Cytological study of pathological changes in Japanese black pine (*Pinus thunbergii*) seedlings after inoculation with pine-wood nematode (*Bursaphelenchus xylophilus*)

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マツノザイセンチュウの接種によるクロマツ幼齢木枯損過程の細胞学的観察

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Résumé

Cytological observations of pathological changes in pine trees after inoculation with pine-wood nematodes were carried out. Pathogenic *Bursaphelenchus xylophilus*, and non-pathogenic *B. mucronatus* were inoculated on the cut surface of 3 or 4 years-old Japanese black pine (Pinus thunbergii) seedlings. From the observations of pathological changes in leading shoots after inoculation, following results were obtained; (1) As the first symptom after inoculation, vacuoles were observed in ray parenchyma cells. The area of ray parenchyma cells having vacuoles increased in wide range in rather short period after inoculation. (2) A part of the vacuolar contents was tannic materials. (3) Vacuoles developed more conspicuously and finally burst with lapse of time after inoculation. Ray parenchyma cells with vacuoles were considered to be in necrobiosis. (4) Vacuoles also were formed in ray parenchyma cells after inoculation of *B. mucronatus*. The area of ray parenchyma cells having vacuoles, however, was limited and almost no vacuole bursts. (5) With the breakdown of vacuoles, unidentified substances which were considered to be transported from parenchyma cells accumulated in tracheids. Some of these materials among them distributed homogeneousely in the xylem. These substances in tracheids have been postulated to lower the water-conducting activity of tracheids. (6) From the above observations, it has been postulated that some factors originating from *B. xylophilus* act on living parenchyma cells and cause the necrosis of them and moreover, the necrosis of parenchyma cells results in secondary changes of transportation of unidentified substances to tracheids.

要　旨

マツノザイセンチュウによるマツ属樹木の枯損現象に関する研究の一環として，細胞学的な観察を行なった。すなわち，3又は4年生クロマツ苗木にマツノザイセンチュウ（病原性），ニセマツノザイセンチュウ（非病原性）を接種した。接種後にクロマツ木部が示した変化を，接種後の時間的経過を追って観察した。得られた結果は以下の通りである。(1)マツノザイセンチュウ接種後初期の段階で，放射柔細胞中に液胞が出現した。また液胞の出現範囲は短期間に，地際近くまで広がった。(2)液胞は内容物の一部として，タンニン系物質を含んでいた。(3)液胞は接種後の時間的経過に伴ない，さらに発達し，ついには崩壊した。この段階の放射柔細胞は，壊死段階にあるものと推定される。(4)ニセマツノザイセンチュウ接種木においても，液胞は出現した。しかしその範囲は比較的狭かった。また液胞はほとんど崩壊しなかった。(5)液胞の崩壊に伴ない，仮道管中に柔細胞から移動したと考えられる物質が蓄積した。これらの中で，木部仮道管中に広範囲に存在する物質があった。これら仮道管中の物質は，水分通導の機能を低下させているものと推定される。

以上の観察結果から，マツノザイセンチュウに起因する何らかの因子が柔細胞に作用し，柔細胞は壊死したものと思われる。さらに柔細胞の壊死は，仮道管中への物質の放出等の二次的な変化をもたらすと推定される。

1. Introduction

Many reports have been published concerning the pine wilt disease after the infection of pine-wood nematodes. Among these, Mamiya[1], Hashimoto[2][3], Sugawa[4][5], Kuroda and others[6], and Sasaki and others[7] have studied this aspect from the anatomical point of view. There are, however, few cytological observations about the process of the host reaction to the parasite-nematodes-. In this report, cytological observations of the process of pine wilt disease after the inoculation of nematodes have been conducted to get the basic information. *Pinus thunbergii* which is very susceptible to the pine-wood nematodes[8] was selected for study. Two *Bursaphelenchus* species—pathogenic *B. xylophilus* and non-pathogenic *B. mucronatus*—were inoculated and comparative study was carried out. Cytological observations were performed with special reference to the time sequence after the inoculation and to the pursuit of the distance from the inoculation points.

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2. Materials and Methods

2.1 Materials

The inoculation experiments were carried out on June 10th, July 31st in 1982 with about 100 seedlings of 3 or 4 years-old Japanese black pine (*Pinus thunbergii*) planted at the nursery of the Experimantal Forest of Kyoto University (Kyoto City). The inoculation of nematodes was carried out as follows. After cutting off an upper part of the leading

shoot elongated in current year, a piece of absorbent cotton was placed on the cut surface, and 1ml of nematode suspension containing 2,000 individuals was poured on it. Then, its inoculation site was covered with Parafilm (American Can Co.), lest the inocula should be washed away in the rain.

One third of pine seedlings were inoculated with pathogenic *Bursaphelenchus xylophilus* (hereafter shown as A group), another one third with non-pathogenic *B. mucronatus* (shown as B group), and the last one third (shown as C) was used as control and inoculated with 1ml of distilled water. These seedlings were harvested with lapse of time and were used for the observations to compare the difference in symptoms among the three treatments.

2.2 Methods

2.2.1 Light microscopy

For the cytological observations, 3cm segments were cut from the seedlings and all segments were fixed in a 10% formalin solution. Radial sections of 30 μm thickness were cut on a freezing microtome. The following staining methods were employed: nuclei -safranin and light green, lipid droplets or unidentified substances in tracheids-Nile blue or Sudan IV, tannic materials-Nitroso reaction[9], starch grains-IKI solution, coloration-no staining. A part of the sections was observed under a phase contrast microscope.

2.2.2 Electron microscopy

For the transmission electron microscopy, small pieces, 1×1×3mm, were fixed in a 3% glutaraldehyde solution, post-fixed in a 1% osmium tetroxide solution, dehydrated in a series of alcohol, and embedded in Epon 812. Ultrathin sections were cut with a diamond knife in a LKB ultramicrotome, and observed under a JEM-100C electron microscope (100 KV). In this study, results only about the bordered pits were reported. For the scanning electron microscopy, wood blocks fixed in a 3% glutaraldehyde solution were washed with phosphate buffer and dried in a desicator with silica-gel. Specimens coated with gold were observed under a JSM-U3 scanning electron microscope (10KV).

3. Results and Discussion

The response of the pine seedlings to the pine-wood nematodes was observed both in xylem and phloem. For the elucidation of the mechanism of the pine wilt disease, it was necessary to study both parts. As the first step of the cytological study, the response observed in the xylem has been reported.

The elements constituting the xylem of *Pinus thunbergii* are as follows; tracheids, epithelial cells and accompaning cells surrounding axial resin canal[10], ray parenchyma cells, epithelial cells surrounding radial resin canal, and ray tracheids. As they are divided into two groups-parenchyma cell and tracheid groups-, the results were shown in each group.

3.1 Changes in parenchyma cells

Changes in ray parenchyma cells which distributed homogeneously in the xylem were observed in the first place. Nuclei of the ray parenchyma cells showed long elliptical shape in healthy trees (Photo. 1a). Other main contents of ray parenchyma cells were reserve

substances-aboundant lipid droplets and few strach grains. The ray parenchyma cells showed several changes after the inoculation with pine-wood nematodes and finally died. Changes of ray parenchyma cells after inoculation were as follows. The first symptom was the conspicuous vacuolization. Ray parenchyma cells of healthy trees show no visible vacuole under a light microscope. Vacuoles showed yellow or brown color in unstained sections. As they changed to cherry-red color after Nitroso reaction, one of the vacuolar contents was revealed to be catechol tannins. The accumulation of tannic materials into vacuoles was reported by Chafe and others[11], and Nobuchi and others.[12] The occurrence of vacuoles in ray parenchyma cells was observed in B and C groups in addition to A group.

In general, it is reported that vacuoles store some materials after response to wounds.[13] The vacuolization observed in present experiments was supposed to be occurred after the response of pine wood to some kinds of wounds. The patterns of response were, however, different in three groups. The area of the occurrence of vacuoles in ray parenchyma cells was measured for the 3 groups with lapse of time after inoculation (Fig. 1). Vacuoles

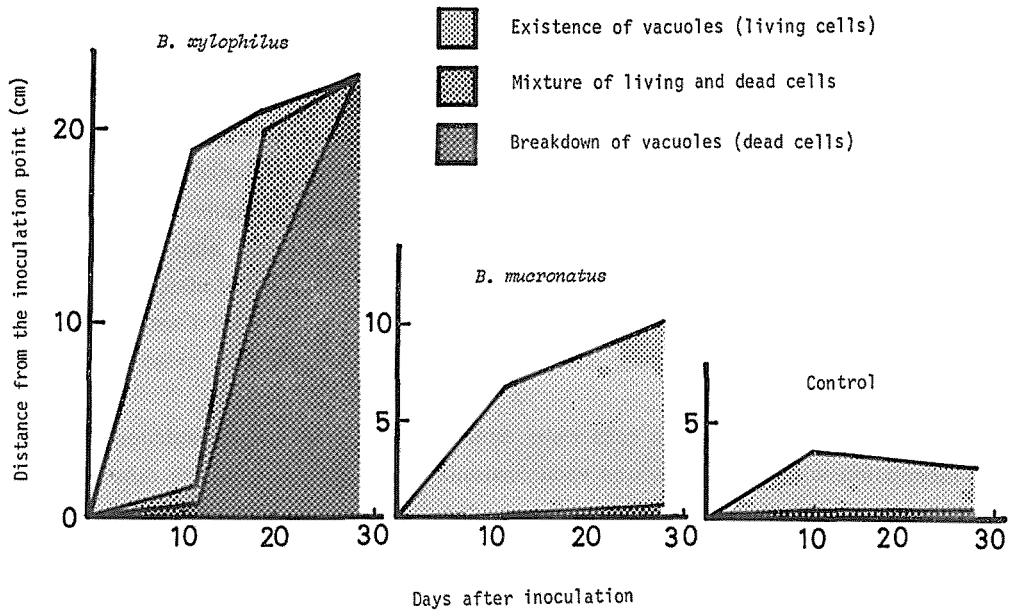

Fig. 1 The area of formation, development, and breakdown of vacuoles in ray parenchyma cells in three groups.

were observed in 3 groups, as mentioned above. In C group, however, the area did not increase with the time after inoculation. The vacuole formation in C group was attributed to the response to mechanical injury since no nematodes were inoculated in C group. As a characteristic of A group conspicuous vacuolization was observed in the lower part of the leading shoot in rather short period after inoculation.

Vacuolization of ray parenchyma cells was accompanied with the decrease in lipid droplets as the reserve substance. Further vacuolization occurred in A group, and finally

tonoplast burst. Nucleus in the ray parenchyma cells having burst vacuoles shrank or changed to debris-like structure (Photo. 1b). The cell lumina also changed their color to yellow or brown after bursting of vacuoles. It has been postulated that these ray parenchyma cells having burst vacuoles have no living functions. The cells having these features were judged to be dead cells and the area of dead cells was measured (Fig. 1). In A group, vacuoles burst in a wide area. In B group, on the other hand, the bursting of vacuoles was limited in a narrow area near the inoculation point. This is quite distinct from A group. Vacuoles have many physiological functions.[14] Stewart[15], Matile and Wiemken[14] reported that vacuole is the place accumulating waste materials formed during metabolic process in cells. In these experiments the occurrence of vacuoles was attributed to be the response to the wounds. As the vacuoles developed and burst in wide range only in A group, some factors originating from pathogenic *B. xylophilus* were considered to be different from the factors in B or C groups. One of the vacuolar contents was tannic materials mentioned above. It is necessary to analyze the exact constituents of vacuoles in the 3 groups.

The results about the occurrence and breakdown of vacuoles were discussed in relation with the distribution patterns of nematodes in the seedlings. The number of nematodes was small in the leading shoot within 2-3 weeks after inoculation. After that a sudden increase in the nematode population occurred.[16] The formation of vacuoles, therefore, was observed before the increase in the nematode population in affected seedlings. Vacuolization, moreover, was observed in almost all ray parenchyma cells which distribute homogeneously in the xylem. Nematodes, on the other hand, were located mainly in resin canals (Photo. 3) as Mamiya[1] reported. From the results mentioned above, it was suggested that the formation of vacuoles occurred without direct contact of nematodes with ray parencyma cells. Some factors originating from the nematode inoculation seemed to cause vacuolization, and factors originating from *B. xylophilus* only seemed to cause the bursting of vacuoles.

Results about ray parenchyma cells were summarized. The first conspicuous symptom after inoculation with nematodes was vacuolization. In A group, vacuoles developed in a wide area and finally burst. One of the vacuolar contents was phenolic substance which is suggested to be the waste matter of the metabolism. It has, therefore, been postulated that ray parenchyma cells do not perform normal physiological function after the breakdown of vacuoles and the spread of vacuolar inclusion in the cells. In B group, vacuolization was observed in a limited area but the vacuoles did not burst. As phenolic substances, if toxic, were separated from cytoplasm by tonoplast in B group, the ray parenchyma cells should continue the metabolic activity.

Epithelial cells and the accompaning cells surrounding the axial resin canal showed same kinds of changes after inoculation. Some differences, however, were observed. The vacuoles formed after inoculation were small in size and spherical (Photo. 4). These cells surrounding the resin canals will be studied especially in relation with the secretion of resinous materials.

3.2 Changes in tracheids

Some anatomical changes were also observed in tracheids which function as the water-conducting system. In healthy trees, nothing was observed in the lumen of tracheids. Unidentified substances existed in the tracheid lumen in the early stage after inoculation (Photo. 5). These substances showed, generally, oil droplet-like structure. They were colorless or pale yellow in unstained sections and were stained with Nile blue. They were soluble in ethanol and their chemical analysis will be the future problem of this investigation. Sasaki and others[7] reported similar kinds of unidentified substances in tracheids in the experiments using 1 or 2 years-old *Pinus densiflora* seedlings. They considered that these substances completely block the water-conducting system in the final stage of the disease.

Unidentified substances were considered to be transported from parenchyma cells although the origin of parenchyma cells—epithelial or ray parenchyma cells—producing these substances was not clarified. These unidentified substances were not homogeneously distributed but unevenly in the xylem, that is, mainly in the late wood or its vicinity. As axial resin canals distributed mainly in late wood or transition zone between early to late wood, the unidentified substances are considered to be transported from parenchyma cells surrounding axial resin canals. Compared to the results of Sasaki and others[7], the distribution patterns of axial resin canals were different. Axial resin canals distribute rather homogeneously in 1 or 2 years-old xylem.

In addition to the unidentified substances, changes in bordered pit structure were also observed in tracheids. Under a phase contrast microscope, the margo of pit membrane appeared homogeneous in healthy trees (Photo. 6a), but the radial lines were observed in the affected xylem (Photo. 6b). To study pit structure at the ultrastructural level, pits were observed under electron microscopes. Under a scanning electron microscope, microfibrils of margo showed fine structure in healthy trees (Photo. 7a). In the affected trees, microfibrils changed to thickened structure and the surface of torus was also covered with some materials (Photo. 7b). The observation of ultrathin sections revealed that some bordered pit-pairs were aspirated but many of them were open. Osmiophilic materials, moveover, were deposited onto the surface of pit membrane (Photo. 8). The electron microscopic observations suggested that some materials were deposited onto the pit membrane after inoculation with nematodes. The patterns of

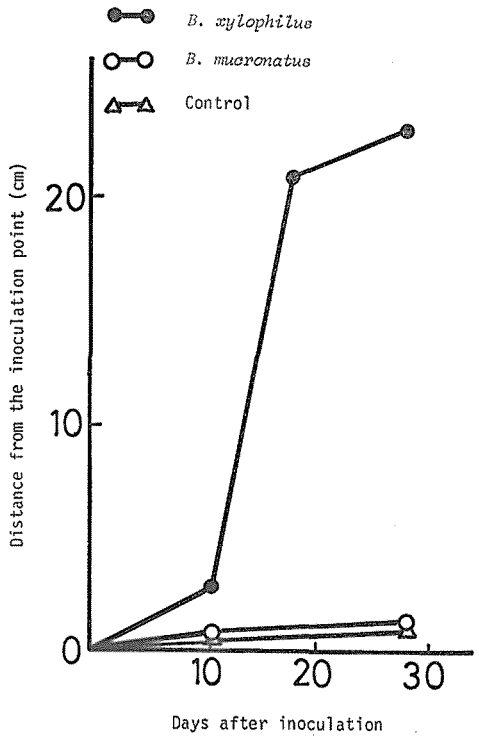

Fig. 2 The area of the changes in bordered pit structure.

radial lines in pit membrane observed under a phase contrast microscope were considered to be caused by the thickening of microfibrils in appearance. The area of the pits having above features was measured under a phase contrast microscope (Fig. 2). In A group, the changes in pit structure developed in a wide area with lapse of time after inoculation. In B or C groups, on the other hand, pit structure changed only in a limited area near the inoculation points.

The results shown in Fig. 2 were discussed in comparison with the data of Fig. 1. It was revealed that the area of changes in pit structure coincided with that of dead ray parenchyma cells. This coincidence showed that the changes in pit structure were occurred at the time of breakdown of vacuoles and the following coloration of ray parenchyma cells. Bordered pits with altered structure were homogeneously distributed in xylem differing from distribution patterns of unidentified substances which distributed unevenly. The substances deposited onto pit membrane were considered to be transported from ray parenchyma cells, because they were distributed homogeneously in xylem. That is, ray parenchyma cells transported some substances to tracheids in the necrotic process after the breakdown of vacuoles. As one of the reasons of pine wilt disease, water stress hypothesis was put forward by Suzuki.[17] In this study the measurements of moisture contents or transpiration stream were not performed. From the observation of pit-pairs, however, it was revealed that many pit-pairs were not aspirated after inoculation of nematodes, and this means that the transportation of materials from ray parenchyma cells to tracheids occurred in the stage of rather heigh moisture content of xylem. After these materials were deposited onto inner surface of tracheids, especially to pit membranes, it is considered that the activity of water conducting function decreased and finally seedlings died.

From the results mentioned above the cytological changes after inoculation with nematodes were as follows. Parenhcyma cells showed conspicuous vacuolization in early stage after inoculation with pine-wood nematodes. In this stage the population growth of nematodes was not observed. Vacuoles developed more conspicuously and finally burst. These changes were considered to be responsible for the death of parenchyma cells. Following the necrosis of parenchyma cells, some materials were transported from parenchyma cells to tracheids. These materials were deposited onto the inner surface of tracheid walls. In inoculation with *B. mucronatus*, on the other hand, vacuolization was also observed but vacuoles did not burst. This clearly shows the different point between *B. xylophilus* and *B. mucronatus*. In the latter, parenchyma cells did not die but continued living and moreover no substances were transported to tracheids. It is a important problem to clarify the factors which originate from inoculation with pathogenic *B. xylophilus* and cause the necrosis of parenchyma cells.

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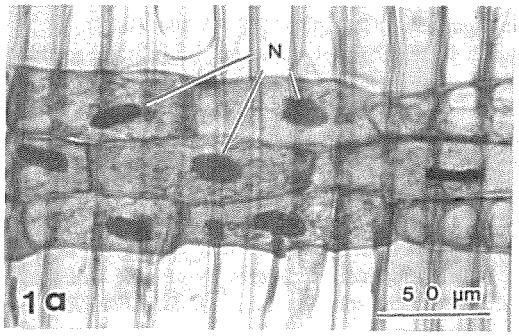
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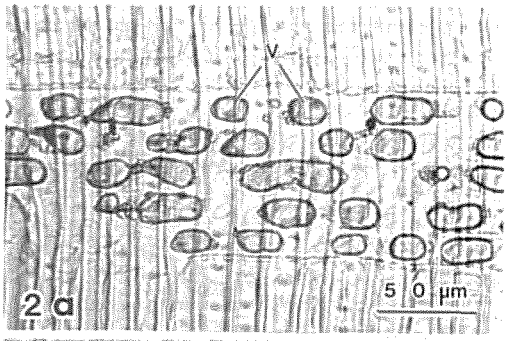
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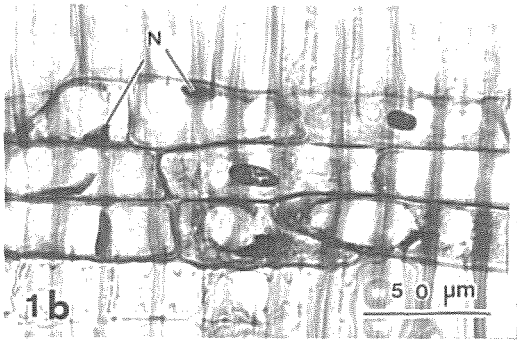
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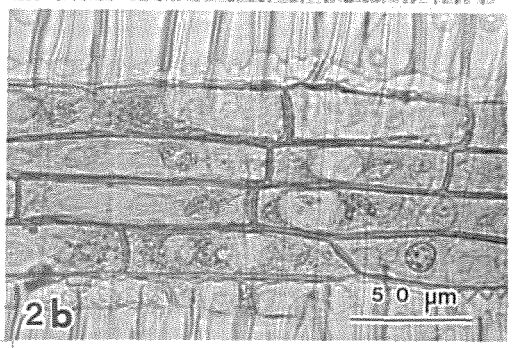
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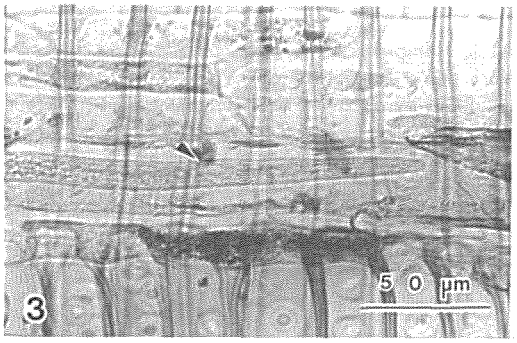
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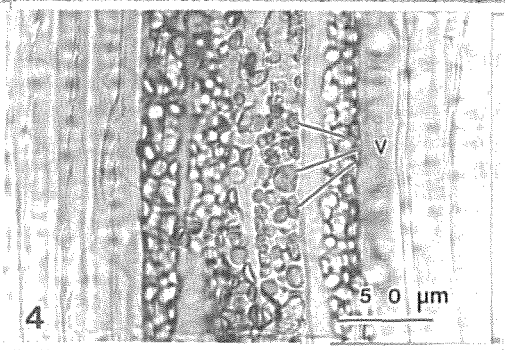
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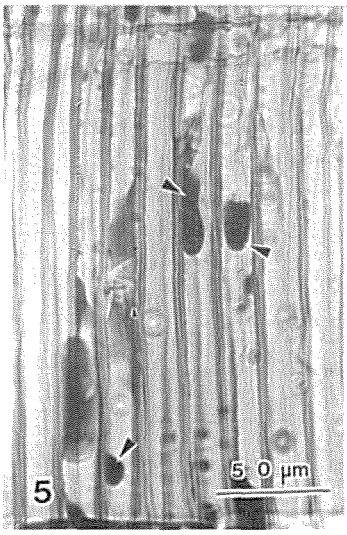
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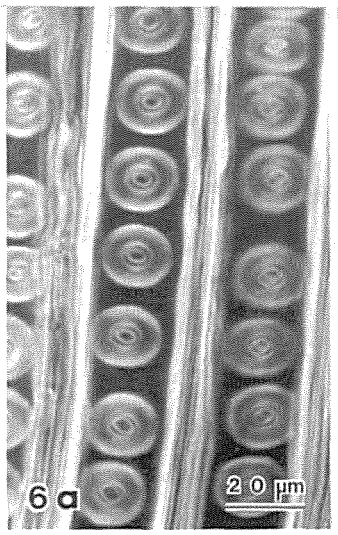
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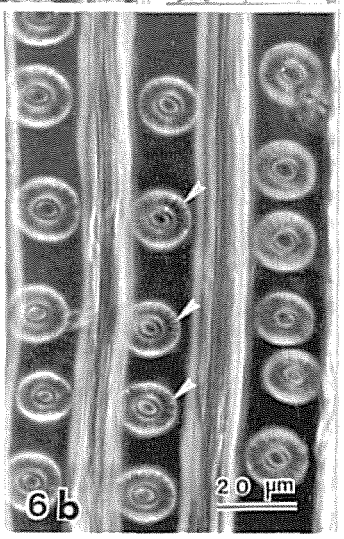
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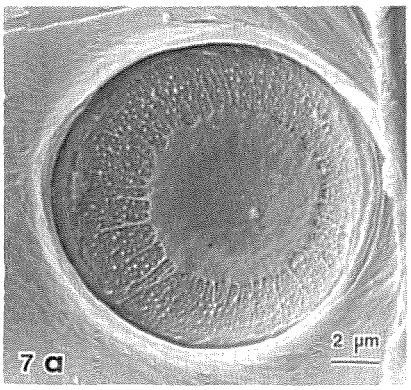

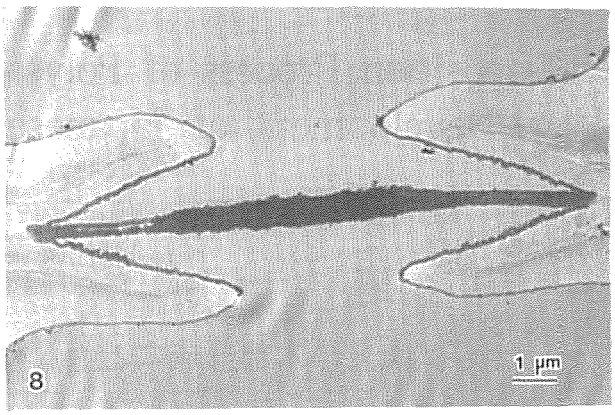

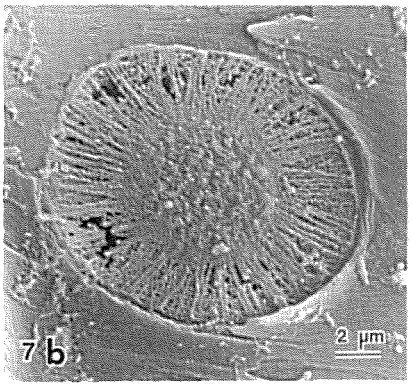

Photo. 1 Nuclei of ray parenchyma cells. Radial sections stained with safranin and light green. (a) Healthy seedling, (b) Seedling inoculated with pine-wood nematodes. N : nucleus.

Photo. 2 Vacuoles in ray parenchyma cells in the seedling inoculated with pine-wood nematodes. (a) Vacuoles of ray parenchyma cells, (b) Breakdown of vacuoles and resulting coloration in ray parenchyma cells. V : vacuole.

Photo. 3 A nematode in the radial resin canal (arrow). Radial section stained with Nile blue.

Photo. 4 Vacuoles in epithelial and accompaning cells surrounding the axial resin canal. Unstained radial section.

Photo. 5 Unidentified substances in tracheid (arrows). Radial section stained with Nile blue.

Photo. 6 Pits of tracheids observed under a phase contrast microscope. (a) Healthy seedling where margo shows homogeneous pattern, (b) Seedling inoculated with pine-wood nematodes. Margo shows radial lines (arrows).

Photo. 7 Scanning electron micrographs of pit membrane. (a) Healthy seedling, (b) Seedling inoculated with pine-wood nematodes.

Photo. 8 A transmission electron micrograph of a bordered pit-pair of the tracheids of the seedling inoculated with pine-wood nematodes. Osmiophilic materials attach to the pit membrane.