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**New Marking Method by Electrical Stimulation
for Studying Xylem Formation III[†]
Identification of the Influenced Tracheids***

By

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木部形成研究のための電氣的刺激による
新しいマーキング法 (第3報)[†]

影響を受けた仮道管の確認*

今川一志** 石田茂雄**

CONTENTS

Introduction	397
Materials and Methods	398
Results	399
1. Occurrence of the influenced tracheids	399
2. Identification of the influenced tracheids	400
2.1. State of the wall formation	401
2.2. State of the lignification	404
Discussion	404
Conclusion	406
Acknowledgement	407
References	707
要 約	407
Explanation of Photographs	408
Photographs	

Introduction

It is significant to understand seasonal development of the xylem in forest trees, in which the xylem cells are newly formed, successively differentiated and finally accumulated to form an annual growth ring. Thus, numerous quantitative investigations have been performed. In such investigations, some experimental methods for obtaining quantitative results have devised. For example, in the punching method, specimens which are periodically punched out from stems are examined microscopically (WHITMORE and ZAHNER 1966, IMAGAWA and ISHIDA 1970, ODA

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and NAKASONE 1979). And infrequently, in the radiological method, labelled¹⁴C is applied (WASEL and FAHN 1968). In the pinning method, pins are inserted through bark into xylem in order to form traumatic tissues as a mark of a given date (WOLTER 1968, YOSHIMURA et al. 1981). In these methods, however, some experimentally unsatisfactory or incomplete points seem to remain to indicate the actual developmental process.

From a such view point, the authors introduced a new marking method, in which electrical stimulation was externally applied to stems for marking a given date in the current growth ring. This electrical method was applied in *Abies* (IMAGAWA and ISHIDA 1981), *Avicennia* (IMAGAWA 1981), and *Alnus* and *Cinnamomum* (IMAGAWA and ISHIDA 1983). As a result, evidence of the possibility for marking a xylem by this new method was provided in these species. By the application of electrical stimulation, certain types of the influenced cells such as crushed cells occurred in the current growth ring. However, the state of such cells at the time when the electrical stimulation was applied are still not clear. In other words, it is not clear which differentiating cells were influenced by the electrical stimulation. If the state at the time of application can not be identified clearly, it is apparent that this method is not useful for practical use. In this study, therefore, the state of each influenced cell at that time are clarified in detail.

Materials and Methods

About 15 year-old Japanese larchs, *Larix leptolepis*, grown in the nursery in the campus of Hokkaido University were used. Two larch trees examined were 22 and 19 cm in diameter at breast height, and 8 and 7 m in height respectively. Electrical stimulation applied in this study is 220 volts by dry batteries for a duration of 5 seconds. Two electrodes in a vertical orientation were inserted through the bark into the mature xylem at a right angle to the stem axis. The stimulation

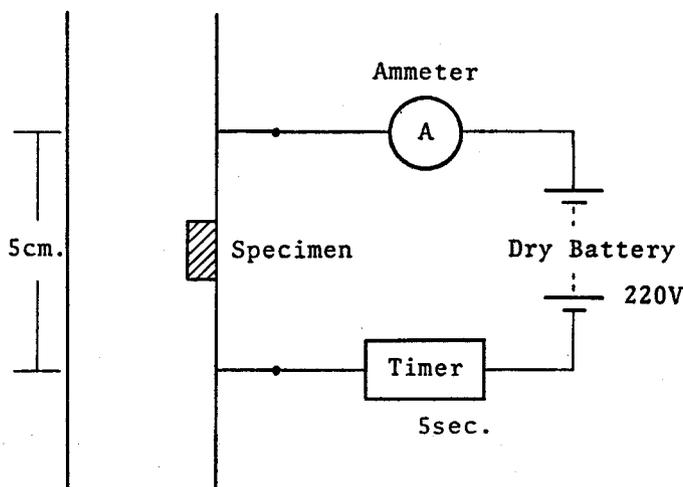


Fig. 1. Schematic diagram of the experimental method.

was applied to the stems through electrodes which were spaced 5 cm apart (Fig. 1). The electrodes were stainless-steel pins (0.5 mm in diameter, 40 mm in length), and they were drawn from the stem immediately after the application. On the 15th of July in 1981, the electrical stimulation was simultaneously applied to 8 positions at about 2.5 m above the ground (Fig. 2). The maximum readings of the electrical currents for 5 seconds were measured with an ammeter. They ranged from 44 to 52 mA (48 mA on the average) in Tree-1 and 41 to 62 mA (53 mA) in Tree-2.

Specimens were collected on the 1st, 2nd, 3rd, 5th, 7th, 9th, 11th and 14th day after the application of the electrical stimulation (Fig. 2). At each collection, one specimen was punched out from the middle portion between the traces of the insertion of the electrodes, in order to avoid traumatic influences in the current xylem by the insertion. Soon after the collections, the specimens were fixed in FAA. A part of each specimen was embedded in celloidin, and the remainder was further finely cut and then embedded in epoxy resin. Usual light microscopical sections were cut from the celloidin embedded specimens, and stained with safranin and fast green. Ultraviolet microscopical sections (0.5 μm in thickness) were cut from the epoxy resin embedded specimens, and mounted with glycerine on quartz slide and cover glasses. In addition, semi-ultrathin sections (2~4 μm in thickness) were cut from the epoxy resin embedded specimens, and stained with basic fusin (HUBER et al. 1968). These sections were observed with a usual light microscope, a polarizing microscope or an UV microscope.

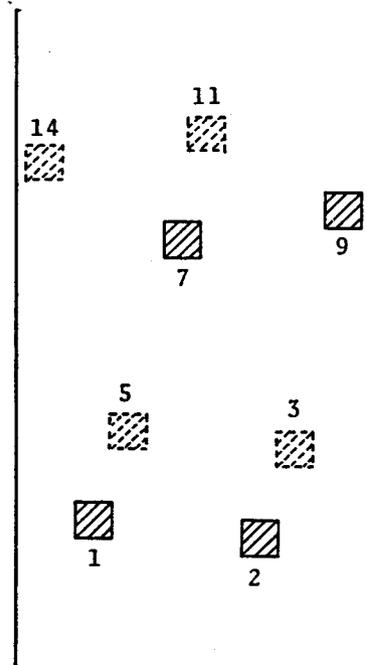


Fig. 2. Positions of the specimens' Figures indicate the sampling day after the application.

Results

The results obtained are explained in two separate parts, i. e., the successive process of the occurrence of the influenced tracheids and the state of them at the time of the application.

1. Occurrence of the influenced tracheids

In order to clarify the process in which the influenced tracheids appeared by the application of the electrical stimulation in the current growth ring, each transverse section of 8 specimens which were collected from Tree-1 over 2 weeks (Photos 1-8). In all the photographs, the bark is located upward, and each figure at the lower left in the photographs indicates the number of days after the application.

On the 1st day after the application (24 hours after), some differentiating

tracheids which were radially crushed slightly are observed in a tangential series (Photo 1). Such crushing tracheids are located more or less far from the cambium. There are several differentiating tracheids between the cambium and these crushing tracheids. The curvature of the radial tiers of the cambial cells appear to be artifacts that were caused during the microscopical preparations. On the 2nd day (Photo 2), completely or incompletely crushed tracheids are located at almost the same position from the cambium as in Photo 1. The completely crushed tracheids are represented as a tangential black-line in Photo 2. There are about 10 partially crushed tracheids on the pith side of the completely crushed tracheids. On the 3rd day, the number of the crushed tracheids increases rapidly (Photo 3). As a result, the crushed tracheids gather to form a tangential wide-band. There are only a few partially crushed tracheids which are located on the pith side of the completely crushed tracheids in each specimen (Photos 4-8).

Although some of the differentiating tracheids were strongly influenced by the electrical stimulation, on the other hand, the cambial cells indicated no changes at least regarding their cell shape such as these differentiating tracheids in the period over 2 weeks. Consequently, it may be concluded that the cambial cells are not influenced by the stimulation. This may be supported by the ordinary cell-production as shown in later specimens. Namely, the number of the newly formed tracheids increased in order, so that each cambium was gradually separated farther from the crushed tracheids.

On the 5th day, ray cells in which deposits were abundantly accumulated are found (Photo 4). Such ray cells are located in either the cambium and the region of the differentiating tracheids. Although the exact mechanism by which the deposits were produced in the ray cells is still not clear, it is assumed that they resulted from the application of the electrical stimulation. In the later specimens, such deposits were accumulated in the ray cells on the pith side of the crushed tracheids (Photo 8), though the deposits are not clearly observed in these photographs (Photos 5-7). The deposits were not chemically analyzed in this study.

As shown distinctly in Photo 8, several tracheids on the cambium side of the crushed tracheids have considerably thinner walls than those of the tracheids produced later. And also they are more or less irregularly arranged and varied in cell shape. Judging from the observations, they seem to arise from their abnormal differentiations after the application.

In Tree-2, the crushed tracheids occurred earlier than the case of Tree-1. They occurred on the 1st day after the application. The appearance of their occurrence was similar to those on the 2nd day in Tree-1 (Photo 2). This difference in rate of their occurrence may probably arise from the difference in extent of the xylem formation. Since the later cell-production in Tree-2 advanced in a disorderly fashion, however, the photographs in which the process of the occurrence of the influenced tracheids are shown are omitted here.

2. *Identification of the influenced tracheids*

As mentioned above, the process in which the influenced tracheids occurred

was considerably clarified. Thus, the state of the influenced tracheids at the time when the electrical stimulation was applied is explained in two separate parts, i. e., the state of the wall formation and the lignification. Since the influences appeared promptly, especially, the earlier specimens must be carefully investigated.

2.1 *State of the wall formation*

Photo 9 shows the transverse section (semi-ultrathin section) on the 1st day after the application in Tree-1. Photo 9 b is the polarized micrograph of Photo 9 a. In the cambial cells, no changes are observed regarding the cell shape, and the artifacts as shown in Photo 1 are also not found. They are regularly arranged in radial tiers. At the middle portion in the photo, some tracheids are shown to be radially crushed. Since they are in a stage of differentiation immediately after the onset of their collapse, they are incompletely crushed and consequently their lumina can be still observed. These crushing tracheids are located solitarily, in a tangential series or in an oblique series. All the crushing tracheids were thin walled and still showed their protoplasts, though they are not distinctly observed in this photo. Some differentiating tracheids including the crushed tracheids are brightened (Photo 9 b). It is well known that at first S-1 layers of differentiating tracheids show birefringence in a transverse section. Therefore, it is clear that the crushing tracheids are in a stage of differentiation following the initiation of the formation of the S-1 layers. Accompanied with the collapse of the tracheids, the rays near them are bent or folded. The tracheids at the bottom in photo 9 a show their cell shape, and their walls are relatively thick,

Photo 10 shows the transverse section on the 1st day in Tree-2 (a) and its polarized micrograph (b). The cambial cells also maintain their native cell shape, and consequently they are regularly arranged in radial tiers. Remarkably influenced tracheids occur following about 10 normal shaped tracheids apart from the cambium. Almost more than a half of them has already crushed completely, and the remainder incompletely. They are tangentially arranged, so that they macroscopically observed as a tangential wide band. In spite of the specimen on the same day as Photo 9, completely crushed tracheids have occurred earlier. In other words, the rate of the occurrence of the influence is considerably different from one another. However, it remains unclarified as to what such difference arose from. It may be assumed that their growth condition or the rate of their xylem formation is varied. Both the completely and incompletely crushed tracheids are seen brightened between cross nicol prisms. In addition, a few tracheids on the cambium side of them also show birefringence. Therefore, it is apparent that such tracheids were already initiated to form the S-1 layers at least.

Since it was clarified that both the completely and incompletely crushed tracheids were in a differentiating stage after the onset of the S-1 layer formation, investigations were further advanced regarding the extent of the wall formation of such influenced tracheids. Photo 11 a shows the transverse section on the 1st day in Tree-2. Photo 11 b shows the radial section at almost the same position as in Photo 11 a. Photo 11 c is the polarized micrograph of Photo 11 b. The

cambiums are all located at the right in each photograph. In both the transverse and the radial section, crushed tracheids can be clearly distinguished. The radial walls of them indicate birefringence (Photo 11 c). On the other hand, the normal shaped tracheids which are located on the cambium side of the crushed tracheids failed to be brightened. As S-1 layer is oriented almost along cell axis, it shows birefringence in the radial section. Therefore, it is considered that the crushed tracheids are in a stage of differentiation after the onset of the accumulation of the S-2 layers. In the normal shaped tracheids on the cambium side of the crushed tracheids, the S-2 layers have still not formed because they indicate no birefringence. Consequently, it is considered that only the tracheids after the onset of the formation of the S-2 layers were influenced by the electrical stimulation, so that they were crushed completely or incompletely. Generally, it seems to be reasonable that the differentiating tracheids with S-2 layers are considerably more resistant to pressure than those with only S-1 layers. In addition, cambial cells are much more weaker than the tracheids with S-2 layers. Nevertheless, such weaker tracheids or cambial cells were not crushed. Far from that, no changes regarding cell shape were observed. However, the causes of such phenomena are still obscure.

If it is definitely confirmed that the crushed tracheids have at least S-2 layers and the tracheids on the cambium side of them have only S-1 layers, this would be highly significant and valuable for practical use of this electrical method. However, cases dissimilar to such phenomenon were rarely found (Photo 12). Photo 12 a shows the radial section on the 2nd day in Tree-1, and Photo 12 b is the polarized micrograph. In Photo 12 a, the crushed tracheids are distinctly observed. And the tracheids on the right side of the crushed ones are also found. They were not crushed and maintained their cell shape. A part of the ray cells may be observed at the upper and lower portion.

In polarizing microscopy, the tracheids on the cambium side of the crushed tracheids show no birefringence (Photo 12 b). This result is similar to those of Photo 11. However, some of the crushed tracheids were considerably different from the specimen on the 1st day in Tree-2. Although the left part of the crushed tracheids is similarly brightened, the remainder on the right side shows no birefringence. In spite of being in the same state regarding the collapse of tracheids, the latter did not indicate any evidence of having S-2 layer. Since the radial sections examined were considerably thin (2~4 μm in thickness), this may result from a deficiency of the thickness for polarizing microscopy. But it seems to be unreasonable to attribute the cause only to the thickness of the section. It may be necessary to investigate further the wall structure of the crushed tracheids by an electron microscope. Eventually, it must be concluded that the crushed tracheids exist which have the S-2 layer or rarely only the S-1 layer. Consequently, the crushed tracheids should be considered to be in a stage of differentiation after the onset of S-1 layer formation.

As the state of the crushed tracheids at the time of the application was explained, non-influenced tracheids with regard to cell shape were investigated further.

Photo 13 shows the transverse section on the 1st day in Tree-2. Photo 14 a shows a part of Photo 13 which is surrounded by a rectangle. Photo 14 b is the polarized micrograph. In Photo 14 a, the normal shaped tracheids and partially transformed tracheids are observed. Namely, the boundary zone between the non-influenced tracheids and the influenced tracheids is shown. The normal shaped tracheids at the lower portion in the photo have comparatively thick walls. They show birefringence depending on their S-3 layers (arrow in Photo 14 b). The transformed tracheids at the upper portion have slightly thinner walls. They seem to lack the S-3 layers because their walls are not brightened. However, the relation between the extent of the transformation and the amount of the accumulation of the S-3 layers could not be clarified in this study. Electron microscopical investigations must be advanced for elucidating the relation. Although such detailed problems remain unsolved in this study, it is reasonable to consider that the transformed tracheids were in a differentiating stage or were immature at the time of the application, and the normal shaped tracheids were mature at that time.

As mentioned earlier, noticeable tracheids with thin walls were located on the cambium side of the crushed tracheids. Photo 15 shows the such tracheids in the transverse section on the 14th day in Tree-2. Photo 16 a shows a part of Photo 15 which is surrounded by a rectangle. Photo 16 b is the polarized micrograph. In Photo 16 a, several thin walled tracheids are observed on the cambium side of the crushed tracheids. They are almost normal in shape, but their walls are thinner and less lignified than those of the tracheids produced later. These thin walled tracheids do not have the S-3 layers, and the tracheids following them have S-3 layers as shown in Photo 16 b. Protoplasms were not observed in the thin walled tracheids and most of the tracheids with S-3 layers had the protoplasms, though they can not be clearly observed in Photo 16. With regard to a loss of protoplasm, they are mature tracheids, because protoplasm usually disappears at the time of the maturation of the wall structure. However, it is apparent that these tracheids have not matured regarding their wall structure judging from polarizing microscopy. Therefore, they seem to have died in immature after the application based on the loss of the protoplasms. Whereas, regarding their position of occurrence, these tracheids correspond to those on the cambium side of the tracheids which were crushed just after the application (Photos 9 and 10). Such tracheids had already obtained S-1 layers at that time, though the layers were not sufficiently accumulated. However, their walls in Photo 16 are relatively thicker than those at that time (Photos 9 and 10). Therefore, in such tracheids, the wall formation seems to have continued for a period after the application. After that, eventually, they seem to die. Based on this presumption, these thin walled tracheids may be the tracheids which were in the stage immediately after the onset of S-1 layer formation at that time. These tracheids must have been located nearer the cambium and had thinner walls than the crushed tracheids. However, the former was not crushed but the latter was crushed. The reason why these thin walled tracheids were not crushed is not clear.

2.2 *State of the lignification*

With regard to lignification, the influenced tracheids were further investigated by an UV microscope. Photo 17 shows the transverse section on the 2nd day in Tree-1. Photo 18 is the UV micrograph (280 $m\mu$ in wave length) corresponding to Photo 17. The tracheids on the cambium side of the crushed tracheids are numbered from near the cambium. Judging from the UV absorption (black in Photo), the lignification begins at the cell corners in No. 3 tracheid. In these numbered tracheid, the UV absorption at the cell corners increases gradually while approaching the crushed tracheids. However, the lignification does not seem to advance sufficiently into the intercellular layers in the walls between each cell corner even in No. 11 tracheid. In the crushed tracheids, on the other hand, such intercellular layers show considerably strong absorption. As a result, each tracheid is distinctly bordered by a black line in Photo 18. This black line indicates the lignified intercellular layer which absorbed UV rays. Consequently, it is considered that the tracheids with lignified intercellular layers at the time of the application were crushed and the tracheids without them were not. Although the reasons why such difference occurred are still not clear, this is apparently important to explain the state of the tracheids which were crushed at that time.

In the specimen on the 7th day, the lignification of tracheids on the cambium side of the crushed tracheids progressed further (Photos 19 and 20). Not only the intercellular layers but also a part of the secondary walls have been considerably lignified. And the walls have also thickened. As a result, these tracheids seem to be more strongly lignified than the crushed tracheids (Photo 20). Therefore, it appears that the future thin-walled tracheids as shown in Photos 15 and 16 were alive and the differentiation continued at least until this time. This may be also substantiated by the existence of the protoplasts in the lumina.

Photo 21 shows the UV micrograph on the 14th day in Tree-1. The crushed tracheids and the thin walled tracheids are seen clearly. The state of the crushed tracheids are similar to those in Photos 17 and 18. Whereas, the state of the thin walled tracheids are extremely different from those in Photos 18 and 20. In such portions, relatively thick walled or considerably thin walled tracheids are found together. The thick walled tracheids are more strongly lignified than the thin walled ones judging from the extent of UV absorption. On the basis of the results, the tracheids in this portion seem to be still living up to this time. On the cambium side of this portion, normal shaped tracheids with lignified and thick walls are located. Although the tracheids in this portion may continue to differentiate to some extent, eventually, it can be hardly expected that they mature and become normal tracheids.

Discussion

By the application of the electrical stimulation, mainly two types of influenced tracheids occurred in the current growth ring. They were crushed tracheids and thin walled tracheids. Both were differentiating tracheids at the time when the

electrical stimulation was applied. On the other hand, no influences were found in the cambial cells. The crushed tracheids seem to have been in the differentiating stage after the initiation of the accumulation of the S-2 layers. In addition, their intercellular layers were fully lignified. It was clarified that the tracheids in such differentiating stage were crushed and died by the electrical stimulation.

When the electrical stimulation was applied, it is presumed that Joule's heat was generated by the electric current which is the true nature of the electrical stimulation applied to the stem. Although the mechanism by which the generation of the heat results in the occurrence of the crushed tracheids is still not evident, it is possible to presume as follows,

It is well known that a collapse often occurs in wood during the drying (PANSIN and ZEEUW 1980). The collapsed wood is in appearance similar to those of the crushed tracheids occurred by the electrical stimulation. According to SIAU (1971), such a collapse occurs in wood when the capillary tension exceeds the compressive strength perpendicular to the grain. The increase of the tension results from too-rapid removal of the capillary water from the cell cavities in wood by high temperature. In addition, since the cell walls are greatly weakened as the temperature increases, the collapse occurs more easily at high temperature. Among some factors from which the collapse of wood results during drying, in conclusion, the most decisive factor seems to be the temperature.

When the electrical stimulation, i. e. electric current was applied to the larch stem examined, it is considered that most of it flowed through the liquid-rich tissues such as the differentiating tracheids or the cambial cells (IMAGAWA and ISHIDA 1982). As a result, it is presumed that Joule's heat was generated in such tissues in which the cells had only weak walls. Therefore, it seems certain that the collapse occurs in such tissues of larch stem. In such a case, it is doubtful that only the crushed tracheids were in a suitable state for collapse. It seems to be reasonable that the tracheids on the cambium side of the crushed tracheids or the cambial cells were in a more suitable state judging from their weaker walls. Nevertheless, such cells with weaker walls were not crushed.

Again, according to SIAU (1971), collapsed wood can be restored to the original shape by subjecting wood to high temperature and humidity for a period of time after it has been dried. In this case the air and water vapor in the wood exerts a pressure inside the cells which reverses the tension forces that originally caused the collapse. Based on his opinion, the weaker cells other than the crushed tracheids may be restored to their original shape by such a mechanism after the collapse, but the crushed tracheids were not restored. Such restoration may occur within 24 hours after the collapse. Or, the collapse may have not occurred in these cells. Or, only the thin walled tracheids on the cambium side of the crushed tracheids may have been crushed, because they were influenced as shown in their disorder differentiation after the electrical stimulation. Moreover, the folded walls of the crushed tracheids may be too firm to be restored to their original shape. Since no specimens were collected within 24 hours, a clear conclusion was not

obtained in this study.

In this electrical method for marking the xylem, the mark such as the crushed tracheids which occurred in the current growth ring were not located in the cambium. They occurred somewhat apart from the cambium. Therefore, this method may be considered to be unfavorable for marking the xylem because no marks could be observed in the cambium. In quantitative investigations on growth ring formation, it may be important to indicate the location of the cambium on a given date. In fact, this was achieved by the pinning method (YOSHIMURA et al. 1981). However, remarkable traumatic tissues as a mark which indicate a given date were formed in the cambium by the insertion of pins. Generally, it is apparent that traumatic tissues can not be formed during normal cell-production. In this electrical method, abnormal cells derived from the cambial cells (cell division) were not formed, but only influenced tracheids which arose from the collapse of the existing differentiating-tracheids occurred. Therefore, it can be expected to continue normal cell-production after the application of the electrical stimulation, because traumatic tissues were not formed by this method. This seems to be extremely favorable and advantageous for investigating successive process of normal growth ring formation.

Many crushed tracheids occurred in a radially wide band by the electrical stimulation. Such an abundance is not necessary for marking the xylem alone. In this study, excessive electrical stimulation (220 volts) were applied in order to mark the xylem clearly from a view point of identification of the state of the influenced tracheids. In the previous study (IMAGAWA and ISHIDA 1981), it was suggested that even a small amount of the stimulation (154 volts) was sufficient to mark the xylem. And the influences were apparently varied by the experimental conditions (IMAGAWA unpublished). Therefore, it is necessary to investigate a suitable stimulation. Moreover, the collections within 24 hours must be done.

Conclusion

The state of the influenced tracheids at the time of the application was identified. The influenced tracheids were observed at an early time in the specimen on the 1st day after the application. However, they were not found in the cambium, but they appeared in a tangential wide-band in the region of differentiating tracheids.

When the electrical stimulation was applied, the crushed tracheids were in a stage of differentiation after the initiation of the formation of at least S-1 layers. Most of them had the S-2 layers. Their intercellular layers were fully lignified. The thin walled tracheids on the cambium side of the crushed tracheids seemed to be in a stage following the initiation of only S-1 layer formation. Although their lignification began at the cell corners, their intercellular layers between each cell corner did not begin. The non-influenced tracheids on the pith side of the crushed tracheids were mature.

The mechanisms by which these influenced tracheids occurred were still obscure

though the state of them at the time of the application was clarified in detail. Since the cambial cells were not influenced, this electrical method seems to be advantageous for marking the xylem because normal cell-production is expected even after the application of the electrical stimulation.

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要 約

電氣的刺激によって影響された仮道管が、刺激時にはどのような状態であったのかを確認するために本研究を進めた。220ボルトの電圧を6秒間、5cm離れた電極に与え、1, 2, 3, 5, 7, 9, 11, 14日後に試料を打抜き、検鏡した。

半径方向に押しつぶされた仮道管はS-1層の沈着開始以降のものであり、その大部分はS-2層をも有していた。そのような仮道管の形成層側にある、薄壁の仮道管はS-1層のみを有

していた。ただし、押しつぶされた仮道管の細胞間層の木化は十分に進んでいたが、薄壁のもののはコーナー部だけで、細胞間層全体にわたっての木化は見られなかった。形成層細胞にはその形状の上からは刺激による影響は認められなかった。

押しつぶされた仮道管の出現状況は、木材の乾燥時にしばしば認められる落ち込み現象で生じた細胞の状況と極めて類似していた。したがって、落ち込みの発生原因を参照しながら、押しつぶされた仮道管の出現機構を若干考察してみた。

Explanations of Photographs

- Photos 1-8.** Successive process of the occurrence of the influenced tracheids in Tree-1.
- Photo 9.** Transverse section on the next day after the application in Tree-1 (a) and its polarized micrograph (b).
- Photo 10.** Transverse section on the next day after the application (a) and its polarized micrograph (b) in Tree-2.
- Photo 11.** Transverse section (a) and radial section (b) on the next day after the application, and its polarized micrograph (c) in Tree-2.
- Photo 12.** Radial section (a) and its polarized micrograph (b) on the second day after the application.
- Photo 13.** Transverse section on the next day after the application in Tree-1.
- Photo 14.** Transverse section (a) and its polarized micrograph (b) showing the region which is bordered in a rectangle in Photo 13.
- Photo 15.** Transverse section on the 14th day after the application in Tree-2.
- Photo 16.** Transverse section (a) and its polarized micrograph (b) showing the region which is bordered in a rectangle in Photo 15.
- Photo 17.** Transverse section on the second day after the application in Tree-1.
- Photo 18.** UV micrograph (280 m μ in wave length) showing the region which is bordered in a rectangle in Photo 17.
- Photo 19.** UV micrograph on the 7th day after the application in Tree-2.
- Photo 20.** UV micrograph showing the region which is bordered in a rectangle in Photo 19.
- Photo 21.** UV micrograph on the 14th day after the application in Tree-1.

