% B at 28–42 min; 50% B at 42–46 min; 50–55% B at 46–60 min; 55–56% B at 60–64 min; 56–66% B at 64–74 min; 66–85% B at 74–90 min; 85–100% B at 90–95 min; 100% B at 95–100 min.

2.4. RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (PCR) Analysis

The RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR) analysis were performed as described by Chen et al. [34]. Seven genes related to the synthesis of triterpenoid (3-hydroxy-3-methylglutaryl-coenzyme A reductase, HMGR; 1-deoxy-D-xylulose 5-phosphate reductoisomerase, DXR; squalene synthase, SQS; UDP-glycosyltransferase, UDP; β -amyrin synthase, β -AS and glucose-1-phosphate adenylyltransferase, GPS) were selected. Relative gene expression was calculated by the $2^{-\Delta\Delta C_t}$ method with 18S ribosomal RNA as an internal control gene [35] (Table 1 presents primer information for the studied genes). For comparison of the relative expression of the mRNA in different N form treatments and sampling times, transcript levels for each gene in all nitrogen treatments and sampling times were normalized to that of the T1 treatment at 30 days after N form treatments.

Table 1. Primers used in quantitative real-time polymerase chain reaction (qRT-PCR) in this study.

Gene Name	Sequence of Primer (5'-3')
HMGR-F	TTTAGCGATGGACATGAGCA
HMGR-R	GGAGTGGCAGAGCGTCAGAGGC
DXR-F	GCTGGTTCAATGTAACTCTTC
DXR-R	CTCTATGACTCCTTGCTCCC
GPS-F	GAGCGAAGTTATTCTGCTG
GPS-R	GTGTAAATGGGAGATGAACG
SQS-F	GAACAGGCTGGATGCGATAC
SQS-R	TCAATTATTTGGTCGTTTGG
β -AS-F	TGGTTCATGGTTTGCACTGGAG
β -AS-R	CTCTCTCAGCCTGTCCAGCATGA
UDP-F	TCTACTATCACCTCGACCTCCT
UDP-R	TTTTTACATCCTGAAATGCCTT
18s-F	AGTATGGTCGCAAGGCTGAAA
18s-R	CAGACAAATCGCTCCACCAA

2.5. Statistical Analysis

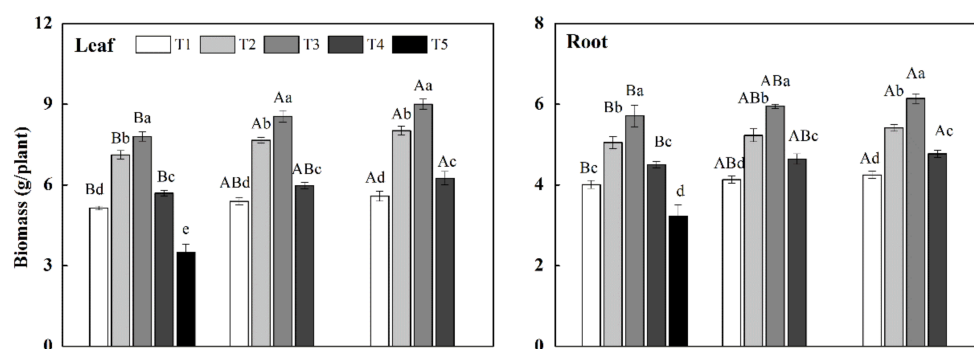
Analysis of variance (ANOVA) was performed to analyze the impacts of nitrogen forms and ratios on growth, triterpenoid accumulation and relative gene expression of triterpenoid biosynthesis, followed by Tukey's test with $p < 0.05$. Relationships among different indexes were evaluated by Pearson's correlation analysis. All statistical analyses were conducted using SPSS 19.0 software (SPSS, Chicago, IL, USA). All values were expressed as mean $\pm$ standard deviation (SD).

3. Results

3.1. Seedling Biomass

Nitrogen form and ratio had a significant effect on biomass production of *C. paliurus* seedlings at all sampling times ($p < 0.05$) (Figure 2), while *C. paliurus* seedlings had died at 52 days after the treatment in T5. Meanwhile, significant differences in each biomass component and whole plant were also found between 30 and 90 days after the treatments. At the end of the experiment (90 days of treatment), total biomass production per seedling among the treatments was in the order of T3 > T2 > T4 > T1 treatment. Compared with T3 treatment, the total biomass per plant reduced by 34.7% in T1, 11.3% in T2 and 26.9% in T4, respectively.

A similar treatment effect on the biomass production of leaf, stem and root was also observed. However, the influence extent of N forms on the biomass varied among the components. Compared with T3 at 90 days after the treatments, the leaf biomass per plant reduced by 38.0% in T1, 10.9% in T2 and 30.6% in T4 respectively, while the stem biomass per plant reduced by 33.6% in T1, 10.9% in T2 and 26.1% in T4. Moreover, the ratio of root to shoot varied from 0.39 to 0.45, and increased by 1.8–11.0% for T1, T3 and T4 treatment as compared to T2 at 90 days after the treatments (Figure 3).

**Figure 2.** Cont.

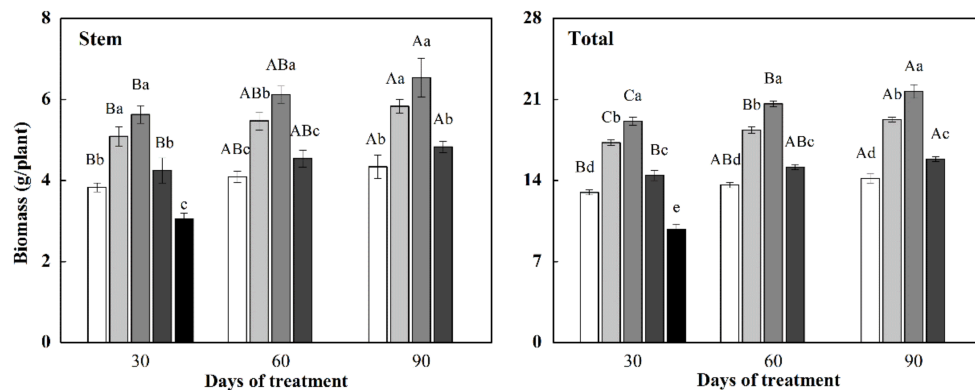


Figure 2. Effects of $\text{NO}_3^-/\text{NH}_4^+$ ratio on biomass in different components of *Cyclocarya paliurus* (Batalin) Iljinsk. seedlings sampled at 30, 60 and 90 days after treatments. Different capital letters indicate significant differences among different $\text{NO}_3^-/\text{NH}_4^+$ ratio treatment for the same sampling time according to Tukey's test ($p < 0.05$). Different small letters indicate significant differences among different sampling time for the same $\text{NO}_3^-/\text{NH}_4^+$ ratio treatment according to Tukey's test ($p < 0.05$). All *C. paliurus* seedlings had died at 52 days after the treatment in T5.

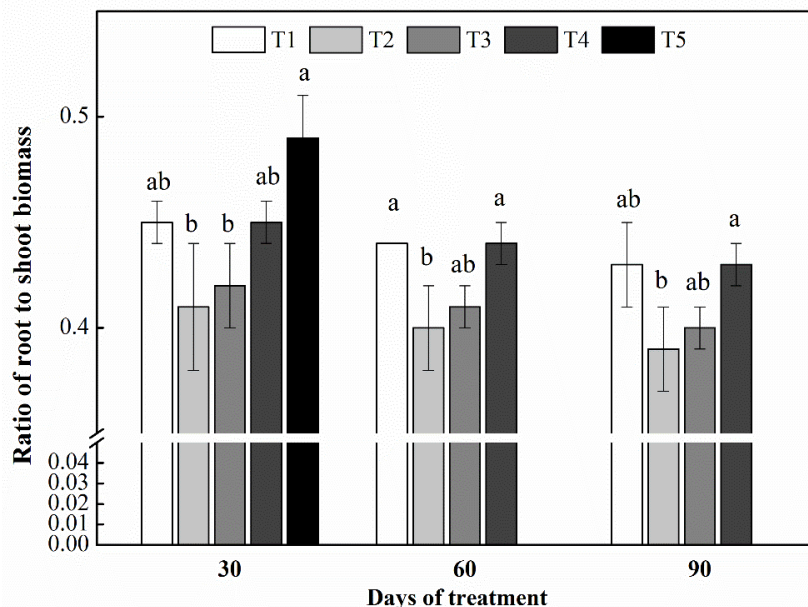


Figure 3. Ratios of root to shoot (including leaf and stem) biomass among the different treatments of nitrogen forms at various sampling times. Different letters indicate significant differences among different $\text{NO}_3^-/\text{NH}_4^+$ ratio treatments for the same sampling time according to Tukey's test ($p < 0.05$). All *C. paliurus* seedlings had died at 52 days after the treatment in T5.

3.2. Triterpenoid Contents

The contents of total triterpenoid and five individual triterpenoids were determined in this study. Analysis of variance indicated triterpenoid contents were significantly influenced by N forms and their ratios ($p < 0.05$) (Figure 4). Among the components evaluated, leaves accumulated the greatest total triterpenoids (ranging from 14.32 to 23.45 mg/g), followed by stems (3.92–5.96 mg/g) and roots (3.38–5.48 mg/g), while the contents of five individual triterpenoids in the various components also followed this pattern. As a general tendency, the contents of total triterpenoid and five individual triterpenoids were enhanced with the prolongation of treatment times except for cyclocaric acid B and pterocaryoside A in roots and stems (Figure 4).

At 90 days after N form treatments, the highest contents of both total triterpenoid and the selected individual triterpenoids in *C. paliurus* leaves were detected in the T1 treatment, while the T3 treatment

resulted in the lowest content (Figure 4). The contents of total triterpenoid and the selected triterpenoids in stems and roots showed a similar variation pattern. Compared with the T1 treatment sampled at 90 days, the contents of total triterpenoid, arjunolic acid, cyclocaric acid B, pterocaryoside A, hederagenin and oleanolic acid in the leaves of T2, T3 and T4 treatments decreased by 11.9–18.3%, 25.0–31.4% and 4.8–8.2%, respectively.

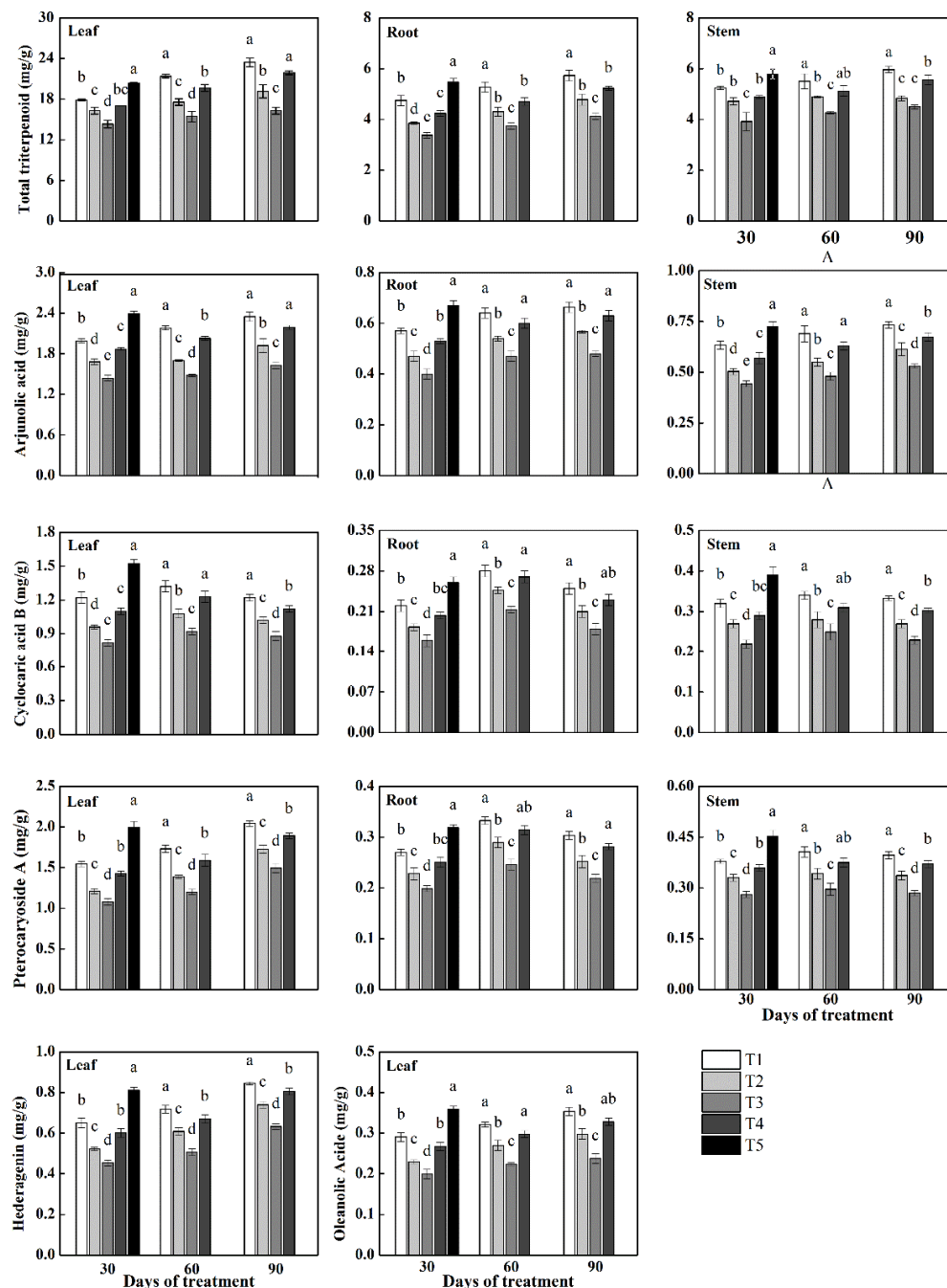


Figure 4. Effects of $\text{NO}_3^-/\text{NH}_4^+$ ratio on the contents of total triterpenoid and five individual triterpenoids in different components of *C. paliurus* seedlings sampled at 30, 60 and 90 days after the treatments. Different letters indicate significant differences among different $\text{NO}_3^-/\text{NH}_4^+$ ratio treatments for the same category according to Tukey's test ($p < 0.05$). All *C. paliurus* seedlings had died at 52 days after the treatment in T5.

3.3. Triterpenoid Accumulation per Plant

In practice, only *C. paliurus* leaves are harvested for making tea or nutraceutical foods. The accumulations of total and selected triterpenoids in the leaves and in whole plant were respectively calculated for different N form treatments based on the component biomass and their corresponding triterpenoid contents (Figure 5). Analysis of variance showed that the accumulations of total triterpenoid and five individual triterpenoids per plant were significantly affected by $\text{NO}_3^-/\text{NH}_4^+$ ratio, and the accumulation of measured phytochemicals per plant showed an increasing tendency with the treatment periods (Figure 5). The quantities of total triterpenoid, arjunolic acid, cyclocaric acid B, and pterocaryoside A accumulated in the leaves per plant accounted for 66.9–74.0%, 65.6–70.8%, 72.4–75.2%, and 74.4–80.7% respectively, while the hederagenin and oleanolic acid were only detected in the leaves.

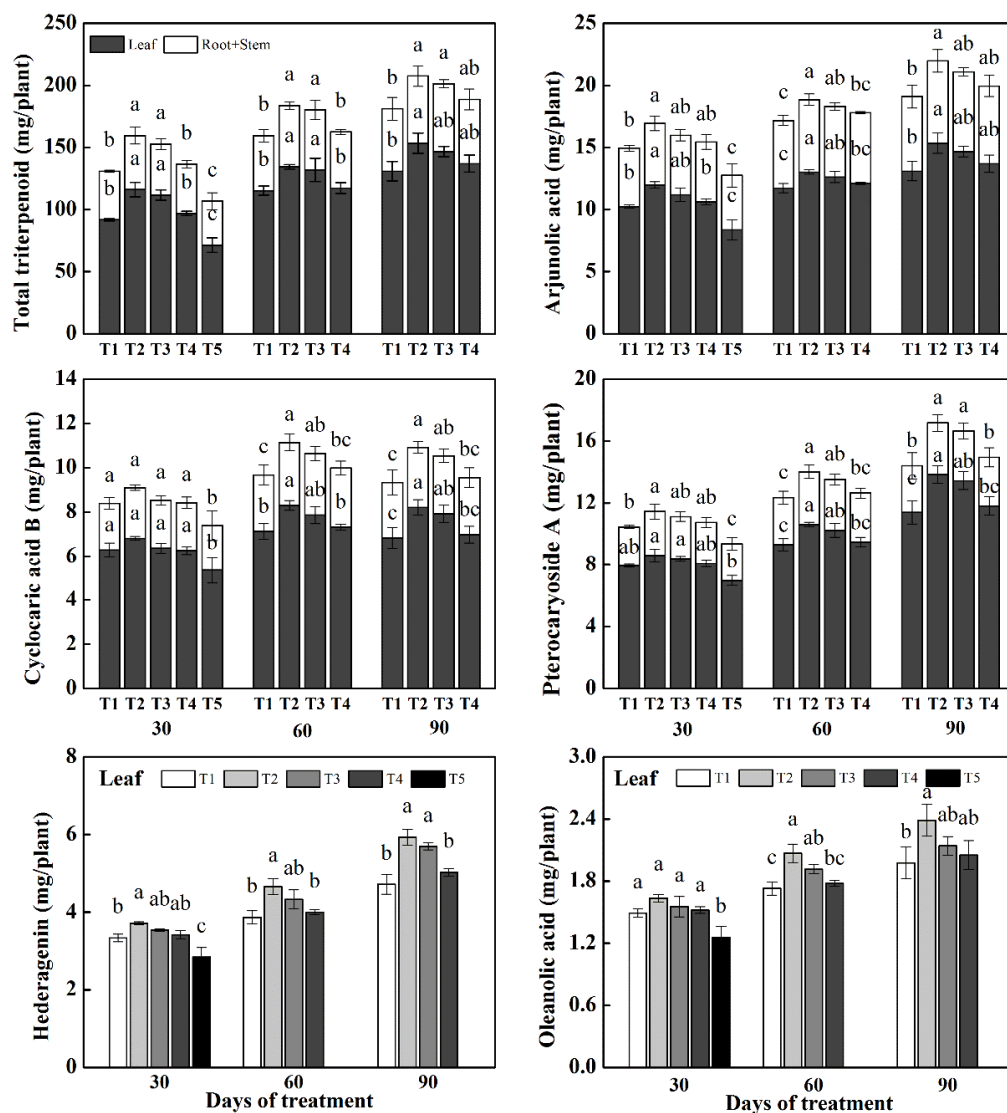


Figure 5. Effects of $\text{NO}_3^-/\text{NH}_4^+$ ratio on the accumulations of total triterpenoid and five individual triterpenoids in leaves and whole plants of *C. paliurus* seedlings sampled at 30, 60 and 90 days after the treatments. Different letters indicate significant differences among different $\text{NO}_3^-/\text{NH}_4^+$ ratio treatments for the same category according to Tukey's test ($p < 0.05$). All *C. paliurus* seedlings had died at 52 days after the treatment in T5.

At the end of the experiment, the highest accumulations of total and selected triterpenoids were achieved in the T2 treatment, while the lowest productions were observed in the T1 treatment. Compared with T1 treatment, the accumulations of total triterpenoid, arjunolic acid, cyclocaric acid B, pterocaryoside A, hederagenin and oleanolic acid in leaves of T2, T3 and T4 treatments increased by 17.3–25.9%, 8.6–20.8% and 2.2–6.8%, respectively, while the accumulations per plant were enhanced by 14.6–25.9%, 8.6–20.8% and 2.4–6.8%.

3.4. Gene Expression Related to Triterpenoid Biosynthesis

The ANOVA indicated that $\text{NO}_3^-/\text{NH}_4^+$ ratios significantly modified relative gene expression of *HMGR*, *DXR*, *GPS*, *SQS*, β -AS and *UDP* in leaves of *C. paliurus* among different treatment periods (Figure 6). Generally, the relative expression of *DXR* and *UDP* displayed an increasing trend over time. Relative expression of *HMGR*, *DXR*, *GPS*, *SQS*, β -AS and *UDP* was consistently the lowest under T3 treatment across the treatment periods. At the N concentration of 238 mg/L, addition of 100% NO_3^- -N (T1) or 100% NH_4^+ -N (T5) significantly upregulated relative expression of all genes evaluated on day 30 (Figure 6), but this upregulation effect was much greater in the T5 treatment (100% NH_4^+ -N supplied), approaching 1.4–13.4 times the T1 treatment (100% NO_3^- -N supplied). Correlation analysis between relative gene expression of triterpenoid biosynthesis pathway (*HMGR*, *DXR*, *GPS*, *SQS*, β -AS and *UDP*) and the content of measured triterpenoids content showed significantly positive relationships ($p < 0.01$, Table 2).

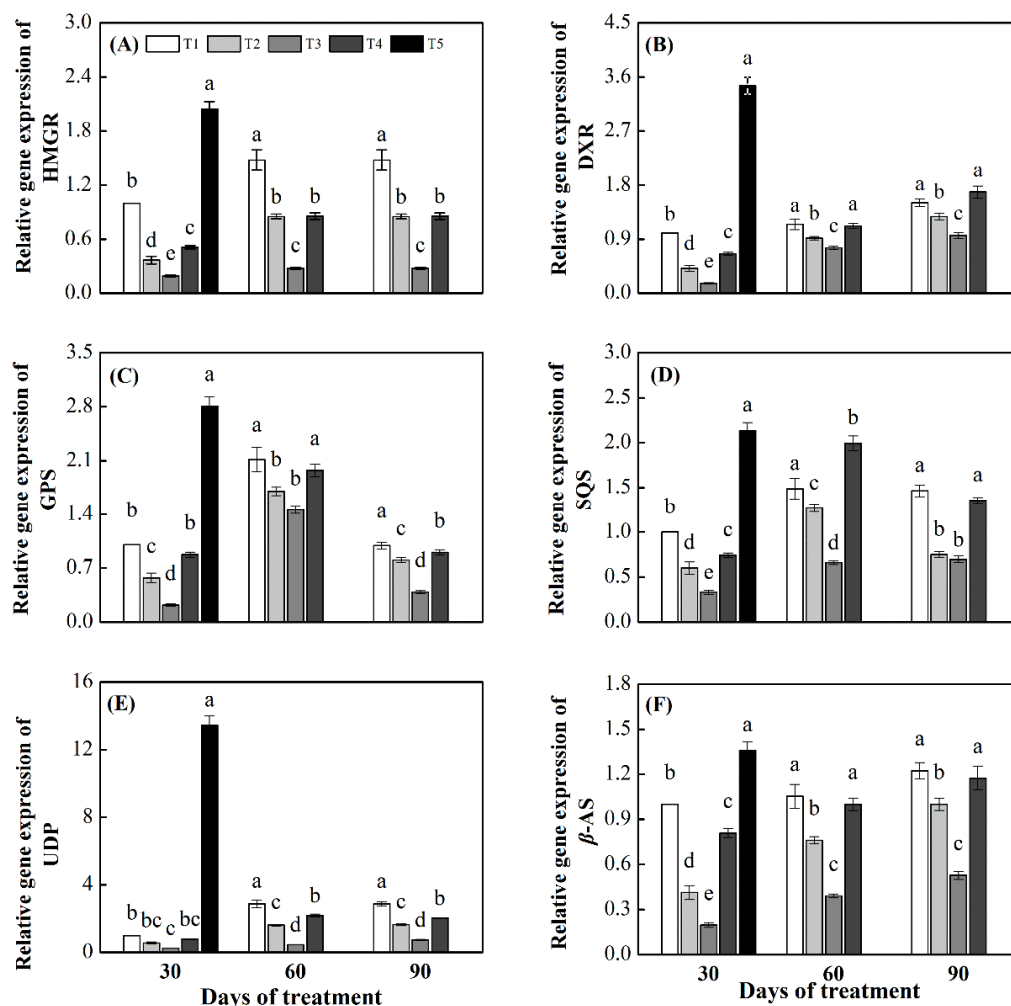


Figure 6. Effects of $\text{NO}_3^-/\text{NH}_4^+$ ratio on relative gene expression of *HMGR* (A), *DXR* (B), *GPS* (C), *SQS* (D), β -AS (F) and *UDP* (E) in *C. paliurus* leaves sampled at 30, 60 and 90 days after treatments. Different

letters indicate significant differences among different $\text{NO}_3^-/\text{NH}_4^+$ ratio treatments for the same sampling time according to Tukey's test ($p < 0.05$). All *C. paliurus* seedlings had died at 52 days after the treatment in T5.

Table 2. Pearson correlation coefficients (r value) between triterpenoid content and gene expression in the leaves across the nitrogen treatment periods ($n = 39$).

Gene Expression	Total Triterpenoid	Arjunolic Acid	Cyclocaric Acid B	Pterocaryoside A	Hederagenin	Oleanolic Acid
HMGR	0.50 **	0.69 **	0.91 **	0.57 **	0.53 **	0.68 **
DXR	0.61 **	0.76 **	0.78 **	0.79 **	0.77 **	0.78 **
GPS	0.43 **	0.54 **	0.81 **	0.44 **	0.41 **	0.58 **
SQS	0.73 **	0.80 **	0.87 **	0.73 **	0.71 **	0.81 **
β -AS	0.71 **	0.74 **	0.64 **	0.82 **	0.81 **	0.77 **
UDP	0.50 **	0.69 **	0.91 **	0.57 **	0.53 **	0.68 **

** indicate correlation is significant at the 0.01 level.

4. Discussion

4.1. Effects of $\text{NO}_3^-/\text{NH}_4^+$ Ratio on Growth and Triterpenoid Content

Nitrate and ammonium are predominant forms of inorganic nitrogen absorbed by the roots of higher plants, but there exists controversy about the relative advantage of one form over the other in terms of various physiological processes or plant species [18,24]. For example, rice, tea and sugarcane, prefer NH_4^+ nutrient to NO_3^- , while NO_3^- is nutrient preferred by maize, sugarcane wheat and poplar [29,36–39]. For most plants, NH_4^+ is toxic and inhibits plant growth by induced nutrient deficiency, when supplied at high concentration without NO_3^- [40]. Despite NH_4^+ can induce toxicity symptoms, higher energy was consumed by of NO_3^- uptake relative to NH_4^+ [41]. Our study demonstrated that growth in mixed NO_3^- and NH_4^+ (T2, T3 and T4) was significantly superior to that in a sole N source (T1 and T5) (Figure 2), consistent with reports from Zhu et al. [27] who found that the growth of *P. vulgaris* was inhibited by a high concentration of either NO_3^- or NH_4^+ . When NH_4^+ is the sole N source, in *C. paliurus* seedlings there occurred a symptom of toxicity, for example, old leaves' margins turning brown and withering, having shorter stems, and even dying at 52 days after treatments. However, the survival and growth of *C. paliurus* seedlings were relatively uninfluenced when NO_3^- is the sole N source, compared to the sole NH_4^+ source (Figure 1). Meanwhile, Figure 1 also shows that the biomass production in T2 ($\text{NO}_3^-/\text{NH}_4^+ = 2.33$) was significantly higher than in T4 ($\text{NO}_3^-/\text{NH}_4^+ = 0.43$) for all components. Thus, it is inferred that *C. paliurus* may prefer NO_3^- to NH_4^+ . Our results confirm that NH_4^+ as the sole nitrogen source inhibited growth compared to NO_3^- nutrition or a mixture of NO_3^- and NH_4^+ . The possible reason is that the NH_4^+ supply affects regulatory processes by which plants adjust their metabolism to nitrogen assimilation [16], such as altering osmotic regulation [42,43], and hormonal regulation between root and shoot [44].

The nitrogen form and ratio had a similar effect on biomass production of root, stem and leaf (Figure 3), consistent with a report by He et al. [45]. However, nitrogen forms (NO_3^- -N and NH_4^+ -N) and their ratios also affect dry matter distribution and carbohydrate consumption. Our results showed that NH_4^+ promotes root growth of *C. paliurus* seedlings, resulting in higher root/shoot ratio compared to NO_3^- nutrition or the mixture of NO_3^- and NH_4^+ (Figure 3), inconsistent with the results reviewed by Guo et al. [17] for agricultural crops (wheat, bean, tomato and maize plants), where NH_4^+ inhibits root growth, and results in higher shoot/root ratio compared to NO_3^- nutrition or the mixture of NO_3^- and NH_4^+ . However, this promoting effect reduced as treatment times were prolonged (Figure 3).

The possible reason is the losses of organic carbon vary due to differences between NO_3^- and NH_4^+ in the metabolism of absorption, assimilation, transportation, and energy cost.

Both plant growth and the biosynthesis of secondary metabolites are stimulated by multiple factors, such as the specific characteristics of visible light qualities [46,47] and N nutrition [17,29,48]. Our results confirmed that a sole concentration of NO_3^- or NH_4^+ (T1 and T5) accumulated more total and individual triterpenoids content (Figure 3), and support the resource availability hypothesis, where plant defense will increase when resources (such as light, water and nutrient) become stressed or limited [49]. Moreover, some hypotheses have also been presented to explain the potential trade-off between plant growth and synthesis of secondary metabolite [50–52], which are supported by the results from our study, where *C. paliurus* seedlings grow better under T2 and T3 treatments, and possess lower triterpenoid contents (Figures 2 and 4), when compared with the T4 treatment. These results suggest that the manipulation of nitrogen form and ratio in the practice is necessary to enhance production of target triterpenoids in *C. paliurus*.

4.2. Effects of $\text{NO}_3^-/\text{NH}_4^+$ Ratio on Relative Gene Expression

Some studies on the effect of N nutrition on metabolism and biosynthesis of secondary metabolites have been conducted in woody plants [29,47,53]. For instance, recent investigations have shown that growth-promoting N nutrition reduced flavonoid accumulation in the leaves of apple trees [53,54], where phenylalanine ammonia-lyase (PAL) activity seems to be downregulated, thus forming a bottle-neck resulting in a generally decreased flavonoid accumulation. Huang et al. [55] reported the metabolic and transcriptional responses of young shoots of *Camellia sinensis* to four N conditions (N-deficiency, nitrate, ammonia, and nitric oxide), and indicated that N-deficiency tea plants accumulated diverse flavonoids, corresponding with higher expression of hub genes including flavonoid dioxygenase (F3H), flavone synthase (FNS), UDPG-flavonoids glucosyl transferase (UGT), basic helix-loop-helix protein 35 (bHLH35), and basic helix-loop-helix protein 35 (bHLH36).

Triterpenoids are synthesized by the isoprenoids pathway in plants, while HMGR and DXR are the key enzymes of MVA and MEP pathways, respectively, in the upstream pathway. *GPS* is a gene involved in the synthesis of glycosides of triterpenoid saponins, whereas *SQS* is the first key enzyme in the branch of triterpene synthesis. The downstream pathway consists of 3 gene families (OSCs, POMD and GT), where UDP belongs to GT, and β -AS belong to OSCs. Li et al. [56] showed that the secondary metabolites content was increased or decreased by modifying the gene expression involved in secondary metabolic pathways under stress conditions in medicinal plants with same genetic background. Moreover, in various plant species, the positive correlations between the activities and gene expression levels of the key enzymes and the accumulation levels of triterpenoid saponins have been proved [7]. Our results indicated that expression of genes involved in the triterpenoid biosynthesis pathway was affected by nitrogen form and ratio (Figure 6). Significantly positive correlations were determined between the relative gene expression (*HMGR*, *DXR*, *GPS*, *SQS*, β -AS, and *UDP*) with total and selected triterpenoid contents (Table 2). Overall, the sole NH_4^+ or NO_3^- nutrition increased the content of triterpenoid by enhancing the relative gene expression of triterpenoid biosynthesis. On the basis of the presented results, an upregulation of measured genes under the sole NH_4^+ or NO_3^- nutrition can be assumed when N nutrition supply is not at N-deficiency or at high concentration, although this has to be confirmed by further studies at the level of more detailed transcripts.

4.3. Effects of $\text{NO}_3^-/\text{NH}_4^+$ Ratio on Triterpenoid Production per Plant

Metabolite profiles of plants are often modified by environmental stress conditions, which may promote the production of bioactive secondary metabolites with beneficial properties in medicinal plants [23,27,57]. However, the potential trade-off between plant growth and carbon-based secondary metabolite (such as triterpenoid) was proved [58], therefore, how to get the best trade-off is quite important in the practices for obtaining the greatest yield of target phytochemicals. When the *C. paliurus* plantations are planted, the management goal is to obtain not only higher quality (e.g., higher

bioactive substance, such as triterpenoid contents in the leaves) but also greater target phytochemical accumulation per area (e.g., economic yield). Given the middle N nutrition supplied (at the N concentration of 238 mg/L) in the present study, the highest leaf biomass production was achieved at the ratio of $\text{NO}_3^-/\text{NH}_4^+ = 1.0$ (T3) (Figure 6), while the sole concentration of NO_3^- (T1) or NH_4^+ (T5) accumulated the highest triterpenoid content in the leaves. However, the maximum yield of total triterpenoids in the leaves per plant was obtained at the ratio of $\text{NO}_3^-/\text{NH}_4^+ = 2.33$ (T2), which could be referenced as the effective ratio of $\text{NO}_3^-/\text{NH}_4^+$ to induce the highest triterpenoid yield in practice. Moreover, regression analysis showed that the polynomial functions best described the relationship of total triterpenoid content in the leaves, total leaf triterpenoid accumulation and leaf biomass per plant to nitrogen forms (Figure 7), with a R^2 ranging from 0.41 to 0.83 ($p < 0.001$). Therefore, results from this study provide a basis for optimizing the $\text{NH}_4^+/\text{NO}_3^-$ ratio in nitrogen fertilization to achieved the triterpenoid yield per area in *C. paliurus* plantations.

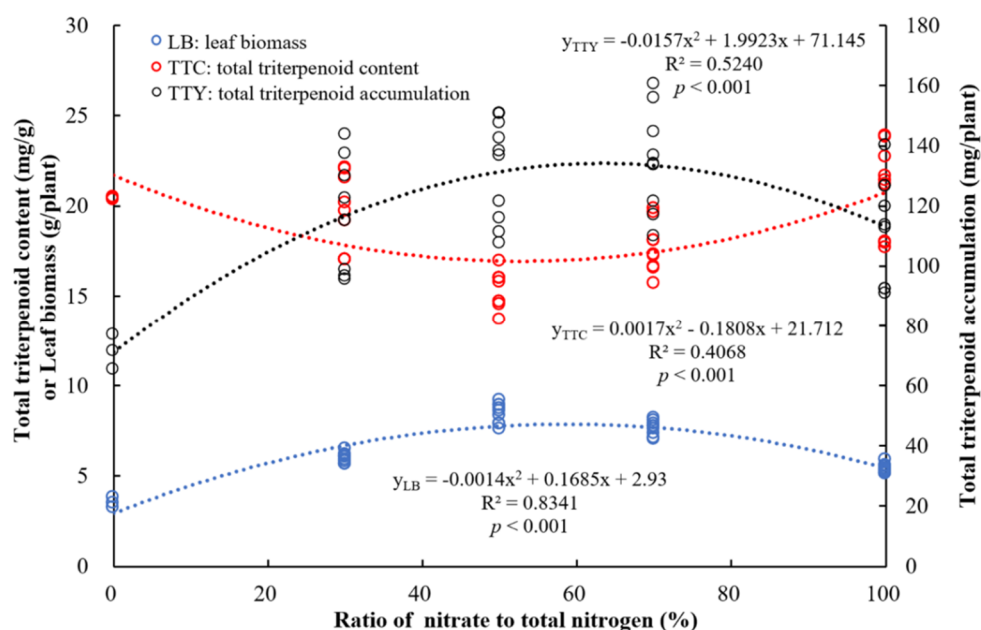


Figure 7. Relationships of total triterpenoid content in the leaves (y_{TTC}), total leaf triterpenoid accumulation (y_{TTY}) and leaf biomass (y_{LB}) per plant to nitrogen forms at the concentration of 238 mg/L (x) across the sampling times ($n = 39$). All *C. paliurus* seedlings had died at 52 days after the treatment in T5.

5. Conclusions

Growth, triterpenoid accumulation and relative gene expression of *C. paliurus* seedlings were significantly influenced by nitrogen forms and their ratios. Given the middle N nutrition supplied (at the N concentration of 238 mg/L), the highest triterpenoid content was achieved in a sole NO_3^- or NH_4^+ nutrition, while the mixed N nutrition with an equal ratio of NO_3^- to NH_4^+ (T3) induced the highest biomass production in *C. paliurus* seedlings. However, the mixed N nutrition with a ratio of NO_3^- to NH_4^+ of 2.33 (T2) produced the highest triterpenoid accumulation per plant. Regression analysis suggests the polynomial functions can best describe the relationship of total triterpenoid content in the leaves, total leaf triterpenoid accumulation, and leaf biomass per plant to nitrogen forms and their ratios. The relative expression of *HMGR*, *DXR*, *GPS*, *SQS*, β -AS and *UDP* involved in triterpenoid biosynthesis were all up-regulated under the sole NH_4^+ or NO_3^- nutrition conditions, which was consistent with the increase of total and selected triterpenoid contents in the leaves. Our results provide a theoretical basis for manipulating nitrogen fertilization to achieve the highest yield of triterpenoids in *C. paliurus* cultivation. However, how to obtain a higher triterpenoid accumulation in *C. paliurus*

plantations through fertilizing with the nitrogen form and ratio needs to be further studied in the field trial with better designed tests.

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