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Comparison of anatomical structure between agarwood made by artificial incense technology and genuine agarwood sold in the market

Libao Zhang [1, a], Ruiling Yuan [1, b], Dan Feng [1, c], Genying Zhao [1, d], Peng Chen [1, 2, e, *], Yueqiu He [3, f], Shahzad Munir[3, g]

[1]Yunnan Academy of Forestry and Grassland Sciences, Kunming, Yunnan 650201, China;

[2]Key Laboratory of Biodiversity and Ecological Security in Gaoligongshan, Yunnan Province, Kunming, Yunnan 650201, China;

[3]Yunnan Agricultural University, Kunming 650201, China.

[a]zlbao666@163.com, [b]1297922529@qq.com, [c]474727338@qq.com, [d]1656857246@qq.com, [e]chenpenglinkeyuan@163.com, [f]ynfh2007@163.com, [g]shahzad_munir@ynau.edu.cn,

*Corresponding author

Abstract

This study aims to ascertain the anatomical structural differences between Aquilaria sinensis produced through various artificial perfuming techniques and traditional Chinese medicine A. sinensis, as well as pseudo-A. sinensis. In addition, we also investigate the methods for authenticating A. sinensis wood and enhance understanding of the accumulation mechanisms of A. sinensis. For the first time, both macro-observation and micro-observation were used to understand the differences in tissue structure among artificial fragrant tested samples for the above. The results showed that the texture, color, surface covering, a trace of crucial oil substance and section structure of the sample was visible under the microscope, and there were noticeable apparent differences between the accurate actual and false samples of A. sinensis. Except for the white wood treatment, dark brown A. sinensis substances were deposited in the phloem and wood ray parenchyma cells of the other samples in the artificial perfuming samples. Further, the perfuming effect of insect-borne bacteria was better than that of the general artificial perfuming treatment. The findings indicate that the authenticity and quality of A. sinensis can be precisely determined through structural identification of its samples, and "insect-borne bacteria" have a better fragrance-making effect than traditional artificial techniques.

Keywords

Aquilaria sinensis, Ultramicro section, Insect-borne bacteria.

1. Introduction

Aquilaria sinensis (Lour.) Gilg belongs to Aquilaria of the Daphne family, and the pathological tissue left after external adverse factors stimulate its trunk is called *A. sinensis* (also known as *A. sinensis*, Ardisia crenata, Gui Xiang, Guanxiang and Ruxiang) [1], generally brown to dark black, and its quality will improve with the deepening of color [2]. According to the origin of *A. sinensis*, it can be divided into three producing areas: China *A. sinensis*, Hui 'an (Viet Nam, Cambodia, Laos·Aquilaria crassna) and Sin Chew (Indonesia, Philippines, Brunei, Malaysia ·Aquilaria malaccensis) [3]. China's agarwood-producing areas are mainly distributed in evergreen, broad-leaved mixed forests in low-altitude mountains and hills such as

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Guangdong, Hainan, Guangxi, Fujian, Yunnan, and Taiwan Province, China. Agarwood has the functions of calming, strengthening the heart, relieving pain, stopping vomiting, relieving asthma, and regulating the central nervous system. Modern pharmacological studies have found that agarwood also improves myocardial ischemia, anti-inflammatory [4], antibacterial [5], anti-tumor [6], anti-depression [7], anti-oxidation [8], regulating blood lipids, relieving heat, and relieving pain [9]. Besides, it also has the potential to treat diabetes as well [10,11]. It also has specific curative impact on diseases such as Alzheimer's disease with rare medicinal plant unique to China [12-14]. It is stipulated by the People's Republic of China (PRC) Pharmacopoeia: 2020 Edition Part I as the only plant source of domestic agarwood [15]. In addition, agarwood is widely used in high-grade spices, cosmetics, and tea [16], and handicrafts with collection value [17]. Only 7%-10% of the trees of *A. sinensis* can make incense under natural conditions [18]. The deterioration of habitat and predatory felling of wild agarwood resources make high-quality agarwood resources more scarce [19]and has been listed as a second-class endangered plant in China.

In recent years, researchers have searched for the symbolic chemical components of *A. sinensis* from different regions through various chemical analysis methods and found that it is necessary to comprehensively and meticulously analyze the chemical components of a large number of *A. sinensis* samples from different areas, and use complex mathematical models to calculate and summarize the evolution laws of compounds during the formation of *A. sinensis* [20,21]. Although modern molecular identification technology can identify agarwood primordium, different provenances, and aroma-forming methods will lead to different chemical components of agarwood. So far, no specific method has been found to effectively distinguish and identify samples from the same source with different aroma-forming methods [22,23]. Presently, the research on morphological identification of *A. sinensis* primarily focuses on comparing wood tissue structure and chemical composition [24], wood structural characteristics and identification methods of true and false agarwood [25,26] and so on. The research shows that it is not reliable to distinguish the authenticity of agarwood simply by observing the apparent morphology of the sample [27]. However, the method of combining macro-structure with micro-structure observation has not yet been reported. Therefore, macroscopic observation is combined with microscopic observation of the microstructure characteristics such as axial parenchyma, wood fiber, catheter and ray to deeply understand the influence of different treatment conditions on *A. sinensis*, and provide reliable primary data for further research on the identification of authenticity of *A. sinensis*.

2. Materials and Methods

2.1. Selection and treatment of agarwood wood

Fungal infected samples, "insect-borne bacteria" samples, insect corrosion samples and PDA (potato dextrose agar), cold drill and white wood blank control samples were selected 6 months after incense. The traditional Chinese medicine *A. sinensis* used in the experiment was purchased from Kunming Sheng 'ai Chinese Medicine Museum, Kunming, China and the pseudo-*A. sinensis* came from the Forest Protection Institute of Yunnan Academy of Forestry and Grassland Sciences laboratory. Immediately after the sample was extracted, the agarwood sample was fixed in 70% FAA (formalin, acetic acid, ethanol) fixed solution for 24 hours. All samples were cut with a scalpel into square wooden blocks with a length of 1cm for later use.

2.2. Main instruments and reagents

The instruments and reagents include a Rotary slicer, paraffin embedding machine, electric blower drying oven, film dryer, film spreading machine, microscope, xylene, fast green, saffron, neutral gum, and anhydrous ethanol.

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2.3. Macrostructure observation of A. sinensis samples

Starting from one side of the sample with a sterile scalpel, the sample is cut in the transverse, longitudinal and chordal ways, as shown in Fig. 1. The differences between samples can be distinguished by observing and comparing the characteristics of texture, color, fragrance, surface covering, trace of essential oil, and section structure.

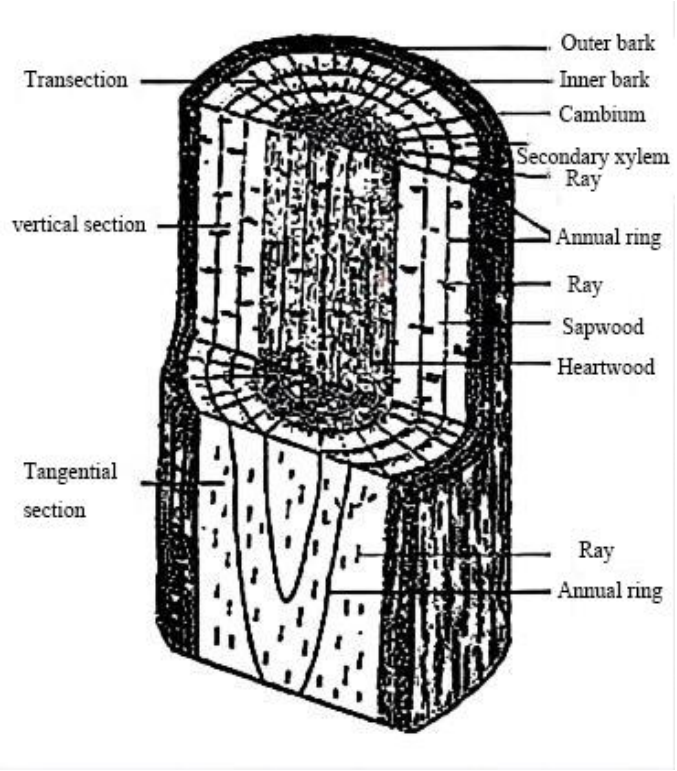

Fig. 1 Schematic diagram of agarwood wood section（The picture comes from the Internet https://anywood.com/news/detail/26194.html）

2.4. Production and observation of ultramicro-slices of agarwood wood

2.4.1. Slice making

The samples were cut into three kinds according to 2.3, namely, transverse, longitudinal and oblique, and put in a fixed solution for marking.

2.4.2. Dehydration

Dehydration is carried out in the following order: 50% ethanol (including 1% saffron), 70% ethanol, 80% ethanol, 95% ethanol, anhydrous ethanol and anhydrous ethanol. The processing time for each step wass 2 h.

2.4.3. Softening, waxing and embedding of wood

After the dehydration treatment of agarwood wood, it is transferred to the wax soaking step for 2 hours each time. The methods are: 4/5 ethanol +1/5 xylene, 3/5 ethanol +2/5 xylene, 2/5 ethanol +3/5 xylene, 1/5 ethanol +4/5 xylene, xylene and xylene. After the completion of wax soaking, the wood is removed and soaked in paraffin for 24 hours. After softening, the wood completely soaked in wax is placed in an embedding box and marked with numbers. Finally, the embedded wood is stored in a biological, low-temperature refrigerator for later use.

2.4.4. Wood slicing, spreading, and baking

The embedded material was taken out and put on a rotary slicer with an inclination of 5 and a slice thickness of 20μm. The water temperature was set of the exhibition to 40°C and preheat it in advance to ensure the proper temperature during the exhibition. A continuous flat slice was selected, and it was gently pinch with tweezers and put it in a spreader to spread it, and then

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use an adhesive glass slide to adsorb the spread slice on the glass slide. The slides were put on the drying machine and the temperature was set to 0℃ to quickly and effectively remove moisture and dry the slices.

2.4.5. Slice dyeing, sealing, and microscopic examination

The dried slices were placed in xylene solution for dewaxing for 30 min, and then the slides were washed with anhydrous alcohol, counterstained with 1% solid green alcohol xylene solution for 1 min, followed by washing with anhydrous alcohol after counterstaining. The washed glass slide was put on the workbench, ensure it surface is clean, and drop 1-2 drops of neutral resin in the center of the slicing part (pay attention to drop the resin at the same position when dropping to avoid bubbles). The cover glass was gently cover on the resin and slice to make it closely fit the glass slide. The the cover glass was gently squeeze so that the resin gradually spreads outward to the whole lens. The sample was placed in a ventilated place to dry for about 1h to completely dry the resin. A cotton swab was dipped in an appropriate amount of xylene, gently wipe the sample's surface to remove excess resin, and then place it under a microscope for microscopic examination.

2.5. Macro Slice Making and Observation

The cross-section, longitudinal section, and tangential section of the sample were observed simultaneously by using tweezers, and the microstructure features such as axial parenchyma, wood fiber, catheter and ray were observed by microscope, to comprehensively compare the structural features of *A. sinensis* samples under different treatment conditions and verify the aroma-forming effect of entomogenous bacteria.

2.6. Data collection and analysis

Image-pro plus 6.0 software was used to analyze the microstructure under a 40-fold microscope, which was repeated three times, and the average value was taken as data. The input data was put into the software of IBM SPSS Statistics 26.0 for difference analysis, and finally the software of Graphpad Prism9.5.1 was used to make a statistical chart.

3. Results and analysis

3.1. Macro observation

The wood of *A. sinensis* is soft and porous[28]. Among them, the section of white timber treated with white wood is yellow and white, and there is no oily substance (Fig. 2.1). The color of all sections in other treatments is yellowish brown, among which the existence of oily substances in the cross-section is directly proportional to the discoloration color, and the darker the cross-section color results in more oily substances. The wood rays in the longitudinal section are thin and closely arranged, and yellow-brown oily substances are filled in them (Fig. 2.2z~7z). Different shades of oily substances accompany tangential section.

The tube holes in the cross-section of *A. sinensis* are single tube holes or radial multiple tube holes, with a large number and relatively small size (Fig. 2.1~7h). Pseudo-agarwood is hard in texture, closely arranged in pores (Fig. 2·8X), pungent in fragrance, and covered with black oily substances on its surface (Fig. 2·8H). The texture of Chinese traditional medicine *A. sinensis* is relatively soft, the stomata are loosely arranged (Fig. 2·7X), and it has a medicinal fragrance, which is combined with a sweet and mellow smell. The surface is dark brown, and the surface is natural, with white wood and rotten wood parts in the section (Fig. 2·7H).

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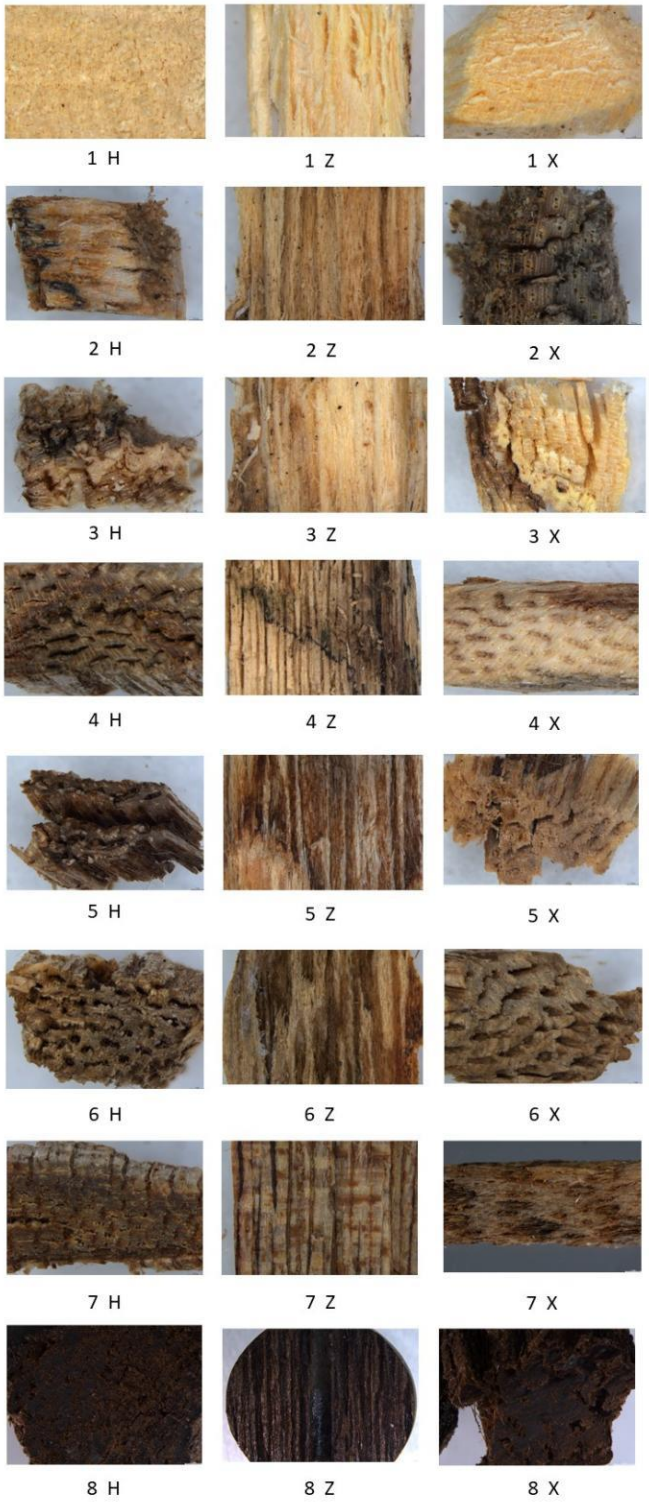

Fig. 2: Macro observation of Aquilaria sinensis wood
1: white wood, 2: PDA, 3: cold drill, 4: insect, 5: Yb-1, 6: Yb-1, insect-borne bacteria, 7: traditional Chinese medicine agarwood, 8: fake agarwood, H: crosscutting, Z: longitudinal cutting, X: string cutting.

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3.2. Ultra-micro section observation

3.2.1. Observation on the structure of agarwood sample

There is no evident growth ring grain on the cross-section of the sample of *A. sinensis* (Fig. 3.1~7). The axial parenchyma is not obvious, showing a ring-shaped structure. The tube hole of the catheter is small, with a diameter of 45~98μm, and its irregular shape is primarily porous. Each tube hole contains several pores, and the wall thickness is typically thin, approximately 3μm, with no infiltrator inside. Wood fibers are polygonal, elongated, and aligned in parallel. Most cells exhibit uniformity in shape and size, but a few displays variation, with an average diameter of approximately 30μm. The phloem is arranged in an island configuration between the double-tube apertures and the clusters of tube holes.

The whole wood tissue of Pseudo-*A. sinensis* is coated with oily substances, and most of the pores are tubular and compound, with an average diameter of 47μm~75μm, and the wall of the pores is about 3μm. There are yellowish. The axial thin-walled tissues show a wing-like structure obviously-brown and irregular infiltrations or gums in the pores. The axial thin-walled tissues show a wing-like structure obviously show a wing-like structure (Fig. 3.8).

In the artificial perfumed sample (Fig. 3.1-6), there is no oily substance in the tube hole of white wood treatment, the wood grain is visible without obvious deformation, and the wood ray width is standard without prominent thickening, thinning, artificial scratches, and drilling (Fig. 3.1). Many yellow-brown oily substance infiltrations gathered in the phloem on the cut surface of other treated wood, and the darker the aroma sample, the more oily substances.

The sample variety of Chinese traditional medicine *A. sinensis* is *A. sinensis*, which has a ring-like tubular structure with a thin axial wall, trim and porous tube holes, a thin tube wall and irregular shape, and long and thin wood fibers arranged in parallel. In the parenchyma of ducts and wood rays, a large number of oily substances invaded the filling body, and the area was large (Fig. 3.1-7).

3.2.2. Ultrastructural observation of true and false agarwood

After calculating the fragrant infected areas of different treatments under the microstructure, the results show obvious noticeable differences (Table 1 and Fig. 4). Among the control samples, the aroma-forming area of argarwood is the best (transverse cutting: 548678.65 μm^2, longitudinal cutting: 1238716.7 μm^2, chord cutting: 1627770.8 μm^2), followed by YB-1 insect-borne infection, insect infection, YB-1 fungal infection, cold drilling, PDA, and finally white wood. The results showed relatively low aroma-forming area of PDA medium and cold drilling treatment. Still, no aroma-forming was found in the white wood treatment. In contrast, the infection area of insect-borne bacteria treatment was significantly higher than that of insect erosion and fungal infection treatment. The comprehensive analysis showed that fungal infection, insect erosion and insect-borne bacteria treatment all accelerated aroma formation, among which the insect-borne bacteria treatment performed best and had the best aroma formation effect.

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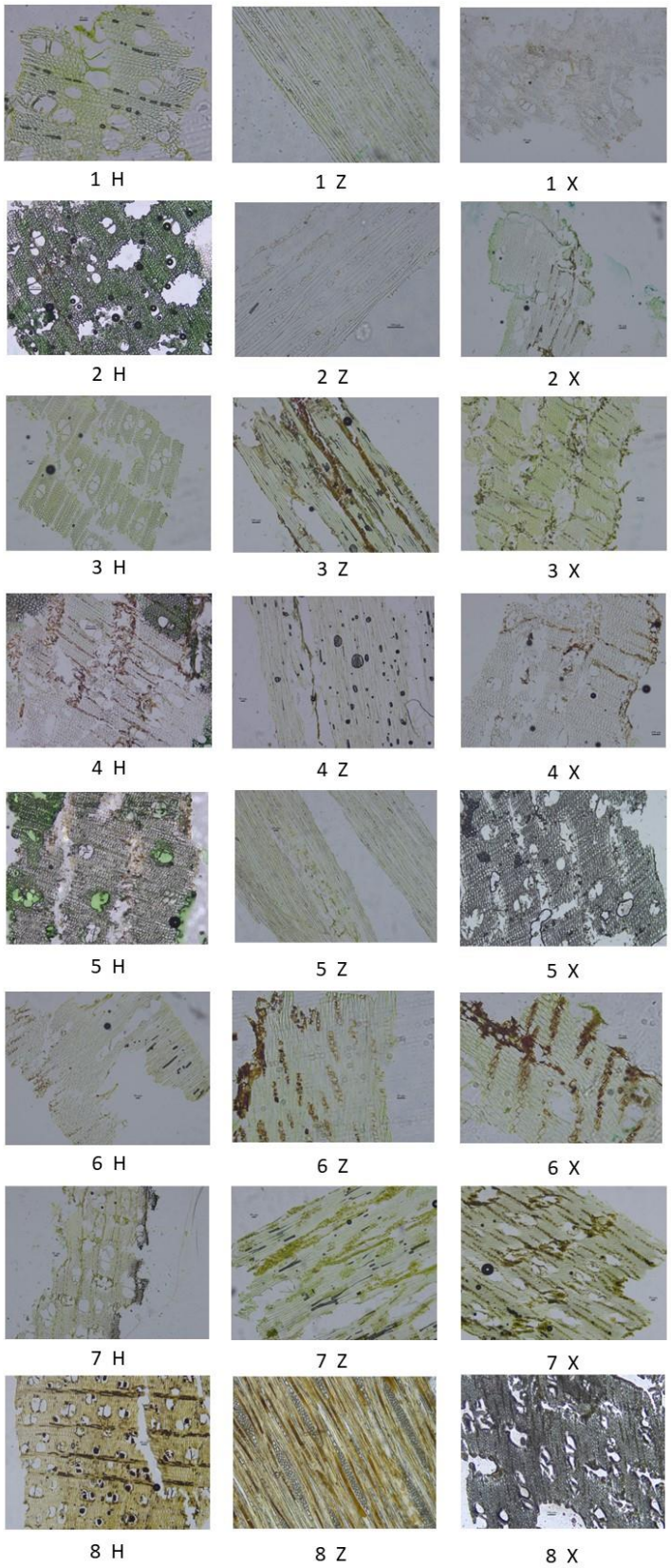

Fig. 3: Ultra-micro section observation of Aquilaria sinensis wood
1: white wood, 2: PDA, 3: cold drill, 4: insect, 5: Yb-1, 6: Yb-1, insect-borne bacteria, 7: traditional Chinese medicine agarwood, 8: fake agarwood, H: crosscutting, Z: longitudinal cutting, X: string cutting.

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Table 1 Aroma-forming area of different treatments under microstructure

project	Cross-cutting fragrant area /μm2	Longitudinal Fragrance-forming Area /μm2	Chord-cut fragrant area /μm2
white wood	0	0	0
PDA	23085.85±55.76f	14463.43±124.91g	27557.3±484.98g
Cold drill	12616.22±54.25g	37514.69±72.88f	72725.44±115.39e
insect	108533.98±440.47d	61303.68±175.36e	280479.73±289.45d
YB-1	58675.55±271.19e	90450.56±249d	54725.67±158.58f
YB-1 insect-borne bacteria	297675.88±237.44b	210492.72±227.59c	303520.88±266.45c
Chinese medicine chenxiang	548678.65±238.65a	1238716.7±223.79a	1627770.8±567.48a
Fake agarwood	196967.69±470.37c	337499.43±170.73b	421736.09±155.54b

Note: Different lowercase letters in the same column indicate significant differences at P<0. 05 level.

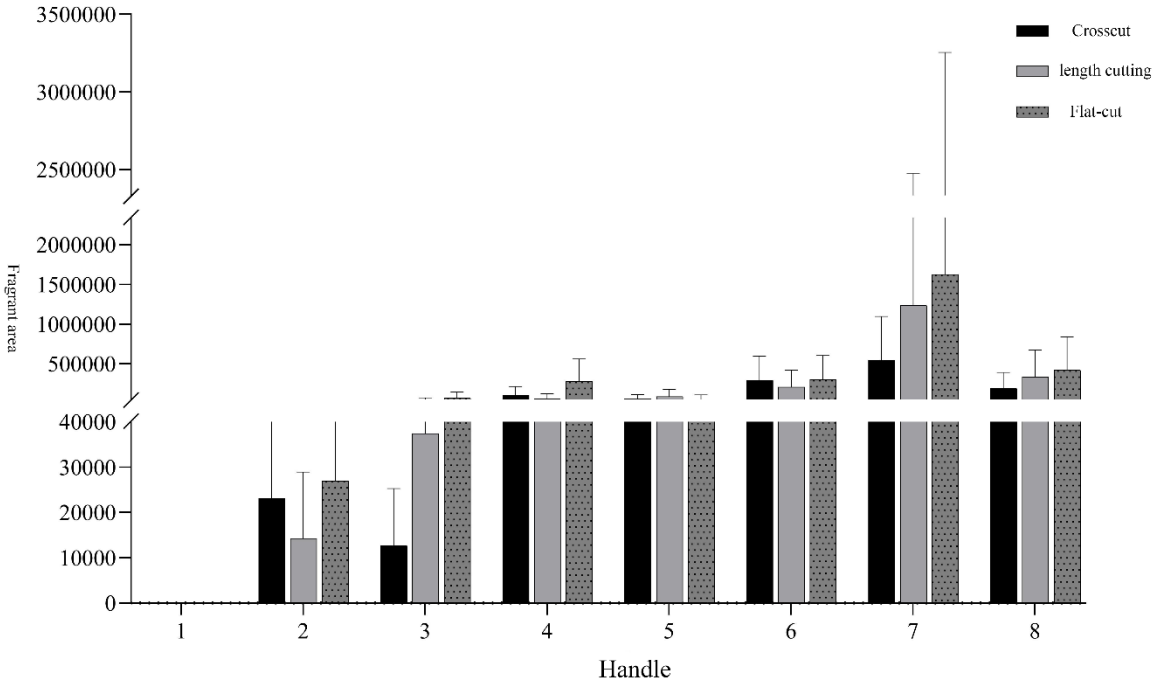

Fig. 4 Microstructure infection area of Aquilaria sinensis wood in different treatments
Note: 1: white wood, 2: PDA, 3: cold drill, 4: insect, 5: YB-1, 6: YB-1, insect-borne bacteria, 7: traditional Chinese medicine agarwood, 8: fake agarwood.

4. Discussion

Observing and comparing the characteristics of *A. sinensis*, such as texture, color, fragrance, surface covering, the trace of essential oil and section structure, help us to consider that *A. sinensis* belongs to the porous material, with relatively soft texture, large pore diameter, thin pore wall, inconspicuous axial parenchyma, and abundant phloem. It can't be fragrant, and only after being injured or stressed by the outside world. There are agarwood substances deposited in phloem and wood ray parenchyma cells, showing dark brown, and evident medicinal fragrance. This is because the accumulation of agarwood is closely related to the strength of the catheter transport function [28]. The degradation and dissolution of starch in cells are caused

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by external damage to the tree [29,30]. The transportation of organic matter is blocked, affecting wound repair and agarwood deposition. At the same time, the wood is weakly acidic, and its humid environment and rich carbohydrates can create conditions for the growth and reproduction of fungi to accelerate fragrance [31,32]. All kinds of artificial aroma-forming treatments show the effect of accelerating aroma-forming, and the "insect-carrying bacteria" combined with fungal infection and insect erosion has been proven to be the best artificial aroma-forming method.

These methods are all biological methods, and their main advantage is the ability to continuously produce agarwood [33]. In addition, the biological method is faster than the physical method, and the safety is higher than the chemical method, and the sesquiterpenes produced are more concentrated and the concentration is higher [31,34]. Studies have shown that after 3 weeks of Fusari-um oxysporum infection, the structure and chemical composition of agarwood are similar to those of wild agarwood, and the content of alcohol-soluble extract, pigment and essential oil is higher [35]. In summary, the effective content of agarwood formed by artificial agarwood is higher. Therefore, a comprehensive and multi-dimensional exploration of the molecular mechanism of agarwood and the development of efficient, high-yield and stable artificial aroma technology not only provide raw materials for the medicinal, aromatic and collection of agarwood industry, but also provide a basis for identifying the authenticity of agarwood. Although the biological induction method has many advantages, the effect of incense formation is also affected by internal and external factors such as climate, soil microorganisms, pests and diseases, and plants in the environment. Existing studies have also shown that biological induction of incense formation is unstable [36].

There are still some things that could be improved in future studies: the characteristics of *A. sinensis* may particular due to certain specific variations and overlap in other places of origin, habitats, and varieties. In the future, by analyzing the differences in the internal chemical components of *A. sinensis*, a reliable method for screening and identifying these different components can be established to quickly classify the provenance, quality control and quality of *A. sinensis*.

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