Nai-Wen Tsao, Ying-Hsuan Sun, Shih-Chang Chien, Fang-Hua Chu, Shang-Tzen Chang, Yueh-Hsiung Kuo and Sheng-Yang Wang*

Content and distribution of lignans in *Taiwania cryptomerioides* Hayata

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Abstract: Taiwania (*Taiwania cryptomerioides* Hayata) is one of the economically important tree species indigenous to Taiwan. Hundreds of secondary metabolites have been identified from its wood, bark, root, and needles with lignans as the dominant ones. This substance group contributes a lot to the color, durability, and bioactivities of Taiwaniana. The present paper is dedicated to the quantification of radial and longitudinal lignan distribution. The extractives begin to accumulate largely in the transition zone (TZ), and reach a maximum after finishing the heartwood (hW) formation. Both dibenzyl-γ-butyrolactone type and arylnaphthalide type lignans were found in sapwood (sW) except for the compound taiwanin A. Clearly, the heartwood formation of Taiwania differs from the hitherto known hW formation types and it is suggested to be denominated as Taiwania-type hW formation, where the biosynthesis of extractives begins in the sW and where their accumulation is clearly elevated in the TZ. A generalized biosynthesis scheme of Taiwanin is presented showing the putative relationships among the most important dimeric lignans that lead to the formation of taiwanin type lignans.

***Corresponding author: Sheng-Yang Wang,** Department of Forestry, National Chung-Hsing University, Taichung 402, Taiwan, Phone: +886-4-22840345, ext-138, e-mail: taiwanfir@dragon.nchu.edu.tw; Agricultural Biotechnology Center, National Chung-Hsing University, Taichung, Taiwan; and Agricultural Biotechnology Research Center, Academia Sinica, Taipei, Taiwan
Nai-Wen Tsao and Ying-Hsuan Sun: Department of Forestry, National Chung-Hsing University, Taichung, Taiwan
Shih-Chang Chien: Experimental Forest Management Office, National Chung-Hsing University, Taichung, Taiwan
Fang-Hua Chu and Shang-Tzen Chang: School of Forestry and Resource Conservation, National Taiwan University, Taipei, Taiwan
Yueh-Hsiung Kuo: Graduate Institute of Chinese Pharmaceutical Science, China Medical University, Taichung, Taiwan; and Department of Biotechnology, Asia University, Taichung, Taiwan

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Introduction

Taiwania (*Taiwania cryptomerioides* Hayata) is one of the economically important tree species indigenous in Taiwan. It was discovered in 1904 by Konishi as discussed in Hayata (1906) and later classified as a new species with an independent genus status under Taxodiaceae in 1906 by Hayata. Nowadays, the Taxodiaceae has merged into the Cupressaceae family (Christenhusz et al. 2011). The existence of this genus can be traced back to the Tertiary (Walther 1999) and it is considered as a "living fossil". Taiwania species are sparsely distributed at high elevation in Taiwan and the mountains around the border between Myanmar and China, where the species found refuge during the most recent ice age. The habitat disturbance due to climate changes and logging activities led to the listing as "vulnerable" according to the IUCN Red List criteria (Farjon 2001).

The extractives of Taiwania have been studied extensively due to the attractive characteristics and excellent durability of its wood. Hundreds of terpenoids, lignans, flavonoids and steroids have been identified from root, bark, wood and needles of Taiwania (Chang et al. 2003; Chien and Kuo 2009). Many of these compounds show the photodiscoloration effect (Chang et al. 1998; Chang et al. 1999a) and they contribute to durability (Chang et al. 1999b; Chang et al. 2001a) of Taiwania heartwood (hW). The essential oils of Taiwania trees smell sweet (Chang et al. 2001b) and they show bioactivities against fungi (Chang et al. 2000a), bacteria (Chang et al. 2000b) and tumor cells (Chang et al. 2000c). Diterpenoids isolated from the hW of Taiwania have antioxidant activities (Wang et al. 2002). The extractives of Taiwania have a high potential for value added production of numerous products.

Lignans are phenylpropanoid dimers and they are dominant in Taiwania extractives. Lignans are known for antiviral, anticancer, anti-inflammatory, anti-oxidative, immunosuppressive, and hepatoprotective activities (Adlercreutz 2007). They also play an important role

in pant defense and in the regulation of plant growth (Willför et al. 2006). While the polyphenols in softwood species contain mainly lignans, in hardwood species flavonoid type polyphenols are dominating. In Taiwania hW, there are two types of lignans, namely the dibenzyl-γ-butyrolactone type and arylnaphthalide type (Chang et al. 2000c). The lignans hinokinin, savinin, taiwanin A, taiwanin C, taiwanin E, and helioxanthin in Taiwania hW contribute a lot to color and durability.

The distribution of extractives in the tree trunk is heterogeneous. Thompson et al. (2006) demonstrated, for example, that the terpenoid contents in the hW, inner sW, and outer sW of loblolly pine are 2.3±0.42%, 0.77±0.31%, and 0.35±0.16%, respectively. The distribution of phenolic extractives in the trunk of *Toxicodendron vernicifluum* examined by Hashida et al. (2014) showed that the phenolic extractives content was the lowest in the sW and increased in the outer hW, and then decreased in the inner hW.

Kampe and Magel (2013) classified the hW formation into two types based on distribution patterns of extractives in stem wood of various trees species. (1) Type I (Robinia-type) hW formation, where the accumulation of phenolic extractives starts in the TZ, while no phenolic precursors were found in the aging sW. (2) Type II (Juglans-type) hW formation, where the phenolic precursors gradual accumulated centripetally with progressive aging of the sW tissues. The extractives that characterize the Type II heartwood were formed in the TZ either by *de novo* biosynthesis or secondary reactions (oxidation or hydrolysis) of precursor substances. These precursors include flavonoids and some lignans such as pinoresinol and dehydrodiconiferyl alcohol, that derived from the direct dimerization of coniferyl alcohol, but not matairesinol, hinokinin, helixanthin, etc., that derived from the secondary reactions. (Dellus et al. 1997; Burtin et al. 1998; Takaku et al. 2001; Mayer et al. 2006; Saito et al. 2008).

Despite the discovery of various ligans in the hW of Taiwania, the hW development and the mechanism of lignan biosynthesis in Taiwania is still unknown. In the present paper, the distributions of lignans in the stem wood should be characterized under consideration in both radial and longitudinal directions. Such distribution patterns of lignans are crucial for prospective identifying the enzymes involved in the lignan biosynthesis.

Materials and methods

Instruments: UV and IR data were acquired on a PerkinElmer 241 polarimeter (PerkinElmer, USA) and a PerkinElmer Spectrum 100 FT-IR spectrometer (PerkinElmer, USA), respectively. NMR spectra were obtained on a Bruker Avance III 400 NMR spectrometer (Bruker, Germany) in $CDCl_3$ (Sigma, USA). HREIMS data were determined with a Thermo/Finnigan Quest MAT 95XL Mass Spectrometer (Thermo, USA). HPLC was carried out with an Agilent 1100 HPLC system equipped with a UV detector (Agilent, USA). A Luna C18 column (150.0×4.6 mm; Phenomenex, Torrance CA, USA) was used and a Phenomenex column (5 μm, 250×10 mm, Phenomenex Co.). Open column chromatography was carried out with silica gel (4×30 cm; 60–80 mesh; Merck).

Three 0.5 cm diameter wood cores (Figure 1a, at 120 cm high) were collected with an increment borer from a 30-year-old Taiwania (tree 1) grown at Huisun Experimental Forest Station of National Chung-Hsing University in August 2014. The tree was 20 m high, with a diameter at breast height (DBH) of 30 cm, and had orange-reddish heartwood (hW). For each of the wood core, the sapwood (sW), intermediate wood (transition zone: TZ), outer hW (IW-1), inner hW (hW-2) and pith (P) were separated as shown in Figure 1a, and their yields of extractives were determined such as the radial distribution of lignan contents. Another Taiwania (tree 2) of the same age was cut down to obtain 25 logs of 0.8 m long each. The logs were designated 1 to 25 from top to bottom of the tree. The TZ and IW-1 portions (1×1×1 cm^3) of logs 2, 5, 7, and 24 were obtained as shown in Figure 2b, and the amount of individual lignans were quantified in longitudinal direction.

Extractives quantification in radial direction: The fresh samples were milled and lyophilized for 72 h to dryness and accurately weighted samples were placed in an Erlenmeyer flask (100 ml) with 25 ml of MeOH (MercK, Germany), and sonicated (Branson 5510, Branson Ultrasonic, Ontario, Canada) for 30 min. The extractives were then decanted, filtered under vacuum, concentrated in a rotary evaporator and lyophilized. Yield of extractives (w/w%) were expressed as mean±SD (n=3).

Lignan extraction and purification: The major lignans were obtained following the extraction and purification protocol of Chang

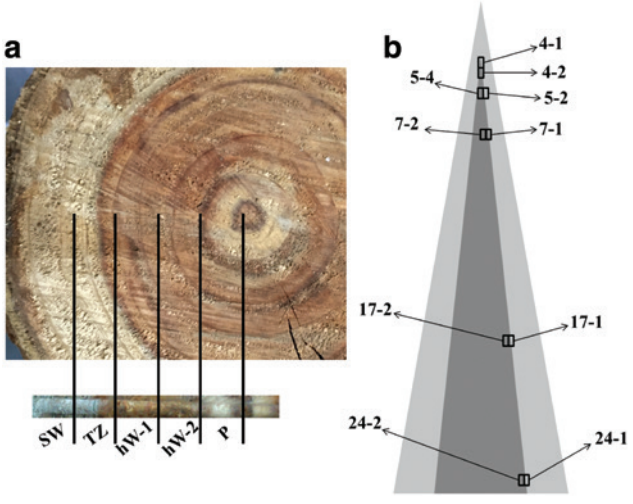

Figure 1: (a) Growth cones and cross section collected from a 30-year-old *T. cryptomerioids*. sW, Sapwood; TZ, transition zone; hW-1, outer heartwood; hW-2, inner heartwood; P, pith. (b) TZ (4-1, 5-2, 7-1, 17-1, and 24-1) and hW-1 (4-2, 5-4, 7-2, and 24-2) sampling from another Taiwania tree with the same age.

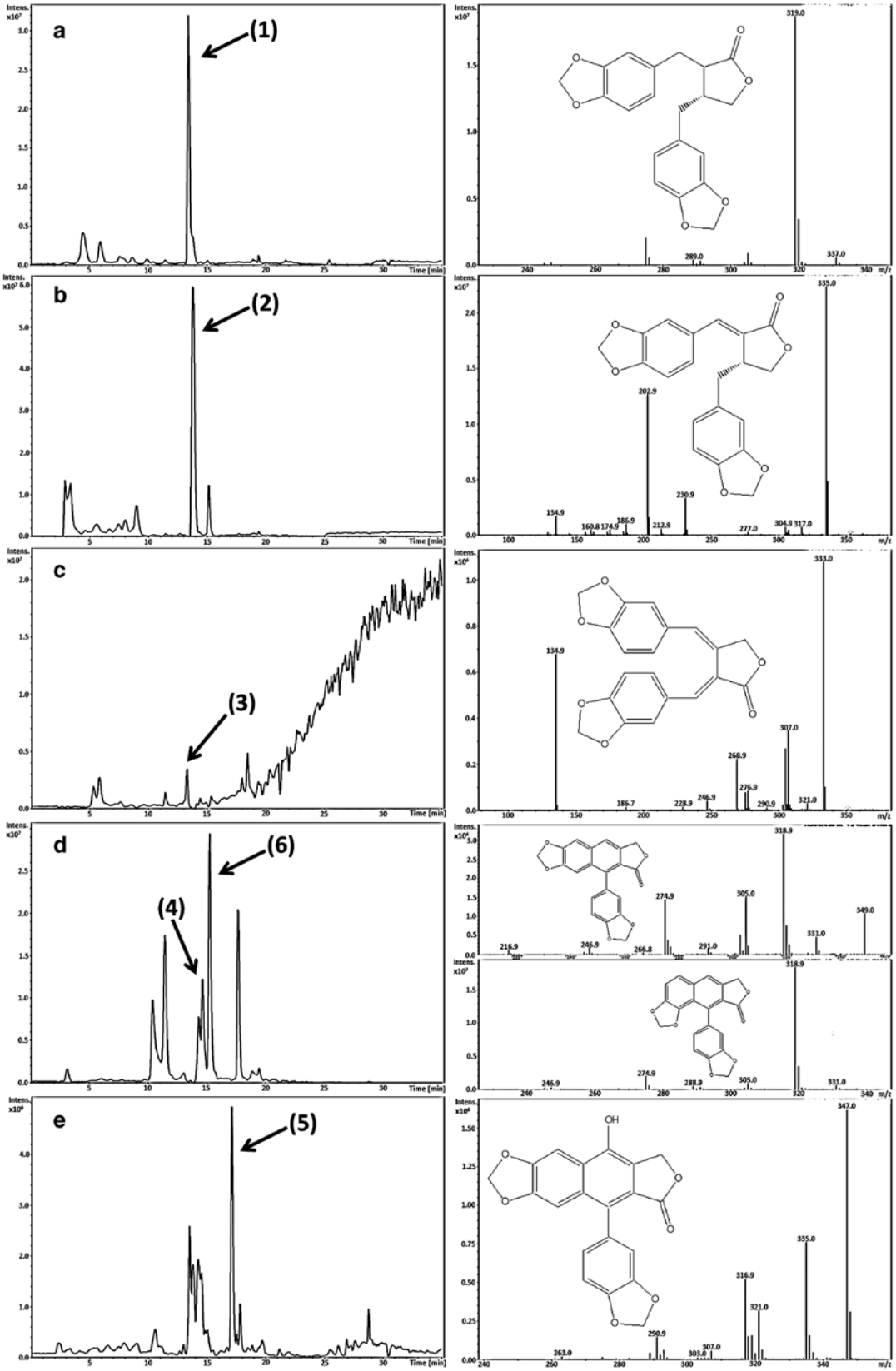

Figure 2: The chromatogram (left panel) and MS spectrum (right panel) of lignans in heartwood in Taiwania. (a) The chromatogram of 355 *m/z* and the spectrum of hinokinin (**1**). (b) The chromatogram of 353 *m/z* and the spectrum of savinin (**2**). (c) The chromatogram of 351 *m/z* and the spectrum of taiwanin A (**3**). (d) The chromatogram of 349 *m/z* and the spectrum of taiwanin C (**4**) and helioxanthin (**6**). (e) The chromatogram of 365 *m/z* and the spectrum of taiwanin E (**5**).

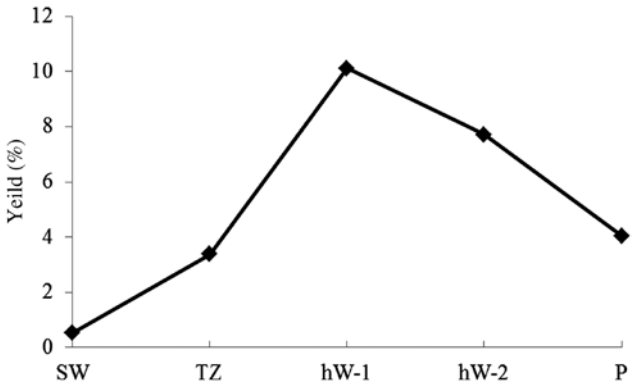

Figure 3: Yields of extractives distributed in a 30-year-old Taiwania. SW, Sapwood; TZ, transition zone; hW-1, outer of heartwood; hW-2, inner of heartwood; P, pith.

et al. (2000c). Briefly, the air-dried hW chips (3 kg) were exhaustively extracted with methanol (MeOH). The extractives were condensed to *ca*. 195 g, and extracted successively with *n*-hexane (n-C_6H_{14}), $CHCl_3$, EtOAc, and MeOH to yield the corresponding soluble fractions in these solvent, and the MeOH insoluble fraction. Taiwanin A was separated from the n-C_6H_{14} by removal of the solvent (Chang et al. 1999a). Savinin, taiwanin C, taiwanin E, helioxanthin, and hinokinin were isolated and purified from the $CHCl_3$ soluble fraction by flash column chromatography and semi-preparative HPLC. The structure and purity of each lignan were confirmed by spectral analysis and comparing the data with those of the literature (Su et al. 1998; Wang et al. 1998; Chang et al. 1999a,b).

Lignan analysis by ultra-high performance liquid chromatography coupled with electrospray ionization – ion trap mass spectrometry (UHPLC-MS): The UHPLC-MS analysis of lignans were conducted on a Thermo Scientific Dionex UltiMate 3000 Rapid Separation Liquid Chromatography (Thermo, USA) coupled with a Bruker amaZon speed mass spectrometry (Bruker, Germany) system. Lignans were separated by a Waters RP-18, 100×2.1 mm 1.7 μm column. The elution

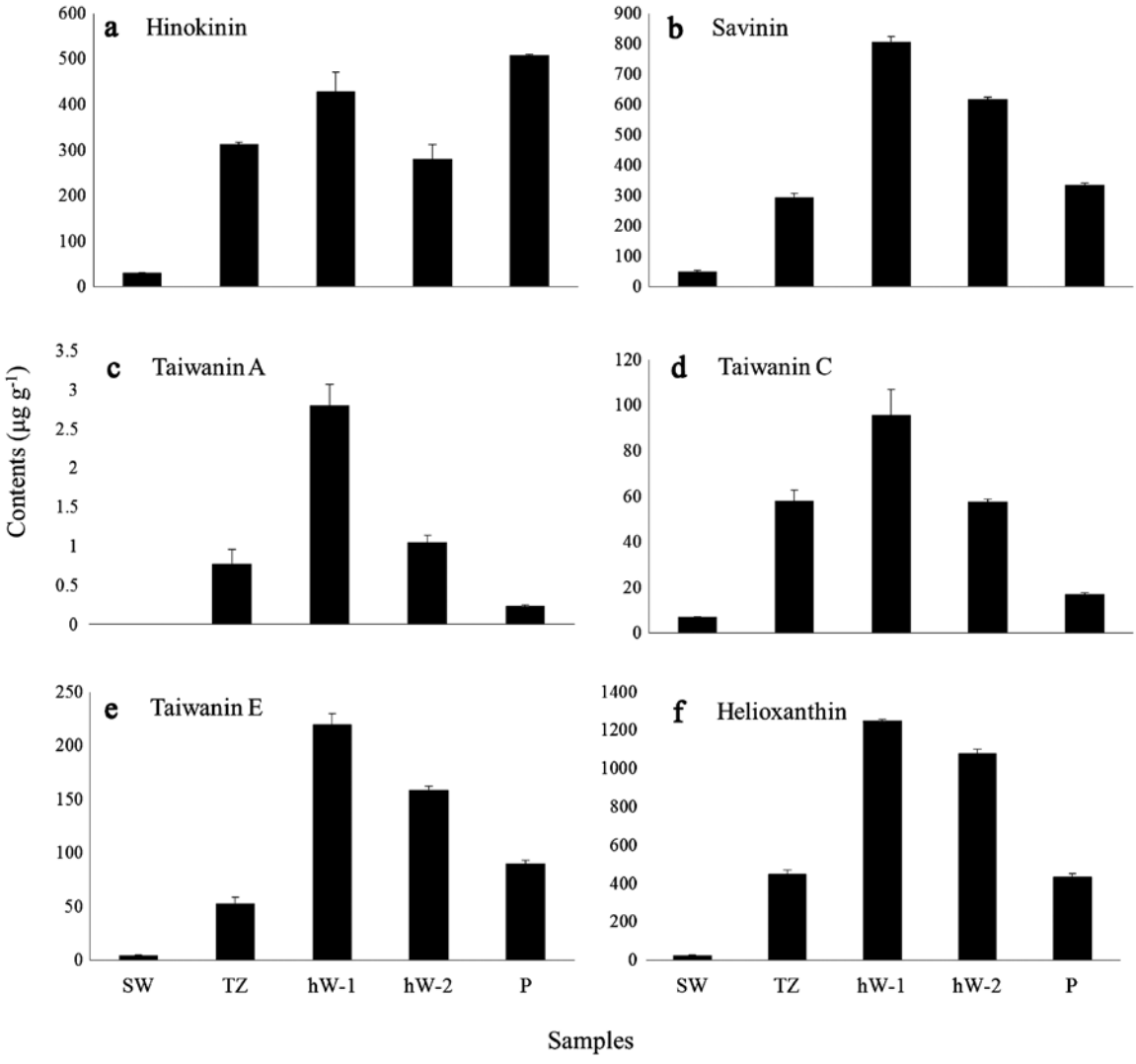

Figure 4: The horizontal distribution of lignans in the cross-section of Taiwania stem wood analyzed by UHPLC-MS. SW, sapwood; TZ, transition zone; hW-1, outer of heartwood; hW-2, inner of heartwood; P, pith.

solvent system was 0.1% (v/v) acetic acid in water (A), MeOH (B) and acetonitrile (Merck, Germany) (ACN, C). The gradient elution program: 0–7 min (40% A and 60% C), 9 min (30% A, 60% B, and 10% C), 11 min (20% A, 70% B, and 10% C), 12 min (20% A, 65% B, and 15% C), 17 min (5% A, 80% B, and 15% C), 20 min (5% A, 75% B, and 20% C), 23 min (100% B), 23–28 min (100% B); (30°C and 0.1 ml min^{-1}). The MS conditions: 4.5 kV for capillary voltage 193.06 kPa nebulizer pressure, 250°C dry gas temperature and 8.0 l min^{-1} dry gas flow. The instrument was operated in the positive ion mode and the lignan indices were detected in the multiple reaction-monitoring (MRM) mode with 351, 353, 355, and 365 *m/z* for the detection of taiwanin A, savinin, hinokinin, and taiwanin E, and 349 *m/z* for both taiwanin C and helioxanthin. Although taiwanin C and helioxanthin has the same *m/z*, they can be detected separately based on their different LC retention time (14.6 min and 15.2 min, respectively, Figure 2). The chromatogram and spectrum of the lignans are shown in Figure 2.

Quantification of lignans: Standard calibration curves (peak area vs. concentration) for each lignan were determined at concentrations of 0.4, 0.8, 1.0, 2.0, 5.0, 10.0 µg ml^{-1}. Quantification was then performed by UHPLC-MS analysis. The peak areas of the index compounds in the chromatogram of the MeOH extracts (with known loading concentration) were then defined, and their contents in the extracts were calculated on the basis of the standard calibration curves. The analyses were performed in triplicate, and the results are presented as mean±SD.

Results and discussion

Radial variation of extractives contents

Figure 3 shows the amounts of extractives distributed in the cross-section of Taiwania. The content was the lowest in sW (0.52%), followed by the TZ (3.8%) and then it was enriched to a content in hW-1 of 10.2%. The contents of extractives in hW-2 (7.9%) and pith (P) (4.1%) are slightly lower. The extractives began to accumulate in TZ, and reached the maximum in the finished hW. The lower yield in P is expected, as P is not a mature wood (Duenisch et al. 2010).

Radial distribution of lignans

Lignans in radial section were performed by UHPLC-MS (Figure 4). Overall, both dibenzyl-γ-butyrolactone type and arylnaphthalide type lignans were found in sW, but taiwanin A was not detected. The most abundant lignan in sW is savinin with the content of 50.5 µg g^{-1} wood, followed by hinokinin (30 µg g^{-1}), helioxanthin (26.1 µg g^{-1}), taiwanin C (7.0 µg g^{-1}), and taiwanin E (4.4 µg g^{-1}). Taiwanin A was already detected in TZ, and reached the highest content (2.8 µg g^{-1}) in hW-1. Similarly, the highest contents of savinin (806.1 µg g^{-1}), taiwanin C (95.5 µg g^{-1}), taiwanin E (220.0 µg g^{-1}), and helioxanthin (1204.8 µg g^{-1}) were also located in hW-1. However, the highest content of hinokinin (507.9 µg g^{-1}) was found in the pith. Our data indicate that the hW formation of Taiwania does not belong neither to type I nor to type II hW formation according to Kampe and Magel (2013) because the presence of major lignans, which are classified as secondary metabolism products, including dibenzyl-γ-butyrolactone type (Hinokinin and savinin) and arylnaphthalide type lignans (taiwanin C, taiwanin E, and helioxanthin), are already present in the sW. Furthermore, the current classification of hW formation (type I and type II) are only based on the results of hardwoods (broad-leaves trees). Therefore, we suggest that the hW formation in Taiwania should be classified as a new "Taiwania-type" type hW formation, with the beginning synthesis of extractives in the sW (and not in TZ) and accumulating further in the TZ.

Figure 5: The presense of heartwood formation in Taiwania. (a) log 3 (b) log 4 (c) log 5. The red asterisk indicate the presence of heartwood.

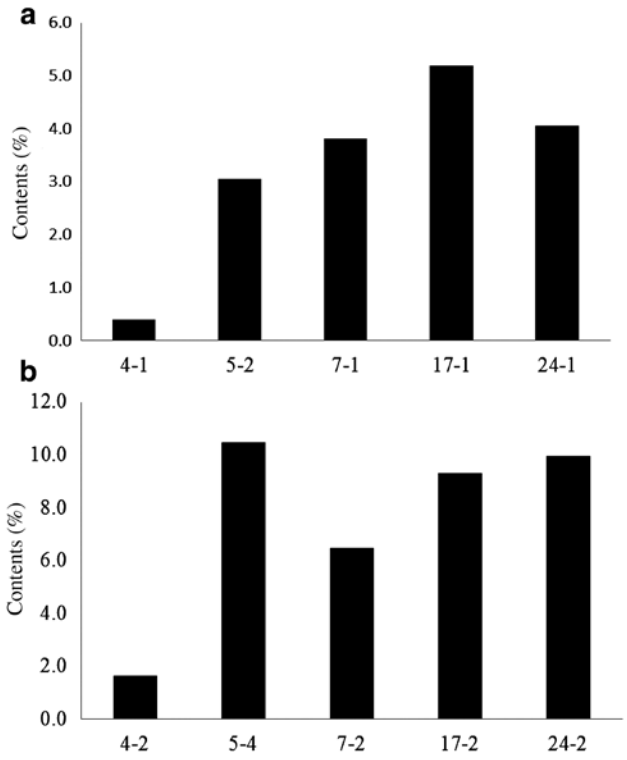

Figure 6: Contents of extractives in (a) TZ samples (4-1, 5-2, 7-1, 17-1, and 24-1) and (b) hW-1 samples (4-2, 5-4, 7-2, and 24-2) of Taiwania. The sampling site was shown in Figure 1 (b).

Longitudinal variation of extractives and lignans

Heartwood formation could not be detected for log 1 to log 3 (Figure 5a), while hW was observed at the 11th annual ring of log 4 (Figure 5b). TZ samples (4-1, 5-2, 7-1, 17-1, and 24-1) and hW-1 samples (4-2, 5-4, 7-2, and 24-2) were obtained and the MeOH extractives for each sample were quantified (Figure 6). Over all, the amounts of extractives of hW-1 samples (4-2, 5-4, 7-2, and 24-2) were higher than that in TZ samples (4-1, 5-2, 7-1, 17-1, and 24-1). Both TZ and hW-1 exhibited a same trend with increasing extractive contents from the top to the bottom. The high extractive contents in 5-4 might be due to the knot in the sample. The lignan distribution shows the same trend as the extractives, i.e. in both TZ and hW-1 samples the amounts of lignans at the bottom of the tree are higher than at the top (Table 1). It is the first report pointing out the beginning hW formation of Taiwania in 11th annual ring.

The chemical interrelation between the lignans

Based on the results of lignan distribution in sW, TZ, and hW, a tentative scheme of the formal interrelation of the Taiwania lignans beginning with the coniferyl alcohol is presented in Figure 7. As stated above, both dibenzyl-γ-butyrolactone type (hinokinin and savinin) and aryl-naphthalide type (taiwanin C, taiwanin E, helioxanthin) lignans are present in sW except taiwanin A (dibenzyl-γ-butyrolactone type lignan) (Figure 4). Extensive literature survey results indicate that the occurrence of taiwanin A is limited to Taiwania, i.e. that only Taiwania may dispose of a specific dehydrogenase (Figure 7 step III) that is responsible for synthesizing taiwanin A from hinokinin or savinin. The activity of this enzyme may be associated with the hW development, because taiwanin A can be found in TZ, hW, and pith (Figure 4c), but not in sW.

Table 1: Quantification of lignans in TZ and hW-1 of Taiwania from the top to bottom of tree by UHPLC-MS analysis.

					Contents (μg g^{-1})	
	Hinokinin	**Savinin**	**Taiwanin A**	**Taiwanin C**	**Taiwanin E**	**Helioxanthin**
Transition zone						
4-1	4.0±0.2	9.3±0.1	tr[a]	8.2±0.1	26.5±0.6	3.5±0.1
5-2	91.8±2.5	94.9±5.3	0.04±0.00	269.2±4.2	645.8±8.7	7.9±2.1
7-1	33.0±1.7	69.9±4.2	0.01±0.00	370.1±7.2	618.9±15.4	3.1±0.1
17-1	74.1±6.6	94.5±1.2	0.34±0.02	516.2±3.6	1080.0±14.3	3.5±0.2
24-1	85.7±1.6	66.2±0.5	tr	303.1±4.2	498.9±16.6	2.1±0.2
Outer heartwood						
4-2	3.7±0.4	43.3±0.9	0.6±0.01	133.4±4.5	150.0±2.0	134.6±0.9
5-4	20.8±1.1	183.0±3.3	27.1±0.6	786.3±18.8	1033.0±19.2	1174.0±55.0
7-2	52.6±1.5	106.2±0.1	0.1±0.0	718.8±10.0	1784.0±39.4	614.8±0.7
17-2	89.2±6.4	153.7±0.8	15.4±0.25	929.6±12.7	2578.0±16.4	621.1±17.5
24-2	121.3±1.4	169.4±2.2	1.0±0.1	1197.0±15.7	2920.0±36.9	581.1±44.1

[a]tr, Traces.

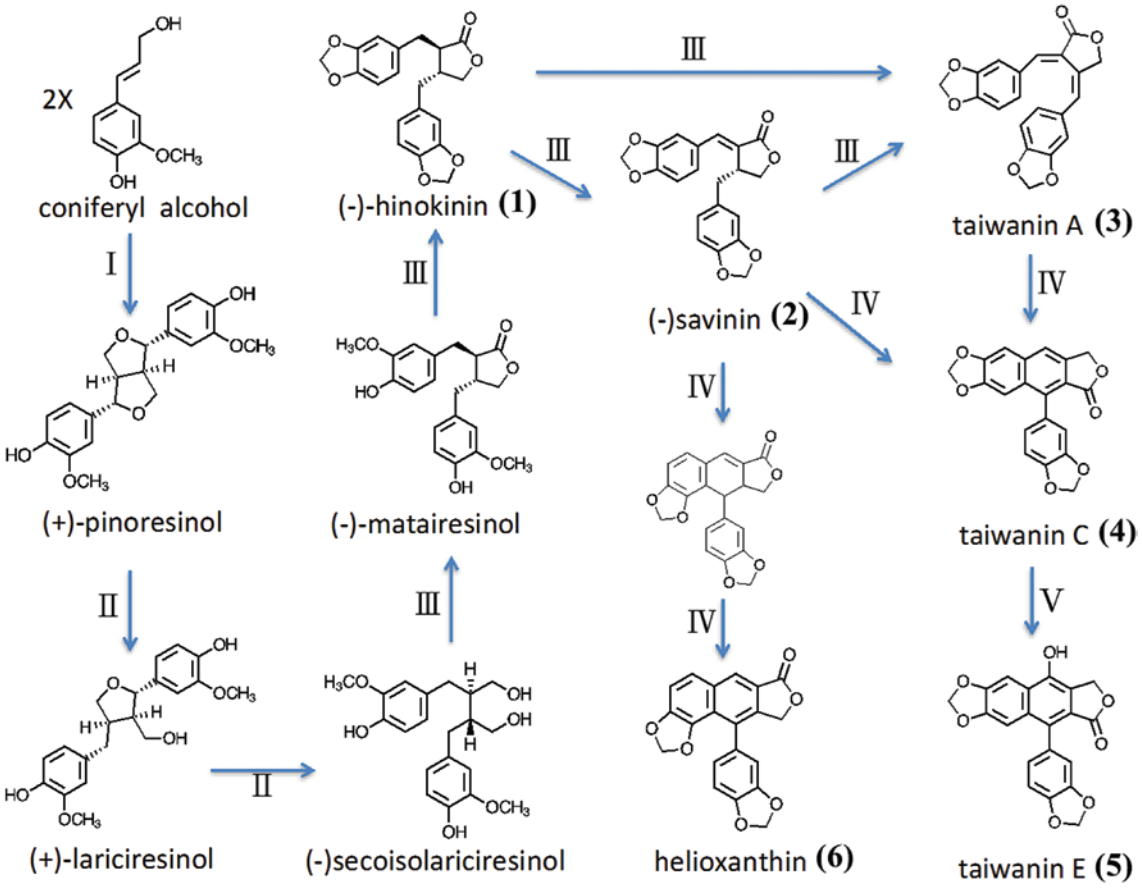

Figure 7: The most plausible chemical relation of some dimeric lignans. The chemical reactions are dimerization (I), reduction (II), dehydrogenation (III), aromatization (IV), and oxidation (V). Hinokinin (**1**), savinin (**2**), taiwanin A (**3**) are dibenzyl-γ-butyrolactone type compounds, and taiwanin C (**4**), taiwanin E (**5**), helioxanthin (**6**) are arylnaphthalide type compounds.

The taiwanin C may be synthesized through the aromatization from taiwanin A or savinin (Figure 7 step IV) and further oxidized to form taiwanin E (Figure 7 step V; Chang et al. 1999a). Both taiwanin C and taiwanin E can be found in all of the tissues surveyed in this study. Such results suggest that the taiwanin C and taiwanin E in the sW were synthesized via the route of savinin aromatization. However, it is also possible that taiwanin A may be synthesized in sW through an alternative dehyrogenase that is active in sW, and then immediately converted to taiwanin C and by the subsequent oxidation to taiwanin E. However, further studies are needed to test these hypotheses. Helioxanthin is the most abundant lignan in the stem wood of Taiwania (Figure 7).

Conclusion

The highest extractive contents can be observed at the hW-1. Characteristic lignans of Taiwania are synthesized already in sW and accumulated significantly in TZ. All lignans are synthesized in sW, except taiwanin A. Taiwania seems to have a new hW formation type which has not yet described in the literature and thus we suggest to call it Taiwania type hW formation.

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