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Integrated Effects of Light Intensity and Fertilization on Growth and Flavonoid Accumulation in *Cyclocarya paliurus*

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ABSTRACT: *Cyclocarya paliurus* has been used for drug formulations and ingredients in functional foods in China. Field studies were conducted to examine the relationships between environmental factors and flavonoid accumulation. A split-plot randomized design was used to establish three shading treatments and three fertilization levels, and growth parameters and flavonoid contents were detected. The greatest biomass production was achieved in intermediate shade and fertilization treatment, and leaf production per seedling increased by 139.5% compared to the treatment without shade and fertilization. Overall, shade and fertilization had a significantly negative effect on contents of total flavonoid, kaempferol, quercetin, and isoquercitrin in leaves of *C. paliurus*. However, the greatest accumulation of total flavonoid in the leaves was observed in intermediate shade and fertilization treatment, achieving 364.4 g/plant. The results suggest that manipulating the field growing conditions and optimizing the silvicultural system would be important for obtaining the greatest yield of targeted health-promoting substances.

KEYWORDS: *Cyclocarya paliurus, shade, biomass production, quercetin, kaempferol, isoquercitrin*

INTRODUCTION

Cyclocarya paliurus (Batal) Iljinskaja, the sole species in its genus, is native to China.[1] The leaves of *C. paliurus* have been a food resource for maritime people for a long time and have also been used for drug formulations in traditional Chinese medicine (TCM) and as an ingredient in functional foods in China.[2−4] Some studies demonstrated that the leaves of *C. paliurus* have beneficial effects in the prevention of hypolipidemia and diabetes mellitus, whereas the extracts from leaves of *C. paliurus* were shown to strongly inhibit protein tyrosine phosphatase 1B (PTP1B) and to inhibit pancreatic lipase (PL) activity.[2,5,6] However, most studies on *C. paliurus* were focused on extract activities and low molecular weight substances, such as triterpenoids, flavonoids, steroids, saponins, and other compounds present in this plant, whereas less attention was paid to the silvics of the species.[1,3,4] Owing to its multiple beneficial effects on human health, a huge production of leaves is required for *C. paliurus* tea production and for medical use. However, there are not enough *C. paliurus* plantations to produce the leaves.[1] Thus, recently, attempts have been made to develop plantations of *C. paliurus* as a functional food or an ingredient to be used in TCM.

The content of phytochemicals in plants is affected by genetic, cultural, harvesting, and environmental factors that occur during the growing period.[7] Thus, factors influencing the phytochemical content and profile in the production of plants are worth considering for both plant and human health. Flavonoids are a large group of phenolic plant constituents, and the bioavailability of flavonoids varies greatly between different subgroups and compounds.[8] Some beneficial bioactivities of flavonoids have been proved, such as antibacterial, anticarcinogenic, antioxidant, antimutagenic, anti-inflammatory, antiallergic, antiobesity, and antidiabetic activities.[6,8,9] Consequently, flavonoids from plants, as functional food ingredients, have become a hot topic for research and development. Previous chemical constituent studies have shown the presence of abundant phenolic compounds in *C. paliurus*, especially flavonoids, and three flavonoid compounds, quercetin, kaempferol, and isoquercitrin, were isolated from the leaves of *C. paliurus*.[4,6,10,11] Fang et al. investigated the genetic and temporal variations of quercetin, kaempferol, and isoquercitrin in leaves of *C. paliurus*, but no information is available on the influences of environmental and cultural factors on phytochemical contents of *C. paliurus* leaves.[4]

Björkman et al. indicated that factors that influence phytochemical content and profile may interact, and studies of plant compounds were restricted by methods allowing only a reductionistic approach.[7] However, it is now possible to design multifactorial experiments that simulate their combined effects, and this will provide important information to ecologists, plant breeders, and agronomists. The objectives of the present study were to investigate the integrated effects of environment and fertilization on accumulation of total flavonoid and key health-promoting flavonoids (quercetin, kaempferol, and isoquercitrin) in the leaves of *C. paliurus* under field conditions. The information provided by this study would be of great value for identifying and increasing the health-promoting effects and establishing optimal cropping strategies of *C. paliurus* plants.

MATERIALS AND METHODS

Plant Material and Experimental Conditions. Seeds of *C. paliurus* were collected in late October 2009 and were subjected to chemical scarification, exogenous gibberellin A3 (GA3) treatments, and stratification treatments in early January 2010, according to the method proposed by Fang et al.[1] After a 3 month stratification treatment, the germinated seeds were sown in containers, and the seedlings in the containers were transplanted into the experimental site at a spacing of 40 × 50 cm in early June 2010.

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The experiment was carried out during the 2010 growing season in Zhenjiang Nursery, Jiangsu Province, China, and the site conditions were the same as described by Fang et al.[4] A split-plot randomized design was used to establish three shading treatments in split plots and three fertilization levels on each seedling in split subplots. With three replications, the trial gave a total of 27 subplots, and each subplot consisted of 20 seedlings.

At approximately 20 days after planting, shading and fertilization treatments were conducted in late June 2010. Three shading treatments were subjected to three light intensity regimens: 100% sunlight (A1, without shading net), about 50% of solar radiation (A2, covered with one layer of shading net at 2 m height), and around 15% of solar radiation (A3, covered with two layers of shading net at 2 m height). Three fertilization levels were 0.0 g/plant (B1), 20.0 g/plant (B2), and 40.0 g/plant (B3), and a commercial inorganic NPK fertilizer (15% N, 15% P_2O_5, and 15% K_2O) was used in this study. The NPK fertilizer was given at two separate times with hole fertilization. The first fertilization comprised 10.0 g/plant for B2 and 20.0 g/plant for B3 and was applied in late June, whereas the second fertilization was added with 10.0 g/plant for B2 and 20.0 g/plant for B3 in late August 2010. Normal agronomic practices (soil tillage and weed control) were provided for all of the treatments during the experimental periods.

A hand-held Agricultural Weather Station (TNHY series model, Zhejiang Top Instrument Co. Ltd., Hangzhou, China) was set up to monitor environmental factors in different shading treatments automatically. Photosynthetic photon flux density (PPFD) was recorded at full sunlight and under shade conditions at intervals of 30 min, whereas air temperature (T) and relative humidity (RH) were measured at intervals of 10 min during the experimental periods.

Growth and Biomass Assessment. Growth and biomass assessments of the seedlings were conducted on October 20, 2010. The height and basal diameter of each seedling were measured for all treatments, and the mean-tree technique was used to assess the biomass.[12] The selection of sample seedlings was based on the mean basal diameter and height of seedlings in each plot, and a total of 27 sample seedlings (3 sample seedlings for each treatment) were selected and excavated. After excavating, the sample seedlings were washed with tap water. Then leaf, stem, and root components of each sample seedling were separated, weighed, and dried at 70 °C. The total dry mass of each seedling was calculated as the sum of leaf, stem, and root dry weights.

All dried samples were sliced and ground into fine powder before extraction. Samples were stored at room temperature prior to analysis of flavonoid contents.

Determination of Flavonoid Content. *Total Flavonoid.* The total flavonoid content was determined by using a colorimetric method with minor modification.[13] In brief, 1.0 g of a sample was placed in a Soxhlet extractor and refluxed with 75% methanol for 2 h at 80 °C. The extract was evaporated to dryness in a rotary vacuum evaporator at <40 °C and then dissolved with methanol. Exactly 0.3 mL of 5% $NaNO_2$ was added to a 1 mL extract in a 10 mL volumetric flask, and the mixture was kept for 5 min at room temperature. Addition of 0.3 mL of 10% $AlCl_3{\cdot}6H_2O$ to the mixture, which was incubated for another 5 min, was followed by the addition of 2 mL of 1 M NaOH. After 15 min of incubation at room temperature for color development, the absorbance at 415 nm was measured. Total flavonoid content was calculated using the standard rutin curve and expressed as milligrams rutin equivalent per gram of dry weight (mg/g dm).

Kaempferol, Quercetin, and Isoquercitrin. For extraction of quercetin and kaempferol, 1.0 g of the dry sample was hydrolyzed with 50 mL of petroleum ether for 2 h and then refluxed with methanol for 4 h at 80 °C. After the extract was evaporated to dryness, the residue was refluxed with a mixture of methanol and HCl (the volume ratio of methanol to HCl is 4:1) at 100 °C for 0.5 h. The extract was cooled to room temperature and adjusted to 25 mL with methanol. Then the extract was filtered through a 0.45 μm organic phase filter for HPLC analysis. However, for analysis of isoquercitrin, 1.0 g of the dry sample was hydrolyzed with 25 mL of 75% methanol in duplicate. After 2 h of refluxing at 80 °C, the extract was cooled to room temperature. Then, the extract was filtered through a 0.45 μm organic phase filter for HPLC analysis.[4]

The analyses of selected compounds were performed on a Waters 2695 Alliance HPLC system (Waters Corp., Milford, MA, USA), equipped with a quaternary pump solvent management system, an autosampler, and an online degasser. The separation was carried out on a Kromasil 100-5 C18 column (250 mm × 4.6 mm, 5 μm) at a column temperature of 35 °C.

One solvent system with two solvents (A, acetonitrile; and B, 0.1% phosphoric acid) was used for quercetin and kaempferol determination. The chromatographic separation was performed by isocratic elution of the mobile phase (mixture of solvents A and B (34:66 v/v) that was filtered under vacuum through a 0.45 μm membrane before use) at a flow rate of 1.0 mL/min. Detection was performed at a wavelength of 365 nm. However, for the isoquercitrin determination, the mobile phase was composed of A (acetonitrile) and B (0.1% phosphoric acid (v/v)) with a gradient elution at the flow rate of 1.0 mL/min: 0−28 min, 14% A; 28−29 min, 90% A; 29−35 min, 90% A; 35−36 min, 14% A; 36−48 min, 14% A. Re-equilibration duration was 15 min between individual runs. Detection was performed at a wavelength of 350 nm.

Identification of kaempferol, quercetin, and isoquercitrin was carried out by comparing their retention times with those of authentic standards. Quercetin and kaempferol were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), whereas isoquercitrin was obtained from Fluka Chemicals Co. (Milwaukee, WI, USA). Quantitative determination was carried out using calibration curves of the standards.[4]

Statistical Analysis. Data are reported as the mean ± standard deviation (SD), and all tests were performed using the SPSS 10.0 statistical software program (SPSS Inc., Chicago, IL, USA). After a Levene test for the homogeneity of variances across data sets, a one-way analysis of variance (ANOVA) was conducted to compare the integrated effects of shading and fertilization on growth, flavonoid contents, and flavonoid accumulation per plant among the treatments. However, a two-way ANOVA, with shading and fertilization as the main fixed factors plus a shading × fertilization interaction term, followed by Duncan's multiple-range test, was performed for each growth and flavonoid variable. All statistical analyses were performed at a 95% confidence level.

RESULTS AND DISCUSSION

Variation in Environmental Factors. The microclimatic parameters were changed in different shading conditions. From July 1 to September 30, values of daily mean air temperature in the three shading treatments were 28.96 ± 7.75 °C for A1, 27.49 ± 6.49 °C for A2, and 26.97 ± 5.48 °C for A3, whereas the daily mean RH values were 75.77 ± 22.57, 81.90 ± 17.90, and 81.93 ± 17.04% for A1, A2, and A3, respectively.

A similar dynamic of daily photosynthetic photon flux density (PPFD, daytime 6 a.m.−6 p.m.) was recorded in the three shading treatments from July 1 to September 30; however, a great difference in the range of PPFD was observed. The distribution of PPFD in treatment A1 was 40.5% in 0−200 μmol/m^2/s, 19.0% in 201−400 μmol/m^2/s, 13.5% in 401−600 μmol/m^2/s, 9.5% in 601−800 μmol/m^2/s, 6.4% in 801−1000 μmol/m^2/s, and 11.0% in >1000 μmol/m^2/s during the 3 months (Figure 1), whereas the greatest PPFD distribution in treatment A3 was in 0−200 μmol/m^2/s (accounting for 95.6%). The PPFD in treatment A2 ranged from 0 to 800 μmol/m^2/s, and the distribution was 63.9% in 0−200 μmol/m^2/s, 22.1% in 201−400 μmol/m^2/s, 13.2% in 401−600 μmol/m^2/s, and 0.8% in 601−800 μmol/m^2/s in the same periods (Figure 1).

However, shading treatments affected not only solar radiation, air temperature, and RH values but also numerous

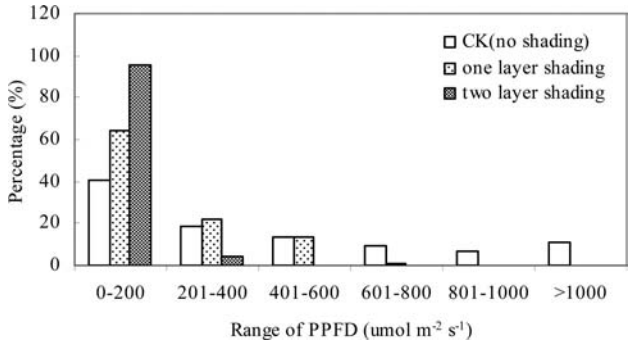

Figure 1. Distribution percentage of photosynthetic photon flux density (PPFD) at different PPFD ranges under three shading treatments during experimental periods (from July 1 to September 30, 2010).

other environmental parameters, such as soil temperature and soil moisture content. Therefore, the monitoring of climatic conditions during growth was not sufficient in this study to identify precisely the environmental factors, and further research is required to unravel the effects of individual environmental factors on growth and flavonoid accumulation of *C. paliurus*.

Variation in Biomass Production. A significant integrated effect of shading and fertilization treatments on biomass production of *C. paliurus* seedlings was detected ($p < 0.05$). The total biomass production per seedling among the treatments was in the order A2B2 > A1B3 > A2B1 > A1B2 > A3B1 > A2B3 > A1B1 > A3B2 > A3B3, and a similar treatment effect on the biomass production of leaf, stem, and root was also observed (Figure 2). Allocation of total biomass to the root, stem, and leaf, for which nine treatments were averaged, was 23.1, 31.7, and 45.2%, respectively. However, there were obvious differences in the allocation ratios of biomass to the components among different treatments. The greatest ratio of root to total biomass was achieved in treatment A1B1 (28.0%), whereas the highest ratios of stem and leaf were observed in treatments A2B3 (38.5%) and A3B3 (51.4%), respectively. Compared to treatment A1B1, the mean leaf production per seedling in treatments A2B2, A1B3, A2B1, A1B2, A3B1, and A2B3 increased by 139.5, 98.4, 74.8, 61.2, 47.6, and 25.0%, respectively, whereas the leaf biomass in A3B2 and A3B3 decreased by 9. 8 and 13.6%, respectively.

Horticultural production has primarily focused on increasing productivity through intensification of fertilizers and water.[14] However, sunlight is one of the major environmental factors for plant growth and yield, whereas the light compensation points and light saturation points were different for various plants.[15] Our study indicated that shading and interaction of shading × fertilization had significant effects on leaf and total biomass production (Table 1). Compared to treatment A3 (two-layer shading), plant growth obviously increased with increasing light intensity and achieved its maximum at intermediate light levels (A2 treatment, one-layer shading), but it slightly declined above the levels (treatment A1, no shading). The response of *C. paliurus* seedlings to light was similar to that of many woody species growing under different light regimens.[16,17] Our results also confirmed the conclusion from a study of *Poa crymophila*, where the biomass decreased as the light reduced, but could be compensated by fertilization.[18] The results from this study also suggested that *C. paliurus* seedlings increased leaf biomass ratio at relatively low light intensity, whereas the seedlings showed an increase in root biomass allocation to favor an increase in water uptake and a decrease in transpiration rate (Figure 2), in agreement with the results from *Rauvolfia* species[17] and freshwater macrophytes.[19]

Fertilizer application has been shown to improve the growth of individual plants and to increase net primary production in a variety of forest ecosystems.[20,21] Our result indicated that no significant difference in biomass production was observed for the fertilization treatment, provided shading was applied. This is in agreement with most earlier studies with shrub and/or tree seedlings, which did not find any growth stimulation in response to increased nutrient supply at low light levels.[22,23] However, the result is in contrast to the study of Hättenschwiler, who indicated that increased soil nutrient availability can stimulate seedling growth of some liana species in the understorey of a lowland tropical rainforest.[24] In forest understoreys, Coomes and Grubb concluded that light alone limits seedling growth in forests on nutrient-rich soils, whereas competition for nutrients becomes increasingly important on infertile soils.[25]

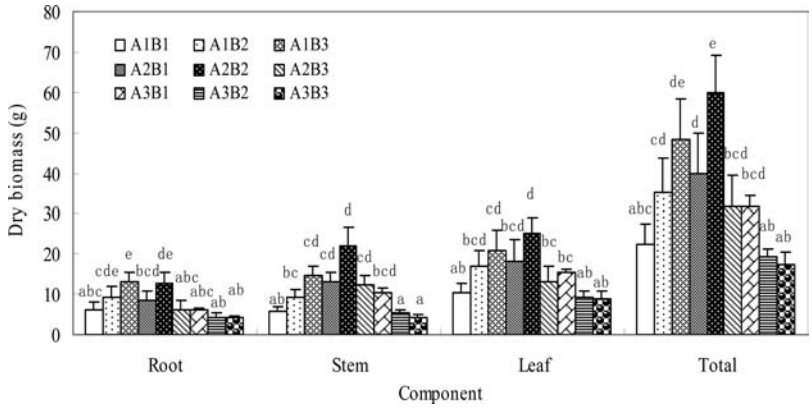

Figure 2. Variation in biomass production of *Cyclocarea paliurus* seedlings in different shading and fertilization treatments (mean ± SD). Different lower case letters indicate significant differences between various treatments within a component ($p < 0.05$ by Duncan's test). A1, A2, and A3 represent no shading, shading with one-layer net, and shading with two-layer nets, respectively, whereas B1, B2, and B3 indicate the addition of inorganic NPK fertilizer at rates of 0.0, 20.0, and 40.0 g/plant, respectively. A1B1 represents the treatment without shading and fertilization, A1B2 represents the treatment without shading and with 20.0 g/plant fertilizer, and so forth.

Table 1. Summary of Significance Levels (Two-Way ANOVA) for the Effects of Shading and Fertilization on Biomass Production, Flavonoid Contents, and Flavonoid Accumulation in Leaves

	significance (p value)[a]									
	biomass (g/plant)		flavonoid content (mg/g dm)				flavonoid accumulation (g/plant dm)			
source	leaf	total	total	isoquercitrin	kaempferol	quercetin	total	isoquercitrin	kaempferol	quercetin
shading (A)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
fertilization (B)	**0.972**	**0.770**	0.010	<0.001	<0.001	<0.001	0.034	**0.273**	0.005	**0.216**
interaction of A × B	0.002	<0.001	**0.555**	<0.001	<0.001	<0.001	<0.001	0.001	0.004	**0.450**

[a]Bold-faced properties are considered not significantly different at a 95% confidence level.

Table 2. Mean Flavonoid Contents in the Different Components of *Cyclocarya paliurus* Seedlings

	flavonoids[a] (mg/g dm)			
component	total flavonoid	isoquercitrin	kaempferol	quercetin
root	6.24 ± 0.67a	0.02 ± 0.03a	0.04 ± 0.01a	0.01 ± 0.01a
stem	4.76 ± 1.47a	0.05 ± 0.04a	0.05 ± 0.02a	0.02 ± 0.01a
leaf	18.91 ± 3.80b	0.82 ± 0.81b	0.68 ± 0.65b	0.28 ± 0.26b

[a]Means ± SD in the same column with different letters are statistically significantly different among the components for each flavonoid ($p < 0.05$ by Duncan's test).

Table 3. Flavonoid Contents in the Leaves of *Cyclocarya paliurus* under Different Treatments

	flavonoids[a] (mg/g dm)			
treatment	total flavonoid	isoquercitrin	kaempferol	quercetin
A1B1	25.63 ± 3.74e	2.67 ± 0.40e	2.72 ± 0.37c	0.93 ± 0.20c
A1B2	22.49 ± 2.96d	1.15 ± 0.10c	1.22 ± 0.35b	0.42 ± 0.09b
A1B3	21.24 ± 1.60cd	1.50 ± 0.13d	0.43 ± 0.08a	0.35 ± 0.12b
A2B1	19.21 ± 1.51bc	0.65 ± 0.05b	0.33 ± 0.04a	0.18 ± 0.06a
A2B2	18.63 ± 1.58bc	0.53 ± 0.04b	0.34 ± 0.03a	0.17 ± 0.01a
A2B3	16.16 ± 2.99ab	0.27 ± 0.02a	0.37 ± 0.06a	0.15 ± 0.01a
A3B1	16.20 ± 1.25ab	0.16 ± 0.01a	0.18 ± 0.05a	0.13 ± 0.04a
A3B2	15.84 ± 1.34ab	0.20 ± 0.02a	0.28 ± 0.05a	0.12 ± 0.03a
A3B3	14.76 ± 1.65a	0.41 ± 0.03a	0.26 ± 0.06a	0.12 ± 0.02a

[a]Means ± SD in the same column with different letters are statistically significantly different among the treatments for each flavonoid ($p < 0.05$ by Duncan's test).

Table 4. Duncan's Multiple-Range Test of Biomass Production and Flavonoid Contents in Leaves after a Two-Way ANOVA

		biomass[a] (g/plant)		flavonoid contents in leaves[a] (mg/g dm)			
treatment	level	leaf	total	total	isoquercitrin	kaempferol	quercetin
shading (A)	A1	16.0b	35.4b	23.120c	1.774c	1.457b	0.567b
	A2	18.8b	43.7b	17.998b	0.484b	0.348a	0.164a
	A3	9.0a	19.2a	15.598a	0.212a	0.241a	0.123a
fertilization (B)	B1	14.7a	31.4a	20.348b	1.161b	1.078c	0.411b
	B2	14.8a	34.4a	18.985ab	0.626a	0.613b	0.237a
	B3	14.3a	32.6a	17.383a	0.682a	0.354a	0.207a

[a]Means in the same column with different letters are statistically significantly different among the treatment levels for each flavonoid ($p < 0.05$ by Duncan's test).

Variation in Flavonoid Content. There were significant differences in the mean flavonoid contents among different components of *C. paliurus* seedlings ($p < 0.05$, Table 2). For all of the flavonoids measured (nine treatments averaged), the highest content was achieved in leaves, whereas no significant difference was found between root and stem ($p < 0.05$, Table 2).

An integrated effect on total flavonoid and selected flavonoid (kaempferol, quercetin, and isoquercitrin) contents in the leaves of *C. paliurus* seedlings was significant among the treatments ($p < 0.05$, Table 3). The highest content of both total flavonoid and selected flavonoids was observed in treatment A1B1, but no significant difference in quercetin and kaempferol was found under shading conditions (Table 3). For example, compared to treatment A1B1, the mean content of total flavonoid in the leaf in treatments A1B2, A1B3, A2B1, A2B2, A2B3, A3B1, A3B2, and A3B3 decreased by 14.0, 20.7, 33.4, 37.6, 58.6, 58.2, 61.8, and 73.6%, respectively, whereas the kaempferol content in the leaves of treatment A1B1 was 2.2,

6.3, 8.2, 8.0, 7.4, 15.1, 9.7, and 10.5 times those of treatments A1B2, A1B3, A2B1, A2B2, A2B3, A3B1, A3B2, and A3B3.

It is well-known that the plant may adjust its secondary metabolite content in response to changing environmental conditions. Cronin and Lodge reported that leaf phenolics in two freshwater macrophytes were 72% higher in unshaded than in shaded plants, whereas leaf phenolic concentrations were 31% higher in fertilized than in unfertilized plants.[19] The secondary metabolites not only play an important role in defenses against herbivores but also are frequently utilized in functional foods as an ingredient or in medicine as a therapeutic agent.[4,17,26] Flavonoid accumulation can be induced by a number of environmental conditions.[14] André et al. indicated that the environmental conditions mainly affected the quantity of phenolic composition but not the quality.[27] In the present study, a two-way ANOVA showed that both shading and fertilization treatments significantly affected the contents of flavonoid in the leaves of *C. paliurus* seedlings, and the significant interaction of shading × fertilization on the flavonoid contents was also observed except for the total flavonoid (Table 1). Overall, shading and fertilization had a significantly negative effect on the accumulation of total flavonoids, kaempferol, quercetin, and isoquercitrin (Table 4), supporting that high photosynthetically active radiation (PAR) triggers flavonoid biosynthesis, whereas light quality also affected flavonoid levels.[28,29] Increased flavonoid accumulation under low N availability has been widely reported, consistent with the present study[30,31] It could be expected that manipulation of light intensity and nutrient availability would be a powerful tool for stimulating secondary plant metabolite accumulation, particularly for crops in intensive management system. However, the response might vary for different crops and secondary plant metabolites. For instance, fertilizer application did not affect the major regulatory factors in polyphenolic formation in apples and flavonoid content in olives.[32,33]

Variation in Flavonoid Accumulation Per Plant. On the basis of the leaf biomass and flavonoid contents, total and selected flavonoid accumulations in the leaves per plant were calculated for nine treatments (Figure 3). In the leaves, the accumulation of total flavonoid ranged from 134.8 to 364.4 g/plant, whereas the accumulations of kaempferol, quercetin, and isoquercitrin were from 2.3 to 29.0 g/plant, from 1.0 to 10.0 g/plant, and from 1.8 to 31.3 g/plant, respectively. One-way ANOVA indicated that an integrated treatment effect on the accumulation of both total and selected flavonoids in leaves per plant was significant ($p < 0.05$, Figure 3).

The greatest accumulation of total flavonoid in the leaves per plant was achieved in treatment A2B2, followed by treatments A1B3 and A1B2, whereas the lowest was found in treatment A3B3. Compared to treatment A1B1, the accumulation of total flavonoid in treatments A1B2 and A1B3 was increased by 42.8 and 65.9%, respectively, whereas the total flavonoid accumulation in other treatments was decreased by 6.7−45.9%. However, the highest accumulation of both kaempferol and quercetin in the leaves per plant was obtained in treatment A1B1, whereas the greatest accumulation of isoquercitrin was achieved in treatment A1B3. Compared to treatment A1B1, the accumulation of isoquercitrin in treatment A1B3 increased by 9.8%, whereas the accumulation in the leaves per plant decreased by 32.6−93.5% in other treatments.

Several theories have been proposed to explain potential trade-offs between growth and secondary metabolite synthesis.[17] Our results support the carbon/nutrient balance

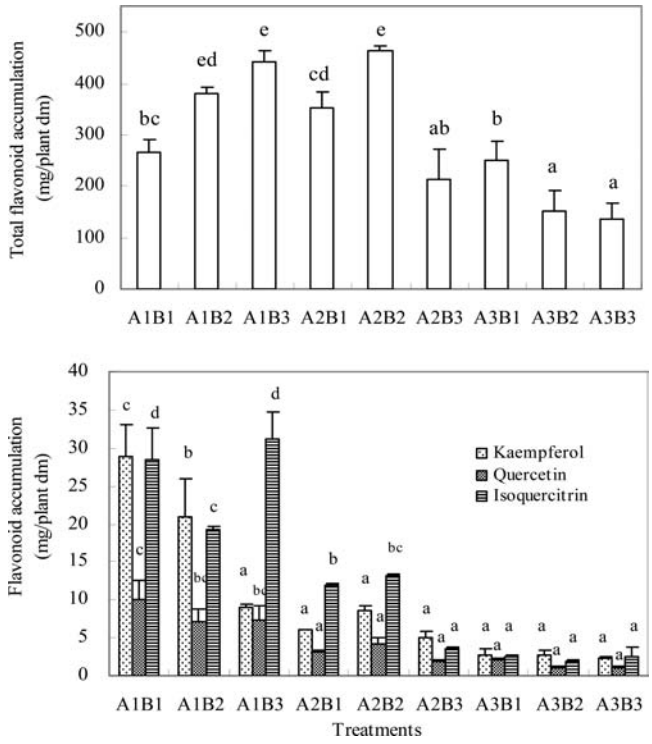

Figure 3. Variation in total and selected flavonoid accumulation in leaves of *Cyclocarya paliurus* per plant among various treatments (mean ± SD). Different lower case letters indicate significant differences between various treatments within the same flavonoid ($p < 0.05$ by Duncan's test). A1, A2, and A3 represent no shading, shading with one-layer net, and shading with two-layer net, respectively, whereas B1, B2, and B3 indicate the addition of inorganic NPK fertilizer at rates of 0.0, 20.0, and 40.0 g/plant, respectively. A1B1 represents the treatment without shading and fertilization, A1B2 represents the treatment without shading and with 20.0 g/plant fertilizer, and so forth.

theory, which suggests that if light becomes limiting (i.e., shade) in nutrient-rich environments, the decline in photosynthesis may limit carbohydrates for growth and carbon-based defenses.[34] However, some researchers reported that with the decrease in light, the carbon-based secondary metabolite (such as phenols, terpenes, etc.) decreased, whereas N-containing secondary metabolites increased in leaves of medicinal plants.[35−37] Our results could not confirm whether there was an increase in N-based secondary metabolites (e.g., alkaloids) in the leaves of *C. paliurus* seedlings with decreasing light intensity because we did not measure these chemicals.

Understanding how different growth rates and environmental factors affect the production of secondary metabolite will be of great importance for optimizing field growth conditions for maximal recovery of phytomedicinal chemicals. Plant compound contents in vegetables were affected by plant density and intercropping systems, whereas the effects of plant density and intercropping system on plant secondary metabolites are the result of a combined effect of all factors included in plant competition, such as decreased availability of light, nutrients, and water.[7] However, more studies under controlled conditions are needed to separate the effects of the multiple interacting factors that influence growth and secondary metabolite levels under various field conditions. To obtain the greatest yield of health-promoting substances per area, we should carefully manipulate field growing conditions, which can improve the

content of targeted secondary metabolites in plants while large-scale dry matter accumulation was maximized. Therefore, results from this study provide a basis for optimizing the silvicultural system of *C. paliurus* to economically produce specific flavonoids for the food and medical industries.

In conclusion, significant differences in growth, flavonoid contents, and flavonoid accumulation per plant were observed in seedlings of *C. paliurus* along experimental gradients of shading and fertilization. The microclimatic parameters were changed in different shading conditions, and both the dry matter production and flavonoid concentrations were significantly affected by environmental conditions. The greatest growth and biomass production were achieved in intermediate shade and fertilization treatment (A2B2). Overall, shading and fertilization had a significantly negative effect on contents of total flavonoid, kaempferol, quercetin, and isoquercitrin in the leaves of *C. paliurus*. However, it is worth pointing out the predominance of light intensity over fertilization effects to explain the variations in growth and flavonoid contents. The leaf flavonoid accumulation per plant depended on potential trade-offs between growth and secondary metabolite synthesis, suggesting that carefully manipulating field growing conditions and optimizing the silvicultural system would be important for obtaining the greatest yield of targeted health-promoting substances per area.

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Notes

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