Preliminary observation of *Aquilaria crassna* wood associated with the formation of aloeswood

Tadashi NOBUCHI and Somkid SIRIPATANADILOK

Aquilaria crassna 材における沈香形成過程の予備的観察

野渕　正，ソムキット・シリパタナディロック

Résumé

Preliminary observation of *Aquilaria crassna* wood related with the formation of aloeswood was carried out. Investigations of the anatomy of this species and the changes of living cells in aging process were first tried. Cytological changes of living parenchyma cells in sapwood following wounding on the trunk were then observed associated with aloeswood formation. Results are summarized as follows. (1) The sample species had included phloem as the other species of the genus *Aquilaria*. The parenchyma cells constituting included phloem were considered to play important roles for aloeswood formation together with the parenchyma cells of xylem. (2) The main reserve substance in parenchyma cells was starch and the amount of lipids was less. In the aging process from cambium to pith starch gradually decreased and lipids slightly increased. The sapwood width of the species was considered to be very wide. (3) Following the days after wounding starch grains showed abrupt decrease. In the next stage living parenchyma cells showed conspicuous vacuolization. (4) In vacuolization process some vacuoles had electron dense substances. Cytoplasmic matrix also showed electron dense condition. (5) Electron dense substances migrated from living cells to neighboring wood fibers. These substances were postulated to be strongly related with the components of aloeswood.

要　　旨

香木である沈香の形成について，*Aquilaria crassna* を用いて予備的観察を行った。すなわち，供試樹種の組織構造的特徴およびエイジングに伴う柔組織生活細胞の変化をまず調べ，その後人為的傷害に対して辺材部柔細胞に生ずる変化を沈香形成と関連するものとして細胞学的に観察した。得られた結果は以下の通りである。（1）供試樹種は *Aquilaria* 属の他の樹種と同様に材内師部を持っていた。材内師部を構成する柔細胞は，木部の柔細胞と同様に，沈香形成に強くかかわっていると推定された。（2）生活柔細胞内の貯蔵物質は主としてデンプンで，脂質はごく小量含まれているにすぎなかった。形成層から内方へのエイジングに伴い，デンプンは徐々に減少し，

脂質はわずかに増加した。供試樹種の辺材幅は極めて広いと判断される。（3）傷害後の時間的経過を追って生活柔細胞の変化を調べたところ，まずデンプンの減少が見られた。次に柔細胞内は顕著に液胞化を示した。（4）液胞化の過程で高電子密度物質を含む液胞が出現し，また，平行して細胞質基質も高電子密度となった。（5）その後高電子密度物質は生活柔細胞から周囲の木部繊維等へと移動した。これらの物質が沈香成分と強くかかわっていると推定される。

1. Introduction

Aloeswood or agarwood used as an incense wood is formed in the trunk of *Aquilaria* trees which range in tropical Asia. The main components of it are reported to be oleoresin[1]. Some scientists[2,3] reported that the formation of aloeswood was caused by invasion of fungi, and others[4,5] considered the main cause was not fungi but mechanical wounding. Recently Siripatanadilok and others[6] also investigated the process of aloeswood formation after treating with pesticide and denied the fungal effect for aloeswood formation. The mechanism of aloeswood formation, however, has been obscure.

In this report, therefore, cytological observation of the process of aloeswood formation has been carried out to get the preliminary information. *Aquilaria crassna* which is widely distributed in Thailand was selected as a sample tree species. Anatomical characteristics and cytological features in the aging process were first investigated to get the fundamental information, and second cytological characteristics of living parenchyma cells which constituted xylem at the time of wounding were observed following the days after wounding.

The authors express their sincere thanks to the personnel of Faculty of Forestry, Kasetsart University for their cooperation in collecting samples. A part of this study was supported by the Kajima Foundation's Research Grant.

2. Materials and methods

2. 1. Materials

Two trees of *A. crassna* which grew in the natural forest of eastern Thailand and two young trees grown at the nursery of Kasetsart University were used as sample trees. Description of sample trees is shown in Table 1.

Table 1. Description of sample trees*

Tree	Location	D.B.H. (cm)	Time of experiment
A	Natural Forest of eastern Thailand	40	Oct. 2, 1990
B		40	Oct.-Nov., 1985
C	Nursery of Kasetsart University (Thailand)	5	Apr.-May, 1991
D		5	Apr.-May, 1991

* The age of all sample trees was unknown

From tree A wood blocks were collected to investigate the anatomy and characteristics of aging process. In tree B mechanical injury was inflicted with a chisel on the trunk where bark and outer sapwood, about 2cm deep from surface, were cut off. Wood blocks 3cm long, 2cm wide (tangential), and 2cm thick (radial) were collected from upper and lower parts of the injury following the days after wounding; that is 2, 4, 14, and 30 days.

From the tree C and D wood blocks were also collected to investigate the relationship between mechanical wounding and aloeswood formation. Wounding and collecting of wood block were basically the same as tree B. The days after wounding were 1, 7, 30 days. The season was rainy season in the experiment of tree A and B and was dry season in that of tree C and D.

In addition to the wood blocks mentioned above aloeswood used as an incense wood was also investigated.

In wounding experiment the surface of the mechanical injury was covered with a pesticide, Santar SM (Sandoz Ltd., Switzerland) immediately after wounding to prevent the invasion of microorganisms.

2. 2. Methods

Wood blocks collected from living trees were immediately fixed with 3% glutaraldehyde.

20 μm thick sections cut from the fixed block were observed after following staining: safranin-light green, I_2-KI, Sudan IV, Sudan black, O_sO_4 and Nitroso reaction[7]. A part of blocks was embedded in celloidin mainly for the anatomical observation.

Ultrathin sectioning was also carried out. Small wood blocks cut from glutaraldehyde-fixed block were post fixed with 1% O_sO_4 and embedded in Epon 812. Ultrathin sections post stained with uranyl acetate and lead citrate were observed under a transmission electron microscope (JEM-100C, 100kV).

For scanning electron microscopy air-dried wood blocks were observed under a scanning electron microscope (JSM-T330A, 10kV) after Au coating.

3. Results and discussion

3. 1. Anatomy of Aquilaria crassna

Transverse, radial and tangential sections of *A. crassna* are shown in Fig. 1. One of the characteristics of this species was the existence of included phloem (foraminate type) as the other species of genus *Aquilaria*. This was confirmed by light microscopy and scanning electron microscopy on sieve tube members (Fig. 2).

In addition to sieve tube members cellular elements constituting included phloem were phloem parenchyma cells (axial), phloem ray parenchyma cells and phloem fibers. Conjunctive tissue was observed associated with included phloem. A phloem ray parenchyma was connected with a xylem ray parenchyma. The cell wall of the former, however, was thinner than that of the latter and was unlignified. In this experiment the comparision of the anatomy between included phloem and normal phloem in bark was not carried out.

Elements constituting xylem part were vessel elements, ray parenchyma cells and wood fibers. Scanty paratracheal parenchyma cells were also observed. Ray parenchyma showed uniseriate

(Fig. 1c) and heterogeneous (Fig. 1b). Wood fibers had many pits (Fig. 13) and they showed bordered pits (Fig. 11). The wood fiber of *A. crassna*, therefore, is considered to be fiber tracheid.

In xylem tissue of sample trees, wood fibers particularly included much amount of air or gas. This was a very feature of this species different from tree species of temperate or warm temperate zones which the authors have observed. The feature of having much amount of air or air bubbles was observed both in tree A and B (sampled in rainy season) and tree C and D (sampled in dry season). Although the physiological roles of the air were not clarified in this experiment it will be one of the important factors which should be considered when investigate the formation of aloeswood.

3. 2. Cytological changes of living parenchyma cells in the aging process from cambium to pith.

Living parenchyma cells are the most important elements concerning aloeswood formation because they are the only cells that are able to biosynthesize components of aloeswood. The characteristics of cell contents of parenchyma cells and their changes in aging process were investigated. Because the differences of cytological features among xylem ray parenchyma, phloem ray parenhcyma and phloem parenchyma in included phloem were not found out, following results are mentioned as the common features among them.

Figure 3 shows a radial section of outer sapwood stained with I_2-KI. The main reserve substance was starch. The amount of lipids in outer sapwood was very little and they showed very small droplets (Fig. 4). When lipid droplets were compared between xylem and phloem parenchyma they were slightly more in the latter.

Next is the characteristics of parenchyma cell contents in inner sapwood. By the way, the length of wood cores taken with an increment borer from tree A was about 14cm which included cambium to inner xylem. The inner sapwood in this report, therefore, means the part of about 14cm from the cambium.

Figure 3b shows starch grains in inner sapwood. It is clear that the amount of starch slightly decreased and that of lipids slightly increased (Fig. 4b). The characteristics that starch decreased and lipids increased following aging are the same tendency reported for the trees of temperate or warm temperate zones[8,9].

The tree investigated, therefore, had a very wide sapwood. According to the book "Commercial Timbers of India"[10] *Aquilaria* was reported to want heartwood. The authors, however, believe that trees, at least those of large size, have the heartwood based on the definition of IAWA[11]. Although the characteristics of heartwood of *A. crassna* were not investigated, this species is postulated to have wide sapwood and pale or non-colored heartwood based on the report of Nobuchi and others[12] in which the relationship between sapwood width and the characteristics of heartwood was investigated.

3. 3. Preliminary observation of the relationship between the changes of living parenchyma cells following wounding and aloeswood formation

In this experiment the changes of living parenchyma cells near injury (5-10mm from the cut

end) were mainly observed.

As mentioned above living cells investigated were xylem and phloem ray parenchyma cells, and phloem parenchyma cells. However, the differences of cytological features among them were not found out so far as observed. The following results are the common characteristics observed among them.

Following the days after wounding discoloration occurred near the injury (Fig. 5). When compared the upper part with the lower the length of discoloration showed slightly longer in the lower part. This tendency is the same result of Nobuchi and others[13].

The first typical change after wounding was the decrease of starch. Differences among sample trees, however, were observed. In tree B almost all starch grains were disappeared from living cells in the sections of 4 days after wounding while small amount of starch still existed in the living cells of 7 days after wounding in tree C and D. The main cause for this difference was considered to be the seasonal factors. It was rainy season in the experiment of tree B and dry season in tree C and D. In rainy season active transportation of assimilates and water conduction might occur though the exact seasonal fluctuation of physiological factors in tropical trees is still under discussion.

The second typical change observed was vacuolization. A transmission electron micrograph of a xylem ray parenchyma cell of 7 days after wounding (tree C) is shown in Fig. 6. As mentioned above the amount of starch decreased in 7 days of tree C and vacuoles occupied the space in a cell. Vacuolization became marked following the days after wounding. Following vacuolization cytoplasmic matrix tended to be osmiophilic observed under a transmission electron microscope.

In addition to the decrease of starch and vacuolization yellowish brown droplets in unstained sections were observed under a light microscope (Fig. 7). These droplets existed in parenchyma cells of 14 days and thereafter in tree B, and in that of 30 days in tree C and D. This feature indicates that yellowish brown droplets appeared after the disappearance of starch grains.

Some histochemical observations were carried out for living cells. Yellowish brown droplets in unstained sections showed color reaction with Sudan IV, O_sO_4 and Nitroso reaction[7], but didn't with Sudan black. Figure 8 shows an example which was stained with O_sO_4 (14 days after wounding, tree B). Cytoplasmic matrix, on the other hand, showed strong reaction with Sudan black (Fig. 9), but it didn't show strong reaction with O_sO_4, Sudan IV and Nitroso reaction.

From histochemical observations it was considered that yellowish brown droplets had phenolic substances in a wide sense rather than lipids because they reacted on O_sO_4 and showed Nitroso reaction. A preliminary investigation of them by a spectrophotometer (Zeiss, UMSP-80) revealed that they had the strong absorption in the range between 270-290nm. This also supports that the droplets contain phenolic substances.

Transmission electron microscopy on the cells in which droplets developed was carried out. Figure 10 shows one example of a phloem ray parenchyma cell. A droplet in light microscopy was revealed to be a vacuole which had very electron opaque or stronger osmiophilic substances than cytoplasmic matrix. It should be noticeable that vacuoles with very electron opaque sub-

stances were not always observed in every living cell.

In the process of vacuolization osmiophilic materials migrated from living cells to wood fibers (Fig. 11). This process is considered to be very important step for aloeswood formation.

Light and scanning electron microscopy on aloeswood used as an incense wood were carried out. Figure 12 shows a light micrograph and Fig. 13 a scanning electron micrograph of an incense wood, together with that of control from wood not wounded. In Fig. 12 much amount of yellowish brown materials was observed in parenchyma cells. They were also observed in wood fibers and vessel elements. From Fig. 12 it is clear that wood fibers included much amount of these materials which were considered to be the main components of aloeswood. From light microscopy yellowish brown materials observed in aloeswood resemble yellowish brown droplets in living parenchyma cells formed after wounding. As shown in Fig. 11 materials inside wood fibers are considered to have migrated from living cells. It is, however, uncertain that materials migrating from living cells to wood fibers are related with osmiophilic substances in vacuoles and/or substances in cytoplasmic matrix. According to the cytological study of biosynthetic process of phenolic substances following wounding in Japanese cedar phenolic substances mainly existed in cytoplasmic matrix from where they migrated to neighboring tracheids[14]. Component of cytoplasmic matrix is one of the important objectives associated with aloeswood formation.

From the preliminary observation mentioned above it has become clear that living parenchyma cells showed abrupt changes in sapwood after mechanical wounding. The main changes were the decrease and disappearance of starch, and vacuolization occurred parallel with it. The vacuolization is an important indicator of aging or necrosis. In the process of vacuolization accumulation of osmiophilic substances in vacuoles and changes of cytoplasmic matrix to osmiophilic condition occurred, which are considered to be closely related with the aloeswood formation. Fungi or hyphae were not observed in sapwood tissues so far as observed in this experiment.

In conclusion the authors propose following point for future research works: The relationship between cytological features and aloeswood components should be investigated including microscopic spectrophotometry.

References

1) VERMA, K. R., MAHESHWARI, M. L. and BHATTACHARYYA, S. C.: Terpenoids-LXII-The constitution of agarospirol, a sesquiturpenoid with a new skeleton. Tetrahedron, **21**, 115-138, 1965
2) BOSE, S. R.: The nature of "Agaru" formation. Sci. Cul., **4**, 89-91, 1938
3) BARUAH, J. N., MATHUR, R. K., JAIN, S. M. and KATAK, J. C. S.: Cultivation and utilization of aromatic plants. Actal, C. and Kapur. B. M. ed., Regional Research Laboratory, Council of Science and Industrial Research, Jammu-Tawi, India, 1982, 662-667.
4) RAHMAN, M. A. and BASAK, A. C.: Agar production in agar tree by artificial inoculation and wounding. Bano Biggyan Patrika, **9**, 87-93, 1980
5) RAHMAN, M. A. and KHISA, S. K.: Agar production in agar tree by artificial inoculation and wounding II. Further evidence in favor of agar formation. Bano Biggyan Patrika, **12**, 57-63, 1984
6) SIRIPATANADILOK, S., CHALERMPONGSE, A. and SANGTHONGPRAOW, S.: Utilization and propagation of agarwood trees (*Aquilaria spp.*), Final report of IFS Research Grant, 1-77, 1991
7) REEVE, R. M.: Histochemical tests for polyphenols in plant tissues. Stain Tech., **26**, 91-96, 1951
8) FREY-WYSSLING, A. and BOSSHARD, H. H.: Cytology of ray cells in sapwood and heartwood. Holz-

forschung, **13**, 129-137, 1959

9) NOBUCHI, T., KURODA, K., IWATA, R. and HARADA, H.: Cytological study of the seasonal features of heartwood formation of sugi (*Cryptomeria japonica* D. Don). Mokuzai Gakkaishi, **28**, 669-676, 1982

10) PEARSON, R. S. and BROWN, H. P.: Commercial timbers of India, Vol. II, A. J. Reprints Agency, 1981, 858-862.

11) INT. ASSOC. WOOD ANAT.: International Glossary of Terms used in Wood Anatomist. Trop. Woods, No. 107, 1-36, 1957

12) NOBUCHI, T., TOKUCHI, N. and HARADA, H.: Variability of heartwood formation and cytological features in broad-leaved trees. Mokuzai Gakkaishi, **33**, 596-604, 1987

13) NOBUCHI, T., AKAMATSU, Y., SATO, K. and HARADA, H.: Early response of ray parenchyma cells following wounding in sugi (*Cryptomeria japonica* D. Don) wood.-Seasonal changes of discoloration and cytological structure-. Bull. Kyoto Univ. Forests, No. 57, 290-299, 1986

14) NOBUCHI, T., NAGASHIMA, K. and SAIKI, H.: Cytological study of biosynthetic process of phenolic substances following wounding in Japanese cedar (Jap.). Proc. the Japan Wood Research Soc., 458, 1991

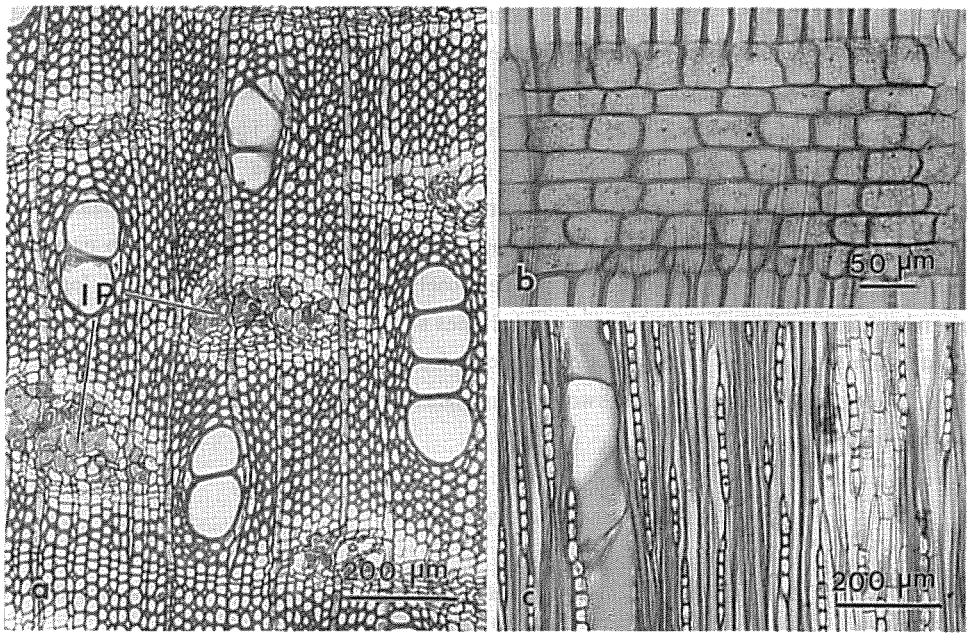

Fig. 1 Light micrographs showing anatomy of *Aquilaria crassna*. (a) a cross section, (b) a radial section, (c) a tangential section. IP: included phloem.

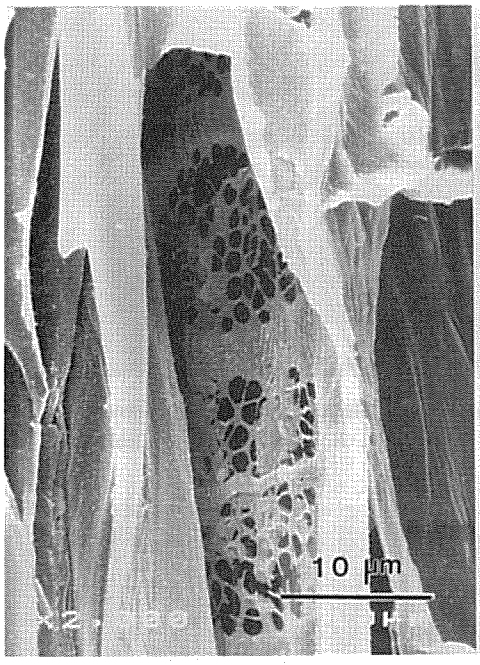

Fig. 2 A scanning electron micrograpgh showing a sieve plate between sieve tube members.

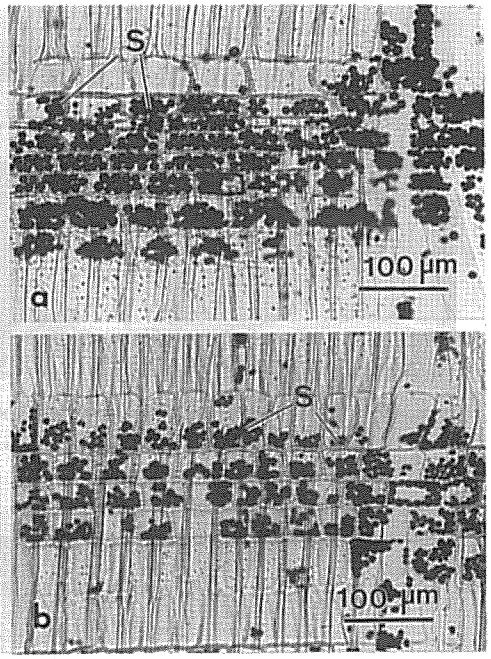

Fig. 3 Radial sections showing starch grains (S) stained with I_2-KI in (a) outer sapwood and (b) inner sapwood.

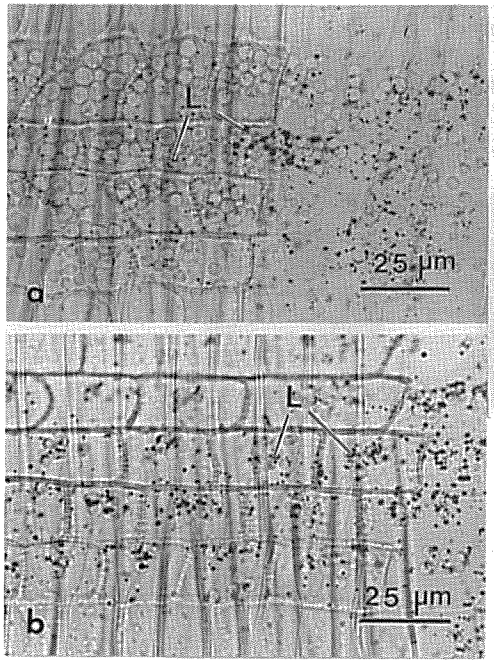

Fig. 4 Radial sections showing lipids (L) stained with Sudan IV in (a) outer sapwood and (b) inner sapwood.

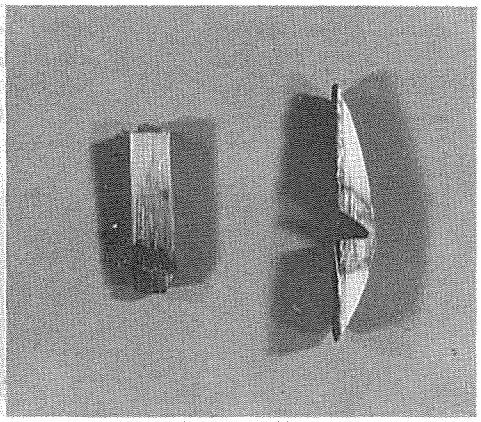

Fig. 5 Wood blocks showing discoloration following wounding. The left block shows dark discoloration in very long time after wounding and the right indicates pale colored in 30 days after wounding (tree C).

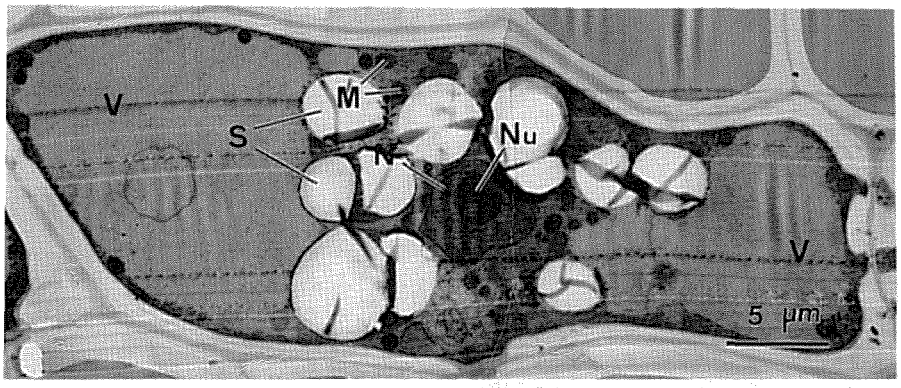

Fig. 6 A transmission electron micrograph showing vacuolization of a xylem parenchyma cell (7 days after wounding, tree C). V: vacuole, N: nucleus, Nu: nucleolus, M: mitochondrion.

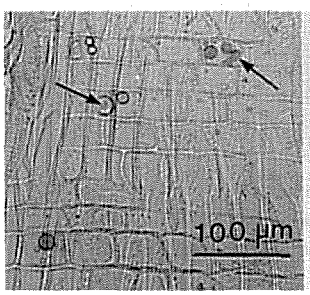

Fig. 7 A light micrograph of unstained section showing yellowish brown droplets (arrows) (7 days after wounding, tree C).

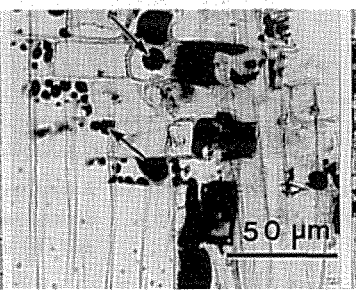

Fig. 8 A light micrograph of a radial section stained with O_sO_4. Arrows indicate droplets reacted on O_sO_4 (30 days after wounding, tree C).

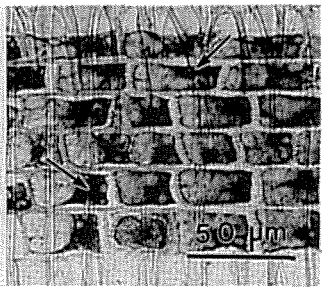

Fig. 9 A light micrograph of a radial section stained with Sudan black. Cytoplasmic matrix (arrows) shows dark color (30 days after wounding, tree C).

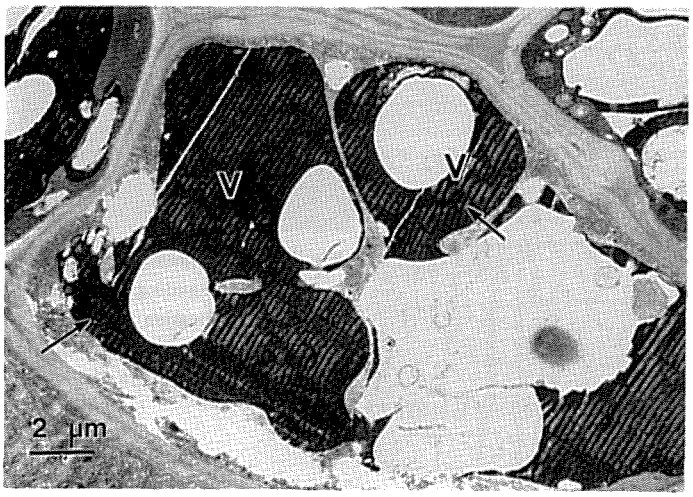

Fig. 10 A transmission electron micrograph showing a ray parenchyma cell of phloem in which osmiophilic substances (arrows) accumulated in vacuoles (30 days after wounding, tree C).

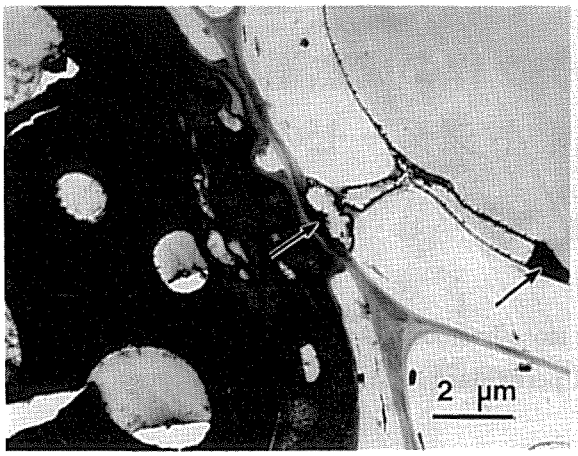

Fig. 11 A transmission electron micrograph showing the migration of electron dense substances (arrows) from a ray parenchyma cell to a neighboring wood fiber (30 days after wounding, tree C).

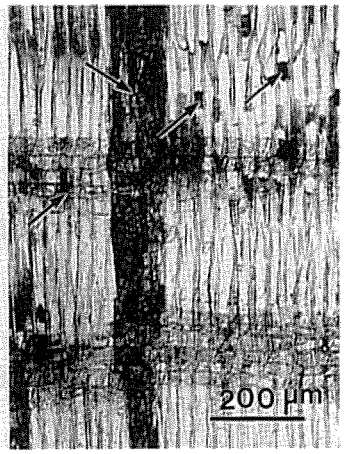

Fig. 12 A radial section cut from aloeswood (an incense wood sample) showing yellowish brown substances in axial and ray parenchyma cells and wood fibers (arrows).

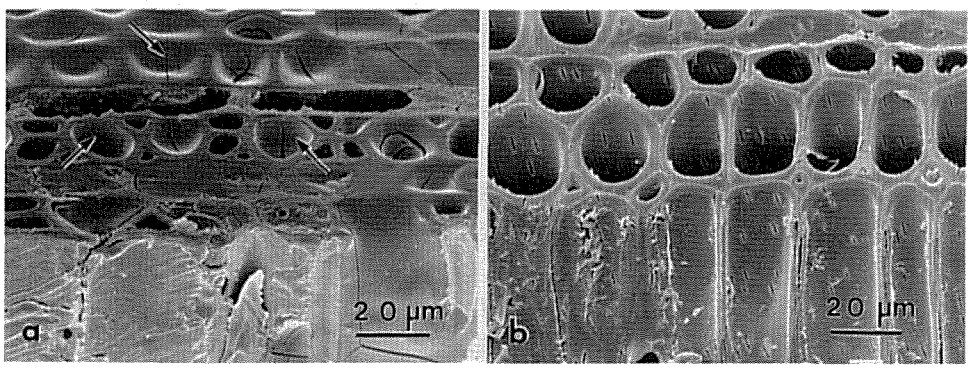

Fig. 13 Scanning electron micrographs showing (a) aloeswood (an incense wood sample) where wood fibers have much amount of substances (arrows). (b) Control taken from a trunk without wounding.