Plant Physiology and Biochemistry 135 (2019) 111–118

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Effects of nitrogen availability on mineral nutrient balance and flavonoid accumulation in *Cyclocarya paliurus*

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ARTICLE INFO

Keywords:
Cyclocarya paliurus
Flavonoids
Nitrogen availability
Mineral nutrient
Enzyme activity
Growth

ABSTRACT

Cyclocarya paliurus has traditionally been used as medicine or nutraceutical foods. This study aims at investigating flavonoid accumulation in *C. paliurus* dependent on nitrogen availability and the following internal mineral nutrient balance under controlled condition. The 1-year-old seedlings of *C. paliurus* were grown in five different nitrogen levels. Along with the N gradient, C/N was significantly decreased, and the concentrations of phosphorus, potassium, calcium and magnesium were changed within plant. In the leaves, the main accumulation organ in *C. paliurus*, the highest flavonoid accumulation was achieved in intermediate N level (N3), which was closely related to flavanone-3-hydroxylase (FHT) activity as they had the similar variation patterns. Correlation analysis suggested that internal mineral nutrient balance can significantly affect flavonoid accumulation, especially for Mg within plant. These data revealed that nitrogen availability and the following altered internal mineral balance can significantly affect flavonoid accumulation. This study can provide the basis for developing new agricultural practices to maintain high yield while still keeping the nutritional value of crop or medicinal plants.

1. Introduction

Cyclocarya paliurus, which is a deciduous tree and widely distributed in sub-tropical regions of China, belongs to the Juglandaceae family (Fang et al., 2011). The leaves of *C. paliurus* have long been used for drug formulations or ingredients of functional foods in China as its medicinal and health benefits (Xie et al., 2010). Studies showed that the health-promoting effects, such as inhibiting protein tyrosine phosphatase 1B, improving mental efficiency and enhancing antihypertensive action, carried by flavonoids in the leaves of *C. paliurus* play an important role (Zhang et al., 2010; Wu et al., 2017). As a valuable woody medicinal tree species, *C. paliurus* has higher content of flavonoids than those of 22 selected vegetables, fruits and medicinal plants (Sultana and Anwar, 2008; Fang et al., 2011).

As the ingredients of dietary or traditional medicine, flavonoids have health-promoting effects on humans, and possess high antioxidant capacities in both vivo and in vitro systems and hence protect humans against degenerative diseases (Yao et al., 2004; André et al., 2009; Taleon et al., 2012). Quercetin and its glycoside derivatives (e.g. isoquercitrin), the most common flavonoids in human diet (Spencer et al., 2004), possess cytoprotective effects, such as reducing the level of lipid peroxidation, protein carbonylation and reactive oxygen species (ROS) (Palazzolo et al., 2012). In addition, kaempferol was also found a strong antioxidant potential, and act as an activating agent of anti-oxidant enzymes, such as glutathione peroxidase and catalase (Rajendran et al., 2014). These flavonoid compositions were shown to have a variety of biological activities. Some mechanisms involves in health-promoting effects have been proposed, but clinical trials are still in progress.

Flavonoids are mostly biosynthesized by plants, and are usually conjugated with glucose presenting as glycosides (Iwashina, 2000). In plants, the main biosynthesis pathway of flavonoid is shikimate pathway, which provides the common precursor of phenylalanine not only for amino acid and protein synthesis, but also for secondary metabolites like flavonoids (Taiz and Zeiger, 2002). Three synthetic stages were contained for shikimate pathway: (1) *p*-coumaroyl CoA was formed by several enzymes including phenylalanine ammonia lyase, cinnamate 4-hydroxylase and 4-coumaroyl:CoA-ligase; (2) dihydroflavone was formed by enzymes of chalcone synthase, chalcone isomerase and flavanone 3-hydroxylase, and branch pathways of flavonol, isoflavone and proanthocyanidins were occurred in this stage; (3) various flavonoid groups were formed by flavonoid 3′-hydoxylase, flavonol synthase and UDPG-flavonoid glucosyl transferase (Gong et al., 2011) (Fig. 1).

It has been widely accepted that biotic and abiotic environmental

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https://doi.org/10.1016/j.plaphy.2018.12.001
Received 6 September 2018; Received in revised form 18 November 2018; Accepted 1 December 2018

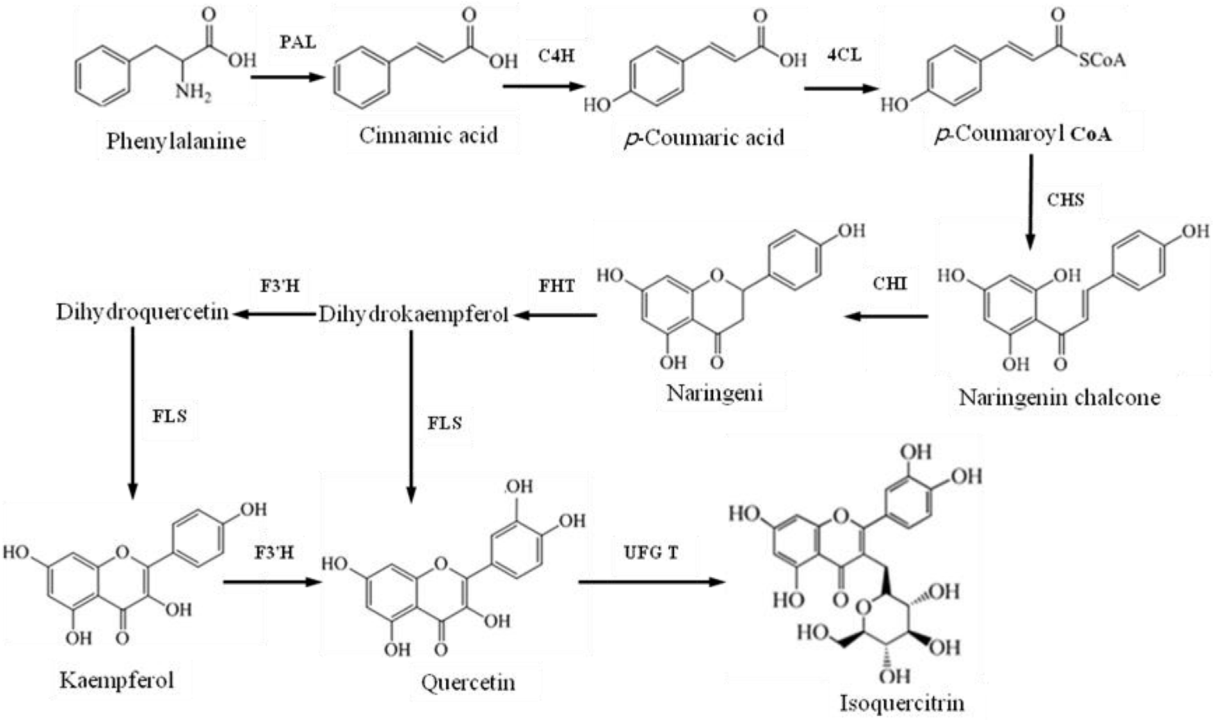

Fig. 1. Biosynthetic pathway of quercetin, isoquercitrin and kaempferol. Phenylalanine ammonia lyase, PAL; cinnamate 4-hydroxylase, C4H; 4- coumaroyl:CoA-ligase, 4CL; chalcone synthase, CHS; chalcone isomerase, CHI; flavanone 3-hydroxylase, FHT; flavonoid 3′-hydoxylase, F3′H; flavonol synthase, FLS; UDPG-flavonoid glucosyl transferase, UFGT.

stresses can induce flavonoid biosynthesis and accumulation in plants. Higher level of flavonoid accumulation inducing by nitrogen deficiency was found in many plants, such as *Potamogeton amplifolius*, *Nuphar advena*, and *Arabidopsis* (Cronin and Lodge, 2003; Lillo et al., 2008). Potassium deficient, however, was found decreasing the flavonoid accumulation in *Chrysanthemum morifolium* in relation to 4-coumaroyl:CoA-ligase activity (Liu et al., 2011). Our previous studies have also proved that fertilization (N/P/K) can decrease the accumulation of flavonoid in *Cyclocarya paliurus* leaves (Deng et al., 2012). Under the conditions of nutrient limitation, UV-B radiation and fungal infection, the mRNA of phenylalanine ammonia lyase and chalcone synthase would increase, and result in an upward flavonoid accumulation within plants (Taiz and Zeiger, 2002). In cultivation systems of medicinal plants, vegetables and crops, fertilization can increase biomass production while decreasing the biosynthesis and accumulation of physiological active substances, including flavonoid and terpene, and further impact quality of raw materials and the production of target secondary metabolites on unit area (Groenbaek et al., 2016). To our knowledge, none of the literature deals with the effects of internal mineral nutrient balance (phosphorus, potassium, calcium and magnesium) on flavonoid production. The present study aims at investigating the content of flavonoid in *C. paliurus* and the yield per plant, dependent on nitrogen availability and the following internal mineral nutrient balance under controlled condition. The information would be of great value for increasing the health-promoting effects and establishing optimal cropping strategies of *C. paliurus* plants.

2. Materials and methods

2.1. Plant material and experimental design

Seeds of *C. paliurus* were collected from natural forests (a selected single tree) of Anji (Zhejiang, China) in late October 2014. The collected seeds were first subjected to chemical scarification, followed by exogenous gibberellin A3 (GA3) treatments, and then stratification treatments using a method described by Fang et al. (2006). After stratification treatment for 3 months, the germinated seeds were first sown in plastic containers (5 cm in diameter, 15 cm in height) and then transplanted to the field (Baima, Nanjing, China) when the seedlings were about 6 cm in height.

In March 2016, the 1-year old plants were retransplanted to experimental containers (18 cm in diameter and 21 cm in height) filled with 1:2 underling soil -perlite in Hefei (Anhui, China) and grown in a controlled phytotron with 620 μmol $m^{-2}{\cdot}s^{-1}$ light intensity, a 12 h photoperiod, a 25 °C/15 °C diurnal/night temperature and a constant relative humidity of 65%. The soil used for cultivating plants was collected from Anhui Agricultural University, with the pH value, total organic matter, available nitrogen, available phosphorus and available potassium of 5.8, 2.1 g/kg, 30.2 mg/kg, 31.4 mg/kg and 75.2 mg/kg, respectively. After 3-week planting, fertilization treatment was conducted. Five fertilization levels were included in this study: 0 g/plant NH_4NO_3 (N1), 1.0 g/plant NH_4NO_3 (N2), 3.4 g/plant NH_4NO_3 (N3), 6.0 g/plant NH_4NO_3 (N4), 10 g/plant NH_4NO_3 (N5). The amounts of phosphorus and potassium in each container were 2.0 g and 1.2 g supplied with $Ca(H_2PO_4)_2{\cdot}H_2O$ and K_2SO_4, respectively. Three replications were included in each treatment, and each replication consisted of 5 seedlings. Plant materials were harvested in the end of the August.

2.2. Carbon and nitrogen

All plants were divided into three organs types: roots, stalks (including branches) and leaves, and then dried (70 °C) and ground. Samples were stored at room temperature until analysis. For measurement, 0.5 mg of tissue samples were warped up with tin can (2 × 5 mm), and the concentrations of total carbon and nitrogen were determined by combusting in an element analyser (EA3000, Euro Vector, Italy).

Table 1
Variations of carbon, nitrogen, and carbon-to-nitrogen ratio (C/N) in root, stalk and leaf of *Cyclocarya paliurus* seedlings under the five nitrogen fertilization levels. Different letters indicate significant differences among nitrogen treatments for the same category according to Duncan's test ($p < 0.05$).

Treatment	Root (%)			Stalk (%)			Leaf (%)		
	nitrogen	carbon	C/N	nitrogen	carbon	C/N	nitrogen	carbon	C/N
N1	1.11a	42.31a	38.14c	0.62a	41.90a	68.05d	2.09a	42.48b	20.42c
N2	1.54 ab	41.63a	27.28b	0.87 ab	41.89a	48.33c	2.78b	43.24b	15.59b
N3	1.81bc	41.28a	23.36 ab	1.07b	42.06a	40.58bc	2.72b	42.39b	15.59b
N4	2.17c	39.31a	18.16a	1.39c	41.55a	30.20a	2.85b	42.57b	14.95b
N5	2.11c	41.04a	19.91a	1.38c	42.22a	30.97 ab	3.23c	39.22a	12.19a

2.3. Phosphorus, potassium, calcium and magnesium

For the measurement of total contents of phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg), all samples were digested in HNO_3 and $HClO_4$ (v/v = 2/1), and the concentrations of the above four mineral nutrients were determined by atomic absorption spectrophotometer (UNICAM 969, USA).

2.4. Flavonoids

The fine ground samples for carbon and nitrogen measurements were used for flavonoids analysis. Samples were extracted using an ultrasonic-assisted method with 75% ethanol after removing the fat soluble impurities with petroleum ether. Total flavonoid content was determined using a colorimetric method with detection at 415 nm (Bao et al., 2005). Flavonoid concentration in the extracts was calculated by referencing to a standard rutin (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China) curve (linearity range: 1.5–52.0 μg rutin/mL, $R^2 > 0.99$) and expressed as milligram rutin equivalent per gram of dry weight (mg/g).

Individual flavonoids, quercetin, isoquercitrin and kaempferol, were determined using high performance liquid chromatograph (HPLC), and all extractions were filtered through a 0.45 μm polytetrafluoroethylene (PTFE) filter prior to HPLC analysis. Quercetin and kaempferol were quantified as aglycones after acid hydrolysis. An Agilent 1200 series system (Waldbronn, Germany) was used, which consisting of an online degasser, a quaternary pump solvent management system, an autosampler, a column heater, an UV/VIS diode array detector (DAD), and a data processing system. Quercetin and kaempferol were separated on an Eclipse Plus C18 column (250 mm × 4.6 mm, 5 μm) at 30 °C, and detected at 365 nm. The mobile phases were methanol (A) and 0.3% phosphoric acid (B) at 55: 45 (V_A: V_B). For isoquercitrin determination, the mobile phases were methanol (A) and 0.5% phosphoric acid (B). The gradient elution include 0–25 min, 15% A; 15–26 min, 15–90% A; 26–36 min, 90% A; 36–37 min, 90-15% A; and 37–45 min, 25% A. The detection wavelength was 350 nm. The standards quercetin, kaempferol (Sigma-Aldrich Inc. St. Louis, USA), and isoquercitrin (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China) were used to obtain an external calibration curve.

2.5. Enzyme activity

To measure the levels of PAL, CHS and FHT in leaves of *C. paliurus*, Plant L-Phenylalanine ammonla-lyase (PAL) ELISA Kit, Plant chalcone synthase (CHS) ELISA Kit, and Plant Flavanone-3-hydroxylase (F3H) ELISA Kit were used, respectively. The purified plant PAL (or CHS, FHT) antibody was used to coat microtiter plate wells, followed by adding PAL to the wells, forming antibody-antigen-enzyme labelled antibody complex. After washing completely, TMB substrate solution was added for color developing at 37 °C for 15 min, and then the reaction was terminated with sulphuric acid solution and the absorbance was performed at 450 nm.

2.6. Tree height growth and biomass accumulation

Tree height of all seedlings was measured monthly (May 21, June 21, July 25, and August 23), while biomass assessment was performed on August 23, 2016. For dry mass measurement, all seedlings were excavated, washed and separated (leaf, stalk and root). Afterwards, all of the components were dried at 70 °C and weighed. Total dry mass of each seedling was calculated as the sum of leaf, stem, and root dry weight.

2.7. Statistical analysis

For the analysis of variance (ANOVA), Duncan's multiple-range test was used to calculate significant differences. All statistical analyses were performed at a 95% confidence level. Calculations were conducted using SPSS (version 16.0, SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Carbon (C), nitrogen (N) and C/N ratio

The contents of carbon and nitrogen in roots, stalks and leaves for each treatment were measured, and the C/N ratios were calculated (Table 1). Variance analysis showed that nitrogen in roots, stalks and leaves were significantly increased with the elevated N availability ($p < 0.05$), and ranged from 1.11% to 2.11%, 0.62%–1.38% and 2.09%–3.23%, respectively. However, the carbon contents in three different organs were relative stable, with a variation range from 39.22% to 42.57%, and there were no significant differences among the five treatments except for leaf carbon in N5 (Table 1). With the elevated N availability, the linear increased nitrogen in different organs resulted in a significantly decreased C/N ratio ($p < 0.05$). Compared with treatment N1, the C/N ratios in root, stalk and leaf in N5 were decreased 47.8%, 54.5% and 40.3%, respectively (Table 1).

3.2. Phosphorus, potassium, calcium and magnesium

Under the different nitrogen fertilization levels, the contents of phosphorus, potassium, calcium and magnesium in roots, stalks and leaves were detected (Fig. 2). The contents of P in roots ranged from 1.19 to 2.91 mg/g, while the P in stalks and leaves were 0.90–2.69 mg/g, and 1.61–3.41 mg/g, respectively. With the elevated nitrogen availability, the contents of P in different organs were significantly decreased ($p < 0.05$), phosphorus in N1 were approximately 51.8% greater than the mean contents of other four treatments. Similar variation pattern was also observed for potassium in stalks and leaves, and ranged from 17.07 to 12.43 mg/g and 39.09 to 29.69%, respectively. Potassium in roots, however, possessed an opposite variation pattern, and the highest contents were achieved in treatments N4 (17.57 mg/g) and N5 (17.49 mg/g). Analysis of variance showed that the magnesium contents in roots, stalks and leaves were significantly increased with the elevated nitrogen availability ($p < 0.05$), with a variation range from 7.21 to 10.36 mg/g, 5.43–7.66 mg/g and 10.48–14.58 mg/g,

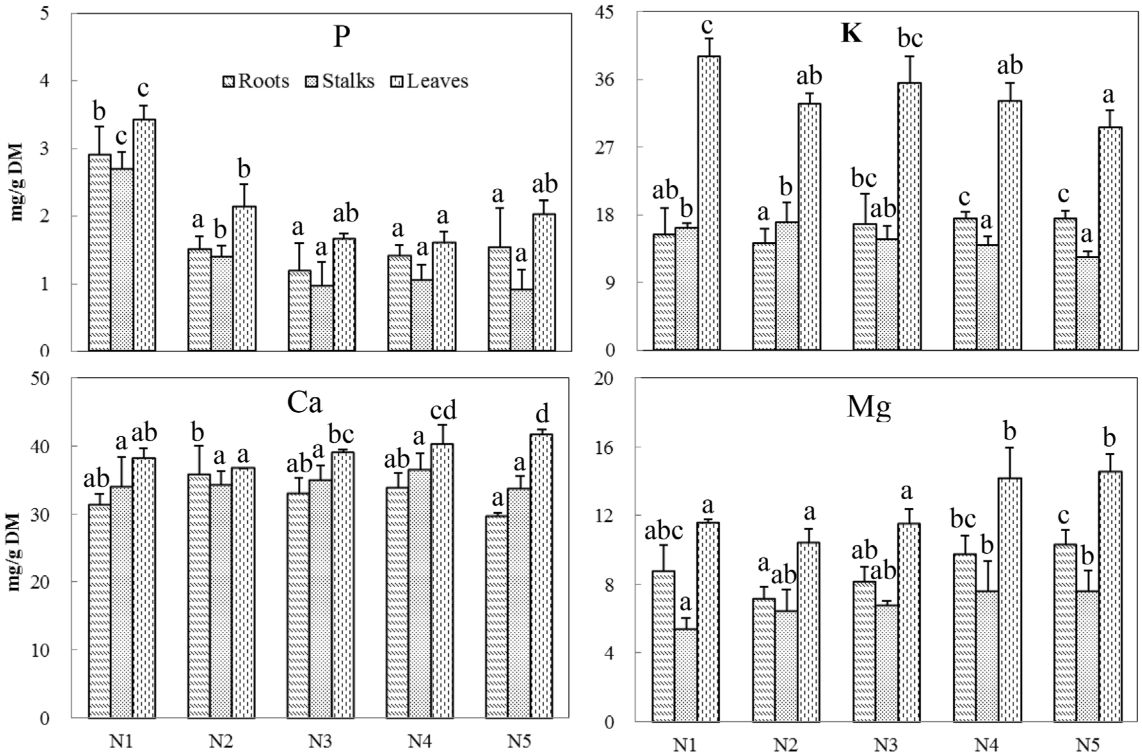

Fig. 2. Concentrations of phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg) in different organs of *Cyclocarya paliurus*. Different letters indicate significant differences among nitrogen treatments according to Duncan's test ($p < 0.05$).

respectively. Furthermore, the contents of calcium in three different organs under the five nitrogen levels had an overall mean of 35.65 mg/g, and ranged from 29.81 to 42.72 mg/g. There was limited variation among different nitrogen levels and only some treatments differed significantly from each other (Fig. 2).

3.3. Tree height and biomass

Tree height growth of all seedlings was determined monthly from May to August 2016. Total increment of tree height indicated the final height measured in August, while the net growth was calculated as the difference value between final height and initial height (measured in May). Analysis of variance showed that total increment of tree height was significantly increased from treatment N1 to N4 ($p < 0.05$), with a variation range from 30.4 to 36.7 cm (Fig. 3a). However, the lowest total increment of tree height was detected in treatment N5 (the highest nitrogen condition), which was only 68.1% as compared to N4. An almost similar variation pattern was observed for net growth of tree height and dry mass accumulation (Fig. 3a and b). The net growth of tree height ranged from 3.1 to 16.3 cm and in the order of N4 > N2 > N3 > N1 > N5. The highest and lowest root biomass was achieved in treatment N4 and N5, respectively, with a 2.72-fold difference (Fig. 3b). A similar variation pattern was also observed for total biomass accumulation, with a variation range from 3.4 to 7.2 g/plant. However, the highest biomass accumulation in stalk and leaf was detected in treatment N3 and N2, respectively.

3.4. Flavonoid content

In this study, the contents of total flavonoid and three individual flavonoids were measured. Analysis of variance indicated that flavonoids contents were significantly influenced by N availability

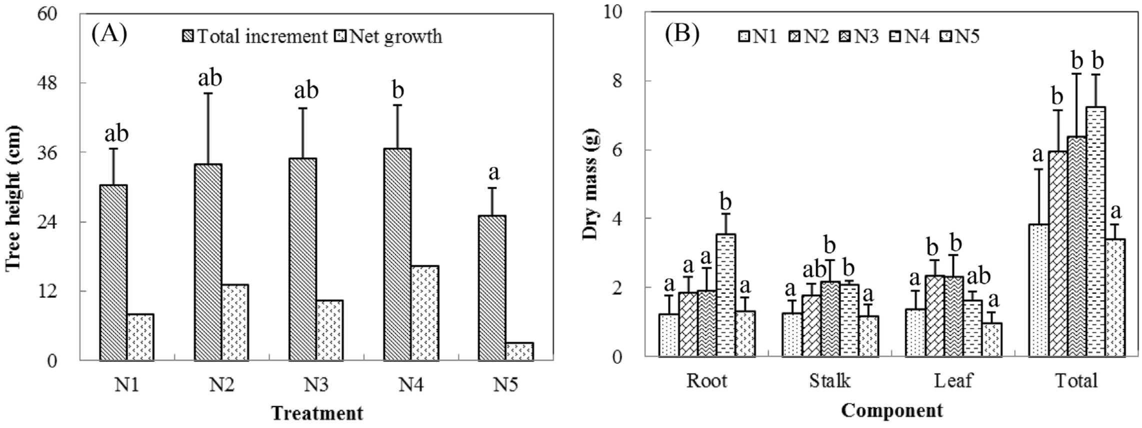

Fig. 3. Effects of nitrogen on tree height growth (net growth was measured from May to August) (A) and biomass accumulation in different organs of *C. paliurus* (B). Different letters indicate significant differences among nitrogen treatments for the same category according to Duncan's test ($p < 0.05$).

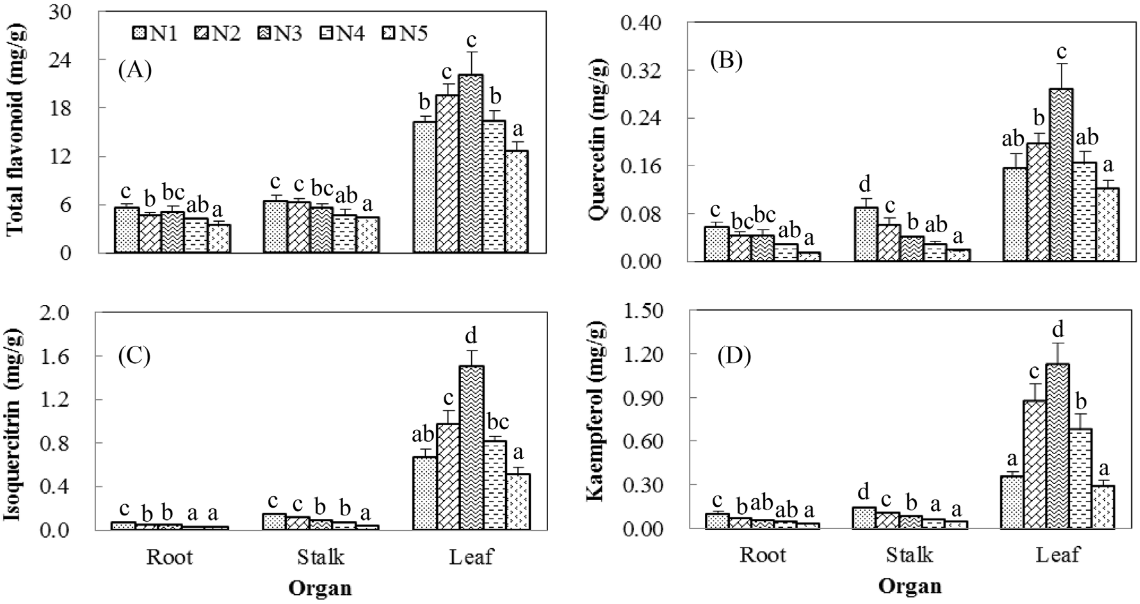

Fig. 4. Effects of nitrogen on the contents of total flavonoid and three individual flavonoids in different organs in *Cyclocarya paliurus* seedlings. Values within each graph followed by the different letters indicate significant differences among nitrogen treatments according to Duncan's test ($p < 0.05$).

($p < 0.05$). The highest total flavonoid contents accumulated in roots and stalks were detected in treatment N1, and linear decreased with the elevated nitrogen availability (Fig. 4a). As compared with N1, the contents of total flavonoid in N5 were decreased 37.2% in roots and 31.6% in stalks, respectively. However, flavonoid accumulation in leaves showed a different variation pattern, that intermediate N availability (N3) resulted in the highest content (22.2 mg/g dry mass), and the lowest content was detected in treatment N5 (12.7 mg/g dm). Furthermore, the variation patterns of the three individual flavonoids were similar to total flavonoid along with the nitrogen gradient (Fig. 4b, c, d). For example, the contents of quercetin in root and stalk were linear decreased from 0.06 to 0.04 mg/g and 0.09 to 0.02 mg/g with the elevated nitrogen availability, respectively, while the highest content of quercetin in leaf achieved in N3. Isoquercitrin, the main individual flavonoid among the three individual flavonoids, had an overall mean content of 0.35 mg/g dm, followed by kaempferol (0.28 mg/g dm) and quercetin (0.09 mg/g dm). Pearson correlation analysis indicated that concentrations of P, K and Mg did not significantly correlated to flavonoid contents in leaves of *C. paliurus*, except for P and kaempferol, and for Ca and total flavonoid (Table 3). However, there were significant negative correlations between Mg and total flavonoid, quercetin and kaempferol ($p < 0.05$).

Of the organ evaluated, leaves were the main position of flavonoid accumulation, while roots had the lowest flavonoid content (Table 2). Variance analysis showed that flavonoid in leaf was significantly higher than root and stalk. Under the five different nitrogen levels, the overall mean contents of total flavonoid in root, stalk and leaf were 4.62, 5.48 and 17.45 mg/g dm, respectively. The contents of quercetin, isoquercitrin and kaempferol in the three different organs ranged from 0.04 to 0.10 mg/g dm, 0.05–0.90 mg/g dm and 0.07–0.67 mg/g dm.

Table 2

Mean flavonoid contents in different organs of *Cyclocarya paliurus* seedlings. Different letters indicate significant differences among the organs for each flavonoid ($p < 0.05$ by Duncan's test).

Organ	Flavonoid content (mg/g)			
	total flavonoid	quercetin	isoquercitrin	kaempferol
root	4.62a	0.04a	0.05a	0.07a
stalk	5.48a	0.05a	0.10a	0.10a
leaf	17.45b	0.19b	0.90b	0.67b

3.5. Enzyme activity

Activities of PAL, CHS and FHT in leaves of *C. paliurus*, the main enzyme of each synthetic stage, were detected under the different nitrogen availability. PAL activity was relatively higher in low nitrogen condition (N1 and N2), while significantly decreased with the elevated N availability (N3, N4 and N5), which approximate preserved activity as 51% was still left (Fig. 5a). FHT activity had a similar variation pattern compared to flavonoid accumulation in leaves, the highest activity was detected in intermediate nitrogen level (N3), and with a 2.05- and 2.98-fold as compared to N1 and N5, respectively (Fig. 5c). For CHS activity, however, a completely opposite variation pattern was observed as compared to FHT. The lowest activity of CHS was detected in treatment N3, and was only 79.2% and 59.4% as compared to N1 and N5, respectively (Fig. 5b).

4. Discussion

4.1. Effects of nitrogen availability on flavonoid accumulation

It is well known that flavonoid biosynthesis and accumulation in plant can be significantly influenced by various environmental factors, such as light intensity, temperature, UV-B radiation and soil nutrients (Xu et al., 2014; Wang et al., 2016). As an important woody medicinal plant in China, *C. paliurus* is widely cultivated because of its application in food and medicinal industrials. However, limited information is available on the effects of nitrogen fertilization on flavonoid accumulation and production. The present study indicated that elevated nitrogen availability leaded to a linear decreased flavonoid contents in roots and stalks (Fig. 4). However, the highest flavonoid content in leaves was found in intermediate nitrogen condition (N3), and the contents of the three individual flavonoids in N3 were significantly higher than other treatments. Our results partially confirmed the common viewpoint that high nitrogen could decrease the flavonoid accumulation in plants (Nguyen and Niemeyer, 2008). Furthermore, this study proved that leaves of *C. paliurus* were the main position of flavonoid accumulation (Table 2).

Nitrogen and Carbon metabolisms are tightly linked in almost every

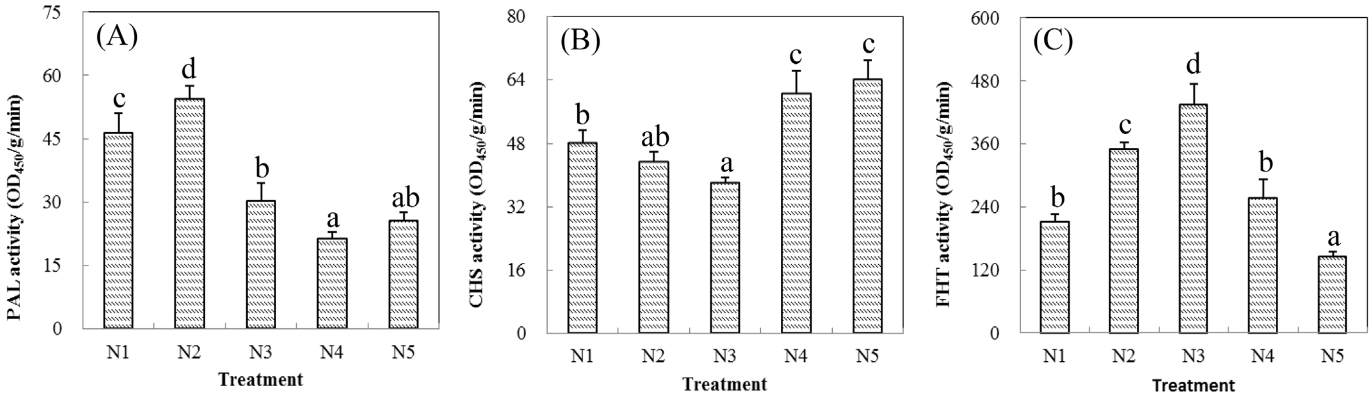

Fig. 5. Enzyme activities of PAL, CHS and FHT in leaves of *Cyclocarya paliurus*.

biochemical pathway within plants (Coruzzi and Bush, 2001). The changed C/N balance because of nitrogen fertilization was proved by carbon-to-nitrogen ratio in this study (Table 1), which was also observed in other plants, such as kale, tobacco and grapevine (Paul and Driscoll, 1997; Grechi et al., 2007; Groenbaek et al., 2016). The altered internal C/N balance could further affect the growth and metabolism of plants in many ways, such as gene expression involved in nitrogen assimilation and biosynthesis of secondary metabolites. Lillo et al. (2008) found that nitrogen depletion was a key factor for controlling gene expression of flavonoid biosynthesis. More recently, Larbat et al. (2016) indicated that nitrogen limitation can influence the partitioning of carbon resources between growth and defense, and resulted in a reduction of plant growth, while some secondary metabolites involved in plant defense accumulated in plant, such as flavonoids. Indeed, nitrogen depletion can increase the levels of amino acid and carbohydrate in leaves of tobacco, and feedback repression the photosynthesis (Paul and Driscoll, 1997). These findings suggested that the changed C/N balance because of nitrogen fertilization could alter the allocation patterns of carbon resource between primary and secondary metabolisms, and which reflected in gene expression, concentration and composition of metabolites and plant morphology. However, it is important to note that there was a different variation pattern for flavonoid content in leaf and non-leaf organs (root and stalk) (Fig. 4). Along with the nitrogen gradient, the highest contents of flavonoid in leaves were found in intermediated nitrogen availability (N3), which suggested that flavonoid biosynthesis and accumulation in *C. paliurus* were also can be inhibited by extremely low nitrogen availability (N 1 and N2 in this study). This result was agreed with the prediction of growth-differentiation balance (GDB) hypothesis (Stamp, 2003, 2004). Based on the predictions of GDB hypothesis, plants should preferentially allocate the limited resources to growth processes over differentiation under low resources availability, and would yield low growth rate and moderate secondary metabolites. When plants experiencing intermediate resource level, growth is limited by the resource, while photosynthesis is less influenced, and the excess pool of assimilates will tend to allocate to secondary metabolisms. At a high resource level, greater proportion of assimilates allocate to growth rather than differentiation.

It is important to note that we should not against the GDB hypothesis based on the different variation patterns in response to nitrogen availability between leaf and non-leaf organs (Fig. 4). As the leaves are the biosynthesis organ of flavonoids and they were capable of long-distance transportation from their synthetic site to distant tissues (root tip) in vascular tissue (Jenkins et al., 2001; Buer et al., 2007). Therefore, effects of N on flavonoid biosynthesis can be reflected only by flavonoid accumulation in leaves or in whole plants.

Activity of enzymes involved in flavonoid biosynthesis pathway was also influenced by nitrogen availability. Phenylalanine ammonia-lyase (PAL), which locates at the intersection of primary and secondary metabolisms and catalyzes the phenylalanine to form cinnamic acid, was most frequently studied. Many biotic and abiotic factors can trigger the activity of PAL, such as nutrient limitation, light intensity and fungal infection (Zhang et al., 2016). As such, it is not surprising that flavonoid biosynthesis is regulated by N availability. In the present study, PAL activity in low nitrogen levels (N1 and N2) was significant higher than in relative high nitrogen levels (N3, N4 and N5) (Fig. 5). Comparable results were also found by Kováčik and Klejdus (2014), that *Matricaria chamomilla* plants cultured in high nitrogen resulted in an increased shoot phenylalanine ammonia-lyase (PAL) activity and most of the flavonoids (four of these six selected compounds) in shoot. However, the greater PAL activity in low nitrogen availability was not resulted in a high flavonoid accumulation in *C. paliurus* leaves. That is, flavonoid accumulation in *C. paliurus* was not significantly correlated with PAL activity, and similar results were also observed in *Pyrus communis* (Steyn et al., 2004) and *Vitis vinifera* (Hiratsuka et al., 2001). However, CHS activity was significant greater in high nitrogen levels (N4 and N5) and the lowest activity was found in N3 (Fig. 5), which was also not significantly correlated with flavonoid accumulation in *C. paliurus* leaves. These results indicated that the upstream genes of flavonoid biosynthetic pathway (PAL and CHS) may not the key enzyme genes. Interestingly, the variation pattern of FHT activity was completely consistent with flavonoid accumulation pattern in leaves of *C. paliurus*, which suggested that FHT may the key enzyme of flavonoid biosynthetic pathway. Some early studies found that the gene expression of FHT can be activated by carbohydrates, and promote flavonoid accumulation (Zheng et al., 2009). Flavonoid accumulation occurred under intermediate nitrogen level condition, and usually accompanied with accumulation of carbohydrates within plants (Stamp, 2003). However, further studies are needed for recovering the regulation effects of nitrogen on flavonoid accumulation.

4.2. Effects of nitrogen availability on internal mineral nutrient balance

Mineral nutrients are indispensable for plant growth and metabolisms, as their important role in structural matters, enzymatic reactions and various physiological regulations. For example, potassium (K) limitation resulted in a decreased stomata conductance and a lowered net photosynthetic rate (Liu et al., 2011). In the present study, concentrations of phosphorus, potassium, calcium and magnesium in different organs were measured. Results indicated that the contents of these mineral nutrients within plants were significantly influenced by nitrogen availability. Indeed, there are antagonistic effects or synergistic effects among different mineral elements in nutrient absorption process, such as nitrogen absorption can decrease the uptake of P and K. That is, effects of nitrogen on flavonoid biosynthesis and accumulation may relate to these mineral elements. To our knowledge, however, none of the literature deals with effects of internal mineral nutrient balance on flavonoid accumulation.

Among the four tested mineral nutrients, effects of P on flavonoid

Table 3
Pearson correlation coefficients (*r* value) between flavonoid content and mineral nutrient (n = 15).

	Flavonoid content (mg/g)			
	total flavonoid	quercetin	isoquercitrin	kaempferol
P	−0.22	−0.36	−0.38	−0.52*
K	0.35	0.28	0.17	0.11
Ca	−0.65**	−0.41	−0.32	−0.45
Mg	−0.70**	−0.51*	−0.43	−0.50*

* and ** indicate significance at $p < 0.05$ and 0.01, respectively. Total flavonoid, the three individual flavonoids and mineral nutrients (P, K, Ca and Mg) represent values in leaves of *C. paliurus*.

accumulation were most frequently studied. Lillo et al. (2008) found that P depletion can activate gene expression of flavonoid biosynthesis pathway. Rouphael et al. (2012) also found flavonoid accumulation in leaves of *Cynara cardunculus* was decreased in high nutrient solution concentration condition. However, our results indicated that there were no significant correlation between flavonoid accumulation in leaves of *C. paliurus* and P concentration within plants except for P and kaempferol (Table 3). This difference suggested that interaction effect between N and P can affect flavonoid accumulation in *C. paliurus*. Of K evaluated, Liu et al. (2011) found K-supply was related to flavonoid accumulation in leaves of *Chrysanthemum morifolium*, which agreed with our result, although concentration of K was not significantly correlated to flavonoid in *C. paliurus*. Sucrose transport in K-deficient leaves was restricted and the leaves tended to accumulate soluble sugars (Liu et al., 2011). Moreover, flavonoid biosynthesis and accumulation were usually occurred when there were surplus carbohydrates within plants (Stamp, 2003). As such, it is not surprise that the positive correlation between K and flavonoid accumulation.

Interestingly, Ca was significant negatively correlated with total flavonoid accumulation ($p < 0.05$, Table 3). Furthermore, Mg was also significant negatively correlated with flavonoid accumulation, except for Mg and isoquercitrin. These results indicated that deficient of Ca and Mg within plants could increase flavonoid accumulation. However, limited information is available on effects of Ca and Mg on flavonoid accumulation. Overall, this study indicated that internal nutrient balance can be influenced by nitrogen availability, and further affected flavonoid accumulation in *C. paliurus*. However, further studies are needed for illuminating the interaction effects of nitrogen and other mineral nutrients on flavonoid accumulation.

4.3. Effects of nitrogen availability on flavonoid production per plant

On the basis of dry mass accumulation and flavonoid contents in different organs, flavonoid productions in the leaves and in whole plant were calculated for each treatment (Fig. 6). In the leaves, total flavonoid production per plant ranged from 12.21 to 48.70 mg/plant, while productions of quercetin, isoquercitrin and kaempferol were from 0.11 to 0.63 mg/plant, from 0.58 to 3.69 mg/plant and from 0.40 to 2.81 mg/plant, respectively. The highest productions of total and three individual flavonoids in leaves were achieved in intermediate nitrogen level (N3), while the lowest productions were observed in high or low nitrogen levels, which was consistent with the variation patterns of flavonoid content in leaves. As compared with flavonoid production in leaves, a similar variation pattern was observed for flavonoid production in whole plant, while the overall mean production of total flavonoid, quercetin, isoquercitrin and kaempferol increased 60.1%, 46.2%, 14.7% and 22.9%, respectively. The data of flavonoid production per plant confirmed the prediction of GDB hypothesis once again, that assimilates allocated to secondary metabolites should peak at intermediate resource availability (Stamp, 2003, 2004).

Plant growth mainly relies on the primary metabolisms, while plant defense relies notably on the synthesis of secondary metabolites, such as flavonoids and alkaloids. In the past decades, plant ecologists focused on the potential trade-off result between growth and defense for studying plant-pathogens relationships (Larbat et al., 2016). This trade-off between growth and defense is also an important aspect for cultivation of crops and medicinal plants. In the present study, the highest total biomass accumulation in relative high nitrogen level (N4), while did not result in the highest flavonoid production per plant (Figs. 3 and 6). Understanding how environmental factors impact growth and accumulation of secondary metabolites will be of great important for optimizing growth conditions for maintaining the quality of raw materials while still keeping a high production of target phytochemicals.

To obtain the greatest yield of target phytochemicals on unit area, subtly regulation of field growing conditions is needed. However, more

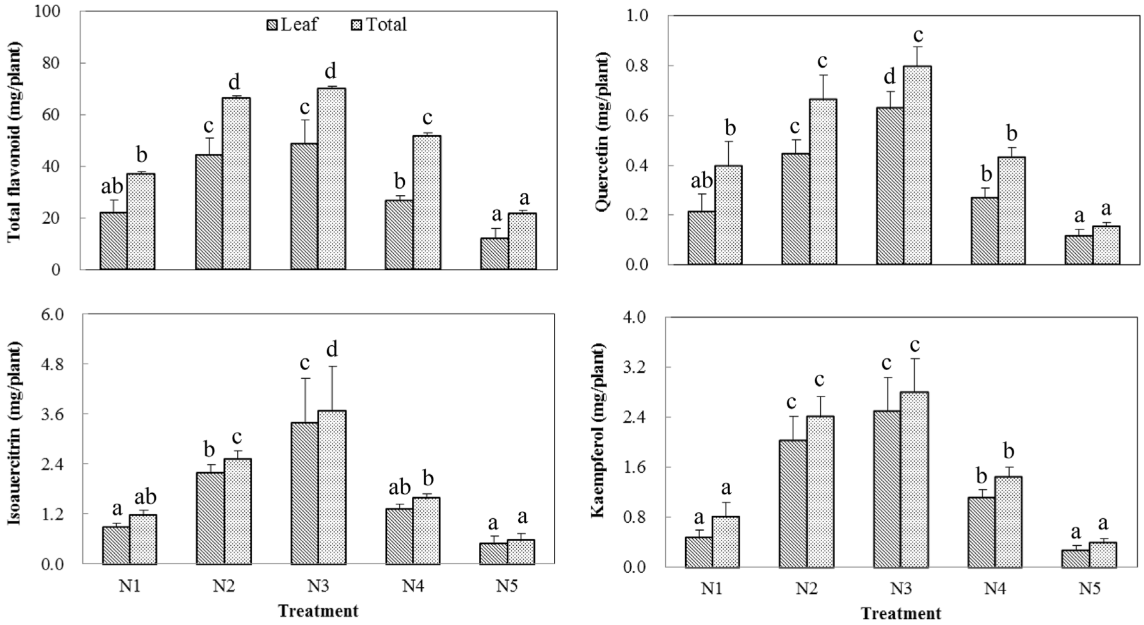

Fig. 6. Accumulation of total and three individual flavonoids in leaves and whole plants of *Cyclocarya paliurus* among the different treatments. Values within each graph followed by the different letters indicate significant differences among nitrogen treatments (lower case) according to Duncan's test ($p < 0.05$).

studies under field conditions are necessary, as environmental factors are different between field condition and phytotron, such as light intensity and air temperature, and the interaction effects of these factors may result in a different conclusion. Overall, this study provide a basis for optimizing the silvicultural system of *C. paliurus* to economically obtain target secondary metabolites.

Our results revealed that internal mineral nutrient balance, growth and flavonoid accumulation of *C. paliurus* were significantly influenced by nitrogen availability. Along with the nitrogen gredient, total N in roots, stalks and leaves were significantly increased, while total carbon was relatively stable, and resulted in a linear decreased C: N ratio. The effects of nitrogen availability on concentrations of phosphorus, potassium, calcium and magnesium were complex in different organs, giving increasing and decreasing responses. Intermediate nitrogen level (N3) induced the highest falvonoid accumulation in leaves, where was the main organ of flavonoid accumulation in *C. paliurus*. There was a similar variation pattern between leaf flavonoid and FHT activity, which suggested that FHT was the key enzyme of flavonoid biosynthetic pathway. Furthermore, correlation analysis indicated that internal mineral nutrient balance can significantly affect flavonoid accumulation, especially for Ca and Mg within plant. However, further studies are needed for illuminating the interaction effects of nitrogen and other mineral nutrients on flavonoid accumulation. Overall, these results revealed that nitrogen availability and the following altered internal mineral balance can significantly affect flavonoid accumulation. The present study can provide the basis for developing new agricultural practices to maintain high yield while still keeping the nutritional value of crop or medicinal plants.

Conflicts of interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Bo Deng: Formal analysis, Funding acquisition, Project administration, Writing – original draft. **Yuanyuan Li:** Data curation, Investigation. **Gang Lei:** Investigation, Software. **Guihua Liu:** Conceptualization, Supervision, Writing – original draft.

Acknowledgements

Financial support for this work were provided through the National Natural Science Foundation of China (31800528), and the project of Introduce and Stability of Talents (yj2016-03).

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