日植病報 55: 170–178 (1989)
Ann. Phytopath. Soc. Japan 55: 170–178 (1989)

Terpenoids Causing Tracheid-Cavitation in *Pinus thunbergii* Infected by the Pine Wood Nematode (*Bursaphelenchus xylophilus*)*

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Abstract

Following invasion by the pine wood nematode, *Bursaphelenchus xylophilus*, cavitation develops in the sapwood of *Pinus thunbergii*. This causes abnormal water conduction in the trunk, and is supposed to result in death of infected trees. Cavitation, assumed to be vapor blockage, can be induced by gas produced inside the tree without induction of air from outside. To detect the substances causing cavitation, constituents of vapor in the cavitation areas were analyzed by gas chromatography using xylem harvested 2 weeks after the inoculation with pine wood nematode. Amount of mono- and sesquiterpenes increased significantly in the nematode-inoculated sample: α-pinene was 2 to 4 times that of healthy tree, β-pinene and several other monoterpenes 2 to 3 times, and longiforene, a sesquiterpene, *ca.* 3 times. Terpene synthesis seemed to be activated before development of cavitation. Monoterpenes excessively produced and exuded in tracheids will vaporize easily under negative pressure in summer months, and will disrupt water columns of the tracheids throughout wide areas within short time. Even when water stress is reduced during night or in autumn, refilling of tracheids with water will be prevented by hydrophobic terpenoids. It is proposed that the vapor blockage of tracheids caused by evaporating terpenoids is responsible for extensive cavitation in pine sapwood within a short period after the invasion of pine wood nematode, and pine trees are eventually killed by water deficit following the cavitation.

(Received September 28, 1988)

Key words: monoterpene, α-pinene, cavitation, vapor blockage, *Bursaphelenchus xylophilus*, pine wilt disease.

INTRODUCTION

Cavitation of tracheids is a remarkable initial symptom of pine wilt disease caused by the pine wood nematode, *Bursaphelenchus xylophilus*[13]. Kuroda *et al.*[16] emphasized that the ultimate death of pine tree is due to water deficit induced by extensive cavitation of sapwood. Kozlowski[14,15] explains that some wilting diseases kill trees by interfering with water translocation rather than by an immediate and direct toxic effects[22]. Substances causing cavitation must be discovered to assess why pine trees are killed by the pine wood nematode.

Cavitation is first recognized as white streaks appearing on the cross surface of trunks of *Pinus thunbergii* from 2 weeks following inoculation with pine wood nematode[16]. Aqueous dye-solution injected into the base of a standing pine does not diffuse into the cavitation areas, suggesting the presence of some hydrophobic substances in those areas. Cavitated areas enlarge drastically and finally reach the cambium by 4 weeks. At that period, water content of the xylem is only 30 to 40% of healthy trees, and necrosis of cambium, phloem and parenchyma

* A part of this study was presented at the 5th International Congress of Plant Pathology, Kyoto, 1988.

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cells become conspicuous in the trunk[16]. Such necrosis appears to be a secondary symptom caused by water deficit after the progression of cavitation.

Zimmermann[32] indicated that vapor blockage was an important aspect of xylem blockage, and that little attention was paid to this in the pathology literatures. Recent investigations on fungal disease of trees[4,5,29] state that vapor blockage is the cause of tracheid cavitation and it leads to water deficit in plants. It is proposed that once a bubble is produced in a tracheid, the bubble must increase size until the entire tracheid is vapor-filled[28,32]. Vapor seems to be more effective in cutting the water columns of tracheids quickly and widely than would either liquids or solids such as resin os degraded cell-substances. Nevertheless, in the case of pine wilt disease, the concept of vapor blockage and following cavitation of tracheids has been completely overlooked in explaining the mechanism of wilting.

The most significant problem concerning the mechanism of vapor blockage is from where and how air or gas is produced in xylem[4,5,32]. Introduction of air bubbles from outside through ray cells is doubtful. Gregory[10] noted that in the absence of a pathway to the atmosphere, such as a wound, it was difficult to envisage how external gas could reach the xylem elements. In the pine wilt disease, cavitation develops in xylem without penetrating cambium and phloem[16], suggesting that vapor is produced inside the trunk. If a certain volume of volatile substances are synthesized inside plants in response to the nematode infection, they can vaporize under negative pressure due to water stress in trunks. The author hypothesizes that vapor which cause cavitation consists of volatile substances excessively synthesized in parenchyma cells after the nematode invasion.

The purpose of the present experiments is to define the substances which directly cause cavitation of tracheids, and to relate this to a discussion why pine wood nematode is harmful and fatal to pine trees. To answer these questions, xylem in the early stage of cavitation was removed from nematode-inoculated trees and analyzed by gas chromatography, in parallel with anatomical observation of the pine tissue.

MATERIALS AND METHODS

Inoculation and sampling. Three 11-year-old Japanese black pines (*Pinus thunbergii*) were inoculated with 10,000 *Bursaphelenchus xylophilus* into a branch at the height of *ca.* 200 cm. Inoculation was done on September 22, 1987, by the same method as previously described[16,27]. One week following inoculation, two of the inoculated pines were injected with acid fuchsin from the bottom of trunks as described earlier, and one day later were harvested. Two weeks after the inoculation, the third pine and a non-inoculated control were harvested. Disks of 2 cm in thickness were cut from each trunk at a height of 50, 150, and 250 cm. Three blocks ($2\times2\times2$ cm) were cut from each disk and prepared for anatomical observation. The residual part of the disks were kept for one month to detect nematode distribution by incubation method[16].

For analysis by gas chromatography, the trunk harvested 2 weeks after inoculation, when the cavitation first became obvious, was stored at -20 C till used.

Anatomy. Sample blocks prepared from the disks were fixed with formalin, acetic acid and water (5:5:90, v/v) for several days. To avoid the elution of ethanol soluble materials, ethanol was not added to the fixation solution. The blocks were then washed with tap water for one day.

To observe the contents of cavitated tracheids, some of the blocks were embedded in gelatin as previously described[16]. Sections of three planes, 30 to 40 μm in thickness, were cut from inoculated and control samples with a sliding microtome, stained with nile blue, and observed with a microscope. Those sections were compared with the cavitation areas observed on the blocks with a stereo microscope.

Gas chromatography. Samples harvested 2 weeks after inoculation, and non-inoculated control were analyzed for xylem components by gas chromatography.

Charcoal powder (60 mesh) was washed with acetone and dichloromethane and activated by heating at 160 C over night. Fifty mg portions of charcoal powder was filled in glass tubes.

Xylem chips, made from cavitated areas of the inoculated sample and from the control sample without inoculation, were ground for 2 min while cooling with flowing water. Five grams of each sample were mixed with 30 ml of distilled water in flasks. The method of steam distillation is a slight modification of Nabeta *et al.*[20] and Sugisawa *et al.*[24] The flask was heated at 90 C for 1 hr while constantly bubbled with nitrogen gas at the flow rate of 100 ml/min. The nitrogen gas coming out from the flask was passed through activated charcoal in the tube. The charcoal trap was heated at 60 C to avoid the aggregation of water drops during the steam distillation.

Volatiles trapped onto the charcoal were eluted with 2 ml portions of CH_2Cl_2, and the solvent was then dried over 2 g of anhydrous Na_2SO_4 in a glass column. The solution was condensed for one night in the refrigerator after the addition of internal standard, dodecane. The residue of powdered xylem was oven dried at 105 C for one day and weighed.

After the solution was concentrated *ca.* 5 times, 1 μl was analyzed with a gas chromatograph (Yanaco G3800) equipped with a flame-ionization detector. The column was a 25 m$\times$0.25 mm i.d., silica capillary column (FFAP). Temperature of the column was 60 C for 10 min and then gradually raised by 5 C/min until 160 C. Temperature of injector and detector was 220 C. The carrier gas used was N_2 at a flow rate of 1.0 ml/min.

Volatile constituents were identified by comparison with the gas chromatograms of standard chemicals included in xylem of the *Pinus* species[31]. The amounts of detected substances were calculated based on internal standard.

To confirm whether metabolic changes, such as *de novo* synthesis of xylem components begin in cells before cavitation or subsequent to development of cavitation because of water shortage, the volatile components in xylem were compared between conspicuously cavitated areas (Plate I-1, arrow a) and non-cavitated areas (Plate I-1, arrow b) within a same disk harvested 2 weeks after the nematode inoculation.

RESULTS

Anatomical alteration in pine tissue

Pine wood nematode was detected throughout the trunk 1 week after inoculation, by the incubation of wood for one month. Cavitation was not clearly detected with naked eyes at this time.

Since the experimental set up was delayed beyond the natural infection season of this disease, the development of cavitation appeared to be slightly retarded as compared with the previous experiment on tracheid blockage which was conducted during August in the same year[16]. Two weeks after nematode inoculation, cavitation areas were still small white spots on the cross surface of the trunk (Plate I-1, arrowheads. Compare with I-2). Such spots distributed across between half to two-third of the cross surface at the height from 50 to 250 cm. The other part was rarely cavitated and the cavitation was restricted to late wood. This stage was judged to be just the beginning of cavitation.

Plate I-3 is a magnified cross surface showing areas of cavitation 2 weeks after the inoculation, and Plate I-4 is a cross section cut from the same area with I-3. Vapor-filled tracheids are not plugged (Plate I-3, 4, arrows) except for some tracheids surrounding a few vertical resin canals (Plate I-4, arrowhead) as in the case of the experiment conducted earlier in the same year[16]. When stained with nile blue, the unidentified plugging material was slightly different in color from leaked resin. This material was observed from 1 week after the inoculation in a very few tracheids.

Droplets which stained with nile blue were scarce in the tracheids, and sometimes no droplets were observed in the cavitated areas, even when care was taken to retain ethanol-soluble contents. In radial sections, it was seen that organelle of ray parenchyma cells and epithelium surrounding resin canals in the cavitated area were deformed and stained differently with nile blue from those in the control sample (Plate I-5, 6).

Components of extracts

Typical gas chromatograms of volatiles extracted by steam distillation from cavitation areas of the sample harvested 2 weeks after the inoculation and from non-inoculated control were shown in Fig. 1. The kinds of monoterpenes in the inoculated sample are nearly identical with those found in the control. In both cases, the following seven monoterpenes were identified by the comparison with standard substances: α-pinene, camphene, β-pinene, myrcene, limonene, β-phellandrene, and p-cymene. In the inoculated sample, all monoterpene peaks except that of limonene were higher than those of the control sample. The most conspicuous increase was seen in α- and β-pinene, myrcene, and β-phellandrene. In addition, many peaks, assumed to be those of sesquiterpenes, were seen in both samples. Although they also increased in the inoculated sample, only longiforene which showed the greatest increase was identified by comparison to a known standard. Other high peaks have not been identified yet.

The amount of these substances in inoculated and uninoculated wood samples were calculated based on the internal standard, dodecane (Fig. 2). Among all monoterpenes, α-pinene increased by 2 to 4 times in the inoculated sample. Of all the monoterpenes in healthy pines, α-pinene presents in the largest in quantity. It is 60% of total monoterpenes in the control, and 70% in the inoculated sample. β-pinene and several other monoterpenes increased by *ca.* 2 times in the inoculated sample. The sesquiterpene longiforene increased to 3 times.

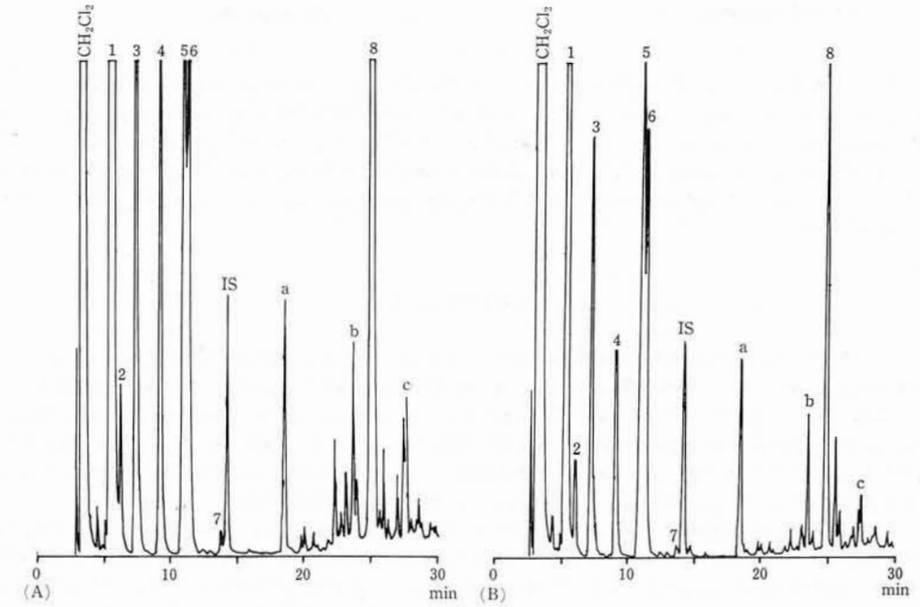

Fig. 1. Gas chromatograms of volatile substances detected in the xylem of *Pinus thunbergii* inoculated with *Bursaphelenchus xylophilus* (A), and control sample without nematode inoculation (B).
Peaks 1: α-pinene, 2: camphene, 3: β-pinene, 4: myrcene, 5: β-phellandrene, 6: limonene, 7: p-cymene, 8: longiforene. a, b, and c: unidentified substances, IS: internal standard, dodecane.

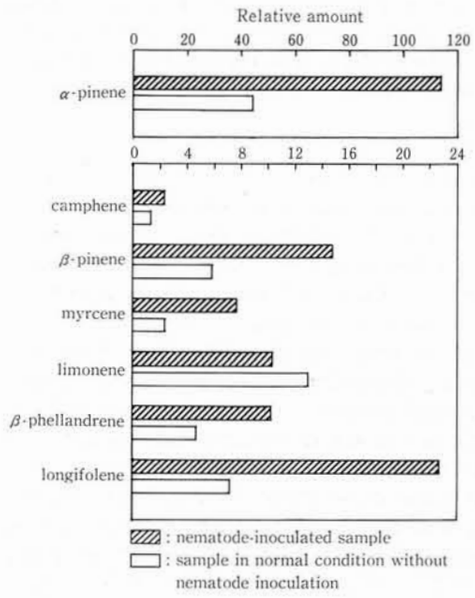

Fig. 2. Increase of mono- and sesquiterpenes in the nematode-inoculated sample. Horizontal axis: relative quantities based on internal standard.

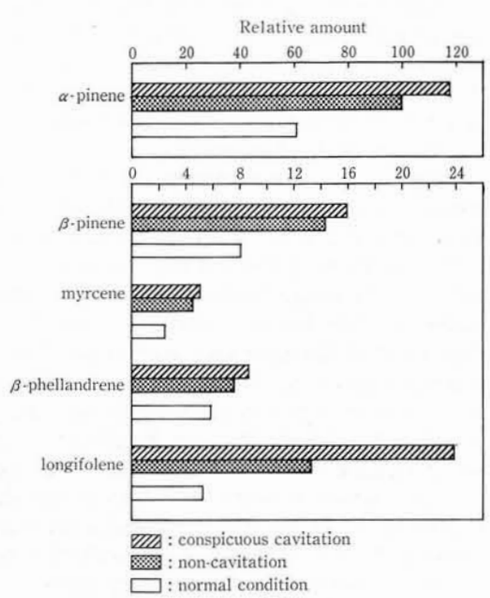

Fig. 3. Comparison of the amount of terpenoids in the conspicuously cavitated area (Plate I-1, a) and non-cavitated area (Plate I-1, b) on the same disk.

When the amount of terpenoids were compared between conspicuously cavitated area and non-cavitated area within a disk of the inoculated sample (Fig. 3), there was a clear-cut increase of monoterpenes in the non-cavitated area also. For instance, the amount of α-pinene reached 81% and β-pinene reached 86% of that detected in the cavitated area. The increase of longiforene, however, was not as conspicuous in the non-cavitated area and was 53% of that in the cavitated area.

DISCUSSION

With the start of cavitation in the sapwood, mono- and sesquiterpenes increased significantly. The main constituents were the same as in healthy trees and unusual volatile chemicals were not detected. Increase of volatile oils has been reported to accompany the dying process of pine trees infected with pine wood nematode and these volatile oils act as attractants of pine sawyer, the vector of the pine wood nematode[11]. However, the origin of these materials and their effects on the physiology of pine trees have never been discussed.

In the present experiment, monoterpenes increased both in cavitated and non-cavitated areas of infected trees. This indicates the metabolic pathway of terpene synthesis is activated prior to the start of cavitation, and monoterpenes are not synthesized as a result of cavitation. Increase of sesquiterpenes seems to be retarded a little from monoterpene synthesis. These observations point to the fact that monoterpenes can contribute the development of cavitation.

Increase of some terpenoids was reported together with a dry zone formation in *Pinus* species after inoculation with blue-stain fungi such as *Ceratocystis*[2,6,19]. On the other hand, terpene synthesis and subsequent formation of lightwood identical with the cavitation area and "dry zone" were reported after paraquat was applied to pine stem[12,30,31]. These observations of

the response of pine to fungal disease, chemical injuries, and the pine wood nematode, suggest a close relationship between terpene synthesis and cavitation of drying or sapwood.

Cavitation development observed during the previous[16] and present experiments suggested the gas filling tracheids is not air induced from outside of bark, but is produced inside trunks. The unresolved question is how the gas is formed. Coutts[4,5] suggested that in the case of fungal disease some substances diffuse from the infected region might be responsible for initiating gas embolism. Nevertheless, volatile substances such as monoterpenes have never been proposed as a candidate for the agent which effectively cuts the water column of the vascular system.

In diseased wood, the plasma membrane of parenchyma cells is assumed to be degraded and hence leaky[21,23]. Excessively produced terpenoids in parenchyma cells will be exuded into adjacent tracheids through pits. High negative xylem pressure of greater than -10 atm is reported during the day-light hours in conifers and water column is maintained by tensile strength of water[32]. In addition, temperature rises above 30 C during mid-day of summer. Under such condition, volatiles should evaporate easily. Following the initial bubble formation, it is assumed that the gas will be expanded instantly and fill many tracheids through the pits as described by Zimmermann[32], and by Tyree and Dixon[28]. These hydrophobic substances prevent the redispersal of water into the cavitated tracheid. The death of pine trees due to the pine wilt disease is retarded by the everyday watering after inoculation[25]. This procedure releases water stress[25,26]. This suggests that if negative pressure is relaxed, cavitation development will be slowed.

Even in healthy trees, seasonal or diurnal cavitation and refilling of cavitated tracheid is assumed to occur[29] based on reports that water contents decrease in many conifers at most 50% in summer especially during the day[3,9], and that cavitation occurs every day in herbaceous plants[18]. Dissolved air in xylem sap may emerge as small bubbles in tracheids and initiate transient cavitation when the xylem is under extensive negative pressure in high temperature. If some tracheids do cavitate transiently in the sapwood of pinewood-nematode inoculated tree and if the synthesis of terpenoids have been activated by nematode inoculation, volatile substances could evaporate and disperse easily in tentatively cavitated tracheids. Even if the water stress is released during night, terpenoids attached to the tracheid walls and pit membrane prevent the refilling of tracheids with water by their hydrophobic character.

Usually volatile oil, or essential oil, is completely distinguished from resin, but is believed to be exuded only from epithelium to resin canal together with resin acid as oleoresin constituents[7,8]. The present experiment proposes that ray parenchyma cells also function as a site of terpene synthesis based on the observation that physiological changes were observed in ray cells, that droplets were exuded from ray cells into cavitation areas, and that cavitation develops as radial streaks along ray tissue. Droplets stained with nile blue and exuded from ray cells into the cavitation area may include volatile oil together with non-volatile substances.

The results of present experiment support the hypothesis that the cavitation, which is assumed to be the lethal factor in pine trees infected by pine wood nematodes, is caused by vapor blockage with volatile substances. These volatiles include monoterpenes and possibly sesquiterpenes synthesized in parenchyma cells. This may also occur in fungal diseases[1,6,10,17], where volatile substances are known to increase after infection and may be the cause of wilting or drying of xylem. Dry zone formation accompanying fungal infection is usually restricted to a small area surrounding the infection zone and is not lethal to the infected tree. Although some terpenoids including monoterpenes are reported as antimicrobial substances or phytoalexin[12,19], in the case of pine wilt disease, monoterpenes seem to play a significant role in killing pine trees instead of preventing nematode activity as phytoalexin.

The mechanism of pine wilt will be explained as follows: 1) Injuries by moving and feeding nematodes stimulate the synthesis of terpenoids in parenchyma cells. Metabolite of the nematode, or enzymatically degraded cell-substances by the nematode activity may contribute to this process. 2) Following the aging of parenchyma cells, terpenoids are exuded through degraded

plasma membrane into adjacent tracheids which may cavitated transiently during the day. 3) Volatile terpenoids evaporate in tracheids under negative pressure and make bubble. 4) Monoterpene gas fills throughout a tracheid and then cavitates neighboring tracheids. 5) Refill of cavitated tracheids with water will be prevented by hydrophobic effects of terpenoids, even when the water stress is released during the night, therefore, permanent cavitation enlarge gradually. 6) Cavitated areas reach to cambium, and the tree dies due to the water deficit.

At present, monoterpenes are cited as the most effective substances for vapor blockage. In order to prove this point, investigation of other substances such as ethylene must be checked to see if they also increase after inoculation and contribute as vapor. The transient cavitation must be confirmed as to whether it precedes the exudation of terpenoids. Together with the discussion on the amount of monoterpenes necessary to dehydrate the lumen of a tracheid, the contribution of sesquiterpenes to cavitation will be investigated. Direct demonstration that monoterpenes cause vapor blockage in tracheids is difficult. Therefore, several indirect experiments are necessary in order to explain the mechanism of cavitation in detail. The author is planning periodical analysis of xylem contents together with observations on cavitation events after the nematode inoculation. It will add much information on the relation between vapor formation and cavitation. Inoculation of pine wood nematode into several *Pinus* species which are resistant to the nematode such as *P. taeda* will also provide useful evidence on this subjects.

The author wishes to thank Prof. R.F. Myers, Rutgers University, U.S.A., and Prof. R. Bolla, University of Missouri, St. Louis, U.S.A., for their critical reading of the manuscript. Thanks are due to the members of the Forest Pathology Laboratory, Kansai Research Center, Forestry and Forest Products Research Institute, for their help during the experiment.

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和 文 摘 要

黒田慶子：マツ材線虫病において仮道管キャビテーションをひき起こすテルペン類

マツノザイセンチュウが侵入すると，マツ樹幹内では仮道管のキャビテーション（空洞化）が起こる。仮道管から水が排除されて通水阻害が進行し，マツは最終的に水不足で枯死するものと判断された。キャビテーションは，気体または蒸気が仮道管に充塡して起こると推定されているが，その気体は外部から導入された大気ではなく，材内で生産される可能性が高い。キャビテーションの原因物質を明らかにするために，キャビテーション部位に含まれる気体の成分をガスクロマトグラフ法により分析した。線虫を接種して2週後の，キャビテーションが始まった直後のクロマツの材内では，モノテルペン，セスキテルペンの量が増加していた。健全木に比べて α-ピネンは 2～4 倍，β-ピネンおよび数種のモノテルペンは 2～3 倍，ロンジフォレンは約 3 倍であった。接種試料では，キャビテーションが起こっていない部位でもモノテルペンの量がすでに増加しており，テルペン合成の活性化はキャビテーションに先駆けて柔細胞内で起こるものと判断された。仮道管は夏期の水ストレスのために極度の負圧状態に置かれ，モノテルペンは柔細胞の壁孔から滲出すると，容易に気化するものと推定された。テルペン類は疎水性であるため，たとえ水ストレスが夜間に緩和されても，仮道管に水が再び入るのを阻害するであろう。これらの結果から，線虫の侵入により合成が促進された揮発性物質，モノテルペン，セスキテルペンは，蒸気として仮道管の水柱を切断し，キャビテーションを短期間にしかも広範囲に起こすのに，重要な役目を果たすものと判断した。

Explanation of plate

Plate I. Cavitation observed in the xylem of *Pinus thunbergii* inoculated with *Bursaphelenchus xylophilus*.

1. Beginning of cavitation shown as white fusiform spots (arrowheads) on the cross cut surface of a pine trunk 2 weeks after the nematode inoculation. Conspicuously cavitated part (arrow a) and non-cavitated part (arrow b) both were used for gas chromatography (see Fig. 3). (×1.0)
2. Disk surface of a pine trunk without nematode inoculation. Cavitation is not observed. (×1.0)
3. Magnified disk surface of cavitation areas 2 weeks after inoculation. Vapor-filled tracheids are observed as a whitish area (arrow). (×24)
4. A cross section of the same area with I-3. Tracheid plugging stained with nile blue is found only around a vertical resin canal (arrowhead). Most part of cavitation area (arrow: the same site with the arrow in I-3) is without plugging. (×26)
5. Normal organelles in ray parenchyma cells of control sample without nematode infection (radial section). (×260)
6. Deformed organelles in ray cells in a cavitation area of nematode inoculated sample. Cell contents are stained differently with nile blue from normal ray cells seen in I-5. This indicates the change in physiological aspects of cells in cavitated areas. (×260)

Plate I