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Bursaphelenchus xylophilus induced pine wilt: Factors associated with resistance

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Abstract

We investigated factors affecting resistance of *Pinus* spp. to *Bursaphelenchus xylophilus*. Nematode migration and multiplication was inhibited in resistant *P. taeda*. As a consequence acceleration of monoterpene synthesis terminated, tracheid-cavitation did not become extensive, and xylem sap ascent was not significantly blocked. In *P. strobus*, inhibition of nematode activity was not as complete as in *P. taeda*, and temporal needle-wilting was seen on some branches. Although this suggest some blockage of xylem water conduction, we were unable to confirm development of cavitation induced by nematode activity. β-myrcene concentration in these species was not related to resistance.

1 Introduction

Resistance to pine wilt disease caused by *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle has been compared among many pine species (OHYAMA et al. 1974; FUTAI and FURUNO 1979; LINIT and TAMURA 1987). In the diploxylon group, *Pinus thunbergii* Parl. and *P. densiflora* Sieb. et Zucc. of subsection *Sylvestres* are highly susceptible to this nematode in Japan. *P. taeda* L. and several species of *Australes* are resistant (TANAKA 1973). *P. strobus* L., belonging to the haploxylon group, is thought to be resistant. Sublethal effects of nematode was demonstrated in *P. strobus* by the temporal reduction of oleoresin flow (LINIT and TAMURA 1987). In these reports, the term "resistant" is used in a broad sense: a host is not affected so seriously by the pathogen and can survive. To avoid a confusion, we also follow this concept, mortality-resistance, here including the case of tolerance (HOLLIDAY 1989). TAMURA and DROPKIN (1984) reported that in resistant species, population growth of the nematodes in the xylem is lower than in susceptible species. Factors determining resistance, however, have not been found. In order to breed resistant pines, such factors must be present and must be under genetic control.

We have shown one aspect of the mechanism of disease development (KURODA et al. 1988; KURODA 1989, 1991; KURODA et al. 1991). Thus in susceptible *P. thunbergii* and *P. densiflora*, cavitation caused by vapor or gas occurs in many conducting tracheids of trunk xylem following nematode infection. As ascent of xylem-sap is blocked in the cavitated area, xylem desiccation progresses drastically. This leads to cambial necrosis about one month after infection. Cavitation is induced by volatile substances with low surface tension (SPERRY and TYREE 1988; KURODA 1991). Such materials increase shortly after nematode infection (KURODA 1991). Wilting is enhanced by severe soil water deficit and temperature over 35 °C during summer in Japan. These results make it possible to consider a detailed investigation into factors involved in resistance. Thus we have compared the nematode distribution in resistant *Pinus taeda, P. strobus* and susceptible *P. thunbergii* to physiological responses including increase in volatile terpenes and blockage of water conduction. We also compared concentrations of β-myrcene which has been reported to stimulate nematode multiplication and to induce susceptibility of pines to the nematode (ISHIKAWA et al. 1987; HINODE et al. 1987).

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2 Material and methods

2.1 Inoculation and sampling

Pinus taeda (4-year-old), *P. strobus* (7-year-old) and *P. thunbergii* (6-year-old) were used. These were field grown at the Forestry and Forest Products Research Institute, Kansai Research Center. A virulent population, S-10, of *B. xylophilus* (KIYOHARA and BOLLA 1990) were cultured on *Botrytis cinerea*. On August 10, 1988, eighteen trees of each species were inoculated with 10000 *B. xylophilus*. Inoculation was made into branches at a height of ca. 130 cm. Each week for 5 weeks, 3 inoculated trees and one uninoculated control tree of each species were harvested. These were cut into ca. 50 cm long bolts. Two xylem blocks were taken from each bolt to check nematode distribution, and the bolts were stored at −30 °C. The remaining inoculated trees were used to check symptom development and mortality through November when susceptible species usually show complete wilt. Specimens of *P. thunbergii* were the same as those used in the previous report (KURODA 1991).

2.2 Distribution of the nematode

The Baermann funnel method (THORNE 1961) was used to estimate the nematode population in trunk xylem. If no nematodes were detected the day after sampling, another sample block from the same site was stored for a month to allow the nemtode population to multiply to a detectable number (incubation method: KURODA 1989).

2.3 Detection of cavitation and volatile terpenes

Cavitation development was evaluated visually on the surface of the wood disk taken at 50cm intervals from the experimental and control trees. As an estimate of the degree of the blockage of xylem-sap ascent, xylem water content (WC) was calculated from fresh weight (FW) and dried weight (DW) (oven dried at 105 °C for one night) of xylem blocks as percent water in dried wood: $WC = [(FW-DW)/DW] \times 100\,\%$. Volatile terpenes contained in xylem were analyzed as follows. Ground xylem chips taken from disks adjacent to those used for cavitation observation were distilled with water and internal standard, tetradecane, for 1 hour at 90 °C. Volatiles were trapped onto charcoal powder and were eluted with CH_2Cl_2. The eluant was analyzed by gas chromatography using a Shimazu GC-15A chromatograph with FID and silica capillary column CBP20 (25 m × 0.2 mm ID). During the analysis, the temperature was held at 50 °C for 10 minutes and then raised at a 7°C/min. ramp to 180 °C. Injector and detector temperature were set at 200 and 220 °C, respectively. Flow rate of the carrier gas, helium, was 0.3 ml/min. Samples from healthy trees were analyzed in parallel with those from inoculated trees. At the same time volatile terpenes were compared the β-myrcene concentration in control and experimental trees of these three species.

3 Results

3.1 Nematode distribution and multiplication

In *P. taeda*, only a very small number of nematodes migrated into the trunk from inoculated sites on branches (Fig. 1 A). Their distribution first became restricted around the base of inoculated branches; and it took 3 weeks to become systemic. During the 5 week post-inoculation period, the population did not increase (Fig. 1 B). Nematodes distributed throughout the trunk of *P. strobus* beginning one week after inoculation (Fig. 1 A). Then the population decreased from 2 to 3 weeks after inoculation (Fig. 1 B). By 5 weeks after inoculation, there was a tendency for a second increase in the nematode population in this

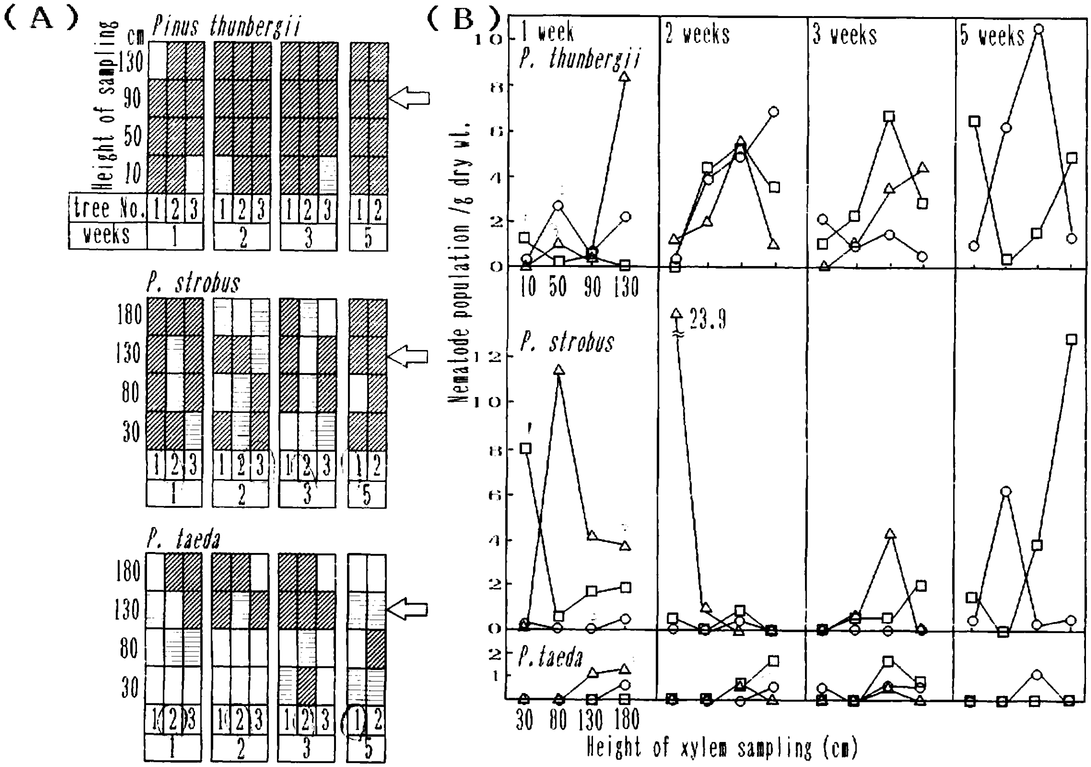

Fig. 1. Migration speed (A) and population growth (B) of *Bursaphelenchus xylophilus* in susceptible *Pinus thunbergii*, and resistant *P. taeda* and *P. strobus* to pine wilt disease for 5 weeks after inoculation in branches. – (A) Arrows: heights of inoculated branches. ▨ : nematode distributed. ▤ : presence of nematode was determined after incubation to increase the nematode population. This indicates that very few nematodes were initially present. □ : nematode does not exist. (B) □, ○, △: nematode population per 1g of oven dried wood, measured at 4 heights of 3 different trees, corresponding to tree No. 1, 2, and 3 in (A), respectively

pine. In susceptible *P. thunbergii*, the nematode was distributed throughout a trunk by one week after inoculation (Fig. 1A), and it gradually increased during the subsequent 4 weeks (Fig. 1B).

3.2 Cavitation and symptom development

As seen on cross cut surfaces of *P. taeda* trunks (Fig. 2), cavitation began as small spots by 2 weeks after nematode inoculation from bases of inoculated branches (Fig. 2A, B). These spots were distributed only within a narrow area on the disk surfaces. The degree of cavitation development varied significantly between trees. Even in the most developed case 5 weeks after infection, cavitation was found only in limited portion of trunks (Fig. 2C). Cavitation in *P. taeda* did not increase laterally across the trunk section as time after infection increased, nor did it extend throughout the trunk. Xylem water content in infected *P. taeda* was the same as in healthy trees. By 6 weeks after infection, only inoculated branches died and there was no indication of other symptom development after that.

In nematode infected and healthy *P. strobus*, cavitation was visible in the central part of trunks (Fig. 3). Cavitation areas increased slightly by 5 weeks in inoculated trees (Fig. 3C) but it was difficult to decide whether this increase was due to nematode activity in the trunks. Spot like cavitation similar to that found in *P. taeda* and *P. thunbergii* (Kuroda

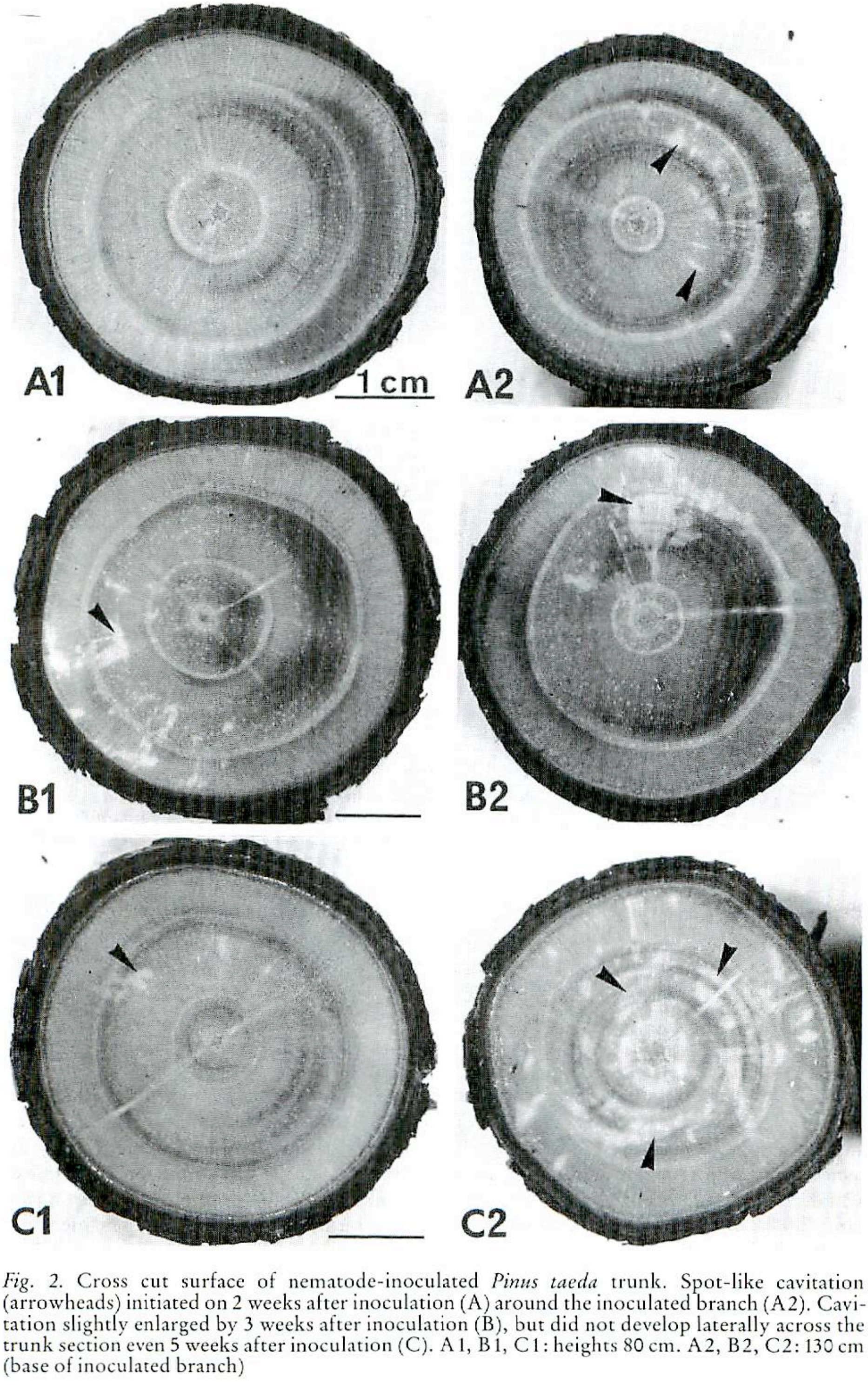

Fig. 2. Cross cut surface of nematode-inoculated *Pinus taeda* trunk. Spot-like cavitation (arrowheads) initiated on 2 weeks after inoculation (A) around the inoculated branch (A2). Cavitation slightly enlarged by 3 weeks after inoculation (B), but did not develop laterally across the trunk section even 5 weeks after inoculation (C). A1, B1, C1: heights 80 cm. A2, B2, C2: 130 cm (base of inoculated branch)

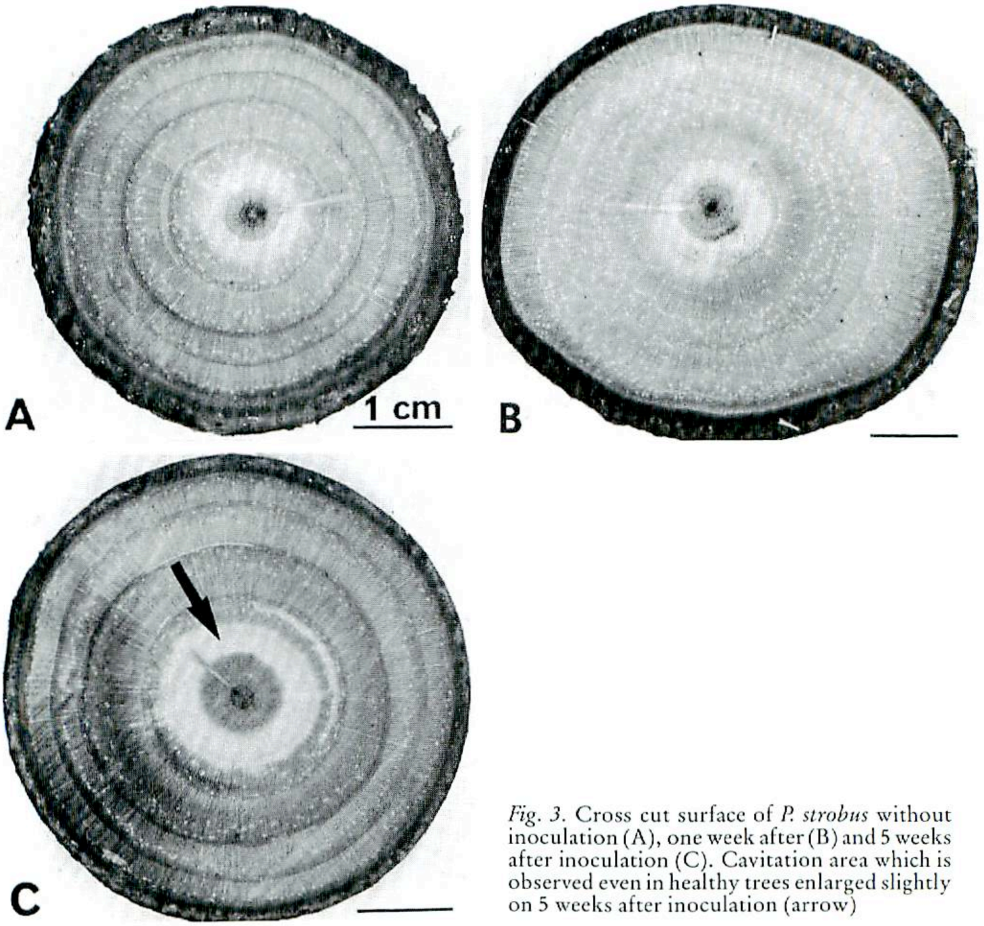

Fig. 3. Cross cut surface of *P. strobus* without inoculation (A), one week after (B) and 5 weeks after inoculation (C). Cavitation area which is observed even in healthy trees enlarged slightly on 5 weeks after inoculation (arrow)

1991) did not develop in *P. strobus*. By 5 weeks after inoculation, some needles showed initial signs of wilting on some branches, but these recovered. Inoculated branches were not killed. We did not observe scar formation such as that reported as a characteristic symptom in *P. strobus* (FUTAI and FURUNO 1979).

3.3 Increase of volatile terpenes

Healthy *P. taeda* contains a slightly higher concentration of volatile terpenes than seen in healthy *P. thunbergii* (KURODA 1989, 1991). Main constituents were the same in these two species. α-pinene had the highest concentration, and β-pinene the next highest. In infected *P. taeda*, total volatile terpenes increased by 1 week after infection, and then gradually decreased beginning 2 weeks after inoculation (Fig. 4) when samples taken from the same height of 80 cm were compared. By 5 weeks after infection, total volatile concentration was on an average the same as that in healthy trees. A tendency for an increase in the concentration of volatiles was seen only around the base of inoculated branches from 3–5 weeks after infection.

Healthy *P. strobus* exuded abundant resin from cut injuries on trunks, and the concentration of volatile terpenes was 2 to 3 times that of *P. thunbergii*. Main constituents were the

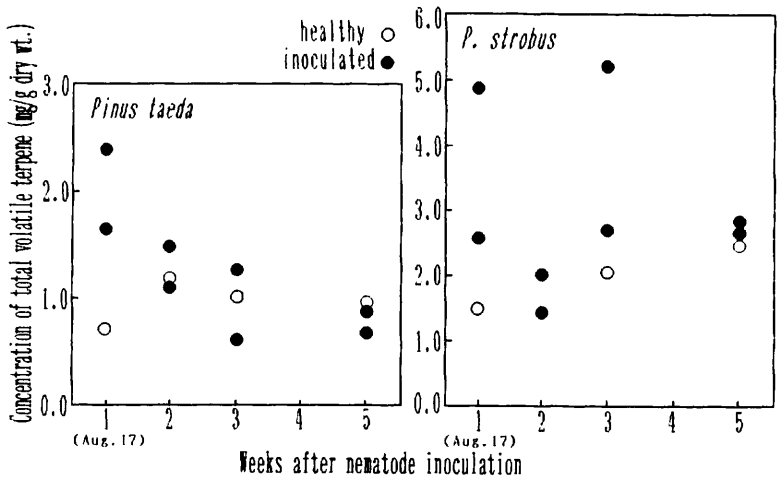

Fig. 4. Variation of total volatile terpene in xylem following nematode-inoculation into *P. taeda* and *P. strobus*. Concentration was compared at height 80 cm, with one healthy and two inoculated trees per each sampling. (Healthy *P. strobus* harvested on Aug. 24 was not used because the trunk was injured)

same, however. β-pinene was present in highest concentration. *P. strobus* kept cortical tissue, including densely-developed resin cysts, even in 7-year-old trees (Fig. 3) as compared to the other two species. Following nematode inoculation, volatile terpenes increased only in some part of the samples harvested 1 and 3 weeks after infection (Fig. 4). Most infected *P. strobus* contained nearly the same amount of volatile terpenes as did healthy trees.

β-myrcene concentration was compared among the three *Pinus* species (Fig. 5). β-myrcene concentration in resistant *P. taeda* and *P. strobus* was no lower than that seen in susceptible *P. thunbergii*. After the nematode inoculation, β-myrcene increased in *P. thunbergii* beginning 2 weeks after inoculation and then it decreased. β-myrcene also increased in *P. strobus* and *P. taeda* beginning one and 2 weeks after inoculation, respectively; it then decreased. Analysis of the variation of β-myrcene concentration during 5 weeks after nematode inoculation indicated that there was no tendency for the concentration to be lower in resistant species.

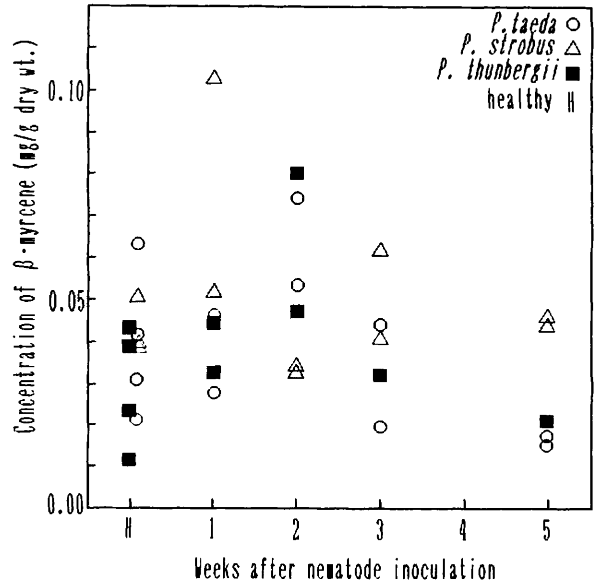

Fig. 5. Concentration of β-myrcene in healthy and nematode-inoculated trees of *P. taeda*, *P. strobus* and *P. thunbergii*

4 Discussion

There were many differences in the responses of *P. taeda* and *P. strobus* to nematode infection, and therefore, factors involved in resistance should be discussed independently on these two species of the diploxylon and haploxylon group, respectively.

We observed that cavitation initiates even in resistant *P. taeda* trunks. Although in the strictest meaning, this species is affected by pine wood nematodes, the effect terminates soon after inoculation in this species, blockage of sap ascent does not become so serious during the dry summer season and the pine survives. Another characteristic in this species is that there is significant inhibition of nematode migration and multiplication as has been reported from other resistant species such as *P. jeffrey* and *P. echinata* (TAMURA and DROPKIN 1984). In our earlier reports, we assumed that stimuli from feeding and migration of nematodes, activated terpene synthesis in susceptible host tissue within a few days after their inoculation (KURODA 1989, 1991). Increase of volatile terpenes in *P. taeda* within a short period probably was due to primary migration of several nematodes. Early decrease of volatiles beginning 2 weeks after infection in this species indicates that nematode-distribution of less than 2 nematodes per gram in a very restricted section of trunks did not activate terpene synthesis. As a result, cavitation development terminates at an initial stage. Death of inoculated branches is caused by a large nematode population which did not move from inoculation sites. We think that a factor which prevents the activity of nematode is very significant to inhibit cavitation development within narrow areas. This factor should be directly related to resistance of *P. taeda* to pine wilt disease.

Low concentration of β-myrcene is reported to be a significant factor in preventing nematode increase (ISHIKAWA et al. 1987), however, we were unable to show a difference in β-myrcene contents between *P. taeda*, a resistant species, and *P. thunbergii* whether the trees were healthy or nematode inoculated. It is hard to imagine that the nematode population increase in naturally growing tree depends on the amount of β-myrcene in host tissue. BENTLEY et al. (1985) found a nematode immobilization-substance, which consist primarily of carbohydrates, in *P. taeda*. Detailed studies on the effects of this substance are very important.

P. strobus is assumed to be less resistant than *P. taeda* since abnormality sometimes is found following nematode infection (FUTAI et al. 1979; TAMURA and DROPKIN 1984; BOLLA et al. 1986). Nematode migration from inoculated branches to the top and base of trunks is very rapid comparable to that seen in *P. thunbergii*. TAMURA et al. (1988) reported that larger numbers of *B. xylophilus* invaded the tissue of *P. strobus* than in susceptible *P. densiflora*. Following the primary distribution of nematodes, however, there was a clear decrease followed by slow increase in the size of the nematode population which differed from the continual increase seen in susceptible species. These changes were not related to β-myrcene concentration. Even in the samples in which volatiles increased, the area of cavitation did not enlarge in comparison with the cavitation area in healthy trees which is assumed to be transient (WHITEHEAD and JARVIS 1981). Abundant terpene production, even in healthy trees, possibly due to an extensive resin canal system in cortical tissue, suggests that the plasmamembrane of parenchyma cells of *P. strobus* is more resistant to the accumulation of toxic terpenes than other species. Temporal wilting of needles in late summer indicated that a certain degree of blockage occurred in water conduction in branches. Cavitation of tracheids may occur at a rate too low to be visually detectable. We hypothesize that disease development is very slow and blockage of water conduction is not significant during extensive drought and high temperature in midsummer of the year of inoculation. *P. strobus* is considered mortality-resistant when environmental conditions are not severe. LINIT and TAMURA (1987) reported that abnormal decrease of oleoresin flow is induced by nematode infection during the first year after inoculation and it continues even into the second year after infection. Based on these observations it is necessary to do long term experiments to learn how long nematodes affect the physiology of *P. strobus* and the subsequent recovery process.

Relative resistance to pine wilt disease can be examined by studying the factors such as nematode distribution and cavitation development throughout a trunk which develop as symptoms of the disease in susceptible species. If these two points are examined in nematode infected trees of many *Pinus* species, the resistance of the tree can be determined, and considerable information will be gained about the factors relating to resistance to pine wilt disease.

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Summary

To find factors involved in the resistance to pine wilt disease caused by ***Bursaphelenchus xylophilus***, early physiological reaction of resistant species, ***Pinus taeda*** and ***P. strobus*** were compared with that of susceptible ***P. thunbergii*** after nematode inoculation. In ***P. taeda***, nematode migration and multiplication was strongly inhibited. Acceleration of monoterpene synthesis and tracheid-cavitation initiated in the restricted areas of trunks but terminated at an early stage, and blockage of xylem sap ascent was not serious enough to kill infected trees. In ***P. strobus***, inhibition of nematode activity was weaker than in ***P. taeda***. Temporal needle-wilting found on a part of branches in late summer suggested slight water blockage in the trunk. But the cavitation development by nematode activity was not confirmed visually on the disks from trunks. β-myrcene concentration in xylem of these species was not related to the resistance.

Résumé

Le dépérissement des pins à Bursaphelenchus xylophilus: facteurs associés à la résistance

Afin d'identifier les facteurs impliqués dans la résistance, la réaction physiologique précoce d'espèces résistantes, *Pinus taeda* et *P. strobus* a été comparée avec celle de l'espèce sensible *P. thunbergii* après inoculation par le nématode. Chez *P. taeda*, la migration du nématode et sa multiplication sont fortement inhibées. l'accélération de la synthèse des monoterpènes et de la cavitation des trachéides initiée dans des zones limitées de tronc mais achevée à un stade précoce, le blocage du flux ascendant de sève dans le xylème, ne sont pas suffisamment importants pour tuer les arbres infectés. Chez *P. strobus*, l'inhibition de l'activité du nématode est plus faible que chez *P. taeda*. Le flétrissement des aiguilles dans le temps, observé sur une partie des branches en fin d'été laisse penser qu'un léger blocage hydrique a lieu dans le tronc. Cependant, le développement de la cavitation par l'activité du nématode n'est pas confirmé visuellement sur des disques du tronc. La concentration en β-myrcène dans le xylème de ces espèces n'est pas liée à la résistance.

Zusammenfassung

Durch Bursaphelenchus xylophilus induzierte Föhren-Welke: Resistenz-assoziierte Faktoren

Auf der Suche nach Faktoren, die mit der Resistenz gegen die durch *Bursaphelenchus xylophilus* verursachte Föhren-Welke in Zusammenhang stehen könnten, wurden physiologische Reaktionen von zwei resistenten *Pinus*-Arten, *Pinus taeda* und *P. strobus*, mit solchen einer anfälligen Art, *P. thunbergii*, nach künstlicher Inokulation mit Nematoden verglichen.

Bei *P. taeda* waren Migration und Vermehrung der Nematoden stark gehemmt. Die Beschleunigung der Synthese von Monoterpenen und die Emboliebildung in Tracheiden begannen in begrenzten Bereichen des Stammes, hörten jedoch in einem frühen Stadium wieder auf. Die Unterbrechung des Xylems war nicht groß genug, um die infizierten Bäume zum Absterben zu bringen.

Bei *P. strobus* war die Hemmung der Nematodenaktivität schwächer als bei *P. taeda*. Vorübergehendes Welken von Nadeln an einem Teil der Äste im Spätsommer ließ schwache Blockaden des Xylemstroms vermuten. Embolien in Tracheiden konnten jedoch bei visueller Untersuchung von Stammscheiben nicht festgestellt werden. Die β-Myrcen-Konzentration im Xylem dieser Arten stand nicht mit der Resistenz in Beziehung.

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