Histological Observations on the Responses of Pine Species, *Pinus strobus* and *P. taeda*, Resistant to *Bursaphelenchus xylophilus* Infection

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Abstract

Bursaphelenchus xylophilus was inoculated into the branches of wilt-resistant *Pinus taeda* and *P. strobus*, and wilt-susceptible *P. thunbergii*. The histology of inoculated pine branches was investigated. Nematode numbers increased in whole inoculated branches of *P. thunbergii*. Nematode numbers decreased in those of resistant species, except in proximity to the inoculation site of *P. taeda*. Histological changes were delayed in the cortex and phloem tissue and cambium of *P. thunbergii* inoculated branches. In resistant species, necrosis and destruction of cortex and phloem tissue and cambium (*P. taeda*) or occlusion of cortical resin canal (*P. strobus*) occurred near the inoculation site rapidly after nematode inoculation. Wound periderm was formed within 3 weeks and 5 weeks after inoculation in *P. taeda* and *P. strobus*, respectively. Wound periderm formation was observed only around the cortical resin canals in *P. thunbergii*. Necrosis of xylem parenchyma was conspicuous in *P. thunbergii*, but not in resistant species. These results suggest that responses of resistant species, wound periderm formation and occlusion of cortical resin canal, trapped the nematode within damaged tissue. It is also suggested that insensitivity of xylem parenchyma to nematode infection acts as a defensive factor.

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Key words: *Bursaphelenchus xylophilus*, histology, nematodes, pine wilt disease, *Pinus* spp., resistance.

INTRODUCTION

Native Japanese black (*Pinus thunbergii* Parl.) and Japanese red (*P. densiflora* Sieb. et Zucc.) pines are susceptible and loblolly pine (*P. taeda* L.) and eastern white pine (*P. strobus* L.) are resistant to infection by pinewood nematode, *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle. Such relative resistance to *B. xylophilus* infection among pine species have been well documented by several researchers^{1,3,5,8,9,13}.

Several histological or chemical investigations suggested that dynamic responses contributed to the resistance mechanisms. Wound periderm formation was observed in pine trees inoculated with the nematode, including *P. strobus*, which had not wilted^{10,11}. Dynamic responses were suggested to contribute to the resistance mechanisms of rigida pine (*P. rigida* × *P. taeda*)¹². In a histochemical study, host responses occurred rapidly in the cortex and phloem of resistant species *P. strobus*, while these responses were delayed in a susceptible species, *P. thunbergii*¹⁴. In the previous report, we found several phenomena in the responses of resistant species, and reported the differences in the responses of stem between *P. taeda* and *P. strobus*⁶.

To reveal the resistant responses, we have investigated the histological changes in nematode-

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Fig. 1. Diagram showing nematode inoculation and sampling.

inoculated branches of pines, resistant species *P. taeda* and *P. strobus* in comparison with the susceptible species *P. thunbergii*.

MATERIALS AND METHODS

Inoculation and sampling. Nematodes used for inoculum, *B. xylophilus* (isolate S-10) were reared on *Botrytis cinerea* Pers. Four-year-old *P. taeda*, seven-year-old *P. strobus*, and eight-year-old *P. thunbergii* trees were grown at the nurseries of the Kansai Research Center, Forestry and Forest Products Research Institute. On August 10th, 1988, five thousand *B. xylophilus* were introduced into each cut wound on two branches of each pine tree. Then, the inoculation sites were wrapped with parafilm. Branches of three inoculated trees and one uninoculated tree were cut-off at 1, 2, and 3 weeks after inoculation. Branches of two inoculated trees were cut-off 5 weeks after inoculation. The branches were then divided into 3 portions, A, B, and C as shown in Fig. 1.

Nematode extraction and anatomy. Nematodes were isolated by a Baerman funnel method, and the number of nematodes were counted. For the histological observation, tissue samples from each portion were fixed in FAA (formalin-acetic acid-50% ethanol, 15 : 5 : 200), dehydrated in alcohol series, and embedded in paraffin. Sections of ca. 20 μ m in thickness were made with a sledge microtome and double-stained with safranin and fast green to observe microscopically.

RESULTS AND DISCUSSION

Development of the disease in whole tree

The two-year-old needles of *P. thunbergii* began to yellow 4 weeks after inoculation. The needles generally discolored and the trees wilted, 5 and 6 weeks following inoculation, respectively. No visible symptoms were observed in *P. taeda* and *P. strobus* until 5 weeks after inoculation. Until the end of the inoculated year, a few inoculated branches of *P. taeda* died, but *P. strobus* displayed neither dead branches nor dead trees.

Distribution of nematodes

Nematode numbers increased in the proximity of inoculation site, portion A, of *P. taeda*, but did not increase apart from the inoculation site, at portion B and C (Fig. 2a). In whole inoculated branches of *P. strobus*, the nematode numbers were as high as those of *P. thunbergii* up to one week after inoculation, but then decreased gradually (Fig. 2b). Nematode numbers increased in whole inoculated branches of *P. thunbergii* (Fig. 2c).

Fig. 2. Population development of *Bursaphelenchus xylophilus* in inoculated branches of (a) *Pinus taeda*, (b) *P. strobus* and (c) *P. thunbergii*. A, B and C denotes the portion of sampling. Vertical bars show the standard deviations.

Table 1. Necrosis and destruction of cortex, phloem and cambium of *B. xylophilus*-inoculated *Pinus* spp.

Species	Portion	Destruction of cortex epithelial cell				Necrosis/destruction of cortex and phloem				Necrosis/cavity formation at cambium			
		1 ^{a)}	2	3	5	1 ^{a)}	2	3	5	1 ^{a)}	2	3	5
<i>P. taeda</i>	A	+ ^{b)}	++	++	++	± ^{b)}	+	++	++	- ^{b)}	+	+	±
	B	+	++	++	++	-	+	+	+	-	±	-	-
	C	+	++	++	+	-	±	++	+	-	±	±	-
<i>P. strobus</i>	A	+	+	+	+	±	±	±	+	-	-	-	-
	B	+	+	+	+	±	±	-	+	-	-	-	-
	C	+	+	+	+	±	±	-	+	-	-	-	-
<i>P. thunbergii</i>	A	+	++	++	++	±	±	+	++	-	-	±	++
	B	+	++	++	++	-	-	±	++	-	-	±	++
	C	+	++	++	++	-	-	±	+	-	-	-	±

a) Weeks after inoculation.

b) -: absent, ±: slightly visible, +, ++: increasingly conspicuous.

Necrosis and destruction of cortex and phloem tissue and cambium

One week after inoculation, destruction of epithelia lining the cortical resin canals was observed in all species (Table 1, Plate I-1). Two weeks after inoculation, destruction of cortex and phloem tissue and cambium in *P. taeda* had already occurred (Plate I-2, 3). Histological changes, other than destruction of epithelia, were not observed until 2 weeks after inoculation in cortex and phloem of *P. thunbergii*. Necrosis and destruction of cortex and phloem tissue was most conspicuous in *P. taeda*, but was the least

Table 2. Protective tissue formation in cortex and phloem of *B. xylophilus*-inoculated *Pinus* spp.

Species	Portion	Wound periderm formation				Wound periderm formation around resin canal				Occlusion of resin canal			
		1 ^{a)}	2	3	5	1 ^{a)}	2	3	5	1 ^{a)}	2	3	5
<i>P. taeda</i>	A	— ^{b)}	—	+	++	— ^{b)}	—	+	+	± ^{b)}	+	+	±
	B	—	—	±	++	—	—	±	++	—	±	±	+
	C	—	—	±	++	—	—	—	++	—	±	±	±
<i>P. strobus</i>	A	—	—	—	+	—	—	—	++	±	+	+	++
	B	—	—	—	+	—	—	—	++	±	+	+	++
	C	—	—	—	+	—	—	±	++	±	±	+	+
<i>P. thunbergii</i>	A	—	—	—	±	—	—	±	—	±	±	+	—
	B	—	—	—	—	—	—	+	+	±	±	+	+
	C	—	—	—	—	—	—	+	+	—	—	±	—

a) Weeks after inoculation.

b) —: absent, ±: slightly visible, +, ++: increasingly conspicuous.

apparent in susceptible *P. thunbergii*, until 3 weeks after inoculation. Cavity formation and cell necrosis were observed in the cambium of *P. taeda* near the inoculation site, portion A, within 2 weeks after inoculation (Table 1). Fewer cavities, however, were observed in the distal portion. Cambial death did not develop in *P. strobus*. Breakdown of *P. thunbergii* cortex and phloem tissue became conspicuous, and advanced cambial death was observed 3 weeks after inoculation. Many nematodes distributed in destroyed cortex and phloem tissue and cavities at cambia, especially of *P. taeda* (Plate I-2, 3).

Responses in the cortex and phloem

Wound periderms were well developed in *P. taeda* 3 weeks after inoculation (Plate I-4), and also in *P. strobus* within 5 weeks, but not formed in *P. thunbergii* except for the periderms surrounding cortical resin canals (Table 2). In all species, wound periderms were formed around cortical resin canal, when cortex epithelial cells were destroyed and necrosis developed in the encircling parenchyma cells (Plate I-5). However, frequency and the degree of wound periderm formation around the resin canal was lower in *P. thunbergii* than that in *P. taeda* and *P. strobus*. Occlusion of cortical resin canal, resulted from the hypertrophy of parenchyma cells and hyperplasia of epithelial cells, became gradually conspicuous in inoculated *P. strobus* (Plate I-6a, b). Occlusion of cortical resin canal and wound periderm formation did not inhibit the initial migration of nematodes. However, they would become effective to trap the nematodes within damaged tissue after their development.

Rapid tissue discoloration was reported in *P. strobus* after the inoculation of *B. xylophilus*^{10,11). Moreover, death of the inner bark tissue and cambium was restricted in parts of trees and no mortality of whole tree was observed in *P. strobus*^{3,14). The findings of Tamura *et al.* suggest that histochemically detectable responses occur rapidly in *P. strobus*, despite of low degree of cortex and phloem tissue destruction^{14). Lesser death in *P. strobus* cortex and phloem may cause delayed wound periderm formation. If localized death of cortex and phloem occurred in *P. strobus*, wound periderm would be formed more rapidly.}}}

Restriction of nematode dense area observed in *P. taeda* corresponded to rapid necrosis and destruction of cortex and phloem tissue and the following wound periderm formation near the inoculation site. Reproduction of dispersed nematodes was not observed in the inoculated branches of *P. taeda* and *P. strobus*. These results suggest that localized death in the cortex and phloem tissue of resistant species, especially of *P. taeda*, results from the excessive response to the invasion of *B. xylophilus*, and concerns with the resistance.

Changes in the xylem

One week after inoculation, epithelium destruction and parenchyma discoloration and necrosis were rarely observed in the xylem of any species (Table 3). Then, only in *P. thunbergii*, these changes became widespread in the inoculated branches, and most of parenchyma had been dead by 5 weeks after inoculation. A high number of xylem ray parenchyma cells became necrotic in *P. thunbergii* before the

Table 3. Necrosis and destruction of *B. xylophilus*-inoculated *Pinus* spp. xylem

Species	Portion	Destruction of epithelial cell				Necrosis of parenchyma cell			
		1 ^{a)}	2	3	5	1 ^{a)}	2	3	5
<i>P. taeda</i>	A	— ^{b)}	+	+	±	— ^{b)}	±	±	+
	B	—	—	±	±	—	±	±	±
	C	—	—	±	±	—	±	±	±
<i>P. strobus</i>	A	±	—	±	+	±	±	—	±
	B	—	—	±	±	±	±	—	±
	C	—	—	±	±	±	±	—	±
<i>P. thunbergii</i>	A	—	±	+	+	—	+	+	++
	B	—	+	±	+	—	+	+	++
	C	—	+	±	+	—	±	±	++

a) Weeks after inoculation.

b) —: absent, ±: slightly visible, +, ++: increasingly conspicuous.

cambial death (Table 1, 3). In *P. taeda* xylem, destruction of epithelial cells and necrosis of parenchyma cells, following the destruction of cortex and phloem tissue and cambium, were found proximal to the inoculation site, portion A, and were still infrequent in the distal portion. In spite of conspicuous destruction of cortex and phloem tissue and cambium, few xylem parenchyma cells died before cambial death in *P. taeda*.

Water deficit is an important factor responsible for whole tree mortality in susceptible species^{4,7)}. Physiological changes in xylem parenchyma which cause cavitation are closely related to the denaturation of xylem parenchyma²⁾, and therefore possibly related to the final death of xylem parenchyma. Death of xylem parenchyma preceded death of cortex and phloem tissue in inoculated branches of susceptible species. This suggests that xylem dysfunction finally induce cambial death in susceptible species.

In the previous studies, cavitation was found only in limited portion of *P. taeda* stem⁶⁾ and of inoculated branches of *P. strobus*¹⁴⁾. Substances which cause tracheid cavitation and progressive water blockage might not increase in the stem of resistant species for the insensitivity of xylem parenchyma to nematode activities⁶⁾. In the present study, death of xylem parenchyma was less in inoculated branches of *P. taeda* and *P. strobus* than those in susceptible species, while cortex and phloem tissue of inoculated branches of *P. taeda* were destroyed in the early stage. These histological observations also suggest that insensitivity of xylem parenchyma to nematode infection contribute to the wilt resistance of *Pinus* spp. Localized death of cortex and phloem tissue and cambium appears to induce the following localized death of xylem parenchyma in inoculated branches of resistant species, especially of *P. taeda*. Further investigation on the chemical factors associated with localized death of cambium and insensitivity of xylem parenchyma observed in resistant species will be necessary to reveal the resistance mechanisms.

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和 文 摘 要

山田利博・伊藤進一郎：マツノザイセンチュウ感染に対する材線虫抵抗性マツ、テーダマツおよびストロブマツの反応の組織学的観察

マツノザイセンチュウを感受性のクロマツ、抵抗性のテーダマツおよびストロブマツの枝に接種し接種枝の解剖観察を行った。線虫数はクロマツでは枝全体で増加したが、抵抗性種ではテーダマツの接種部近傍を除き次第に減少した。クロマツの皮層、篩部や形成層では病変の現れるのが遅かったが、抵抗性種では早くから、皮層、篩部や形成層の壊死・破壊、皮層樹脂道の閉塞がみられた。傷害周皮の形成はテーダマツで接種3週後、ストロブマツで5週後にみられたが、クロマツでは殆どなかった。木部柔組織の壊死はクロマツでは顕著であったのに対し、抵抗性種では少なかった。傷害周皮形成および皮層樹脂道の閉塞、線虫加害に対する木部の感受性の低さが抵抗性要因として働くことが示唆された。

Explanation of plate

Plate I

1. Breakdown of cortex epithelial cells (arrows) in *P. thunbergii*. Bar represents 200 μ m.
2. Cavity formation (arrows) at cambium of *P. taeda*. Bar represents 100 μ m.
3. Destruction of *P. taeda* cortex tissue. Arrow indicates the invasion of the cortex tissue by the nematode and cavity formation. Bar represents 100 μ m.
4. Wound periderm (arrow) formation around the necrotic tissue of *P. taeda* cortex and phloem. Bar represents 200 μ m.
5. Wound periderm (arrow) formation in *P. thunbergii* encircling the cortical resin canal. Bar represents 200 μ m.
6. Hyperplasia of cortex epithelial cells (arrows) (a, b) and occlusion of cortical resin canal (b) of *P. strobus*. Bars represent 100 μ m.

Plate I