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Mini review

Pine wilt disease caused by the pine wood nematode: the induced resistance of pine trees by the avirulent isolates of nematode

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Abstract

Since the beginning of the 20th century, pine trees in Japan have been seriously damaged by the pine wilt disease. This disease is caused by the pine wood nematode, *Bursaphelenchus xylophilus*, which is transmitted by the Japanese pine sawyer, *Monochamus alternatus*. The control of disease depends to a large extent on chemicals, but the public is now demanding environmentally friendly control methods. The virulence of *B. xylophilus* varies very widely. Pre-inoculation of young pine trees in a nursery with avirulent *B. xylophilus* has induced systemic resistance of trees against a subsequent inoculation with virulent *B. xylophilus*. This induced resistance was considered a hopeful means for developing a biological control for the disease. The induced resistance by the avirulent nematodes was also expressed in mature pine trees in a forest where the disease was naturally epidemic. However, the effects of induced resistance were not satisfactory for practical biological control. Since the inoculation with higher concentrations of the avirulent *B. xylophilus* induced the resistance more effectively, the pre-inoculation method will need to be improved to develop the biological control. The induced resistance of pine trees by avirulent *B. xylophilus* should be one of the candidate biological control methods against pine wilt disease. This induced resistance also provides an experimental system to clarify physiological interactions between the nematodes and pine trees.

Pine wilt disease caused by the pine wood nematode

Three species of two-leaf pines (genus *Pinus*: Subgenus *Pinus*) are native to Japan: the Japanese red pine, *Pinus densiflora*; Japanese black pine, *Pinus thunbergii*; and Ryukyu pine, *Pinus luchuensis*. In the past, the forests of these species were mostly resources for organic fertilizers and firewood. Now that we mostly use synthetic fertilizers and fossil fuels, the economic value of pine trees has decreased. Of course, there are still some economically important pine forests that produce not only timber but also Matsutake mushrooms, *Tricholoma matsutake*, which is a symbiotic fungus of *P. densiflora* and very expensive. The Japanese, however, have a sentimental feeling for pine trees. Miniature 'bonsai' pine trees are present in the small gardens of people's homes, other ornamental pines occur on golf courses, in Japanese gardens and so on. Pine trees are planted along coastal areas to prevent sand movements and damage by salt, and on steep slopes to prevent erosion because they are highly tolerant of salt and poor soil fertility. Moreover, pine trees are planted as green belts in residential areas to keep our environment quiet and clean.

Since the beginning of the 20th century, pine trees in Japan have been seriously damaged by pine wilt disease (Mamiya, 1988). The pathogens that caused pine wilt disease were unknown until it was learnt that a nematode was causing the disease (Kiyohara and Tokushige, 1971). The pine wood nematode was identified as *Bursaphelenchus lignicolus*

(Mamiya and Kiyohara, 1972), but this was subsequently found synonymous with *B. xylophilus* (Nickle et al., 1981). It was confirmed that the nematodes are transmitted from the infected dead pine trees to healthy pines mainly by the adults of the Japanese pine sawyer (cerambycid beetle), *Monochamus alternatus*, which emerge once a year in early summer (Mamiya and Enda, 1972; Kobayashi et al., 1984). The distinguishing symptom of pine wilt disease is the sudden wilting of leaves after the infection of the trees by nematodes. Focusing on this symptom, the decrease in xylem water conduction has been studied to clarify the mechanism of death of pine trees by the nematodes (Fukuda, 1997; Ikeda and Kiyohara, 1995; Kuroda, 1991) though the ultimate cause is still unknown.

B. xylophilus is probably native to North America because it is less pathogenic to some of the native pine trees in that region (Bergdahl, 1988; Dropkin et al., 1981). The nematodes in Japan are presently considered invading pests from North America because of their morphological (Nickle et al., 1981) and genetic similarity (Beckenbach et al., 1999; Webster et al., 1990) and hybridization among North American and Japanese isolates of nematodes (Guiran and Bruguier, 1989; Kiyohara et al., 1995). In recent years, the nematode was found in the Chinese mainland in 1982, in Taiwan in 1985 and on the Korean Peninsula in 1988 (Enda, 1997). The nematode was recently found in Portugal, Europe (Mota et al., 1999). The pine wilt disease caused by *B. xylophilus* has thus become a threat to Asian and European pine forests.

Damages from and control of the disease in Japan

Tremendous numbers of pine trees have been lost by the pine wilt disease since its typical symptoms were first observed in 1905 in Japan (Mamiya, 1988). However, the natural and social environments surrounding the pine wilt disease have not been constant during this time. The time may be divided into four periods: establishment of the disease in southern Japan (in the 1930s); spread to central Japan (until 1970); spread to northern Japan and the rapid increase and decrease of damage (until 1990); and the present (from 1990). The damages from the disease and its control until the late 1990s will be summarized as follows (see also Mamiya, 1988). The first outbreak of pine wilt disease in Japan in 1905 at Nagasaki, southern Japan, was controlled by eradicating dead pine trees. However, no effort was made to control the disease reported in Sasebo, near Nagasaki in 1925 because it occurred in a navy base. After that, the disease spread throughout southern Japan. Surveys of the amount of dead pine trees caused by the disease were started in 1932 (Kishi, 1995). The annual losses were nearly 10,000 m^3 in 1932 and approximately 30,000 m^3 until 1937. In the confusion of World War II, attempts to control the disease were abandoned, resulting in a rapid increase in annual losses of more than 1,000,000 (up to 1,230,000) m^3 during 1947–1950. After that, extensive control mostly by eradication of dead trees decreased the annual losses to 500,000 m^3 during the mid 1950s–1960s. However, the areas of forests infected with the disease continued to increase, and the disease spread to most of central Japan. Changes in Japanese society from the 1960s also changed the management of pine forests drastically, including the disease control strategy. Popularization of the use of fossil fuels and synthetic fertilizers decreased the economic value of pine forests. High growth of the Japanese economy attracted labour from rural communities to urban areas. Thus, most pine forests were not managed adequately, including neglect of disease control. As a result, the annual losses rapidly increased, exceeding 2,000,000 (up to 2,430,000) m^3 during the peak of the outbreaks (1978–1981). By 1982 the disease had spread throughout northern Japan, except in the northernmost part and cold districts of Honshu Island, and Hokkaido, the northernmost main island. A national project to control the pine wilt disease started in 1977. Pesticides were used in addition to eradication of dead trees after the pathogen and its vector were identified (Kiyohara and Tokushige, 1971; Mamiya and Enda, 1972). Pesticides were used broadly (Ikeda, 1984), except in forests near residences or in those used for water-sources. The rising cost, decreasing number and advancing age of workers in rural areas might also have promoted the use of pesticides. Insecticides were sprayed upon crowns of pine trees from the air and ground against vector insects for large areas of pine forests. Nematicides were injected into pine trunks against pathogenic nematodes for individual important trees such as those in famous temple grounds. Infected dead trees were fumigated after cutting. The active ingredients of these pesticides were fenitrothion and carbaryl for spraying, morantel tartrate and mesulfenphos for injection, and metam-ammonium for fumigation. The annual losses were reduced to 1,000,000 m^3 by the late 1980s, and annual losses had decreased to 720,000 m^3 by 1999 (Anonymous, 2000). However, the public has recently become critical or even opposed to the use of pesticides, especially aerial spraying. The scope of aerial spraying to control the pine wilt disease, supported by the Japanese government, decreased from 136,000 ha in the year of the peak 1981, to 37,000 ha in 1999, covering only about 10% of target forests (data from Forestry Agency, Japan). Neglect of disease control, however, could lead to a rapid increase in damage again such as seen in the late 1940s and late 1970s. Indeed, some well-managed pine forests such as those used for producing Matsutake mushrooms or those growing in tourist areas are suffering from the disease which has spread from surrounding infected forests. More environmentally friendly control methods are now being demanded. Various control measures have been studied, such as the biological control of vector insects, breeding of resistance pine trees, and so on (Kishi, 1995).

Induced resistance of pine trees against pine wilt disease is considered one of the most attractive means of biological control of the disease. It may be worth mentioning some recent activities of local communities. Control of pine wilt disease without pesticides needs a lot of labour. Some pine forests are managed by volunteers from local communities, who eradicate dead pine trees from forests, cut bush and understory and re-plant pine trees. The Japanese government has programmes to support these communities.

Induced resistance of pine trees by avirulent nematodes

Induced resistance of trees against fungal and insect pests is one of the remarkable phenomena in recent forest protection (Christiansen et al., 1999; Kaitaniemi et al., 1999) and crop plant pathology. Kiyohara, who has led many studies on pine wilt disease and *B. xylophilus*, reported that the virulence of the nematode to pine trees varied widely (Kiyohara, 1977). As early as 1980, he showed that pre-inoculation with avirulent nematodes induced resistance of *P. thunbergii* to a post-inoculation of virulent nematodes (Kiyohara, 1981). Subsequently, a series of studies on induced resistance was undertaken and the results were published in a series of papers (in Japanese) and later reviewed (Kiyohara, 1984a; 1989).

The basic methodology of Kiyohara's studies will be described. Experiments were done in the Kyushu branch of Forestry and Forest Products Research Institute (FFPRI) and in the surrounding Kumamoto prefecture, a southern part of Japan. One experiment was done in the headquarters of FFPRI, Ibaraki prefecture, central Japan. In the Kyushu branch of FFPRI, the annual mean temperature and annual precipitation were about 16 °C and 2000 mm, respectively; mean temperature and total precipitation of the period for main symptom development of the disease (June–September) were about 25 °C and 1100 mm, respectively. At the headquarters of FFPRI, the annual temperature and precipitation were about 14 °C and 1200 mm, respectively, and those in that period (June–September) were about 23 °C and 550 mm, respectively. Experiments were conducted under natural conditions. Young pine trees grown in nurseries or 1- to 2-year-old seedlings in pots were used. Nematodes, in aqueous suspensions, were inoculated into scars made by a knife on the tree trunks. *B. xylophilus* were isolated from many regions and sources of dead pine trees and vector insects, then subcultured on the fungus, *Botrytis cinerea*. Inoculation of 5-year-old *P. thunbergii* with 30,000 nematodes of these isolates per tree (Kiyohara, 1977) and 2- to 3-year-old *P. thunbergii* and *P. densiflora* with 5000 nematodes per tree (Ibaraki et al., 1978; Kiyohara, 1989; Kiyohara and Bolla, 1990; Kiyohara et al., 1977; 1983) demonstrated wide varieties of nematode virulence. Virulence of further isolates was also examined and showed wide variation. Among these isolates, three avirulent isolates named K-48, C14-5 and Ok-2, produced a mortality level of trees less than 10% and two virulent isolates named S6-1 and T-4, killed more than 90% of the trees. Sample trees were pre-inoculated with 30,000 avirulent nematodes and then inoculated with 10,000 virulent nematodes. Virulent nematodes were inoculated in the middle of July when the environmental conditions are favorable for disease development. The expression of induced resistance in pine trees was determined when sequential inoculating of trees or seedlings with avirulent and virulent isolates showed higher survival rates than inoculating with a virulent isolate alone. Survival rates of pine trees were recorded in the winter of the inoculated year. The number of sample trees in each treatment in the experiments was 10 and over. Experiments other than under these conditions will be described in the text or the legends for the tables and figures.

The degree of induced resistance of pine trees by pre-inoculation with the avirulent isolate K-48 to pine wilt disease caused by subsequent inoculation with the virulent isolate S6-1, increased with the concentration of avirulent nematodes (Kiyohara, 1983; 1984a) (Figure 1). Inoculation with avirulent and virulent

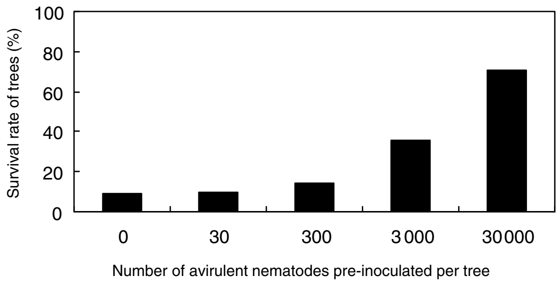

Figure 1. Effect of pre-inoculation with an avirulent isolate (K-48) of pine wood nematode, *Bursaphelenchus xylophilus*, on the survival of 13-year-old *Pinus thunbergii* subsequently inoculated with a virulent isolate (S6-1) of the nematode. All trees were inoculated with 10,000 virulent nematodes 60 days after the pre-inoculation with 0, 30, 300, 3000 or 30,000 nematodes per tree. Survival of trees was observed in September of the next year of inoculation. *n* in the range 10–14. Data from Kiyohara (1984a).

isolates (K-48 and S6-1, respectively) at the same time did not induce the resistance of 4- to 5-year-old *P. thunbergii*: the survival rate of inoculated trees was 0% (Kiyohara, 1981; 1984a). However, pre-inoculation with K-48 induced resistance of 4- to 5-year-old *P. thunbergii* when done 5–120 days before the inoculation with S6-1: the survival rates of inoculated trees 5, 10, 21, 30, 60, 90 and 120 days before inoculation with S6-1 were 60%, 75%, 90%, 95%, 60%, 75% and 60%, whereas it was only 5% in S6-1 alone (Kiyohara, 1989). Induced resistance was systemic as pre-inoculation of K-48, on the lower stems of 4-year-old *P. thunbergii* induced resistance to wilt caused by inoculation with S6-1, on the distal parts of the trees 30 days after the pre-inoculation: the survival rates were 85% vs. 15% in S6-1 alone (Kiyohara, 1984a). The multiple pre-inoculation of 10,000 nematodes of the avirulent isolate C14-5, at different times (middle May and middle June, totally 20,000 nematodes per tree) induced the resistance of 4-year-old *P. thunbergii* more effectively than single pre-inoculation of 10,000 avirulent nematodes (middle May) to post inoculation with S6-1: the survival rates were 50% vs. 35% and 12% in S6-1 alone (Kiyohara and Kusunoki, 1987). When 10,000 nematodes of C14-5, were pre-inoculated to 4-year-old *P. thunbergii*, the multiple pre-inoculation at different locations (5000 nematodes per each inoculation) on the same plant induced resistance more effectively than the single pre-inoculation (10,000 nematodes per inoculation) to post-inoculation with S6-1, 60 days after the pre-inoculation: the survival rates were 50% vs. 35% and 12% in S6-1 alone (Kiyohara and Kusunoki, 1987). Induced resistance by pre-inoculation with the avirulent isolate Ok-2, was demonstrated in 1- to 2-year old seedlings of four species of *Pinus* spp. to post-inoculation with S6-1, 30 days after the pre-inoculation indicating that this resistance was not species specific: the survival rates in Ok-2 + S6-1 vs. S6-1 alone were 53% vs. 10% in *P. thunbergii*, 60% vs. 10% in *P. densiflora*, 53% vs. 0% in *P. taedae* and 67% vs. 33% in *P. massoniana* (Kiyohara, 1984a,b).

Higher water content in the soil in the nursery probably enhanced the degree of induced resistance because survival rate of 2-year-old *P. thunbergii* were higher in thin plantings than in dense plantings when pre-inoculated with 10,000 nematodes of the avirulent isolate C14-5. Subsequently, inoculation with 5000 nematodes of the virulent isolate T-4, 30 days after the pre-inoculation produced survival rates in C14-5 + T-4 vs. T-4 alone of 43% vs. 7% in thin plantings and 20% vs. 3% in dense plantings. This experiment was conducted in the headquarters of FFPRI, and the survival was observed in middle September (Kiyohara and Yamada, 1996). Resistance of young *P. thunbergii* (2- to 7-year-old) was not induced by pre-inoculation with 30,000 heat-killed or disrupted nematodes of the avirulent isolate K-48. Inoculation with the virulent isolate S6-1, 30 days after the pre-inoculation produced survival rates of 10% in heat-killed, 5% in disrupted and 5% in S6-1 alone (Kiyohara, 1984a). The pre-inoculation of 30,000 nematodes of *Bursaphelenchus mucronatus* or other four unidentified species of *Bursaphelenchus* spp. did not induce resistance in 5-year-old *P. thunbergii* to subsequent inoculation with the virulent isolate S6-1, 40 days after the pre-inoculation. The survival rates were 15–25% in *B. mucronatus* and these four species of *Bursaphelenchus* spp. and 15% in S6-1 alone (Kiyohara, 1982). However, in a preliminary experiment, 2-year-old seedlings of *P. thunbergii* inoculated with some species of bacteria and fungi as well as six species of *Bursaphelenchus* spp. rather than *B. xylophilus*, showed high survival rates when they were subsequently inoculated with 5000 nematodes of the virulent isolate S6-1 (Kiyohara et al., 1989). These organisms did not affect the activity of *B. xylophilus* (Kiyohara, unpubl.). This induction of resistance by inoculating fungi, nematodes or bacteria was demonstrated in 1-year-old seedlings of *P. densiflora* in pots (Kiyohara et al., 1990) (Table 1).

Table 1. Comparison of ability to induce resistance against *Bursaphelenchus xylophilus* in 1-year-old seedlings of *Pinus densiflora* among different species or isolates of fungi, nematodes and bacteria (Modified from Kiyohara et al., 1990)[1]

Inducer	Source of isolation[2]	*n*	Survival rate (%)
Fungi			
Cladosporium sp.	a	9	67
Coleosporium sp.	a	9	56
Colletotrichum sp.	a	9	78
Lophodermium sp.	a	9	67
Pestalotiopsis sp.	a	9	44
Phomopsis sp.	a	9	56
Rhizosphaera sp.	a	9	44
Septoria sp.	a	9	67
Unidentified sp. 1	a	9	67
Nematodes			
Aphelenchoides sp.	b	9	56
Bursaphelenchus xylophilus[3]	c	9	67
B. mucronatus	c	6	83
Bursaphelenchus sp. 1	d	9	56
Bursaphelenchus sp. 2	d	9	67
Bursaphelenchus sp. 3	e	8	50
Bursaphelenchus sp. 4	f	9	56
Bursaphelenchus sp. 5	g	7	56
Bacteria			
Unidentified 1	h	9	44
Unidentified 2	h	9	33
Unidentified 3	i	9	56
Unidentified 4	i	9	67
Unidentified 5	j	9	44
Unidentified 6	j	9	44
Unidentified 7	j	7	56
Sterilized water (control)		9	11

[1]Inoculation of microorganisms was conducted on 21 April 1989, and a virulent isolate (S6-1) of *B. xylophilus*, was subsequently inoculated (5000 nematodes per seedling) on 27 June 1989; water suspension of fungal spores or bacteria were used for inoculation of seedlings; if spores were not made, the fungal mats on the medium were attached to the scars on the trunk; inoculum potentials of inducers were not described.
[2]a: leaves of *Pinus thunbergii*; b: rhizosphere of *P. thunbergii*; c: trunks of *P. densiflora*; d: trunks of *P. luchuensis*; e: a trunk of a kind of camphor tree, *Machilus japonica*; f: a weevil, *Niphades variegatus*; g: a cerambycid beetle, *Acalolepta fraudatrix*; h: *B. mucronatus*; i: *Bursaphelenchus* sp. 4; j: *Bursaphelenchus* sp. 5.
[3]An avirulent isolate of *B. xylophilus*, C14-5.

Biological control of disease

Kiyohara and fellow researchers identified some characteristics associated with the induced resistance in pine trees by avirulent nematodes. However, to apply this induced resistance phenomenon to the biological control of pine wilt disease, resistance had to be demonstrated in mature pine trees in forest stands. It was also necessary to determine the effects of inoculating avirulent nematodes on trees in situations where the disease was naturally epidemic. Long-term observation on the effects on tree survival of inoculating with avirulent nematodes was necessary because it had been reported that pine wood nematode populations had been maintained in *P. sylvestris* for more than 10 years in northern Vermont, USA, without showing wilt symptoms (Bergdahl and Halik, 1999). To solve these problems, three experiments were conducted, each of which was started from 1996, 1997 (Kiyohara et al., 1999; Kosaka et al., 1998) and 1998 (Kosaka et al., 2001). Thirty-year-old *P. densiflora* trees in a countryside forest around the headquarters of FFPRI were used in the experiments from 1996 and 1997. The pine wilt disease was not epidemic in this forest. In the experiment from 1998, a mixed pine forest of *P. thunbergii* and *P. densiflora* near the headquarters of FFPRI was used. The pine wilt disease was naturally epidemic in this forest. About 300 pine trees aged from 10 to 30 years grew in the forest. A new avirulent isolate, OKD-1 and a new virulent isolate, Ka-4 were also used in the experiments. OKD-1 was originally isolated from a wilted pine tree, but lost its virulence after continuous subcultures (Kawazu et al., 1996). Virulence of OKD-1, was the same or lower than the isolates mentioned previously, and that of the virulent isolate Ka-4, was the same or higher (Kiyohara, unpubl.).

Induced resistance by the pre-inoculation of avirulent isolates into mature pine trees was demonstrated in the experiment started in 1996 (Figure 2). Pine trees inoculated with the avirulent isolate C14-5 or OKD-1, and subsequently with the virulent isolate T4, showed higher survival rates (ca. 80% in C14-5+T4 and 60% in OKD-1+T4) than those exposed to T4 alone (ca. 50%), in the autumn of the year of inoculation. However, these survival rates became similar and less than 20% after the next year of inoculation. Thus, induced resistance was effective in only delaying the occurrence of disease symptoms, and not in survival of trees. The results of the experiment should be stable not later than two years after the inoculation because only one tree inoculated with the avirulent isolate OKD-1 was dead from 1998 to 1999. Some pathogenicity was shown in mature pine trees to the avirulent isolate C14-5, killing 22% of inoculated trees even in the autumn of the year of inoculation, whereas another avirulent isolate OKD-1 alone, caused almost no mortality of trees in the autumn. Although pine trees inoculated with the

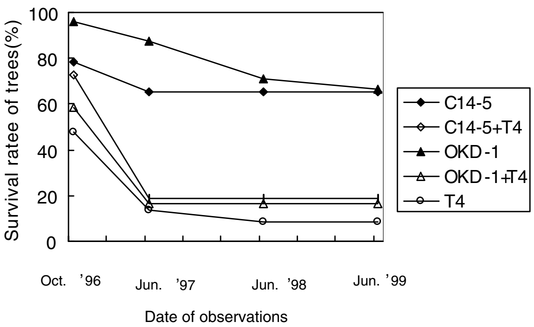

Figure 2. Effect of inoculation of an avirulent isolate (C14-5 or OKD-1) and/or a virulent isolate (T-4) of the pine wood nematode, *Bursaphelenchus xylophilus*, on the survival of 30-year-old *Pinus densiflora*. Inoculation with 20,000 avirulent nematodes per tree were conducted on 3 June 1996, and that with 10,000 virulent nematodes on 4 July 1996. *n* in the range 22–24. Data were added to Kiyohara et al. (1999) and Kosaka et al. (1998).

Table 2. Effect of inoculum concentration of an avirulent isolate of the pine wood nematode, *Bursaphelenchus xylophilus*, on the induced resistance of 30-year-old *Pinus densiflora* to pine wilt disease caused by a subsequent inoculation with the virulent isolate of nematodes (Data were added to Kiyohara et al., 1999)

No. of inoculated nematodes/tree			Survival rate of trees (%)	
Avirulent[1]	Virulent[2]	*n*	20 October 1997	18 June 1998
20,000	10,000	16	75	31
20,000	—[3]	16	100	100
50,000	10,000	16	81	56
50,000	—	16	100	94
—	10,000	15	40	27

[1]OKD-1 isolate was used for pre-inoculation on 12 June 1997.
[2]Ka-4 isolate was used for post inoculation on 9 July 1997.
[3]Not inoculated.

avirulent isolate C14-5 or OKD-1 alone, showed a similar survival rate (ca. 80%) two years after the inoculation in 1998, the avirulent isolate OKD-1 had to be used for experiments from 1997 because it did not cause early death of trees.

In the experiment started in 1997, pre-inoculation with 50,000 nematodes of the avirulent isolate OKD-1, induced resistance in mature pine trees more effectively than that with 20,000 nematodes, but some trees showed only delayed symptom development (Table 2). Thus, 81% of trees pre-inoculated with 50,000 nematodes of OKD-1 were alive in the autumn of the inoculated year, but this survival rate decreased to 56% in the following year.

In the experiment which started in 1998, 20,000 nematodes of OKD-1, were inoculated in May into about half of the pine trees in the forest. Inoculated trees showed a higher survival rate than the untreated control trees in 1998 (93% vs. 86% observed in May 1999). The surviving trees, which had been inoculated with the avirulent nematodes in 1998 and got over the pine wilt disease in this year, were inoculated again with the same amount of avirulent nematodes in May 1999. Survival rates observed in May 2000 were similar between inoculated trees (81%) and untreated control trees (83%). These results suggest that the effect of inoculation with avirulent nematodes on pine trees may have been influenced by a slight difference in the year-to-year climatic conditions.

These experiments confirm that the inoculation of mature pine trees with avirulent nematodes temporarily induced resistance to the pine wilt disease under natural conditions. These results, however, are still not satisfactory for practical biological control of the disease. It is necessary to determine the conditions where the induced resistance of pine trees is consistently effective and of longer duration. The inoculation with higher concentrations of avirulent nematodes and with multiple inoculations may be more effective in inducing resistance of pine trees to the wilt. Exploration of new isolates of avirulent nematodes that induce resistance of pine trees more effectively to the pine wilt disease may also be necessary. A chemical inducer, DL-β-aminobutyric acid, was reported to be effective against some nematode diseases of cereals (Oka and Cohen, 2000). In pine seedlings, the expression of stilbene synthase gene, which is related to the formation of stilbenoids such as pinosylvin that has potential to kill nematodes, was demonstrated by spraying sodium salicylate or its analogous to the leaves (Kuroda and Kuroda, 1998). Thus, alternative methods should be assessed for their effective induction of the resistance of pine trees against pine wilt disease.

Concluding remarks

The mechanisms of induced resistance as well as pathogenesis by *B. xylophilus* to pine trees have not been elucidated. However, some observations provide keys to solve the mechanisms. Reproductive potential of avirulent isolates of *B. xylophilus* on fungal cultures were lower than that of virulent isolates (Kiyohara and Dozono, 1986; Kiyohara and Bolla, 1990). An avirulent isolate of *B. xylophilus* showed differences of multiplication and proportion of 3rd stage dispersal juveniles from the virulent isolates when they were incubated on pine bolts inoculated with a fungus after autoclaving (Aikawa and Kosaka, 1998). Avirulent isolates of *B. xylophilus* in a glass tube moved more slowly than the virulent isolates (Iwahori and Futai, 1995; Kaneko et al., 1998). These lower potentials for reproduction and movement of avirulent isolates of *B. xylophilus* were demonstrated more clearly and conspicuously in living pine trees (Kiyohara, 1981; Kiyohara and Bolla, 1990) and seedlings (Ichihara et al., 2000). When a virulent isolate of *B. xylophilus* was inoculated to pine trees after inoculation of the avirulent isolate, overall nematode reproduction was constrained to the level of avirulent isolate alone that was far lower than the virulent isolate alone (Kiyohara, 1984a). Later, it was shown that nematode reproduction was an important factor causing pine tree mortality because inoculation with either virgin female or with male nematodes did not cause mortality of trees (Kiyohara and Yamada, 1995). In tomato plants, prior inoculation with a root-knot nematode, *Meloidogyne incognita*, decreased reproduction rate of *M. hapla* on the plant roots that were subsequently inoculated (Ogallo and McClure, 1995). Relationships between reduced reproduction rate of *M. hapla* and the amount of defense-related peroxidases in tomato plants inoculated with *M. incognita* have been described (Ogallo and McClure, 1996). In pine wilt disease, accumulation of secondary metabolites which have nematicidal activity, especially inhibiting nematode reproduction and movement, should be studied in resistance-induced pine trees by inoculation with avirulent nematodes. Another remarkable observation was that the cambium of pine seedlings where the resistance had been induced by an avirulent isolate of *B. xylophilus* was not killed by subsequently inoculated virulent nematodes, and water conduction of the seedlings was maintained only in a very thin layer of xylem near the living cambium (Fukuda et al., 1997). Based on these observations, a hypothesis suggests that one of the key determinants of death or survival of pine trees after infection of virulent nematodes is in the cambial zone (Fukuda, 1997).

The production of large amounts of avirulent *B. xylophilus* is considered easy and economical, because several hundred thousands of the avirulent nematodes can be obtained when they are cultured on *B. cinerea* growing on autoclaved polished barleycorns (Kiyohara, unpubl.). Induced resistance by the avirulent nematodes provides an experimental system to help clarify these nematode-related physiological mechanisms of pines as well as potential biological control of the pine wilt disease.

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