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Vessel Network Tracing by Wire Insertion and Pigment Injection

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ワイヤー挿入および染料注入による道管ネットワークの追跡

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Résumé

Vessels, which characterize the hardwood, are too long to be traced along their longitudinal direction. The actual three-dimensional analysis is very troublesome procedure, although some methods such as serial sections have been tried. New methods, that is, insertion of thin wire and injection of pigments into vessel lumens, were proposed for the quantitative analysis of the network. By these methods, vessels could be identified one by one on both ends which were cut transversely at the distance of 5 cm or longer.

Wire insertion was fit to investigate the specimen of which vessels were large in diameter and relatively straight, and applicable to not only sapwood but heartwood vessels which were generally plugged by tyloses. On the contrary, pigment injection was very effective to the smaller vessels, to say nothing of larger ones. However, the plugged vessels which were common in the heartwood were impossible to be injected by pigments. Pigment injection was also shown to be very useful when it was combined with the resin casting. The troublesome procedures of three-dimensional analysis on the vessel network are expected to be improved dramatically by these methods.

和 文 要 旨

広葉樹に特徴的な組織である道管は，軸方向に非常に長いため追跡するのが困難である。これまで，その3次元的な解析には連続切片の作製など，非常に手間のかかる手法がとられてきた。本研究では，道管の内腔に細いワイヤーを挿入する手法と，染料を注入する手法により道管のネットワークを定量的に解析することを試みた。これらの方法では，軸方向に5 cm以上離れた両木口面での道管の位置を一本一本追跡できた。

ワイヤー挿入は径の大きく，比較的通直な道管についての調査に適している。この方法は，辺材部だけではなく，一般にチロースにより閉塞されている心材部の道管の解析にも適用できる。一方，染料の注入は大きい径の道管は言うまでもなく，環孔材の晩材部の道管のように径の小さな道管にも有効である。しかし，ワイヤーの挿入とは異なり，チロースによって閉塞された道管の追跡は不可能である。染料の注入は，樹脂鋳型法を併用することによりさらに有効なものとなる。

これらの手法を基に，道管ネットワークの３次元解析についてこれまで行われてきた非常に手間のかかる手法が，大きく改善されることが期待される。

INTRODUCTION

The vessel consists of individual vessel elements lined up end to end. The structure of the vessel element has been clarified by microscopic examinations. But three-dimensional vessel distribution has been still unknown clearly, because the vessels, which are small in diameter and long, are difficult to be traced in longitudinal direction by the microscope.

Three-dimensional vessel network have been analyzed by a series of transverse sections[1)2)] and cinematographic methods[3)4)]. Kucěra and Bosshard measured the structure quantitatively[5)6)]. But these reconstructed figures were 1-5 cm in longitudinal length. The length seem to be not enough to discuss vessel distribution. Although the resin casting method developed by Fujii[7)] is a fine method for visualizing vessels and their connection with other tissues under a scanning microscope, surveying on wide areas may be difficult. In this paper we tried wire insertion and pigment injection as new methods and will discuss results by these methods.

MATERIALS AND METHODS

Wood Specimens

Larger vessels are considered to be surely applied by wire insertion and pigment injection, so some ring-porous woods such as *Castanea crenata, Quercus serrata, Paulownia tomentosa* were used, which had large pores of 100-400 μm in diameter at their earlywood. For the diffuse-porous woods, *Juglans sieboldiana* and *Eucalyptus tereticornis* were used, which had relatively large vessels of about 150 μm. Although wood specimen of 30 cm long in *Q. serrata* was traced by both methods, other species (*C. crenata, P. tomentosa, J. sieboldiana and E. tereticornis*), which were smoothed or sectioned by a sliding microtome, were offered to the further experiments.

Heartwood samples of *C. crenata, J. sieboldiana* and *P. tomentosa*, and fresh samples of *P. tomentosa* and *E. tereticornis* taken from a living tree were cut about 5 cm in their longitudinal direction. In the latter blocks the polarity from the apex to the root was recorded. One radial surface was smoothed along their wood grain by a sliding microtome and then their both transverse ends were sectioned. Sections of 50 μm thick from these ends were treated by the ordinary preparation for the microscopy. Vessel distribution on both cut ends were recorded on photographs from these sections. The positions of each vessel were got by using personal computer and Luzex Ⅲ.

Wire Insertion

Wire of 0.2 mm in diameter (copper), 0.1 mm (platinum or tungsten) and 0.05 mm (tungsten) were cut appropriately. Wire with proper thickness and flexibility was selected and inserted into a vessel lumen under a binocular. When wire was stopped up on the way

to the opposite end, it was pulled up a little and pushed again. After repeating the procedures carefully, wire was passed through the lumen. The positions of each vessel at both ends were marked on photographs of both transverse ends. Succeedingly, wire was inserted reversely from the exit end to the entrance one to confirm their correspondence. Larger vessels at the beginning of an annual ring were examined first and then tried to smaller ones. The number of vessels examined was about 60 per one annual ring.

Internal Inspection of Specimen Blocks Inserted with Several Pieces of Wire

The wood blocks inserted with several pieces of wire were examined by the transmission of soft X-rays, because the distribution of vessels was examined only on the surfaces of wood block in wire insertion. Blocks of about 5 mm thick in radial direction were trimmed, and put on the imaging plate (Fujix BAS3000 IP-BAS UR by Fuji Photo Film Co., Ltd.) in the Softex chamber (7.5 kV, 4 mA, 30 sec. exposure)(Softex-CMR by Softex Co., Ltd.). The imaging plate has pixel areas of 50 μm square and very wide dynamic range for the gray level than the ordinary photographic films[8]. Soft X-ray figures of the wood blocks recorded on the plate were read by R-AXIS system (Rigaku Co., Ltd.) and displayed on CRT.

Pigment Injection

Drawing inks of red, orange, yellow, green, blue and black from Rotring color ink series were prepared in syringes. On the other hand, a setting pin was sharpened by sand paper and curved at the point (Fig. 1). One cut end of specimen blocks which was smoothed by a microtome was sealed by the transparent cellophane tape. Under a binocular the film was made holes on the vessels by sticking the pin into vessel lumens. In the diffuse-porous wood or the latewood, where each vessel was difficult to be holed, the film was opened by a razor blade at small rectangular areas or narrow lines along radial or tangential direction.

A small droplet of pigment was put on the hole or the open area by a syringe. Pigment was added again when it was sucked into vessel lumens. The wood block was several times reversed and observed under a binocular. When the pigment appeared on the vessels at the other cut end, the pigment supply was stopped. The position of each vessel at both ends was marked on photographs in the same way as in the wire insertion. After the injection of one pigment, the film was pealed off and

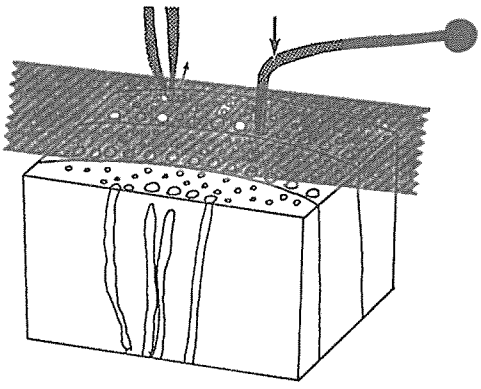

Fig. 1 A schematic illustration for the pigment injection. The surface of a specimen block was covered by a cellophane tape and then opened on the vessels, being made holes by a point of a pin or cut off by a razor blade. A small droplet of pigments was put on the opened pores.

specimen block were dried in an oven at 60 ℃. Then, new film was pushed again on the surface and bored at another vessels. Another pigment injection was repeated.

Internal Inspection of Specimen Blocks Injected by Pigments

The vessel network in the specimen blocks was examined by two methods. One was the saw-cutting of injected block every 10 mm length and the cut ends were smoothed by a razor or a microtome knife. Vessels marked by various colored pigments were traced on the surface of cut ends using a binocular or a video-microscope (OVM-1000N by Olympus Optical Co., Ltd.).

The other method was the application of the resin casting. The specimen blocks were soaked in styrene monomer containing 0.1 % azobis-isobutyronitrile and polymerized at 60℃. The embedded block was treated by sodium hypo-chlorite and sulfuric acid solutions to remove lignin and polysaccharides, and one of its tangential surfaces was opened. Resin castings of vessel lumens which were pigmentized were observed by a binocular or a video-microscope.

RESULTS AND DISCUSSIONS

Wire Insertion

Almost all vessels at the earlywood in *C. crenata* and in *J. sieboldiana* could be inserted with proper wires. In using the tungsten wire of 50 μm in diameter, we could insert it into vessels as small as 80 μm. As we could pass the wire through the *Q. serrata* wood having 30 cm long, this method will be possible to be carried out at longer distance. Tyloses plugging vessel lumens were not obstacle to the procedure. On the other hand in *P. tomentosa*, which has vessels with large diameter, wire was considered to break through the vessel wall, for it sticked out from another tissue on the exit surface. It might be the reason that the vessel walls are relatively thin and the confluent parenchyma is developed remarkably. Therefore *P. tomentosa* could not be investigated by this methods.

The following descriptions are data and its interpretation in *C. crenata* and *J. sieboldiana* by this method.

Internal Inspection of Specimen Blocks Inserted with Several Pieces of Wire

Fig. 2 shows the result of transmission of soft X-rays obtained from *C. crenata*. This pattern is not so clear because the gray level is not even and white striations indicate not only the inserted wire but also high density part of the wood. But one can surely distinguish the former from the latter, because the former has a clear outline (arrowheads in Fig. 2B) and the latter unclear. Even the wire of 0.05 mm in diameter can be distinguished more clearly in Fig. 2B. Although the resolution of the imaging plate is generally said to be coarser than the reading slit area (50×50μm in this case), the tungsten wire of 0.05 mm, that is 50 μm in diameter, could be traced certainly. By the transmission of soft X-rays we found the course of the vessels was relatively straight in the range of 5 cm longitudinal distance.

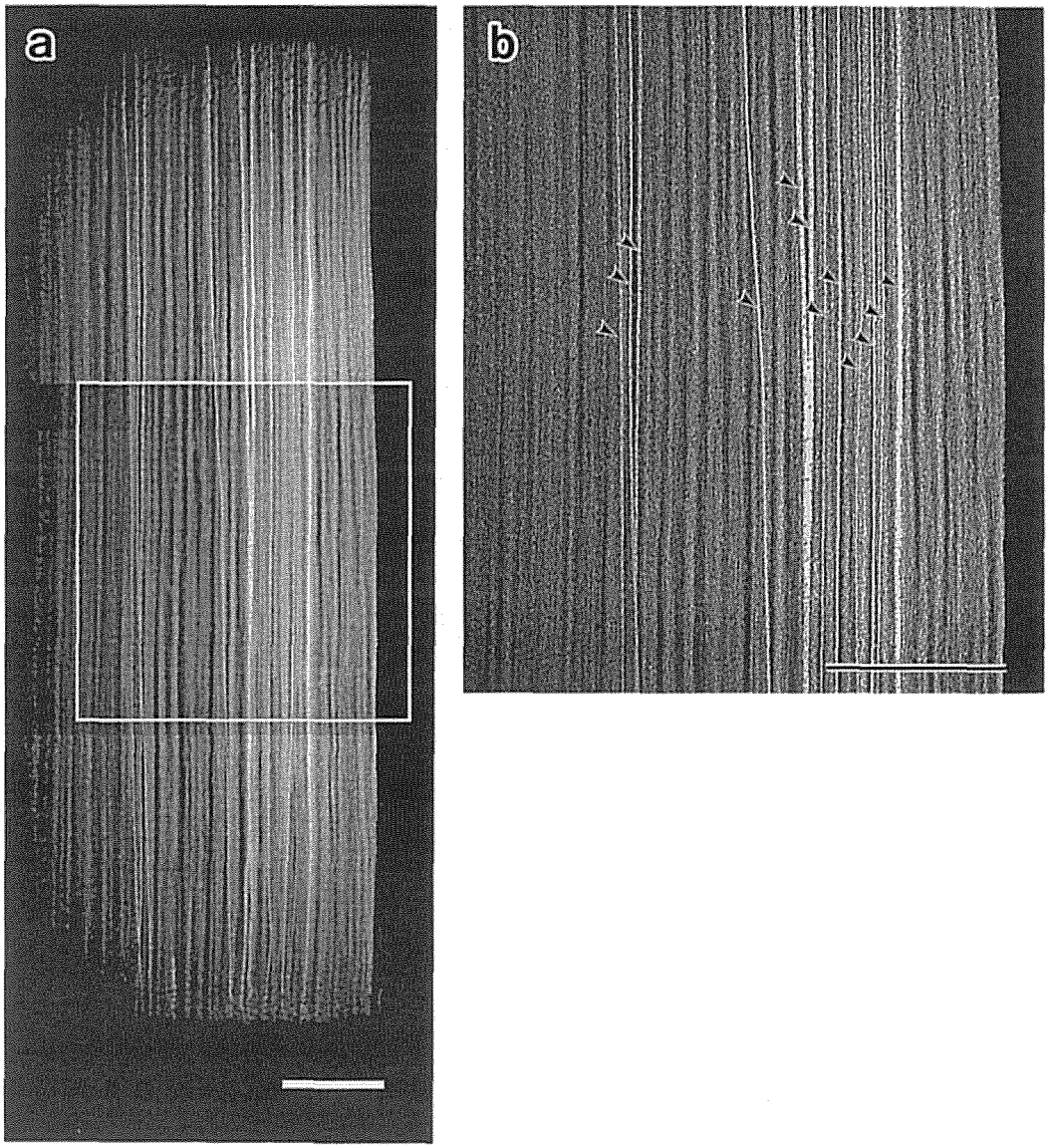

Fig. 2 Sofx X-ray figures for the block of *Castanea crenata* which has 5 mm thick. a: Whole figure of specimen. b: Magnified figure of box of a. Arrowheads point out the location of inserted wire. There are 3 kinds of wire (0.2 mm, 0.1 mm and 0.05 mm in diameter). The number of inserted wire is 12. Bars: 5 mm.

The Position of Each Vessel at Both Cut Ends

The reliable standard must be applied to the quantitative three-dimensional analysis of wood structure. As the wood specimens used here were not collected in this viewpoint, the tree axis of the specimen was unknown. So new standards were tried. The annual ring boundary was suitable for the standard in the radial direction. On the other hand a

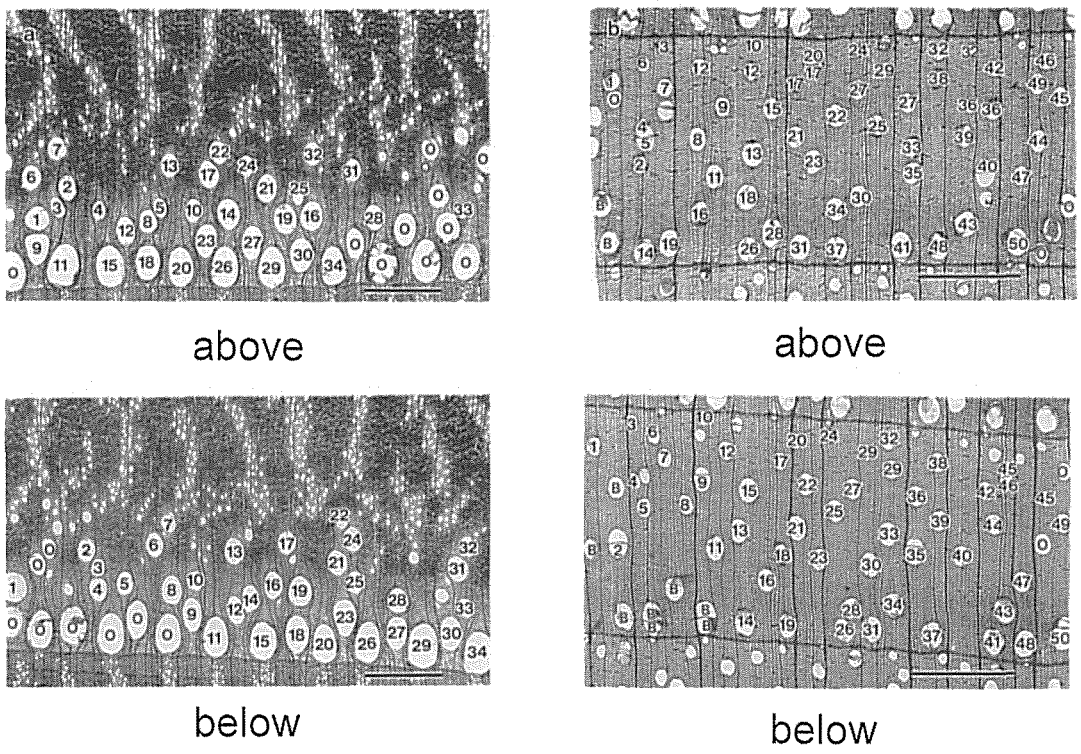

Fig. 3 Position of each vessel at both ends. a: *Castanea crenata*. b: *Juglans sieboldiana*. O: These vessels could be inserted, but at the other end, they are located out of the photograph. B: These vessels also could be inserted, but lead to the part out of the block. Bars: 1 mm.

tangential standard was difficult to be found out on the wood tissue. In this paper the relative positions of vessels were used.

The examples of *C. crenata* and *J. sieboldiana* are shown in Fig. 3. We tried to throw light on the tendency of the course of vessels, being based on the displacements in the relative position of vessels at both transverse surfaces. The position of each vessel was defined by the center of gravity on the pore. The coordinate of the position at both ends was expressed by the following way. Radial positions were set as a distance from the annual ring boundary to pores along the direction of the ray (length of line P_1A or P_2C in Fig. 4). Tangential positions were set as

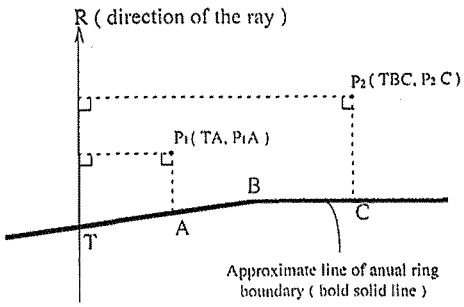

Fig. 4 Diagram of the method for defining the coordinates of pores. Radial position is a distance from the annual ring boundary to each pore along the direction of the ray (P_1A and P_2C). Tangential position is a length from a temporary standard to each pore along the annual ring boundary (TA and TBC). P_1, P_2: Center of gravity on the pore. T: Temporary standard.

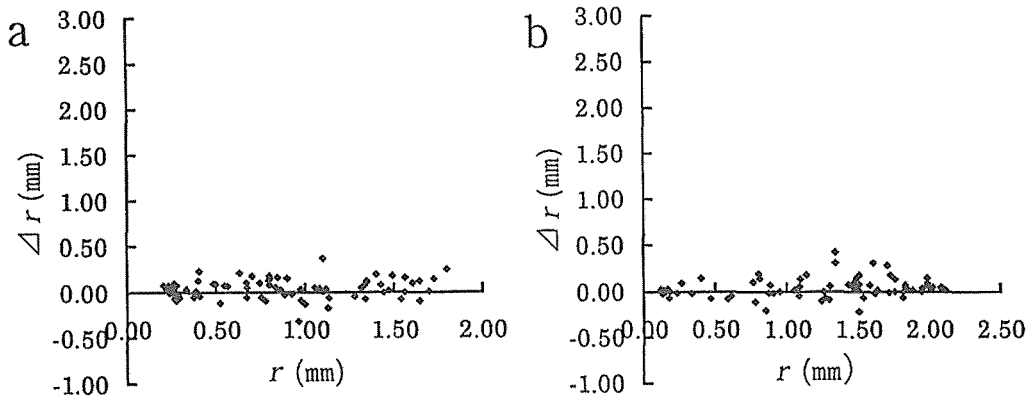

Fig. 5 The displacements (Δr) in radial direction. X-axis is radial position at one end. a: Earlywood in *Castanea crenata*. b: *Juglans sieboldiana*.

a length from a temporary standard to pores along the annual ring boundary (length of line TA or TBC in Fig. 4). The displacement in each direction was defined as the value which was left the coordinate of pore position at one end from that at the other end.

Fig. 5 shows the displacement (Δ r) in radial direction. The x-axis is radial distance from the annual ring boundary at one end. These indicate that Δ r is very small and at random. By this reason we regarded vessels didn't move in this direction with a tendency.

Fig. 6 is the graphs of the displacement (Δ t) in tangential direction. The x-axis was decided by the same way in graphs of Δ r. There are few differences for the displacement in radial direction wherever one chooses one or the other end. The displacement in this direction varies by the origin set in determination of the coordinates of pore positions. But the values of whole displacements increase or decrease with an equal value. In Figs. 6A, B, C the values were adjusted, and the average displacement of the vessels formed at the beginning of the season was set about zero.

Earlywood Vessels in C. crenata

At earlywood in *C. crenata*, Δ r was, as above-mentioned, very small and at random. We considered the displacement in this direction was too small to be analyzed by this method. Fig. 6A indicates Δ t. By this graph one can see that the vessel distribution consists of 4 layers in radial distance from the annual ring boundary. The first layer (green marks in Fig. 7) : This layer had the vessels with large diameter. Vessels in this layer were parallel, for the values of their displacements were almost equal. The second layer (orange marks in Fig. 7) : Vessels in this layer were parallel or inclined to them in the first layer. In the following description an inclination of a vessel means a deviation from the vessels in the first layer. The third layer (red marks in Fig. 7) : The vessels with various inclinations were mixed as the second layer. But the average inclination of vessels in this layer was larger than that in the second layer. The fourth layer (yellow

marks in Fig. 7) : Vessels in this layer were small and located at the transition from earlywood to latewood. In proportion as the distance from the annual ring boundary was large, the inclination of vessels reversed and became about zero. Fig. 6B indicated that there were only three layers in this annual ring. This annual ring was narrower than above-mentioned. Therefore the number of layers might be decided by the width of the earlywood. The direction of inclination in each layer was always S-helix.

J. sieboldiana

The displacement in radial direction was ignored based on the similar viewpoint in *C. crenate*. Fig. 6C shows the displacement in tangential direction. The tendency in *J. sieboldiana* was different from that at the earlywood in *C. crenata*. The nearest vessels to the initial boundary were almost parallel each other. Up to 1 mm point from the boundary there was gradual increase in the inclination of vessels. The displacements were constant at more than 1 mm point although with a variation. The direction of inclination in the vessels was always S-helix same as the results of the earlywood vessels in *C. crenata*

Pigment Injection

Pigment injection was succeeded only on the sapwood where vessel lumens were not plugged by tyloses. Especially, the current annual ring, which was obtained from the living tree and dried immediately after collection, was proper to be penetrated into with pigment solutions. In the wood of *Q. serrata* of 30 cm long, pigment passed through pore zone vessels of the outermost annual ring. Diffuse and small vessels of

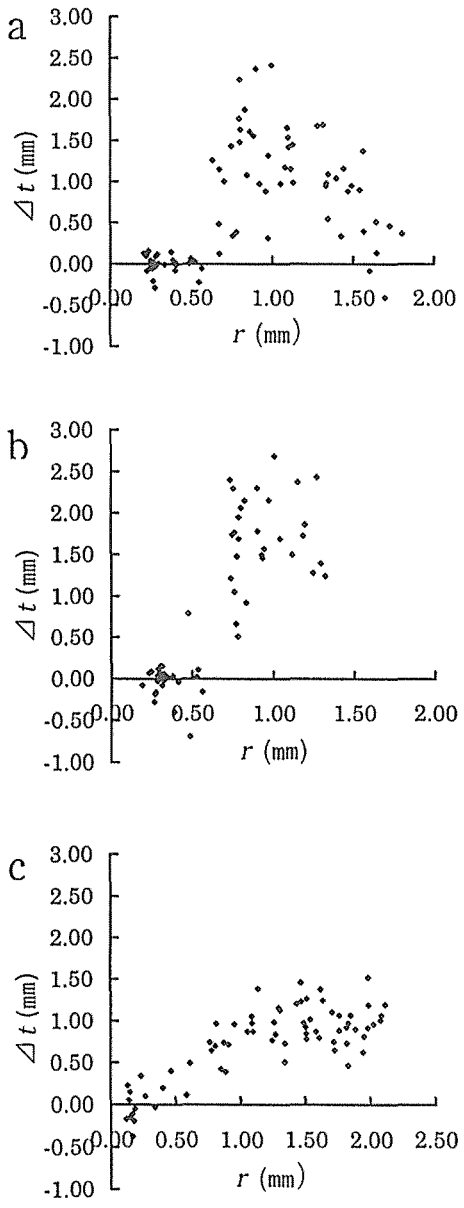

Fig. 6 The displacements (Δr) in tangential direction. X-axis is radial position at one end. If Δt is plus, the inclination is S-helix to the general direction of the vessels formed at the beginning of the season. a: Earlywood in *Castanea crenata*. b: Earlywood in C. *crenata*, which has narrow annual ring. c: *Juglans sieboldiana*.

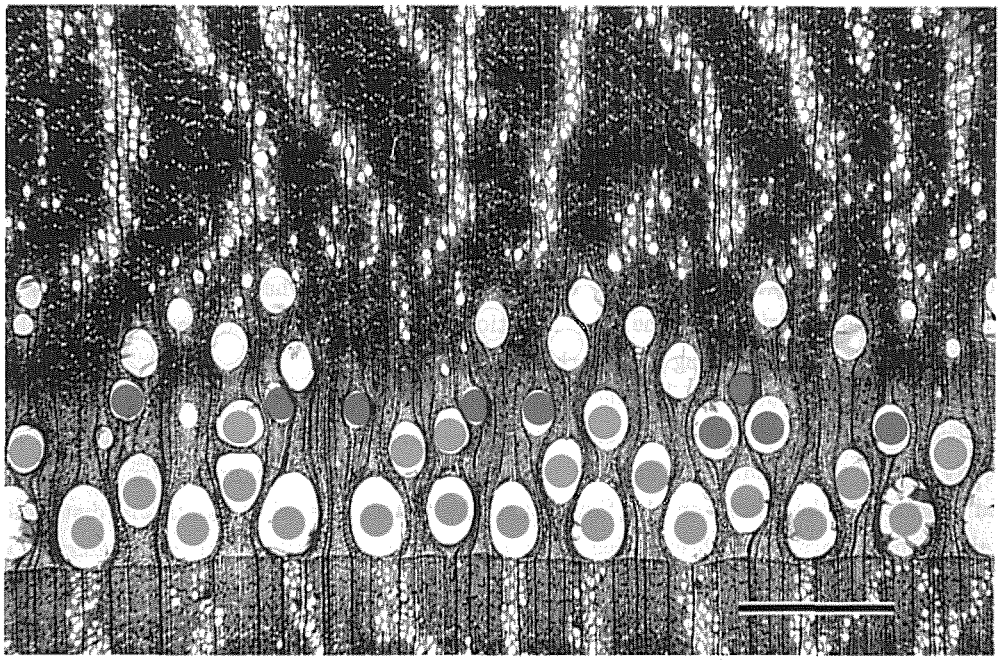

Fig. 7 A transverse section showing four groups of vessels distinguished by the layers as to tangential inclination in *Castanea crenata*. Bar: 1 mm.

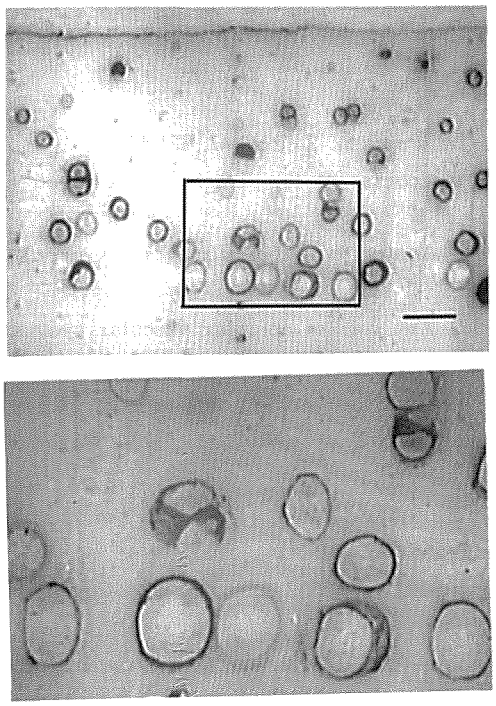

Fig. 8 A set of video-microscopic photographs on a transverse section of a saw-cut end of *Paulownia tomentosa* block. Vessels on the current annual ring can be identified by various colored pigments injected before the saw-cutting. b: Magnified view of box a. Bar: 1 mm.

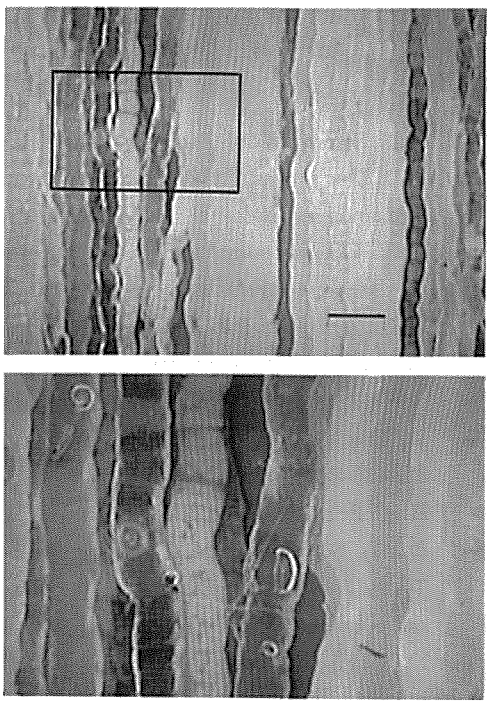

Fig. 9 Video-microscopic photographs on the tangential view of *Paulownia tomentosa* in which some vessels were injected by pigments and cast by styrene resin. Vessel lumens, especially pigmentized ones, are shown like a pillar. b: Magnified view of box of a. In this view, elements can be observed on a vessel in addition to the interwinded vessels. Bar: 1 mm.

E. tereticornis, small ones at the latewood of *P. tomentosa* can be traced by the method, to say nothing of the large vessels at the pore zone. The grouped ones were pigmentized together. The injections were practiced carefully. But the pigment often overflowed from the exit end of vessels and made dirty around the end. Internal vessel network marked by pigments could be traced easily after saw-cuttings (Fig. 8). If the pigment injection is combined with the wire insertion, a very long specimen will be traced. That is, some sapwood vessels are beforehand marked by pigmentation and then the specimen is divided into many short blocks by transverse saw-cuttings. The vessel network in each block can be analyzed more precisely by the wire insertion, and the data of adjacent blocks will be reconstructed by corresponding the marked vessels.

Clearer result was obtained by the resin casting. Because vessels contained in the block have been identified one by one on both transverse surfaces, we could observe their network directly in the tangential view under a binocular or a video microscope (Fig. 9). Pigmentized vessels will be traced on the figure just like pillars. Vessel elements are distinguished along the pillars. The three-dimensional arrangement of pillars are well reserved, because their both ends were connected with the resin layers just like a floor and a ceiling which enveloped the block in the embedding procedure.

As the conclusion the combined procedures of wire insertion, pigment injection, saw-cutting, and resin casting have been expected to bring us the new development of three-dimensional analysis of vessel network.

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