Distribution Differences of Shikimic Acid in Cultivated Illicium *lanceolatum*

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***Abstract*—Shikimic acid is an important intermediate to oseltamivir phosphate which is the most effective drug for treating bird flu disease. This study was performed to determine the shikimic acid distribution of *Illicium lanceolatum* which was affected by its age, light condition and ecotype. The results indicated that the age of trees had an optimal effect on its content of leaves and it maintained at the highest level as trees grew. Whereas the content in root and branch declined with increasing age. The 50% light condition promoted accumulation of schikimic acid and the leaf contained the highest level of that regardless of the light conditions. The Wu'ning ecotype produced the largest amount of that in all organs. The result that the shikimic acid content in the leaves was higher than in the root indicated that the leaves can substitute for the root which was a traditional medicinal part. Possible mechanisms for the enhanced content are discussed. Findings from this study have provided the scientific bases for promoting shikimic acid industry, and developing a high efficiency artificial cultivation system of *I.lanceolatum*.**

***Index Terms*—Shikimic acid, *Illicium lanceolatum*, organ, age, light condition, ecotype**

I. Introduction

Shikimic acid is used as the raw material to synthesize many bioactive derivatives and it plays a key role in the biosynthesis of many important natural products including aromatic amino acids, alkaloids, phenolics, and phenylpropanoids [1]. It is very effective in anti-infection, pain-killing, and suppression of blood clot formation and strokes. It is also an intermediate for anti-virus and anti-cancer drugs [2–3].

Shikimic acid is a high valued compound used as a key starting material for the synthesis of the neuramidase inhibitor GS4104, which was developed under the name Tamiflu® for treatment of antiviral infections [4–8]. In the pharmaceutical industry, lack of supply of shikimic acid has become the bottle neck for making the drug to meet the increasing social needs [6, 9–11]. An excellent alternative to the isolation of shikimic acid from fruits of the *Illicium* plant is the fermentative production by metabolic engineered microorganisms [5]. Whereas, chemical synthesis process and microbial fermentation technology for producing shikimic acid both involve complex procedures which are results in high cost. Thus extraction of the compound from plants seems to be an affordable way to solve the supply problem [8, 10–13].

Shikimic acid was first isolated in 1885 [14], from parts of the Japanese tree, shikimino-ki (*Illicium anisatum*). However, shikimic acid to study its properties, medicinal function, it is also present in the seeds of the fruit of the star aniseed (*Illicium verum*) [1]. Several researches have been done on and extraction procedure and technology [15–20]. Some studies have identified that *Illicium* plants produced the larger amount of shikimic acid among all plant species [8, 17, 19]. So, more studies were done on characterization of shikimic acid content of *Illicium verum* [12–13, 18, 21]. Those studies have laid the foundation for exploring this plant resource. *Illicium* plants grow very slow, it is impossible to use the existing plant materials to produce enough raw materials for the pharmaceutical industry. In order to solve this problem, selecting, breeding and large scale cultivation of high shikimic acid content and fast-growing plants have to be developed which will supplement or even replace obtaining shikimic acid from wild plants [21].

Illicium lanceolatum is an important traditional Chinese herb in the genius of *Illicium*. Its bark from trunk and roots are known to be effective in promoting blood circulation, pain killing, expelling wind and removing damness [22–23]. Modern pharmacology studies demonstrated that its crude extracts and active compounds possess wide pharmacological actions and it is the major source of shikimic acid [2–3, 8, 24–25]. So in recent years, it is considered as the most promising natural resources for producing this compound [26-31]. *I.lanceolatum* has wide ecological amplitude and very spotty distribution, so its wild population is dwindling fast due to the traditional aggressive harvest style.

To satisfy the demand of global market for shikimic acid, especially as the raw material for making oseltamivir during regional or global epidemic periods of bird flu, it is an urgent task to make large scale domestication, cultivation and protection of this species, and develop a cultivation management system that can efficiently produce shikimic acid.

However, no data is available on shikimic acid production from *I.lanceolatum* trees. During the prior phase of this project, different genotypes have been collected. In this study, the content of shikimic acid was determined for different organs of trees that were growing under varied light conditions. The relationships between shikimic acid accumulation and age, organs, ecotypes of trees during period of fast growth were established. This results will provide scientific bases for exploration of the medicinal values of this species.

II. Materials and Methods

A. Description of the Experimental Site

The experiment was conducted in Forest Nursery, Tianmu Mountain, Lin'an city, Zhejiang Province, China. The geographical coordinates of this location are 118°51'-

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119°52' E, 29°56'-30°23'N, the elevation is 47 m. It is in the Mid-North Asia tropical monsoon climate zone. The annual average temperature is 15.4°C, and the air temperature in January is above 3.2°C, average air temperature in July is 29.9°C, and the annual precipitation is 1,250-1,600 mm. The experimental site was chosen on the relatively flat land in a hilly area. It has acidic yellow loam clay soil, organic matter content at 23.95 $g\cdot kg^{-1}$, total nitrogen content at 0.44 $g\cdot kg^{-1}$, total phosphorus at 10.09 $g\cdot kg^{-1}$, total potassium at 6.31 $g\cdot kg^{-1}$, alkali-hydrolyzable nitrogen at 217.62 $mg\cdot kg^{-1}$, available phosphorus content at 363.97 $mg\cdot kg^{-1}$, and available potassium at 40.51 $mg\cdot kg^{-1}$. The soil pH value was 4.62.

B. Preparation of Experimental Plants

Four ecotypes of *I.lanceolatum* respectively from Li'nan city, Zhejiang province (LA), Kai'hua city, Zhejiang province (KH), Wu'ning city, Jiangxi province (WN) and Nan'ping city, Fujian province (NP) in China were tested in this study. Seedlings were raised in the original location for each ecotype.

Fruits of each ecotype were harvested from healthy wild plants in each location during Sep–Oct, 2005 and 2006. Air-dried seeds of each ecotype were stored in moist sand at 4 °C. In mid-April, the following year, seeds were sown in the field at 10 m of inter-row and 1 m of in-row spacing.

The growth soil medium was a mixture of garden soil and organic fertilizer (2:1), the average planting density was 189 $plants\cdot m^{-2}$. The seedbed was mulched with straw immediately after seeded, and shading (50% sunlight) was provided during the summer season. Irrigation was applied at seed emergence stage and seedlings were thinned to the same density. When they were 2 years old, the plants were interplanted with different trees. According to differences in light environment for growth, plants were divided into three groups: group 1 of full light where plants were growing under full natural light; group 2 of half shadow where plants were growing under *Acer albopurpurascens* trees with 50% of crown density; and group 3 of shadow where the plants were intercropping with *Acer buergerianum* with 80% crown density of canopy. Plants growing under full light was considered as control.

The experiment was done using four-year old plants. The IL-1700 quantum radiation meter was used to measure the relative light transmittance ratio at 10:00 am for five consecutive sunny days in Sep., 2009. Based on light transmittance ratio, the three light conditions were assigned as 100% light, 50% light, and 20% light respectively.

Root, stems, leaves were collected from different ages of *I.lanceolatum* trees at the fast growing seasons in July-August in 2007, 2009 and 2010 respectively and placed in an oven for 2h at 105°C, and then dried for 12h at about 75°C. Dried plant materials were ground to a fine powder and passed through a 70-mesh sieve.

C. Analysis of Shikimic Acid Content

Shikimic acid was analyzed using a Venusil ZORBAX NH2 column (5μm，4.6×150mm), with a mobile phase of acetonitrile-1.0% phosphoric acid (94:6) running at a flow rate of 1.0 mL·min-1, with column temperature of 30°C. The detection wavelength was chosen at 210 nm [17]. Reagents were all chromatography grade (Fisher Scientific, USA). A shikimic acid standard was purchased from Sigma (USA), the chemical has a melting point of 185°C and 99% purity.

D. Data Analysis

Statistical analyses were conducted using Microsoft Excel 2003 (11.0, Microsoft Corporation, Washigton state, USA) and SPSS16.0 (SPSS Corporation, Chicago, USA). One-way analysis of variance (ANOVA) were conducted to compare the effect of different organs, ages and ecotypes on content of shikimic acid. The correlation between ages, light conditions and the content of shikimic acid were also analyzed.

III. RESULTS

A. Shikimic Acid Content in Different Ages of I.lanceolatum Trees

I.lanceolatum trees grow very slow. To study the effect of age on the accumulation of shikimic acid in different organs of plants, its content was determined in each organ from 1-4 years old trees of the ecotype from Zhejiang Tianmu Mountain, China (Table I). Table I showed that there was significant difference between the different years of groups ($p < 0.05$). For young trees of 1–3 years old, root shikimic acid content declined as trees grew, that of the one year old (1a) trees differed significantly from the 2–3 years old groups (2a and 3a) ($p < 0.05$). But the four years old tree produced the largest amount of shikimic acid content (26.82mg/g) in roots, which was at a significantly difference level from the other age trees ($p < 0.05$). Leaf content increased continuously with increasing age of trees, it reached the most rapid stage of shikimic acid accumulation in the 3a trees when the content was 53.55mg/g, i.e., 1.77 -1.73 fold of the 1a or 2a old trees. Whereas branch content declined with increasing age. For shikimic acid content of branches, there was no significant difference between the 1 and 2 year old trees (1a and 2a), neither between the 3 and 4 years old groups (3a and 4a). But, groups of 1a and 2a contained a significant higher level of shikimic acid compared to the 3a and 4a groups ($p < 0.05$), respectively. For the leaves, there was also no significant difference between the 1 and 2 year old trees, while the significant difference existed between the rest of the other ages of plants ($p < 0.05$).

When compared between different organs (Table I), the shikimic acid content in roots from young trees (1a *p* 3a) contained consistently lower level than branches and leaves, and with the significant difference between roots and branches, leaves ($p < 0.05$), respectively. For the four years old tree, it was only lower than leaves but not branches. The content in branches from the 1a and 2a trees was slightly higher than that in leaf, but for the 3a and 4a old trees which branch content was lower than the leaf and only contained 43.4% or 36.2% of leaves from the same trees. Table I showed that the shikimic acid content of different organs existed significant difference ($p<0.05$) among the same age of plants, while that of branches and leaves for one year old plants had no significant difference.

These results indicate that the age of trees had an optimal effect on shikimic acid content of leaves and it maintained at the highest level as trees grew. The four years old tree contained the highest level of shikimic acid in all the organs except branches with the lowest content of 20.95mg/g.

Significant correlations were found between the age of trees and shikimic acid production in all organs and its correlation coefficient ranged from 0.8419 to 0.8744 (Fig. 1).

TABLE I: THE CONTENT OF SHIKIMIC ACID FOR DIFFERENT ORGANS OF DIFFERENT YEARS OLD SEEDLINGS

Seedlings age	shikimic acid content(mg/g)		
	root	branch	leaf
1 year old	11.83±0.57aA	33.62±2.62aB	30.25±2.25aB
2 years old	8.31±0.11bA	34.5±2.511aB	31.03±0.97aC
3 years old	2.73±0.67cA	23.27±0.73bB	53.55±2.55bC
4 years old	26.82±1.12dA	20.95±0.05bB	57.82±1.18cC

*The capital letters indicate the difference on the 0.05 significance level between different organs; and the lower-case letters indicate the difference on the 0.05 significance level between different ages.

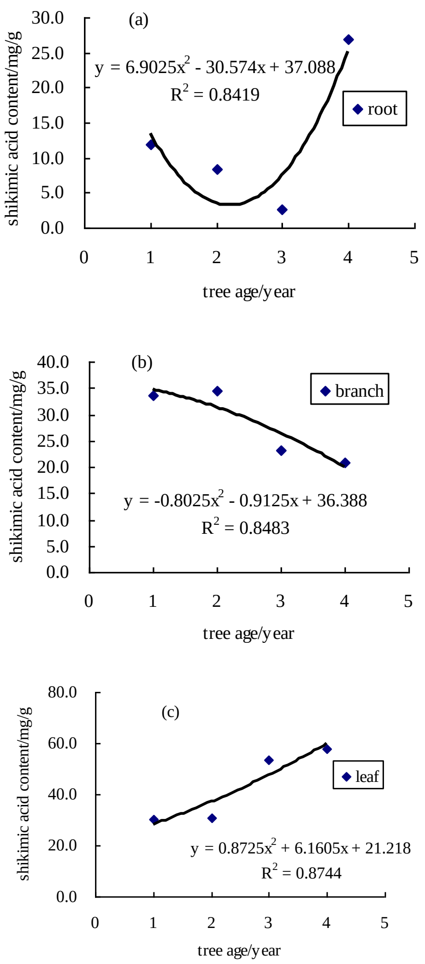

Fig. 1. The correlation between the age of trees and shikimic acid content of organs

B. Variation of Shikimic Acid Accumulaiton under Different Light Conditions

The results showed that the shikimic acid content of trees was also effected by the different light condition under which the trees grown (Table II). For the ecotypes from Zhejaing Tianmu Moutain, the shikimic acid content in roots decreased in the following order: 100% light (average 26.7mg/g) >50% light (average 15.3mg/g) >20% light (average 10.2mg/g). And that of branch bark also decreased in the following order: 100% light (average 51.2mg/g) >50% light (average 49.7mg/g) >20% light (average 26.8mg/g). However, the shikimic acid content in branches and leaves was consistently higher in the 50% light condition followed by 100% light and then 20% light condition. In the 50% light condition, the average content was 29.0mg/g in branches and 52.8mg/g in leaves. The content under the 50% light condition was 1.53-3.37 fold in branches, 0.97-1.85 fold in branch bark, and 1.20-1.50 fold in leaves respectively, of trees that were exposed to 100% or 20% light condition.

Analysis of variance indicated that shikimic acid content of root, branch and leaf for 4 years old of Illicium lanceolatum plants existed the significant differences under different illumination conditions(p<0.05). The branch bark content of plants had no significant difference between 50% and 100% light conditions, but all had significant differences with that of 20% illumination conditions respectively (p<0.05). These results indicate that 50% light environment is optimal for accumulation of shikimic acid in branch, branch bark, and leaf.

Shikimic acid content in different organs was also varied among the three light treatments. It decreased in the following order: branch bark>leaf>root>branch in the 100% natural light condition; leaf>branch bark>branch>root in the 50% light treatment and leaf>branch bark>root>branch in the 20% light treatment. Conclusively, branch bark and leaf organs contained the highest level of shikimic acid regardless of the light conditions and it was lower in roots and branches. This is the pattern of shikimic acid abundance in the whole tree. Analysis of variance also indicated that there was no significant difference in shikimic acid content between root and branch under 20% light conditions, while it was existed among the other organs (p<0.05). There was no significant difference between branch bark and leaf under 50% light conditions, while it was existed among the other organs (p<0.05). And there was significant difference among different organs under 100% light conditions (p<0.05).

Under 100% natural light, branch bark contained the highest level of shikimic acid, but leaves contained the highest concentration in the 50% and 20% light treatments. Thus partial shading enhanced shikimic accumulation in leaves and which in roots was lower and more stable. Therefore, cultivation managements should be used to effectively enhance shikimic acid accumulation in branch bark and leaves which are the primary targets for yield improvement.

Significant correlations were also found between the light intensity and shikimic acid production in all organs and its correlation coefficient ranged from 0.9946 to 1.0000 (Fig. 2).

TABLE II: THE SHIKIMIC ACID CONTENT OF DIFFERENT ORGANS FOR 4 YEARS OLD SEEDLINGS UNDER DIFFERENT LIGHT CONDITIONS

Light condition	shikimic acid content(mg/g)			
	root	branch	branch bark	leaf
20%light	10.2±0.43aA	8.636±0.08aA	26.8±1.10aB	35.13±1.04aC
50%light	15.3±0.61bA	28.968±0.78bB	49.7±0.63bC	52.83±1.42bC
100%light	26.7±0.22cA	19.040±0.54cB	51.2±0.90bC	44.04±1.07cD

*The capital letters indicate the difference on the 0.05 significance level between different organs; and the lower-case letters indicate the difference on the 0.05 significance level between different light intensities.

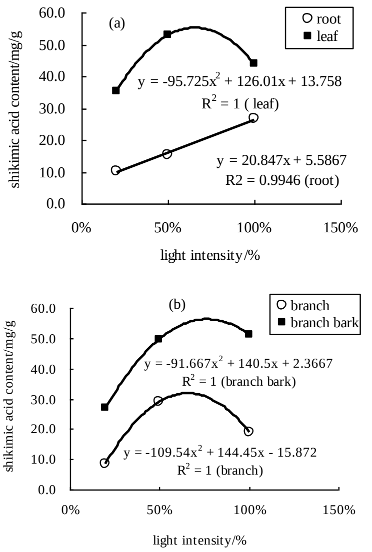

Fig. 2. The correlation between light intensity and shikimic acid content of different organs.

C. The Shikimic Acid Content of Different Age of Branches for the Same Tree

Table III showed the shikimic acid content in different ages of branches on the 4 years old trees under 50% light condition. The branches content declined significantly with its increasing age. The content in 1-4 years old branches was 17.12mg/g, 14.93mg/g, 7.94mg/g and 6.91mg/g, respectively. The 1-2 years old branches contained significantly higher shikimic acid than the 3-4 years old ones ($p<0.05$). Analysis of variance indicated that there were significant differences in shikimic acid content among different ages of branches ($p<0.05$). Fig.3. showed that the significant correlation between the shikimic acid content and different ages of branches was existed and its correlation coefficient was 0.9247. The disparity of shikimic acid content in different branches on the same tree could be caused by the uneven allocation of nutrients during the fast growing season. This showed that the different growth seasons probably made a potential impact on the shikimic acid content of different ages of branches and it needs to be further study.

TABLE III: THE SHIKIMIC ACID CONTENT OF DIFFERENT AGES OF BRANCHES FOR 4 YEARS OLD SEEDLINGS

different ages of branch	shikimic acid content(mg/g)
1 year old	17.12±0.22a
2 years old	14.93±0.83b
3 years old	7.94±0.26c
4 years old	6.91±0.21d

D. Effect of Ecotypes on the Shikimic Acid Content

Results from this study indicate that the shikimic acid of organs in the 2 years old trees differed significantly among ecotypes. The shikimic acid content was 2.90-4.45mg/g in roots, 2.25-3.00mg/g in branches, 18.54-23.89mg/g in branch bark, and 13.14-22.08mg/g in leaves (Table IV). When the ecotypes were ranked according to the shikimic acid content level, it was WN>LA>KH>NP according to roots, WN>KH>LA>NP according to branches, LA> WN>NP>KH according to branch bark, and WN>LA>KH>NP according to leaves. In total, the WN ecotype from Jiangxi produced the highest amount of shikimic acid in all organs, followed by LA and KH ecotype from Zhejiang, and then the NP ecotype from Fujian. Analysis of variance indicated that there was no significant differences in shikimic acid content of branch bark and leaf between KH and NP ecotype, and also between LA and WN ecotype, but the significant differences in shikimic acid content of branch bark and leaf existed among the other ecotypes($p<0.05$). The shikimic acid content of branch showed the significant differences between WN and NP ecotype($p<0.05$). For the shikimic acid content of root, there was significant differences between WN and the other ecotypes ($p<0.05$), but no significant differences between LA, NP and KH ecotypes, respectively.

It was also shown that among all the ecotypes, shikimic acid content decreased consistently in the following order: branch bark>leaf>root>branch. Analysis of variance also indicated that for LA, NP and KH ecotypes, there was no significant differences in shikimic acid content between root and branch, but that of this two organs all had significant differences with leaf and branch bark respectively($p<0.05$). For LA, NP and KH ecotypes, there was significant differences in shikimic acid content between leaf and branch bark ($p<0.05$). For WN ecotype, there was no significant differences in shikimic acid content between root and branch, and also between leaf and branch bark. No significant difference was found in root and branch content among all the ecotypes ($p>0.05$). It is very likely that the variation in the shikimic acid content associate with nitrogen status in those organs.

The significant correlation in shikimic acid content between leaf and other organs was also found and its correlation coefficient ranged from 0.5738 to 0.8596 (Fig. 3).

The collection of leaf samples was easier than root and other organs samples of plants, so the correlation in shikimic acid content between the leaf and other different organs was analysed to guide the accumulation measures of shikimic acid by the high efficient cultivation of this plants (Fig. 3).

TABLE IV: THE SHIKIMIC ACID CONTENT OF DIFFERENT ORGANS FOR 2 YEARS OLD SEEDLINGS OF FOUR ECOTYPES

	root	branch	branch bark	leaf
LA	3.43±0.63aA	2.70±0.50aA	23.89±1.89aB	19.04±1.04aC
NP	2.90±0.30aA	2.25±0.25abA	19.95±1.05bB	13.14±2.54bC
KH	3.42±0.42aA	2.83±0.23abA	18.54±1.46bB	14.54±2.54bC
WN	4.45±0.65bA	3.00±0.20bA	23.03±0.97aB	22.08±1.18aB

*The capital letters indicate the difference on the 0.05 significance level between different organs; and the lower-case letters indicate the difference on the 0.05 significance level between different provenances.

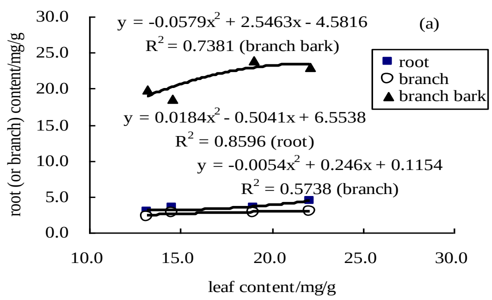

Fig. 3. The correlation in shikimic acid content between leaf and other organs.

E. Effect of Nutrient Content in Different Organs on the Shikimic Acid Content

Nitrogen is one essential mineral nutrient in plants. Leaf nitrogen content and the utilization efficiency affect plant photosynthesis and growth. Leaf nitrogen content is also used as a measurement for plant adaptability to environmental condition [26]. As described above, shikimic acid was mainly contained in branches and leaves of the *I.lanceolatum* trees. For high efficiency production, it is very important to explore the impact of plant nitrogen content on leaf photosynthetic capacity and shikimic acid production. As shown in Table 1, according to the analysis of the nitrogen level for organs and the corresponding shikimic acid content of trees at different ages, the two factors have a significant and positive correlation ($p<0.01$). The effect of the age of the branches on shikimic acid content was also associated with branch nitrogen content ($p<0.01$) (Table I).

IV. Discussion

Shikimic acid is the precursor for many alkaloids, it is used as the raw material for mass production of biological active compounds. Recently, shikimic acid was used to make oseltamivir which is the only clinically proven active bird flu medicine [1–2]. Due to the medicinal value of shikimic acid, much efforts were devoted to developing and clarifying the medicinal mechanism of the compound and hence the pharmaceutical usage potential [5–9, 11–13, 18, 20]. Thus more researches have been mainly focused on evaluation, screening of high yield plants, and optimization and validation of extraction technology [6–8, 10, 13, 18, 20]. Nowadays, shikimic acid was extracted mainly from fruits of Chinese star anise (*I.verum*). This species has a very limited growth regions and harvest season [12–13], thus it cannot be used to produce large amounts of shikimic acid. There is a need to systematically evaluate production of shikimic acid using this plant species.

I.lanceolatum, a species in the genus of *Illiciaceae*, can provide an important source of shikimic acid. There is a very promising market for this product [27–29]. However the real situation is that those trees grow extremely slow, they are shade-living and scattered in the wild, and there is a lack of wild germplasm resources. Breeding, selection and intensive cultivation of high shikimic acid content and fast-growing trees will be the major way to explore this species. In this study, the effect of growth light environment, tree age and organs nitrogen status on shikimic acid accumulation was evaluated, and the major factors were identified. We have found that shikimic acid content in organs is significantly affected by the age of trees under cultivation. The shikimic acid content in leaves elevated continuously as trees grew. Furthermore, leaf contained the highest shikimic acid level among all the organs from all the trees examined. This results indicated that the leaves can substitute for the root which was a traditional medicinal part.

Additionally, this study found that shikimic acid accumulation was also affected by the light condition trees were exposed to. Compared to 100% light or 20% light, the 50% light treatment was the most appropriate light condition producing the highest level of shikimic acid in branches, branch barks, and leaf. When grown under 100% sunlight, the trees grew more slowly, forming smaller canopy and lower biomass yield. Content of shikimic acid in roots and branch bark from the trees under 100% sunlight was slightly higher than that from the 50% sunlight treated. As *I.lanceolatum* is a shade-living plants, the strong irradiation under 100% sunlight could induce certain stresses for the fast growing trees, and hence trigger the biosynthesis of some intermediates for shikimic acid. Shikimic acid production is considered as a mechanism for plants to adapt to stressful environmental conditions [32]. According to this study, providing appropriate level of shade or interplanting *I.lanceolatum* with other trees, can effectively increase the shikimic acid content in branch bark and leaves. Promoting branch and leaf growth is the most effective way to obtain high shikimic acid yield.

When comparing different ecotypes, it was found that the WN trees contained the highest level of shikimic acid, followed by the LA and KH plants from Zhejiang province. Therefore, the WN and LA population should be the priority to be selected for high shikimic acid yield.

The growth of the tree and the accumulation of shikimic acid both were sensitive to the amount of sunlight plants receive, the soil moisture content and fertility, and other environmental factors. Therefore, it is very important to implement the best field cultivation management of sunlight and fertilizer to enhance production of the compound. Thus, mechanisms relating to how the shikimic acid production is affected by light, soil moisture and other condition should be more studied.

Tolerance to high solar irradiation is an important aspect of stress tolerance for landscape plants, particularly for species native to understory conditions. Several researches have been done on growth responses of *I.taxa* to light, nutrient [26, 33–34], but few studies done on shikimic acid content of *I.taxa* and the relationships between shikimic acid content and environmental factors [24]. Olsen *et al.* (2002) investigated the effect of two light intensity treatments, 45% and 100% full sunlight, on gas-exchange parameters of five *I.taxa* and found that optimal growth occurs in shaded conditions [33]. Olsen (2001) also made a second study investigated the influence of light intensity and rate of nitrogen application on the growth of *I.taxa* [26]. He suggested all taxa be grown in low light to optimize growth and fertilizer efficiency. Griffin et al. (2004) also evaluated the differential tolerance to high solar irradiation and underlying photosynthetic characteristics of eleven commercially available taxa of *Illicium L.* grown under full sun or 50% shade [34]. All of these studies laid the research foundation for the development of medicinal value of *Illicium L.*

I.lanceolatum is a popular aromatic and medicinal plant in China. Liang *et al.* (2011) made the first report on the chemistry, antinociceptive and anti-inflammatory activities of *I.lanceolatum* [25]. His results indicate that the essential oil may contain the bioactive components of *I.lanceolatum*. Li et al. (2007) also studied the antibacterial activity of herb extract of *I.lanceolatum* and found that the extracts of the dry fruit of *I.lanceolatum* have obvious inhibitory effect against fungus, bacillus and coccus, the most obvious effect of antifungal [24].

In this study, content of shikimic acid in different organs of trees during the fast growing season was evaluated. More studies will be carried out on seasonal changes of shikimic acid content. There were some reports on the plasticity of plant growth under different light conditions, and responses to water stress and the photosynthetic physiological properties of different genotypes [29]. Those studies on *I.lanceolatum* have provided the scientific foundation for developing the species into a sustainable production source of shikimic acid.

Conflict of Interest

The authors declare no conflict of interest.

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