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Mechanism of cavitation development in the pine wilt disease

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Abstract

Volatile terpenes increase in xylem tissue after infection of *Pinus thunbergii* with the pine wood nematode (*Bursaphelenchus xylophilus*). The role of these terpenes in tracheid cavitation, which blocks xylem-sap ascent and leads to water deficit in pine trees, was assessed. Volatile terpene concentration increased long before initiation of tracheid cavitation. After the volatile terpenes reached the highest concentration, severe cavitation developed. Direct injection of α-pinene into healthy pine trunks formed artificial cavitation in xylem. These observations support the hypothesis that excessively produced volatiles, which are hydrophobic and have lower surface tension than water can promote tracheid cavitation in pine wilt disease.

1 Introduction

Pine wilt disease has caused significant damage to pine forests in Japan over several decades (MAMIYA 1988). Symptom development is very drastic. Usually, the pathogenic nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle, kills susceptible *Pinus* species within a few months after infection. Following the discovery of the nematode (KIYOHARA and TOKUSHIGE 1971), this disease has been extensively studied (KIYOHARA and SUZUKI 1978). The mechanisms of pathogenesis, however, has not been elucidated.

I have undertaken studies on early physiological changes occurring in pine trees following nematode infection. The first symptom of the disease is partially interrupted water-conduction in sapwood; this starts within two weeks after nematode infection (TAMURA et al. 1987). This blockage of sap ascent is caused by cavitation or embolism of tracheids (KURODA et al. 1988). It is evident that infected pine trees die from water shortage caused by cavitation. There is a significant increase in monoterpenes in the cavitated (or embolized) area during early stages of infection which led KURODA (1989) to hypothesize a mechanism of tracheid cavitation induced by volatile terpenes (Fig. 1): Xylem sap in pine trunks is kept under tension during summer to respond to a water deficit in soil and a high transpiration rate (ZIMMERMANN 1983). Volatile substances are excessively produced as a reaction to the nematode activity and are exuded from parenchyma. Such volatile substances help to cut water columns under tension, leading to dehydration of tracheids. Hydrophobic terpenes prevent refilling of tracheids with water even when the water stress is released during the night. These reactions lead to gradual enlargement of the area of cavitation and water deficit progresses in the tree. Transient cavitation of tracheids due to water stress, which is a common event in healthy trees (TYREE and SPERRY 1988), may occur before the evaporation of terpenes.

Although blockage of water conduction by gas is assumed to be very effective in interrupting sap ascent over a wide cross sectional area, such a phenomenon has been little investigated in wilting diseases of trees (ZIMMERMANN 1983). To test my hypothesis, I have investigated the relationship between the start of cavitation and increase of volatile terpenes in pine tissue.

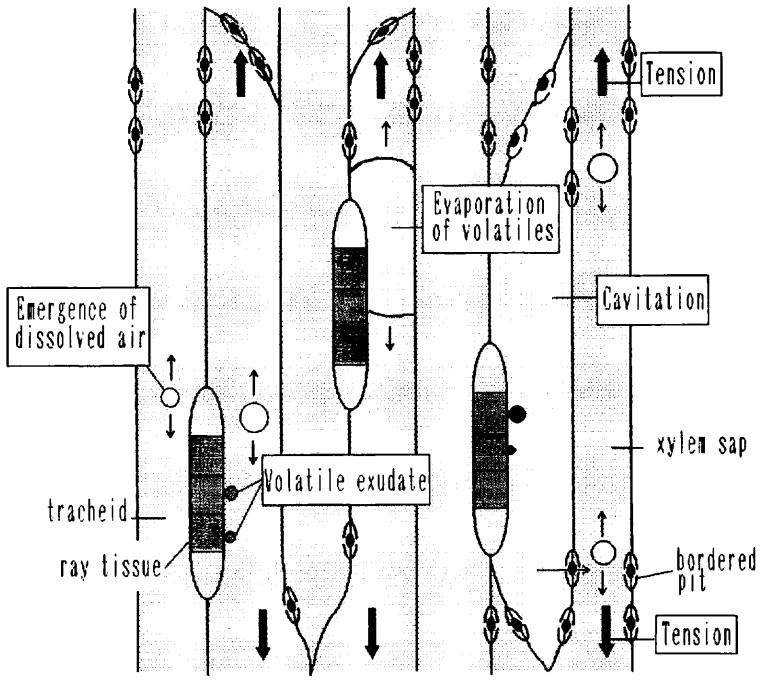

Fig. 1. Diagrammatic representation of the cavitation hypothesis (tangential section of pine xylem)

2 Materials and methods

2.1 Inoculation, sampling and detection of nematode distribution

Six-year-old Japanese black pines (*Pinus thunbergii* Parl.) growing in the nursery of Forestry and Forest Products Research Institute, Kansai Research Center were used. A population of 10000 pine wood nematodes cultured on *Botrytis cinerea* Pers. were inoculated into the pines via a cut wound made on branches at the height of ca. 90 cm. Eighteen trees were inoculated on August 10, 1988. Three days later, 3 inoculated trees were cut followed weekly by groups of 3 trees. A non-inoculated healthy tree was harvested on each of the tree harvest dates. Sample trees were cut into ca. 40 cm long bolts. Nematode populations in xylem were counted at 40 cm intervals by Baermann funnel technique (THORNE 1961). Distribution of nematodes during early period after inoculation was also examined by incubation method (KURODA et al. 1988). The bolts were stored at −30°C until used for the detection of cavitation and analysis of volatile substances in cavitation areas.

2.2 Detection of cavitation and gas chromatography

Two cm thick disks were cut from each frozen bolt. The cut surfaces were examined with a stereo-microscope and visual tracheid cavitation was evaluated.

Volatile substances associated with phloem and xylem were analyzed separately. In case of xylem, 2 to 3 cm thick disks were ground, and ca. 5 g (fresh weight) was used for distillation (SUGISAWA et al. 1984). Volatiles were trapped onto charcoal and eluted with CH_2Cl_2. The solution was condensed and analyzed with a gas chromatograph (Shimazu GC-15A) with a FID. Silica capillary columns, FFAP (25 m × 0.25 mm ID) or CBP20 (25 m × 0.2 mm ID) were used. Column temperature was held at 60°C for 8 minutes and then raised at

a 5°C/min. ramp until 180°C was reached. Injector and detector temperatures were set at 200° and 220°C, respectively. Helium was used for carrier gas at a flow rate of 0.3 ml/min. As an internal standard, 0.3 mg of dodecane was added just before distillation to the mixture of ground sample and water. Non-inoculated trees were analyzed in parallel with inoculated trees.

2.3 Direct injection of a monoterpene

To test whether monoterpenes have an ability to cause cavitation in conducting tracheids, α-pinene was directly injected into healthy *P. thunbergii*. Small cut wounds which reach to xylem were made on trunks and branches with a knife. Ten μg of α-pinene was injected into wounds on branches and the wounds were sealed. Also, cotton soaked in α-pinene was attached to the cut wound on stems. For comparison, water was injected into wounds on control trees. Four days after injection, sample trees were cut down and formation of cavitated areas was checked after freezing. The bases of some branches were soaked in acid-fuchsin (1 %) over night, and water blockage was checked based on uptake of the dye in the xylem.

3 Results

3.1 Development of water blockage and other symptoms

Fig. 2 A to D shows the development of cavitation during the 5 weeks following pine wood nematode inoculation. Water blockage attributable to cavitation first became visible two weeks after inoculation. This was seen as a scattering of white spots on cross cut surfaces throughout the trunk of the infected trees (Fig. 2B). The maximum total area of spots in a cross section was seen at the base of the inoculated branch. Beginning three weeks after inoculation, the cavitation area became drastically enlarged (Fig. 2C), and there was an obvious decrease in xylem water content. By five weeks after inoculation, xylem water content was only one third of that seen in healthy trees, and necrosis of cambium had already started in the infected trees (Fig. 2D).

Nematodes were distributed throughout the trunk within one week after inoculation. A sudden increase in nematode population size occurred 5 weeks after inoculation. Discoloration of old needles started 3 weeks after inoculation in some inoculated trees. Complete wilting was not observed even 5 weeks after inoculation even though resin exudation from cut surface had stopped completely. Symptom development was essentially the same as in 11-year-old *P. thunbergii* used in earlier experiments (KURODA et al. 1988).

3.2 Increase of volatile terpenes

Total concentration of volatile terpenes, including monoterpenes and sesquiterpenes, varied in xylem during 5 weeks after nematode inoculation as indicated in Fig. 3. Variation in terpene contents was rather large even within a single tree. Therefore, the values for different dates were compared using samples taken from the same height on the tree. Three days after inoculation, total volatile concentration was 2 to 3 times of that in healthy trees on that date. The main constituent was α-pinene (Table 1). Other monoterpenes such as camphene, β-pinene, β-myrcene, limonene, β-phellandrene, and a sesquiterpene, longifolene, also increased in inoculated trees.

One week after inoculation, volatile terpene concentration was less than that on 3 days after inoculation (Fig. 3, Table 1). Volatiles attained the highest concentration 2 to 3 weeks after inoculation; there was a tendency of decrease after that. Even in healthy trees, a natural gradual increase of volatiles was observed from August to September. In samples taken one and two weeks after inoculation, volatile concentration was highest at the base

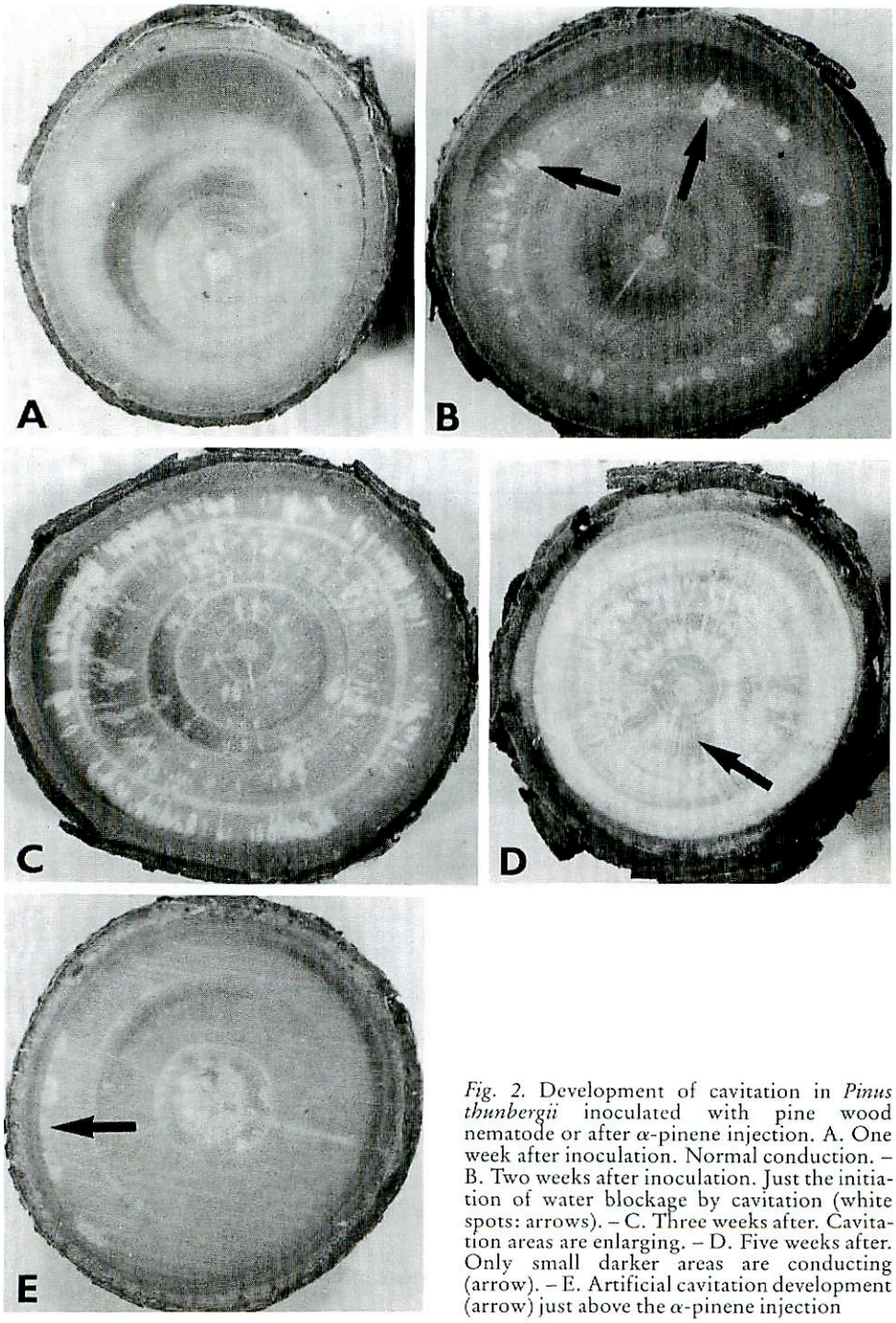

Fig. 2. Development of cavitation in *Pinus thunbergii* inoculated with pine wood nematode or after α-pinene injection. A. One week after inoculation. Normal conduction. – B. Two weeks after inoculation. Just the initiation of water blockage by cavitation (white spots: arrows). – C. Three weeks after. Cavitation areas are enlarging. – D. Five weeks after. Only small darker areas are conducting (arrow). – E. Artificial cavitation development (arrow) just above the α-pinene injection

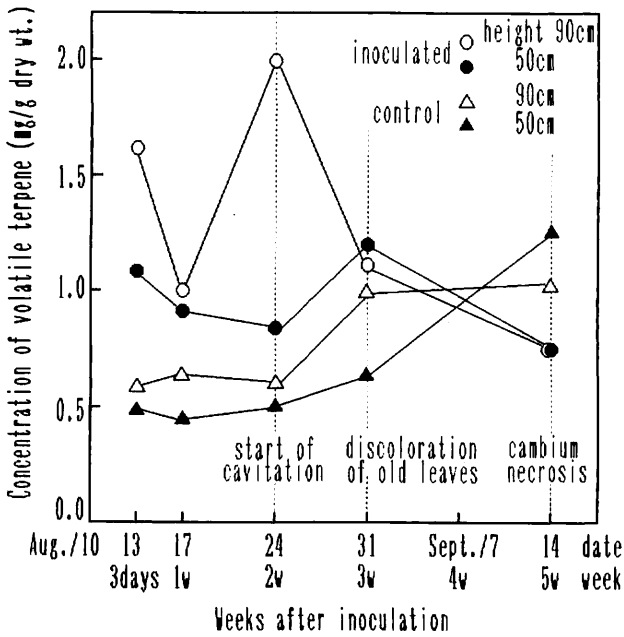

Fig. 3. Increase of volatile terpene in xylem for 5 weeks after nematode infection

of inoculated branches. This site coincides with that at which cavitation became conspicuous beginning two weeks after inoculation.

Volatile terpenes were also abundant in phloem. But there was no significant increase in inoculated trees.

Table 1. **Variation of terpene contents in Pinus thunbergii during 5 weeks following nematode inoculation**

Terpenes (mg/g dry wt.)	3 days		1 week		2 weeks		3 weeks		5 weeks	
	cont[1]	ino[2]	cont	ino	cont	ino	cont	ino	cont	ino
α-pinene	0.385	1.201	0.442	0.588	0.417	1.321	0.670	0.769	0.593	0.469
camphene	0.006	0.027	0.004	0.014	0.009	0.025	0.015	0.021	0.012	0.009
β-pinene	0.076	0.162	0.068	0.056	0.034	0.153	0.136	0.122	0.084	0.079
β-myrcene	0.013	0.037	0.013	0.033	0.024	0.114	0.050	0.033	0.038	0.021
limonene	0.015	0.111	0.012	0.129	–[3]	0.163	0.017	0.039	0.105	0.025
β-phellandrene	0.044	0.052	0.040	0.062	0.078	0.158	0.076	0.100	0.103	0.127
cymene	0.001	0.001	–	–	–	0.002	0.001	–	–	–
Longifolene	0.034	0.037	0.040	0.050	0.027	0.074	0.028	0.031	0.079	0.042
total volatiles	0.575	1.631	0.619	0.994	0.562	2.010	0.993	1.115	1.014	0.772
ratio (ino/cont)[4]		2.84		1.61		3.41		1.12		0.76

[1] Non-inoculated sample taken from height 90 cm.
[2] Nematode inoculated sample taken from height 90 cm.
[3] The peak of limonene was included in β-phellandrene.
[4] Total value of volatile terpenes in the inoculated sample was divided by that of the control.

3.3 Effects of α-pinene injection

Fig. 2E indicates the artificial cavitation caused by injection of α-pinene into a healthy pine trunk. Cavitation was easily visible above the injected wounds. Water injection did not lead to cavitation. Blockage of water conduction was also seen as a white area surrounded by conducting xylem dyed with acid fuchsin in the dye uptake experiments.

4 Discussion

The increase of volatile terpene concentration 3 days after nematode inoculation occurs much earlier than does water blockage by cavitation. The activation of excessive terpene synthesis appears to start in pine cells immediately after the invasion by nematodes especially at the base of inoculated branches. When total volatiles reached maximum concentration two weeks after infection, a small area of cavitation was initiated. These observations indicate that such early increase of terpenes is not the result of water blockage, and support the hypothesis that volatile terpenes can contribute to the initiation of tracheid cavitation.

When extensive development of water blockage was observed 3 weeks after inoculation, concentration of volatile substances already had decreased. This means that either terpene synthesis decreased, and/or the newly produced volatile terpenes were modified or dispersed from production centers by this time. Terpenes are thought to be produced in parenchyma cells including epithelial cells around resin canals (HILLIS 1987). Secondary products, such as phenolic compounds and terpenes, are toxic for living cells (STEWART 1966). If excess amount of these substances accumulate in a cell, membranes will degrade and release them into surrounding tracheids (STEWART 1966). In this experiment, the period of membrane degradation appeared to be from 2 to 3 weeks after infection, just before development of drastic cavitation.

I did not anticipate such early physiological changes in pine cells occurring only several days after nematode infection. Activities of nematodes within the first few days after infection has received little study because such a small population of nematodes was not believed to have significant effects on pine physiology. Judging from a report that pine wood nematodes move 25 to 50 cm per day (HASHIMOTO 1973), stimuli by the nematodes moving from the inoculation sites seems to be very significant for inducing the first physiological changes of pine cells. To understand physiological changes in pines so soon after infection, much work has to be done on nematode activity early after infection. Volatiles gradually increase even in healthy trees indicating a slight change in terpene content, but at concentrations which should not be harmful to pine cells. Increase during summer may be caused by extensive water stress.

Direct injection of α-pinene demonstrated that volatile substances can cause cavitation in pine trees. Transient cavitation is assumed to happen daily in healthy trees (TYREE and SPERRY 1988). Such cavitation is thought to be a result of water evaporation in tracheids or emergence of air bubbles from solution in the tissue surrounding the water conduit because of negative pressure or tension caused by water deficit and high transpiration rate. SPERRY and TYREE (1988) demonstrated that direct injection of butanol or tween causes cavitation in healthy maple twigs. They explained that the water column was cut and cavitation developed by air-seeding because these experimentally used substances have lower surface tension than water. From the results, they concluded that cavitation develops when a fluid tension is greater than the surface tension of xylem sap. In the case of pine wilt disease, the water column in trunks will be disrupted at the point where volatile terpenes with low surface tension are exuded. In healthy trees, cavitation will disappear by the refilling to tracheids with water when water stress is relaxed. This is the significant difference from irreversible cavitation in pine wilt disease. As explained in previous reports (KURODA et al. 1988; KURODA 1989), hydrophobic terpenes, which prevent the refilling with water, more effectively develop the sustained cavitation. Ethanol, which may promote cavitation, was reported to increase in pine wilt disease (IKEDA and ODA 1980). But alone it cannot be the main causal substance of cavitation because ethanol does not increase until after the start of cavitation.

Terpenes such as α- and β-pinene are known to increase in the wood of wounded conifers (HILLIS 1987), and they inhibit fungal growth (KILE and TURNBULL 1974). After infection by fungi, terpenes increase around the site of infection, and a dry zone is formed in

that area (CHENICLET 1987; CROTEAU et al. 1987). In many cases, invasion of fungi is limited to the infection site in the xylem. In pine wilt, on the other hand, the pathogenic nematode is mobile and distributes throughout the pine within a short period after infection. Terpene synthesis can, therefore, be elicited throughout the tree if the nematode can escape the initial resistance response. This can lead to development of cavitation throughout the entire tree. The infected pine trees will die because of water shortage.

The causal substances of abnormal water relation in pine wilt disease have not been investigated following the reports by SUZUKI and KIYOHARA (1978) and IKEDA and SUZAKI (1984) on abnormal water status in this disease, probably because the water blockage by cavitation was not considered to have effects on disease development. Recently, some blue-stain fungi, responsible for the mortality of lodgepole pine (*Pinus contorta* Dougl.), were reported to inhibit sap flow completely and to cause distinct dry whitish area (YAMAOKA 1989). This suggests that the development of cavitation and water blockage also occurs after fungal infection of pines.

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Summary

Following infection of *Pinus thunbergii* with pine wood nematode, water conduction is progressively blocked in xylem due to tracheid cavitation. The present experiment demonstrated that volatile terpenes increased in xylem tissue of pines within a few days after the pines were infected with the nematode. This increase occurred much earlier than the initiation of tracheid cavitation beginning 2 weeks after infection. Volatile terpenes reached the highest concentraion 2 to 3 weeks after infection. Cavitation areas enlarged drastically beginning 3 weeks after infection. Direct injection of α-pinene, one of the main constituents of volatile terpenes in pine trees, into healthy pine trunks caused cavitation in xylem. These facts support the hypothesis that excessively produced volatiles, which are hydrophobic and have lower surface tension than water, can cause tracheid cavitation in pine wilt disease.

Résumé

Mécanisme de cavitation dans le flétrissement du Pin

A la suite de l'infection de *Pinus thunbergii* par le nématode des pins, la conduction de l'eau est progessivement bloquée dans le xylème par la cavitation des tracheides. L'expérimentation rapportée ici montre que les terpènes volatiles augmentent en quelques jours dans les tissus ligneux après que les pins aient été infectés par le nématode. Cette augmentation a lieu beaucoup plus tôt que la cavitation des tracheides qui commence 2 semaines après l'infection. Les terpènes volatiles atteignent la concentration la plus élevée 2 à 3 semaines après l'infection. Les zones cavitées s'agrandissent fortement après 3 semaines. L'injection directe d' α-pinène, l'un des principaux constituants des terpènes volatiles des pins, dans le tronc d'arbres sains, provoque la cavitation du xylème. Ces faits confortent l'hypothèse selon laquelle les substances volatiles produites en abondance, qui sont hydrophobes et qui ont une tension de surface plus faible que l'eau, peuvent provoquer la cavitation des tracheides dans le cas du flétrissement des pins.

Zusammenfassung

Die Entwicklung von Embolien im Holz bei der Kiefernwelke

Der Gehalt flüchtiger Terpene nimmt im Xylem von *Pinus thunbergii* nach Infektion mit *Bursaphelenchus xylophilus* zu. Die Rolle dieser Terpene für die Verödung von Teilen des Xylems, wodurch der Wassertransport blockiert wird, was bei den Kiefern zu Wassermangel führt, wird abgeschätzt. Die Konzentration flüchtiger Terpene steigt lange bevor die Verödung der Tracheiden eintritt. Nachdem die flüchtigen Terpene ihre höchste Konzentration erreicht haben, entwikkeln sich umfangreiche Embolien. Die direkte Injektion von α-Pinen in gesunde Kiefernstämme

führte zu künstlichen Embolien im Xylem. Diese Beobachtung stützen die Hypothese, nach der im Übermaß gebildete gasförmige Substanzen, die hydrophob sind und eine niedrigere Oberflächenspannung besitzen als Wasser, die Verödung von Tracheiden bei der Kiefernwelke fördern können.

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