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Morphological, Physiological, Biochemical and Metabolite Analyses of Parenchyma Cells Reveal Heartwood Formation Mechanism of *Schima superba*

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Abstract: A sapwood tree is a species in which the sapwood does not differ significantly from the heartwood and cannot be classified by shades of color. It is generally accepted that heartwood has a higher economic value than sapwood, but most of the studies related to heartwood formation have focused on heartwood trees, with less research on sapwood trees. In this paper, we take the sapwood tree *Schima superba* as the research object and analyze the physiological and biochemical changes in the process of heartwood formation by studying the anatomical structure of parenchyma cells, and then further explore the main categories of metabolites and compositional changes. The results showed that during heartwood formation, the parenchyma cells become inactive and the nucleus disappears, while at the same time, the storage substance starch is gradually degraded under the action of enzymes and transformed into secondary metabolites, which include terpenoids, phenols and alkaloids. The accumulation of white and colorless compounds in large quantities in the heartwood, which has some effect on the heartwood color, is an important reason why the heartwood in *Schima superba* shows normal formation but no difference in color from the sapwood. This study fills a gap in the mechanism of heartwood formation in sapwood trees.

Keywords: sapwood tree; *Schima superba*; heartwood formation; color; parenchyma cells; metabolite

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1. Introduction

At the macro level, there are differences in the wood color of different tree species. However, there are some tree species whose sapwood does not differ significantly from the heartwood and cannot be classified by color shade, also known as sapwood trees, e.g., *Schima superba*, *Picea asperata* and *Abies fabri* [1,2]. Among them, *Schima superba* is the main establishment species of subtropical evergreen broadleaf forests in China and is the most dominant species for efficient biological fire protection, ecological landscaping and ecological protection afforestation in southern China [3]. As the *Schima superba* is a sapwood tree, the center and periphery of the trunk have no difference in color. Because we cannot distinguish between the heartwood and sapwood through their color depth, heartwood formation and the formation mechanism still remain to be elucidated.

The ultramorphological and structural changes that occur during the programmed death of physiologically active parenchyma cells in tree xylem play a very important role in the formation and transformation of heartwood [4–8]. Parenchyma cell organelles and cellular structure are altered during heartwood formation and cell viability is manifested mainly through changes in organelles (nucleus, vesicles, mitochondria) and storage substance (starch grains, lipid droplets) [9–12]. During programmed cell death (PCD) of parenchyma cells, nuclei are widely present in parenchyma cells of sapwood and are one of the most important markers characterizing cell activity [13,14]. Song et al. found

that the nucleus deformation index of parenchyma cells of wood rays in the sapwood of *Cunninghamia lanceolata* gradually increased from the formation layer to the transition zone [15]. A study by Yang showed that the survival rate of ray cells gradually decreased from the outer sapwood to the transition zone [16]. Gartner et al. also found that the frequency, volume ratio and activity of ray parenchyma cell nuclei in *Pseudotsuga menziesii* showed an increasing trend from the pith to the bark [17]. In addition, the starch grains of parenchyma cells in the sapwood also play a role in heartwood formation, serving as a storage substance that provides energy and raw materials for cellular metabolism. Chen et al. found that starch grains gradually decreased and eventually disappeared with the sapwood-to-heartwood transformation, by studying the active ray parenchyma cells during heartwood formation in *Taiwania cryptomerioides*. The cumulative transformation of starch grains became an important feature of the sapwood-to-heartwood transformation process [18]. However, most scholars have focused on heartwood trees [19,20] as well as conifers [15,16,21] when investigating anatomical variations of parenchyma cells during heartwood formation, while studies on the role of parenchyma cells in the heartwood formation of broadleaf sapwood trees have been limited.

Apoptosis of parenchyma cells is accompanied by enhanced specific enzyme activity, which promotes heartwood formation [1]. Plant antioxidant enzymes are crucial for sustaining plant life processes [22–24]. In the context of increased phenylalanine ammonia-lyase (PAL) activity (the main source of phenolic compound formation), the increase in polyphenol oxidase (PPO) activity is associated with the synthesis of complex substances of a phenolic nature in sapwood [25–27]. Differentially expressed genes associated with terpenoid biosynthesis pathway synthases were all up-regulated in the transition zone during heartwood formation in *Taiwania cryptomerioides* [28]. As primary metabolites, such as starch, decrease and enzyme activity increases, primary metabolites are eventually converted to heartwood material [29]. Studies have shown that inclusions such as starch grains in ray parenchyma cells show a gradual degradation from sapwood to heartwood during cell death [30]. At the same time, large amounts of organic solvent extracts, known as heartwood extractives, accumulate in the cell cavities or tissues at the heartwood [31,32]. Studies on *Taiwania cryptomerioides* heartwood also found a gradual loss of starch grains and concomitant deposition of extractives into radial and axial cell lumens starting from the external transition zone [18]. Thus, heartwood formation is accompanied by biosynthesis and accumulation of secondary metabolites [30,33,34]. Metabolites are the end products of gene expression and protein regulation. During heartwood formation, parenchyma cells are subjected to constant changes in metabolites by other influences, such as other tissues around the xylem and the surrounding environment. Phenols and flavonoids are widespread secondary metabolites in plants. It was found that aromatic compounds such as flavonoids were more abundant in the heartwood of *Sitka spruce*, which contained 70% more extractives than the sapwood [31]. Shao et al. detected 607 metabolites in *Taxus chinensis*, with elevated levels of 146 metabolites in the heartwood, which were mainly involved in the metabolic pathways of secondary metabolites such as flavonoids and phenylpropanoids, as well as biosynthesis [35]. Yang et al. analyzed metabolomics of the heartwood and sapwood of *Cunninghamia lanceolata* of different ages and showed that 21 phenolics, including flavonoids, coumarins and their derivatives, accumulated in the heartwood [36]. What makes some tree species easy to distinguish is the accumulation of colored material in the heartwood. Bosshard suggested that light-colored heartwood species result from the absence of colored extractives or the presence of small amounts of colored extractives within the heartwood [37]. However, for sapwood trees, whose color distinction between heartwood and sapwood is not clear, the accumulation of secondary metabolites is still unknown and the association between secondary metabolites and heartwood color formation remains obscure.

In conclusion, complex biochemical changes occur during heartwood formation. Storage substances such as starch, which are present in large quantities in sapwood, are degraded in the transition zone and further converted into secondary metabolites by the

action of enzymes. This involves a process of cellular activity changes, physiological and biochemical changes and metabolite changes. In this regard, scholars have carried out some studies, but most of these focus on the tree species with obvious difference between sapwood color and heartwood color. The heartwood formation mechanism of sapwood trees is still unclear and needs further exploration. Therefore, this paper takes *Schima superba* as the research object, focusing on the characteristics of its heartwood and sapwood whose color distinction is not obvious, and the research objectives were to (i) explore radial variations in the anatomical configuration of parenchyma cells to reveal heartwood formation; (ii) analyze the physiological and biochemical changes of heartwood and sapwood and the law of change of metabolites, and explore the enzyme-catalyzed secondary metabolites generated by the heartwood and sapwood transformation process; and (iii) further explore the association between secondary metabolite accumulation and heartwood color and to elucidate the mechanism of heartwood formation in sapwood tree. This study fills in the blank of the sapwood tree heartwood formation mechanism, provides a theoretical basis for the comprehensive development and utilization of sapwood tree heartwood resources and has realistic research significance for future artificial regulation of sapwood tree heartwood formation and improvement of timber properties.

2. Materials and Methods

2.1. Sample Collection

Schima superba was the research subject for this investigation. The samples were collected from Guangxi Gaofeng Forest Park, located in Yongwu Road, Xingning District, Nanning City, Guangxi Zhuang Autonomous Region, between longitude 107°45′~108°51′ E and latitude 22°13′~23°32′ N, which is rich in forest resources. The sampling site is a pure forest of *Schima superba*, 24 years old, with a simple forest structure. Three 24-year-old normal-growing *Schima superba* without obvious defects were selected as experimental samples. Complete wood cores (bark-pith-bark) were taken from each tree in a north–south direction using a growth cone (5.15 mm diameter), and a portion of the samples were immediately added to liquid nitrogen, transferred to the laboratory on dry ice and stored in an ultra-low refrigerator at −80 °C; a portion was brought back to the laboratory to be air-dried for use in subsequent experiments.

2.2. Macro Color Measurement of Wood

The colorimetric determination was carried out using a CM-2300D spectrophotometer (Konica Minolta (China) Investment Co., Ltd., Japan, Shanghai, China). After calibration of the standard whiteboard, the values of the color parameters—lightness value (L*), red and green chromaticity index (a*) and yellow and blue chromaticity index (b*)—were determined. L* indicates the brightness of the color, a*(+) indicates red, a*(-) indicates green, B*(+) is yellow and B*(-) is blue. Each sample was measured three times and the measurements averaged. The total color difference (ΔE) of the wood was calculated according to the following formula:

$$\Delta E = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}} \quad (1)$$

where ΔE is the color difference in the radial direction of the wood and ΔL*, Δa* and Δb* are the differences in L*, a* and b* between annual growth rings from cambium to pith, respectively.

2.3. Morphological Changes in Parenchyma Cells during Heartwood Formation

The wood samples were divided into 24 growth rings and each growth ring was observed and measured. Samples were immersed in FAA fixative for 48 h and 15 μm-thick sections were cut using a slide-away slicer. Acetocarmine staining was used to visualize the parenchyma nucleus and I_2-IK staining to visualize starch grains. All sections were observed under a light microscope (Nikon E100, Nikon, Sendai-shi, Japan).

2.4. Physiological and Biochemical Changes during Heartwood Formation

2.4.1. Enzyme Activity

Stem samples for determination of enzyme activity were collected from the trees and immediately added to liquid nitrogen and preserved using dry ice. The samples were sent to the Shanghai Enzyme-linked Biotechnology Co., Ltd. (Shanghai, China) for determination of malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL) using an enzyme-linked immunoadsorbent assay (ELISA) kit and microplate reader (Cytation3, BioTeK, Winooski, VT, USA). All detection procedures followed the kit instructions by using microplate reader [38,39].

2.4.2. Total Phenol Content

The total phenol content of *Schima superba* extracts was determined according to the Folin–Ciocalteu method [40], with some modifications. The sapwood, transition wood and heartwood were dried and crushed and 0.1 g of powder was taken from each plant, which was extracted with 10 mL of methanol solution by ultrasonic extraction at room temperature for 240 min and then passed through 0.22 μL of organic filtration membrane to obtain the sample solution.

Plotting the standard curve: accurately suck 0, 0.05, 0.10, 0.15, 0.20, 0.25 mL of 0.1 $mg{\cdot}mL^{-1}$ gallic acid standard solution in a test tube, add methanol solution to 0.25 mL, then add 0.25 mL of Folin phenol reagent, mix well and add 1 mL of 15% Na_2CO_3 solution, mix well and then react for 15 min away from light. The absorbance was measured at 760 nm and the linear equation was determined by plotting the standard curve with the absorbance as the vertical coordinate and the mass of the standard as the horizontal coordinate.

Sample Determination: take 0.25 mL of the sample solution, follow the procedure in the standard curve preparation, measure the absorbance value at 760 nm and calculate the total phenol content of the sample through the standard curve of gallic acid. Instead of the sample solution, 0.25 mL of methanol solution was used as blank.

2.4.3. Total Flavonoid Content

The total flavonoid content of *Schima superba* extracts was determined according to the method described by Hong and Kim [40], with some modifications. Plotting the standard curve: accurately absorb 0, 0.1, 0.2, 0.3, 0.4, 0.5 mL of 0.1 $mg{\cdot}mL^{-1}$ rutin standard solution in a test tube, add methanol solution to 0.5 mL, add 0.5 mL of 5% $NaNO_2$, shake well and let stand for 6 min, add 0.5 mL of 10% $Al(NO_3)_3$, shake well and let stand for 6 min, add 4 mL of 4% NaOH, shake well and let stand for 15 min. The absorbance was measured at 510 nm. To draw the standard curve and obtain the linear regression equation, the absorbance was taken as the vertical coordinate and the mass of rutin was taken as the horizontal coordinate.

Sample Determination: take 0.5 mL of sample solution, follow the procedure in the standard curve preparation, measure the absorbance value at 510 nm and calculate the total flavonoid content of the sample through the standard curve of rutin. Instead of the sample solution, 0.5 mL of methanol solution was used as blank.

2.5. Metabolite Changes during Heartwood Formation

The three key zones of *Schima superba*, sapwood, transition zone and heartwood, were dried and crushed and 0.1 g of powder was taken from each zone, which was extracted with 10 mL of methanol solution by ultrasonication at room temperature for 240 min, while the methanol extract was obtained by passing through 0.22 μL of organic system filter membrane.

The metabolite compositions of different zones of *Schima superba* were determined using gas chromatography–mass spectrometry (GC-MS) (Thermo Fisher Scientific, Waltham, MA, USA). A triple quadrupole GC–MS was used and the capillary column for the determination was TG-5SIMS (30 m × 0.25 mm × 250 μm), using high-purity helium as carrier gas and high-purity argon as collision gas, with an injection volume of 1 μL. The warming

procedure was as follows: the initial column temperature was 90 °C, held for 1 min, the first stage was warmed up to 200 °C at a rate of 5 °C·min^{-1}, held for 5 min and the second stage was warmed up to 280 °C at a rate of 4 min. The second stage was ramped up to 280 °C at a rate of 5 °C·min^{-1} for 5 min with a solvent delay time of 4 min.

Ultra performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) (Thermo Fisher Scientific, USA) was used to identify the chemical constituents of the three zones, sapwood, transition zone and heartwood, of *Schima superba*. UPLC conditions: UPLC column was an ACQUITY UPLCBEHC18 column (1.7 μm, 2.1 mm × 50 mm), mobile phases: A was 0.1% formic acid in water and B was methanol; the gradient elution was 0–1 min, 10%–20%B, 1–3 min, 30%–45%B, 10–17 min, 45%–70%B, 17.1–19 min, 70%–90%B, 19.1–19 min, 70%–90%B, 19.1–19 min, 30%–90%B. 1–3 min, 20%–30%B, 3–10 min, 30%–45%B, 10–17 min, 45%–70%B, 17.1–19 min, 70%–90%B, 19.1–20 min, 10%B; flow rate: 0.4 mL·min^{-1}; column temperature: 40 °C; injection volume: 0.2 μL; mass spectrometry conditions: ESI ion source; scanning mode: MRM; capillary voltage: 2500 KV; ion transfer tube temperature: 100 °C; desolvent temperature: 350 °C; desolvent gas flow rate: 690 L·h^{-1}.

2.6. Statistical Analysis

Excel 2010 and SPSS 11.0 software were used for data statistics, ANOVA and correlation analysis of the experimental results. The analysis of variance significance was carried out using the Duncan method at a significant level of $p < 0.05$. Correlation graphs were plotted using OriginPro 8.0.

3. Results and Analysis

3.1. Macroscopic Color Change of Schima superba

The macroscopic color radial variation of *Schima superba* is shown in Figure 1. From the figure, it can be seen that the values of L*, a* and b* of each growth ring of *Schima superba* have a small range of variation and have no obvious pattern of change, and the value of total color difference represented by ΔE is low. It shows that from cambium to pith, there is little difference in the color of each growth ring and it is not possible to distinguish between heartwood and sapwood as a regional division from the macroscopic point of view, which is in line with the characteristics of the sapwood tree. Therefore, we next further explored the heartwood formation of *Schima superba* in terms of changes in the anatomical morphology of parenchyma cells.

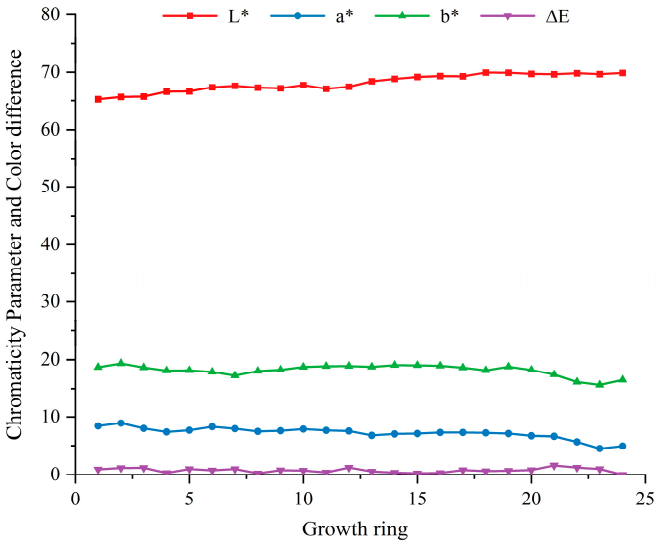

Figure 1. Radial variation of wood color. L*: lightness value. a*: red and green chromaticity index. b*: yellow and blue chromaticity index. ΔE: radial color difference of wood. The horizontal coordinate numbers 1–24 correspond to 24 growth rings from the pith to the outer side of the wood.

3.2. Morphological Changes in Parenchyma Cells during Heartwood Formation

3.2.1. Changes in the Number of Starch Grains

Heartwood formation is accompanied by cytological changes such as aging and PCD from year to year; therefore, the PCD clearly defines the transformation of sapwood to heartwood. Shain and Hillis found that parenchyma cells were less active the farther they were from the forming layer and that the highest peaks of metabolism in the transition zone accompanied many of the processes of heartwood transformation [41]. Among them, starch grains, as one of the main forms of energy storage in parenchyma cells, can provide energy and material base for the cells; therefore, the number of starch grains can reflect the activity of the cells to some extent [4].

Figure 2 shows the staining of starch grains in different growth rings of *Schima superba*. After staining with I_2-KI, the starch grains in parenchyma cells were stained brownish-black, as shown in the figure, and the 24th growth ring (Figure 2a,b) contained a large number of well-filled starch grains occupying most of the entire cell lumen, which proved that they were physiologically and metabolically active. In the 20th growth ring (Figure 2c,d) there was a gradual decrease in the number of starch grains and a certain degree of shrinkage. In the 15th growth ring (Figure 2e,f), the reduction in the number of starch grains was intensified, with sparse rows and further reduction in size. In the 11th growth ring (Figure 2g,h), starch grains disappeared completely. Continuing toward the pith, starch grains also completely disappeared in the first growth ring (Figure 2i,j).

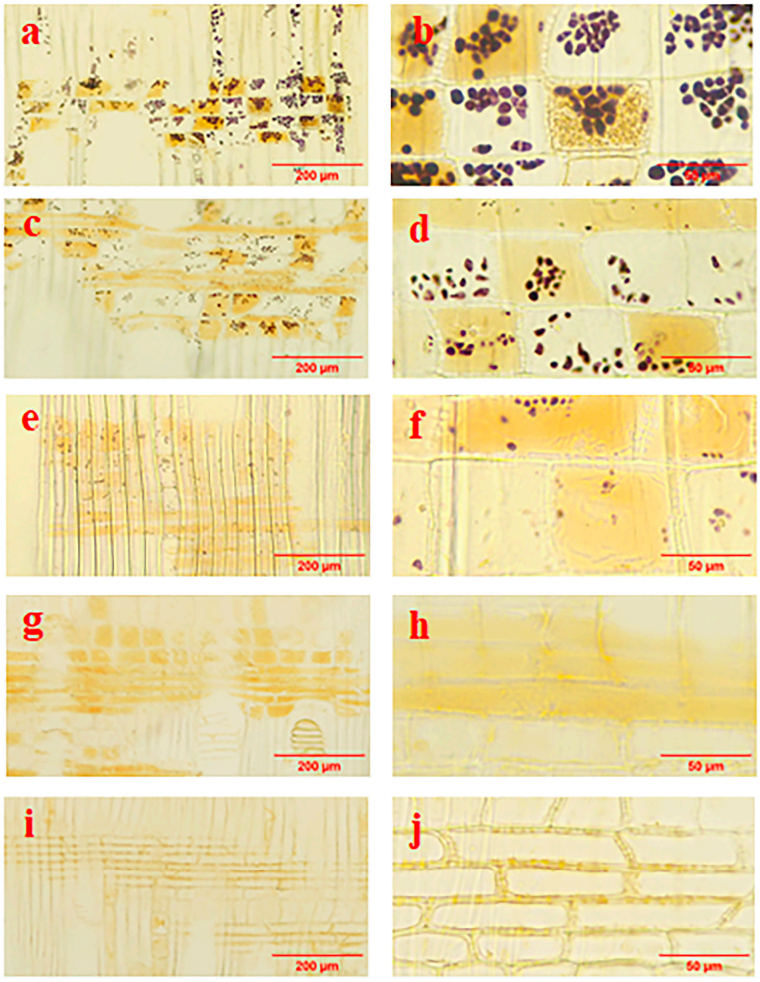

Figure 2. Color development of starch grains in parenchyma cells. (**a**,**b**) show the 24th growth ring starch grains' morphology. (**c**,**d**) show the 20th growth ring starch grains' morphology. (**e**,**f**) show

the 15th growth ring starch grains' morphology. (**g**,**h**) show the 11th growth ring starch grains' morphology. (**i**,**j**) show the first growth ring starch grains' morphology.

Figure 3 shows the number statistics of parenchyma cell starch grains in 24 growth rings of *Schima superba*. As can be seen from the figure, the 24th growth ring contained the highest number of starch grains, and from the 24th growth ring toward the pith, the number of starch grains gradually decreased until the 11th growth ring, when the number of starch grains dropped to zero, indicating that the starch grains completely disappeared. That is, from cambium to pith, the starch grains are gradually degraded, with the 11th growth ring as the demarcation point, and then toward the pith, no starch grains are present.

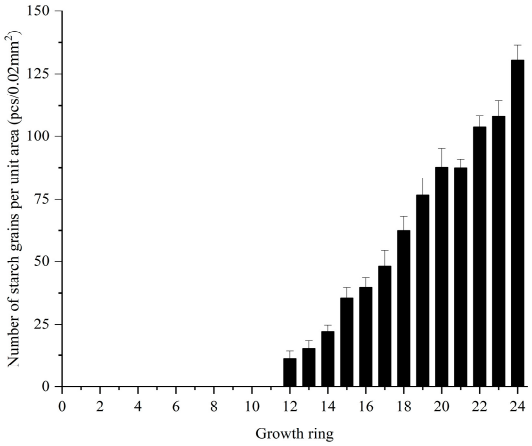

Figure 3. Number of starch grains in parenchyma cells. The horizontal coordinate numbers 1–24 correspond to 24 growth rings from the pith to the outer side of the wood.

3.2.2. Morphological Changes of Parenchyma Nucleus

The disappearance of the nucleus is usually taken as a sign of PCD. During the process of heartwood formation, the nucleus viability of parenchyma cells gradually declined, and by the transition zone the viability had been minimized. By the time the heartwood was reached, the viable cells had completely disappeared, indicating the death of parenchyma cells. Therefore, Nakaba et al. characterized the morphology of parenchyma cells, including the nucleus morphology, as an indicator of the PCD [21].

Figure 4 shows the nucleus morphology of parenchyma cells from different growth rings of *Schima superba*. As can be seen from the figure, the nucleus morphology of the 24th growth ring showed fusiform shape and was located in the center of the cell (Figure 4a). By the 20th growth ring, the nucleus becomes ovoid (Figure 4b) and its length is reduced. Toward the pith, by the 15th growth ring, the nucleus tended to be progressively more rounded (Figure 4c), with the length/width decreasing and the nucleus closer to the inner wall of the cell lumen compared to the previous ones. By the 13th growth ring, the nucleus appeared rounded and close to the inner wall of the cell lumen (Figure 4d). Continuing toward the pith, by the 11th growth ring, some nuclei were degraded and reduced in number (Figure 4e). In the 10th growth ring, the nucleus completely disappeared (Figure 4f). Nucleus shape changed according to the following rules: fusiform, oval, tending to be rounded and close to the inner wall, rounded and gradually degraded, disappeared.

Figure 5 shows the statistics of the length, width and length/width of parenchyma nuclei from different growth rings of *Schima superba*. As it can be seen in Figure 5, the length of the 24th growth ring was greatest from cambium to pith and the general trend of the nucleus length (Figure 5a) was gradually decreasing. Nucleus width (Figure 5b) was roughly at one level, with little overall variation. The nucleus length/width (Figure 5c) also became progressively smaller. The length, width and nucleus length/width were all mutated down to zero at the 11th growth ring, whereas the length/width of nucleus directly reflects the degree of cellular activity in parenchyma cells. Therefore, the cellular

activity of *Schima superba* parenchyma cells showed a decreasing trend from cambium to pith (up to the 11th growth ring) and from the 11th growth ring toward the pith, which was devoid of cellular activity.

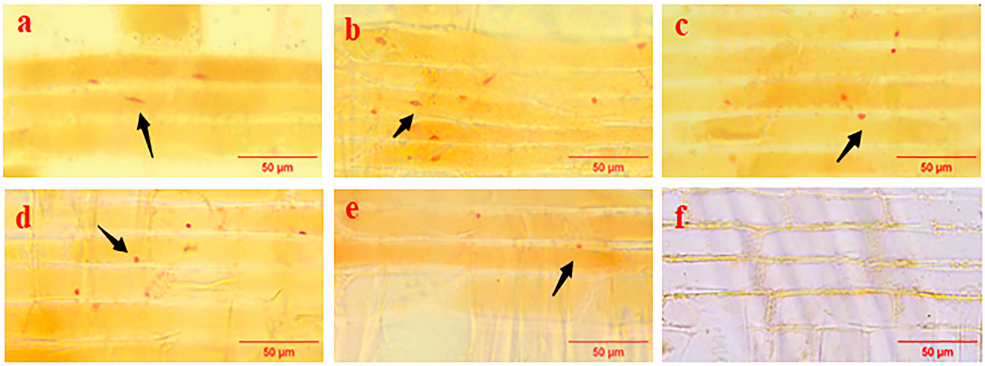

Figure 4. Color development of parenchyma nucleus. (**a**–**f**) are the parenchyma nuclei of the 24th, 20th, 15th, 13th, 11th and 10th growth rings, respectively.

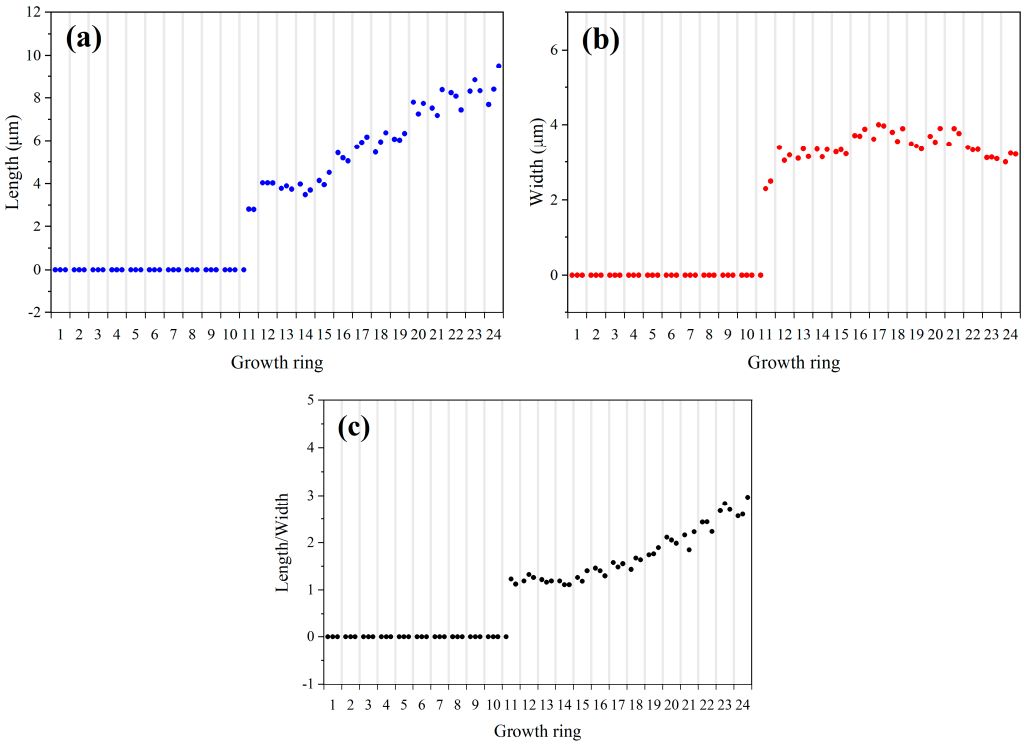

Figure 5. Morphological index statistics of parenchyma nucleus. (**a**) depicts the length of parenchyma nuclei in different growth rings. (**b**) depicts the width of parenchyma nuclei in different growth rings. (**c**) depicts the length/width of parenchyma nuclei in different growth rings. The horizontal coordinate numbers 1−24 correspond to 24 growth rings from the pith to the outer side of the wood.

3.2.3. The Formation of Heartwood in *Schima superba*

Figure 6 is a diagram of the anatomical and structural changes in parenchyma cells of *Schima superba*, which is based on the above statistics on the staining, number and morphology of the starch grains and nuclei of parenchyma cells, which were analyzed in a comprehensive manner. From cambium to pith, the starch grains were more numerous and full on the outside, and towards the pith, the number of starch grains gradually decreased and their size became smaller until they disappeared in the 11th growth ring. Nucleus shape changed according to the following rules: fusiform, oval, tending to be rounded and close to the inner wall, rounded and gradually degraded, disappeared.

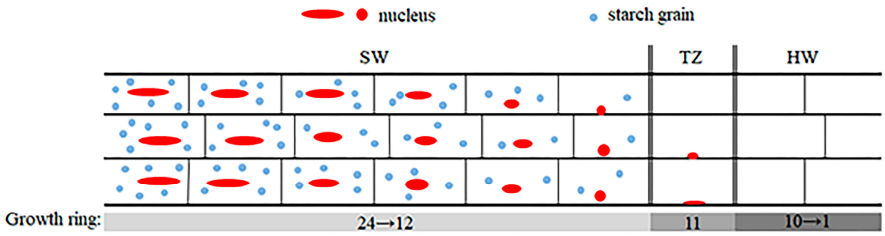

Figure 6. Anatomical changes of parenchyma cells. SW: sapwood. TZ: transition zone. HW: heartwood.

Parenchyma cells start from the cambium, and the closer they are to the pith, the less active their cells become, until programmed death occurs in the heartwood, so that the PCD clearly defines the sapwood-to-heartwood transformation. The starch grains and nuclei of parenchyma cells underwent a sudden change in the 11th growth ring, and then in the direction of the pith, the starch grains and nuclei disappeared, suggesting the existence of a heartwood component in *Schima superba*. Therefore, *Schima superba* samples were divided into three sections, classifying the 12th–24th growth rings as sapwood, the 11th growth ring as transition zone and the 1st–10th growth rings as heartwood.

3.3. Physiological and Biochemical Changes during Heartwood Formation

3.3.1. Enzyme Activity

During heartwood formation, starch in the sapwood is continuously transferred through the ray cells to the transition zone, where it is degraded to soluble carbohydrates while enzyme activity is increased for further conversion to heartwood material. Genes controlling the expression of specific enzymes in the sapwood and transition zone during heartwood formation are up-regulated, which results in enhanced specific enzyme activity related to the synthesis of heartwood substances such as phenols and terpenoids.

Figure 7 shows the changes in enzyme activities during heartwood formation in *Schima superba*. As can be seen from the figure, there were significant differences in the MDA content and the activities of SOD, CAT, POD, PPO and PAL between the outer sapwood, inner sapwood and transition zone of *Schima superba*. The activities of all enzymes were highest in the transition zone, second highest in the inner sapwood and at their lowest in the outer sapwood. Since the enzyme activity increases with the heartwood material formation, this indicates that the transition zone is where heartwood formation most rapidly occurs.

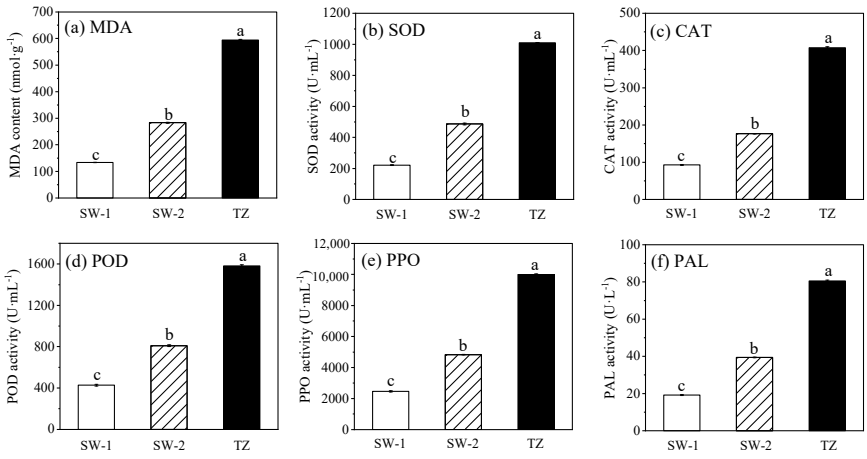

Figure 7. Changes of enzyme activity during heartwood processing. (**a**) shows the MDA content. (**b**) shows the SOD activity. (**c**) shows the CAT activity. (**d**) shows the POD activity. (**e**) shows the PPO activity. (**f**) shows the PAL activity. SW−1: the outer sapwood. SW−2: the inner sapwood. TZ: the transition zone. The letters in the figure represent the results of Duncan and different letters represent significant differences ($p < 0.05$).

3.3.2. The Content of Total Phenols and Total Flavonoids

The most important manifestation of heartwood formation in trees is the deposition of large amounts of heartwood material in the xylem ducts and parenchyma cells. Studies have shown that during cell death, primary metabolites such as starch grains in parenchyma cells show a gradual degradation from sapwood to heartwood, culminating in the heartwood material formation. The cell cavities or tissues at the heartwood accumulate large amounts of organic solvent extracts, known as heartwood extractives [32].

Among phytochemicals, flavonoids are the most common large group of polyphenols. Flavonoids are more soluble in alcoholic solvents and have lower extraction rates in extractants with water as the solvent, which is more polar, so methanol was chosen as the solvent for the experiments. Figure 8 shows the changes in the total phenol and total flavonoid content of secondary metabolites during the heartwood formation of *Schima superba*. As can be seen from the figure, the highest content of flavonoids and phenols was found in the heartwood of *Schima superba*, followed by the transition zone and the least in the sapwood. The total phenol and flavonoid content differed significantly among sapwood, transition zone and heartwood, with total phenol and total flavonoid content gradually increasing from sapwood to heartwood.

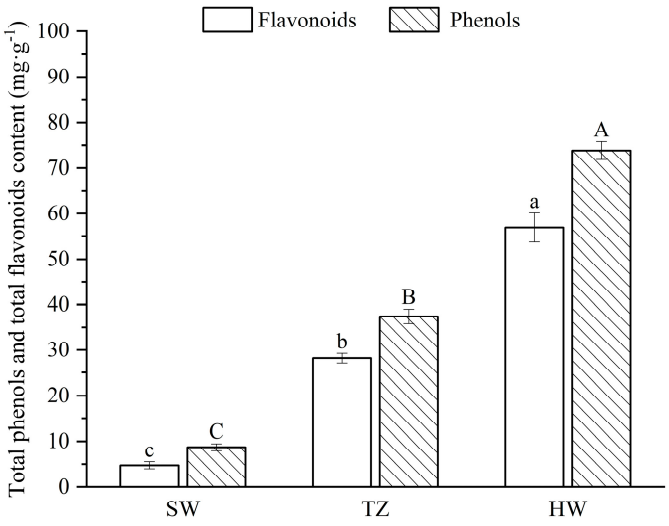

Figure 8. Changes of total phenol and flavonoid content. SW: sapwood. TZ: transition zone. HW: heartwood. The letters in the figure represent the results of Duncan and different letters represent significant differences ($p < 0.05$).

3.3.3. Relationships between Parenchyma Cell Morphology, Metabolites and Color Parameters during Heartwood Formation of *Schima superba*

Based on the above results of wood color parameters, parenchyma cell structural characteristics and physiological and biochemical indices, a comparative analysis of the relationships between parenchyma cell morphology, metabolites and color parameters in heartwood formation of *Schima superba* was carried out (Figure 9). As can be seen from the figure, there is a strong link and high correlation between the structural characteristics of the parenchyma cells and the total phenol and total flavonoid content. There was a negative correlation between the structural characteristics of parenchyma cells and total phenol and total flavonoid content, and a very strong positive correlation between the number of parenchyma cell starch grains and the nucleus length/width, as well as between total phenol and total flavonoid content. The weak correlation between the wood color correlation index and the structural characteristics of parenchyma cells, total phenol content and total flavonoid content proved the difference between the macroscopic color presentation of the wood of the sapwood tree *Schima superba* and the heartwood formation inside the cells. Therefore, in order to explore more deeply the heartwood formation of

the sapwood tree *Schima superba* and the reasons for its lack of color development, the metabolites in the heartwood formation process were further investigated.

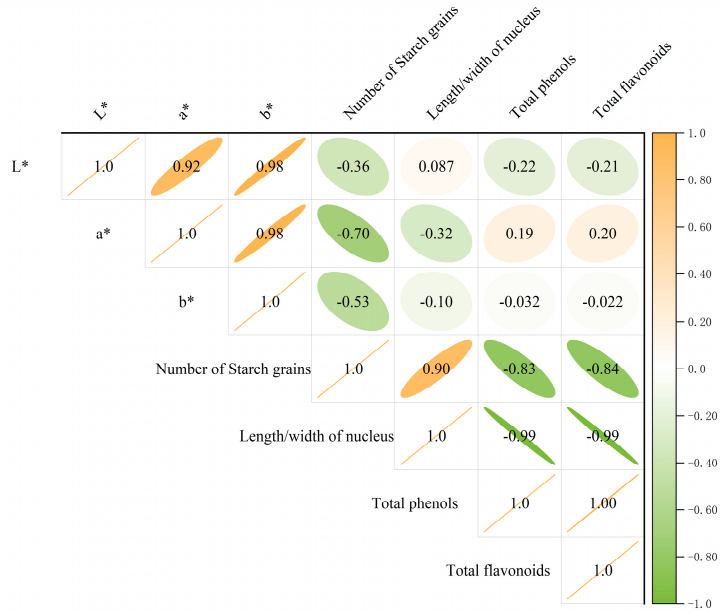

Figure 9. Relationships between parenchyma cell morphology, metabolites and color parameters in heartwood formation. L*: lightness value. a*: red and green chromaticity index. b*: yellow and blue chromaticity index.

3.4. Metabolite Changes during Heartwood Formation

3.4.1. GC–MS Analysis of Extracts from *Schima superba*

The peaks in the total ion flow diagram were retrieved using a mass spectrometry data system and further checked using standard mass spectrometry diagrams to derive the major metabolites of the *Schima superba* metabolite. The results, analyzed by GC–MS, showed that the *Schima superba* extracts mainly contained phenols, terpenoids, lipids, alkaloids, carboxylic acids, alcohols and amino acids.

Table 1 shows the comparison of major metabolite species and their relative contents in different zones of *Schima superba*. As can be seen from the table, the measured phenols coniferyl alcohol, 2′,6′-dihydroxy-4′-methoxyacetophenone, 2,4-di-tert-butylphenol and isorhamnetin were not found in the sapwood, and in the transition zone, only a small amount of 2,4-di-tert-butylphenol. The content of phenols in the heartwood was much higher than that in the sapwood and transition zone. For terpenoids, only trace amounts of phytane were found in the sapwood and transition zone, while beta-ionone and thioguanine were not found in the sapwood and transition zone, and terpenoids were much higher in the heartwood than in the transition zone and sapwood. The alkaloid skatole is found only in the heartwood. The lipids methyl butyrate and isopropyl dodecanoate are highly abundant in the sapwood.

Table 1. Species and relative contents of main metabolites in different zones of *Schima superba*.

NO.	Classification	Compounds	Formula	Molecular Weight (Da)	Peak Area Percentage (%)		
					SW	TZ	HW
1	Phenols	Coniferyl Alcohol	$C_{10}H_{12}O_3$	180.20	-	-	5.41
2		2′,6′-Dihydroxy-4′-Methoxyacetophenone	$C_9H_{10}O_4$	182.17	-	-	2.95
3		2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	206.32	-	0.07	2.93
4		Isorhamnetin	$C_{16}H_{12}O_7$	316.26	-	-	1.59

Table 1. *Cont.*

NO.	Classification	Compounds	Formula	Molecular Weight (Da)	Peak Area Percentage (%)		
					SW	TZ	HW
5	Terpenoids	Beta-Ionone	$C_{13}H_{20}O$	192.30	-	-	1.38
6		Phytane	$C_{20}H_{42}$	282.50	0.04	0.18	1.16
7		Thioguanine	$C_5H_5N_5S$	167.19	-	-	0.86
8	Alkaloids	Skatole	C_9H_9N	131.17	-	-	1.55
9	Lipids	Methyl butyrate	$C_5H_{10}O_2$	102.13	7.14	-	-
10		Isopropyl dodecanoate	$C_{15}H_{30}O_2$	242.40	1.13	-	-

SW: sapwood; TZ: transition zone; HW: heartwood.

3.4.2. UPLC–MS/MS Analysis of Extracts from *Schima superba*

In order to gain a better understanding of the heartwood substances' formation during heartwood formation in *Schima superba*, their metabolites were examined using UPLC–MS/MS. The metabolites were detected in both positive and negative ionic modes and the preliminary characterization of the acquired chromatographic peak substances through the database yielded that the *Schima superba* metabolites mainly consisted of compounds such as terpenoids, phenols, alkaloids, lipids, coumarins, organic acids and lignans, with terpenoids being the most abundant, followed by phenols (Figure 10).

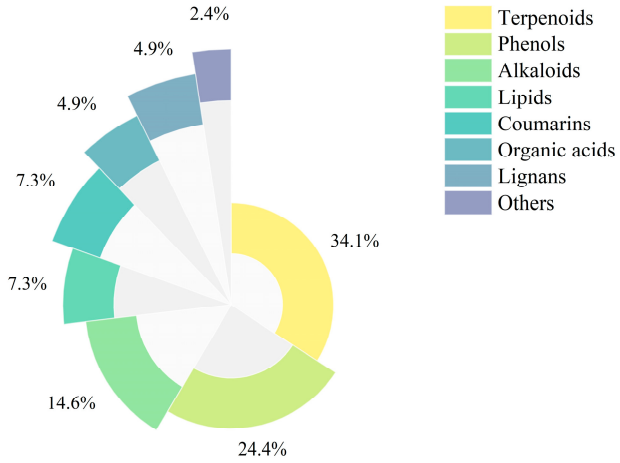

Figure 10. Classification of the identified metabolites.

The type and composition of metabolites directly affects the properties of the wood. Using the size of the peak area as the response value, the relative content of the substances was ranked, and the top 10 substances with the highest content in the heartwood are listed in Table 2. The three metabolites with the highest relative amounts were bakkenolide A, melamine and 4-ethyl-2-methoxyphenol. It is the accumulation of colored material that makes heartwood easy to distinguish from sapwood. Bosshard proposed that light-colored heartwood differs from colored heartwood in that it does not contain colored extracts or only contains a small amount of colored extracts [37]. From Table 2, it was found that melamine, 4-ethyl-2-methoxyphenol and homovanillic acid, which are very high in relative abundance in the heartwood of *Schima superba*, are white or colorless compounds. On the basis of this analysis of their changes in the heartwood formation process, it can be seen from Figure 11 that melamine, 4-ethyl-2-methoxyphenol and homovanillic acid, as secondary metabolites of *Schima superba*, are present in large quantities in the heartwood and have a certain effect on the presentation of the heartwood color.

Table 2. The 10 metabolites with the highest relative content in *Schima superba* heartwood.

NO.	Rt (min)	Compounds	Formula	Molecular Weight (Da)	Peak Area
1	7.695	Bakkenolide A	$C_{15}H_{22}O_2$	235.1682	1,075,835.728
2	12.391	Melamine	$C_3H_6N_6$	127.0729	620,495.897
3	3.262	4-Ethyl-2-methoxyphenol	$C_9H_{12}O_2$	211.0971	534,145.486
4	1.019	Limonin	$C_{26}H_{30}O_8$	471.2011	448,018.769
5	8.771	Homovanillic Acid	$C_9H_{10}O_4$	181.0499	385,446.775
6	13.582	Quillaic acid	$C_{30}H_{46}O_5$	487.3397	163,758.184
7	7.695	Doxofylline	$C_{11}H_{14}N_4O_4$	267.1101	144,042.467
8	1.565	8-Epiiridotrial glucoside	$C_{16}H_{24}O_8$	367.1367	136,495.749
9	8.824	Nigakilactone N	$C_{21}H_{30}O_7$	395.2057	130,933.374
10	4.801	Manoalide	$C_{25}H_{36}O_5$	417.2619	113,622.510

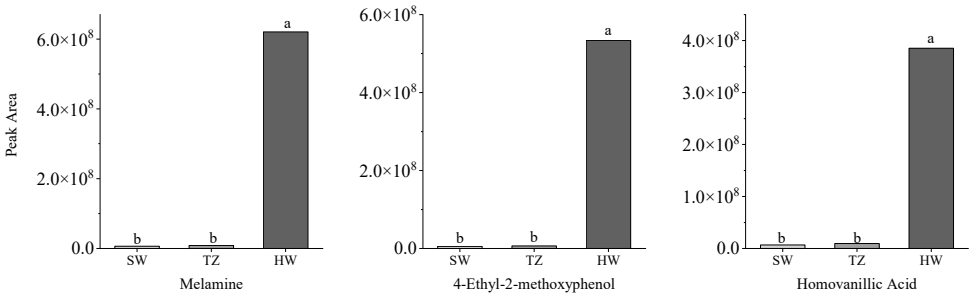

Figure 11. The relative content of melamine, 4-ethyl-2-methoxyphenol and homovanillic acid in *Schima superba*. SW: sapwood. TZ: transition zone. HW: heartwood. The letters in the figure represent the results of Duncan and different letters represent significant differences ($p < 0.05$).

4. Discussion

Sapwood and heartwood are usually distinguished by the lighter color of the sapwood and the darker color of the heartwood, but there are some tree species whose sapwood and heartwood cannot be classified by the darkness of their color. Therefore, the International Association of Wood Anatomists (IAWA) defines sapwood and heartwood as follows. The sapwood of a living tree contains living cells and storage substance such as starch grains and oils, while the heartwood is the central portion of the tree that does not contain living cells and in which the storage substance has been eliminated or converted to extractives in the heartwood.

In this study, the sapwood tree *Schima superba* was taken as the object of study. Firstly, the macroscopic color of its wood was measured, and the results showed that macroscopically, from cambium to pith, the color of the growth ring did not differ much and it was not possible to differentiate between the heartwood and the sapwood zone division macroscopically, which was in line with the characteristics of the sapwood tree. Therefore, we further explored the heartwood formation of *Schima superba* from the anatomical and morphological variations of parenchyma cells. During tree growth and development, a series of physiological changes, such as parenchyma cell decline and apoptosis, all play a crucial role in the heartwood formation. The nucleus being one of the most important markers characterizing cellular activity, we showed in *Schima superba* a gradual decrease in the nucleus length/width from cambium to pith until reaching zero. Nucleus shape changed according to the following rules: fusiform, oval, tending to be rounded and close to the inner wall, rounded and gradually degraded, disappeared. This shows that the activity of the parenchyma cells decreases progressively in the radial direction. The nucleus eventually disappears after heartwood generation and the same pattern exists in other tree species [21]. At the same time, starch grains were abundantly present in parenchyma cells of the 12th–20th growth rings of *Schima superba*, with a plunge occurring in the 11th growth ring and the complete disappearance of starch grains in the 1st–10th growth rings.

This is similar to the findings reported by Mishra et al. on the reduction in starch content of *Eucalyptus bosistoana* from sapwood to heartwood [19].

Although it is not possible to macroscopically discriminate heartwood from sapwood in *Schima superba*, a series of anatomical structural variations in parenchyma cells suggests that the heartwood zone exists in *Schima superba*. We divided the *Schima superba* samples into three zones: sapwood (12th–20th growth rings), transition zone (11th growth ring) and heartwood (1st–10th growth rings).

During heartwood formation, starch in the sapwood is continuously transferred through the ray cells to the transition zone, where it is degraded to soluble carbohydrates while enzyme activity is increased for further conversion to heartwood material. Genes controlling the expression of specific enzymes in the sapwood and transition zone during heartwood formation are up-regulated, resulting in enhanced activity of specific enzymes related to heartwood material synthesis. The experimental results showed that the MDA content and the enzyme activities of SOD, CAT, POD, PPO and PAL were much higher in the transition zone than in the sapwood, which was consistent with the findings of Yang et al. [42] and Moshchenskaya et al. [25]. Among them, SOD is the most important protective enzyme in the plant defense system. In the early stage of heartwood formation, cell activity was reduced and constantly stressed, but in order to maintain the balance of cell membrane structure, more SOD was accelerated to clear harmful reactive oxygen species in the tree. Therefore, SOD activity in the transition zone was much higher than in the sapwood. The total phenol and total flavonoid content of the sapwood, transition zone and heartwood of *Schima superba* were then determined, and the results showed that the total phenol and total flavonoid content differed significantly among the sapwood, transition zone and heartwood and that the total phenol and total flavonoid content gradually increased from the sapwood to the heartwood. Carbon compounds are increasingly used in the in situ synthesis of phenol extracts in the later stages of heartwood formation when mitochondrial respiration is blocked [43]. Thus, biosynthesis of heartwood components occurs during the transformation of sapwood into heartwood, with the transition zone being its primary site.

The result of heartwood formation is that there is a large amount of heartwood material deposited in the parenchyma cells, ducts, fibers and tubular cells of the xylem, which are plant secondary metabolites, produced by secondary metabolic processes. The result of the adaptation of woody plants to the ecological environment over a long period of evolution, these are also the main characteristic changes that distinguish heartwood from sapwood. In general, wood with a short growth period is usually pale, and as the stand ages, extractives are converted to insoluble polymers that are deposited in the heartwood, thus exhibiting a natural color [44]. Therefore, in order to explore more deeply the heartwood formation of the sapwood tree *Schima superba* and the reasons for its lack of color development, the metabolites in the heartwood formation process were further investigated. Bosshard proposed that light-colored heartwood differs from colored heartwood in that it does not contain colored extracts or only contains a small amount of colored extracts [37]. It was found that melamine, 4-ethyl-2-methoxyphenol and homovanillic acid, all of which are white or colorless compounds with very high relative abundance in the heartwood, are present in large amounts in the heartwood as secondary metabolites of *Schima superba* and have some influence on the presentation of the heartwood color. Therefore, the presence of large amounts of colorless and white extractives and only small amounts of colorful extractives is an important reason for the normal formation of heartwood in *Schima superba* that does not differ in color from the sapwood.

5. Conclusions

Heartwood is present in the sapwood tree *Schima superba*, but the color difference between heartwood and sapwood is not significant. In the process of heartwood formation, the parenchyma cells become inactive and the nucleus disappears, while at the same time, the storage substance starch is gradually degraded under the action of enzymes and trans-

formed into secondary metabolites, which include terpenoids, phenols and alkaloids. The accumulation of white and colorless compounds in large quantities at the heartwood, which has some effect on the heartwood color, is an important reason for the normal formation of heartwood in *Schima superba* that exhibits no difference in color from the sapwood.

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