病原性の異なるマツノザイセンチュウおよびニセマツノザイセンチュウを接種したクロマツ木部の解剖学的変化および通導阻害

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論 文

Cavitation and Cytological Changes in Xylem of Pine Seedlings Inoculated with Virulent and Avirulent Isolates of *Bursaphelenchus xylophilus* and *B. mucronatus*

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FUKUDA, Kenji, HOGETSU, Taizo, and SUZUKI, Kazuo: **Cavitation and cytological changes in xylem of pine seedlings inoculated with virulent and avirulent isolates of *Bursaphelenchus xylophilus* and *B. mucronatus* J. Jpn. For. Soc. 74: 289～299, 1992** The occurrence of cavitation and cytological changes in xylem parenchymatous cells of Japanese black pine (*Pinus thunbergii*) seedlings were investigated after inoculation with a virulent isolate (S6-1) and an avirulent isolate (C14-5) of the pine-wood nematode (*Bursaphelenchus xylophilus*), and the nonpathogenic species, *B. mucronatus*. Cavitation in tracheids and cytological changes such as the disappearance of lipid droplets and the denaturation of the cytoplasm in xylem parenchymatous cells occurred in wide areas in seedlings inoculated with the virulent isolate, resulting in water deficiency and the death of the seedlings. Meanwhile, in the cases of the avirulent and the nonpathogenic nematodes, cavitation and cytological changes were restricted spatially, with the cambia and newly-formed outer xylem unaffected, so that the seedlings did not show water deficiency. In every case, cytological changes in the xylem parenchymatous cells occurred before the cavitation development and the increase of nematodes. On the other hand, treatment with oxalic acid, which caused denaturation of parenchymatous cells and cavitation over a wide area, induced chlorosis of the older needles and wilting of the whole tree. These symptoms closely resembled the typical symptoms of the pine wilt disease. From the above results, relationships between cell denaturation in xylem parenchyma, cavitation, and nematode distribution during the symptom development are discussed.

福田健二・寶月岱造・鈴木和夫：**病原性の異なるマツノザイセンチュウおよびニセマツノザイセンチュウを接種したクロマツ木部の解剖学的変化および通導阻害 日林誌 74: 289～299, 1992** マツ材線虫病における病原性発現のメカニズムを明らかにするため，病原性の異なる2系統のマツノザイセンチュウ（*Bursaphelenchus xylophilus*）と，ニセマツノザイセンチュウ（*B. mucronatus*）をクロマツ苗に接種し，木部柔細胞の細胞学的変化と通導阻害(キャビテーション)とを時間的，空間的に比較した。強病原性のS6-1系統を接種した場合，木部放射組織柔細胞および軸方向柔細胞の脂質の消失，細胞質の変性，およびそれらに続いて木部通導阻害が広範囲に生じ，形成層が壊死して苗は枯死した。弱病原性のC14-5系統接種および非病原性のニセマツノザイセンチュウ接種では，細胞生理の変化と通導阻害は形成層近傍を除く限られた範囲にのみ生じ，葉の水分生理状態に変化はなかった。一方，キャビテーションを誘導するとされる蓚酸水溶液で処理した苗は，広範囲に木部柔細胞の変性と通導阻害を生じ，旧葉の変色，当年枝の萎凋という，材線虫病特有の病徴を現した。以上のことから，マツ材線虫病では木部柔細胞の変性，通導阻害の順に病徴が進展し，形成層の壊死と通導阻害が広く生じた場合に枯死にいたることが明らかにされた。

I. Introduction

In the course of development of the pine wilt disease, caused by the pine-wood nematode (*Bursaphelenchus xylophilus* (STEINER et BUHRER) NICKLE), dysfunction of water conduction in infected trees (IKEDA and SUZAKI, 1984; SASAKI *et al.*, 1984; TAMURA *et al.*, 1987; KURODA *et al.*, 1988) results in water deficiency in the leaves that causes changes in water relation parameters and cessation of photosynthesis and transpira-

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tion (FUKUDA *et al.*, 1992). Cavitation and embolism in tracheids of nematode-infected trees are assumed to be brought about by cytological changes in xylem parenchymatous cells (KURODA, 1989). From some anatomical studies, "denaturation" and death of parenchymatous cells, which are observed as changes in the stainability of cells with safranin and fast green, have been pointed out as one of the earliest symptoms of the disease (SUGAWA, 1982; MAMIYA, 1984). It has been shown that lipids in xylem ray and axial parenchymatous cells become stained differently by Sudan III, Sudan IV, or Nile blue in the course of the disease development (HASHIMOTO, 1980; KURODA, 1989). These phenomenon may be useful as indicators of cell senescence and an increase in secondary metabolites. IKEDA and SUZAKI (1984) suggested that the death of ray parenchymatous cells in affected xylem make it unable to refill the cavitated tracheids. KURODA (1989) hypothesized that physiological changes in terpenoid synthesis of xylem parenchymatous cells were responsible for cavitation in tracheids. On the other hand, SASAKI *et al.* (1984) hypothesized that oleoresin leakage from resin canals in xylem is responsible for the tracheid blockage, and NOBUCHI *et al.* (1984) observed vacuolation in xylem parenchymatous cells and occlusion of the pit membrane of tracheids. The relationship, however, between these cytological changes and the dysfunction of water conduction have not been clarified yet. To clarify the relationship, the sequence of initiation and spreading of these symptoms must be investigated minutely.

KIYOHARA (1989) found that some isolates of pine-wood nematodes seldom kill pine trees. Furthermore, there is a nonpathogenic nematode species, *B. mucronatus* MAMIYA et ENDA, which is closely related to the pine-wood nematode (MAMIYA and ENDA, 1979). We thought these avirulent and nonpathogenic nematode isolates might be useful for studying the mechanism of the symptom development and pathogenesis in pine wilt disease.

In this study, we examined cytological changes in xylem parenchyma, and the process of dysfunction of water conduction after inoculation with three kinds of nematodes with different pathogenicity, a virulent isolate (S6-1) and an avirulent isolate (C14-5) of *B. xylophilus*, and a nonpathogenic species, *B. mucronatus*. In addition, the treatment with an oxalic acid solution which induces cavitation in trees (COUTTS, 1977; SPERRY and TYREE, 1988) were included in our comparisons of cytological changes, water conduction, and other symptom developments with those in the pine wilt disease.

II. Materials and Methods

Three-year-old Japanese black pine (*Pinus thunbergii* PARL.) seedlings were used for our investigation. They were planted in clay pots and grown in a green house in the Koishikawa Arboretum, Faculty of Agriculture, University of Tokyo, Tokyo. The seedlings were watered twice a week. Five seedlings were used for each treatment.

Three thousand nematodes of virulent S6-1 or avirulent C14-5 isolate of *B. xylophilus*, or nonpathogenic *B. mucronatus* were inoculated in a notch on the stem at 15 cm above the ground on July 27, 1989.

Treatment with oxalic acid also was made on July 27, 1989, after SPERRY and TYREE (1988). A plastic collar was attached water-tightly around the stem at about 10 cm above the ground, and filled with 500 ml of an aqueous solution of 100 mM oxalic acid. Then, a notch was cut on the main stem with a razor blade under the surface of the solution, to make the seedlings absorb the solution from the notch.

To investigate disease development, resin exudation was checked by making wounds on different one-year-old branches by the tip of a scalpel at 3 or 4 days intervals. To monitor changes in the water status of the seedlings, base water potentials just before sunrise ($\phi_{w(max)}$) was measured in some leaves on the main stem with a pressure chamber (Model 600, PMS instrument, Co., Corvallis, USA.) each 3 or 4 days after the inoculation.

Every week after the inoculations for 3 weeks, one seedling of each treatment was harvested. The last two seedlings of each treatment were harvested 4 weeks after the inoculations. Dysfunction of water conduction was examined by the acid fuchsin absorbed overnight from the roots (SASAKI *et al.*, 1984). Dysfunctional areas were observed under a dissecting microscope as white patches in the red-dyed conducting xylem.

Then, stem segments for anatomical observations were taken at every 10 cm height above the ground and

fixed in FAA, and the rest of the stems were used for nematode isolation by the Baermann funnel technique. The fixed segments were sectioned by a freezing microtome. To check the physiological changes in parenchymatous cells, sections were stained with Nile blue, Sudan III, or safranin fast-green. Physiological changes in cells should be investigated by histochemical methods such as a comparison of enzyme activity in cells or the detection of some specific metabolites, however, in this study, as a preliminary investigation, the staining of lipids with Sudan III or Nile blue were used as well as an ordinary double staining with safranin and fast green. Usually, Nile blue (1%) is used for staining neutral lipids red and acidic lipids blue, however, in this study, Nile blue (0.02%) were used to stain acidic lipids (JENSEN, 1962). Using this staining, we found stainability of parenchyma cytoplasm drastically changed after the nematode infection. This phenomenon was used for checking the physiological changes in parenchymatous cells. For observation of bordered pit pairs, stem segments were dehydrated by ethanol series, critical-point dried, split with a razor blade to expose the pit membranes, and then observed under a scanning electron microscope (SEM, S-4000, Hitachi, Tokyo).

III. Results

1. Symptom development and water status of the seedlings

In seedlings inoculated with the virulent isolate S6-1, cessation of resin exudation was observed 10 days after the inoculation. Base water potentials ($\psi_{w(max)}$) in one of the seedlings in each treatment are shown in Fig. 1 as typical examples. A drastic decrease of $\psi_{w(max)}$ was observed in all seedlings from 2 to 3 weeks after the inoculations. At the times of $\psi_{w(max)}$ decrease, seedlings showed chlorosis and necrosis of needles, and they died 3 to 4 weeks after the inoculations. In the case of the avirulent C14-5 inoculations, resin exudation decreased after 2 or 3 weeks, however, neither $\psi_{w(max)}$ decreased (Fig. 1; representation of one of them), nor chlorosis of older needles appeared. In the case of *B. mucronatus* inoculations, resin exudation decreased slightly and to occasional, and no other symptoms appeared.

Every seedling injected with oxalic acid showed drastic decreases of $\psi_{w(max)}$ from 2 to 4 weeks after the treatment (Fig. 1). Chlorosis of older needles became obvious from 2 weeks after the treatment, and the seedlings were dead 4 weeks after the treatment.

2. Dysfunction of water conduction and nematode distribution in the seedlings

The development of the dysfunction of water conduction in xylem after nematode inoculations or oxalic acid injections was examined (Plate I, Table 2) in relation to the nematode distribution (Table 1).

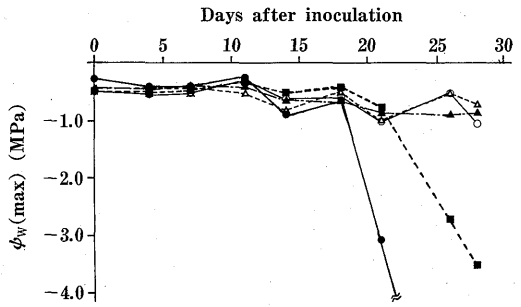

Fig. 1. Typical changes of base water potential ($\psi_{w(max)}$) in *Pinus thunbergii* seedlings after inoculations with nematodes with different pathogenicities and oxalic acid treatments

Legend: ○, Control; ●, *Bursaphelenchus xylophilus*, S6-1; ▲, *B. xylophilus*, C14-5; △, *B. mucronatus*; ■, Oxalic acid.

Notes: These data were obtained from one of the two seedlings in each treatment observed for 4 weeks. Other seedlings showed the same trends as shown here.

Table 1. Nematode distributions in the inoculated seedlings

	S 6-1 Weeks after inoculation				C 14-5 Weeks after inoculation				*B. mucronatus* Weeks after inoculation			
	1	2	3	4	1	2	3	4	1	2	3	4
Seedling size(cm)	77	65	70	70	70	65	55	70	82	60	60	70
Heights(cm)												
60	−	++	+++	++	−	−		−	−	−	−	−
50	++	+	+++	++	−	−	−	−	−	−	−	−
40	+	+	+++	+++	−	−	−	−	−	−	−	−
30	+	+	+++	+++	+	+	+	+	−	−	−	−
20	+++	+	+++	+++	+	+	+	++	−	++	−	−
10	++	++	+++	++	+	+	+	+	+	+	+	+
0	+	+	+++	++	+	+	+	+	−	−	−	+
Roots		+	+++	++		+	−	+	−	−	−	−

−, Nematodes not isolated; +, Less than 10 nematodes/g fresh weight; ++, 10～100 nematodes/g fresh weight; +++, More than 100 nematodes/g fresh weight.

Table 2. Dysfunction of water conduction in the inoculated seedlings

Heights (cm)	S 6-1 Weeks after inoculation				C 14-5 Weeks after inoculation				*B. mucronatus* Weeks after inoculation				Oxalic acid Weeks after inoculation			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
60	−	−	++++	++++	−	−		−	−	−	−	−	−	++++	++++	++++
50	−	+	++++	++++	−	−	−	−	−	−	−	−	−	++++	++++	++++
40	−	+	++++	++++	−	−	−	−	*	−	−	−	++	++++	+++	++++
30	−	+	++++	++++	−	*	−	−	*	*	−	−	++	++++	+++	++++
20	++	++	++++	++++	−	++	++	+++	+	+	−	−	+++	+++	+++	++++
10	−	++	++++	++++	++	+	+++	+++	*	+	++	++	+++	++	++	++++
0	−	++	++++	++++	−	+	++	+++	−	*	++	*	−	−	−	+++
Roots	−	+++	++++	++++	−	++	++	+++	−	−	+	*	−	−	−	+++

Relative areas of dysfunctions in cross sections.
−, Dysfunction not observed; *, One small area of dysfunction; +, 1～10% of the cross-sectional area; ++, 10～50% of cross-sectional area; +++, 50～99% of cross-sectional area; ++++, No water conduction.

The distribution of S6-1 already were seen widely in the seedling one week after the inoculation. Its population showed rapid growth 3 weeks after the inoculation. The distributions of C14-5 and *B. mucronatus* were restricted, and their populations did not grow as much as those of the S6-1 isolate even 4 weeks after the inoculation.

Dysfunctions of tracheids were observed from 1 week after the inoculation with S6-1 as white patches around the inoculation sites (Plate I). Thereafter, the dysfunctional area spread from the root to one-year-old main stem (Table 2). Water conduction completely stopped 3 weeks after the inoculations. In this period, necrosis of cambium, phloem, and cortex had begun (Plate I).

In C14-5 inoculated seedlings, dysfunction was observed 1 week after the inoculation only near the inoculation site (Plate I). Thereafter, dysfunction areas enlarged, especially in roots and in stems below the inoculation points, but the cambia were intact, and the newly-formed xylem near the cambia was still conducting (Plate I). Water conduction was maintained throughout the experiment in this case (Table 2).

In every seedling inoculated with *B. mucronatus*, dysfunction of the tracheids was found only in restricted areas near the inoculation sites, and they neither enlarged to the opposite sides of the stems, nor to the next internode from the inoculation sites (Plate I, Table 2).

In the case of the oxalic acid treatment, dysfunction occurred in the stems at 10 to 40 cm heights 1 week

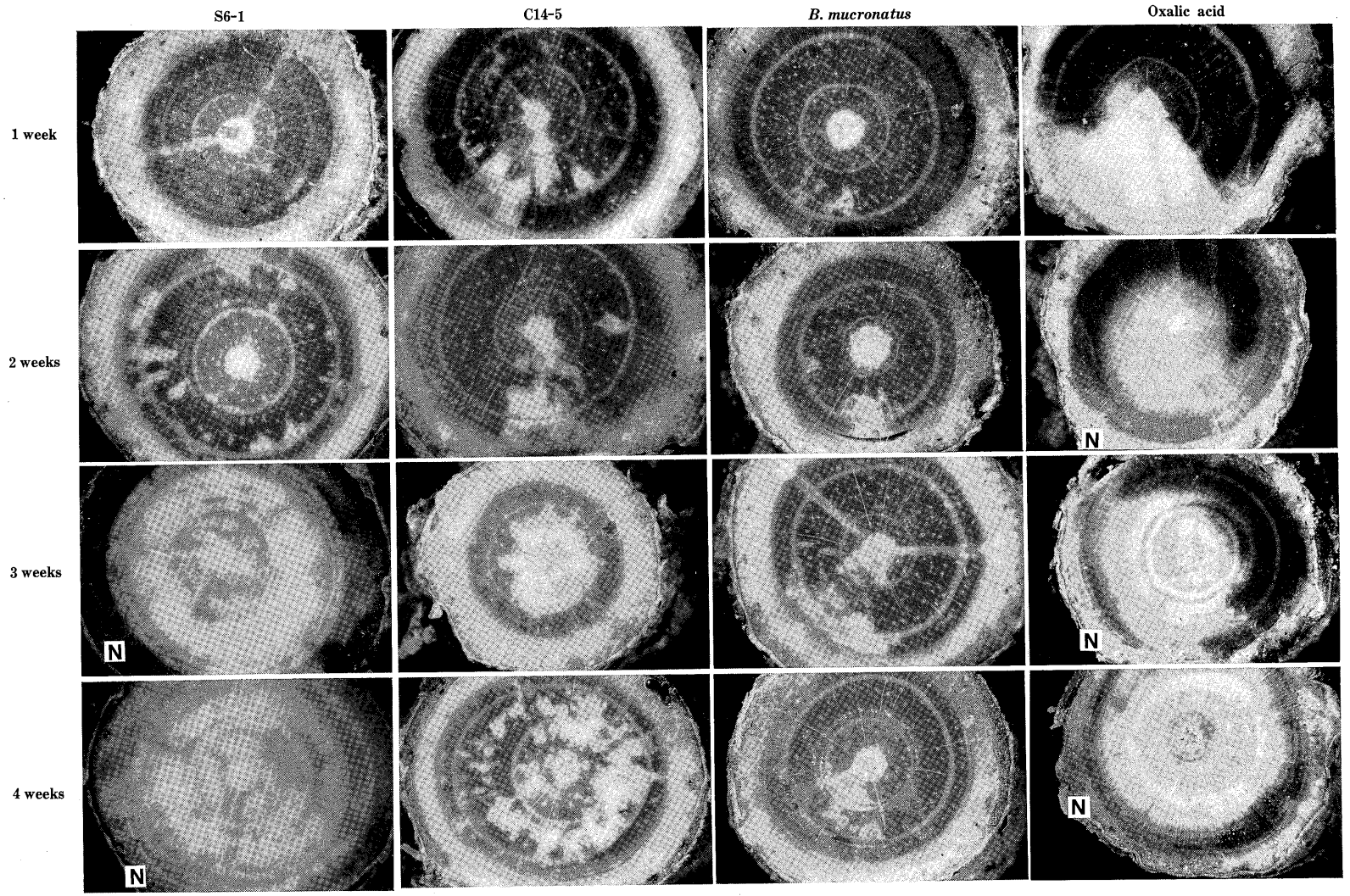

Plate I. Dysfunction of water conduction in *Pinus thunbergii* at a 20 cm height observed by the acid fuchsin method after nematode inoculations or oxalic acid treatments

S6-1, C14-5, *B. mucronatus*: seedlings inoculated with S6-1, C14-5, or *Bursaphelenchus mucronatus*; Oxalic acid: seedlings treated with oxalic acid. N: Necrosis of cambium and cortex.

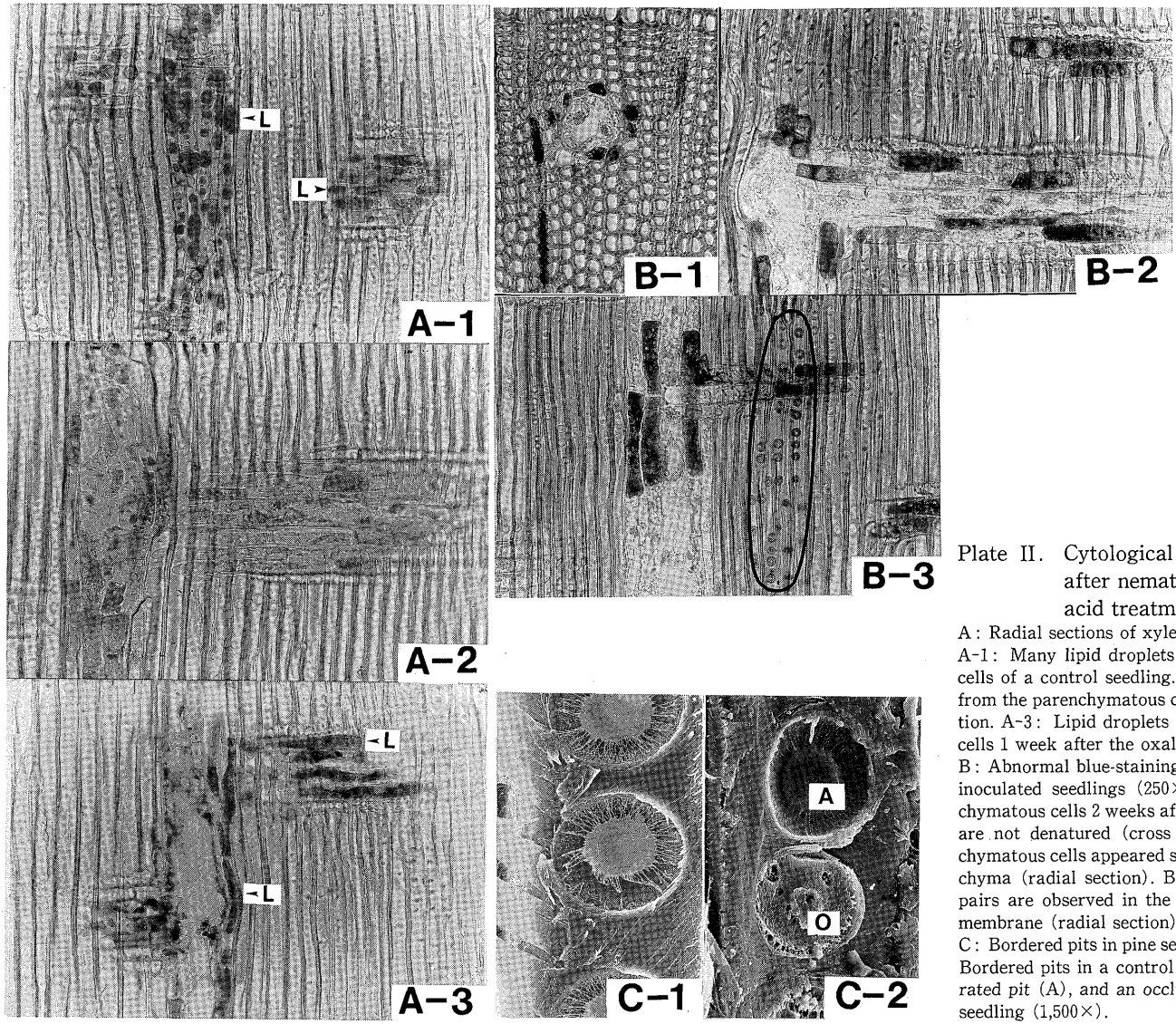

Plate II. Cytological changes in *Pinus thunbergii* after nematodes inoculations and oxalic acid treatments

A: Radial sections of xylem stained with Sudan III (250×). A-1: Many lipid droplets (L) are seen in parenchymatous cells of a control seedling. A-2: Lipid droplets disappeared from the parenchymatous cells 1 week after the S6-1 inoculation. A-3: Lipid droplets are seen in dead parenchymatous cells 1 week after the oxalic acid treatment.

B: Abnormal blue-staining of parenchymatous cells in S6-1 inoculated seedlings (250×). B-1: Denatured axial parenchymatous cells 2 weeks after the inoculation. Epithelial cells are not denatured (cross section). B-2: Denatured parenchymatous cells appeared scatteringly in ray and axial parenchyma (radial section). B-3: Blue-staining of bordered pit pairs are observed in the circle indicating occlusion of pit membrane (radial section).

C: Bordered pits in pine seedlings observed under SEM. C-1: Bordered pits in a control seedling (1,300×). C-2: An aspirated pit (A), and an occluded pit (O) in a S6-1 inoculated seedling (1,500×).

Table 3. Disappearances of lipid droplets in xylem cells observed by Sudan III

Heights (cm)	S 6-1 Weeks		C 14-5 Weeks		*B. mucronatus* Weeks	
	1	2	1	2	1	2
60	++		−		−	−
50	++	+	−	−	−	−
40	++	++	−	−	−	−
30	+	++	+	+	+	+
20	++	+++	++	++	+	+
10	+	+	+	++	+	+
0	+	++	+	++	+	−

−, Every parenchymatous cell contained lipid droplets; +, Droplets disappeared from less than 50% of the cells; ++, Droplets disappeared from more than 50% of the cells; +++, Droplets disappeared from all cells.

Table 4. Denaturation of xylem cells observed by Nile blue

Heights (cm)	S 6-1 Weeks		C 14-5 Weeks		*B. mucronatus* Weeks	
	1	2	1	2	1	2
60	−		−		−	−
50	−	+	−	−	−	−
40	−	+	−	−	−	−
30	−	++	−	+	−	−
20	+	++	−	++	+	−
10	−	++	+	++	−	+
0	−	++	−	++	−	+

−, No denaturation; +, Denaturation in less than 50% of the cells; ++, Denaturation in more than 50% of the cells; +++, Denaturation in all cells.

after the injections (Plate I, Table 2). Thereafter, dysfunction developed throughout the stems and the seedlings died in the fourth week.

3. Cytological changes in the tissue

In healthy tree xylem, parenchymatous cells, such as ray and axial parenchyma cells and epithelial cells, except for immature ray cells, contained many lipid droplets which were stained yellow with Sudan III (Plate II ; A-1). The first symptom in the xylem following each nematode inoculation was the disappearance of these lipid droplets from the parenchymatous cells (Plate II ; A-2). To discuss the relationship between the disappearance of lipid droplets and the development of dysfunction in water conduction, the decrease in the lipid droplets was examined in the first 1 weeks (Table 3).

One week after S6-1 inoculations, lipid droplets disappeared from most parenchymatous cells throughout the stems. However, epithelial cells around axial and radial resin canals contained many droplets even 2 or 3 weeks after the inoculations. Disappearance of lipid occurred in wider areas than dysfunctions of water conduction (Table 2). In the case of the C14-5 inoculations, the lipid disappearances also occurred more widely than the dysfunction areas, but the disappearances did not spread throughout the seedlings. In the case of *B. mucronatus* inoculations, lipids disappeared only near the inoculation sites, where dysfunctions of water conduction occurred. In the case of the oxalic acid treatments, xylem ray cells around the notches were dead and had deformed nuclei with lipid droplets remaining (Plate II ; A-3). On the opposite side of the notches, lipid disappeared from parenchymatous cells as in nematode-inoculated seedlings.

In addition to the staining of lipid, Nile blue indicated a drastic change of stainability in parenchymatous cells after the inoculations. In healthy pine trees, cytoplasm of phloem and cortex parenchyma cells scarcely were stained blue, and that of xylem parenchyma cells was not stained blue. After the nematode inoculations, cytoplasm of many xylem and cortex parenchyma cells became stained blue (Plate II ; B). This blue staining of cytoplasm corresponded to the change of color in cells stained with safranin and fast-green.

One week after the S6-1 inoculations, blue staining of xylem ray- and axial parenchyma cells were found scattered near the inoculation sites (Plate II : B-2, Table 4). Some showed granulation of cytoplasm and collapse of nuclei. At that time, the blue-staining was not observed in epithelial cells, although some adjacent parenchymatous cells were blue-stained (Plate II ; B-1). Two weeks after the inoculations, the blue-stained cells increased and were distributed more widely (Table 4). Some of these cells turned brown. Three weeks after the inoculations, most of parenchymatous cells in xylem, phloem, and cortex turned brown. In every cross section in the first 2 weeks, the blue-staining of xylem parenchymatous cells were observed in wider areas than in the dysfunctions of water conduction.

In the case of C14-5 inoculations, in which dysfunction of water conduction occurred in restricted areas of the inner xylem (Plate I), the blue-stained cells were observed only in and adjacent to the dysfunctional

areas at the same heights of dysfunction (Tables 2, 4). Blue-staining rarely was observed in newly-formed xylem adjacent to cambia, and was observed widely in cortex cells throughout the stems. In the case of *B. mucronatus*, in which water conduction was not affected in most tracheids (Plate I, Table 2), only some cells near the inoculation sites were blue-stained (Table 4). In oxalic acid-treated seedlings, the blue-stained cells were observed throughout the stems on the sides opposite the injection notches.

In tracheids of nematode-inoculated seedlings, some bordered pit pairs were occluded with some substances which were stained blue with Nile blue (Plate II ; B-3). Occlusions of pit membranes also were observed under a SEM (Plate II ; C-2 : "O"). However, most tracheids in dysfunctional area did not show such pit occlusions, and those pits that were occluded always were aspirated at the same time. Pit aspirations frequently were observed without occlusions in seedlings after nematode inoculations and oxalic acid-treatments (Plate II ; C-2 : "A"). These aspirated or occluded pits were observed rarely in control seedlings (Plate II ; C-1).

IV. Discussion

The main difference in the symptom development among the seedlings inoculated with the three types of nematodes of different pathogenicity and a chemical treatment was the severity of the dysfunction in water conduction. Virulent S6-1 and oxalic acid-treatment caused severe dysfunction which finally resulted in water deficiency of the leaves. However, avirulent C14-5 and nonpathogenic *B. mucronatus* caused dysfunctions in the restricted areas so that physiological changes in leaves and seedling deaths never occurred. These results agreed with those of IKEDA *et al.* (1990) and FUKUDA *et al.* (1992), in which the water potentials did not decrease after the inoculations of C14-5 or *B. mucronatus*. In the dysfunctional area of xylem, aspiration of bordered pits which indicate cavitation was observed more widely than occlusions by solid or liquid substances. Therefore, the dysfunction of water conduction in inoculated seedlings was demonstrated to be caused by cavitation by gaseous substances at first (IKEDA and SUZAKI, 1984 ; KURODA, 1989 ; KURODA *et al.*, 1988).

We also found that the distribution and the population growth of nematodes were restricted in the case of C14-5 and *B. mucronatus* in contrast to the case of S6-1, and they exactly corresponded to the development of the dysfunction area in xylem. ODANI *et al.* (1985) also reported a restricted distribution of *B. mucronatus* in Japanese black pine seedlings. The dispersal and reproduction of nematodes were demonstrated to have close relationships to cavitation development.

Another great difference between S6-1 and the other two types of nematodes was found in the cambium destruction. C14-5 and *B. mucronatus* could not destroy cambial zones except for the inoculation sites. In those seedlings, water conduction was not affected in newly-differentiated tracheids near the cambia. A similar phenomenon also was reported by SASAKI *et al.* (1984) in survived seedlings after inoculations with virulent nematodes. In woods mechanically wounded or inoculated with fungi, pathological heartwood or the dry zone, that is, cavitation area, progresses further in the inner rather than in the outer sapwood (COUTTS, 1976 ; YAZAWA *et al.*, 1969). These facts suggest that the inner xylem is easier to be cavitated than the outer xylem. If cambial death is caused by cavitation in neighboring tracheids, it is possible that the pathogenicity of nematodes depends on their ability to induce cavitation in wide areas including newly differentiated tracheids adjacent to the cambium. The other possibility is that pathogenicity depends on the ability of nematodes to affect the cambium. Cambial death should induce physiological changes in adjacent ray cells and subsequently induce cavitation. Cavitation in newly differentiated xylem and the death of the cambia always were observed at the same time in this study. Which of these is the cause or the result is most important for making clear the mechanism of pine wilt disease.

Disappearance of lipid droplets from xylem parenchymatous cells, which was the first cytological symptom after the nematode infection, is thought to be a response to signals triggered by a small number of nematodes, because it occurred widely before the increase of the nematode population. In epithelial cells of *Pinus pinaster* AIT., lipid droplets disappeared during terpenoid synthesis after wounding and fungus inoculation (CHENICLET, 1987 ; WALTER *et al.*, 1989). Terpenoids such as monoterpenes increase in

nematode-infected wood, and are suspected to be the causal agent of gas embolism in pine-wood nematode-infected xylem (KURODA, 1989). Thus, the disappearance of lipid droplets in xylem parenchymatous cells by nematode infections seems to be related to the increase of terpenoid synthesis.

Parenchymatous cells of nematode-infected pine trees exhibit changes in stainability with Nile blue or safranin-fast green. Because granulation of cytoplasm and collapse of nuclei occur in these cells, such changes represent physiological changes in the cells which would be followed by cell deaths. These denaturations in xylem parenchymatous cells appeared at the same height as the cavitation in all types of nematode inoculations and oxalic acid treatment, and were observed more widely than cavitations in cross sections. This fact indicates that the physiological changes and deaths of xylem parenchymatous cells have an important role in the occurrence of cavitation. We found that denaturation had started already in ray and axial parenchyma cells before epithelial cells were affected. This fact suggests that denaturations are not caused by a direct contact with the nematodes because nematodes are known to move through resin canals destructing epithelia (MAMIYA, 1984). Thus, denaturation is probably an active response of the host tissue to some stimulus, such as hypersensitive cell death, which is known to have a scattered pattern of cell deaths (DOKE, 1982). In the response of *Cryptomeria* wood to a fungal infection, blue-staining of cells by Nile blue occur, and blue-stained substances containing antifungal phenolics are secreted into tracheids and deposits into pit membranes (YAMADA *et al.*, 1988). The similarity of internal symptoms caused by pine wilt disease, such as cavitation, resinosis, terpenoid synthesis, and cell death, and host responses of coniferous woods against infection by wood-inhabiting fungi (BOLLA *et al.*, 1984; MYERS, 1988; KURODA, 1989) supports the above hypothesis. To explain the mechanism of cavitation and pathogenesis of the disease, it must be clarified what is happening in the denatured parenchymatous cells.

The restricted occurrence of denaturation in xylem was supposed to be the factor that prevented the wide development of cavitation in seedlings inoculated with C14-5 and *B. mucronatus*. Our results demonstrated that the restricted distribution and small population growth of avirulent and the nonpathogenic nematodes resulted in the restricted occurrence of denaturation of the xylem cells. Nematodes are known to be distributed wider in the cortex than in the xylem in the early stage of the disease (MAMIYA, 1984) and in the case of resistant pine species inoculated with virulent nematodes (TAMURA and DROPKIN, 1984). Judging from these facts, the nematode movements in cortex, phloem, xylem and across the cambium must be investigated more minutely, because they must be the key factor in determining the ability of nematodes to induce cavitation in xylem, to affect the cambia, and finally to kill the tree.

In the seedlings treated with oxalic acid, parenchyma necrosis around the notch without lipid disappearance indicated rapid cell death caused by the toxicity of oxalic acid. However, lipid disappearance and denaturation on the opposite side were the same as those in nematode inoculations. These sequential changes of the parenchymatous cells; namely, lipid disappearance, denaturation of cytoplasm, and cavitation in tracheids, are thought to be a general response of pine tissue to injury or infection. The fact that the chlorosis of older needles by the oxalic acid-treatment was similar to that in S6-1 inoculations suggests that this characteristic symptom of pine wilt disease is induced by cambial death or cavitation in the outer xylem which were the common internal symptoms of the two.

In conclusion, the process of disease development by virulent nematodes was thought to be as follows: In the first stage, the disappearance of lipid droplets in xylem parenchymatous cells are induced by a small number of nematodes, and this might be related to terpenoid synthesis in pine tissue. In the second stage, denaturation and death of the cells occurs, resulting in cavitation in the neighboring tracheids. In some cases, it results in the occlusion of tracheid lumina and pit membranes by released substances. These cytological symptoms and cavitation progress successively in this stage. In the final stage, these internal symptoms spread throughout the seedlings, nematode populations grow, cambium is killed, and water conduction completely ceases. At this time, water deficiency in leaves, cessation of photosynthesis and transpiration, and chlorosis of older leaves take place (FUKUDA *et al.*, 1992). In the avirulent C14-5 isolate or *B. mucronatus* inoculated pines, the internal symptoms of the first and the second stages also occur. However, they are restricted to cortex and inner xylem, or to the vicinities of the inoculation sites. These restricted develop-

ments of cytological symptoms corresponded to the restricted cavitation and nematode distributions. Thus, the final stage observed after the inoculations with virulent nematodes never appears in the case of avirulent and nonpathogenic nematodes inoculations. The factor which restricts nematode movement and cytological symptoms should be investigated further.

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