PINE WILT DISEASE AND THE DECLINE OF PINE FORESTS

A GLOBAL ISSUE

KAZUYOSHI FUTAI

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By

Kazuyoshi Futai

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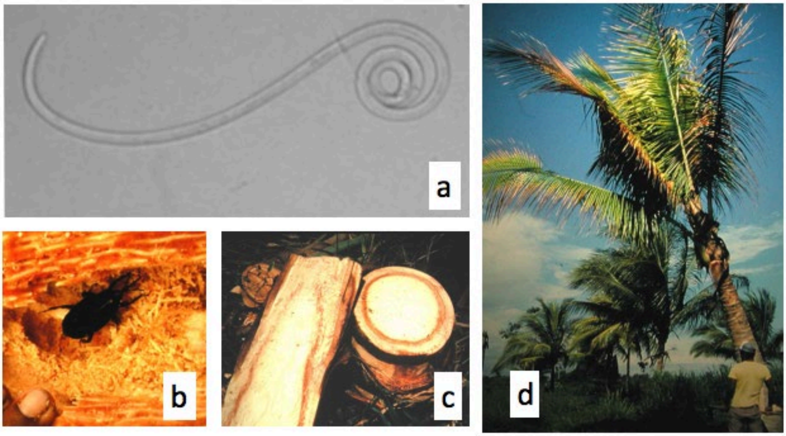

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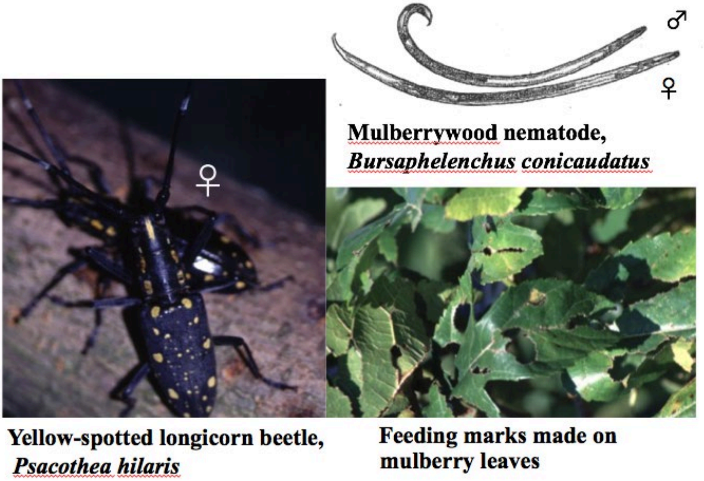

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PROLOGUE

FAMILIAR ENVIRONMENT NO LONGER: DISAPPEARANCE OF PINE FORESTS

Disappearance of pine forests: A case report

Kyoto the old capital of Japan for 1000 years until 1868, now home to nearly 1.5 million, is situated in a basin surrounded by gently sloping mountains. The Kamigamo Experimental Station of the Kyoto University Forest, where my research began is situated in the northern part of Kyoto. The area of the experimental station is covered with 60% natural secondary forest and has an area of about 50 hectares. About 60 years ago, Japanese red pine (red pine hereafter), *Pinus densiflora*, was the main tree species of the forest, along with cypress and hardwood. Since the mid-1960s, pine wilt disease (PWD) has killed many trees and devastated the forest (Fig. 1).

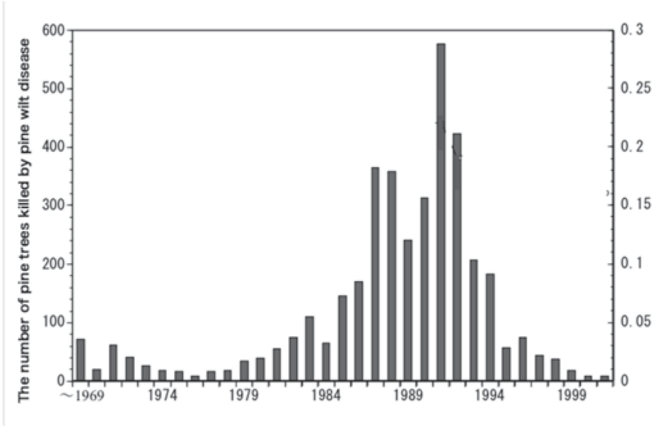

Fig. 1 Annual pine loss at the Kamigamo Experimental Station of the Kyoto University Forest.

University forest officers since 1969 made enormous efforts to control pine wilt disease by cutting down dead pine trees and removing them from the forests each year. However, PWD could not be managed, and the disease spread so extensively that the red pine trees could no longer be found within the experimental station. When I started my studies on PWD in 1977, the forest damage due to this disease was already serious, though many red pines remained in the station. In the 1970s, I enjoyed the relaxing experience of walking and driving through the pine trees. The red pine trees disappeared from the forest and it became almost impossible to find a pine tree even for experiments. How did the red pine forests that were everywhere totally disappear from the experimental station? To answer this question, I studied how PWD had spread in the station. After looking for a stand where the red pine trees were still growing, I finally found one stand.

I counted all the surviving red pine trees at this stand and plotted their locations on a map in 1995. I found 178 red pine trees in a forest area of 1.8 ha. Then I overlaid the distribution of the pine trees killed over the preceding ten years in the area. The study showed that 810 red pine trees were distributed in 1985, suggesting that almost 78% of the trees had been killed by PWD thus far (Fig. 2). All of the university forest rangers were surprised. Even those engaged in the control of PWD every year could not remember the forest being densely covered with red pine trees 10 years earlier. It was hard to understand the progress of PWD without accurate statistics of the live and dead pine trees at the Experimental Station. So I collected the data and made a figure (Fig. 3) showing the reduction in red pine trees and also the change in mortality ratio.

Fig. 3 shows that the mortality rate remained low in the early stage of PWD, then increased and reached a peak (25%) resulting in serious damage. Finally, the rate decreased along with the decrease in the number of surviving trees. The mortality rate of 25% means that one-quarter of the pine trees that were surviving in early summer were dead by late autumn. This severe damage continued in the pine forest from 1992 to 1994 and the numbers of surviving trees dropped to 20% in ten years (Futai, 1999).

Fig. 2 Almost 78% of the Japanese red pine trees at Kamigamo Experimental Station were killed in 10 years.

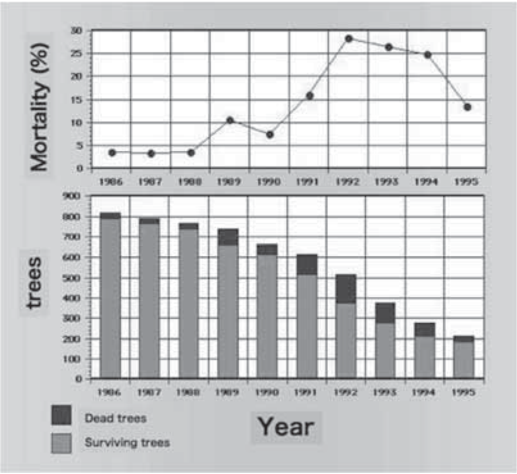

Fig. 3 Changes in the number of surviving and dead pine trees (bottom), and mortality ratio (top) at Kamigamo Experimental Station.

Loss of pine forests

Most of the low mountains surrounding the Kyoto basin were covered with beautiful red pine forests before the serious devastation caused by PWD. Local residents enjoyed visiting nearby mountains, harvesting miscellaneous trees (bush) for their rituals, and collecting edible mushrooms such as the precious pine mushrooms, matsutake, in autumn. However, the red pine forests of the mountains have almost disappeared. Being one of the world's leading forest countries, with forests covering about two-thirds of the total country area, 251,000 square km, and 10% i.e., 21,000 square km are said to be woods composed of the red pine or black pine, *Pinus thunbergii*. However, more than a quarter of the trees have been affected by PWD. Therefore, it is difficult to estimate how many healthy pine forests remain. A survey of the production of matsutake by a forest officer over a wide area of red pine forests in Kyoto Prefecture revealed that there were more than 1000 square km of pine tree-dominated forests in Kyoto Prefecture. However, most of such pine forests were devastated by PWD and replaced with various species of broad-leaved trees. The surviving JRP trees appear to be buried in the broad-leaved forest. Primary pine forests dominated by red pine and black pine decreased sharply to less than 50 square km. In other words, it had decreased to one-twentieth of the area in 30 years. However, this dramatic decrease in pine forests was not recorded in the annual governmental report probably due to the government's inadequate knowledge about the decline of pine forests.

For example, according to the governmental report, the area of man-made pine forests in Kyoto Prefecture in 1990 was about 100 square km, which was similar to that in 1980. However, the area with pine trees killed by PWD during those ten years was at least 400,000 cubic meters (equivalent to about 1,200,000 pine trees). This discrepancy may have been due to the way the area was recorded: thee forest with at least one surviving pine tree was recorded as a pine forest irrespective of the number of remaining pine trees. Therefore, the real damage and loss of JRP could not be grasped.

When the number of dead pine trees first appearing as brown spots on the green mountainside, and the discolored pines started covering the whole mountainside (Fig. 4), people finally realized something undesirable was happening and became concerned. In Japan, the mild climate conditions prevailing from spring to summer and high precipitation during the rainy season accelerate the replacement of dead pines with other tree species. Within a few years, only the skeleton of the dead pines remained leaving stems and branches on the forest floor, and soon even the bleached trees were swallowed in the sea of green trees. Before long, people forgot that many of their nearby mountains used to be covered with red pine trees or black pine trees.

Fig. 4 A number of dead pine trees appeared as discolored spots on a mountainside. (photo courtesy of Dr. Furuno, T.)

Pine forests spread with human activity

Until PWD became an epidemic, pine trees had been growing on many nearby mountains and were considered a part of the natural scene. I wondered if it was also so in ancient times. It is possible to determine the species of plants growing in the area and their dominance by identifying the species and the abundance of the pollen buried in the stratum.

This field of study is called Palynology. A palynologist reviewed the results of pollen analyses reported from various districts in Japan and presumed that the red pine and the black pine began to propagate roughly 1,500 years ago, and finally became dominant in the Japanese vegetation 500 years ago. When paddy rice cultivation began 3,000 years ago in Japan, the population in those days was sustained with sufficient food obtained from naturally available paddy fields. A stable food supply achieved through agriculture brought about a boom in the population and then urged the expansion of paddy fields by reclamation of the surrounding forests.

The inhabitants cleared the forests covering mountain slopes by cutting and burning trees for 1,500 years. Then they started intensive slash-and-burn agriculture by cultivating buckwheat, millets (foxtail millet, sorghum), beans, and tubers. After several years of slash-and-burn cultivation, soil nutrients were exploited. The agricultural land with poor soil was abandoned, and this accelerated the invasion of red pine trees. The red pine trees have symbiotic relationships with mycorrhizal fungi on their fine roots and thereby can grow on land with poor soil (the Mycorrhizal symbiosis is described later). The accumulation of fallen leaves makes the soil nutritious. As a result, saprophytic fungi begin to spread. Therefore, the symbiotic mycorrhizal fungi colonizing the roots of pine trees are suppressed by the active saprophytes. When the mycorrhizal symbiosis is compromised, water and nutrient availability is reduced and the host pine tree is replaced by the surrounding competing tree. Without human intervention, the red pine forest eventually becomes a climax forest such as an evergreen broadleaf forest.

Red pine trees flourish on mountainsides where the surface is exposed after deforestation and in abandoned wastelands surrounding newly-developed farms. Pollen of the red pine trees exceeded 50% of all pollen examined from about 500 years ago. This was the time when the techniques of agriculture such as the two-crop system, use of fertilizers, and equipment in irrigation facilities developed rapidly.

The introduction of the monetary-based system promoted the cultivation of commercial crops. Fallen leaves were collected for use as compost for the fields, which accelerated the exploitation of the forest. This made the site more comfortable for the red pine, which resulted in its dominance.

The Edo period or period of Tokugawa Shogun continued for 265 years from 1603 until the transition of power to the Meiji emperor, in 1868 when the capital was moved from Kyoto to Tokyo. The Tokugawa government promoted the development of new paddy fields, and the total area of paddy fields in Japan nearly doubled by the early 18th century. This reclamation of land for agriculture undoubtedly led to the destruction of the forests. On the other hand, various projects were carried out to protect coastal regions from erosion including the planting of Japanese black pine trees in different regions in the 17th century. Thus, man-made black pine forests gradually took over Japan’s coastal lines.

The first record of Pine Wilt Disease

PWD devastated the flourishing red pine and black pine forests. The first record of the PWD in Japan dates back to 1905. In 1913, an entomologist, Munemoto Yano, reported the occurrence of withered pine trees in the vicinity of Nagasaki city (①: see Fig. 5). The aspects of the pine disease, such as symptoms, seasonal symptom development, and spreading manner of the disease, he reported are totally in accordance with what we currently refer to as the pine wilt disease.

At almost the same time, another record of high mass mortality involving pine trees was reported in Fukuoka Prefecture (②) in Kyushu. A few years later, a large number of black pine trees that were planted along Fukiagehama Beach in Kagoshima Prefecture (③) to safeguard against salty wind died suddenly for unknown reasons. In 1915, many old pine trees were killed at a shrine in Ako City (④), Hyogo Prefecture, and massive pine extinction spread to the mainland. In the 1920s, the pine forest damage gradually spread into surrounding prefectures in southwestern Japan.

During World War II, Japan’s forests became seriously devastated and became a hotbed for the sudden increase in pine tree mortality after the war. There are several possible explanations for this sudden increase. The access to the pine forests surrounding naval ports and other military installations was severely limited. Moreover, the dead pine trees were left in the woods without any treatment and might have become an infection source. However, the major reason was the abandonment of steady efforts to keep their own mountain clean due to the lack of labor and harsh living conditions during the war.

Fig. 5 Early pine wilt occurrence sites in southwestern Japan.

PWD-damaged pine logs were moved from place to place with the movement of a large number of pine logs during and after the war. As a result, PWD spread north to over 27 prefectures from Kyushu to the Kanto area. The annual loss of pine trees caused by PWD reached 720,000 cubic meters (equivalent to about 2,000,000 trees). The General Headquarters of the occupation forces (GHQ) became concerned about the severity of PWD and were worried that the devastating scene of the pine trees in the mountains might further upset the Japanese people, already depressed in defeat. Therefore, the GHQ summoned an American entomologist, Dr. R.L. Furniss (USDA) to investigate the damaged forests and submit a proposal on the control of the disease. The method ultimately suggested was simple: cut down the dead pine trees, peel off their bark, and burn the bark. The forest agency of Japan carried out this powerful PWD control under the authority of GHQ. The result was remarkable, and the spread of PWD stopped after 1950. This simple method achieved remarkable success because of social situations and abundant availability of labor. Each local government thoroughly implemented the method under the absolute authority of GHQ, and the dead pine logs sold as firewood**,** like hotcakes.

How pine forests became seriously devastated

Japan was in a post-war reconstruction period from 1945 to 1955. Later, Japan celebrated a period of rapid economic growth, with a significant change in lifestyle, which rapidly promoted the fuel and fertilizer revolution.

People used to go to the red pine forests in the nearby mountains and scrape the floor to collect fallen needles, which were useful as fuel or fertilizer. The forest floor of the red pine forests was generally without litter sedimentation and thus poor in soil nutrients. Thereby, ecological succession became stagnant and maintained the pioneer red pine forests at the subclimax condition. As new fuel and fertilizer became available, fallen pine needles accumulated on the forest floor, resulting in the eutrophication of red pine forests.

The decline of the precious Matsutake production could be ascribed to this eutrophication of the red pine forests. On the other hand, the red pine and black pine trees were used as raw materials for pulp production. Large-scale pine afforestation was carried out, and the area of pine forests increased rapidly from the end of the 1920s to the 1930s.

However, broad-leaved trees began to be used as a natural material for pulp production in place of pines, and the stronger yen currency stimulated the import of much cheaper material (wood). The economic value of the pine forest was thus reduced and the forests were left unmanaged. The increase in the abandoned and/or eutrophicated pine forests that appeared in this period were unsuitable for the growth of the red pine trees, which are adapted to poor soil conditions. The red pine trees were constantly challenged with physiological stresses because of competition with broad-leaved trees for water and nutrients. These stresses reduced the resistance of pine trees against pests and pathogens, and damage of the pine wilt became widespread and severe.

Before the epidemic of pine wilt, the pine accounting for total timber production was almost the same as those of Japanese cedar and cypress. However, the timber production of pines decreased with the severity of pine wilt damage. This is clearly shown by the comparison of the proportion of the pine trees cut for total timber production in the areas where pine wilt damage is severe compared with that where the damage is still small. For example, the damage caused by pine wilt during the 10 years from 1986 to 1995 was 410,000 m^3 in Hyogo Prefecture in western Japan, but it was 97,000 m^3 in Iwate Prefecture in northeastern Japan where the damage was still small. In this case, the proportion of pine trees in total timber production in Hyogo dropped to less than half, but there was almost no change in the production in Iwate Prefecture (Fig. 6).

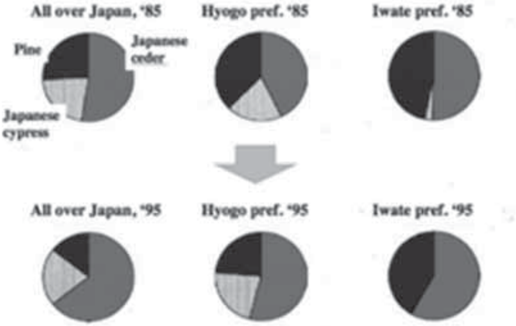

Fig. 6 Changes in the proportion of three major tree species in total timber production from 1985 to 1995.

I. EXPLORING THE TRUE CAUSE OF MASSIVE PINE DEATH

1. So-called "Pine-eating beetles" – Relationship between wood-boring beetles and trees

How "Pine-eating beetles" received this name

When the dead pine trees are cut, the bark falls off and exposes a large number of insect larvae and various tunnels they made (Fig. 7).

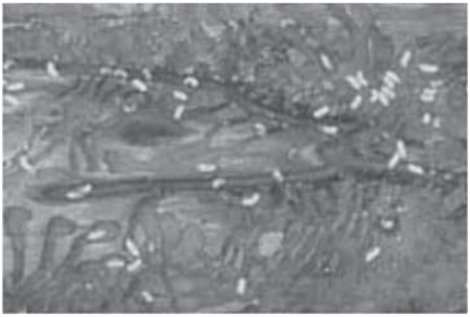

Fig. 7 Many larvae and tunnels of beetles under the bark of a tree.

Most of the insects called wood-boring beetles belong to several species and families. The most common three families are longhorn beetles, bark beetles, and weevils (Fig. 8). These insects bore holes, make tunnels under the bark, and then move into the wood. They then feed on wood tissues and/or fungi growing on them.

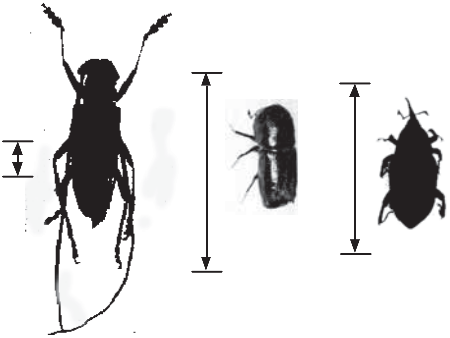

Fig. 8 The most common wood boring beetles (from left to right, a longhorn beetle, a bark beetle, and a weevil). Arrows mean 1 cm in length.

Since most of the dead pine logs harbor numerous wood-boring beetles, they were considered as a causative organism of the massive death of pines. Scientists ascribed pine death to these insects and targeted them for disease control before and after World War II.

Even 40 years after the nematode was identified as the true causal agent of the disease, the Japanese media still blame the “matsukuimushi” (Japanese word for pine eating beetle) as the cause of pine wilt disease damage. However, “matsukuimushi” is neither a scientific Japanese name nor a scientific entity, but merely a common name for the insects infesting dead pine logs. Incidentally, this word was first used by a newspaper reporter, who attended a meeting on pest control of pines held in Hyogo Prefecture, in 1941. Why did so many researchers consider “matsukuimushi” as the causal agent of the massive pine death? For a long time, whether or not the beetles damaged the living trees remained a controversial issue.

Primary forest pests and secondary forest pests.

A tree pest beetle known as the sugi* bark borer (Fig. 9 left) causes serious damage to Japanese cedar (*Cryptomeria japonica*) and Japanese cypress (*Chamaecyparis obtusa*) trees (Ito and Kobayashi, 1991). Female beetles insert their ovipositor through cracks in the bark of healthy cedar or cypress trees and lay their eggs. The larvae hatch after 8 to 18 days and stay for a while in the outer bark, then go through the inner bark and feed on the cambium tissues. When attacked, the healthy host trees exude resin to cover the wounds made by the beetles. The larvae might be hampered by the sticky resin and thereby might be killed. The beetles that can break through active host resistance and damage healthy trees are called primary pests, while those that can attack only trees or logs with reduced resistance such as withering or felled trees are called secondary pests. Wood-boring beetles can be classified into these two categories.

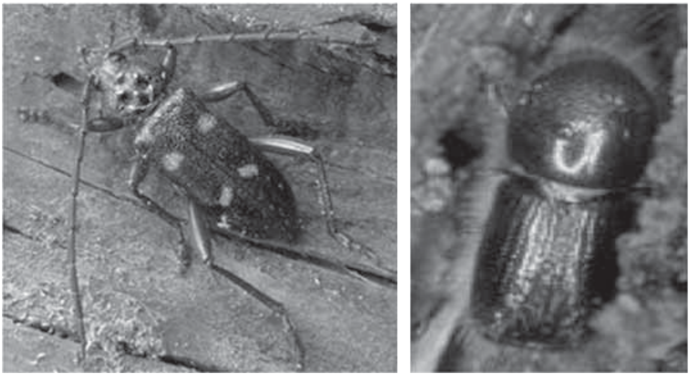

Fig. 9 Tree pest beetles. Left: Sugi* bark borer (*Semanotus japonicus*), Right: Spruce bark beetle (*Ips typographus*)

* Sugi is the Japanese name for *Cryptomeria japonica*

However, when the roots are cut off due to strong typhoon winds or their leaves are consumed by an abnormally high number of herbivores, many trees become weakened or die. Then the numbers of secondary pests increase abnormally and begin to attack the surrounding healthy trees. This phenomenon is called “a phase transition from secondary to primary pest”. Most of the wood-boring beetles are secondary pests, but such phase transitions have been reported for several species of beetles. Spruce bark beetle (*Ips typographus*, Fig.9 right), a famous pest insect of Yezo spruce (*Picea jezoensis*) and Sakhalin fir (*Abies sachalinensis*) in Hokkaido island, is principally a secondary pest. However, this beetle becomes a primary pest when a large number of trees are felled by strong wind or when the tree logs are left at a felling site (Furuta, 1989). Then, what is the cause of the difference between the primary and the secondary pests?

Tree wood is composed of three polymers; cellulose, hemicellulose, and lignin. All of these polymers, mainly made of carbon, oxygen, and hydrogen, are indigestible and persistent. However, wood contains little nitrogen, which is essential for organisms to produce protein. The ratio of carbon to nitrogen called the “C/N ratio” is an indicator of the quality as a food resource, production efficiency, and decomposition degree.

Generally, the quality as food is in parallel with the nitrogen ratio, a lower C/N ratio means higher food quality. For instance, fallen leaves have a low C/N ratio ranging from 40 to 170 depending on the tree species. Tree wood has a higher C/N ratio, ranging from 350 to 1,250 (Cowling 1970). Generally, wood contains lots of carbon, but hardly any nitrogen. Therefore, tree wood is not a useful food resource. Even the wood-boring insects cannot live on wood alone as a dietary source and have overcome this challenge in several ways.

A strategy of feeding on living tissue
– In the case of bark beetles –

Bark beetles, a group of wood-boring beetles eat the inner bark, that is, living tissue, including the cambium. These living tissues contain cytoplasmic matrices and nutrients. However, the host trees do not expose their important tissues unnecessarily to pest attack; they have accumulated repellant or toxic substances such as monoterpenes and/or polyphenols to protect themselves from beetle attacks.

Resin secretion is the most effective defense mechanism that coniferous trees have. The larvae of Sugi bark borer feeding on inner bark tissues are captured by the sticky resin, and thereby, most of them are eliminated. To overcome this host resistance, some primary bark beetles have developed a strategy called “mass attack,” in which a large number of beetles attack a host trunk together (Fig. 10 top right). The aggregation pheromone, which is a signaling substance, makes the mass attack possible (Clark et al. 2012).

A few beetles stay under the bark of the host tree and release the aggregation pheromone. Due to the release of the pheromone, numerous beetles of the same species are attracted to the tree resulting in a mass attack. In a mass attack, the amount of resin each beetle is exposed to is reduced thereby resulting in reduced resistance. Thus, it becomes possible for bark beetles to feed on nutritious inner bark tissues without being exposed to the dangerous host resin. The mountain pine beetle (Fig. 10 top left) in western North America and the southern pine beetle in the southern USA are known to have adopted this strategy.

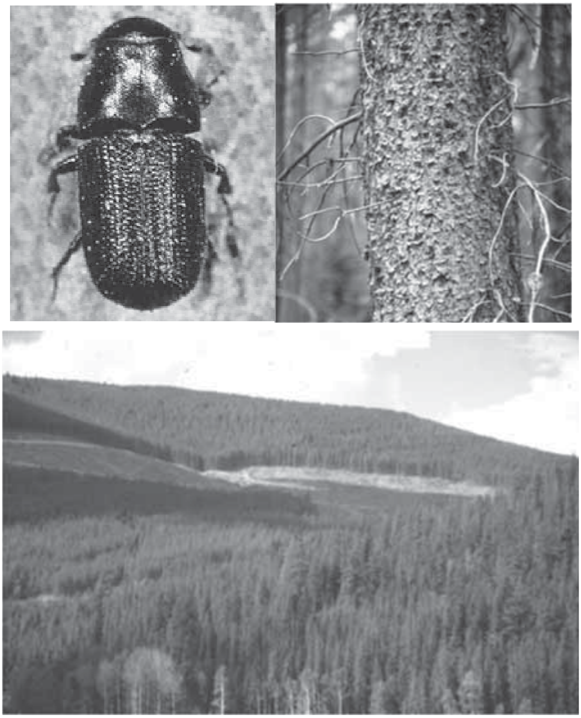

Fig. 10 The mountain pine beetle (MPB). Top left: adult beetle, top right: the trunk of a lodgepole pine with mass attack by MPB. The bottom figure: wide areas of Canadian lodgepole pine forest damaged by MPB. *See centerfold for this image in color.*

This group of beetles also adopted a distinct strategy of cooperating with pathogenic fungi to suppress the resistance of the host tree (Safranyik et al. 1973). Blue-stain fungi belong to a group of Ascomycetes that attacks sapwood at a relatively early stage after the tree dies and discolors the sapwood blue and black. The mountain pine beetle, for example, keeps blue-stain fungi such as *Grosmannia clavigerum* or *Ophiostoma montium* in sac-like mycangia on its maxillary and transports the fungi to new host trees (Whitney and Farris, 1970). The fungus that is carried depends on the species of the beetle.

Fungi moving into the host trunk by the beetle, attack the surrounding living tissues, spread over the sapwood in one month, and inhibit water conduction within the host stem. Thus, blue-stain fungi suppress the host resistance and provide the beetle a suitable breeding ground, while the beetles ensure consistent transport and continuity of breeding for the fungi (Yamaoka, et al. 1995).

There are “three major forest diseases in the world” that appear in textbooks on forest pathology. They are Dutch elm disease, white pine blister rust, and chestnut blight, and these three forest diseases have caused severe damage to indigenous trees in newly introduced locations.

Dutch Elm Disease

Generally, indigenous trees have never encountered such diseases, and consequently have no resistance to them. Among the three major diseases, *Ophiostoma ulmi,* the pathogen of Dutch elm disease, was transported to the new host tree by some bark beetle species (3 domestic species in Europe, an invasive European, and a domestic bark beetle in North America), and resulted in an epidemic (Fig. 11).

Fig. 11 Dutch elm disease. Top: An elm tree damaged by Dutch elm disease, Bottom lefr: European elm bark beetle (*Scolytus multistriatus*), Bottom right: spores (conidia) of *Ophiostoma ulmi*

Therefore, the relationship between the blue-stain fungus (*O. ulmi*) and certain bark beetles is similar to that between the blue stain fungus and North American pine beetles, such as mountain pine beetle and southern pine beetle. However, the main tactic of attacking healthy trees by these beetles is the behavior of the beetle itself, “mass attack”, while Dutch elm disease, the blue stain fungus with strong pathogenicity kills the trees, and ensures the propagation of the next generation of the vector beetles.

Bark beetles in Dutch elm disease were found to rely more on the accompanying blue-stain fungus than these beetles. Thus, the spread of Dutch elm disease is strongly influenced by the pathogenicity of the accompanying fungus. *O. novo-ulmi*, which is more pathogenic than *O. ulmi*, emerged during the disease spread and accelerated the expansion of damage in Europe. Meanwhile, in North America, the pathogenic fungus was brought in with elm logs, in the 1930s. Later, a new American strain of blue-stain fungus appeared and intensified the damage.

The American strain of blue-stain fungus enhanced the pathogenicity re-landed from North America to Europe and killed numerous elm trees by overwhelming the host resistance that had been produced by long breeding efforts.

Bark beetles that did not adopt the tactic of "mass attack"

Bark beetles that lacked the habit of mass attack are considered a secondary pest. The number of bark beetles increased abnormally and attacked the surrounding healthy trees due to large amounts of withering trees because of strong winds or an outbreak of herbivorous insects. This is mentioned above as "a phase transition from secondary to primary pest". Spruce bark beetles (*Ips typographus*) and larch bark beetles (*Ips cembrae*) are secondary pests, but *Ips* beetles have stronger primary pest traits than other secondary pests. These bark beetles also are deeply related with the blue-stain fungus. Still, they do not have the special organ, "mycangia", to carry their partner fungus, suggesting that they do not have the intimate relationship that the primary bark beetles have.

Beetles that use mushrooms as partners for nutrient absorption.

Most animals that have no wood-degrading enzymes cannot utilize wood as a carbon resource. On the other hand, many wood-rotting fungi can use persistent substances of wood. White-rot fungi can degrade cellulose, hemicellulose, and lignin to the same degree, while brown-rot fungi can degrade only cellulose and hemicellulose. Scarlet bracket-fungus, purple pore bracket, artist's bracket, and the like belong to the former group, while train wrecker and rusty gilled polypore to the latter group.

Fungi inhabiting the wood compensate for nitrogen deficiency by incorporating nitrogen from the outside of the wood by expanding their mycelia, or some fungi, like the Oyster mushroom, capture nematodes using their specialized mycelial traps and use them as nitrogen resources (Thorn and Barron 1984). Some bacteria with nitrogen-fixing ability also inhabit the wood by taking advantage of their capabilities (Griffiths et al. 1993: Perry 1994: Crawford et al. 1997). Some insects obtain carbon from wood material degraded by fungi, and nitrogen from fungal mycelia or bacteria.

By using their mycelial network, fungi inhabiting wood accumulate nitrogen and phosphorus that are present in extremely small amounts in the wood and concentrate them in their cells. Insects feeding on decaying materials absorb nutrients concentrated in these microorganisms at the same time. For instance, the larvae of giant stag beetles (*Dorcus hopei*), which is a popular pet for children, feed on the used mushroom cultivation logs (Araya 1993) (Fig. 12). Other beetle larvae that feed on rotting wood such as longicorn beetles and weevils also make use of the wood with these microorganisms.

Fig. 12 In the rotten wood, the larvae of giant stag beetles (*Dorcus hopei*) (left) feed on the mycelia of white-rot fungi such as turkey tail (*Trametes versicolor*) (right).

Avoiding the scramble for food – In the case of ambrosia beetles

Many wood-boring beetles called bark beetles utilize living tissues under the bark which is exceptionally rich in nutrients. However, the life under the bark is very competitive for common nutritive tissues among various species of insects. The part under the bark is close to the surface and easily exposed to parasitic insects’ attack from the outside. It is safer deep in the wood than right under the bark, but nitrogen deficiency is severe.

The insects that utilize rotting wood overcome nitrogen deficiency by eating nitrogen-rich microorganisms along with the wood. However, they must fight with competitors and natural enemies present in that environment. Fresh wood of the declining tree, on the other hand, is a safer environment with far fewer competitors and natural enemies. The “Ambrosia beetle” has adopted a more advanced way of utilizing a safer space deep in the wood of the declining tree. These beetles bring bait fungi deep into the wood and cultivate them as food and overcome the problem of nitrogen deficiency.

Wood-boring beetles include about 7,000 species of Family Scolytidae and about 1,500 species of Family Platipodidae. Among them, adults of all species of Platipodidae and 10 genera of Scolitydae have storage organs (mycangia) on specific parts of their bodies and keep spores of bait fungi. These beetles dig tunnels deep into the wood of the withering tree, inoculate the spores on the tunnel wall, and cultivate them for their progeny (Endoh, R. et al. 2011). The fungi that the next generation larvae feed on are called ambrosia fungi, and these wood-boring beetles are called “ambrosia beetles” (Fig. 13).

Fig. 13 The oak ambrosia beetle (*Platypus quercivorus*) and the dieback of oak trees by the infection of a pathogenic fungus, *Raffaelea quercivora*, vectored by the beetle. *See centerfold for this image in color.*

The word “ambrosia” coming from a Greek myth, means “food of God,” for immortality.

Besides the ambrosia beetles, several groups of insects, such as wood wasps (horntail), leafcutter ants, and fungus-related termites, have also evolved their fungus-cultivating systems.

2. New developments in clarifying the cause of PWD

The beetle falsely accused of causing PWD

My story has departed considerably from the main subject. The conflict among researchers on the relationship between the beetles and fungi lies in the beetle's ability to kill trees. Researchers were suspicious, but could not ignore the general opinion that wood-boring beetles are responsible for killing pine trees, and have been conducting extensive studies. The above-mentioned examples suggested that wood-boring insects might weaken the pine trees to the point of death. Thus, the suspects were considered to be the insects living in the pine trees just starting to show wilting symptoms. Based on this, eight wood-boring beetles were indeed listed as the candidates for possible causal agents of massive pine death in 1942. They are four Scolytidae beetles (bark beetles), three Curculionidae beetles (weevils), and a Cerambicidae beetle (Japanese pine sawyer). Since then, there were conflicting opinions over the causal agent of the massive pine death between two opposing points of view. Some scientists regarded the pine-wood boring beetles as being primary insect pests, while others regarded them as secondary pests that laid their eggs only on such pines that had been weakened due to other causes.

Several experiments have been carried out to answer this question. Three species of insects whose eggs and/or larvae were frequently found under the bark of the dead pine trees in most of the devastating pine forests were studied. For instance, a species of pine bark beetle (*Cryphalus fulvus*), a species of pine bark weevil (*Shirahoshizo* sp.), and the Japanese pine sawyer (*Monochamus alternatus*) were forced to lay their eggs on the healthy pine stems, or their eggs were artificially inoculated under the bark of a healthy pine stem, to examine their harmful effects.

The results of the experiments, strongly suggested that these pine bark beetles are not the primary pests, but typical secondary pests and cannot kill healthy pines. That is, “The beetle was falsely accused.” The studies on the causal agent of pine wilt disease had to return to the starting point of basic research.

New Clues – What field research revealed

In 1964, researchers at the Japanese Forest Experiment Station established a 2.4 hectares survey area in a national forest within the Chiba forestry office’s jurisdiction. More than 1800 pine trees were planted in this survey area for researchers. After observation of the seasonal changes, disease symptoms of the trees, and the feeding marks of wood-boring beetles, 300 trees were cut down in the following July, September, and October of 1965. The amount of resin exudation and the degree of damage by wood-boring beetles in each tree were examined. Focusing on the exudation amount of resin, many trees seemed healthy, but their resin exudation on the cut surface of their stumps was markedly reduced.

Moreover, the trunks of more than half of the trees were not infested with wood-boring beetles. Many such trees with the decline of resin exudation, were ill before the attack of wood-boring beetles. Thus, the hypotheses on the massive pine deaths to the wood-boring beetles had to be fundamentally reviewed. A research project team started work in 1968 to reveal the actual causal agent of the enormous pine death. Not only entomologists but also tree pathologists, tree physiologists, and pedologists, were involved in this team.

Launch of research project

The research of this team were summarized in the interim report in the “Forest Pests” vol. 9 (1970). According to the report, the following studies were carried out to elucidate the causal relationship between the attack of wood-boring beetles and the physiological disorder of pine trees: (1) Surveillance on the amount of damage and the change in the pest insect fauna in the specific research forest, (2) survey on the roots of pines, (3) survey on the infesting microorganisms (4) study on the influence of the blue-stain fungus that infest pine stems, and (5) studies on the influence of chemical and physical traits of soil, and that of meteorological factors on the occurrence of pine wilt.

To determine which conditioned pine trees are vulnerable to the attacks from wood-boring beetles, this research project team established a simple method for examination of tree health by utilizing the amount of resin exudation from artificially-made wounds and studied the changes in the physiological functions and the chemical tree constituents after withering.

Also, this team studied a pre-treatment method for victim pines against the attack of wood-boring beetles and a regeneration method after massive pine death. The scientists switched the research targets from wood-boring beetles to other unknown factors and widened their research net for both biotic and abiotic factors. This brought a big transformation from the fixed regime of research teams composed of entomologists to a large project team involving scientists from various fields.

Many tree pathologists who joined this research project assumed that microorganisms must be the real causal agent of the massive pine death. They especially concentrated their efforts on examination of microorganisms isolated from withering pines in which resin exudation just began to decrease or had ceased, and thereby attracted wood-boring beetles for egg-laying on their stems.

Koch’s postulates

Sample materials were obtained from various parts of the tree for investigation. Small pieces were cut, placed on the nutrient medium, and various fungi and bacteria grew out of the small pieces of wood after incubation. Then, such microorganisms detected from withering pines were selected, and each of them was transferred aseptically to another medium one by one (known as “**isolation**”). By observing the microorganisms thus isolated under a microscope, and examining the color and morphology of the colonies formed on the medium, or investigating the nutritive requirements based on growth on various media, microbiologists can determine the species of the organisms (“**identification**”). When a microorganism is reported to be a pathogenic microorganism or closely related species, then its pathogenicity is examined by inoculating it onto pine trees. This series of operations is based on the rules to prove that the discovered microorganism is a pathogen. This method initially developed by the German microbiologist H. Robert Koch the founder of modern bacteriology who identified the causal agents of tuberculosis, anthrax, and so on, is called **Koch's postulate**

As the next step in Koch's postulate, researchers had to examine the pathogenicity of a microorganism by inoculating it to the host organism and confirm the disease development ("**confirmation of pathogenicity**"). Then they tried to re-isolate the same species of the microorganism from the diseased host ("**re-isolation**"). (The last postulate was added by US plant bacteriologist, E. F. Smith). Japanese researchers participating in the project were vigorously exploring pathogenic microorganisms according to the old well-known postulates.

In the course of exploring the real causal agent of the massive pine death, tree pathologists discovered several candidates of pathogens in its aboveground parts, such as leaves and branches. Still, these were not involved in the withering of pine trees observed in the field. From roots, pathogenic fungi such as *Rhizina undulata* (pine fire fungus), *Cylindrocarpon* sp., *Armillaria mellea*, and so on were detected. These fungi were found less frequently in the forests devastated by the pine wilt, so they were not considered to be true pathogens of the pine wilt.

Rhizina undulata (Fig. 14), for example, is resistant to high temperatures and propagates rigorously in the soil after a bonfire or fire in the pine forest, causing the successive death of pine trees. However, the expansion of pine death is limited within the area of fungal concentric development. It is also different from the peculiar spreading pattern of the pine death due to the pine wilt disease where the withering disease spreads out was from tree to tree and from forest to forest.

Fig. 14 A candidate pathogen of pine wilt, the pine fire fungus (*Rhizina undulata*)

Breakthrough

Dr. Yozan Tokushige and Dr. Tomoya Kiyohara were plant-pathologists working at the Kyushu branch of the Forest Experiment Station, Ministry of Agriculture, and were involved in the research project of the pine wilt disease. Two years before the project research started, they had already isolated repeatedly the candidate causal pathogens from roots and surrounding soil of dead pines at the pine forests severely infested with this disease and tried to inoculate pine seedlings with the isolates. At that time, Tokushige assumed that a soil microorganism infecting the pine from its roots was a candidate pathogen.

Tokushige was paying particular attention to *Phytophthora cinnammomi*, known to infect more than 900 species of plants, including pines, and to cause disease in trees on the North American Pacific coast. He could not isolate this fungus, but instead, he obtained more common soil fungi, such as *Pythium*, blue stain fungus, *Pestalotia*, and *Fusarium*. Tokushige and Kiyohara tried to isolate candidate fungi and inoculate them to pine seedlings day after day. However, their efforts to detect the real pathogen of the pine wilt disease were in vain. Watching many Petri dishes piled high on the laboratory bench, they sometimes might have sighed deeply. One day in the early autumn of 1968, when the project started, Tokushige was exhausted with microscopic observation and then found something strange in the Petri dish. Tokushige recalled his excitement, and said: “When I was routinely looking at the Petri dish, my eyes became stuck to one point in the medium, where I saw small creatures wriggling at the tip of the pinewood placed in the center of the medium which was the isolation source.”

At first, he thought they might be mites, but they moved differently. To confirm their identity, he observed them under a stereomicroscope. The enlarged “wiggling creatures” had a long and narrow body with a slimy and glistening surface and slid on the agar medium like a snake. It was undeniably a species of nematode. Surprised, he looked all over the medium and found many nematodes on it. Due to their large number, Tokushige assumed that they had propagated on the medium on which hyphae had been growing.

The unexpected real culprit

When fungi are cultured on the medium, many hyphae grow upward from the surface of the medium, giving an appearance of a dense mycelial forest under a stereo-microscope. However, in the part where nematodes are propagating, these hyphae disappear, and the surface of the culture medium becomes slimy as if contaminated with bacteria. After that, Tokushige re-examined other Petri dishes that he had excluded from the fungal isolation due to bacterial contamination and found the same nematodes in many of the dishes.

There was little possibility of nematodes contaminating such a large number of Petri dishes during the steps of fungal isolation. Tokushige and Kiyohara concluded that “these nematodes must have inhabited the small pieces of pine woods placed on the medium to isolate microorganisms.” Therefore, they cut down the pine trees killed by pine wilt on the premises of the Kyushu branch of the Forest Experiment Station and examined each part of the trees. As expected, the two pathologists found nematodes on every part of the dead trees.

Dr. Tomoya Kiyohara and Dr. Yasuharu Mamiya who worked at the central laboratory of a Governmental Forestry Institute, collaborated to identify the newly found nematode (Fig. 15) because they were specialists in plant-parasitic nematology. Detailed taxonomic examination revealed that this nematode was an unclassified nematode of the genus *Bursaphelenchus*. The nematode was then classified as a new species, *B. lignicolus*, and was also given a common name, "pinewood nematode" (Mamiya &Kiyohara 1972). (In 1981, the scientific name of the pinewood nematode was changed to *B. xylophilus*).

The genus *Bursaphelenchus* is included in the order Aphelenchida, which is a cosmopolitan group of nematodes, and some of them are associated with insects. Others are closely associated with plants, as parasites of their roots and/or above-ground parts. Still, others are associated with fungi. About 40 *Bursaphelenchus* species had been described before the discovery of *B. lignicolus* in 1970. Because of the special concern on pine wilt disease, many nematologists studied this particular genus. They discovered many new species, and more than 130 species have been identified in this genus.

Tokushige and Kiyohara collected many pine wood samples from various districts in Kyushu island. They detected the nematode from most of these samples and confirmed its close relationship with dead pine trees. However, they did not believe that the newly discovered nematode was the pathogen of the pine wilt disease, because most of the *Bursaphelenchus* species described before that time were mycophagous (feeding on fungal mycelia), and therefore were not regarded as plant parasites. So, if they had followed such common knowledge, they would not have inoculated the pine trees with nematodes.

They had confirmed that the nematode always accompanied dead pines. This fact suits **Koch's first postulate,** that is, the pathogen must be found in abundance in all organisms suffering from the disease, but are not to be found in healthy organisms. They succeeded in culturing this nematode on the fungus *Pestalotia* sp., isolated from the branches of dead pine. This success fulfills **Koch's second postulate**, that is, the pathogen must be isolated from a diseased organism and grown in pure cultures. Thus, they inoculated the nematodes on pine trees planted on the premises of their institute.

They reared newly discovered nematodes on fungal culture and used them to inoculate three black pine and five red pine trees planted in an arboretum and an experimental forest, respectively. According to Kiyohara, the inoculation was carried out secretly; without even the director knowing. It was an inoculation test that was conducted on a trial basis, in a way that "it will probably not kill the trees". Further, it was only necessary for Tokushige and Kiyohara to adopt the methods they had been using to identify the pathogenicity of the microorganisms separated from the dead pines.

Before they obtained the results of the inoculation test, the scientists decided to report about this newly found nematode. However, there is no doubt that they were both skeptical about the pathogenicity of the nematode. In fact, in the outline of the interim report of the research project published in 1970, Tokushige reviewed the role of micro-organisms in the withering of pines. He reported on the newly-detected nematode in detail: "The nematode belonging to the genus *Bursaphelenchus*, was detected on damaged trees in various districts over Kyushu island, as well as infested roots, stems, and branches of pine trees. These nematodes were recovered from phloem tissues, xylem tracheids, resin tracts, and the pith tissue of withering pines. However, he made the following skeptical remark: "Large quantities of the nematodes were detected frequently from pines whose resin exudation had ceased, and occasionally from those with reduced resin exudation. However, they were scarcely found on fresh pine trees with active resin exudation. Therefore, this nematode seems to inhabit weakened pine trees."

Common sense might have led to the speculation that, if the newly found nematode was the real pathogen, it should always be detected from diseased pines whose resin exudation had become abnormally low. However, the nematodes were rarely separated from such trees that had begun to show reduced resin exudation.

Fig. 15 Newly-found *Bursaphelenchus* nematode (the pinewood nematode)

It became necessary to determine whether the host pine infected with the pine wilt disease, had reduced resin exudation before the number of pathogenic nematodes increased in the pine tree. However, this took a long time.

The results fulfill Koch's third and fourth postulate

On a late summer day in 1969, Kiyohara jumped into the laboratory terribly excited. "They are killed! Many of the trees inoculated with nematodes have been killed." Kiyohara reported to his boss, Tokushige, that the pine trees which had been inoculated with newly-found *Bursaphelenchus* nematodes were suddenly dead. Five of the eight inoculated pines rapidly withered, and their needles turned reddish-brown in color and died. Thus, the results of their trial inoculation fulfilled **Koch's third postulate**, that is, the cultured microorganism should cause disease when introduced into a healthy organism. "Even though it was for research, I killed five good pine trees growing in our institute without permission. I was prepared to see the director angry, but I was surprised that he gave me words of praise,” said Kiyohara reminiscing about those days with a bitter smile. Of course, they recovered some nematodes from the dead pines and confirmed that they were the same species of nematodes as they had inoculated which agrees with **Koch’s fourth postulate**, that is, the microorganism must be re-isolated from the inoculated, diseased host and identified as being identical to the original specific causative agent.

Confirmation of the results

Having been surprised with the result of the inoculation trial, Tokushige and Kiyohara then decided to carry out a full-scale inoculation test. In the following year, they carried out large-scale inoculation tests with the newly discovered *Bursaphelenchus* nematode. The study of eight field experiments with the variables of time of nematode inoculation, location, and method of inoculation. The primary inoculation method used throughout these experiments was to drill holes reaching the center of the trunk, with a blade12 mm in diameter from three directions at the ground height of the pine trees. Into each hole thus prepared, one ml of nematode suspension with specified density was injected. Control trees were injected with the same amount of sterile water in the same way. After inoculation, the holes opened for injection were sealed with a stopper of polystyrene. Moreover, the disease development of inoculated trees was estimated at regular intervals from the external symptoms and the amount of resin exudation as an index. Through these experiments, Tokushige and Kiyohara tried to verify whether the *Bursaphelenchus* nematode[1] that they found was the real pathogen of pine wilt (Kiyohara & Tokushige, 1971).

Based on their confidence that the nematode inoculation could simulate infections in the field, they examined precisely the conditions under which the pine wilt disease occurs outdoors. The results obtained during their inoculation experiments strongly suggest that the pinewood nematode itself is pathogenic and that the nematode infects a pine tree from any wound made on it. It was also clarified that the infection time, infection

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[1] Later, this nematode was named “pinewood nematode”.

density (nematode density), resistance of the host pine, etc. are critical for the outcome of the disease. After these experiments, many other researchers repeated similar inoculation tests and obtained consistent results. The achievements of Tokushige and Kiyohara (1971) have been widely recognized. In their experiments, even small seedlings of 3-year-old Japanese black pine developed the pine wilt disease when inoculated with pinewood nematodes. Experiments are easy to perform with such young pine seedlings and a large number of seedlings can be handled in one experiment. Thereafter, these pine seedlings have been used to study the mechanism of the pine wilt and also for selective breeding of pine wilt resistance. Thus, the experiments they conducted provided essential knowledge for further research.

How do small pinewood nematodes only 1 mm long infect pine trees in the field, and why can such large pine trees die so quickly one after another? Although the causal organism of the disease was identified, the details for its transmission and control remain to be studied.

3. How the pine wilt spread and became an epidemic

Starting point of my studies on the pine wilt disease

I first learned about the pine wilt in 1973 at the laboratory of Nematology of the National Institute of Agricultural Sciences**,** Kita (North) Ward, Tokyo. I stayed there for three months and started my studies in plant-parasitic nematology under the supervision of Dr. Ichinohe, director of the laboratory and known expert of plant-parasitic nematodes in Japan. He asked me to accompany him on his visit to the Forestry Research Institute where he was to obtain information on the pine wilt disease (PWD). He interviewed Dr. Mamiya, who had already been in the spotlight as one of the discoverers and nomenclators of pinewood nematode. Through the interview and a presentation at Ichinohe's seminar, I got an overview of "Pine wilt disease." I was overwhelmed that this forest epidemic was caused by such an interrelationship with several organisms and that the damage caused by this forest disease was so incredible and far-reaching.

I had intended to study nematodes at graduate school but had not considered the pinewood nematodes as a research target. At that time, the causal organism of PWD and its infection chain had already been elucidated, and it seemed unlikely that I could discover something new. Dr. K. Tanaka, a tree pathologist at the Forestry Research Institute, Kansai branch, gave me further advice. The difference in resistance to pine wilt among various pine species was still not clear, and inoculation of various pine species with the pinewood nematodes could become an interesting research project.

Before I introduce my studies on the resistance to pine wilt among host pine species, I must explain how the pine wilt spreads from place to place. As described previously, Drs. Tokushige and Kiyohara observed that inoculation of healthy pines with pinewood nematodes caused rapid withering of pine needles and symptoms of death like that seen in the field.

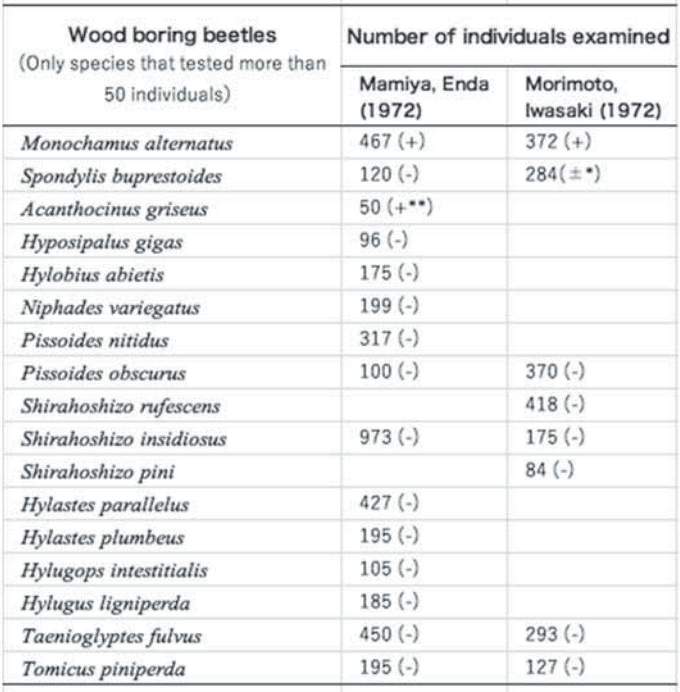
The newly discovered *Bursaphelenchus* nematode also met all of Koch's four postulates to establish a causative relationship between the organism and disease. This nematode was identified as the actual pathogen of PWD. However, field surveys in Miyazaki Prefecture revealed that PWD spread to a distance of 20 km from the infected forest within a year. Moreover, PWD was reported to be expanding about 10 km each year in Ibaraki and Shizuoka prefectures. However, the pinewood nematode is only about 1 mm in length, and it could not possibly spread pine wilt even with maximum motility. This nematode must certainly have a carrier to travel long distances.

Search for the nematode carrie

Many *Bursaphelenchus* species have close relationships with insects, especially with wood-boring beetles, and attach to the beetles to be carried from one habitat to another (phoretic relationship) (Rühm 1956). Pinewood nematode must also have a vector (insects) to transmit them from one pine tree to another. Moreover, the vector was assumed to be one of the insects that gathered on the withering or dead pines, and the search for the vector insect began.

Dr. A. Nobuchi, a Japanese taxonomist of wood-boring beetles, classified insects found in pine forests into seven groups: 1) insects feeding on pines; 2) insects feeding on plants other than pines; 3) insects feeding on dead or rotten plants; 4) insects tentatively staying at or passing through pine forests; 5) insects wandering on the ground or in the soil; 6) insects feeding on carcasses feeders and 7) natural enemies of forest insects. This classification is practical and useful, though it involves the mixing of two criteria: food type and insect behavior patterns. As for the group feeding on

Table 1 Studies to **determine** the vector of the pinewood nematode.



Wood boring beetles (Only species that tested more than 50 individuals)	**Number of individuals examined**	
	Mamiya, Enda (1972)	**Morimoto, Iwasaki (1972)**
Monochamus alternatus	467 (+)	372 (+)
Spondylis buprestoides	120 (-)	284(±*)
Acanthocinus griseus	50 (+**)	
Hyposipalus gigas	96 (-)	
Hylobius abietis	175 (-)	
Niphades variegatus	199 (-)	
Pissoides nitidus	317 (-)	
Pissoides obscurus	100 (-)	370 (-)
Shirahoshizo rufescens		418 (-)
Shirahoshizo insidiosus	973 (-)	175 (-)
Shirahoshizo pini		84 (-)
Hylastes parallelus	427 (-)	
Hylastes plumbeus	195 (-)	
Hylugops intestitialis	105 (-)	
Hylugus ligniperda	185 (-)	
Taenioglyptes fulvus	450 (-)	293 (-)
Tomicus piniperda	195 (-)	127 (-)

* Only one beetle harbored the pinewood nematode.

** The number and carrying rate of pinewood nematodes were extremely low.

pines, 218 spp. of insects**,** including Hemiptera, Lepidoptera, Coleoptera, and Diptera, are included in the list of Nobuchi. The list also includes 49 species of pests as causal agents of forestry problems, such as seven species of leaf herbivores, two species of gall-inducing insects (pests that produce galls), and 27 species of wood-boring insects. The studies to determine the vector of the pinewood nematode, investigations were focused on insects, particularly on wood-boring beetles. Two teams that were in charge of the investigations clarified that of the many insects gathering to withering or dead pines, only those belonging to Cerambycidae carry the pinewood nematode. Further studies revealed that a sawyer beetle, *Monochamus alternatus* (Fig. 16), is an essential vector for this nematode (Table 1).

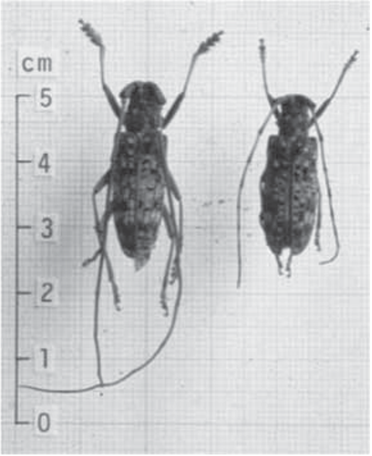


Fig. 16 Pine sawyer, *Monocham alternatus*. (Left: male, Right: female)

Medusa’s Head

Using the Baermann funnel method (Fig.17), the numbers of nematodes carried by each vector insect can be determined, and pinewood nematodes from the insects’ bodies can also be extracted by this method.

To find which organ of the insect harbors these nematodes, we have to carefully dissect the insect body under a stereomicroscope and examine which organ possesses the nematodes. Dissection of insects revealed that pinewood nematodes were lurking in the insect's tracheal system. The openings of the tracheal system are called "spiracles" and are present in two pairs in the thorax of insects and eight pairs in their abdomens, but in the *Monochamus* beetle's abdomen, there are only seven pairs. Out of nine pairs of spiracles, the pinewood nematodes enter the vector body through two pairs of spiracles in the thorax and the forward-most pair in the abdomen. The pinewood nematodes at a particular stage, "the dispersal fourth larvae." usually enter into the vector's body. Nematodes degenerate a feeding organ called a stylet seen in the buccal cavity, and lack a pump organ called the median bulb (Fig. 18).

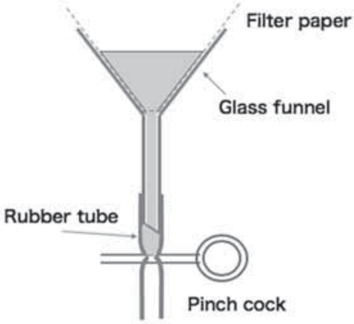


Fig. 17 The Baermann funnel method

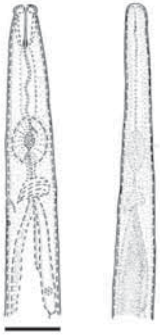


Fig. 18 Two forms of juveniles of the pinewood nematode. Left: reproductive form, Right: dispersal form.

Nematodes lurking in the vector’s trachea are in the “resting stage”. Therefore, they are not parasitic on the vector, the *Monochamus* beetle, but are only passengers who use this insect as an aircraft. This aircraft sometimes may become overcrowded. If this happens, the vector beetle will be adversely affected, and its lifespan will be shortened (Togashi,1982). One *Monochamus* beetle can carry more than 200,000 nematodes at a time if overloaded.

============== **A column article ①** ==============

The pathogenic nematode of gill-knot disease is a parasitic passenger

For vector insects, nematodes are not always safe and harmless passengers who just use them as carriers. Many parasitic nematodes exploit nutrients from the body of the insect. For example, when oyster mushrooms (*Pleurotus ostreatus*) are cultivated on a bed log, which caused “gill-knot disease,” in which a nematode *Iotonchium* produces nematode knots on the gills of mushroom’s fruiting bodies (Fig. 19). A graduate student, Tsuda revealed the tripartite relationship between mushrooms, mycetophilid gnats, and nematodes (Tsuda et al. 1996). The nematode is transmitted from mushroom to mushroom via a small mushroom fly (mycetophilid gnats) in this relationship, growing by the exploitation of nutrients from the fly’s body. The nematode in this way produces future generations of nematodes in the fly’s body. The progeny of nematodes invade the ovaries of the mushroom fly and causes atrophy. The female fly that does not notice the parasites tries to lay her eggs between the gills of *Pleurotus ostreatus*, but lays the nematodes instead of her eggs. The nematode uses mushroom flies not only for expanding its habitat but also as a nutritional resource.

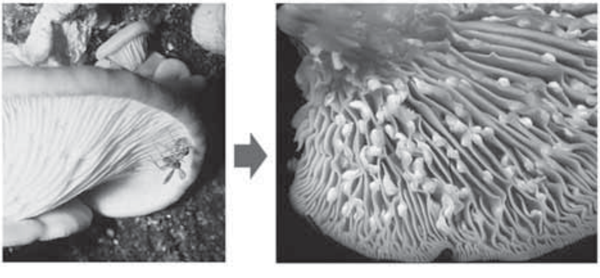


Fig. 19 Gill-knot disease caused by the infection of *Iotonchium* nematode vectored by a small mushroom fly.

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I dissected a beetle to observe the pinewood nematodes lurking inside it. When I examined the body of a beetle in water, the tracheal system, which I carefully took out of the beetle's body, started to move like a snake in the head of the Medusa. I could hardly believe my eyes. Many nematodes resting in the tracheal system were stimulated by the water and began to move actively, moving the trachea like a living organism (Fig. 20). A sharp scanning electron microscope (SEM) photo of pinewood nematodes packed in the trachea (Fig.21), which surprised researchers in this field, was published by a plant nematologist (Kondo, E. 1986).

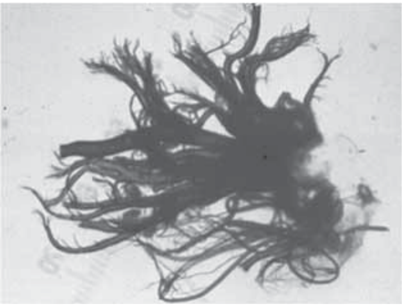


Fig. 20 The beetle's tracheal system started to move looking like snakes of Medusa.

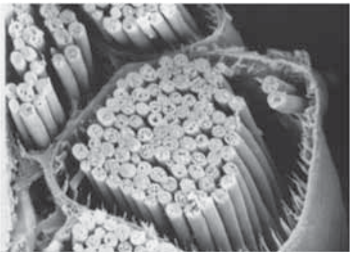


Fig. 21 Beetle's trachea tube packed with many dispersal form pinewood nematodes. (Photo courtesy of Dr. Kondo, E.)

Furthermore, careful observation revealed that most of the nematodes had their heads facing the inside of the beetle's body. The following study showed that these nematodes eventually turned around in the trachea and their heads faced outwards. These findings demonstrated that the nematodes go inside the beetle and then move out onto the pine after carried there by the beetle.

The site of nematode invasion on the host pine

The results of the inoculation tests by Tokushige and Kiyohara showed that the trees were not killed after inoculation of the intact branches and trunks of Japanese red (JRP) and black pines (JBP) with many pinewood nematodes. This nematode cannot invade the host tree that has no wounds. Some kind of injury is necessary for the nematodes to enter the host tree. Here are two possible wounds related to the vector beetle's behavior**: one is** the feeding marks made on the shoots or the twigs for feeding (maturation feeding), and **the other** is the oviposition scars formed on the trunk for laying eggs. The *Monochamus* beetle can only lay eggs on the withering trees that have already ceased to exude resin. Therefore, the site of entry for the pinewood nematodes into healthy pine trees is not the oviposition scar of the vector beetle, but their feeding wounds. Some species of insects stop feeding once they become adults, i.e., the larvae of butterflies feed on multiple plants, but only consume nectar as an energy source and concentrate on reproduction once they become adults. On the other hand, *Monochamus* beetles thrive on the bark of young pine shoots even after becoming adults (Fig. 22) because the gonads are still underdeveloped. Their gonads mature while they feed on the bark of the pine shoots, and the next generation is produced (Katsuyama et al. 1989). The feeding behavior of the beetles is essential for their sexual maturation and enables the accompanying nematodes to invade the host pine resulting in the rapid death of the tree.

This relationship between the pinewood nematode and its vector, the *Monochamus* beetle, is tragic for the pine tree but is a truly clever symbiotic relationship for the pinewood nematode and its vector *Monochamus* beetle. The *Monochamus* beetle delivers pinewood nematodes to healthy pine trees and creates the feeding wound for their invasion into the host trees. The nematodes in return provide the beetle a suitable place for oviposition by weakening the pines, thereby reducing resin exudation.

I have seen a video showing numerous nematodes coming out of the spiracles of the *Monochamus* beetle feeding on a pine shoot. They crawl onto the surface of the beetle's body and gather in white clumps at the end of the beetle's abdomen. The nematode mass is smeared on the feeding wound when the beetle's tail end touches the surface of the shoot.

Fig. 22 An adult *Monochamus* beetle feeding on young pine shoot. (Photo courtesy of Dr. T. Furuno). *See centerfold for this image in color.*

The life cycle of *Monochamus* beetle and the infection chain of pine wilt disease

The discovery of the vector of the pathogenic nematode has revealed an infectious chain of pine wilt. This is explained in the figure below (Fig. 23).

The vector beetle, *Monochamus*, emerges every May to July, from dead pine trees killed in the previous year carrying many pathogenic pinewood nematodes within its body. Some of the nematodes can reach the tip of the beetle's antennae when the number of nematodes in the tracheal system of the beetle is very high. The insects that emerged from dead pine trees are adult, but their sex glands (ovary and testis) are not matured yet. Therefore, they must continue to eat the bark of young shoots of healthy pine trees even after emergence to develop sexually. A large number of nematodes lurking in the tracheal system of the *Monochamus* beetle during this time migrate to the feeding scars (maturation feeding scars) marked on the young shoots, intruding into the pine tree. Invading pathogenic nematodes spread within the tree, and later, pine trees become diseased and begin to show wilt symptoms, which results in the decline of resin exudation and emission of volatiles, including ethanol and monoterpenes. Pines emitting volatile substances attract sexually mature males and females of *Monochamus* beetles. They then mate on the trunk, lay eggs and leave the next generation behind.

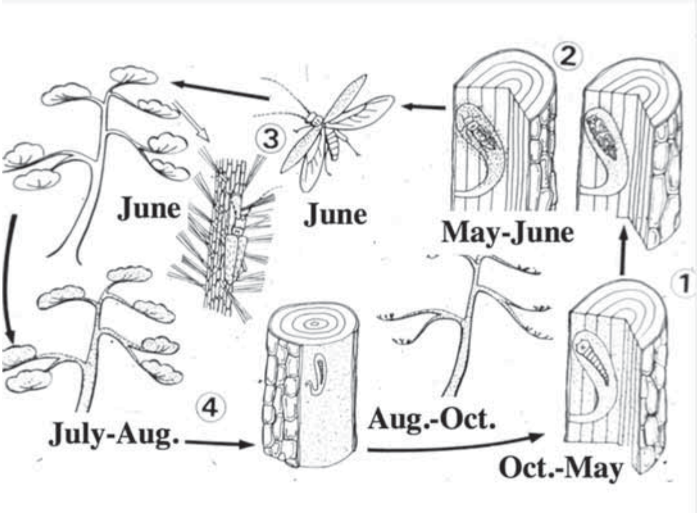


Fig. 23 The disease cycle (the infection chain) of Pine Wilt (drawn by Shuji Kontani)

The wilting symptoms progress further during long periods of high temperatures and dry summers. The color of the needles changes from yellow to reddish-brown in late summer to autumn, and it becomes apparent that the pine tree is dead. Pinewood nematodes proliferate rapidly in the tree during this time, increasing in density up to 20,000-30,000 per g of wood. The eggs of the *Monochamus* beetle that were laid beneath the bark, on the other hand, hatch within a week and become first-instar larvae. First-instar larvae repeatedly molt to the second- and the third-instar larvae by feeding on the wood tissues. Thus, the long-horned beetle, *Monochamus*, feeds on the tissues beneath the bark of dead or dying pines during the larval period.

When it becomes an adult, the beetle feeds on the bark and/or woody tissues of healthy young pine shoots. The larva moving around in the tunnel excavated in the wood becomes 4th instar larva in early autumn, and extends the tunnel deep into the wood tissue. The larva makes a large space (room) at the end of the tunnel in preparation for overwintering and plugs the entrance of the room with wood debris (frass) to protect the site (pupal chamber) from natural enemies.

The next spring, as the temperature rises, the larva after having overwintered resumes growth, eventually transforming into a pupa around May. The pupa becomes an adult in about two weeks in the completely withered pine. The newly-emerged *Monochamus* beetle flies to a healthy pine tree; it carries many pathogenic nematodes within its body. Another infection cycle begins when the *Monochamus* beetle starts eating a healthy pine shoot. Many sophisticated biological relationships remain hidden in this infection cycle.

II. BIOLOGICAL RELATIONSHIPS IN PINE WILT DISEASE

1. Biology of the pinewood nematode

My encounter with the nematode

When asked what my specialty is, the most honest answer would be studying “pine wilt disease.” At some universities, I have given lectures on microbial ecology, so I sometimes say “microbial ecology,” is my specialty, albeit sparingly.

When I was in my senior year at college, I chose to study at the Laboratory of Applied Botany, Department of Agriculture and Forestry Biology. This laboratory had two groups, a plant physiology group led by a professor specializing in flowering physiology, and a microbial ecology group led by an associate professor specializing in the developmental mechanism of ‘matsutake’ or Japanese pine mushrooms.

I had a valuable opportunity to study aquatic entomology during my first summer in college. I learned the idea of “organisms as environmental indicators.” I thought this idea could be applied to the land ecosystem, where some organisms could be used as environmental indicators, as is the case of aquatic insects in the water ecosystem. The nematode as an organism is ubiquitous in various habitats on the Earth and is extremely rich in species variety and abundance. I had read this in several books. Therefore, it may be possible to scientifically clarify the soil pollution problem using soil nematodes as indicators. It was simply age of controversy over the issues of environmental pollution.

Dr. Minoru Hamada, Associate Prof., allowed me to study nematodes, unmistakably animals, in the laboratory of plant sciences. He had been exploring the mechanism of the occurrence of the pine mushroom, known as matsutake in Japanese, for a long time, and also had an excellent understanding of the importance of soil animals. Under his guidance, the research themes chosen by the students were extremely diverse, i.e., classification of fungi, lichens, soil algae, hornworts, physiology, and ecology of fungi and bacteria. Although many researchers shared their interests in the relationships between microorganisms and other organisms, these sober themes received little attention from the public. Whenever I had the chance, I would explain how exciting these relationships are, especially while talking to my students and proposing their research themes.

Our studies have been developed on themes of interactions between various organisms related to pine wilt, the mycorrhizal symbiosis between fungi and plants, the tripartite relationship between insects, nematodes, and microorganisms and/or plants, etc. under such research environment. It is difficult to name our research group because of diverse research fields. In the end, I dare to call it a microbial ecology group.

I would like to make a brief introduction about the nematodes that we studied as a research subject. Roundworms and pinworms are the most common human parasites, and these small parasites belong to the group of nematodes. The word nematode is derived from the Greek “nema” meaning thread. In English, it is called an eelworm. In both cases, it means that it has a thread-like body or is like an elongated worm, like an eel. These worms range in length from 0.3 mm for small soil nematodes to over 8 m, as is the case for whale parasites.

The distinctive feature of the nematode is probably the fantastic diversity of its habitat among various animals. Nematodes inhabit the soil, freshwater, seawater, and other living organisms’ bodies even if we only discuss the main habitats. They can also live in extreme environments, i.e., Antarctic ice, deep in sea mud, in the soil at the high altitude of the Himalayan Mountains, in the desert sand, in hot springs, and in the vinegar that is in the process of production. Diversification of the species is promoted, and more than 15,000 species have been described so far in diverse habitats. It is estimated that more than 500,000 species exist, including those that have not been described. Some researchers estimate that free-living nematodes (non-parasitic) from the ocean alone exceed 100 million species. The species of nematodes are diverse and can be roughly divided into two groups, depending on the nutrient sources they require: free-living nematodes and parasitic nematodes. The former group feeds on microbes or prey on other animals, including other nematodes, while the latter group parasitizes animals or plants.

The size of the nematodes varies; several animal parasitic nematodes are quite large (the parasite of the sperm whale is 8~9 m in length). In contrast, most free-living and plant-parasitic nematodes are small, about 1 mm in length. The width of the body is even smaller, and you can hardly see it with your naked eye when it is not moving. The body of the nematode is extremely simple made up of about 1,000 cells, consisting of the digestive system connected from the oral cavity to the esophagus, the intestines, the rectum, and to the anus, with the ovaries and testis extending along with the digestive system. An elongated sack-like body wall encloses the digestive and reproductive organs. Males and females have their respective external genitalia, and easy to distinguish between males and females. Nematodes also have excretory systems, nervous systems, and sensory systems, but do not have specialized organs for circulatory and respiratory systems. Nematodes usually breathe aerobically, taking oxygen from the atmosphere through the body's surface. The oxygen that has dissolved in the body's fluids diffuses throughout the body.

The form of the oral cavity of a nematode suggests what the worm feeds on (i.e., its food habits). For example, the oral cavities of free-living nematodes that feed mainly on bacteria have a simple cylindrical shape, but the free-living predatory nematodes have large oral cavities with toothed projections on the inner walls of these oral cavities to seize the prey and bite and swallow it. Mycophagous nematodes that feed on fungal hyphae and plant-parasitic nematodes have a sharp needle called a "stylet" in the oral part. They use the stylet to pierce the cell walls of plants and mycelium and absorb its contents as their required nutrients (Fig. 24).

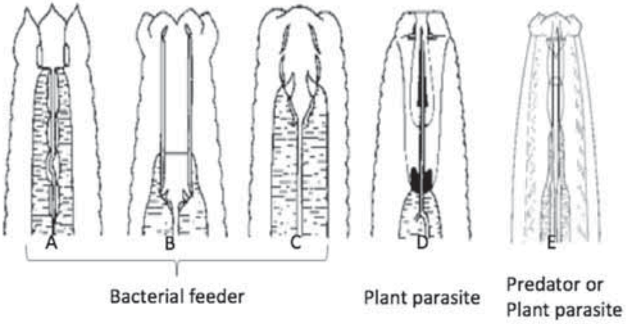


Fig. 24 Some types of the oral cavity of a nematode (from Kanzaki 2014)

Introduction to plant-parasitic nematodes

The phylogenetic classification of nematodes has been undergoing major changes since the introduction of molecular biological methods. The upper taxonomical groups of plant-parasitic nematodes were also modified, but not at the level of the "order". Most plant-parasitic nematodes are assigned to the order Tylenchida, but some belong to the order Aphelenchida or Dorylaimida.

The nematodes of the order Tylenchida mainly infest the roots of plants and feed on only plant cells as a nutrient source. However, many species of the order Aphelenchida infest the above-ground parts of plants: the leaves, stems, buds, and also fungal mycelial cells. The plant-parasitic nematodes of the order Dorylaimida are a minority as plant-parasitic nematodes. Still, some Dorylaimida species are an essential pathogen because they damage plants by transmitting plant viruses. More than 4,100 species of nematodes have been described as plant parasites to date. However, it is not generally understood that nematodes bring about as much damage to crops, fruit trees, and forest trees as caused by insect pests. This may partly be because the nematode is small, i.e., about 1 mm long, and its body is translucent and hard to notice. It is also hard to detect them because they live in soil or inside plant tissue. Moreover, it may partly be because the damage caused by the nematode appears only in inconspicuous forms, such as wilting, atrophy, yellowing, growth attenuation making the plant smaller, and yield decrease.

However, the damage to plant crops due to plant-parasitic nematodes is enormous. It is estimated that crops equivalent to $170 billion are lost annually on a global scale due to nematode damage (Elling, A. 2013). Besides, 50,000 tons of nematicides are used annually to control plant-parasitic nematodes. However, the killing of trees much larger than any crop plant by nematode infection is a little-known fact. The fact that the pathogen of PWD is a nematode species is quite a surprise, but this is not the first example of nematodes killing trees.

A peculiar plant-parasitic nematode

The peculiar plant-parasitic nematode called “red ring nematode” has a characteristically slender body. Among the “thread-like” nematodes, this nematode is particularly slender, having a width of 15.5 μm and length of 800 to 1,350 μm. This nematode is carried by a large weevil 4 to 6 cm in length, between the host trees, i.e., coco-palm and African oil palm.

When the nematodes are carried by the weevil, they stay in the beetle’s body cavity in a dormant state called “dauer larvae.” They do not feed on the weevil’s body fluid (i.e., they do not parasitize the weevil) but merely enjoy the free ride. Weevils lay eggs at the base of a palm leaf or in a wound made in a trunk, and nematodes invade the palm tree through wounds via the weevil’s ovipositor. The leaves start to turn yellow from the lower leaves of the crown a month after the invasion, and the whole leaf turns yellow and hangs down, resulting in death after two months. The dead coconut palm trunk in the cross-section shows a yellowish-brown to a reddish-brown ring 2 to 6 cm in width. It can be seen 2 to 6 cm inside of the epidermis, and the tissue of the ring contains about 10,000 nematodes per gram of tissue. The yellowish-brown to reddish- brown ring also extends from the base to the top of the coconut trunk (Fig. 25). This disease, first reported in 1905 from Trinidad (Caribbean), is called “red ring disease” because of the symptoms (Gerber et al. 1990).

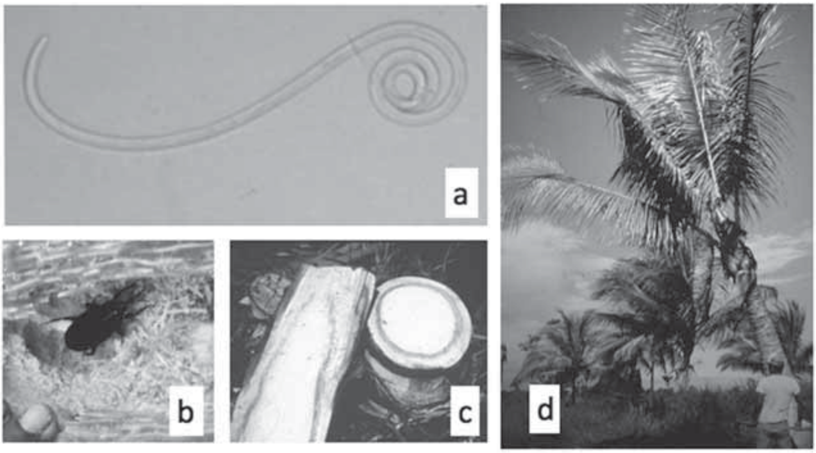


Fig. 25 Red ring disease.

a: the red ring nematode, *Bursaphelenchus cocophilus*
b: the vector beetle (weevil),
c: a reddish-brown ring shown on the palm trunk,
d: a withering coco-palm.

See centerfold for this image in color.

The annual loss at plantations due to red ring disease was estimated to be 10-15% of the total production. This disease is the most severe threat to the coconut and oil palm plantations in the tropical regions of Latin America. Red ring nematode belongs to the same family Aphelenchidae as the pinewood nematode and was formerly called *Rhadinaphelenchus cocophilus*. Later it was moved to the same genus, *Bursaphelenchus*, as pinewood nematode because of the similarity in morphological characters (Baujard 1989). However, *Bursaphelenchus* spp. including the pinewood nematode propagate both on fungal mycelia and plant cells, while the red ring nematode is an obligate parasite and proliferates only on plant cells. This crucial difference in feeding habits did not justify the change in the taxonomic position of this nematode, but subsequent molecular studies using DNA sequencing confirmed its taxonomic status and phylogenetic relationship (Ye, W. et al. 2007, Kanzaki, N. et al. 2014).

How the red ring nematode secures its vector

The weevil, the vector of the red ring nematodes, flies to healthy palms to lay its eggs. The coco palms and oil palms start pruning leaves and harvesting fruit 4 to 7 years after germination. Weevils are attracted at this time by the chemicals released from the wounds made by pruning and harvesting; they then fly there to lay eggs. Thus, both the coco and oil palms remain healthy at the time the female weevil lays its eggs. The red ring nematodes invade from the oviposition scar and kill these healthy palms. Similarly, the vector *Monochamus* beetle makes a feeding wound, the pinewood nematode invades host tissues through the wound and reproduces its progeny. The nematode causes the pine tree to become diseased and stop resin exudation because of its virulence. The diseased tree also emits attractants that lure the vector beetle to the tree which is a safe place for the beetle to lay its eggs. Thus, the pinewood nematode can secure the vector beetles for their progeny. The red ring nematode, on the other hand, invades host tissues when its vector weevil lays its eggs on palm trees. Therefore, the red ring nematodes can secure the vector from the beginning of the infection. Thus, how do the *Bursaphelenchus* species most of which have little virulence, obtain their vectors?

Living small and sparingly

Before the discovery of the pinewood nematode, all of the species of the *Bursaphelenchus* were not considered to be plant parasites**,** but to be mycophagous. The pinewood nematode makes the pine tree diseased, to which the vector beetle is attracted. Thus, the nematode provides a place for the vector beetle to lay eggs, while the vector beetle helps the nematode move to a new host.

Then, how do other nonpathogenic *Bursaphelenchus* species continue their life cycle? We have to examine the life cycles of the non-pathogenic *Bursaphelenchus* species to answer this question.

B. mucronatus is a far less pathogenic species related to the pathogenic pinewood nematode, *B. xylophilus*. If *B. mucronatus* infects a healthy pine and survives on the tree, and the vector beetle never visits the tree because the fresh pine never emits attractants for the vector beetle. Thus, the nematode's progeny are extinguished. There are a few withering pine trees in most pine forests that cannot obtain sufficient light (these are referred to as suppressed trees) because they are under the crowns of surrounding trees. Few pines also have been damaged due to other causes such as intense herbivory, various microbial pathogens, strong winds, and heavy snow. The pine longicorn beetle, *Monochamus*, initially lays its eggs on such withering pines. There were few withered pines before the spread of the pine wilt. Therefore, the population of the *Monochamus* beetle was also very low, or it was a rather scarce species. Therefore, this beetle was prized among longhorn beetle collectors.

Less pathogenic *B. mucronatus* must have been passed down for generations by invading the withering host via the oviposition scar of the vector, the *Monochamus* beetle. *B. mucronatus* was widely distributed in Japanese pine forests before the pinewood nematode *B. xylophilus* spread north from Kyushu and became predominant in Japan. The original form of the relationship between the genus *Bursaphelenchus*, the vector beetles, and the host pine species may be similar to that between *B. mucronatus* and the *Monochamus* beetle. An interesting example supporting this view follows.

Mulberry-wood nematode

Dr. N.Maehara, a researcher of forest pests at the Forestry and Forest Products Research Institute (FFPRI) formerly associated with my laboratory, investigated the yellow-spotted longicorn beetle (*Psacothea hilaris*) that feeds on the plants of the Moraceae family, e.g., mulberry and fig trees. He discovered a new species of *Bursaphelenchus* nematode lurking in the insects' tracheal system of the beetle like the pinewood nematode that is carried by the *Monochamus* beetle.

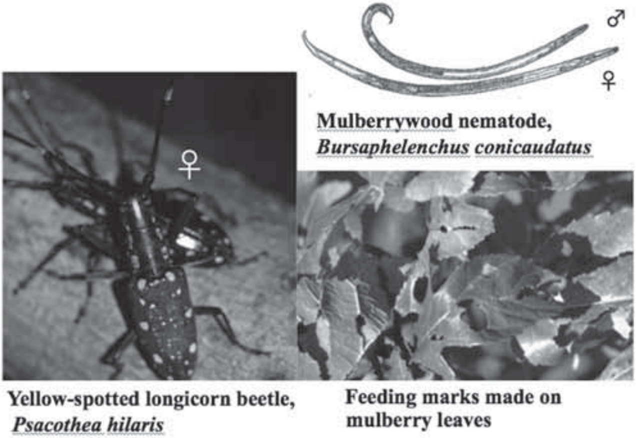
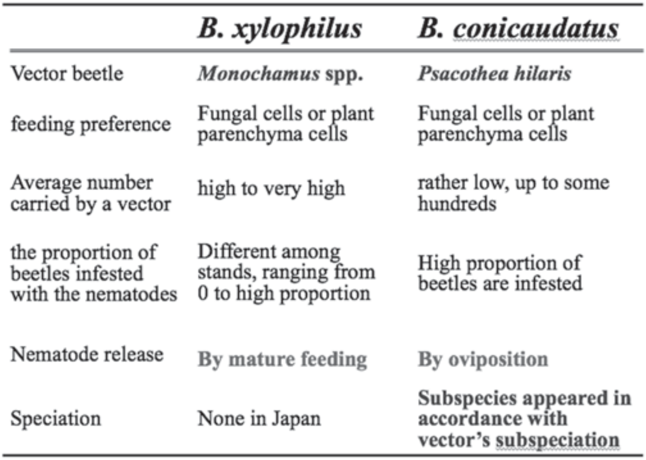


Fig. 26 Mulberry-wood nematode and its vector beetle (Photo courtesy of Dr. Kanzaki). *See centerfold for this image in color*.

However, this nematode (called mulberry-wood nematode hereafter) has no virulence on the mulberry and fig trees, unlike the pinewood nematode. Therefore, it is inconvenient for the mulberry-wood nematode to infect a healthy tree because it would break the relationship with the vector, the *Psacothea* longicorn beetle. How does this nematode invade the mulberry and fig trees? The *Psacothtea* beetle feeds on the host plant leaves but not on the bark of young shoots, even after becoming an adult (post maturation feeding) (Fig. 26) like the *Monochamus* beetle. Dr. Kanzaki who was also a former member of my laboratory and is working at FFPRI extended Maehara's work. He reared a *Psacothea* beetle in a plastic container giving it a mulberry leaf to feed on and a thick mulberry branch for ovipositing. He examined the leaf and branch for nematodes. The result was clear and straightforward, and mulberry-wood nematodes were collected only from the branch where the female beetle laid her eggs. *Psacothea* beetle lays eggs on a withering tree or a withering part of the mulberry or fig, and the mulberry-wood nematodes invade the host body from the beetle's oviposition scars. In this way, the mulberry-wood nematode evades the defense responses exerted by the host tree against intruders, which ensures nematode colonization and propagation in the host wood tissues. In return, nematode proliferation is limited to the area surrounding the beetle's tunnel, where the larvae of the beetle move actively, and the host tissues are discolored and covered with flourishing fungal hyphae. The mulberry-wood nematodes propagate around the beetle's tunnel and feed on the fungi proliferating there. The mulberry-wood nematode unlike the pinewood nematode cannot reproduce widely in the dying or dead host tree, but it ensures the transmission to the next mulberry or fig tree by perpetually living near the *Psacothea* beetle (Kanzaki & Futai, 2001). On the other hand, the pinewood nematodes spread and grow to every corner of the invaded tree because it acquires virulence to kill the pine tree. If so, how do the pinewood nematodes that have moved far away from the beetle's tunnel find and reach the vector, the *Monochamus* beetle when it ecloses in the following spring?

Table 2 Comparison between pinewood and mulberry-wood nematode



	B. xylophilus	***B. conicaudatus***
Vector beetle	***Monochamus*** **spp.**	***Psacothea hilaris***
feeding preference	Fungal cells or plant parenchyma cells	Fungal cells or plant parenchyma cells
Average number carried by a vector	high to very high	rather low, up to some hundreds
the proportion of beetles infested with the nematodes	Different among stands, ranging from 0 to high proportion	High proportion of beetles are infested
Nematode release	**By mature feeding**	**By oviposition**
Speciation	None in Japan	**Subspecies appeared in accordance with vector's subspeciation**

Life cycle of the pinewood nematode

The pinewood nematode is transmitted by the *Monochamus* beetle from a dead pine to the young shoots of living pines. Therefore, healthy living pines become damaged one after the other, and this is the mechanism of the epidemic expansion of pine wilt disease. I will show you a smart trick played by the pinewood nematode carried by the *Monochamus* beetle. Before explaining the method, I will show you the more detailed life cycle of the pinewood nematode. The female pinewood nematode produces about 100 eggs during its oviposition period of 30 days when cultured on a fungus grey mould (*Botrytis cinerea*). Cleavage proceeds in the oviposit cocoon-shaped egg and becomes a 1st stage larva, but this larva remains in the egg as it is, molts once, and hatches as a 2nd stage larva. The 2nd stage larva then molts three times and becomes an adult. Under suitable conditions (25 ～28℃), it takes only four days for an egg just oviposited to become an adult female to produce eggs. The population of the pinewood nematode reaches hundreds of thousands after several weeks when cultured on a fungus grown in a 50mL flask.

The pinewood nematode actively increases in population when endowed with food and space. The nematode, at this stage, is in the propagative form. However, when the food is exhausted, and the living environment deteriorates, the number of nematodes decreases rapidly. At the same time, a particular type of juvenile, dispersal 3rd stage juvenile, appears among the surviving nematode population, and its proportion among the population gradually increases. The nematode at this particular stage stores dark lipid granules in their body and they are slightly larger than the propagative 3rd stage juvenile. When the pinewood nematodes are cultured under these food-deficient conditions, the propagative form juveniles die rapidly and reduce in number, while the dispersal form 3rd stage juveniles survive these harsh conditions. Ishibashi and Kondoh (1977) revealed that the dispersal 3rd stage larvae survive more than 70% for six months at 20 C. The nematodes isolated from the wood tissues around the pupal chamber of the *Monochamus* beetle are at this particular stage.

However, to move on to the *Monochamus* vector’s body, and thereby to be transmitted to the next pine tree, the nematode has to molt once more and proceed into a more specialized stage, the dispersal 4th juvenile stage. The nematode at this stage has a dome-shaped head and loses the lip region and neck structure at the very tip of the head. This specialized stage juvenile also loses the stylet to pierce the cell walls of the fungal hyphae or plant cells to suck up the contents. The organs that act as a pump and suck the collected food (= median bulb) and for digestion (= the intestine) also disappear (Fig. 17). Moreover, the body surface is also covered with sticky material for being carried conveniently by the vector beetle. The pinewood nematodes at this stage are only carried by the *Monochamus* beetle, and they do not parasitize this insect at all, and there is no need for organs involved in feeding and digestion. That said, what mechanism induces this particular stage of the juvenile?

============= **A column article ②** ==============

Are pinewood nematodes attracted to the pupal chamber of the *Monochamus* vector?

When the *Monochamus* beetle was detected as a vector of the pinewood nematodes, it was elucidated that the nematodes are densely distributed around the pupal chamber of this beetle (Mamiya, 1972). Well, how do these nematodes gather around the area? To reach good-smelling food, you can direct your body in the direction of the pleasant smell and move to get closer to it. However, here is another way, too. While moving around randomly, when the tempting smell is intensified, you can reduce the frequency with which you change direction and/or lower your movement speed. On the other hand, when the smell is weakened, you can increase the rate you change direction and/or increase your movement speed, and you will reach the food as a result, though this is an inefficient method in comparison with the former one. Thus, there are two ways for the motile organisms to migrate to external stimuli. One is called "topotaxis," and refers to the directed movement or orientation of a motile organism towards the source of a stimulus. The other is "kinesis", which refers to the movement of a motile organism in response to a stimulus, but the movement is non-directional. Depending on the intensity of the stimulus, the speed of movement (orthokinesis) or the frequency of turning (klinokinesis) is changed, resulting in aggregations. The migrating behavior of lower organisms, i.e., the pinewood nematode, is often the latter method. Therefore, the pinewood nematodes do not directionally gather around the pupal chamber of the *Monochamus* beetle, but eventually, gather (settle in) at that place.

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2. Airliner and passengers

A secret that induces a particular juvenile stage

Dr. Maehara, who discovered a new *Bursaphelenchus* nematode in the yellow-spotted longicorn beetle, solved this secret using a simple model system. The artificial pupal chamber was a model system shown in Fig. 27. This chamber was made of pinewood blocks (2.5 x 2.5 x 5 cm) of 12-year-old Japanese red pine. A hole (1.7 x 1.2 x 4 cm) was bored into each block to simulate a *Monochamus* pupal chamber. Each block was placed into a small wide-mouth bottle, and was plugged with a silicon-rubber stopper, and sterilized at 121℃ for 30 min. Then a blue-stain fungus was inoculated into the woodblock as bait for the pinewood nematodes. When the fungus grew well, the nematodes inoculated on it propagated extensively. Later, the larva of *Monochamus* reared aseptically from an egg and on an artificial diet was introduced into the hole bored into the block. Among the different stages of *Monochamus* beetles, only when a late-stage pupa or an adult beetle was introduced, the particular stage (the dispersal 4th stage) of juvenile nematodes was generated among the nematode population and moved onto the beetle’s body.

The dispersal 3rd stage juvenile, the precursor of the durable stage (the dispersal 4th stage juvenile) of the pinewood nematode, appears even in the absence of the *Monochamus* beetle. Stimulation to induce the 3rd stage juvenile occurs due to food depletion and environmental deterioration under the overpopulated conditions of the pupal chamber. Even when the number of dispersal 3rd stage larvae increases, the next durable larvae do not appear. The presence of the later stage of the pupa or the adult of the *Monochamus* is necessary to generate the sustainable stage of the nematode. The dispersal 3rd stage juveniles induced by environmental degradation enter the particular durable stage due to specific chemical signals emitted from the late pupa or the adult of the *Monochamus* and transfer into the vector beetle’s body.

The pinewood nematodes spread throughout the dead pine tree, aggregate around the pupal chamber of the vector beetle, and are carried to the next healthy pine by *Monochamus*. There are some necessary conditions for the pinewood nematodes to aggregate around the pupal chamber of the *Monochamus* beetle. The excretions and secretions of the longhorn beetle provide rich nutrients and appropriate humidity to the wall of the pupal chamber, promoting the proliferation of microbes, i.e., blue-stain fungi. The pinewood nematode feeds on these fungi resulting in colonization and reproduction of these nematodes. Thus, the pinewood nematodes aggregate and propagate in the wood tissues surrounding the pupal chamber, where the matured 4th instar *Monochamus* larvae overwinter and pupate in the following spring. This aggregation of the pinewood nematodes results in an extremely high nematode density compared to other sites.

This is how the pinewood nematode secures a way for transmission to the next breeding site, and the continuity of the species is maintained.

Observation of the inside of the beetle's pupal chamber

The inner surface of the pupal chamber of the *Monochamus* can be observed with a stereomicroscope. There would appear mycelium of blue-stain fungus growing abundantly, nematodes with slimy skin crawling around and between the mycelium, and more giant mites and springtails walking around in search of prey. There were small fruit bodies (ascocarps) of blue-stain fungus in certain places. If you are fortunate, you can see the peculiar behavior of the nematodes as they crawl on the fruit bodies, rising to their apexes, and twisting and shaking their bodies. This behavior is called "nictating" and is considered to be the behavior of the nematode awaiting its host and vector, like the crew of a shipwreck waving for help on the mast. Respiratory activity increases and a large amount of CO_2 gas is emitted when the pupa of the *Monochamus* emerges to become an adult. Nematodes that entered a durable stage are attracted to this CO_2 gas and move onto the beetle.

Tokushige and Kiyohara's inoculation experiments suggested that the larger the number of nematodes invading the host tree, the more likely the tree will get diseased. Therefore, a single *Monochamus* beetle carries more nematodes, it is more likely to develop pine wilt disease. The number of nematodes carried by a beetle can be considered as an indicator of the harmfulness of each beetle.

What determines the number of nematodes carried by a single beetle?

The number of nematodes carried by a single *Monochamus* beetle within its trachea varies significantly among the individual beetles. For instance, the number varies with the study area, and even within the same stand. The number of nematodes carried by a beetle also differs depending on the tree from which the beetle has emerged. Furthermore, the number of nematodes carried by a beetle varies even among the beetles that have emerged from the same tree.

The distribution of the pinewood nematodes is uneven in the dead pine trees, which are overwhelmingly large compared to the size of the nematodes. Thus, the number of nematodes carried by a single beetle differed depending on the part of the tree where the beetle has made its pupal chamber. The number of nematodes carried by a beetle is reported to decrease when the water content of the pupal chamber declines to less than 20%. Furthermore, the *Monochamus* beetle stays in the pupal chamber for a few days even after emergence, and the nematodes that have been waiting around the pupal chamber move into the beetle's body.

Thus, the period the adult beetle stays in the chamber also affects the number of nematodes the beetle carries, and the longer stay period results in a higher number of nematodes. Because this nematode feeds on fungi in the dead pine tree, the species of fungi propagating in the wood, their degree of propagation and the distribution manner in the tree affect the population of the nematode. Thus, the fungal factor also determines the density of nematodes in the wood, and thereby determines the number of nematodes held by a *Monochamus* beetle emerging from that pupal chamber. One or two species of fungi were inoculated into the artificial pupal chamber (Fig. 27), and the pinewood nematodes were cultured on these fungi. Then larva of the *Monochamus* beetle was placed into the artificial pupal chamber to examine the number of the nematodes held by the beetle after emergence.

The relationship between the number of nematodes recovered from the beetle and the species of the fungi inoculated, inoculation order of the fungi, and their combination were examined.

A blue-stain fungus, *Ophiostoma* sp., was used as a suitable bait for the nematode in the experiment. A *Trichoderma* fungus that is highly competitive against the blue-stain fungus and is less appropriate bait for the nematodes, and a parasitic fungus, *Verticillium*, that kills nematodes, were used as the antagonistic fungi. The wood was inoculated with one of these fungi alone or with another fungus simultaneously, or at different times to change the degree of dominance of each fungus in the chamber. Then the nematodes were inoculated on them to reproduce. Inoculation of three fungi, i.e., the blue-stain fungus, *Trichoderma*, and *Verticillium* creates an environment of suitable food, an environment of less ideal food, and a hostile environment, respectively. The simultaneous inoculation of these fungi would alter the environment and is expected to affect the propagation of the nematodes. For instance, the simultaneous inoculation of the blue-stain fungus with *Trichoderma* may result in the suppression of the growth of the former due to the latter. On the other hand, inoculation of the two species of fungi at different times generally suppress the growth of the later-inoculated fungus by occupying the breeding niche with the early-inoculated fungus. Table 3 shows the total number of pinewood nematodes propagated in the pupal chamber under the different fungal settings, and the number of nematodes transferred to the beetle's body. The pinewood nematodes hardly propagated in the artificial pupal chamber when the wood was inoculated with the *Verticillium* fungus, and few nematodes were recovered from the *Monochamus* beetle's body. This inhibition was not affected by the presence of the blue-stain fungus whatever the order of inoculation (Table 3).

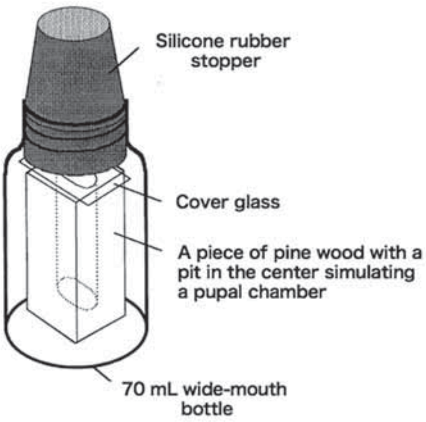


Fig. 27 The artificial pupal chamber

Table 3 Effect of fungi on the propagation of pinewood nematode around pupal chamber and its transfer to vector beetle (Maehara and Futai 1997)

Species of fungi and order of inoculation	**repetetion**	**Number of nematodes propagated**	**Number of nematodes transferred to the vector beetle**
Ophiostoma	10	655,790	2759
Trichoderma	9	11,915	35
Verticillium	8	0.3	0
Ophiostoma +* *Trichoderma*	12	16,482	86
Trichoderma →* *Ophiostoma*	9	12,172	88
Ophiostoma → *Trichoderma*	12	36,187	2979
Ophiostoma + *Verticillium*	5	3.6	1.2
Verticillium → *Ophiostoma*	6	0.7	0.3
Ophiostoma → *Verticillium*	5	0.6	0

＊A + B means to inoculate A and B fungi at the same time, and A → B means to inoculate fungus A first and then fungus B at a later time.

On the other hand, the relationship between the blue-stain fungus and the *Trichoderma* was somewhat more complicated. When the wood was inoculated with *Trichoderma* alone, or together with the blue-stain fungus, *Trichoderma* was superior in competitive ability and dominated the entire chamber wood resulting in the suppression of the reproduction of the pinewood nematodes to a low density. Then, the number of nematodes that transferred to the *Monochamus* beetle became even lower. Therefore, vigorous reproduction of the nematodes is ensured only when the blue-stain fungus predominantly propagates throughout the pupal chamber. Such a situation appears when the blue-stain fungus is inoculated alone or before *Trichoderma*. This will increase the number of nematodes carried by a vector beetle. Thus, the species of fungi proliferating around the pupal chamber of the *Monochamus* beetle has a significant influence on the number of nematodes carried by the beetle.

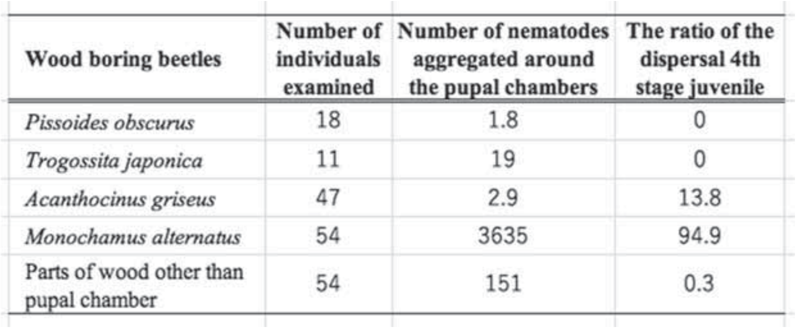
Why is it only the *Monochamus* beetle that carries the pinewood nematodes?

The process of holding the pinewood nematodes by the *Monochamus* beetle and transmitting them from dead pine to healthy pine became clear. It was also revealed how the number of pinewood nematodes carried by a *Monochamus* beetle is determined. But a simple question arose. Various species of insects, i.e., bark beetles, weevils, longicorn beetles, etc. cohabit in dead pine trees. Why is it only the *Monochamus* beetle that holds the pinewood nematodes and not other beetles? Table 4 shows the results of the survey conducted by Dr. Maehara regarding the holding of nematodes by vectors. Among various insects cohabiting with the *Monochamus* beetle, he selected a weevil species *Pissoides* sp., a small longicorn beetle *Acanthocinus* sp., and a predatory beetle *Torogossita* sp., and compared the number of nematodes aggregating around their pupal chambers with those around the *Monochamus* pupal chamber.

Almost no pinewood nematodes were collected from the pupal chambers of the three species of beetles examined except the *Monochamus* beetle or pine wood nematodes specifically aggregated around the pupal chamber of the *Monochamus* beetle only. There are two reasons why the pinewood nematodes are carried by the *Monochamus* beetle; 1) the pinewood nematodes aggregate specifically around the pupal chamber of the

Monochamus beetle, and 2) the late-stage pupa or adult of the *Monochamus* releases chemical stimulants that induces the dispersal 4th stage juvenile nematodes and enables them to move into the beetle's body.

Table 4 The number of nematodes aggregated around the pupal chamber of four boring wood beetles and the proportion of dispersal 4th stage juveniles (Maehara & Futai, 2002)



Wood boring beetles	Number of individuals examined	Number of nematodes aggregated around the pupal chambers	The ratio of the dispersal 4th stage juvenile
Pissoides obscurus	18	1.8	0
Trogossita japonica	11	19	0
Acanthocinus griseus	47	2.9	13.8
Monochamus alternatus	54	3635	94.9
Parts of wood other than pupal chamber	54	151	0.3

Pupation, Eclosion, and Emergence

The *Monochamus* beetle creates a pupal chamber in the dead pine and overwinters as the 4th stage larva in it. It literally becomes a pupa in this chamber as the temperature rises from the next spring to early summer. The length of the pupal stage varies with the temperature, ranging from one week in the warm to nearly three weeks in the cold.

Since the developmental process of insects is affected by temperature, the emergence time of the *Monochamus* beetle may differ depending on the year and the location. To control the *Monochamus* beetles, it is essential to apply the insecticide at the time the vector beetle emerges from the dead pine tree. Therefore, it is important to accurately estimate the time of emergence to determine the appropriate time to apply the insecticide. The applied insecticides will kill the vector beetles when they have emerged from the dead pine tree, but will not kill the vector beetles that are still in the tree. It is crucial to accurately judge the changes in temperature in the specific area each year to control pine wilt disease by killing the vector beetles with insecticides. However, even within the same forest, the thermal conditions at the site of the pupal chamber would depend on the level of exposure to the sun, e.g., is it on the upper side directly exposed to the sun or is it on the inside or lower side of the tree that is not reached by the irradiation. Thus, there is considerable variation in the time of the actual emergence of each *Monochamus* beetle. The emergence time estimated based on the meteorological thermal conditions should be considered only a guideline.

Therefore, just fixing the date for spraying an insecticide according to the calendar every year for human convenience or arbitrary scheduling the insecticide spraying date is far from a valid method of controlling pine wilt disease. Implementation of pesticide application without considering the emergence time of the *Monochamus* beetle may be one of the reasons why many projects to control pine wilt disease have failed in various places.

As the pupa of the *Monochamus* beetle pupates in the pupal chamber, first the whole body is white and its eyes are red. With time the body becomes amber-colored, and the eyes become darker in color. The darkening color is a sign that the beetle will soon eclose. When the pupa ecloses, the adult beetle body is first white, and its elytron (a hardened forewing) is still soft. Then coloration of the body progresses, and the whole body becomes stiff over time completing the transition to an adult beetle. Before its body becomes stiff, the *Monochamus* beetle stay in the pupal chamber for 3 to 7 days. During this period, the pinewood nematodes gathered around the chamber move into the beetle’s respiratory organ, the trachea.

The *Monochamus* beetle with a big, robust jaw can hold a lot of pinewood nematodes in its trachea while making a round hole the size of the beetle’s body to escape from the dead pine tree (Fig. 28).

Fig. 28 *Monochamus* beetle is trying to escape by opening a circular hole the size of its body. (by courtesy of M. Yamasaki)

After leaving the dead tree, the vector beetle flies to a healthy pine tree and begins to gnaw at young, succulent branches. Only after this feeding activity does this longhorn beetle develop its gonads (sex glands) and becomes sexually mature (Katsuyama et al. 1989). This vector beetle vigorously gnaws at the branches. The pine trees killed by pine wilt disease are cut down, and many feeding wounds can be found by careful examination of the branches of the crown. These feeding wounds provide entry portals for the nematode. However, pinewood nematodes are at a resting stage of a particular and durable type in the trachea of the *Monochamus* vector. What does it mean that the nematodes transfer to the pine tree while still asleep? Some interesting studies have been conducted in this regard.

When and how does the pinewood nematode exit the vector beetle?

It is interesting to know what time the pinewood nematodes exit the body of the *Monochamus* beetle. How do the pathogenic nematodes transmit to a pine tree from the *Monochamus* beetle? The infection occurs after the emergence of the *Monochamus* and the timing is essential from the viewpoint of disease control. In other words, this is important information needed to determine the exact time to apply the insecticide to kill the *Monochamus* vector for effective control of the pine wilt disease.

Dr. Kishi, at the Ibaraki Prefectural Forest Research Institute, put a *Monochamus* beetle in a Baermann funnel apparatus to study the invasion of the pinewood nematodes into a host pine (Fig. 29). He fed the beetle a thin branch of pine and replaced the branch at regular intervals. He regarded the number of nematodes that emerged from the branch as the number of nematodes that had invaded them.

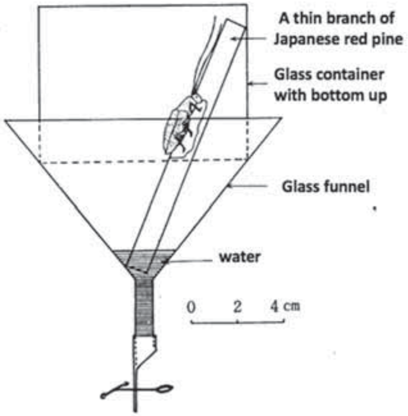


Fig. 29 Equipment for investigating the separation process of pinewood nematodes exiting the vector beetle's body (from Kishi 1995)

By this method, however, the nematodes that once invaded the pine branch might pass through the branch and drop into the water below. Therefore, the number of nematodes that have invaded the pine branches may be underestimated. Still, this is a quick and straightforward method for tracing the invasion pattern of the nematodes to pine trees. In Dr. Kishi's seven-year study, the time of the nematode's departure from the beetle's body peaked between one and two weeks after the beetle's emergence. While in other research reports, the pinewood nematodes scarcely exit in the first week after the emergence of the *Monochamus*, and in many cases, the departure peaks around two weeks after the beetle's emergence (Mineo 1983, Linit 1989). The assumption that the peak departure is around two weeks after the appearance of the *Monochamusis* is consistent with the following experimental results (Mineo and Kontani, 1975). They compared the pathogenicity of the *Monochamus* beetles in different days after emergence against 3-years potted black pine seedlings. No black pine seedlings died when the beetle was allowed to eat within one week after its appearance but 40 to 90% of the seedlings were killed when the beetles were used 2 to 7 weeks after emergence. These studies were conducted to determine the appropriate time to control pine wilting. Still, the results encouraged subsequent researches on a hidden relationship between the pathogenic nematode, the beetle, and the host pine tree.

The gonads of the *Monochamus* beetle are not yet developed immediately after emergence. Therefore, the beetle gnaws at the young, succulent branches of healthy pines. The mating and oviposition of the beetles begin ten days after emergence, and the pinewood nematodes remain in the vector beetle during the first ten days after emergence when the beetle feeds on healthy pine branches. Do the pinewood nematodes invade the host pine tree from the feeding wounds made by the beetles on the pine branches? Most of the nematodes remained in the beetle's body for a week after the emergence of the longhorn beetle. This seemingly contradictory behavior of the nematode can be explained as follows: the pinewood nematode is said to have sneaked into the timber imported from North America and came to Japan about a century ago. Most pine species distributed in North America are resistant to the pinewood nematode. Actually, the North American pine species were hardly killed in my nematode inoculation experiments using various pine species.

The relationship between the North American pine species and the pinewood nematode native to North America, is identical to the relationship between the Japanese red pine/black pine and the non-pathogenic *Bursaphelenchus mucronatus* native to Japan. It is also similar to the relationship between the mulberry wood nematode and its host plants, mulberry and fig trees in Japan. Host trees in such relationships are resistant or immune to these nematodes, and the host tree would not be diseased even when infected with these nematodes while the vector is feeding on a healthy host. Therefore, vector insects cannot lay their eggs on these healthy trees. The mulberry wood nematode transmits to the host tree when the vector beetle, *Psacothtea*, lays its eggs on a weakened host tree or weakened part of the host. The non-pathogenic nematode, *B. mucronatus*, is also mostly transmitted to the host pine when the vector beetle lays its eggs on the tree (Togashi, in personal communication). Of course, the pine must have already been weakened due to suppression or other diseases.

The pinewood nematodes may have had similar relationships with the various native pine species in North America. The pinewood nematode is transmitted to North American pines only when the vector beetle lays its eggs onto the weakened pine trees. The nematodes scarcely exit from the *Monochamus* beetles when they start actively feeding on healthy pines in Japan. The number of nematodes leaving the beetle increases markedly about ten days after emergence when the *Monochamus* beetle begins its reproductive behaviors, i.e., mating and oviposition. It appears contradictory because the feeding scar must be the invasion portal for the nematodes. Therefore, it is reasonable to consider this peculiar behavior as a remnant of the life cycle in North America.

The odor emitted from the feeding wound made on a pine branch

The volatile substances emanating from the feeding wound made by the *Monochamus* beetle can be assumed to trigger the pinewood nematodes for the transfer to the host pine tree from a dormant state deep in the trachea of the beetle.

Watanabe and his colleagues studied the effects of host substances on the pinewood nematodes, especially the volatile terpenes contained in the branches of the pine species. They revealed that the nematodes were attracted to α-pinene, β-myrcene, limonene, β-phellandrene, etc., and most intensively to β-myrcene. The resistant pine species contained a lower concentration of β-myrcene, while the susceptible ones contained a higher density. They assumed that the lower density of β-myrcene and movement of a small number of nematodes to the host tree is the decisive factor of host resistance (Ishikawa et al. 1987). However, even when I artificially inoculated the resistant pine species with the pinewood nematodes, the pines were not killed (Futai and Furuno, 1979). Therefore, it is not possible to explain the resistance of each pine species with the hypothesis that resistant pine species have low concentrations of attractants and, therefore, fewer nematodes migrate to the host tree. In my *in vitro* experiments, the nematodes aggregated to the resistant pine shoots as much as to the susceptible ones (as described below).

The pinewood nematodes used in these experiments were the propagative juveniles cultured on the fungal hyphae in the flask. On the other hand, the nematodes carried by the *Monochamus* beetle and transfer to the host pine tree are at a unique stage (dispersal stage). Therefore, the pinewood nematodes at this stage may respond differently to the host attractants than the propagative ones. It is necessary to conduct our experiments under more natural conditions. In this regard, the research reported by Professor M. Linit and his student W. Stamps at Missouri University is intriguing.

Change in the pinewood nematodes' preference

Linit and Stamps observed the attractiveness of several chemicals like linoleic acid (a fatty acid), α-pinene, β-myrcene (terpenes), and toluene, monoolein (hydrocarbons). Linoleic acid and monoolein are found in the living life residue of the *Monochamus* beetle, i.e., the beetle's excrement. The larva has a habit of spreading the living residue on the wall when the 4th stage larva of the *Monochamus* makes its pupal chamber. Because of the *Monochamus* larva's habits, the concentration of the fatty acid around the pupal chamber is high (Giblin-Davis, 1993). Therefore, the fatty acid plays an essential role in finding the vector beetle in the dead pine wood by the pinewood nematode. Further, a hydrocarbon, toluene, is a component of the adult beetle's cuticle.

Monoterpenes, on the other hand, are chemical constituents of pine trees, and these chemical constituents dramatically change when pine trees are diseased and then killed. The concentrations of chemicals in healthy pines change from two to four weeks after the tree is killed and the particular substances begin to appear (Bolla et al.1989). Stamps and Linit (1998) examined how these chemical substances attract the dispersal larvae of the pinewood nematodes in the trachea of the *Monochamus*. They used straightforward and suitable equipment (Fig. 30) to trap the *Monochamus* between the net and the lid of the petri dish and won't touch the water. The pinewood nematodes, on the contrary, exiting the beetle fall into the water stored under the net. They could count the number of living pinewood nematodes exiting the beetle after a given time. The chemical substance to be tested was dropped onto the surface of the water beneath the net. Further, they created a ventilation window with a net on the lid. Employing this ventilation window, the test substance would not fill the container and impair the reaction of the nematodes.

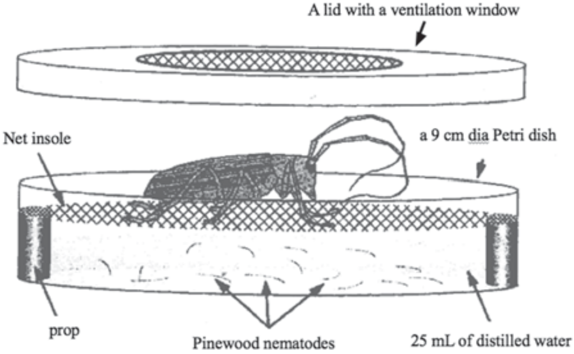
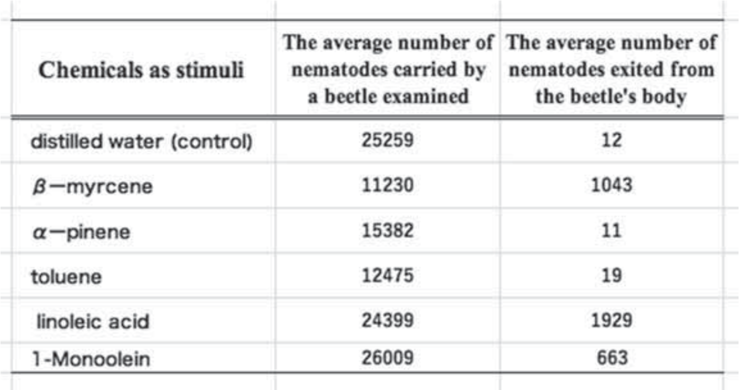


Fig. 30 Equipment for investigating the effects of chemical stimuli on the separation of pinewood nematodes out of the vector beetle's body (from Stamp and Linit 1998)

The dispersal stage nematodes carried by the beetle were strongly attracted to β-myrcene, linoleic acid, and monoolein in this experiment (Table 5).

The concentration of the substance used affected the attraction, and β-myrcene and linoleic acid had the highest attraction activity at a concentration of 10.4 molars. They compared the responses of the nematodes to the five chemicals mentioned above at 10.4 molars concentration between the propagative juveniles and the dispersal juveniles. The propagative juveniles were strongly attracted to the vector beetle-related chemicals, linoleic acid, and monoolein, but scarcely attracted to the host-related β-myrcene.

Table 5 Withdrawal of pinewood nematodes from the body of *Monochamus* beetle by stimulation of several chemicals



Chemicals as stimuli	The average number of nematodes carried by a beetle examined	The average number of nematodes exited from the beetle's body
distilled water (control)	25259	12
β—myrcene	11230	1043
α—pinene	15382	11
toluene	12475	19
linoleic acid	24399	1929
1-Monoolein	26009	663

The dispersal juveniles, on the contrary, were hardly attracted to linoleic acid and monoolein. Still, they were strongly attracted to β-myrcene, a particular substance of host pines, and toluene, the component of the adult beetle cuticle. Thus, the propagative juveniles and the dispersal juveniles at different stages of the life cycle of the pinewood nematode had different responses to the vector beetle-related and the host pine-related chemicals. The propagative juveniles' behavior seems adaptive to their stage because the propagative juveniles aggregate around the beetle's pupal chamber were markedly attracted to the chemicals accumulated around the chamber. The behavior of the dispersal juveniles appears peculiar because they were attracted to both the host pine-related and the vector beetle-related compounds. This seemingly inexplicable result leads to more interesting facts.

Storage materials in the nematode body

The pinewood nematodes lurking in the *Monochamus* beetle's tracheae remain in the body during the first 10 days after the beetle's emergence. However, β -myrcene which is attractive to the nematode is vigorously emitted from the vector's feeding wounds and stimulates the nematodes in the vector's tracheae even during this period. The nematodes start leaving the vector body ten days after the emergence of the beetle, even without the pine-derived attractant β-myrcene. Thus, it seems that the nematode's departure from the *Monochamus* beetle is controlled not only by external stimuli but also by internal factors of the nematode itself. What are the internal factors? The nutrient that supports the nematode that ceases feeding activity like the dispersal juveniles of the pinewood nematode is the neutral lipids stored in the body (VanGundy1965). Kondoh and Ishibashi (1978) proposed that the dispersal juveniles of the pinewood nematode obtain the energy necessary for survival and transformation to become adults by converting storage lipids into glycogen (Fig.31). The stored neutral lipids are consumed at a constant rate (1% a day) even in the body of the dispersal juveniles. Linit and Stamps focused on the storage lipids as the internal factor for the nematodes' departure from the vector beetle, *Monochamus*. Therefore, they used the equipment mentioned above to determine the number of nematodes leaving the beetle on different days after emergence. However, nothing was added to the distilled water receiving the detached nematodes in this case. Thus, they tried to compare the amount of neutral lipids stored in each individual between the nematodes exiting the beetle and those remaining in the beetle. They stained each nematode with oil red dye to stain the lipids stored in its intestine, then took pictures with a video camera attached to a microscope, and recorded them on a computer (Fig. 32). They measured the area of the entire body of the nematode and the area stained with oil red using image analysis software and calculated the ratio. These experiments were conducted where external stimuli were swept away. They examined the amount of the neutral lipids in the nematodes exiting from the vector beetle 1 day to 70 days after emergence at 1-week intervals.

Fig. 31 A dispersal juvenile separated from the *Monochamus* beetle, some time after eclosion. Its intestinal storage lipids were depleted and body color was transparent (upper). On the other hand, the dispersal juvenile separated from the beetle just after emergence had a darkish body color due to storage lipids filling the intestines (lower)

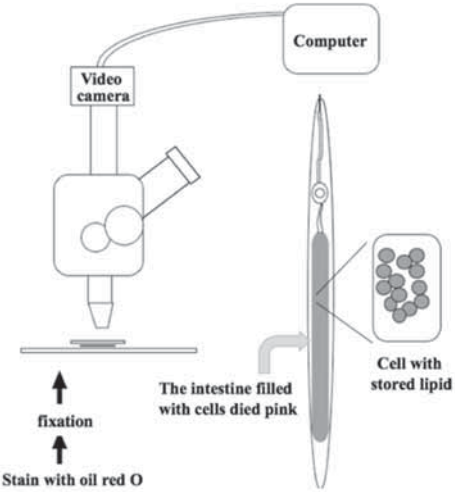


Fig. 32 A method used by Stamp and Linit to measure stored lipids in dispersal juveniles.

The image of the nematode stained with oil red was photographed by a video camera loaded in a microscope, connected to a computer, and the area of the stored lipid portion dyed pink was calculated using image analysis software. They also examined the number of nematodes remaining in the beetle at the same intervals. The number decreased with time. Nematodes that exited the beetle's body generally had fewer lipids than those remaining in the beetle's body. This difference was especially remarkable until three weeks after the emergence of the vector beetle. Linit and Stamps also found that the amount of neutral lipids in the nematodes decreased at a constant rate over time. This result suggests that using the changes in the body lipids as a clock, the pinewood nematodes in the vector beetle's trachea exit spontaneously (Stamps and Linit, 1998b). Linit and Stamps, in their experiment, put small pieces of filter paper immersed with one of the five chemicals mentioned above on an agar plate and inoculated the plate with pinewood nematodes to select them. They also compared the amount of neutral lipids stored in the intestine of the nematodes aggregated to each chemical substance. The amount of the storage lipids was higher in the nematodes aggregated to the vector beetle-related chemicals, toluene, and monoolein. On the contrary, the storage lipids were lost in the nematodes aggregated to the host pine-related chemicals, β-myrcene (Stamps and Linit, 2001). From the results obtained by Linit and Stamps, the exit of the pinewood nematode from the *Monochamus* beetle's body and transfer to the host pine tree can be summarized as follows.

Assumed mechanism of nematode transfer from vector to host pine

The intestine of the nematodes invading the trachea of the *Monochamus* beetle is filled with the storage neutral lipids. The nematodes in this state are strongly attracted to the vector beetle-related chemicals but do not respond to the host pine-related compounds. Therefore, they do not leave the vector's body. The lipids stored in the intestines of the pinewood nematodes decrease with time at a constant rate, and a spontaneous exit begins with the reduction in the amount of lipids stored in the individuals.

A change in the response of the nematode to the surrounding smells occurs simultaneously, and the nematode responds sensitively to the β-myrcene emitted from the pine, and the departure from the insect body is further advanced.

After moving to the vector beetle, the dispersal juveniles of the pinewood nematodes would be attracted to the beetle's body for a while. After a specific time, they become strongly attracted to the host pine, thereby the pinewood nematode transfers from the vector beetle to the feeding wound marked on the pine branch. Thus, a delicate mechanism hidden in the nematode behavior is responsible for the infection cycle of pine wilt disease with high efficiency.

The nematode exits the beetle's body spontaneously after a certain period. The beetle stays in its pupal chamber for a few days after becoming an adult. During this period, the pinewood nematodes that have aggregated around the wall of the chamber invade the beetle's respiratory system, the trachea. Because the pinewood nematodes actively enter the beetle's trachea and their heads face the inside of the beetle's body (Kondoh, 1986). Aikawa, a scientist at the FFPRI, used the *Monochamus* beetles a day after emergence and macerated them to observe the tracheal fragments contained in them. He then examined which way the nematodes involved in the fragments were facing, the inside of the beetle's body, or outside of the body. At first glance, the crushed trachea looked like a small tube, and it seems to be impossible to distinguish which is the front, and which is the back. The cilia (fine hairs) are densely present inside the tracheal fragments, and the hair ends are always facing the outside of the vector's body in careful observation. This fact enables him to distinguish between the front and the back of the trachea. Based on this finding, Aikawa (2000) examined the direction of the nematodes in the beetle's trachea. He found that most of the pinewood nematodes in the beetle just after emergence were facing the inside of the beetle's body. The more days after emergence, the more the nematodes turn their bodies facing their heads toward the outside. Thus, the hypothesis proposed by Linit and his colleagues is supported by this spontaneous exit of the pinewood nematodes from the vector beetle's body according to their internal biological clocks.

3. What determines the pathogenicity of two pinewood nematodes – A comparison with the less-pathogenic pinewood nematode

The pinewood nematodes that exited the vector beetle invade the host pine tree from feed wounds marked on the branch, and exerted their pathogenicity. Several questions have arisen about the process of infection. For instance, what keeps the nematodes from leaving the wound and scattering away? Can they enter the injury without any difficulty? How does the pine tree respond to this invader? Above all, how do small nematodes kill a huge pine tree within a few months after infection?

A less pathogenic pinewood nematode, *Bursaphelenchus mucronatus*

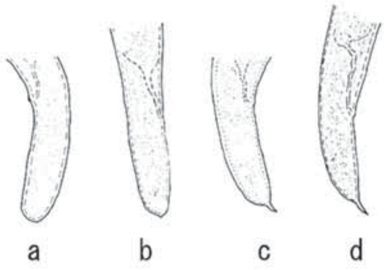


Fig. 33 The shape of female tail tips of three *Bursaphelenchus* species.
a, b: *B. xylophilus*,
c: *B. mucronatus* (Japanese),
d: *B. mucronatus* (French)

To determine what causes the pathogenicity, here we will compare the deadly pinewood nematode, *B. xylophilus* (*Bx*) with another species of the pinewood nematode, *B. mucronatus* (*Bm*) we call “pseudo-pinewood nematode”. This pinewood nematode is closely related to *Bx* and inhabits the withered pine tree similarly but has weak pathogenicity. The morphology of *Bm* is similar to that of *Bx* but is distinguishable from the adult female with mucro at the tail end (Fig. 33). However, the small morphological difference does not seem to be related to their pathogenicity. Detailed examination of the differences between the two nematode species may explain the cause of the pathogenicity.

Comparison of pathogenicity between two species of *Bursaphelenchus*

The virulence of the two species *Bx* and *Bm* was examined by inoculation tests conducted under the same conditions. Three-year-old Japanese black and Japanese red pine trees in the nursery of the university forest were inoculated with one of the two nematodes separately. The difference in pathogenicity of the two pinewood nematode species (Fig. 34) was reflected in their propagation within the Japanese red pine and black pine seedlings. Pathogenic Bx proliferated vigorously, resulting in rapid population growth, while the less pathogenic Bm, hardly propagated.

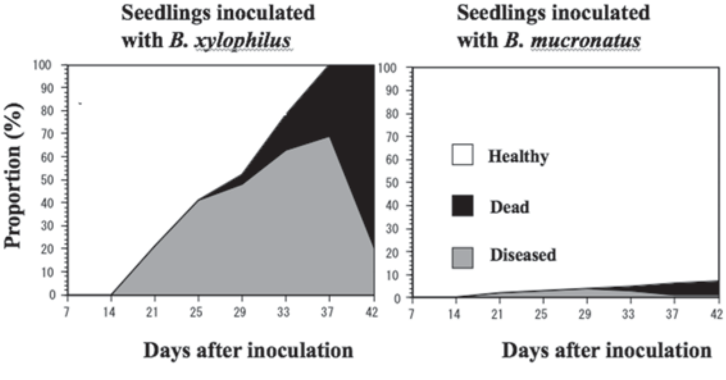


Fig. 34 Comparison of the pathogenicity between two species of *Bursaphelenchus*

There might be a fundamental difference in propagation ability between the two species of pinewood nematodes in the Japanese two pine species. Even though some pine seedlings died after the inoculation of the less-pathogenic *Bm*, the nematode also succeeded in propagation in the seedlings. This suggested the relationship between the pathogenicity of the nematodes and their reproductive ability. In other words, whether or not the nematodes increase in the tree may be actively involved in the cause of pine death.

However, the proliferation of nematodes may have occurred as a result of pine death rather than as a cause of pine death. Therefore, we need to compare the differences in the essential propagative ability of the two pinewood nematode species.

Comparison of the velocity of embryonic development

Both *Bx* and *Bm* are oviparous (animals that reproduce by laying their eggs) and lay oval eggs with a major diameter of 60 microns and a minor diameter of 20 microns (Fig. 35). The laid eggs repeatedly cleave, pass through several stages, and become the 1st-instar larvae in the eggs. The 1st instar larvae move actively in the eggs and eventually molt (the process of shedding old cuticles) to become 2nd instar larvae, and then hatch after a specific time (Mamiya 1975). In other words, these nematodes are already second-instar larvae at the time they hatch. The difference between the two species of pinewood nematodes may be due to the difference in velocity of embryonic development. To determine the velocity, we need to follow the development of each egg from the time they were laid under a stereomicroscope continuously. Then the data obtained by observation of many eggs are averaged to determine the specific developmental rate of each species. Moreover, embryonic development needs to be observed under several temperature conditions. This direct observation method is inefficient and laborious. A more efficient method would be to synchronize the starting point of growth, that is, to observe eggs laid at the same time.

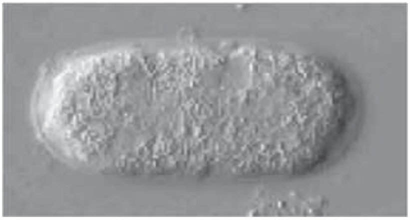


Fig. 35 The egg of the pinewood nematode

How to collect nematode eggs

A photo presented by a tree pathologist, Dr. K. Tanaka, Kansai branch FFPRI, showing many eggs of the pinewood nematode encouraged me to seek a solution. Tanaka used small equipment to collect nematode eggs, and I also tried to make a similar device to collect eggs. However, the nematode eggs stuck to the glass wall of the device, and I could not obtain a sufficient number of eggs. The eggs of the pinewood nematodes stuck to the glass wall, while the adults and larvae did not stick to the wall. This difference was the key to the solution! I realized it might be possible to separate the nematodes and collect the eggs by suspending the nematode culture in water. After gently pouring the solution onto a watch glass and letting it stand for a while, the supernatant was washed with tap water, leaving many nematode eggs on the surface of the watch glass. Thereby many eggs could be collected without any difficulty. However, the problem was that some eggs had just emerged while some eggs were older (laid hours earlier and about to hatch). Therefore, the growth of the eggs was not synchronized. Such eggs could not be used to determine the rate of hatching.

Hatching curve, hatchability (hatching rate), and embryonic development

My solution to this problem was the “hatching curve.” After about 200 to 300 nematode eggs were stuck to the bottom of the watch glass, I poured tap water that had been adjusted to a designated temperature onto the watch glass, and adjusted the temperature to six thermal conditions (11, 15, 21, 26, 29, and 32 ℃). I counted the cumulative number of eggs hatched under each thermal condition and at regular time intervals in two replications. I counted the number of hatched eggs every 8 hours under five thermal conditions: 15, 21, 26, 29, and 32 ℃, except for the eggs at 11 ℃ whose development was prolonged and were observed every 24 hours. The embryonic development of the pinewood nematode progressed rapidly at 26, 29, and 32 ℃, and most eggs hatched within 24 hrs after the first egg hatched, while at 15 and 21 ℃ the development progressed rather slowly, and it took three days for all the eggs to hatch. Furthermore, the room temperature was 25℃. To minimize the change in the water temperature of the watch glass during the observation, I took two to three watch glasses out of the incubator adjusted to the specified temperature and counted the number of hatched eggs as quickly as possible. I repeated this procedure every eight hours for three days, to obtain an accurate hatching curve. This was a very tiring task, but it was well worth it. Figure 36 shows the hatching curves obtained. In these cumulative hatching curves, the part where the hatching rate increases linearly was extended, and the intersection with the 100% hatching rate line was determined. A perpendicular line was drawn from this intersection to a time axis (horizontal axis), and the theoretical date of 100% hatchability (the day by which all eggs hatched) for each thermal condition was determined by the intersection with the time axis (horizontal axis). Of course, with the temperature rise, the time to reach 100% hatchability became shorter. Then, the reciprocals of the time needed to reach100% hatchability were calculated. These values correspond to the growth velocity of the embryo of the pinewood nematode under each thermal condition.

Plotting of these values to coordinates with the temperature on the horizontal axis and the growth velocity on the vertical axis and points are aligned in an upward straight line. This straight line indicates that the developmental velocity becomes faster with the rise in temperature.

Since the growth rate is zero at the intersection of this straight line and the horizontal axis (temperature axis), the nematode stops growing at levels below this temperature (Fig. 37), this temperature is called developmental zero.

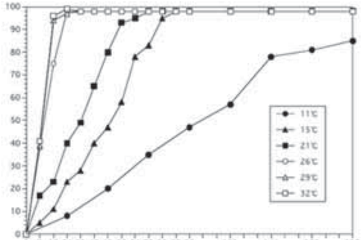


Fig. 36 Cumulative hatching curves of the pinewood nematodes

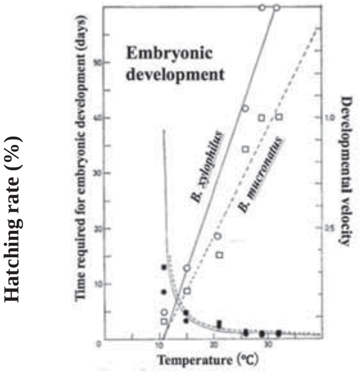


Fig. 37 Effect of temperature on the velocity of embryonic development of *B. xylphilus* and *B. mucronatus*

Comparison of the characteristics of the two species of the pinewood nematode, the pathogenic *Bx*, and the less pathogenic *Bm*, there was no significant difference in the developmental zero temperatures, but the developmental rate was faster in the pathogenic *Bx* than the less pathogenic *Bm*. However, the developmental rate obtained above was just for embryonic growth. Is there any difference between the two species of nematodes in their developmental velocity after emergence from the eggs (postembryonic development)?

Comparison of the velocity of the postembryonic development

To determine the velocity of the postembryonic development of the pinewood nematodes, I used the second stage larvae that emerged from the eggs, because their growth was synchronized within approximately one day of each other. I collected many eggs of the pinewood nematode using the same method mentioned above and immersed them in the water (25 ℃), and let them stand still for one day. Most of them hatched, and I obtained a countless number of 2nd stage larvae. These 2nd stage larvae were surface sterilized and inoculated in a given number on a mycelial mat of fungus *Botrytis cinerea* grown in a test tube. To determine the velocity of the postembryonic development, I had to find out how long it took for these nematodes to become adults. Although it was synchronized by using the 2nd stage larvae at the starting point, their growth was not synchronized as they became adults in a staggered fashion. How was this problem solved? All nematodes in each test tube were harvested at regular intervals, and the number of adults and other stages of larvae were counted separately. All the nematodes were 2nd stage larvae at the time of inoculation, and they gradually grew, and a certain percentage of them eventually became adults. The percentage of adults in the population of the nematodes gradually increased with time. Therefore, the changes in proportion could be used to determine the time required for the 2nd stage larvae to become adults. The proportion of adults remained at zero for some time after the inoculation of the 2nd stage larvae and then increased gradually and peaked. When the adults began to oviposit their eggs, and the 2nd stage larvae emerged rapidly, the proportion of adults quickly fell. Therefore, the time needed for the postembryonic development was estimated from the time of inoculation and the time when the proportion of adults peaked (Fig. 38).

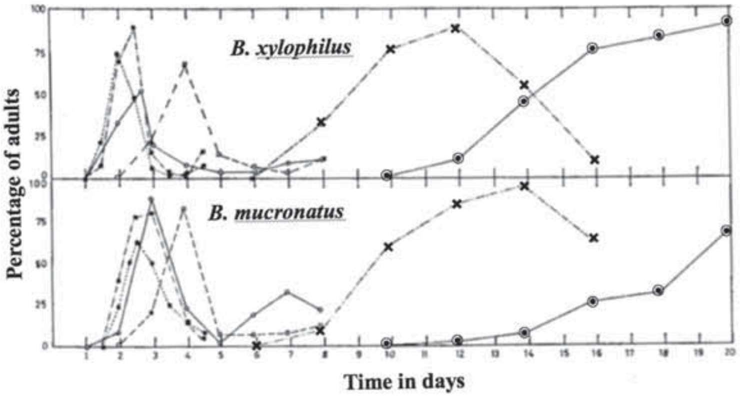


Fig. 38 The percentage of adults in the population of *B. xylophilus* and *B. mucronatus* at different temperatures.

◉——◉ 11°C, x -·-·- x 15°C, □- - -□ 21°C, ○——○ 26°C, ■-·-·-■ 29°C, ●······● 32 °C

As is the case with embryonic development, the number of days needed for postembryonic development at six thermal conditions were used to determine the developmental velocity at each temperature and to obtain the developmental zero temperature for the postembryonic development of two species of pinewood nematodes (Fig. 39). As for the postembryonic development thus obtained, there was no significant difference in the developmental zero temperature of the two nematode species with different pathogenicity. Still, the developmental velocity was higher in the pathogenic *B. xylophilus* than in the less pathogenic *B. mucronatus* at any temperature. Thus, the developmental traits of two pinewood nematodes have been clarified at two developmental stages, i.e., the embryonic and postembryonic stages. Based on these findings, I compared the growth patterns of the two pinewood nematode species.

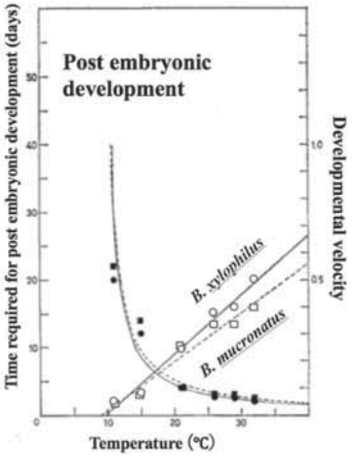


Fig. 39 Effect of temperature on the velocity of **post embryonic** development of *B. xylophilus* and *B. mucronatus*

Population growth patterns of two pinewood nematode species

The pinewood nematodes were cultured on grey mould fungus, *Botrytis cinerea*, grown on potato dextrose agar (PDA) medium in a surface-sterilized test tube. The nematode cultures prepared by inoculating 140 nematodes per test tube were incubated at six temperatures, i.e., 11,15, 21, 26, 29, and 32 ℃, and the population growth of the two species of the pinewood nematode was compared. From the start of the test every two days up to 20 days, five test tubes of each nematode species were harvested from each temperature to determine the average density at each measurement. This experiment required preparation of 600 fungus cultures of *Botrytis cinerea* with uniform growth at the same time seven days before the nematode inoculation. For this experiment, the nematodes had to be at the same stage, and the culture of nematodes had to begin at the same time to obtain a large number of eggs one day before the start of the experiment. For this purpose, the nematodes were reared on 9 cm diameter Petri dishes, and nematode eggs were collected from days 5 to 7. Since fungus *B. cinerea* was grown in advance in a rich PDA medium, there was a high possibility of contamination of other bacteria and fungi in the culture. To prevent contamination, I carried out all inoculations carefully and aseptically and sterilized the nematodes with antibiotics.

I separated the nematodes from the cultures in each test tube with a Baermann funnel and counted the number of nematodes in each tube under a stereomicroscope. This experiment consisted of several complicated and tedious procedures, and the results obtained were interesting.

The numbers of the two species of pinewood nematodes I counted were plotted on the coordinates with the number of days after the start of the experiment on the horizontal axis. The number of nematodes was expressed logarithmically on the vertical axis. Under all temperature conditions examined, and for both nematode species, straight lines were rising to the right, and these straight lines gradually changed their slopes into horizontal lines (Fig. 40). In other words, these two nematodes proliferated exponentially, and eventually reached a saturated density that was maintained for a certain period. The slope of the straight line represents the velocity of the population growth (growth rate).

The population growth rate of the pathogenic *B. xylophilus* (*B.x*) increased with the increase in culture temperature, but in the less pathogenic *B.mucronatus* (*B.m*), the growth rate was highest at 29 ℃. *B. x* had a higher growth rate than *B.m* at all five temperatures except 11℃. The difference in the population growth rate between the two nematodes was in accordance with the difference in their developmental rate described above.

On the other hand, under temperature conditions suitable for growth (21, 26, and 29℃), the saturation densities of *B. m* were higher than those of *B. x*, which indicates a difference in the growth characteristics between these two nematodes (Futai, 1980a). Here we recall the exciting theoretical concept of population biology, called “r- and K-selection,” proposed by MacArthur and Wilson (1967). The terms, r, and K are taken from the well-known mathematical model of population growth: dN/dt=rN(1-N/K), where N is the population, r is the maximum growth rate, and K is the carrying capacity of the given environment. According to their central idea, when certain organisms live in an unstable environment, their population is unlikely to reach the point of carrying capacity (K), where density-dependent factors regulate the population. The population is growing exponentially with intrinsic reproductive rate r, hence the name r-strategist. Some organisms, on the contrary, live in a stable environment and are not severely affected by unpredictable environmental events. Thus, their population almost reaches the carrying capacity K, hence the name K-strategist.

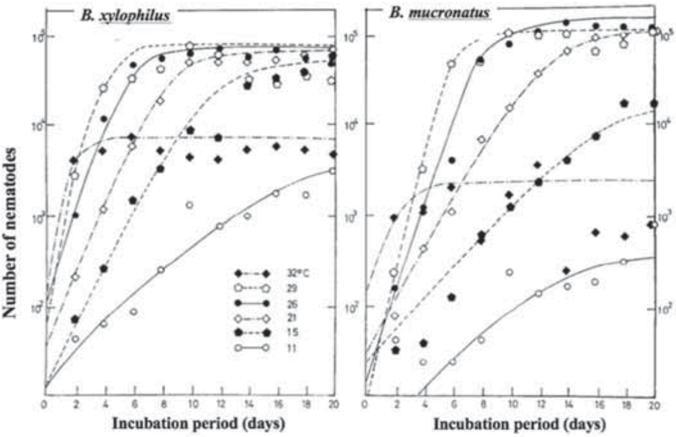


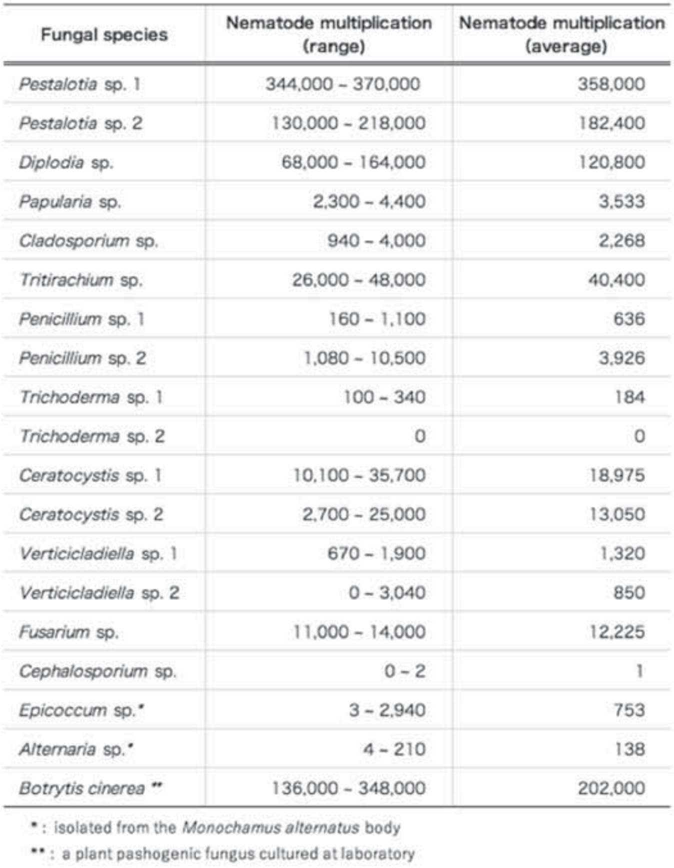
Fig. 40 The effect of temperature on the population growth of *B. xylophilus* (left) and *B. mucronatus* (right)

The “**r**- and **K**-selection theory” was popular in the 1970s and 1980s, but lost importance in the early 1990s. However, this theory was relevant for explaining the difference in population growth traits between the two species of pinewood nematode closely related to each other but which live in entirely different manners. *B. x* parasitic to the pine species, but *B. m* only lives on dying or dead pine trees. The pathogenic nematodes, *B. x* have to be exposed to severe host responses before host resistance is broken. Therefore, population growth cannot reach carrying capacity. They propagate efficiently, and thereby break the host resistance whenever possible. Thus, their population growth velocity is faster than that of less pathogenic *B. m*. On the other hand, *B. m* does not need to propagate rapidly because the dying or dead tree is a relatively safe place for them. They need to endure the competition with other organisms for shared resources (bait fungi) and increase their population as much as possible. Thus, their saturation density became higher than the pathogenic *B. x*.

What does the nematode consume in the pine tree?

Various species of fungi rapidly invade the pine tree when it begins to die or is dead. Both the pinewood nematode *B. xylophilus* and the less-pathogenic *B. mucronatus* can feed on some fungi, but the degree of proliferation depends on the fungal species. Several scientists examined several fungal candidates for suitability as food for the pathogenic pinewood nematode. These fungi were collected from dead or healthy pines, vector beetle’s bodies, the beetle’s pupal chambers, etc. (Table 6). Some of the fungi were suitable for *B. xylophilus* population growth, while some were unsuitable irrespective of the source. Various fungi live on the pine tree in the field, and fungi like *Pestalotia* sp. and *Rhizosphaera* sp. live in the wood or beneath the bark of healthy pine trees. The pine tree infected with the pine wilt disease started withering, and precedent fungi are replaced with *Ceratocystis* sp., *Verticicladiella, Diplodia,* etc. Later, a kind of blue mould, *Trichoderma* sp., *Mariananaea* sp., *Penicillium* sp. appeared. These early fungi are then replaced by white-rot fungi like *Phlebiopsis castanea*, *Gleophyllam sepiorium,* and brown-rot fungi, such as *Cryptoporus volvatus*, *Inonotus valatus*, etc. Among these fungi, some are suitable for the propagation of pinewood nematodes, while others are not (Table 6). After a pine tree is infected with the pinewood nematode and ends up diseased and dying, it is probably the appropriate bait fungi to support the population of these nematodes.

Table 6 Fungal species isolated from living or dead pines and the multiplication of the pinewood nematode on them



Fungal species	Nematode multiplication (range)	Nematode multiplication (average)
Pestalotia sp. 1	344,000 – 370,000	358,000
Pestalotia sp. 2	130,000 – 218,000	182,400
Diplodia sp.	68,000 – 164,000	120,800
Papularia sp.	2,300 – 4,400	3,533
Cladosporium sp.	940 – 4,000	2,268
Tritirachium sp.	26,000 – 48,000	40,400
Penicillium sp. 1	160 – 1,100	636
Penicillium sp. 2	1,080 – 10,500	3,926
Trichoderma sp. 1	100 – 340	184
Trichoderma sp. 2	0	0
Ceratocystis sp. 1	10,100 – 35,700	18,975
Ceratocystis sp. 2	2,700 – 25,000	13,050
Verticicladiella sp. 1	670 – 1,900	1,320
Verticicladiella sp. 2	0 – 3,040	850
Fusarium sp.	11,000 – 14,000	12,225
Cephalosporium sp.	0 – 2	1
Epicoccum sp.*	3 – 2,940	753
Alternaria sp.*	4 – 210	138
Botrytis cinerea **	136,000 – 348,000	202,000

* : isolated from the *Monochamus alternatus* body

** : a plant pashogenic fungus cultured at laboratory

(Kobayashi, Sasaki, and Mamiya 1974, 1975)

What does the nematode eat after invading a healthy pine tree from the vector beetle's feed scar? Soon after the invasion, these nematodes are distributed mainly on the outside of the cambium, that is, in the cortex layer consisting of parenchyma cells. The nematodes mainly use the resin canals, the tubular space created between the cells, for early migration in pine tissues. Around these resin canals, there are epithelium cells that secrete resin into the canals. Nematodes feed on the parenchyma cells and these epithelium cells. The evidence actively supporting this idea is that both *B. xylophilus*, and *B. mucronatus* can propagate well on plant callus cells. The plant callus tissue is a lump of amorphous parenchyma cells formed from a cut surface or when a piece of the cut plant is cultured on the medium supplemented with plant hormones. The plant callus tissues are dedifferentiated and have lost many of the plant's original properties. Still they are used by many researchers because the cells are easy to observe directly.

One of my previous students, Iwahori (currently Professor of Ryukoku University) was a typical plant lover, who handled plants quite skillfully. I advised him to compare the propagation of the pinewood nematode on several plant callus tissues. He used the callus tissues of Japanese red pine, black pine, loblolly pine, massoniana pine, and alfalfa. Even though it is not easy to culture the callus tissues of any one plant species, he prepared a large number of uniformly grown callus tissues for four pine species and alfalfa. Both the pinewood nematodes *B. xylophilus* and *B. mucronatus* proliferated well on the callus tissues of all of the five plant species he examined.

Thus, there was no difference either in the host resistance of the four pine species examined or in the pathogenicity of the two pinewood nematodes studied. However, the results of the experiment provided sufficient data to confirm that the pinewood nematodes can feed on living plant cells, especially pine cells (Iwahori & Futai, 1990). The pinewood nematode feeds on living pine cells, i.e., the parenchyma cells and the epithelium cells, soon after the invasion. Various species of fungi invade the pine tree when it begins to die and then dies. The first group of fungi appear when the tree dies. Unlike the decaying fungi, these fungi cannot utilize the cell wall components, such as cellulose and lignin. Instead they use the starch, sugars, amino acids, etc. that remain in the host cell cytoplasm. Blue stain fungi *Ceratocystis* spp. and *Ophiostoma* spp. belong to this group and are famous for their association with insects. These fungi seem to be the feed for the pinewood nematodes to maintain their population after a pine tree dies.

================== **A column article ③** ==================

● **Difference in the egg surface between *B. xylophilus* and *B. mucronatus***

The sticky nature of the egg surface enabled the collection of a large number of eggs of the pinewood nematode and led to studies of embryonic development and population growth.

This adhesive nature was also found in the eggs of the less-pathogenic pinewood nematode *B. mucronatus*. This nature also enabled us to study the growth characteristics of this nematode in the same way as of *B. xylophilus*. One day, I used a polystyrene petri dish to collect the eggs of the pinewood nematodes instead of the glass petri dish. I found that the *B. mucronatus* eggs were not sticking well to the bottom of the polystyrene petri dish, while the *B. xylophilus* eggs stuck to the bottom, as was the case with the glass Petri dishes. The surface properties of the eggs of these two nematode species were different.

The surface structure was examined with a scanning electron microscope to explain the difference in adhesive traits between the two nematode species. The surface of both nematode eggs was smooth, and no difference was observed between the two nematode species. Thus, the difference in adhesive traits was attributed to the difference in the chemical characteristics of the egg surface. Therefore, we investigated the properties of the egg surface using fluorescein isothiocyanate (FITC)-labeled lectins. Lectins are sugar-binding proteins that coagulate the blood cells of animals and are sometimes called plant hemagglutinin. Lectins aggregate these cells by binding to sugar chains distributed on the surface of animal and plant cells. Lectins can identify the surface sugar chain of an individual cell by strict binding specificity between lectins and the type of sugar. The fluorescent-labeled lectin was used because the fluorescence of the surface makes it possible to discriminate with the naked eye to determine whether it is bound or not. The egg surface properties of two nematode species, three types of lectins, i.e., concanavalin A (which binds to glucose and D-mannose), wheat germ lectin (WGA), (which binds to N-acetylglucosamine), and soybean lectin (SBA), (which binds to N-acetyl D-galactosamine and galactose), were compared. Concanavalin A bound well to the eggs of both species, but the WGA and SBA bound well to the *B. xylophilus* eggs, but hardly bound to the *B. mucronatus* eggs. Thus, the chemical properties of the egg surface of the two species of pinewood nematode are different (Fukushige, H. and Futai, K., 1985).

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4. Host resistance and nematode behavior

Mechanism of host resistance

The pinewood nematode, which is the causal organism of pine wilt disease, infects not only the trees of the pine species, but also firs (*Abies* spp.), spruces (*Picea* spp.), larches (*Larix* spp.), and Himalayan cedar (*Cedrusdeodara*) (Ebine, 1980, 1981). However, in Japan, natural infection in the field resulting in mass death of infected trees is mostly limited to pine species, i.e., Japanese red pine, Japanese black pine and, luchu pine (*Pinus luchuensis*). Pine wilt disease occurs only in pine trees because the pathogenic pinewood nematode invades the host tree via the maturation feeding scar of the *Monochamus* beetle. The *Monochamus* beetle feeds on the tree as a prerequisite for nematode infection. The tree species eaten by the maturating *Monochamus* beetle in the field are limited to the family Pinaceae. All of these tree species recorded to be infected and killed by pine wilt disease belong to the family Pinaceae.

To be a suitable host tree for the *Monochamus* beetle, the tree must be preferred by the adult *Monochamus* beetle not only as a feeding target but also as a suitable site for laying eggs, where the larvae of the offspring can grow. Given these conditions, only pinaceous tree species can become the host, and only these tree species are infected by pine wilt disease. Therefore, two dominant tree species planted in Japanese forests, Japanese cedar (*Cryptomeria*) and Japanese cypress (*Chamaecyparis*) are not at risk of becoming infected by this disease. An artificial inoculation test with *B. xylophilus* conducted by Kiyohara and Tokushige (1971) has revealed that these two coniferous trees are resistant to infection by the pinewood nematodes.

Pine species susceptible and those resistant to pine wilt disease

Kiyohara and Tokushige (1970), conducted inoculation experiments to examine the differences in resistance to pine wilt disease among certain pine species. Whether or not certain pine species are resistant to pine wilt disease in the field depends primarily on the host preference of the *Monochamus* beetle. There are two possible reasons why trees of some pine species do not wither in the field despite the surrounding rage of pine wilt disease. First, the *Monochamus* beetle does not feed on these trees (unsuitable as a host of the *Monochamus* beetle), and second, the beetle may feed on these trees, and leave pathogenic nematodes invading the trees, but the pine species still does not wither due to resistance to nematodes.

There was a stand of slash pine (*Pinus elliottii*) native to the Florida Peninsula adjacent to the native Japanese black pine forest in the Shirahama Experimental Station at the Kyoto University Forest in Wakayama Prefecture. The native black pine forest was gradually damaged due to the spreading of pine wilt disease, but none of the slash pine trees became withered. Dr. Furuno and Uenaka compared the number of feeding scars of the *Monochamus* beetle on the twigs of the two pine stands and found an almost equal number of scars (Furuno et al. 1993). Thus, the *Monochamus* beetle visits two adjacent pine stands of Japanese black pine and slash pine at the same frequency and feeds on them equally. The difference in resistance against pine wilt disease among pine species is not due to the difference in host preference of the *Monochamus* beetle, but due to the difference in host resistance to the pathogenic pinewood nematodes.

What the results of the inoculation tests suggest

As noted by the tree pathologist, Dr. Tanaka at the Kansai Branch of FFPRI,[2] various aspects of pine wilt disease have already been studied, but the host resistance against pine wilt disease has not been studied well and is still disputable. I inoculated the seedlings of various pine species with pinewood nematodes in the nursery at the Kamigamo Experimental Station, Kyoto University Forests. Dr. Furuno, chief of the Experimental Station and a specialist in forest protection sciences, and I used 29 pine species and one interspecific hybrid for the inoculation experiments (Futai and Furuno, 1979).

The nematodes that had propagated on the fungal culture of *Botrytis cinerea* were used as the inoculum for the inoculation test. The inoculum density of the nematodes was adjusted to 2,000 per seedling and inoculated as a suspension. Because two American pine species, *P. taeda*, native to Florida Peninsula, and *P. strobus*, native to Eastern United States, have been reported as resistant, some seedlings of these species were inoculated with the pinewood nematodes at three sites, and each site received 2,000 nematodes, so there were 6,000 nematodes in total. Mimicking the feeding scar of the *Monochamus* beetle as an invasion route of the pinewood nematode in the field, I peeled off the bark of the seedling trunk with a knife to reach the xylem, fixed a piece of absorbent cotton at that site, and injected a nematode suspension into the cotton with a pipette.

The amount of the resin exuded from a pierced needle hole was examined at two weeks and five weeks after the nematode inoculation. The first symptom of the pine tree infected with the pinewood nematode was a decrease or cessation of resin exudation, as recorded previously. This symptom is very sensitive and useful to know that the tree is infected with pine wilt disease. The external symptoms were also observed once or twice a month to follow the progress of the disease after the inoculation of nematodes. As a result of inoculation, the amount of resin exudation was reduced irrespective of the external symptoms. This suggests that even when the pine seedlings survive pine wilt disease without any external

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[2] FFPRI: Forestry and Forest Products Research Institute.

symptoms, there is some physiological disturbance. The progress of the external symptoms is shown in Figure 41. Some characteristic trends in the withering patterns among the pine species were also observed. The most exciting trend was that the withering patterns among pine trees appeared to correspond to their taxonomical classification. There are differences in the mortality ratio from pine wilt disease among strains, even in one pine species.

Taxonomy of pine species and the result of the inoculation test

About 120 species of the genus *Pinus* are distributed exclusively in the Northern hemisphere. Several classification systems of pine species have been proposed by taxonomists. Critchfield and Little reported a system (Critchfieldand Little, 1966) that reflects the differences in the withering patterns among the pine species (Fig. 41). The genus *Pinus* is separated into two subgenera, Pinus (two-needled pine) and Strobus (five-needled pine), and many species of the subgenus Strobus were susceptible and easily killed by the nematode. On the other hand, the species of the subsection australes or contortae, both belonging to the subgenus Pinus, were resistant (rarely killed). Two australes species, *Pinus taeda* (loblolly pine) and *Pinus elliottii* (slash pine) were hardly killed during my inoculation tests.

Among the same subgenus *Pinus*, many species of the subsection Sylvestre to which the Japanese red pine and black pine belong, are highly susceptible. However, this subsection also includes resistant species such as *Pinus reinosa* (red pine) and *P. taiwanensis* (Taiwan red pine).

Among various pine species, those belonging to the subgenus Strobus (five-needled pine) are also called soft pine because their wood is softer than that of the pines belonging to the subgenus Pinus, the two-needled pine (hard pine). The surface of the young branch of the soft pine is also exceptionally smooth. The lesions that develop in the internal tissues due to the pinewood nematode infection also appear as the external symptoms because of the smooth bark surface (Fig.42 left). I tried to detect similar symptoms on the seedlings of other pine species in my inoculation experiments and found some scalding on the bark of a young shoot of the Japanese black pine seedling (Fig.42 right). Usually, lesions occurring in the internal tissues of a pine tree are undetectable, because the surface of the pine trunk and branches are covered with rough and thick bark. However, these exceptional findings enabled us to understand what happens in the internal tissues infected with the pinewood nematodes.

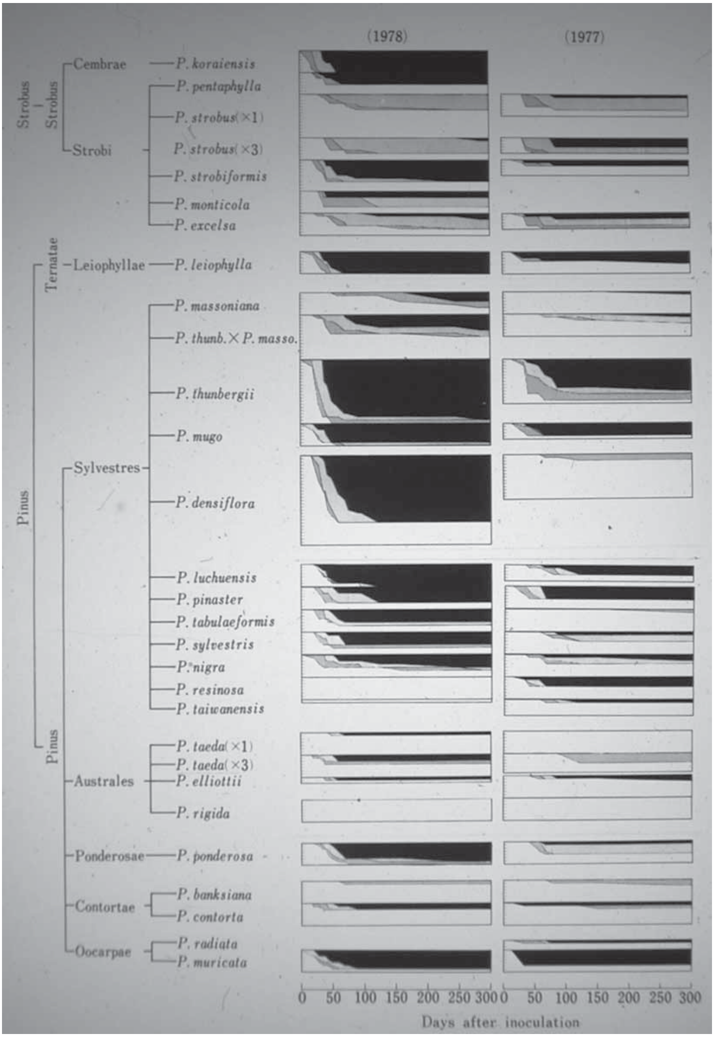


Fig. 41 Symptom progression of various pine species inoculated with pinewood nematodes

Fig.42 The external lesions developed on the bark surface. left: *Pinus strobus*, right: *Pinus thtunbergii*

These external symptoms, such as lesions and scalding, are related to tissue browning and the resulting cell death of the callus on which the pinewood nematodes propagate.

Differences in the resistance against pine wilt disease among families of a given pine species

Various pine species were inoculated in 1977 and 1978. There were differences in the results between the two years among the pine species tested. The results of the inoculation test of Japanese red pine (*P. densiflora*) in particular showed distinct differences with the years. Fourteen trees from two families of *P. densiflora* were inoculated with the pinewood nematodes, and two trees were killed in 1977. The next year, seven trees from four families and 12 trees from another family were used for the inoculation tests. As for the three families, five to seven trees (71 to 100%) of the seven inoculated ones were killed. Among the other two families, three of the seven and two of the twelve inoculated trees (43 and 17%) died, showing some resistance to the disease. Thus, in certain pine species, such as Japanese red pine, the resistance against pine wilt disease varies among families in the same species, ranging from resistant to highly susceptible. Here, we need to identify the term “family.” Japanese researchers of forest tree breeding have been searching for pine trees with excellent qualities of strength among the many pine forests of Japan. They collect the seeds of candidate trees and breed offspring which have the traits of the mother tree. The offspring of one mother tree is referred to as its “family.” The significant difference in resistance to pine wilt disease among the same species suggests the need to create pine forests resistant to the disease by carefully selecting the resistant families and planting them. The selective breeding of pine wilt-resistant lines began in 1976 as a country-wide project. In the first two years, 97,000 candidate trees that exhibited superior resistance were selected from the surviving pine trees in the forests heavily damaged by pine wilt disease from 14 prefectures in western Japan. Large-scale breeding studies were carried out in seven years, from 1978 to1985. Toda (1997) reported the procedure. First, they collected scions from the selected candidate trees and grafted them onto stocks to grow clone seedlings. Then they inoculated the seedlings with the pinewood nematodes (first certification test) and determined the survival rate. The researchers used the seedlings of *Pinus taeda* (loblolly pine) as resistant controls. The families that showed equal or higher survival rates than *P. taeda* seedlings were selected as superior trees. The scions collected from the mother trees of the families selected were grafted to the stocks, and to make 20 clone seedlings for each mother tree. The seedlings thus selected were used for the 2nd inoculation test (secondary certification test). In the 2nd test, the criteria were lowered for Japanese black pine to obtain as many candidate families as possible.

The seedlings that survived the 2nd inoculation test grew into adult trees, and the seeds were taken from the trees and bred for use as resistant seedlings for afforestation. However, it took a long time to grow the seedlings into mature trees. Thus, a simplified method was also used as an alternative method. That is, when the clones survived at a high ratio, the seeds were obtained directly from their mother tree, grown into seedlings, and inoculated with the pinewood nematodes. The seedlings that survived the inoculation were used for afforestation as resistant seedlings. As a result, 92 clones of Japanese red pine and 16 clones of black pine endured two inoculation tests with the pathogenic nematodes. These clones were distributed for public use in afforestation as resistance families. There was a big difference between the number of resistant clones obtained between Japanese red pine and black pine. There is a big difference in resistance to pine wilt disease between Japanese red and black pines, and the black pine has relatively lower resistance than the red pine. This difference in resistance was revealed when the seedlings of these two pine species were planted in the same nursery and inoculated with the pathogenic nematodes at the same time, the symptoms progressed more quickly in the black pine, and more seedlings died (Futai, 1980c).

Appearance of growth decline in the surviving trees

Inoculation tests were conducted using various pine species at Kamigamo Experimental Station, and additional tests were conducted on the pine species at Shirahama Experimental Station in Wakayama Prefecture. While conducting these tests, I noticed the peculiar tree shape of the black pine that survived the inoculation with the pinewood nematodes. The needles were densely packed at the tip of the treetop or the end of each branch.

The elongation growth of each branch and the main axis was significantly reduced the year after inoculation, and needles that were usually deployed in sequence on a well-expanded branch were clumped tightly between the shortened nodes, giving an abnormal tree shape. I also examined other surviving black pines and found that many of them had reduced growth the year after inoculation. Furthermore, the survey of other tree species, revealed that the growth of *Pinus densiflora* and that of *Pinus taiwanensis* were attenuated 50% and 20%, respectively (Furuno & Futai,1986). After inoculation with the pinewood nematode, many pine species showed a temporary reduction in resin exudation, even when they did not wither. The phenomenon of growth attenuation also suggests that the pine tree species becomes physiologically abnormal in function after infection with this nematode, and the tree species may be killed if their resistance is low. Thus, the resistance and the sensitivity may be a phase of the continuous physiological responses of the host pine tree against the pinewood nematode.

Nematode aggregation to pine sap

The pinewood nematodes are in a dormant state in the trachea of the *Monochamus* beetle. They are attracted to the volatile substances emanating from the feeding wound inflicted on the young pine branch by the beetle and transferred to the host pine tree (Stamps and Linit 1998a). The pinewood nematode may not be attracted to the volatile substance in the resistant pine species. Host resistance may be affected by such nematode preference for the host volatiles. To test this hypothesis, I conducted simple experiments to examine nematode aggregation to pine sap. After removing the needles, I cut the shoots of the Japanese black pine (8~10 mm in diameter) into segments 10 mm in length. Then I placed the shoot segment 3 cm from the center of a 1.5% plain agar plate to diffuse the sap into the agar in Petri dishes 9 cm in diameter. After 8 hours, I removed the pine segment and placed a given number of nematodes at the center of the agar plate as follows: I extracted the nematodes reared on *Botrytis cinerea* in the agar plate using the Baerman funnel technique, and excluded small larvae with a sieve (325 mesh /inch). The nematode suspensions were made so that each aliquot contained a given number of nematodes. Water was sucked out of these suspensions, and nematodes were gathered on tiny cotton swabs with the equipment (Fig. 43 left top). I placed a cotton swab with a known number of nematodes face down in the center of an agar plate on which pine sap was diffused 3 cm away from the center (Figure 43 left bottom). After incubation at 25 ° C for 1, 2, 3, 4, 5, or 6 hours, I removed six agar discs, each 10 mm in diameter, 1, 2, and 3 cm away from the center of the plate in opposite directions—one in the place where the pine sap spreads, the other on the other side.

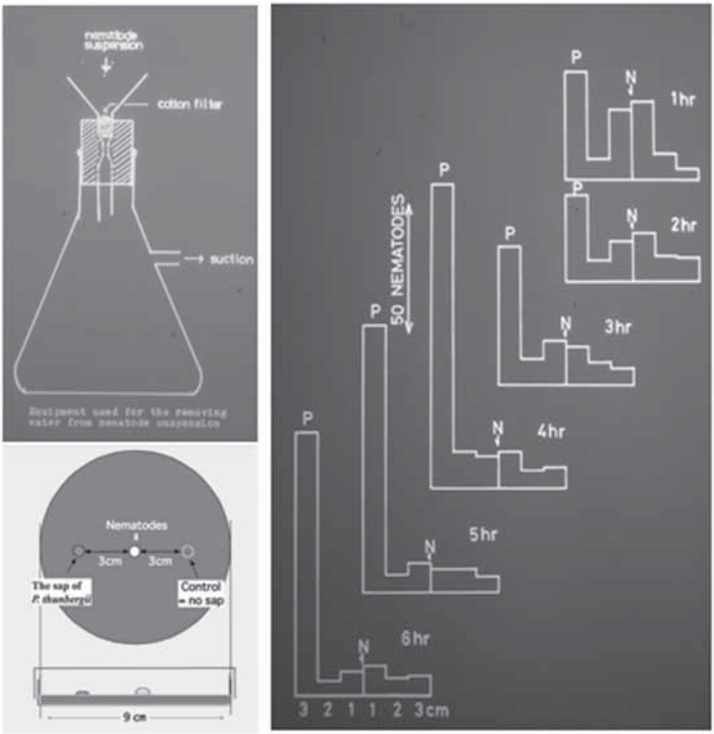


Fig. 43 Experiment for the aggregation of the pinewood nematodes to the sap of *Pinus thunbergii*. Left top: Equipment to obtain the designated density of nematodes while removing water as much as possible. Left bottom: Arrangement of black pine sap and a small cotton lump containing many nematodes on an agar plate. Right: Process of nematode aggregation to pine sap.

Next, I collected the nematodes on each agar disc and counted them. Figure 43 (right) shows the time it takes for the nematodes to aggregate in the pine sap. The nematodes aggregated in the sap 1 hour after the start of the experiment and peaked 4 hours after the start. Then, I continuously observed the nematode behavior under a stereomicroscope and noticed that nematodes moved randomly and did not preferably move toward the pine sap. Upon reaching the pine sap, the nematodes remained there.

Thus, the preliminary experiment revealed that the pinewood nematodes aggregate to the substances exuded from the cross-section of the shoot of the black pine. This experimental system may be useful for determining whether the differences in the resistance to pine wilt found among the pine species are reflected in the aggregation behaviour of the nematodes related to the sap of the corresponding pine species. In the next experiment, I compared the preferences of the pathogenic pinewood nematode, *B. xylophilus*, and the less pathogenic pinewood nematode, *B. mucronatus* about the sap obtained from eight pine species and one oak species. Two shoot segments of the black pine were used as controls, and two of the other pine species or oaks were placed 3 cm apart from the center of a 1.5% agar plate in a Petri dish of 9 cm in diameter. They were spaced equally so that each of the two segments was standing opposite each other around the center of the agar plate (Fig. 44 top).

I removed the shoot segments of the black pine and those of the test species after eight hours and placed about 3,000 nematodes at the center of the agar plate in the same way as mentioned above. All the Petri dishes were incubated at 25 ^{0}C for 12 hours, and then agar disks, 10 mm in diameter, were taken out from where the shoot segments of pine or oak were placed. The nematodes were recovered from these agar disks and counted. The preference of another mycophagous nematode, *Aphelenchus* sp., for the sap of the oak was compared with that for the sap of the black pine under the same conditions.

Based on the number of nematodes aggregated to the saps from eight pine or oak species, the relative preferences of two pinewood nematode species and one *Aphelenchus* species were evaluated using the following index:

$$\textbf{The index of relative preference} =$$

$$\log\left(\frac{\text{the number of nematodes aggregated to the sap}}{\text{the number of nematodes aggregated to } \textit{P. thunbergii} \text{ sap}}\right)$$

The experimental test of the preferential aggregation of three species of nematodes to the sap of eight pine and one oak species produced unexpected results (Fig. 44, bottom), as follows:

(1) The largest number of pathogenic pinewood nematodes aggregated to the sap of the highly resistant *Pinus taeda*. Thus, the resistance of each pine species to the pathogenic pinewood nematode was not reflected in the preferential behavior of the nematode.

(2) The preferences of the pathogenic pinewood nematode and the less pathogenic species were surprisingly similar. The only difference between the two pinewood nematode species was the response to the strobus pine. The pathogenic species showed a strong preference for the sap of black pine, while the less pathogenic species showed a preference for the sap of the strobus pine.

(3) The aggregation behavior of two nematode species to the oak sap was even more impressive. Only one-tenth of the pathogenic pinewood nematode aggregated to the pine sap aggregated to the oak sap, while *Aphelenchus* species aggregated more to the oak sap.

From these facts, it may be concluded that both pathogenic and less pathogenic pinewood nematodes prefer to aggregate to the sap of the pine species. Still, no relationship was seen between the preferences among their aggregation to the host sap and the resistance of the host trees (Futai, 1980b).

Mono-terpene, β-myrcene was reported to be strongly attractive (Ishikawa et al., 1986) and to have a propagation promoting effect (Hinode et al.,1987) on the pinewood nematode. From these findings, host resistance was attributed to the small amount of β-myrcene in the resistant pine species, which made it less attractive. However, my earlier findings suggested otherwise, I sought to get more accurate data using the agar plate method. However, the sap of the host trees used in this experiment volatilize and /or diffuse into the agar plate over time, and it would be better to use the shoot of each tree species instead of the sap to simulate the natural condition.

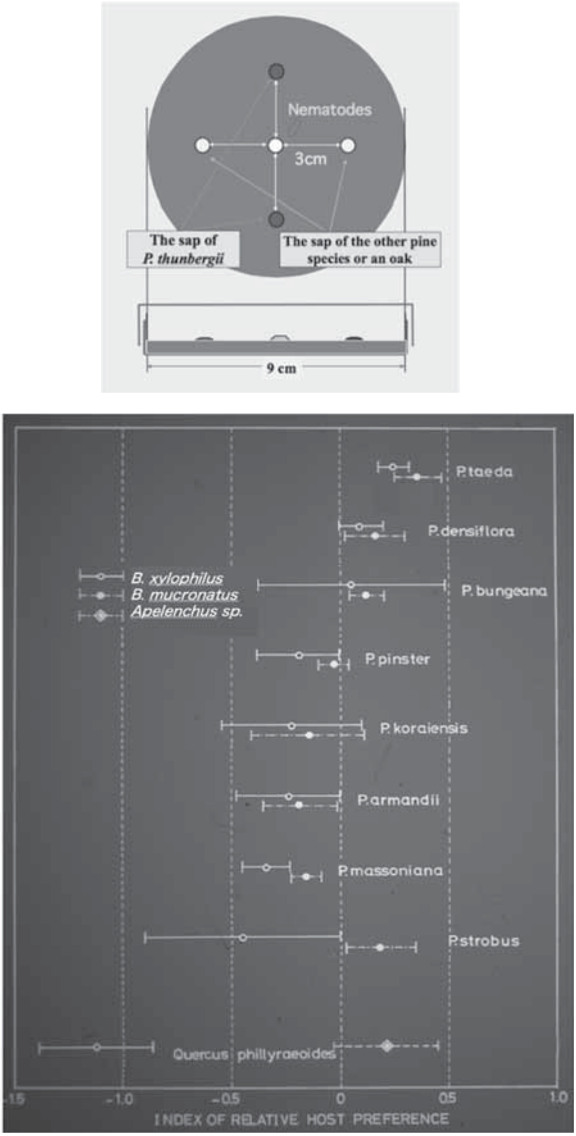


Fig. 44 Preferential aggregation of three nematode species to the sap of 8 pine species and an oak. Top: Arrangement of the sap of pine species to be tested and the sap of *P. thunbergii* as controls. Bottom: Experimental results are shown as the index of relative preference (see text).

Invasion into pine tree tissues

First, I performed an experiment using the shoot segments of black pine (*P. thunbergii*) and loblolly pine (*P. taeda*). I arranged a total of 4 shoot segments so that two black pine and two loblolly pine shoot segments were facing each other at a position 3 cm from the center of the agar plate. Pinewood nematodes were placed at the center of the plate and incubated overnight. Under these conditions, the surface of the cross-section of the shoot segment was exposed to the nematodes, as in the case of feeding wounds made on the young shoots by the *Monochamus* beetle. I was expecting far more pinewood nematodes to be gathered under the shoots of the more susceptible black pine than the resistant loblolly pine. The next morning, a small number of nematodes were observed in the area under the black pine segment under the stereomicroscope. At the same time, a much larger number of nematodes were observed under the segment of loblolly pine. Although it was a preliminary test, I prepared ten Petri dishes of the same combination of two host pines. I inspected the ten Petri dishes successively. Although there were some differences between the Petri dishes, the number of nematodes gathered under the black pine segment was smaller than that under the loblolly pine segment. The pinewood nematodes may be attracted more strongly to the loblolly pine segment than to the black pine segment, but the number of nematodes gathered under the segment of black pine was too small. After trial and error, I considered that the nematodes that had gathered in the segments of black pine invaded the inside of the segments, while the nematodes that had gathered in the segments of loblolly pine found it difficult to invade the segments so that they might have remained under the segment. For confirmation, I arranged two black pine and two loblolly pine segments on an agar plate. Then I inoculated the nematodes in the middle, incubated them overnight, and recovered the nematodes from both the segments and corresponding agar disks under them, and counted their number.

My predictions ultimately proved true. The number of nematodes left under the segment, i.e., the number of nematodes recovered from the agar disk cut out from immediately below the segment, was much higher in loblolly pine than in black pine. The number of nematodes recovered from the segment itself was much higher in black pine than in loblolly pine. The sum of the number of nematodes remaining under the segment and the number of nematodes invading the segment was regarded as the total number of nematodes aggregated to this segment. The rate of the total nematodes aggregated to specific shoot segments to the total number of nematodes inoculated at the center of the agar plate was regarded as an "aggregation rate" to the segment. The number of nematodes invading the segment to that of the total nematodes aggregated to the segment was regarded as the "invasion rate." It was available as an index of the ease of invasion into the segment of the tree species.

The most exciting moment in my research would be when I was showered with many exciting ideas all at once and some of the hypotheses tested proved to be true.

First, I examined the numbers of pinewood nematodes aggregating to and invading the shoot segments of black pine and oak species (*Quercus phyllyraeoides*). I used two mycophagous species of nematodes, *Aphelenchus* sp. and *Aphelenchoides* sp., both of them mycophagous and relatively close to *B. xylophilus*, as controls.

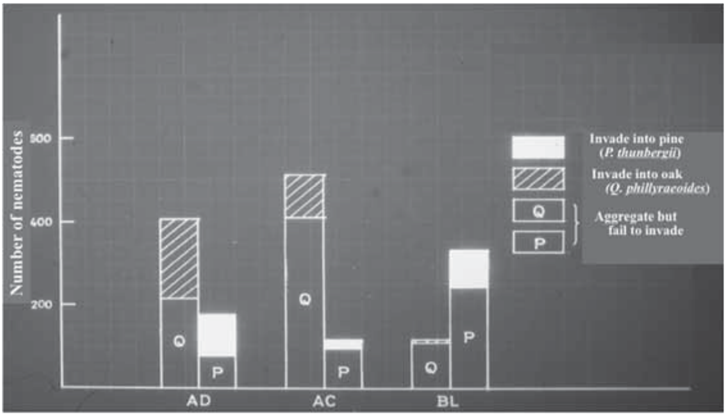


Fig. 45 Preferential assembly and invasion of *Bx* and two mycophagous nematodes to the segments of black pine and the oak. (BL=*Bx*, AC= *Aphelenchus* sp., AD=*Aphelenchoides* sp.)

As shown in Fig. 45, *B. xylophilus* aggregated and invaded the shoot segments of black pine at a higher rate than those of oak. This may be due to the structure of black pine, which made it easy to invaded by nematodes. However, *Aphelenchus* sp. preferably aggregated to the shoot segments of oak rather than those of black pine, and invaded the oak segments at higher rates. *Aphelenchoides* nematodes aggregated to the shoot segment of oak, but similarly invaded the shoot segments of both pine and oak. Thus, the preferential assembly and invasion of *B. xylophilus* into the shoot segments of black pine indicated a high affinity of this nematode for black pine.

I used the shoots of 17 pine species to compare the rates of aggregation and the invasion to their segments. The shoot segments of black pine were used as controls, and two segments of black pine and the other pine species to be tested were placed on an agar plate in the same way as mentioned above. Young shoots of pine species were used in this experiment, and it was easy to divide them into bark and xylem. The nematodes that had invaded the segments were collected separately from the bark and the xylem, and the number of nematodes that had invaded each segment part was examined.

The number of nematodes that had aggregated to each segment to the total number of 3,000 nematodes inoculated in the center of the Petri dish was calculated as the aggregation rate. Furthermore, among the total number of nematodes that had aggregated, the number that invaded the bark and the xylem of the segment were counted. The ratio of invasion into each site was separately determined. The values determined for each tree species were plotted on the coordinates where the aggregation rate was plotted on the x-axis, and the invasion rate was plotted on the y-axis. The values obtained for bark and xylem were plotted on separate coordinates. The experiments were conducted in June and September, and the nematodes used were not only the pathogenic *B. xylophilus* but also the less pathogenic *B. mucronatus*. Here, I will show only the data for *B. xylophilus* responding to the bark of 17 pine species in June (Fig.46). This is because June is the time when *B. xylophilus* invades the host pine in the field, and the bark consisting of parenchyma cells is the site of nematode invasion.

The data obtained for 17 pine species were vertically aligned in this figure. The aggregation rate of *B. xylophilus* did not vary horizontally among the pine species, but the invasion rate into their bark varied. Although the results of the experiments conducted in September are not shown here, the data of each pine species are aligned horizontally. The difference in the invasion rates became smaller in September than in June, but the difference in the aggregation rates became larger among the 17 pine species examined. Thus, these experiments revealed several essential properties of the chemical constituents of the pine tree species that affected the aggregation and invasion behavior of *B. xylophilus* to the host tissue. They can be summarized as follows:

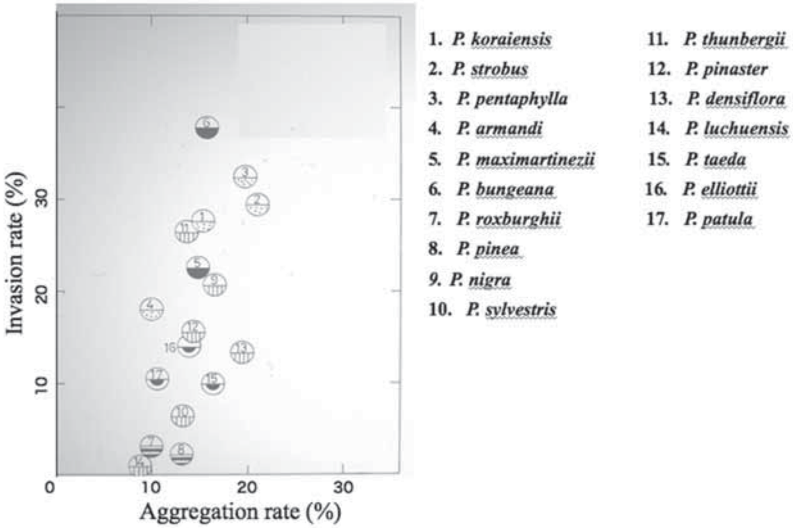


Fig. 46 Response of *B .xylophilus* to the bark of 17 pine species

(1) The chemical constituents of the host pine species that affected the aggregation and invasion behavior of *B. xylophilus* seemed to differ among pine species. However, they were similar in closely related pine species, because the data of closely related pine species in the

classification system (pine species represented in the same pattern) were located close to each other in the coordinate.

(2) The invasion behavior of *B. xylophilus* differed with the host bark and wood.

(3) The chemical constituents of the host pine may change from season to season.

(4) The chemical constituents affecting the *B. xylophilus* aggregation to the host tissue may be different from those affecting the *B. xylophilus* invasion into the host.

(5) More importantly, the pine species with high rates of *B. xylophilus* invasion into the bark in June are generally susceptible to pine wilt disease. This suggests that the host resistance is involved when *B. xylophilus* invades the pine tree from the *Monochamus* beetle's feeding wounds, and the intensity of **the resistance against *B. xylophilus* invasion may explain the resistance of each pine species to pine wilt disease** (Futai, 1985a).

The density of nematode inoculum was always 3,000 in these experiments. However, the nematode density carried by the *Monochamus* beetle in the field shows considerable variation ranging from 0 to 200,000. This variation of nematode density may affect the nematode invasion into the host pine species.

Influence of nematode density on their invasion of host tissues

The number of nematodes inoculated in the center of the Petri dish (9 cm in diameter) was adjusted to five levels, 1,600, 2,500, 4,000, 6,300, and 10,000, and five pine species: Japanese black pine (*Pinus thunbergii*), Japanese red pine (*P. densiflora*), maritime pine (*P. pinaster*), loblolly pine (*P. taeda*), and eastern white pine (*P. strobus*) were examined. The young shoots of these pine species, 7-9 mm in diameter, were sectioned into segments of 1 cm in length, and two segments were arranged to face each other at a distance of 3 cm from the center of the agar plate. All the agar plates prepared were allowed to stand at 25°C. The nematodes were recovered separately from the bark and the wood of the segments, and the agar disks under each segment after 18 hours. To reveal the influence of nematode density on the aggregation and invasion rates, I plotted the number of nematodes aggregating to the segments (x) and the number invading the bark or the wood of the segment (y) in log-transformed coordinates.

The aggregation and invasion to the host shoot segments between the pathogenic *B. xylophilus* and the less-pathogenic *B. mucronatus* were compared. For the five pine species, the invasion rates for the bark and the wood were calculated separately. However, the results were too confusing, so the focus was on how the pathogenic *B. xylophilus* has invaded the bark of these five pine species. This data was plotted on a log-transformed coordinate. Regardless of the species of pine, the higher the inoculation density, the greater the number of nematodes that aggregated to the host segment (x), and accordingly, the higher the number of nematodes that invaded the bark (y). For all of the pine species examined, the data were aligned from the lower left to the upper right on the coordinates. Figure 47 shows an example of, *B. xylophilus* to the segments of Japanese red pine (*P. densiflora*).

The data were subjected to linear regression, which gave a straight line. The value of y-intercept (y_0) of the regression line represents the theoretical invasion rate (y/x) when a single nematode reaches the segment (the specific invasion rate of the nematode to the pine species under conditions free from the density effect). The nematode can easily invade the host if y_0 is 1.0 or more. The invasion is difficult if the value y_0 is lower than 1.0. This value for the bark was the lowest in the resistant loblolly pine (0.3) and was relatively high for the susceptible Japanese black pine (1.8) and Japanese red pine (1.5) among the five pine species examined. The figures were 12.5 for the maritime pine and 3.7 for the eastern white pine. The reason for such high y_0 values in these two species was a bit surprising. However, considering the slopes of the regression lines, another feature of the invasion behavior became apparent. The invasion rate is 10% when 10 out of 100 aggregated nematodes invade the pinewood, and similarly 10% when 100 out of 1000 aggregated nematodes invade the pine wood. Linear regression analysis on a log-transformed coordinate between the numbers of the pinewood nematodes aggregating to the host segments and those invading these segments was conducted. The connection of the two data on a logarithmic coordinate gave a straight line of inclination one.

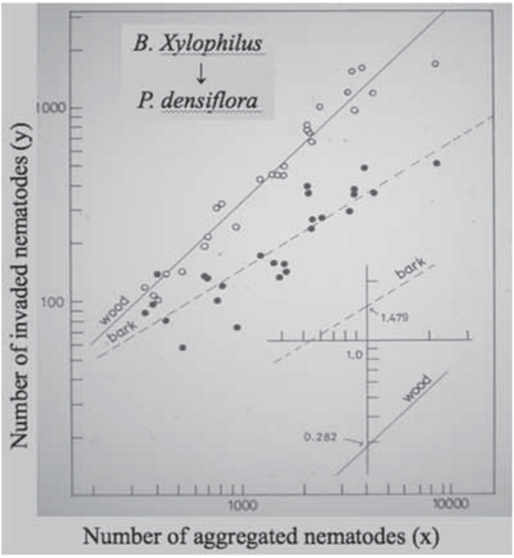
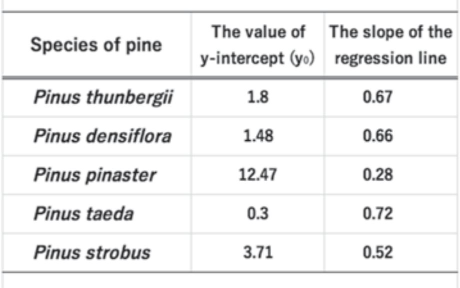


Fig. 47 The relationship between the number of nematodes aggregated to the host segment and the number of nematodes that had invaded its bark or wood.

Thus, the slope of the regression line represents the effect of the density of the nematodes aggregated to the segment on the invasion rate of the nematodes into the segment. When the value of the slope is unity, the invasion rate must be constant irrespective of the density of the aggregated nematodes. If the invasion rate decreases with the increase in the number of aggregated nematodes, the slope will be less than one, and vice versa.

The slope of the regression line was 0.67 for Japanese black pine, 0.28 for the maritime pine, and 0.52 for the eastern white pine. Therefore, in the case of the maritime pine（$y_0 = 12.5$、the slope = 0.28), there was no inherent resistance (resistance against a single nematode) to the pinewood nematodes. Still, as the number of nematodes increased, the resistance increased rapidly, and thereby the invasion rate decreased. Thus, to evaluate the possibility of an invasion of the pinewood nematodes into a certain part of some pine species, we must consider two indices; (1) the specific invasion rate of the nematodes under conditions free from the density effect, shown as y_0 of the regression line, and (2) the density effect on the invasion rate (change in the invasion rate with the increase in the number of nematodes), shown as the slope of the regression line. Table 7 summarizes the two parameters (y-intercept and slope) of the regression line calculated between the number of the pinewood nematodes aggregated to the segments of the five pine species that were examined and those that invaded the bark (Futai, 1985b).

Table 7 Specific invasion rates (y_0) of the pinewood nematodes into the barks of five pine species, and the density effect on it.



Species of pine	The value of y-intercept (y_0)	The slope of the regression line
Pinus thunbergii	1.8	0.67
Pinus densiflora	1.48	0.66
Pinus pinaster	12.47	0.28
Pinus taeda	0.3	0.72
Pinus strobus	3.71	0.52

The effect of the number of aggregating nematodes* on the invasion rate of the nematodes into the segments was examined by plotting the invasion rate (y/x) instead of the number of invading nematodes (y) on the vertical axis of the logarithmic coordinates. Fig. 48 shows how the invasion rates of the bark of the five pines species changed with the density of the aggregating nematodes. The invasion rate of the pinewood nematodes into the bark of any five pine species examined decreased with the increase in density of the nematodes aggregating.

Furthermore, in the case of the maritime pine, the invasion rate dropped sharply with the increase in density. In contrast, the invasion rate was lower in the loblolly pine than in the other four pine species, regardless of the density. Note that at a density of 2,000 to 3,000, i.e., the density I adopted for my inoculation tests (Futai and Furuno 1979), the invasion rate into the bark was the highest in the susceptible Japanese black pine and the lowest in the resistant loblolly pine.

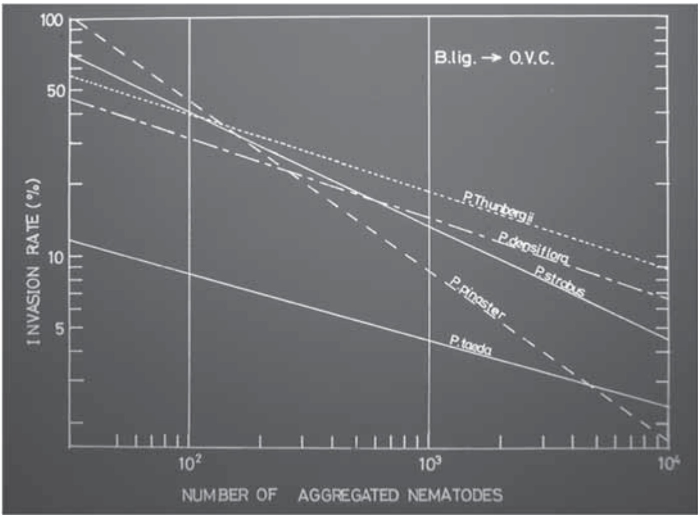


Fig. 48 The density effect of the aggregated nematodes on the invasion rates of the nematodes into the segments of five pine species

The aggregating and invading behavior of the pinewood nematodes related to the shoot segments of various pine species shown via *in vitro* experiments simulated the transfer behavior of the nematodes to the beetle's feeding scar on the young shoot and the invasion behavior related to the tree tissues via the scar, respectively. So, the pine shoot characteristics control the aggregation and the invasion of the pinewood nematodes. Some physical factors such as the bark hardness that changes with the season are considered possible factors. Moreover, the chemical constituents are probably the first.

Chemical constituents that control nematode behavior

The experimental system on agar plates adopted in my previous experiments was suitable for examining the response of the nematodes to various substances. I decided to investigate the behavior of nematodes on pine constituents using the same agar plate arena system.

The whole plan consisted of two parts. The first was an experiment to extract the chemical constituents from the pine shoot segment. I examined the aggregation and invasion behaviors of the pinewood nematodes to the remaining segments from which the substances had been extracted. The second experiment was to investigate the aggregation behavior of nematodes related to the extracted substances. Since these two experiments were complementary, there should be consistency in the results.

The Japanese black pine was used as a susceptible host and the loblolly pine as a resistant host. I collected young shoots, 8 to 10 mm in diameter, of both pine species, then cut these shoots into 1 cm long segments after removing the needles. The preparations were carried out according to the procedure used in previous experiments. In this experiment, however, I divided many shoot segments thus prepared into three groups, and immersed them in three different solvents. The first group was soaked in ethyl ether for 6 hours, the second group was soaked in ethyl alcohol for 6 hours, and the third group was soaked in distilled water for two days. Ethyl ether is a solvent for extracting hydrophobic substances while distilled water is used for extracting hydrophilic substances, and ethyl alcohol has properties between the two. Thus, loblolly pine and black pine segments were immersed in these solvents to elute the hydrophobic and hydrophilic components into the solvents, and thereby removed respective components from the segments. The shoot segments thus prepared were all allowed to stand under airflow for 12 hours, and the solvents were removed. Thus, two segments from which the contents were removed and two intact segments were arranged on an agar plate, 9 cm in diameter, facing each other across the center. As in the previous experiments, 3,000 pinewood nematodes were inoculated at the center of these four segments. All the agar plates thus prepared were allowed to stand at 25ºC for 12 hours. Then, I recovered the nematodes separately from the bark, the wood of the segments, and from the agar disks under each segment. Then their numbers were counted and analyzed (Fig. 49).

Each cumulative bar consists of three bars, the top black bar represents the number of nematodes that invaded the bark, the middle vertical stripe bar depicts nematodes invading the xylem, and the bottom white bar represents nematodes aggregating to the segment but not invading it. The height of the cumulative bar represents the total number of nematodes assembled to the segment. Two cumulative bars, one for the number of nematodes that responded to solvent-treated segments and another that reacted to the corresponding control (intact) segments were presented in pairs.

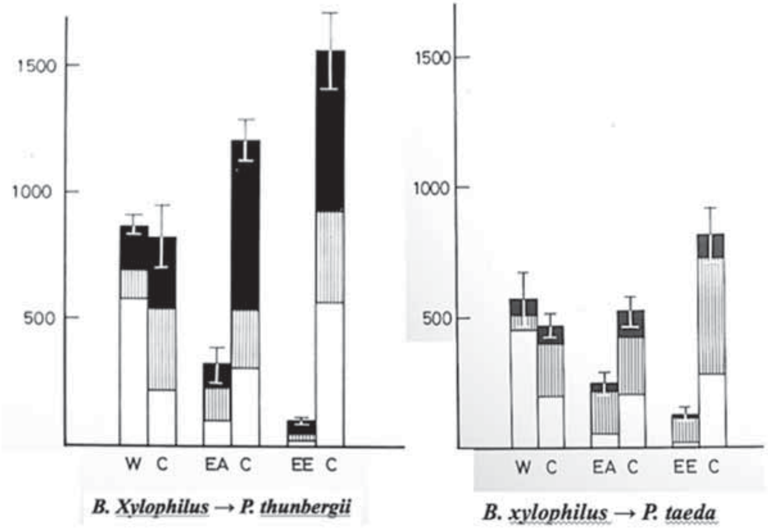


Fig. 49 Aggregation and invasion of the pinewood nematodes to the segments of *Pinus thunbergii* and those of *P. taeda* previously immersed in distilled water (W), ethyl alcohol (EA), and ethyl ether (EE) and to those with no treatment (C).

The number of nematodes aggregated to the ether-treated segments was significantly reduced when compared with the heights of the two cumulative bars in pairs in black pine and loblolly pine. On the contrary, approximately equal numbers of nematodes gathered to the segments treated with distilled water and the control segments. However, the number of nematodes aggregated to the segments treated with ethyl alcohol demonstrated an intermediate value. Therefore, the substances attracting nematodes toward the segment were highly soluble in ether, considerably soluble in ethyl alcohol, and poorly soluble in distilled water, i.e., they were hydrophobic (poorly soluble in water) substances. The segments that lost the attracting materials showed less nematode aggregation.

In the next step, the total numbers of nematodes that had aggregated at the segment and those invading the bark and xylem were examined. However, since the total number of nematodes that had aggregated to each segment was significantly different between the three extraction treatments, it is difficult to compare the difficulty of invasion between them (Fig. 49). Therefore, the number of nematodes invading each site was evaluated by the ratio (invasion rate) to the total number of nematodes that had aggregated to each segment (Fig. 50). This figure is a bit strange. Although the number of nematodes aggregated to the ether-treated segments was significantly reduced, once gathered at the segments, the nematodes invaded at almost the same rate as those that invaded the intact control segments.

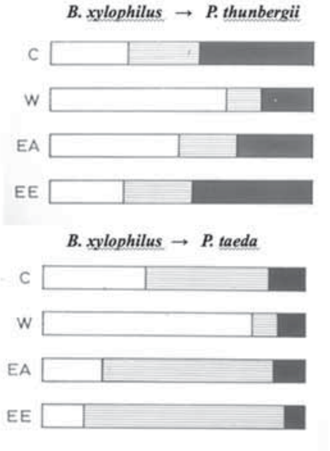


Fig. 50 Invasion rates of the pinewood nematodes into the pine segments previously immersed in distilled water (W), ethyl alcohol (EA), and ethyl ether (EE) and to those with no treatment (C)

On the other hand, the extraction of the pine segment with distilled water did not reduce the aggregation of the pinewood nematodes. Still, it markedly reduced the number of nematodes invading their bark and xylem. These findings suggest that the substances that promote the nematode invasion of the bark and xylem may be dissolved in water (hydrophilic).

The results of the experiments mentioned above showed the differences in the aggregation and invasion rates of the pinewood nematodes (*B. xylophilus*) among the segments of the 17 pine species (Fig. 46) and predicted that separate host substances controlled the aggregation and invasion of the pine segments by the nematodes. The experimental results that were obtained here clearly support the prediction. Furthermore, Fig. 50 shows that irrespective of the solvent used to treat the segments of the two pines, the pinewood nematodes easily invaded the bark of the Japanese black pine, but not the bark of the loblolly pine.

Pursuing substances affecting the behavior of nematodes (Futai, 1979)

In the first series of experiments, I investigated the behavior of the pinewood nematodes related to the pine shoot segments treated with ethyl ether, ethyl alcohol, or distilled water. Thus, I could only indirectly reveal the effects of the host substances on the behavior of the pinewood nematodes by removing them using solvents. To directly demonstrate how host substances control nematode behavior, I examined the nematode behavior for the extracted host chemicals in the second experiment as follows. First, the shoots of *P. thunbergii* and *P. taeda* were cut into small pieces. Then they were divided into the bark and the xylem, finely chopped, and then dipped in 100 mL of ether for 6 hours, and 100 mL of distilled water for 48 hours. After filtering these ether and distilled water extracts, many paper discs, 8 mm in diameter, were immersed in the filtrates for an hour. Then the solvents, ethyl ether, and distilled water were evaporated under airflow. The pine substances held in paper discs were the ether or distilled water extracts of the bark, and the ether or distilled water extracts of the wood. The paper discs immersed in distilled water were used as controls.

These 12 discs (4 kinds of pine extracts x 2 discs, and four control discs) were arranged on an agar plate of 9 cm in diameter, with two discs holding the same extracts facing each other across the center (Fig. 51 right). At the center of 12 discs, 3,000 pinewood nematodes were inoculated in the same manner as mentioned above. The number of nematodes aggregated to each paper disc was counted after 12 hours of incubation at 25 °C (Fig. 51 left). The number of nematodes gathered at each paper disc was displayed as the ratio to those gathered at the control disc.

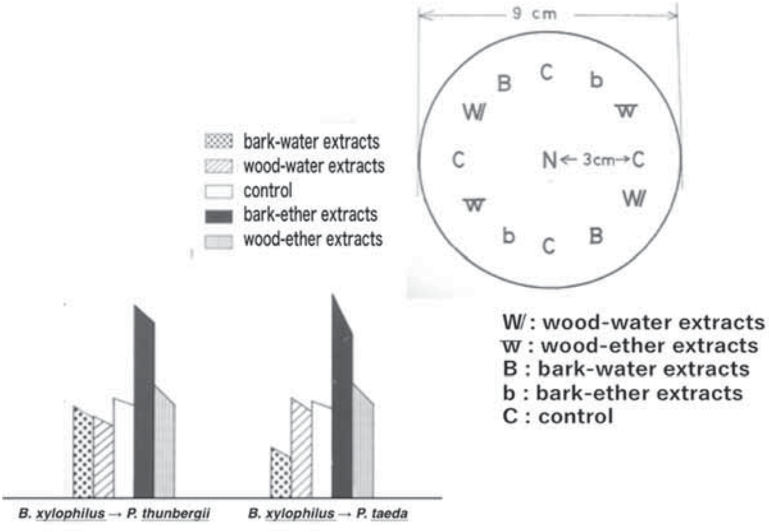


Fig. 51 Right: Arrangement of paper discs holding various pine extracts on an agar plate to examine the preference of the pinewood nematode for pine extracts. N: nematode inoculum. Left: The aggregation of the pinewood nematodes to paper disks holding different extracts from *P. thunbergii* and *P. taeda*. The distance from the height of the right shoulder of each column to that of the left one is the standard error of 10 replications.

Many nematodes aggregated to the ether extracts of the bark of both the black and loblolly pines. This fact was consistent with the results mentioned above, in which the number of nematodes gathered at the segments of the black pine and loblolly pines markedly decreased when soaked in ethyl ether (the hydrophobic substances were removed). Interestingly, the Figure showed that, in the case of loblolly pine, the number of nematodes aggregated to the water extract of the bark was less than the number aggregated to the control paper discs.

Taxis and kinesis - Explanation of nematode aggregation to the control discs

As mentioned in the column article ②, the animal movement in response to an external stimulus can be classified into two types. The movement directed by the stimulus is called taxis, while the movement that is not directed by a stimulus is called kinesis.

Nematodes were placed in the center of the agar plate on which the pine segments or the paper discs with the pine extracts were placed. At first, the nematodes moved in all directions. After a while, however, many nematodes were found gathered at the segments or the paper discs. The aggregation of the pinewood nematodes to these stimulation sources is not taxis, but kinesis.

There are two main types of gatherings for kinesis. In “orthokinesis” the speed of movement is dependent upon the intensity of the stimulus, while in “klinokinesis” the frequency or rate of turning is proportional to the intensity of the stimulus. Still, I could not determine whether the movement of the nematodes toward the pine stimuli was orthokinesis or klinokinesis.

In the previous experiments, many pinewood nematodes gathered to the control paper discs which did not contain any extracts. This aggregation must be due to “thigmotaxis,” which uses physical contact as a stimulus. Therefore, the pinewood nematodes gathered to the paper discs soaking up the pine extracts or to the pine segments as a result of combined responses of thigmotaxis and kinesis to the chemical stimulus.

The pinewood nematodes aggregated to the control paper discs via contact stimulus, but far fewer nematodes - half of those responding to the control discs - aggregated to the discs containing the water-soluble substance in the bark of the loblolly pine. Therefore, the water-soluble material in the bark of the loblolly pine has the property of repelling this nematode. You might recall the results of the experiments, as mentioned above, where I compared the invasion rates of the pinewood nematode into the shoot segments of the five pine species under various nematode densities (Fig.48). The invasion rate of the nematodes into the bark of the loblolly pine was much lower than that into the bark and xylem of other pine species over a wide range of nematode densities. Thus, the resistance of the loblolly pine to pinewood nematodes seemed to be exerted when the nematode invaded the bark and was attributable to the water-soluble component contained in the bark. Later, a substance that immobilizes the pinewood nematode was discovered in the water-soluble component of the loblolly pine (Bentley et al. 1985).

I found differences in resistance among various pine species and revealed their mechanisms. However, many issues remain unresolved. For instance, the primary factor controlling the nematode invasion of the pine tree could not be elucidated because I could not construct a confirmation system. Also, I could not determine the water-soluble resistant substance contained in the bark of the loblolly pine.

III. HOW THE TINY NEMATODE KILLS HUGE PINE TREES

1. Dynamic host resistance

Solving the issues

After the pine tree is invaded by pathogenic pinewood nematodes via the feeding wounds made by the *Monochamus* beetle, it withers and dies within a few months. How does the huge pine tree become diseased and die so quickly after the infection by tiny nematodes only one millimeter in size? The mechanisms underlying the disease development and pine death due to infection by nematodes are significant issues to be solved. Many researchers have tried to solve this problem from various points of view and have proposed different hypotheses.

Several physiological responses appear in the process of becoming diseased and withering to death after infection by the pinewood nematodes. In the next section, I discuss the processes of symptom development in three stages one by one.

Responses of the host tissue at the early stage of the disease

The first stage of the disease process is represented by early symptoms appearing on the host tissues caused by the activity of the small number of nematodes that have invaded the tree. The young shoots are invaded by the pinewood nematode via the beetle’s feeding wound. The nematodes use the resin canals as their migration path in the tree after the invasion. The resin canal is a tunnel-like structure and it is formed in the intercellular space surrounded by the epithelial cells which secrete resin. The vertical resin canals extending axially in the branches and trunks are relatively large, 100 to 200 microns in diameter, provide the passage of the nematodes that are 25 microns in width.

The epithelium cells have thin walls and the cells at the site of movement of the pinewood nematodes are destroyed. Other parenchyma tissues also showed degeneration and necrosis (Mamiya 1985). The artificial inoculation of the pinewood nematodes caused the immediate cessation of resin secretion. There was a temporary generation of ethylene, a plant hormone associated with plant maturation and senescence (Mori and Inoue, 1986). During this period, very few nematodes moved within the tree although they may have dispersed throughout the tree.

The early symptoms of cessation of resin secretion and the generation of ethylene decline with time. Thus, the development of the disease appears to have been inhibited. However, the host tissue shows a rapid reaction (or hypersensitive response) to the nematode activity that progresses quietly during this period. The reaction by the host tissue produces bitter components, or protective substances such as polyphenols, which are accumulated in the vacuoles of parenchymal cells, and eventually, the vacuoles burst, the cells become necrotic, and the cell contents leak extracellularly (Nobuchi et al. 1984).

What happens in the pine tree from infection to death

The contents of the parenchymal cells leak out and flow into the tracheids (water-conducting cavities), gradually impairing the tracheids. This causes cavitation that plugs the tracheids with air bubbles. Over time, the water supply to the crown is stopped, and the pine tree withers to die. Drought and high temperatures accelerate the water stress thereby resulting in withering and death of the pine tree. Even when the pine tree is infected with the non-pathogenic pinewood nematodes, a considerable portion of the xylem becomes blocked, but the water passage is secured because the unblocked area remains at the outermost parts of the xylem (Fukuda, 1997).

Change in color of the water suspension after nematode extraction

The seedlings of Japanese black pine and loblolly pine were inoculated with the nematodes to compare the movement and propagation of the pinewood nematodes in both pine species. Shoots of the seedlings were harvested after a certain time, their surface washed, and a 15 cm portion from the inoculation point of each shoot was cut into five segments 3 cm long.

Each segment was sliced into small pieces and was placed in a Baerman funnel with water overnight. The number of nematodes obtained in the funnel was counted. The nematodes in the water suspension in a Syracuse counting dish were counted under a stereomicroscope. This procedure was repeated every day, and I noticed that the color of the water used for nematode extraction ranged from pale yellow to brown. Close observation revealed that the color of the nematode in the solution after extraction from the susceptible black pine was dark, while that from the resistant loblolly pine was light (Fig. 52).

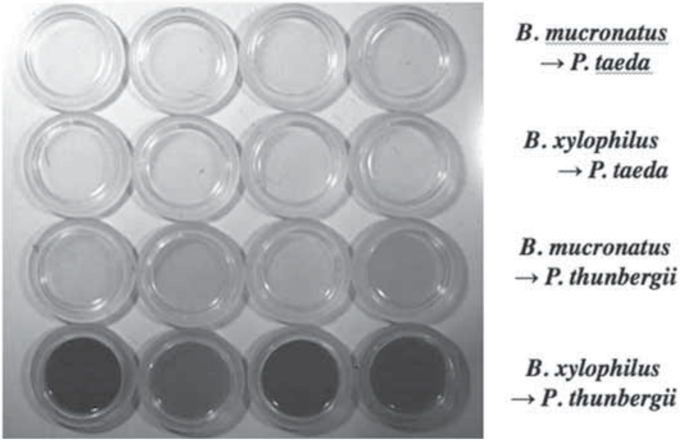


Fig. 52 Discoloration of the water extracted from pine tissues infected with two species of *Bursaphelenchus* nematodes.

When the counting dish was left to stand after counting the number of nematodes, the color of the solution changed from yellow to dark brown within a few days. Therefore, the solution after extraction must contain some substance that is autoxidized with oxygen in the air. The addition of a small amount of reducing agent to the dark brown liquid revived the pale yellow color. Without a doubt, the substances are easily oxidized.

The absorption spectrum of the solution shows a peak near the wavelength of 280 nm, which is characteristic of polyphenolic substances. Polyphenolic substances also have the feature of shifting the absorption peak to the long-wavelength side when the solution is made alkaline. Therefore, after the addition of a small amount of alkaline sodium hydroxide, the peak shifted to 292 nm.

Dynamic resistance shown by the pine tissues infected with pine wilt disease

The polyphenolic substances cause tissue browning and accumulate in injured or pathogen-infected plant tissues (Kosuge 1969). The pinewood nematode also causes the browning of the pine tissue (Mamiya1980, Myers1986), indicating the accumulation of polyphenolic substances. Condensed tannin has been studied in relation to the host resistance against pine wilt disease among the various polyphenolics associated with pines (Saito 1970). Therefore, the changes in tannin content were examined in detail. Temporal and spatial changes in the amount of tannin in the tree were observed after artificial inoculation of nematodes.

The pinewood nematodes were inoculated at the cut end of the pine shoot of 3-year black pine-potted seedlings to examine the relationship between nematode propagation and the intensity of the host response. Fifteen-cm-long stems were harvested and cut into five 3-cm-long segments at 2, 6, 13, 20, and 27 days after inoculation. The number of nematodes and the amount of tannin in each extraction solution were obtained (Fig. 53).

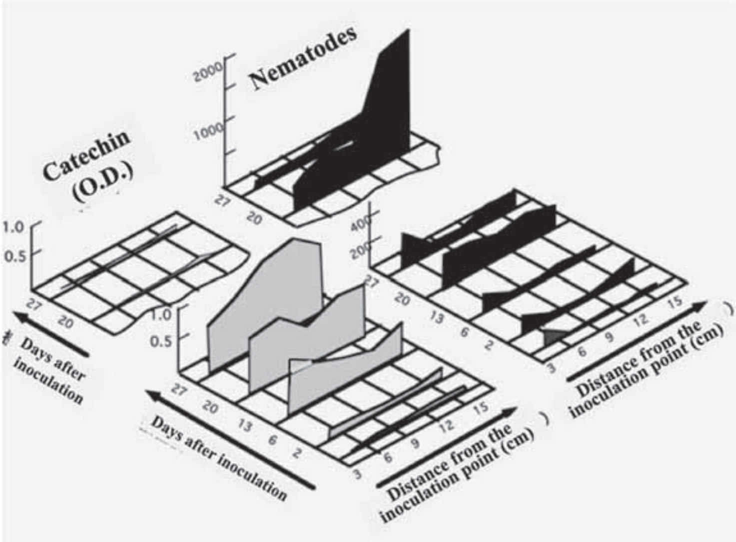


Fig. 53 Temporal and spatial changes in the amount of tannin and the population of the pinewood nematodes in the pine tree.

Almost no population growth was observed except near the inoculation point from which many nematodes were isolated until the 13th day after the nematode inoculation. However, the concentration of tannin (shown as catechin in Fig. 53) in the shoot increased from the second week onward, and this tendency continued until the 3rd and 4th weeks (Futai 1984). On the other hand, the nematode density slightly increased in the shoot at the 3rd week but remained relatively low at the 4th week after inoculation. However, the number of nematodes increased markedly in the shoots of the withering seedlings in the third week, and the amount of tannin dropped to an undetectable level. An increase in the amount of tannin was unlikely to be the result of an increase in the number of nematodes. Rather, the amount of tannin increased in advance to counter the increase in the number of nematodes. The marked increase in the number of nematodes with the decrease in the amount of tannin supports this idea. Therefore, an increase in the amount of tannin seems to be a positive defense response to pathogen invasion, rather than a phenomenon observed during the withering process.

================== **A column article ④** ===================

The solution obtained with small Baermann funnels!

Researchers in plant nematology have been using the Baermann funnel technique to extract nematodes from soil samples or plant materials. The sample is wrapped in cloth such as muslin, and submerged in water in a funnel connected to a rubber tube at the attenuated lower end, and then clipped with a pinchcock. After about 12 hours, nematodes can be collected from the bottom of the rubber tube. Usually, researchers use a glass funnel 9~12 cm in diameter at the top. However, such large funnels are unnecessary to isolate nematodes from the bark and xylem of small pine shoot segments. Instead, because nematodes had to be extracted from many samples as quickly as possible, many funnels were required. Thus, I designed a stand to hold 20 small funnels with an opening diameter of 3 cm, and made 15 such funnel stands. In this way, it became possible to extract nematodes separately from up to 300 samples at once (Fig. 54).

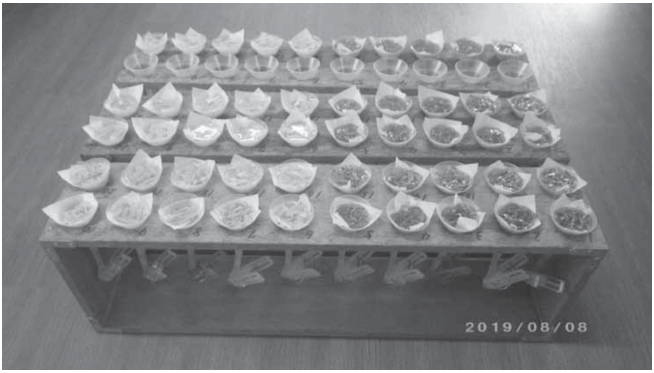


Fig. 54 Small Baermann funnels settled on wooden stands (photo by Ishiguro, H.)

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In these experiments, not only the pathogenic pinewood nematode (*B. xylophilus*) but also the less pathogenic pinewood nematode (*B. mucronatus*) were used for comparison. The results of the inoculation experiment with *B. mucronatus* were not elaborated to avoid complications. Briefly, the inoculation of the same number of *B. mucronatus* nematodes did not result in a notable increase in the amount of tannin in the pine shoot.

Then, we need to determine which tissue of the pine seedling is responsible for the change in the amount of tannin. Collaborative research with Dr. Nobuchi, an expert in the wood structure and specializing in the heartwood formation of trees expanded the research on pine wilt into other areas (Nobuchi et al. 1984).

2. Cellular responses in the host

Nematode infection and cellular responses in the host

The less pathogenic pinewood nematode, *B. mucronatus*, and the closely related pathogenic *B. xylophilus* show many similarities in their life histories i.e., the proliferation in the dead pine trees and utilization of the longhorn beetles as the vector. Although the two are separate species, the F1 hybrid can be produced by artificial crossing of the two species. Comparative studies on these two nematode species may help us understand the pathogenicity of the pinewood nematode, *B. xylophilus* and has been a basic strategy of my research on pine wilt disease.

For our collaborative research, 3- or 4-year black pine-potted seedlings were used as materials. First, the internode of the main shoot of the current year was cut out and 2,000 nematodes of pathogenic or less pathogenic species were inoculated onto the cut surface of the tip of the main shoot. In this way, the movement of nematodes could be confined to the downward direction. Two days, and 1, 2, 3, and four weeks after the inoculation, the main shoots that were 15 cm long, were cut out from the inoculation point and the shoots were cut into five 3 cm long segments. Subsequently, each segment was longitudinally bisected with a razor blade. One was immediately immersed in a fixative, and a tissue section was created for microscopic observation. The other was used to determine the number of nematodes distributed at each segment. The cytological changes in the stem tissues of the pine seedlings concerning the time after the inoculation and the distance from the inoculation point were examined by Dr. Nobuchi.

Cellular responses against nematode infection

Dr. Nobuchi observed the pine tissues infected with the pinewood nematode (*B. xylophilus*). Vacuoles appeared in xylem radial parenchyma cells relatively early after inoculation with the nematodes. This phenomenon spread rapidly downward from the nematode inoculation point. The vacuoles contain the tannin-like substance because staining the tissue with nitroso reagent changes the color of the vacuoles to pink-red (Fig. 55).

My previous experiments revealed the presence of tannin in the pine shoot and its increase after infection with the pinewood nematode. The presence of tannin and its increase in amount in the pine shoot was confirmed by histological studies. These vacuoles became more massive with time after the nematode infection and finally collapsed. These parenchyma cells were probably in the necrotic phase, and eventually, the cell contents leaked from the parenchyma cells and flowed into the tracheid canals (Hara and Futai, 2001). The substances that leaked from the parenchyma cells spread widely and deposited all over the tracheid canals, thereby impaired their water conducting function (Hara et al. 2006) (Fig. 56). The rational basis leading to such an idea is as follows.

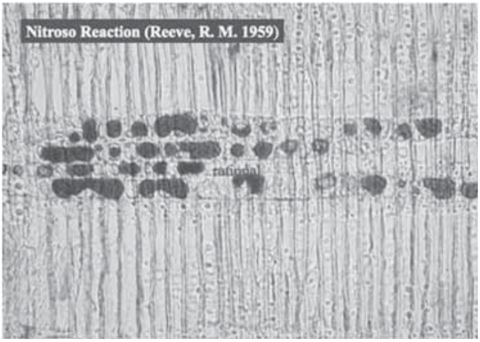

Fig. 55 A histochemical study confirmed the accumulation of tannin in the vacuoles of ray parenchyma cells after nematode infection of pine tissues.

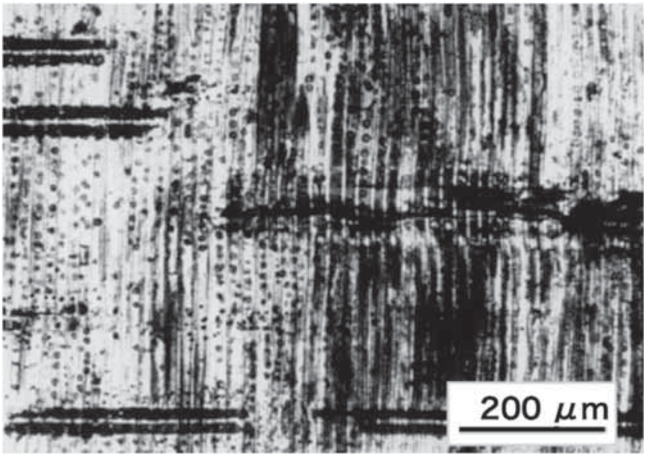

Fig. 56 Some abnormal substances leaking from parenchyma cells and covering many bordered pits, resulting in blockage of the water-conducting system. Sudan black was used to stain the lipid substance. (Photo by Hara, N.)

About 90% of the wood cells are a single type of dead cells, tracheids, in softwood in pine species except for the ray parenchyma cells that are in contact orthogonally with the tracheids (Fig. 57 a). Observation of the wood section of a pine seedling shoot (a section cut longitudinally passing through the center of the stem) under a phase-contrast microscope reveals many small pores on the flanks of the tracheids (Fig. 57 b). These pores are bordered pits and water moves from one tracheid to another through these pores. A bordered pit consists of a fibrous membrane, margo, and a valvate structure, torus; the torus is situated at the center of the pit, hanging from the margo. Water is directed through the mesh-like margo (Fig. 57 c). In the section of the pine seedling inoculated with the pinewood nematode, the bordered pits were somewhat abnormal, having jagged lines radially extending around the torus. On the other hand, the bordered pits of a healthy seedling have a uniform-looking torus perimeter. There is a fine fiber network (margo), through which water moves between tracheids. SEM observation revealed that the margo mesh fiber is very fine, and appears uniform in the healthy tree, but the mesh fiber had a jagged line and became very thick in the nematode-inoculated pine seedling. The margo and torus were densely covered with abnormal substances. The margo mesh fibers have thickened somewhat as expected (Fig. 57 d).

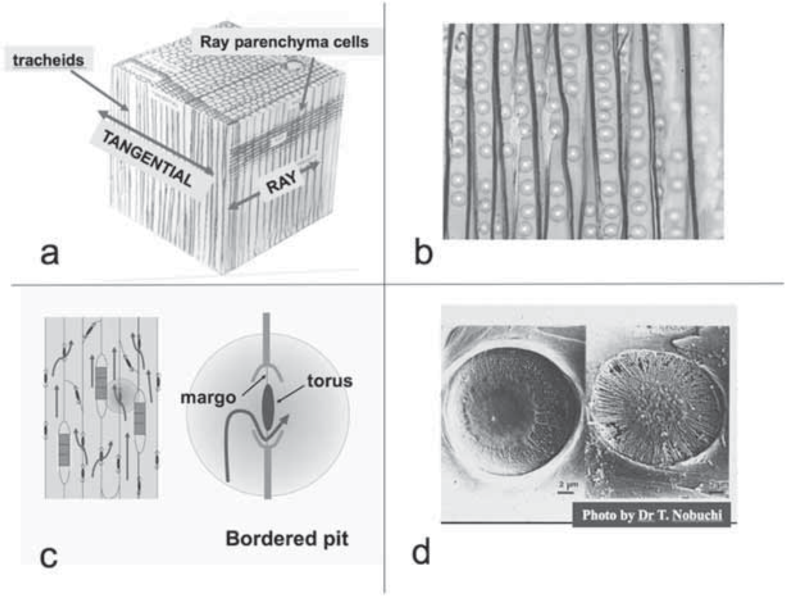

Fig. 57 Pinewood structure and microstructure of water passage system. a: The wood structure of pine. b: The bordered pits on the flanks of the tracheid. c: The fine structure of a bordered pit, with a membrane that consists of margo, mesh structure and torus, a valve structure, and the torus is hanging from the margo. Water goes through the mesh, margo. d: This photo compares the two bordered pits, the left pit is a normal pit and the right pit is a pit of a nematode-infected pine tree, that is heavily covered with abnormal substance(s). These abnormal substances must disturb water conduction by blocking water movement by malfunction valving (torus).

The mechanism leading to wilting to death

Water flows upward from root to leaf by moving from tracheid to tracheid in conifers including pines. The narrowest part of this water passage is the fiber pores (margo) of the bordered pits, and if abnormal substances are deposited there (Fig. 57 d), water conduction will become impaired. The torus of the pits also functions as a valve, and when air bubbles are generated in any of the tracheids, the torus prevents the bubbles from spreading to the adjacent tracheids. Such a function of the torus also must be impaired with the deposition of abnormal substances.

Water exists in the form of a bundle of thin water threads in the pine tree connecting the root and the stomata in the needles. The water threads are moved upward by the pulling-up force generated through transpiration. Accordingly, the air bubbles cut such thin threads of water thereby stopping the upward flow of water. Therefore, it is necessary to separate the air-bubble-impaired tracheid from the healthy tracheids to maintain water flow. The valve function of the torus plays an important role for that purpose. The fibrous membrane structure, margo, enables the torus to move flexibly and helps the torus to function as a valve. However, in the bordered pits of the nematode-infected pine tree, the margo fibers are covered with abnormal substances (Fig. 57-d). Their flexibility is compromised, which impairs the normal valve function of the torus. As a result, the air bubbles clog the tracheid tubes, which may stop the passage of water. This is my hypothesis regarding the mechanism of the withering of pine trees due to the tiny nematode.

Inoculation with the less-pathogenic *B. mucronatus* brings about similar cytological changes in the pine seedlings. Vacuoles appeared in the parenchyma cells near the inoculation site, but the parenchyma cells with vacuoles did not expand so much, and no vacuoles collapsed. Therefore, the cytological changes occurring with the collapse of the vacuoles are specific responses of the host pines to the infection with the pathogenic pinewood nematodes.

3. Host cell suicide reaction

Learning from the host reactions in potato blight

What causes the enlargement of the vacuoles and their collapse? Why does this collapse of vacuoles occur only in the pine seedling infected with the pathogenic nematodes? Many phenomena involved in the host-parasite relationship can now be explained at the molecular and gene levels. However, most of these studies were focused on plant diseases caused by fungi, bacteria, or viruses, and not on the diseases caused by plant-parasitic nematodes. A review of the host responses shown by the potato tuber tissues infected with *Phytophthora infestans* may help us understand pine wilt disease.

Potato tubers infected with an incompatible race of *P. infestans* (a strain that cannot cause the host disease) showed the following reactions. Host reactions that occurred within a few minutes after the cell wall components (glucans) were released from the fungus, including cytoplasmic condensation, production of reactive oxygen species, depolarization of cell membrane potential, and leakage of electrolytes. The substance that triggers this dynamic host resistance is called an elicitor. Lipid peroxidation of the host cells progressed, the protoplasm separation ability was lost, and the host cells died (a hypersensitive cell death) eventually 30 minutes after the infection. Later, the phenylalanine ammonia-lyase gene that is involved in the formation of a cell wall component, lignin, and the formation of flavonoids begins to be expressed, and its activity increases 2 to 3 hours after infection. Then, the cells become brown, and a low-molecular defense substance called a phytoalexin, accumulates, and the mycelium is trapped in these cells and stops growing about 6 hours after infection.

On the other hand, when a compatible race of *P. infestans* (a strain that causes host disease) infects the potato plant, the cell wall substance of the fungus functions as an elicitor and triggers a host resistant response. However, these responses are not evoked by the host because the pathogen continuously secretes a substance that suppresses the host resistance reaction (this is called a suppressor), leading to the establishment and persistence of the infection.

The plant reactions occur to cope with infection by pathogens in a similar way. What is the first critical difference between the host reaction that occurs with a compatible race, and that with an incompatible race? The plant infected with an incompatible race shows oxidative burst that produces a reactive oxygen radical, but not that infected with a compatible race (Doke 1985). Thus, the determinant of the first step in host resistance is the generation of active oxygen. Once active oxygen is produced, the physiological changes such as lipid peroxidation, electrolyte leakage, and tissue browning will occur.

Oxidative burst fail to prevent pine wilt disease

The pine tree may also show general defense responses to pine wilt disease. A series of host reactions occurs in the case of potato blight when the potato is infected with an incompatible race of the pathogen, and the host plant does not become diseased by infection. However, in pine wilt disease, the host pine tree is killed, so the infection process seems to correspond to the relation between the potato and the compatible race of pathogen. I think this contradiction may be explicable by assuming the following. The above-mentioned infection responses expressed by Japanese red and black pine are not for plant-parasitic nematodes, but for pathogens like fungi and bacteria that are encountered most frequently. The Japanese pine species encountering a foreign pathogen for the first time, inevitably tries to protect itself by using resistance reactions prepared for other pathogens.

The pine trees tries to resist the invasion by the nematodes, but the unsuccessful response consequently leads to its death. The host pine tree recognizes the nematode as an incompatible foreign body and tries to suppress its activity by exerting inherent resistant reactions, but fails to prevent nematode activity, and thereby succumbs to the disease. This is the reality behind pine death after pine wilt infection.

Cytological observations of black pine seedlings infected with the pinewood nematode revealed the disruption of vacuoles, and also suggested the leakage of cell contents. Therefore, the leakage of cell contents was ascertained via experimentation as follows. Plant tissues from the main shoot of the pine seedling were excised at various times after nematode inoculation at different distances from the inoculation point. Then, each tissue was immersed in de-ionized water in a small container. The electrical conductivity of the liquid in each container was measured after a certain period. Cell fluids are electrolytes that contain various ions and have high electrical conductivity. The electrical conductivity of the de-ionized water containing the plant tissue must increase if there is leakage of cell fluid from the plant tissues. As expected, leakage of cell contents (increased electrical conductivity) was observed after nematode inoculation over time, and the range expanded outward from the inoculation point. Lipid peroxidation measured at the same time revealed a change similar to the leakage of cell contents. This suggested that lipid peroxidation of cells also progressed at the site of pinewood nematode infection. Therefore, I examined the amount of active oxygen generated in the bark, which is entirely composed of parenchymal cells. The generation of active oxygen occurred before the lipid peroxidation and the cell fluid leakage. These responses are consistent with the resistance responses when plants show exertion against infectious pathogens. I reported these results at the annual meeting of the Japanese Forestry Society (1987). Since then, other researchers have obtained similar results. Accordingly, there is no doubt that the pathological events described here occur during the pine wilt infection process.

How is active oxygen generated in the tissues of red pine and black pine after nematode infection? When plants encounter harmful stress stimuli (stressor), the activities of various enzymes that induce resistance are enhanced, the expression of new genes is promoted, and at the same time or earlier than that, active oxygen is generated.

Active oxygen has two functions; the direct function of eradicating invaders such as bacteria and fungi with intense oxidative action, and the other function is as a signaling substance that evokes subsequent reactions for resistance. However, the active oxygen itself is highly reactive, so the excess active oxygen oxidizes the host’s cell components, and thereby causes some problems. For example, the unsaturated lipids, which are the components of the biological membrane (i.e., cell membrane, vacuolar membrane, etc.) are easily oxidized by the active oxygen. The functions of the biological membrane become impaired once the molecules of unsaturated lipids are peroxidized. Over a long evolutionary period, organisms have developed various mechanisms to maintain the concentration of intracellular active oxygen at a low level. Molecules called scavengers effectively eliminate active oxygen. Enzymes such as superoxide dismutase and catalase, and anti-oxidative substances such as polyphenols, flavonoids, and carotene are among them. Tannin is a polyphenol that functions as a scavenger and it may be increased in the pine shoots due to the increase in active oxygen.

Cytological defense responses and resulting suicide of host cells

After the nematode infects the pine tissue, a tannin-like substance accumulates in the vacuole of the pine cell and eventually, the vacuole collapses. These cytological changes are summarized as follows: After the pine tissue is infected by pinewood nematodes, a large amount of active oxygen is generated (this is called oxidative burst) to eliminate the invading nematodes. The generation of active oxygen functions as a signaling substance and induces resistance. These initial defense reactions are attempts to confine the nematodes at the infection site, but the nematode’s movement breaks the host defense reactions. Accordingly, as the nematodes move, active oxygen is produced continuously and at a high concentration. Production of scavengers, i.e., tannins are accelerated to eliminate the harmful active oxygen, but it cannot keep up with the amount of active oxygen generated, and the peroxidation of membrane lipids caused by excessive active oxygen proceeds, leading to the collapse of the vacuoles. The cells themselves start to die, and their contents leak out of the cells and flow into the tracheids. Then, the cell contents stick to the margo of the bordered pits, thereby impairing the function of bordered pits, and blocking the passage of water. All of this occurs in a short time resulting in the death of the huge pine tree. This is my hypothesis on the sudden death of the huge pine tree caused by the tiny nematodes (Fig. 58).

Thus, pine wilt may appear to be a suicide phenomenon due to hypersensitive reactions.

The damage to Japanese forests caused by pine wilt disease markedly increased during and after World War II. A research project started in 1968 to contain the disease led to the discovery of the pathogenic nematode and its vector beetle. The overall picture of this forest epidemic including the mechanisms of the resistance of the host pine species and the mechanism of the onset of the disease leading to tree death that was revealed through the vast studies is as follows.

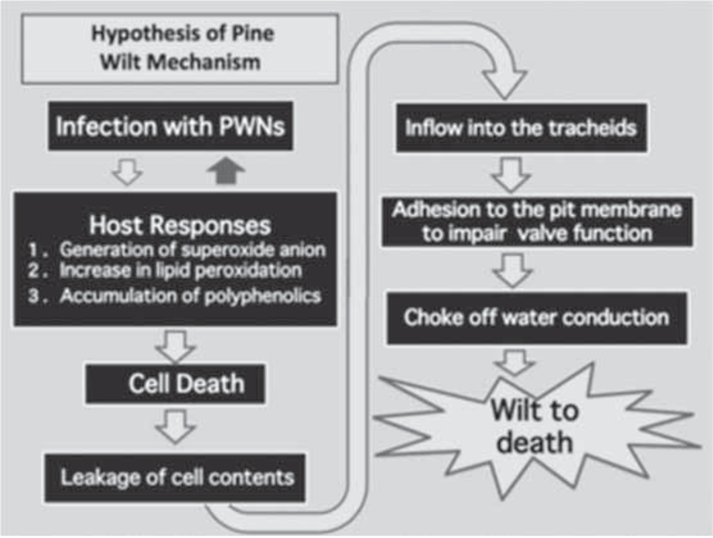

Fig. 58 A hypothesis for the mechanism of sudden pine death.

The *Monochamus* beetle emerges each May to July from the pine tree that died the previous year and carries a large number of pathogenic nematodes (pinewood nematodes) in its tracheal system. This beetle feeds on the bark of pine shoots to attain full development of the gonads (maturation feeding) because it is rich in nutrients. A large number of nematodes lurking in the tracheal system of the beetle are awakened with the progression of the biological clock by the consumption of stored lipids. These nematodes are transferred to the feeding scars made by the beetle on the pine shoots and then invade the pine tree. Pathogenic nematodes that invade the pine tree first move through the resin canal in the bark and then spread throughout the pine tree. The difficulty to invade the bark tissue via the beetle’s feeding scar and the difficulty of subsequent movement and propagation within the pine tissues determines the strength of the resistance of each pine species. The host pine tissue eventually activates a chain reaction of dynamic resistance to suppress the nematode dispersion in the pine tree. However, the nematodes move quickly and break through the defense reactions triggered by the host pine, and then spread throughout the tree. After the movement and proliferation of the nematodes, the cytological damage due to the defense reactions spread and impair the water passage leading to wilting.

The mechanism of pine wilt at a molecular level can be explained by a domino reaction that starts with the generation of active oxygen and triggers tissue-level chain reactions. This domino reaction is essentially a tissue resistance to invaders but is ineffective against pinewood nematodes, and eventually spreads the damage.

The period from nematode infection to disease onset is short; the needles fade and become brown from late summer to autumn. In the process of disease progression, the volatiles emitted from the dying pine tree attract sexually matured male and female beetles to the tree. The beetles mate, and then the female lays the eggs under the pine bark, thus leaving the next generation. The hatched larvae repeat the process of molting while eating the tissues and wood materials under the bark. In early autumn, the larva reaches the 4th instar and digs a tunnel deeply, forms a pupal chamber, and thus prepares for winter. The walls of this chamber are rich in organic substances, such as the residue of beetles (excrement and food waste), and there is adequate humidity, so various kinds of mold grow vigorously. The nematodes become widely distributed, propagate in the tree gradually settling around the pupal chamber and wait for the next spring, living on the mold. Then, the density of nematodes around the pupal chamber is determined by the suitability of the fungi for the propagation of nematodes. The number of nematodes held by the beetles that will emerge in the following year is determined as well.

The beetle larva pupates around May when the temperature rises. At this time, the dispersal-stage nematodes that have become widely distributed in the dead pine tree gather around the pupal chamber, and enter a more specialized stage called “dauer larvae” around the time of the beetle’s emergence. The emerged beetle stays in the chamber until its body hardens, the dauer larvae transfer to the beetle’s body. The beetle flies from the dead pine tree to the next healthy pine, thereby spreading the disease.

================== A column article ⑤ ===================

Sex pheromones of the pinewood nematode

Since the first inoculation experiments conducted by Kiyohara and Tokushige (1971), the relationship between the inoculum density of the nematode and the mortality rate of inoculated seedlings has been attracting the interest of many researchers. The concern is simply how many nematodes are needed to kill a pine tree. In the inoculation experiments of Kiyohara and Tokushige, 30, 300, 1,000, 3,000, and 30,000 nematodes were inoculated per pine tree. The mortality rates for each inoculation density were 5, 20, 25, 50, and 80%, respectively. This showed that the mortality rate was in proportion to the inoculation density.

However, since sex pheromones of the pinewood nematode effectively promote sexual interaction (Kiyohara, 1982), even when only a few nematodes invade the pine tree, they may be able to increase their population. Thus, as long as the nematodes can break through the host resistance that they encounter after the invasion, the host pine tree can be killed even when the number of nematodes is initially small. On the other hand, the higher the inoculation density, the higher the mortality rate. Such a proportional relationship arises because the invasion density of the nematode, as well as the chance for population growth, increases with the increase in inoculation density (Futai 1985b).

Let me introduce Dr. Kiyohara's interesting experiment on the sexual attraction of the pinewood nematode (1982). He picked up the 4th instar juveniles from a large number of pinewood nematodes of various stages isolated from the culture, which was indeed a painstaking task to perform under a stereomicroscope. Then these 4th-instar larvae were cultured individually at 25 ℃. After 24 hours, virgin females and unmated males were obtained. A strip of 1% agar 30 mm long, 6 mm wide, and 1.2 mm thick was placed in the center of a Petri dish. Parallel lines were drawn on the bottom of the dish to divide the agar strip into five equal parts.

In the first experiment, 20 males or virgin females were released onto a piece of agar and left for 10 minutes, so that 20 nematodes were randomly distributed on the agar piece. Kiyohara put one virgin female (one male lacking a mate in the opposite case) on one end of the agar strip, and after 5, 10, and 15 minutes, he examined where the 20 males (or females) ended up. The substances secreted or excreted from the body of the nematode into the agar substrate were examined for pheromone (attracting) activity in the second experiment. The gas emitted from the nematode body was examined for pheromone activity in the third experiment. The males were strongly attracted to virgin females and the substances released from their bodies in all three experiments. Conversely, females were strongly attracted to unmated males and substances secreted by their bodies. However, neither males nor females were attracted to same-sex individuals and their substances.

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IV. THE INFLUENCE OF ENVIRONMENTAL FACTORS ON THE SPREAD OF PINE WILT DISEASE

1. Pine wilt disease and the disappearance of mycorrhizal fungi

Disappearance of gypsy mushrooms from the forest in my neighborhood

There are various species of mushrooms in red pine forests. Many of them have a symbiotic relationship with the roots of red pine trees, and those peculiar to red pine forests are called mycorrhizal fungi. The native pine mushroom (matsutake, *Tricholoma matsutake*) is the most expensive mycorrhizal mushroom in Japan. There are other mycorrhizal mushrooms, such as boletes, russula, coral fungi, and cortinar. The gypsy mushroom (*Cortinarius caperatus*) is an edible mycorrhizal fungus widely distributed over the northern hemisphere. It is popular among European mushroom foragers. This is also regarded as a precious edible mushroom in various districts due to its crispy texture but is not as popular as matsutake in Japan. This mushroom used to be found every fall in the small forest of red pines and oaks near my home and made me a happy mushroom collector. It became difficult to collect this mushroom with time because the red pine trees in the forest were dying one after another due to pine wilt disease. The red pine trees were killed by pine wilt, and the gypsy mushrooms growing along with the pine trees, also inevitably started disappearing.

Decrease in matsutake production and the increase in diseased pine trees

Having experience with the pine wilt disease, I used to believe that the reduction of matsutake was associated with the decrease in pine forests due to pine wilt disease. However, the researchers of matsutake had a totallly different idea about the cause of the decrease in matsutake.

The primary cause of the severe reduction in matsutake growth in Japan is considered to be due to the fuel revolution (from charcoal fuel to petroleum fuel) and the fertilizer revolution (from organic fertilizer to chemical fertilizer) that began in the mid-1950s (see chapter 1). The fallen leaves and dead branches from pine forests used to be harvested for fuel and fertilizer; the floor of the pine forests was always kept clean (without organic deposits) before the fuel and fertilizer revolutions. Furthermore, the leaves on the soil were frequently raked and the soil was kept lean and nutrient-poor. Such operations consequently were advantageous to the pine tree, a mycorrhizal plant. The mycorrhizal fungi generally are more active under poor soil conditions with few competitors such as rotting fungi, which are usually dominant in the organic-rich deposited layer. Many other plants cannot grow under poor soil conditions in the absence of a mycorrhizal partner. Thus, plant succession stops at the pioneering stage (pine forest) and does not progress further. With the modernization of society, few people were harvesting the organic matter, fallen leaves, and dead twigs from pine forests after the fuel and fertilizer revolutions. The remains were left in the pine forests, leading to the eutrophication of pine stands. The deposits of the organic layer enhanced the activity of the decaying fungi and the environment became harsh for the mycorrhizal fungi. Naturally, the activity of the matsutake mycelium has declined, and the growth of the fruit bodies of matsutake was also reduced. Under the harsh environment for matsutake, pine trees become physiologically weakened, and thus, become vulnerable to pine wilt disease. This is the explanation of the cause of pine wilt by researchers of matsutake mushrooms.

The relationship between mycorrhizal symbiosis and pine wilt disease, examined by field experiments is summarized below.

Fertilization of soil impairs mycorrhizal relationships

An experiment was performed in the coastal area of Tottori Prefecture, where black pine trees had been planted steadily for protection against both wind and sand, resulting in the formation of a stunning forest. However, pine wilt disease invaded the pine forests and caused severe damage. Some forestry scientists consider that pine trees growing in the coastal area had insufficient resistance to pine wilt disease due to the lack of nutrients necessary for growth; thus causing the spread of severe pine wilt damage over the coastal area. The experiment was conducted in two 20m x 20m plots in the coastal black pine forest, and fertilizer was added to one plot once a year for three years, while the other plot with no treatment served as a control. The amount of fertilizer given at one time was about half of the standard amount applied to the average forest land. The results were quite different from those expected by forestry scientists. The fertilized plot was damaged more quickly, and more black pine trees were killed than in the control plot (Fig. 59). In the next step, many black pine seedlings were planted in both the fertilized plot and the adjacent non-fertilized plot and the mycorrhiza development of these seedlings was compared between the two plots. In the plots with repeated fertilization, the mycorrhiza development of the black pine seedlings was inferior compared to the control plot.

Fertilization directly suppresses mycorrhizal development or enhances the growth of competitors, such as decaying fungi, thereby disturbing mycorrhizal symbiosis. In any case, the suppression of the mycorrhizal growth caused by fertilization impairs the mycorrhizal function of the nutrient and water supply. Thus, it is not surprising that “pine wilt disease” increased by the fertilization process. The so-called “soil scraping”, carried out by “the matsutake farmers”, is an effort to keep the forest soil in poor nutrition to activate the mycorrhizal activity of the matsutake.

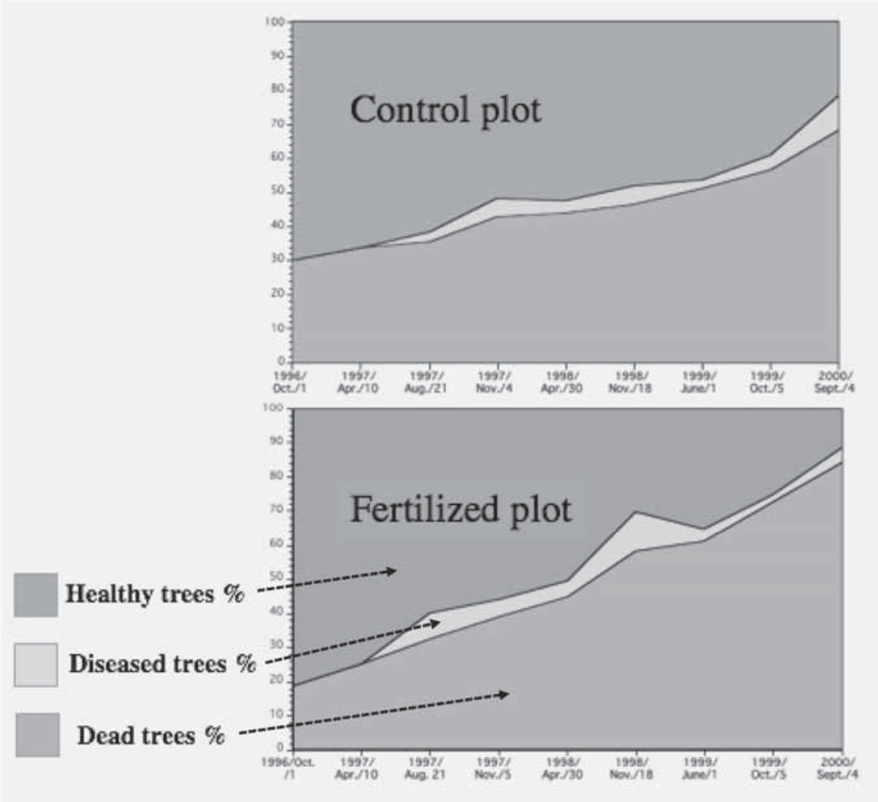

Fig. 59 Comparison of the progress of the pine wilt damage between fertilized and control plots

Coexisting tree species affect the mortality rate of the red pines by pine wilt disease

Nakamura and his colleagues (1995) at Hiroshima University selected four sites with various tree species coexisting with the red pine seedlings. The coexisting species growing at site 1, site 2, and site 3 were a species of alder (*Alnus sieboldiana*), a species of bloom (*Sarothamus scoparius*), and two naturally associated species, long stalk holly (*Ilex pedunculosa*) and Japanese eurya (*Eurya japonica*), respectively. Site 4 was a pure stand of red pine. Among these coexisting species, the alder has a symbiotic relationship with the *Frankia*, while the bloom has a symbiotic relationship with the *Rhizobium*, in their roots. For this reason, these two plant species often grow even on lean soil and are often planted as fertilizer trees to make the wastelands appear greener. The remaining two species growing at site 3 were naturally associated species usually found in red pine forests.

The pine seedlings growing had quite different fates after the inoculation with the nematodes at the four sites. The mortality rates of red pine seedlings 16 weeks after inoculation were 48, 33, 14, and 18% at the sites with coexisting alder, bloom, two naturally associated species, and pure red pine, respectively according to Nakamura and his colleagues. Higher mortality rates at two sites of alder and bloom could be attributed to the increase in the amount of soil nitrogen due to their symbiotic nitrogen-fixing bacteria. The increased amount of nitrogen caused damage to the mycorrhizal symbiosis of red pine seedlings and thus made the seedings vulnerable to pine wilt disease.

================= A column article ⑥ ==================

● Mycorrhizal fungi and mycorrhizal symbiosis

Mycorrhizal fungi that have a symbiotic relationship with pine roots are mostly Basidiomycota fungi and generate mushrooms. The mycelium of these mycorrhizal fungi attaches to the fine roots of pine species. The mycelium forms a coating layer (sheath, or mantle), which covers the surface of the fine roots of the pine is ectomycorrhiza. A part of the hyphae penetrates the root tissue, and the hyphae are weaved between pine cells. The fungus forms a hyphal network (Hartig Net) and provides water and nutrients to pine cells and receives photosynthates from pine cells (Fig. 60). Nutrients and water are supplied from the mycorrhizal fungus to the host pine root in this symbiotic relationship. In return, the fungus receives the sugar produced by the pine tree by photosynthesis. Not all mushrooms found on the pine forest floor are mycorrhizal mushrooms. Some saprophytes use fallen leaves and decayed wood as nutrients, while others are parasites on the roots or wood of the pine tree and exploit the nutrients in a one-sided fashion. The number of mushrooms generated in one forest varies depending on the tree species making up the forest. Some forests produce only about 100 g mushrooms per hectare per year, while others provide more than 800 kg. In Japan, the Japanese cedar (*Cryptomeria japonica*) and Japanese cypress (*Chamaecyparis obtusa*) have been planted as tree species for afforestation, but the symbiotic fungi that form mycorrhiza with these tree species are endomycorrhizal fungi called “AM fungi” of the phylum Glomeromycota. AM fungi do not form mushrooms, and mushrooms rarely occur in the forests of Japanese cedar and Japanese cypress. The ratio of mycorrhizal fungi in generated mushrooms varies from 15% to 100% depending on the tree species that make up the forest.

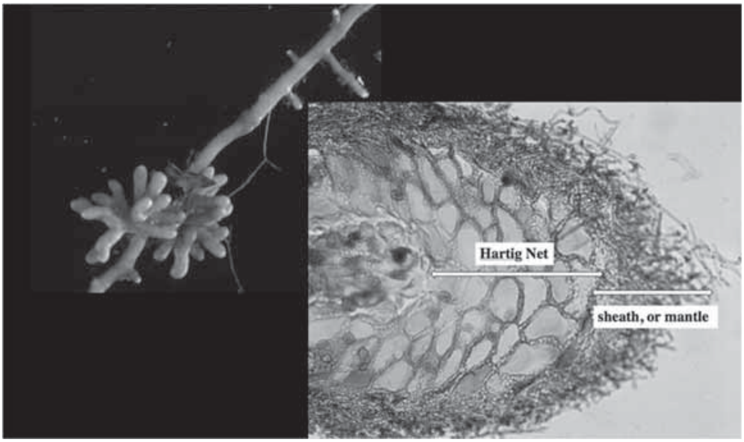

Fig. 60 The ectomycorrhiza formed on the root of Japanese red pine (left), and its cross-section (right).

Mycorrhizal fungi consume about 6-30% of the net production spawned by photosynthetic plant partners. Not all of them have transformed into mushrooms, but the photosynthetic products are also changed into the hyphal biomass and spread around the soil as mycorrhizal fungi. The amount is several to 10 times as large as the biomass that we see as mushrooms, so there is large biomass of mycorrhizal fungi in the soil.

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Effects of mycorrhizal fungi on the physiology of the red pine

The red pine can grow on fields of lean soil by mycorrhizal fungi. What effects do the ectomycorrhizal fungi have on the pine tree? Their supportive effect is best shown at the stage when small pine seeds germinate and grow as seedlings. They will be exposed to a dry condition before their roots can reach the layer replete with enough water, and will die due to slow growth. With the aid of mycorrhizal fungi, the seedlings can obtain nutrients and enough water for growth. The length of the hyphae expands into the soil, and the red pine easily absorbs enough moisture and nutrients when coexisting with mycorrhizal fungi. For instance, a mycorrhizal fungus, Jersey cow mushroom (*Suillus bovinus*) is often found in pine forests and the hyphae of this fungus that stretch from the mycorrhizal root to the outside soil, are several hundred to a thousand times longer than the pine roots. Thus, mycorrhizal fungi can expand the absorption range and supply of water and nutrients to the roots of the host plants. Their mycelium is thin and the surface area is more than ten times as large as the roots of the same weight. A large surface area also guarantees a high absorption capacity. The red pine has root hairs on the surface of the fine root and the diameter of the fine root is about 2 mm, which is more than 100 times thicker than the mycelium, so it cannot enter the fine gaps in the soil. The mycelium extends from the mycorrhiza, penetrates the gaps of the soil, and absorbs moisture and nutrients. This property of mycorrhizal fungi is particularly useful during the dry season. This is because the water becomes isolated deep in the soil gap as the drought progresses, so that the fine roots are no longer close to the water there. Among the nutrients that mycorrhizal fungi give to the plant, phosphorus is the most vital. Though there is a large amount of phosphorus in the soil, the amount of phosphorus available to plants is extremely limited. Mycorrhizal fungi collect and absorb phosphorus from a wide area of soil by its hyphal extension and by their ability to decompose insoluble and inorganic phosphorus.

Thus, mycorrhizal fungi promote the intake of nutrients. Various other direct and indirect benefits of mycorrhizal symbiosis to trees are known. When trees are stressed, mycorrhizal symbiosis is also adversely affected, but mycorrhizal fungi mitigate this stress. Two stresses are closely related to pine wilt disease: stress caused by pathogenic microorganisms, and stress caused by drought. Air pollution stress will be discussed in the next section.

Stress caused by disease and mycorrhizal symbiosis

The ectomycorrhizal fungus prevents the invasion of pathogenic fungi through its mycelial sheath and suppresses the propagation of soil pathogenic fungi by secreting substances and increases the survival rate of pine seedlings. Many microorganisms, including antagonistic bacteria around the mycorrhiza, are gathered and help suppress the invasion of pathogenic fungi. The mycorrhizal symbiosis also mitigates the disease of the over-ground parts of the plants. Pine wilt disease is caused by pathogenic nematodes that end up infecting the pine tree crowns. Using red pine seedlings, we investigated whether mycorrhizal symbiosis is involved in the pine resistance to this disease.

The seedlings of *Pinus densiflora* inoculated with either mycorrhizal fungus, truffle-like fungus (*Rhizopogon roseolus*), or slippery jack (sticky bun, *Suillus luteus*), became healthy. Subsequent inoculation of these seedlings with the pinewood nematodes gave a mortality rate of only about 25% of the total number of seedlings. On the other hand, the mortality rate of the seedlings not inoculated with mycorrhizal fungi was 50% (Kikuchi, J. et al. 1991).

Mycorrhizal symbiosis improves the physiological state of the pine seedlings, thereby mitigating the damage caused by the disease. Pine wilt disease triggered the symptoms of water stress. Therefore mycorrhizal symbiosis may improve the water absorption capacity of the pine seedlings, which might raise the resistance of the seedlings. Another possible explanation is that infection by mycorrhizal fungi induced the systemic resistance of pine seedlings but not to an extent to interfere with mycorrhizal symbiosis, thus keeping the pines from being infected by pathogenic fungi, bacteria, or pinewood nematode.

Drought resistance and mitigation provided by mycorrhizal fungi

Fig. 61 The red pine trees remaining on the mountain ridges after the pine forests were devastated by pine wilt disease.

In the experimental station at the Kyoto University Forest in Yamaguchi Prefecture, located in the westernmost part of Honshu Island, various local varieties of black pine and red pine had been planted in line along the slope of the mountains. The pine forest on this slope was severely damaged by pine wilt disease invasion in the area. I noticed a strange bias in the distribution of damaged trees on my first visit (Fig. 62).

Obviously, there were far more dead trees distributed along the lower part of the slope, and this biased distribution of dead pines might be related to mycorrhizal symbiosis.

The soil moisture and the mycorrhiza state of the pine trees in the upper, middle, and lower parts of the slope of this flat mountain were investigated. The amount of water available to the plant was smaller in the upper part than in the lower part of the slope. Moreover, the amount of open water was remarkably reduced during the dry season. The pine trees planted on the upper part of the slope were exposed to intense drought stress. However, the mycorrhizal roots of Japanese red pine and black pine growing on the upper part of the slope were more developed than the pines growing on the lower part of the slope. The pine trees growing in areas exposed to drought stress mitigate the effect of the stress by developing mycorrhiza. The pine trees

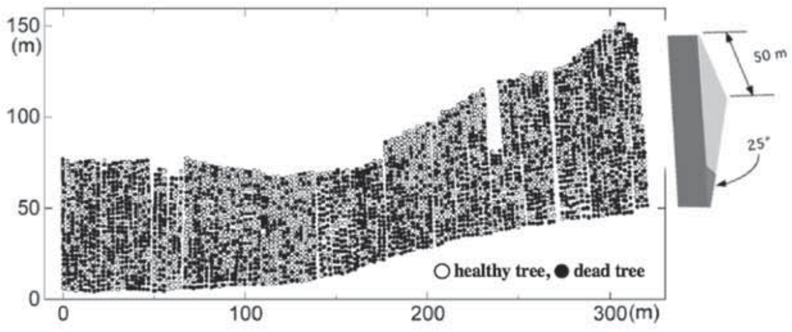

Fig. 62 The distribution of damaged pine trees on a slope of the lower mountain.

growing on the lower part of the slope have sufficient nutrients and moisture and can grow without the help of active mycorrhizal fungi. Therefore, mycorrhiza development of the pine trees was very poor on the lower part of the slope, while on the upper part of the slope where the water supply tends to be depleted continuously, mycorrhizal development was an indispensable prerequisite for the pine trees. Usually, the water balance of pine trees works well both on the upper and lower parts of the slope. However, the growth of the pine trees is severely affected by long periods of drought in the summer, and its influence extends to the lower part of the hill. The pine trees growing on the lower part of the slope were subjected to more significant stress than those growing on the upper part. This is because the mycorrhizae are poorly developed on the pine trees in the lower part of the slope (Akema & Futai, 2005). What happens to the pine forest on the slope when infected with pinewood nematodes? The pine trees growing in the lower part of the hill are vulnerable to drought stress and would be more damaged by pine wilt disease. It is probably for the same reason that red pine trees survive on the ridges of the mountain where the pine wilt caused devastation (Fig. 61). Symbiotic fungi have an intimate relationship with trees while exchanging nutrients, moisture, etc., and mitigate various stresses. The adaptive benefits of the symbiotic relationship for both partners may be much more critical than has been revealed so far.

2. Effects of air pollution on pine wilt disease

A hypothesis attributing pine wilt disease to air pollution

Even after the pinewood nematode was publicized as a pathogen leading to pine wilt disease, some people believed that pine trees were weakened due to air pollutants and that pests like the pinewood nematode were secondary pathogens of the weakened pine trees. This presumption spread widely from the 1960s to 1970s because vast areas of Japan were covered with severe air pollution in those days. Pesticide sprays targeting the vector beetles were considered to be harmful to the environment, so there was opposition in various places. When pesticides were applied taking into account their environmental impact, it became inevitably incomplete, with a high incidence of damage recurring. The people who advocated air pollution as the cause of pine wilt disease criticized the use of insecticides. “The control method that assumed that the pinewood nematode is a pathogen and the *Monochamus* beetle is its carrier failed to suppress the disease. This indicated that the new hypothesis on pine wilt disease was incorrect.”

However, identifying the cause of a disease is an issue completely different from judging the suitability of the method to control the disease. It is essential to determine the cause scientifically and establish a suitable control method. It is unscientific to become emotional and fail to see the real cause. This is also against the standpoint of environmental protection.

Soon after becoming an undergraduate student in the 1970s, I had an opportunity to familiarize myself with the research on aquatic insects. Regarding this research, I was interested in the idea of using aquatic organisms as an indicator of water pollution. In those days, the public was raising concern about environmental pollution problems. Aiming to become a biologist, I could not be indifferent to such problems. However, when I conducted the inoculation of the pinewood nematode on many pine species, the pathogenicity of the nematode was obvious and unquestionable.

Furthermore, my inoculation test was conducted at an experimental station in the university forests, located in the suburban mountains without air pollution. Thus, the massive death of pine trees was not attributable to air pollution, but due to the pathogenic nematode. I also observed the actual occurrence of the disease in the field and studied the actual situation of the damage expansion. I came to believe that the pinewood nematode is a pathogen, and the spread of the damage is due to the *Monochamus* beetle which carries the nematodes from tree to tree.

Most researchers have studied pine wilt disease according to this established theory and are convinced that it is reasonable to ignore the air pollution theory (an approach that attributes the massive pine death to air pollution). Scientists were shunning the work of other scientists studying pine wilt disease from the viewpoint of air pollution, because such research methods were not only considered to deviate from the central theme of the study of this disease, but also thought to confuse the people working to control pine wilt disease. However, the experiment conducted by Tanaka (1975) under such circumstances at the Forestry Experiment Station was very interesting. Black pine seedlings were exposed to sulfur dioxide gas and later inoculated with pathogenic nematodes. The progression of the disease was about one week faster than in the seedlings inoculated with pathogenic nematodes without sulfur dioxide gas exposure. Moreover, some seedlings were killed when exposed to sulfur dioxide gas and later inoculated with the less-pathogenic pinewood nematode, *B. mucronatus*. These results strongly suggest that air pollution may act as a trigger although it might not be a major cause of pine wilt disease. Thus, the relationship between air pollution and pine wilt disease is a critical issue. I was not interested in studying pine wilt disease in relation to air pollution, because air pollution had already been studied, and seemed to be a stale topic. However, I could not keep on avoiding this topic for long.

Unexpected discoveries

New themes are always borne under new situations. One day, a student said he wanted to investigate the relationship between acid rain and pine wilt disease but I had given the student a completely different subject for his graduation project. After he had pursued my proposed experiments without success, one by one, I finally accepted his proposal and allowed him to work on the topic of his choice. It was already the end of November when the nematodes were inoculated, which was significantly later than the season of pine wilt in the field. Third-year potted black pine seedlings grown in a greenhouse were used as the plant materials. The seedlings were kept in the greenhouse even after nematode inoculation. It was known that almost no pine trees become diseased when the nematodes are inoculated after September (Kiyohara & Tokushige, 1971). Although the greenhouse was warmed, the night temperature dropped considerably. The progression of the disease would be suppressed, even when the pinewood nematodes succeed in infecting at the temperature below 18 degrees. The development and growth of the nematode would be greatly retarded, and they would become less active at a lower temperature. The pine seedlings may not become diseased even after inoculation of the nematode. The high probability of such a failure crossed my mind, but quite to my surprise, the study proved to be extremely fruitful.

In the inoculation test, each pine seedling was inoculated with 1000 nematodes of either pathogenic or less pathogenic species. A small amount of deionized water was also inoculated as a control. Then, a dilute sulfuric acid solution of pH 2 or pH 4 was sprayed to simulate acid rain (SAR) once every three days starting from the day after the nematode inoculation. Acid rain at pH 4 is often observed outdoors, and this acidity level adversely affects crop yields or causes damage to sensitive plants. The acid rain with a pH of 2 is 100 times more acidic than that with a pH of 4, and the high acidity is harmful to humans, animals, and plants, and is rarely observed in natural conditions. Thus, the inoculation sources were the pathogenic *B. xylophilus*, the less pathogenic *B. mucronatus*, and ion-exchanged water was used as the control. Moreover, each inoculated black pine seedling received either of three types of acid rain treatment (pH2 SAR, pH4 SAR, pH7 tap water). There were nine treatments in total. Only about 80 seedlings were available for this experiment and only nine seedlings were used for each treatment. This number of seedlings was not enough for comparison of the results between treatments.

The pine seedlings were sprayed with SAR using a simple sprayer, treating each seedling carefully by hand. This method was used to treat the above-ground parts of the black pine seedlings just before the SAR liquid dripped from the plant surface (dripping point), so that the direct effects on the seedlings alone could be examined.

Besides this direct impact on the plants, acid rain can affect the plant through the action of aluminum ions in the soil. The soil becomes acidified when acid rain continues to fall on the ground, and over time, aluminum in the form of silicate ion dissolves and exhibits toxicity to the plant cells by disturbing the cell division of the root tip cells, and inhibiting nutrient uptake. Accordingly, if SAR is inadvertently applied, the plant will suffer from both the direct and indirect adverse effects through the ionized aluminum in the soil. Thus, it becomes difficult to estimate the direct impact of acid rain on the plants.

The SAR had a pH of 2 which irritated the human skin and eyes. Therefore, the student spraying SAR over black pine seedlings, wore protective gear (Fig. 63 left).

His tireless efforts and devotion produced exciting results. The seedlings inoculated with non-pathogenic *B. mucronatus*, and those inoculated with ion-exchanged water did not die even when subsequently exposed to pH 2 or pH 4 SAR. This means that the pine seedlings did not die from the acid rain (pH 2) alone. Most black pine seedlings inoculated with pathogenic *B. xylophilus* stopped resin secretion indicating the occurrence of physiological disturbance. However, when subsequently sprayed with tap water, no seedlings were killed during the experiment. The progression of the disease may have been suppressed because the inoculation time was too late. However, the seedlings sprayed with pH 2 or pH 4 SAR showed wilt symptoms on the needles, and some individual seedlings died (Fig. 63 right). If the same experiment had been conducted in July to August when pine

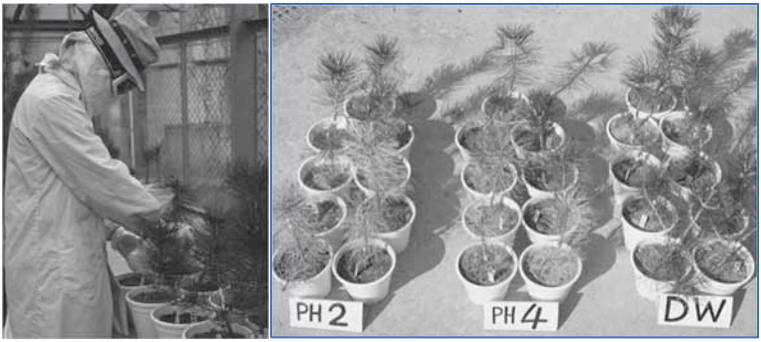

Fig. 63 Left: A student wearing a hat, goggles and a mask sprays acid rain on pine seedlings. Right: The seedlings sprayed with pH 2 or pH 4 sulfuric acid solution showed wilting or died, while those sprayed with tap water were not killed.

wilt occurs outdoors, most pine seedlings inoculated with pathogenic nematodes would have died with or without the acid rain spray. The experiment may have ended without realizing the effects of acid rain. However, the inductive effect of acid rain on the development of disease symptoms was revealed by inoculation of pathogenic nematodes in late autumn. The results we obtained were similar to those obtained by Tanaka using sulfur dioxide gas (Tanaka 1975).

The next year, the experiments on the relationship between acid rain and pine wilt disease were scaled up by automating the spraying of acid rain (Fig. 64 left) and creating a system to observe the water flow continuously in the stem of the tree exposed to acid rain (Fig. 64 right).

The effects of acid rain on the development of symptoms of pine wilt seem to be both promotive and suppressive. The SAR treatment promoted nematode propagation in pine trees, but partly suppressed their invasion into the pine tree (Asai and Futai 2001, a, b).

Fig. 64 Left: The automated acid rain spraying system. Right: The computer-aided sap flow measurement system.

These experiments also suggested that the pine trees were not killed by the acid rain treatment alone. However, this did not mean that air pollution and acid rain have little impact on the pine trees. This idea was supported by the following studies on the giant black pine trees that died of pine wilt disease at the Kyoto Imperial Palace.

The story of the centennial pine trees killed by pine wilt disease at Kyoto Gyoen

“Kyoto Gyoen National Garden” with a total area of 92 ha, stretching 700 m east to west and 1300 m north to south, is situated in the center of Kyoto City. It was the residential area of the Imperial Family and the Court Nobles for over 1000 years from 794 to 1869 when the capital of Japan was moved to Tokyo. The unattended houses of the Court Nobles were removed, and the three Imperial Palaces surrounded by earthen walls were preserved. Known as “Gosho”, to the locals, meaning the place where nobles live, it is a beautiful place for the public to jog or take a walk and enjoy nature freely as long as they do not harm the environment.

Fig. 65 The black pine trees planted at Kyoto Gyoen

There are many trees inside and outside of the white earthen walls that surround the three Imperial palaces. The main garden trees are black pine trees, and about 3,000 black pine trees had been planted with a few red pines among them (Fig. 65). However, 50-100 pine trees were killed every year because of pine wilt disease in recent years. The pine trees at Kyoto Gyoen, which are more than 2 km away from the nearest pine forests, were unlikely to have been frequently attacked by *Monochamus* beetles from the outside. Thus, Kyoto Gyoen is an excellent place to investigate the damage expansion pattern in a forest. In October, 1991, I went to the Imperial Household Office to obtain permission to examine the dead pine trees, but that was the not the office in charge of the management. I finally got permission from the Gyoen Office of the Ministry of the Environment, and examined the distribution of dead pine trees with the cooperation of the office.

All of the dead pine trees examined were around 100 years old, and were of a colossal size. These black pine trees were planted when the Meiji government converted the area into a national garden. These pine trees had grown vigorously because they were planted in a vast site spaced at regular intervals, and had been fertilized sufficiently. We could understand this fact when examining the annual rings of the dead pine trees which would show a wide annual ring width. By examining the annual ring width, it would be possible to reveal the physiological conditions of the pine trees at the time they were killed by the pine wilt disease.

I decided to measure the annual rings of the pine trees killed by pine wilt disease at Kyoto Gyoen, and was able to obtain several wooden disks from the management office. They were cut from the basal part of the trunks of big dead pine trees to measure the annual ring width. Two straight lines passing through the center of the annual rings were drawn, crossing at right angles on a wooden disk. Along the two lines, the annual ring width of each year was measured, starting from the outermost annual ring (the annual ring of the year when the tree died) to the center of the disk, in four directions. The growth width (diameter growth) for each year was estimated from the average value of four rounds.

The measurements showed that the annual ring width of many of the dead pine trees suddenly decreased the year the tree died, or the previous year. None of the pine trees examined showed a gradual decline in diameter growth due to constant stress. Many pine trees had been growing steadily, but once infected with the pinewood nematodes, they suddenly fell into physiological anomaly and died.

There was another concern from the beginning of the annual ring analysis. In the dead pine discs examined, we found a period of about ten years when the annual rings between the two bands of relatively wide annual rings were very narrow and difficult to measure the width (Fig. 66). It was unlikely that the narrow width was caused by inhibition of growth due to shading by neighboring trees because the dead trees occurred in various places with different light conditions within the large Kyoto Gyoen area.

The narrow annual rings were formed in the 1960s to early 1970s in all the wooden disks examined. This suggests that abnormal environmental conditions existed from the 1960s to the 1970s and that all the pine trees distributed in the vast area of Kyoto Gyoen were physiologically suppressed at that time simultaneously.

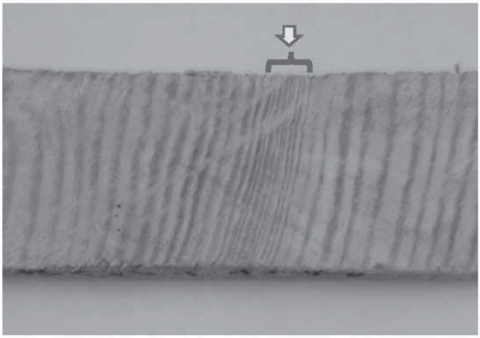

Fig. 66 A band of narrow annual rings sandwiched between ordinary wide rings.

Such a stressful condition over a wide area could only be attributed to air pollution. Therefore, it was necessary to examine the concentration of sulfur dioxide and nitrogen oxides in the atmosphere at that time. The air pollution data for Kyoto City was available only after 1971 when the observation system was established. However, 14 observation stations were already established in 1965. They were situated in various areas throughout Japan including the cities of Tokyo, Yokohama, Kawasaki, Yokkaichi and Sakai. Comparison of the data of atmospheric sulfite in Kyoto since 1971 with the data from these areas during this time revealed that the concentration of sulfite in Kyoto showed the same trend as that reported for the other areas. Thus, it is clear that air pollution in the 1960s-1970s was not a local phenomenon limited to the industrial zone, but an event widespread in Japan that had an equal effect on areas that seemed isolated as in Kyoto which is situated in a basin. While studying air pollution data in Japan, I became interested in the atmospheric conditions at Kyoto Gyoen during the 100 years that the pine trees were growing. There seemed to be no such data at first, but I found that Mr. S. Fujita of the Central Research Institute of Electric Power Industry (CRIEPI) had published data on the changes in sulfur dioxide and nitrogen oxide emissions since 1905 (Fujita 1993).

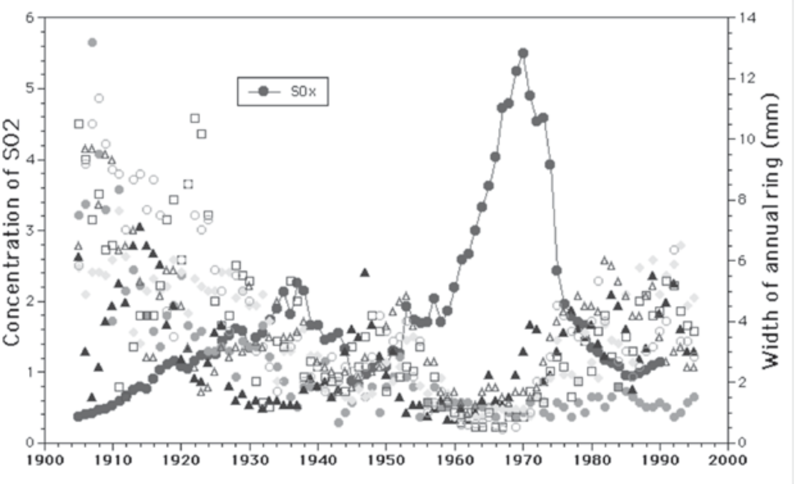

Fig. 67 Relationship between atmospheric sulfur dioxide concentration and annual ring width of seven Japanese black pines planted at Kyoto Gyoen area. Black circle indicates the SO_2 concentration, and each colored symbol indicates a different tree ring width

Based on his original data, the atmospheric sulfur dioxide concentration showed a high correlation coefficient value of 0.86 with the amount of sulfur dioxide emitted. In other words, the data on the amount of sulfur dioxide emitted well corresponds to its concentration in the atmosphere. Thus, the estimated trends regarding sulfur dioxide in the atmosphere since the beginning of the 20th century were superimposed on the annual ring width data on the pines that died in Kyoto Gyoen (Fig. 67). The growth of many of the black pine trees in Kyoto Gyoen was suppressed from the 1960s to the early 1970s, and at that time, the concentration of atmospheric sulfate around Kyoto City reached its peak.

Air pollution and acid rain have a significant impact on the physiology of pine trees and can attenuate their growth. We have to take this into account when considering pine wilt disease. However, the black pine trees of Kyoto Gyoen did not seem to have died from air pollution. These black pines survived the 1960s to the early 1970s, when their growth recovered after marked suppression, and growth was normalized. However, from 1991 to 1993, they suddenly died. The primary cause of pine wilt disease was the pinewood nematode and not air pollution. The investigation of dead pine trees at Kyoto Gyoen deviated from the original purpose and brought exciting results.

Mycorrhizal symbiosis and air pollution

The trees affected by air pollution show some or all of the following symptoms: (1) visible damage on the leaves; (2) attenuated growth of a part of or the whole plant; (3) morphological abnormalities occur visually or at the microscopic level, and (4) physiological and metabolic abnormalities appear.

Air pollutants also affect the microorganisms and their symbiotic relationship with such trees, in various ways. Foliar (phylloplane) and endophytic microbes that live in the host plant’s tissue can be directly affected by air pollutants (Asai et al. 1998). Alternatively, the leaves of trees exposed to air pollutants exhibit structural or chemical changes in their cuticle layer, and the habitat of microorganisms in the host may be impaired. Furthermore, air pollution stress applied to the above-ground part of trees affects the root physiology, thereby indirectly affecting the rhizosphere microorganisms and symbiotic mycorrhizal fungi. The acid deposition over the years also acidifies the soil causing the ionization of metals, i.e., aluminum and manganese. Therefore, microorganisms that coexist with trees in the soil are also directly affected by acid deposition. The eastern white pine (*Pinus strobus*) treated with simulated acid rain (SAR) has a lower rate of mycorrhizal fungal infection in host roots (Stroo& Alexander, 1985). Moreover, even if the SAR is carefully applied only to the above-ground part of the black pine (*P. thunbergii*), the infection rate of mycorrhizal fungi (Maehara et al. 1993) decreased.

The host tree must supply sufficient carbohydrates (an energy source) to its fungal partner to maintain mycorrhizal symbiosis and its normal functions. However, as photosynthesis regulates the supply of carbohydrates to symbionts, the normal functioning of photosynthesis is affected by air pollutants, which results in impaired mycorrhizal symbiosis. The air pollution damage to mycorrhizal symbiosis is also reflected in a decreased production in the fruit body (mushroom) of mycorrhizal fungi. For example, the incidence of fruiting bodies has decreased in Europe’s heavily polluted forests (Arnolds 1988, Jansen et al. 1988). Furthermore, 45 to 50% of the mushrooms in healthy forests are mycorrhizal fungi, but this value drops to about 10% in polluted forests (De Vries et al. 1985).

In this way, air pollution directly or indirectly affects microorganisms that are symbiotic with trees, and the symbiotic relationship itself.

Mycorrhizal symbiosis reduces the adverse effects on plants under environmental stress. The growth of roots in Loblolly pine seedlings exposed to ozone and sulfurous acid gas is markedly suppressed compared to the shoots. The mycorrhizal fungi infecting the roots, reduce the damage caused by these air pollutants and promote root growth (Mahoney et al. 1985). This example is for ectomycorrhizas, but similar findings have also been reported for the arbuscular mycorrhiza (AM), though the host plant is not a woody plant. The yield decrease of soybean exposed to ozone was 48%, but the yield reduction was 25% when the roots of soybean are colonized by the AM fungus, *Glomus geosporum*. Similarly, in trees of Japanese cedar, Japanese cypress, juniper species, etc., which have a symbiotic relationship with arbuscular mycorrhizal fungi, it is assumed that the damage caused by air pollution may be mitigated by the symbiotic relationship with AM fungi. The serious problem here is that air pollution has a detrimental effect on the mycorrhizal symbiosis itself which has a protective function for the host plant.

3. A great hindrance to the control of pine wilt disease: asymptomatic carrier trees

Spreading of PWD to cool temperature areas and the delay in symptom appearance

The first incidence of pine wilt disease was reported on Kyushu Island at the beginning of the 20th century. It then spread to Hyogo Prefecture on the main island, Honshu during the mid-1910s to mid-1920s, and gradually expanded to Southwestern Japan. In Southwestern Japan, where the symptoms of pine wilt disease progressed quickly due to the relatively high temperatures, most trees died within a year.

During the reconstruction period after the Second World War, large amounts of supplies were transported throughout Japan. Mixed with such supplies, the pine logs killed by pine wilt disease, which harbored the pathogenic nematodes and their vector beetles, also traveled long distances. Thus, the pine wilt disease expanded into the Greater Tokyo Area from 1947 to 1948. In the late 1970s, the summers were hot and dry for two consecutive seasons. As a result, the damage caused by pine wilt markedly increased in various parts of Japan, and the disease invaded and settled in cool areas in Northeastern Japan, which had not been invaded until then.

As mentioned earlier, thermal conditions have a decisive influence both on the growth and reproduction of the pathogenic nematode and the vector beetle. Temperature also affects the physiology of the pine trees, and the rate of transpiration increases with the increase in temperature. Besides, if drought due to high temperatures continue~~s~~, the supply of water from the soil will be stagnated, and there will be water stress in the pine tree. Thus, the increase in temperature accelerates the progression of pine wilt disease.

Recently, pine wilt disease has spread to Northeastern Japan and to higher-altitude regions, where thermal conditions are considerably lower than in Southwestern Japan. Thus, even when the pine trees are infected with this disease, the symptoms may not appear in the same year, but may appear the following spring or later (Jinno et al. 1987). In this way, the delay in the appearance of symptoms is due to the cool temperature conditions.

Delayed withering may also occur when the time of infection is delayed when the number of nematodes infected is small, or when the resistance of the host pine is slightly high. The phenomenon in which the symptoms appear one year after the infection is called “Toshikoshi-gare“, which means “withering due to the infection in the previous year.” However, we can determine the time of the infection from the appearance of a withering tree, only before the pine wilt season of that year begins (the end of May, in central Japan or further south). Once the pine wilt season starts, it becomes impossible to distinguish the withering trees infected in the previous year from those newly infected in the current year.

Thus, many pine trees were infected with the pinewood nematodes in a particular year, and then survived until the next spring or later without any apparent symptoms. Such trees are “latent carriers” or “asymptomatic carriers”. These trees have been neglected in the control procedure, and become ill after the start of the next pine wilt season, or later. To clarify the existence of the latent carriers, it is necessary to continue to diagnose the symptoms of the same pine trees and identify the time of the onset of the disease.

Discovery of “hysteresis” by Dr. Togashi

I was strongly impressed by the phenomenon of “hysteresis” reported by Dr. Togashi (doctoral thesis, 1988), which then motivated me to investigate the latently infected trees.

He set up his experimental site at a pine stand on the coast of Ishikawa Prefecture (facing the Japan Sea), and every year he thoroughly eradicated all the pine trees withered in the stand before the next pine wilt season the following year. In this way, he tried to prevent the pine trees in the stand from being attacked by the *Monochamus* beetle that newly emerged within the stand. However, in the following year, pine wilt death occurred again in the vicinity of the stumps of trees that were killed the previous year. Dr.Togashi mathematically proved that the distribution of damaged trees overlapped over two consecutive years, and called this phenomenon a state of “hysteresis” (the state of a system that cannot be predicted solely based on environmental variables, but requires knowledge of the system’s history).

However, he did not mention the mechanism by which the hysteresis occurs. This phenomenon must lead to a valuable clue to revealing the mechanism behind the quick spread of pine wilt. Thus, I asked some forest officers who had engaged in eradicating dead pine trees at the Kamigamo Experimental Station whether pine wilt recurred or not in the vicinity of the dead trees killed the previous year even after they completely eradicated the dead trees. All officers responded, “Yes, the withering trees recurred near the damaged trees of the previous year.” I analyzed the overlap of the distribution of withered trees in two consecutive years, from 1985 to 1987, in an area of 7.7 ha, and concluded that the distribution of pine trees killed by pine wilt disease overlap in two consecutive years. I, therefore, assumed the possibility of two mechanisms to explain the overlapping distribution of damaged trees over two consecutive years (Futai and Okamoto, 1989): (1) the *Monochamus* beetle visited not only the dead or withering tree but also surrounding ones, and infection of pathogenic nematodes, resulted in delayed death the following year, or (2) healthy trees around the dead tree were infected with pathogenic nematodes through the root system.

These are various hypotheses, but to determine the actual mechanism, we have to examine on a regular basis the health condition of all pine trees involved in a given stand. It is also desirable to specify the position of the first diseased tree and then analyze temporal and spatial changes in the distribution of diseased trees in the stand. The symptoms of disease must be detected as early as possible. A small area of Korean pine (*Pinus koraiensis*), which I will introduce in the next section, was the perfect stand for this purpose.

Long-term health check at the Korean pine stand

The Kamigamo Experimental Station has a collection of various pine species from foreign countries and replants them in the forest area of the station as arboretum forests. A small (678 m^2) stand of pine was among them with 72 trees, all of them were 45-year-old Korean pine. Many pine trees were killed every year due to pine wilt in the surrounding red pine forest, but in the stand of Korean pine, no dead trees appeared until 1989.

Fig. 68 Changes in standing tree density before and after damage caused by pine wilt disease in a Korean pine forest. Left: in May,’92, right: in April.’94. *See centerfold for this image in color.*

However, Korean pine was one of the most susceptible species according to our inoculation tests (Futai& Furuno 1979). Therefore, it was surprising that there were no dead pines in the stand, but by the end of 1990, there were two dead trees appeared in this stand. After that, I started to examine the progress of the disease in this small stand by repeatedly checking the health of each tree (Fig. 68).

The easiest way to check the pine trees for health continuously is to examine the amount of resin exuding from pine. I measured the amount of resin exuded from a needle hole of a thumbtack pierced into the trunk, divided the amounts of the resin exuded into five levels, and allotted a resin index from 0 (no resin exuded) to 4 (resin vigorously exuded) to each level. I also investigated the changes in the leaf color, the spawning marks of the *Monochamus* beetle left on the trunk, and the wood debris that the beetle larvae dropped outside the tree. All of these investigations were simple and mainly based on observations. However, this Korean-pine stand was situated on a slope at an inclination of 25 degrees and research on this slope was not an easy task. I repeated the survey of the resin twice a month for 4 years, making a total of 60 observations. Accumulation of the resin index of each tree obtained for each survey was plotted against the time axis. A straight line rises to the right for individuals that are always healthy and vigorously secreting resin. However, the straight line becomes horizontal when resin secretion stops and the condition continues. The progression of symptoms was not always so simple. In some cases, the resin exudation of the pine tree decreased temporarily. Then the tree regained vitality, and the resin exudation became active again. In other cases, the resin exudation repeatedly stopped and resumed, and eventually, stopped completely. Thus, there were various types of symptom progression (Fig. 69). Among the trees examined, the most impressive was the tree whose resin exudation had already entirely stopped in December 1990, when the survey began. This tree looked quite healthy until July of the following year. If it were not for my continuous checking of its health, the tree would be regarded as having been newly infected in 1991 and then to have become sick.

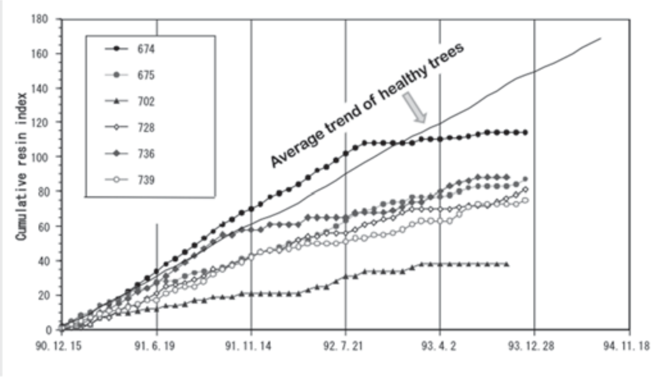

Fig. 69 Cumulative resin index curves for some damaged Korean-pines

Wilt symptoms hardly appear in the pine needles. Once the color turns yellow, the symptoms of wilt have reached the final stages, or the tree has already died. Surprisingly, the *Monochamus* beetle knew the healthy-looking tree was abnormal and laid its eggs on the trunk in June. This oviposition was the earliest one at the stand that year.

When a pine tree is infected late in summer, or infected with a small number of nematodes, the progress of the disease might be delayed. Such trees start to become diseased in the early pine wilt season the next year and emit volatiles such as ethanol and terpenes that attract newly-emerged *Monochamus* beetles from the neighboring pine forests. Thus, such trees (the latent carrier, or asymptomatic carrier) cause the recurrence of “pine wilt” at the pine stand from which all withered trees had been eradicated, and increase the number of asymptomatic carriers in the forest with time. I have proposed this idea as the **“Chain infection model”**.

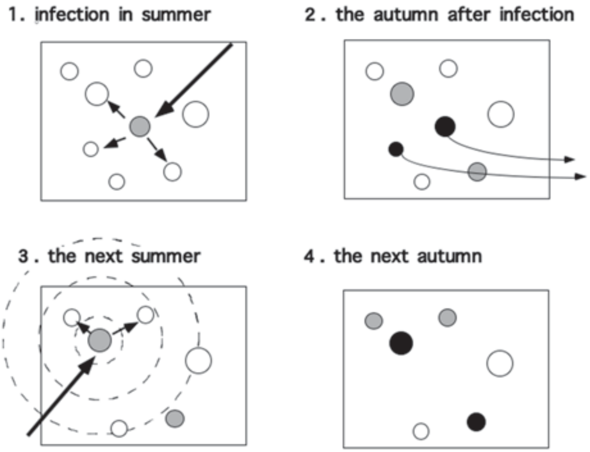

Fig. 70 “Chain infection model” for the spread of pine wilt disease that is mediated by an asymptomatic carrier tree. Solid and shaded circles represent dead and asymptomatic carrier trees, respectively. Invasion from outside of the stand and translocation within the stand of vector beetles are shown by large and small arrows, respectively. Long thin arrows in the top-right (2) mean the removal of dead trees from the stand. Dotted circles in bottom-left (3) represent diffusion of volatiles from a diseased tree.

In the third year of this survey, in May 1992, I found 20 pine trees with one branch withering. When I cut off the dead branches and searched for the presence of pathogenic nematodes, I detected them on 13 branches. Subsequently, 7 of these 13 pines died by the end of the year. From these findings, another scenario of disease progression was derived, that is, “delayed symptom progression”, in which the pinewood nematode does not kill the entire pine tree within the year of infection, but kills only the invaded branches where it stays, or lurks in the host tree, and the next year or even later, as the temperature rises, the nematode population increases eventually leading to the death of the whole tree. All of these trees with a withering branch were distributed in the vicinity of the trees that died the previous year. This fact supports the “**chain infection model**” where dead trees occur in the same place over the years starting with a tree latently infected (Fig. 70).

The health check conducted over four years on 72 trees at a small stand of Korean pine revealed that even when dead trees were removed from the stand, the pathogenic nematodes remained hidden in the surrounding trees, and became active at the beginning of the following season. The appearance of these individuals remains entirely healthy. In some of these individuals, however, resin secretion declined the next spring and stopped in the early summer. Thus, this field experiment revealed one of the mechanisms by which withering trees recur around the stump of the tree killed the previous year.

Directional behavior of the *Monochamus* beetle toward trees with a latent infection

Pine wilt occurs only in the tree species of the pinaceous family, I explained this as follows. The pathogenic pinewood nematode invades the host tree via the maturation feeding scar of the *Monochamus* beetle. Moreover, the tree species selected by an adult *Monochamus* beetle as a suitable host for the maturation feeding is limited to pinaceous trees. Thus, only these tree species can be infected by pine wilt disease. *Monochamus* beetles also select the withering or newly-dead pine trees whose resin exudation has declined or ceased, as the suitable spawning target. However, how can they find these physiologically abnormal and diseased trees among so many pine trees in the pine forest?

The *Monochamus* beetle emerged from a dead pine tree moves to the tip of the branch or the treetop, and then flies from there at human walking speed. This flight is considered a random flight. However, once it reaches a pine tree, it stops flying, settles on the one-year or current year branch, and eats off the bark (maturation feeding). The maturation feeding of the *Monochamus* adults lasts about ten days or more until their gonads are fully developed. During this period, they do not fly so often. The *Monochamus* adults fly toward the withering or newly-dead pine tree when their gonads have matured. They move actively on the trunk of the tree at night, mate when a male and female encounter one another, and the female begins to lay eggs. The average number of eggs laid by one female is 60, and the maximum is over 200.

How to find a spawning target tree

The flight toward the spawning target tree is a directed (orientated) one. A chemist, Dr. Toshiya Ikeda and an entomologist Dr. Kyugo Oda from the Forest Experiment Station (currently renamed Forestry and Forest Products Research Institute) clarified the localization mechanism of the *Monochamus* beetle to the trees for spawning. When a pine tree is infected with pinewood nematodes and its physiological state becomes abnormal, the tree emits volatile gases, i.e., ethanol and terpene (Ikeda and Oda 1980). Sexually mature males and females of the *Monochamus* beetle fly toward the abnormal trees in response to these volatile gases. Since these trees already have reduced resin secretion, the laid eggs can safely hatch, and the hatched larvae can grow without the risk of dying surrounded by resin.

I wondered whether the latent carrier trees infected with pinewood nematodes in the previous year but become diseased in the early summer of the following year (latent carriers) also become spawning targets for the *Monochamus* beetle. If so, the latent carrier trees with a healthy appearance escape the eradication control, thereby remaining in the forest. These trees show withering symptoms in the early summer of the following year in the vicinity of the dead tree (recurrence of Pine Wilt Disease). Furthermore, these trees emit volatiles and attract the mature *Monochamus* beetle for oviposition when the trees become diseased. Thus, the latent carrier trees not only nullify the eradication efforts and seriously impair the motivation of control personnel, but also promote the further spreading of this disease.

A model experiment in a net cage

To explain the spreading mechanism of pine wilt disease, I assumed a chain infection model, where the disease is supposed to start from a tree with a latent infection. However, a model is not enough to explain the spreading mechanism and experimental evidence is essential. Most notably, the model is to prove that the asymptomatic carrier trees attract mature beetles for their oviposition. I conducted an experiment in a cage from July through August 1994 to examine the behavior of vector beetles around an asymptomatic host tree. The size of the cage was 4m long, 2.7m wide, and 1.4m high and I placed 42 potted 3-year-old Japanese black pine (*P. thunbergii*) seedlings in the cage, 35cm apart from each other. I divided 36 seedlings into four blocks and used the remaining six pots as a partition. A seedling inoculated with about 5,000 pinewood nematodes in October of the previous year was put in the center of one of the four blocks of the seedlings and simulated an asymptomatic carrier tree (Fig. 71).

After marking on the back of each beetle with a paint marker for individual identification, five males and five females of *M. alternatus* were released from the center of the cage. Two and four days after releasing the beetles, I recorded which seedling the beetles were located. This experiment was replicated eight times by changing the position of the simulated asymptomatic carrier seedling from one block to another. All seedlings with feeding marks were replaced with new trees in each replication.

The Chi-square test was applied to determine the difference between the number of beetles aggregated in the block containing the asymptomatic carrier seedling, and the number in the other three blocks. Figure 71 displays an example of the results of the replicated cage experiments, which shows the aggregative distribution of vector beetles around the seedling inoculated with pinewood nematodes.

The number of beetles that aggregated in the block containing the asymptomatic carrier seedling was higher than the number in the other three blocks. Subsequent statistical analysis for all replicates ascertained that the number of beetles aggregated in the block where the asymptomatic carrier seedlings were situated was significantly higher ($P < 0.05$) than the number of beetles distributed in the three other blocks.

Shibata (1986) also found that the *Monochamus* beetles gathered in healthy pines surrounding a withering pine in the field similar to my cage experiment. However, it should be noted that the kind of tree that attracted beetles in Shibata's report was a pine tree that developed withering symptoms during the year it was infected, while what I'm concerned about was a tree with a latent infection that had been infected the previous year and remained asymptomatic until the early summer of the following year or later while becoming withered and attracting the newly-emerged *Monochamus*.

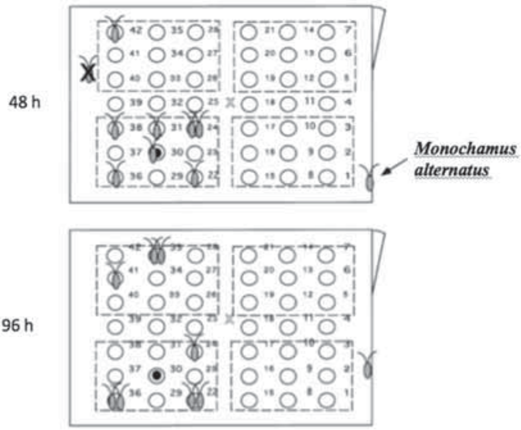

Fig. 71 Arrangement of 42 black pine seedlings in a cage (length x width x height = 4 x 2.7 x 1.4 m) for the study of vector beetle (*Monochamusalaternatus*) behavior. ◉: a simulated asymptomatic carrier seedling, which was moved from one block to another for each replication, ◯: 3-year-old pine seedling, X: the point from which the vector beetles were released.

The cage experiments also revealed maturation feeding scars made by many beetles distributed in the trees around the simulated latent carrier. Thus, the next latently infected trees also will occur around the dead pine trees from the previous year, resulting in an overlap in the distribution of damaged trees in successive years.

Even when the dead trees that are the sources of disease spread are entirely removed from the forest, the trees with asymptomatic infection may remain there due to their latency and would not halt the spread of the disease. Moreover, these latent carrier trees attract the *Monocohamus* beetles from surrounding pine forests without pest control. Thus, latent carrier trees could lead to a recurrence of pine wilt disease resulting in a chain of infection.

When I suggested the possibility that acid rain or the latent carrier trees may enhance the development of pine wilt disease, some people criticized my ideas and said that “If your ideas are made public, the people who are engaged in pine wilt control would be confused about the main cause of the disease. Also, they may find it difficult to control these factors and give up entirely.” However, what causes problems in the implementation of pine wilt control is not the possibility of such factors accelerating the development of the disease, but the insufficient education of the pine wilt control workers. The people responsible for controlling pine wilt need to understand the influence of environmental factors such as acid rain and the role of latent carrier trees so that they can provide appropriate control measures based on the understanding.

When healthy pines are killed even after intensive control procedures, such as thorough eradication of dead pine trees or spraying insecticides to prevent them from being attacked by *Monochamus* beetles, the people in charge of pine wilt control may lose interest in controlling the disease. However, if they understand that trees with latent infection remain in the forest after the control and cause the recurrence of the disease, they may be motivated to continue their efforts to control the disease.

V. GLOBAL CONCERNS REGARDING PINE WILT

1. The situation in North America

The pinewood nematode is distributed not only in Japan but also in North America. However, the pinewood nematode has little pathogenicity to the pine species native to North America. Presumably, the pinewood nematode (*B. xylophilus*) survives in North America with a life cycle similar to that of *B. mucronatus* (less-pathogenic to the pine species) in Japan.

Rutherford et al. (1987), in Canada, reported an interesting view: certain North American native pine trees are susceptible to the pinewood nematode (i.e., become withered when infected). However, as a result of being affected by this nematode, such pine species as Jack pine (*P. banksiana*) were forced to be distributed only in northern Canada and the alpine region, which is cold, even for North America. Therefore, except for these regions, only such species that are resistant to this nematode could survive and remain healthy. Jack pine, which they had regarded as susceptible to this nematode, was not necessarily susceptible in our inoculation test (Futai and Furuno, 1979). There are several species of the *Monochamus* beetles closely related to the Japanese species of *M. alternatus* in North America (*M. carolinensis, M. mutator, M. scutellatus, M. oregonensis, M. titillator, M. notatus,* in Canada: *M. carolinensis, M. marmorator, M. mutator, M. obtusus, M. scutellatus, M. titillator* in the United States). Therefore, there is ample possibility for North American pine species to become infected with pinewood nematodes. Thus, only resistant pine species remain outside of the colder areas, where these beetles were not distributed.

Monochamus spp., the possible carrier of pathogenic nematodes, are widely distributed in East Asia, North America, and Europe. Therefore, the pine species distributed in these regions may also be damaged. The pine wilt damage caused by the pinewood nematode has spread to China, South

Korea, and Taiwan. *P. densiflora* (Japanese red pine), *P. massoniana* (Masson's pine) and *P. koraiensis* (Korean pine) have been withering in these countries, and the disease has become a serious problem. Furthermore, after the disease spread to Portugal in Europe in 1999, it has become a global concern.

2. Nomenclature of the pinewood nematode

When the pinewood nematode-like nematode was detected from a dead Austrian pine (*P. nigra*) in Missouri, pine wilt disease became a problem in the United States. Immediately after the detection of the nematode, nematode taxonomists carefully examined the relationship between this nematode and the pinewood nematode (*Bursaphelenchus lignicolus*) described by Mamiya et al. and clarified that they are identical species. Furthermore, they found that this nematode is the same species as that described as a new species, *Aphelenchoides xylophilus*, detected from the longleaf pine (*P. palustris*) in 1934 in the United States. Since this species was later transferred to the genus *Bursaphelenchus*, the scientific name of the nematode was changed to *Bursaphelenchus xylophilus*. To resolve the nomenclature of the nematode, Dr. Mamiya also worked with the nematode taxonomists, and they carried out further taxonomical examinations. As a result, *B. xylophilus* became the official name for the pinewood nematode instead of *B. lignicolus* (Nickle et al. 1981).

Japanese black pine and other imported pine species such as Scots pine (*P. sylvestris*), and Austrian pine (*P. nigra*) were used as Christmas trees, in a landscape setting, as windbreaks, and so on in the United States, the saplings of these pine species were planted in a vast field. Some of these pine saplings died due to pine wilt disease and became a problem at the end of the 1970s. A national survey was conducted to examine the distribution of the pinewood nematode in the USA, and the nematode was detected in 34 states. However, it was only in some central and southern states that pinewood nematode infection caused the death of pine species. Furthermore, most trees that harbored the pinewood nematodes had either died or were weakened due to other causes. Thus, most native pine species in the USA seemed to be resistant to the pinewood nematode.

3. Pine wilt problem in Europe

Massive death of maritime pine in France

In 1979, in Europe, many pine trees died in a vast maritime pine (*Pinus pinaster*) forest on the southwestern coast of France, and nematodes of the genus *Bursaphelenchus* were discovered from these dead pine trees. Researchers initially worried that the newly-found pinewood-nematode-like nematodes might cause massive pine death. Soon, it became clear that it was a closely related species with low pathogenicity, and the people concerned were relieved. Dr. DeGuiran, a nematologist who was on the French research team, sent me a letter asking me to send him the Japanese pinewood nematode so he could compare it with the newly-found French *Bursaphelenchus* nematode. In response to his request, we exchanged nematodes. Thus, we unexpectedly obtained French nematodes and investigated the morphology and their pathogenicity to the pine seedlings. We found that French nematodes had almost no pathogenicity, and the morphology was slightly different from that of the Japanese pinewood nematode.

In this way, most researchers assumed that European forests are free from the pinewood nematode, but the situation of pine wilt changed dramatically.

Detection of pinewood nematode at a Scandinavian port directly affected North American forestry

Pinewood nematodes were detected in 1984 from the pinewood chips imported from the United States and Canada at a port in Finland. The Finnish government immediately banned the import of coniferous wood from the United States, Canada, and Japan, where the distribution of this nematode was confirmed. Sweden and Norway in 1985 implemented similar import bans following Finland. The two North American countries, the USA and Canada were exporting large amounts of timber to Scandinavian countries started suffering a major negative impact. More than 13,000 people, mainly forestry workers, lost their jobs and suffered more than $60 million in the United States alone. The European Plant Protection Organization (EPPO) in 1986 also designated the pinewood nematode as an A1 rank quarantine pest, which is given to sever possible quarantine pests that are absent from the European and Mediterranean region, following the import ban in Scandinavian countries.

The ban on imports of North American coniferous wood by European countries was a major concern to the United States and Canada. Both countries conducted an intensive nationwide survey to examine the actual distribution of the pinewood nematodes. A governmental agency, FIDS (Forest Insect and Disease Survey) in Canada started the survey in 1985. The main objective of the survey throughout Canada was to collect samples of pinewood nematodes from potential host trees and potential insect vectors (FIDS, 1988). A total of 3,706 trees, including 2,773 dead trees and 5,619 insects, including 1,294 *Monochamus* beetles were investigated by a survey team. This survey yielded surprising results: The pinewood nematodes were detected in all Canadian provinces except Prince Edward Island. However, in every case in Canada, the pinewood nematodes were not detected from collectively dead pine trees, which was the case in Japan, but only from one or two isolated trees. This field survey also revealed that there are two types of pinewood nematodes in Canada; one is the r-form, whose female has a round tail tip, and the other is the m-form, whose female has a tail tip with tiny mucro (protrusion). The m-form pinewood nematode was universally distributed throughout Canada, while the r-form nematode was distributed only in the northern and eastern provinces in Canada. There was also a difference in host species between the two forms of nematodes: the r-form was obtained only from the pine species, while the m-form was detected more often from fir (*Abies* spp.) or spruce (*Picea* spp.) trees than from pine. The pinewood nematodes were detected from the six pine species, Scots pine (*P. sylvestris*), jack pine (*P.banksiana*), resinosa pine (*P. resinosa*), ponderosa pine (*P. ponderosa*), white pine (*P.strobus*), and lodgepole pine (*P. contorta*) in Canada. This nematode was also detected from six other coniferous species, such as the balsam fir, spruce, and douglas fir. However, these coniferous trees were weakened or withered, due to other causes.

The Canadian government decided to invite a researcher from Japan to assess whether North American conifers and European conifers were susceptible to pinewood nematodes. Dr. Mamiya, one of the pioneers of Japanese pine wilt research, was asked to send a Japanese researcher and introduced me to the Canadian government as a candidate. I accepted their invitation and stayed in Victoria, the capital of British Columbia to investigate the pine wilt disease. I studied the pathogenicity of three isolates of the pinewood nematode, the Canadian r- and m-forms, and one Japanese r-form, called S10, against three coniferous species, Scots pine, western larch (*Larix occidentalis*), and black spruce (*Picea mariana*) at the Pacific Forestry Centre for a year, from May 1987 to May 1988. The results of the study were published (Futai & Sutherland, 1989).

Negotiations between two North American and European countries on softwood trade

In July 1987, Dr. Jack Sutherland, the director of the laboratory asked me to accompany him to Ottawa for a meeting. I accompanied the Canadian team, including governmental officers and researchers, and a delegation from the Swedish government to the meeting in Ottawa to discuss the pinewood nematode issues. Also, on the schedule was a visit to a site in the Eastern Canadian city of Fredericton, where the pinewood nematode had been discovered. I was pleased to accompany Jack on this trip because it was an excellent opportunity to see the Canadian forests.

Both the bilateral conference held at the Ministry of Foreign Affairs in Ottawa and the field visit in Fredericton involved a series of urgent discussions among researchers with national interests. Then in 1992, an “International Conference on Trade Issues Related to Pine Wilt Disease” was held in Brussels, Belgium. Government officers, researchers from European countries, two North American countries, myself and another Japanese researcher, as well as one Chinese scientist, also attended the conference. The meetings continued from 9:00 am to 10:00 pm for 5 days. It was a physically and mentally exhausting schedule. Every day, after the enthusiastic discussions, the contents of the day's session were summarized, and on the final day, all the participants joined together to summarize the discussions on the agenda. After the meeting, the participants went downtown and sat at the table with their counterparts who they had been having heated discussions with until then, and enjoyed a late dinner with wine together like old friends. This conference taught me a lot, including rules for productive discussions, rational ways to organize the results of meetings, and how to switch between public and private sectors.

Several international academic conferences held later on also addressed this trade problem, and each time the discussion was heated, but the issues were always straightforward. Scandinavian countries claimed that if the disease invades Europe, it would be a serious hazard because there are some susceptible pine species and possible vectors for this nematode, *Monochamus* beetles. The USA and Canada on the other hand insisted that the temperature in Scandinavian countries is too cold for the disease to develop, so there would be no fear of pine wilt disease developing there. They also argued that this reasoning is strongly proven by the fact that there had been no outbreak of pine wilt disease despite the long-lasting trade of softwood between North American and Scandinavian countries. Furthermore, the USA and Canada also asserted that the pinewood nematodes might already have inhabited Europe, but the disease did not occur simply because environmental conditions and temperature were not conducive to the development of the disease.

Pine wilt disease finally invades Europe!

The situation suddenly changed in 1999. I received an e-mail from Dr. John Webster, a professor at Simon Fraser University, who was a leading scientist studying pine wilt disease in Canada. He announced that the pinewood nematode was just detected in Portugal, in Europe. An intensive survey conducted for two years after the detection of the nematode revealed that the range of damage due to pine wilt disease had been limited to the

Setubal Peninsula about 50 km south of Lisbon. However, when I visited the pine wilt-infested area of Setubal, I felt that this epidemic disease had already been firmly established in a sparse mixed forest of maritime pine (*P. pinaster*) and Italian stone pine (*P. pinea*). The Setubal district is one of the leading industrial zones in Portugal, and a port city facing the Sado River estuary. In Japan, when pine wilt disease jumps to remote areas, it often begins to spread from a port area. Thus, the situation in Setubal seemed to be similar to that in Japan. The concerns of the European countries became a real issue.

4. Pine Wilt Disease in east Asian countries

In China

The first instance of pine wilt disease in China occurred at the Sun Yat-sen Mausoleum, situated at the foot of Mount Zijin (Purple Mountain) in Nanjing, in 1982. The tomb area, Mount Zijin, is covered with Masson's pines (*Pinus massoniana*), but the trees first infected with pine wilt disease were not the Masson's pine, but five Japanese black pines (*Pinus thunbergii*) planted in the approach to the area. The disease then spread to the surrounding Masson's pines. The Chinese government, which took the situation very seriously, embarked on a nationwide survey. The Chinese Academy of Forestry's nematologist, Dr. Yang Bao Jun, since then has been in charge of leading the government research on the pine wilt.

Her research revealed that the damage due to pine wilt disease had spread from Jiangsu Province, where Nanjing is located, to the surrounding provinces, Anhui, Guangdong, Zhejiang, and Shandong Provinces. In Jiangsu Province, where pine wilt was first discovered, the damaged area of pine wilt increased from 200ha to 9500ha, and the number of pine trees annually killed by the disease increased from 260 to 37,500 between 1982 and 1995. Various methods have been used in an attempt to control pine wilt disease in China, such as cutting down every dead pine tree, use of fumigants for dead pine logs, air-spray of insecticides for pine forests, breeding for resistant trees, and introduction of natural enemies. However, none of these has had a decisive or successful effect. Transporting pine logs infested with *Monochamus* beetles seemed to have played a critical role in spreading pine wilt disease in China.

Forest resources are limited, compared to the vast land area in China. Therefore, the native Masson pine trees are valuable forest resources. Regarding Masson pine trees, it is well known that the resistance to pine wilt disease differs with the distribution area. In general, Masson pines in southern Guangdong and Fujian provinces are resistant to pine wilt, while the pines distributed in the more northern Zeonjiang and Anhui provinces are more sensitive. There was mass mortality of the Masson pines in Hong Kong, due to pine wilt disease in the early 1980s. The local government implemented urgent control measures, such as cutting down and burning dead pine logs, and so on to check the spread of the disease. Since then, severe outbreaks of pine wilt disease have not occurred in Hong Kong.

Luchu pine was damaged both in Taiwan and Okinawa

The inhabitation of the pinewood nematode was also confirmed in Taipei, Taiwan, in 1985. However, forest damage caused by pine wilt disease was reported much earlier in the mid-1970s. A newly-introduced pine species, Luchu pine (*P. luchuensis*), suffered from pine wilt in Taiwan. Luchu pine is closely related to the Japanese red pine and black pine, and was found to be high susceptible to the pinewood nematode in our inoculation experiments. The Luchu pines once abundant on the Okinawa islands, their original distribution area, were also killed seriously by pine wilt disease recently, and this was a shock to everyone concerned.

Pine wilt disease in Korea

Pine trees are sacred and appreciated in Korean culture. In South Korea, pine forests cover 23.5% of the total forest area and 15.1% of the country (Shin, 2008).

Pinewood nematodes were detected from dead black pine in Busan, Korea in 1988 and since then, the disease spread northward along the highway and the coastal area. This pathogen invaded the country with the import of goods from abroad, and the movement of people and goods seems to have facilitated the subsequent spread of pine wilt disease in Korea.

5. Ban on conifer trading and the molecular phylogeny of pinewood nematodes

Need to develop reliable identification methods

Since the Scandinavian countries banned the trade of North American coniferous timber, conferences over quarantine issues were held between the politicians of the countries on both sides of the Atlantic Ocean. This political situation required the reexamination of the taxonomy of the pinewood nematode and a reliable identification method.

As mentioned above, the pinewood nematode-like *Bursaphelenchus* nematodes were detected from a large number of dead trees generated in the coastal Maritime pine forests of Southwestern France. This nematode could be crossed with the pinewood nematode and produce offspring, so it was first reported to be the same species as the pinewood nematode (DeGuiran & Boubria 1981). Thus, North American scientists insisted that the pinewood nematodes already existed in Europe, making it inappropriate to ban imports of conifers from North America. However, Dr. Mamiya, who investigated the pinewood nematode-like *Bursaphelenchus* species in France, reported that the French nematode was identical to the Japanese less-pathogenic pinewood nematode *B. mucronatus*. French nematologists reported that the pinewood nematode (*B. xylophilus*) and the less pathogenic pinewood nematode are the same species. This controversy led to confusion in the identification of the two species. Eventually, the American nematologist Nickle, the Japanese nematologist Mamiya and others carried out a detailed comparison, and based on the fact that these two species cannot produce good offspring, concluded that these two are separate species (Nickle et al. 1981).

Later, the French nematologist De Guiran carried out careful mating tests between 3 species of pinewood nematode (American pathogenic, Japanese less-pathogenic, and French pinewood nematode), and got an interesting result. Although it was not possible to produce offspring from the American pathogenic and the Japanese less-pathogenic pinewood nematodes, it was possible to produce offspring between the French *Bursaphelenchus* species and either American pathogenic or Japanese less-pathogenic pinewood nematodes. Based on the result of the mating experiments, Dr. De Guiran proposed treating the American pathogenic and Japanese less-pathogenic pinewood nematodes together as superspecies.[4]

He also proposed the hypothesis that both American pathogenic pinewood nematodes, and the Japanese less-pathogenic pinewood nematodes were born from a common ancestral species that might have originated in Western Europe. One group spread westward to the North American continent and became the pathogenic *B. xylophilus*. The other group expanded eastward on the Eurasian continent and reached Japan to become the less-pathogenic *B. mucronatus*. It took a long time for the two groups of nematodes to establish themselves in the distant locations; therefore, in North America and Japan, the two groups have become two separate species that cannot cross-fertilize. To provide a definite basis for such discussions, many researchers have proceeded with a molecular phylogenetic analysis of this group of nematodes. Dr. Webster and his colleagues in Canada, and Dr. Bolla in the USA, and French scientists, Tares, and Abad, conducted a detailed molecular analysis to reveal the phylogenetic relationship among geographical isolates of the *Bursaphelenchus* species.

A molecular phylogenetic analysis

Our laboratory also studied the phylogenetic relationship among the different isolates of the pinewood nematode obtained from various countries. We made special efforts to reveal the relationship between two Japanese isolates, the pathogenic pinewood nematode (*B. xylophilus*) and the less-pathogenic one (*B. mucronatus*), as well as other *Bursaphelenchus* nematodes from various countries. We succeeded in clarifying the phylogenetic status

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[4] A group of entirely or essentially allopatric taxa (that is, with nonoverlapping distributions) that were once races of a single species but which now have achieved species status.

of the two Japanese pinewood nematodes in the phylogenetic tree of this nematode genus (Fig. 72).

Eleven Japanese, five North American, and one Chinese isolate were used to examine the phylogenetic relationship for the pathogenic pinewood nematode, *B. xylophilus*, as well as three Japanese, one Chinese, and one French isolate for the non-pathogenic *B. mucronatus*.

The genes used for the sake of comparison were three sub-units of cellular granules called ribosomes, and the genes of the ITS region sandwiched between them (referred to collectively as ribosomal genes). The ribosome is an indispensable granule for organisms that synthesize proteins. Since it exists in all organisms, from bacteria to higher organisms, it is possible to identify the phylogenetic relationships between organisms by comparing their ribosomal genes. Besides, the ITS region is a site that is not expressed as a protein or RNA, and mutations tend to accumulate. Thus, the ITS region is also useful for examining phylogenetic relationships between closely related organisms, such as different populations of the same species,

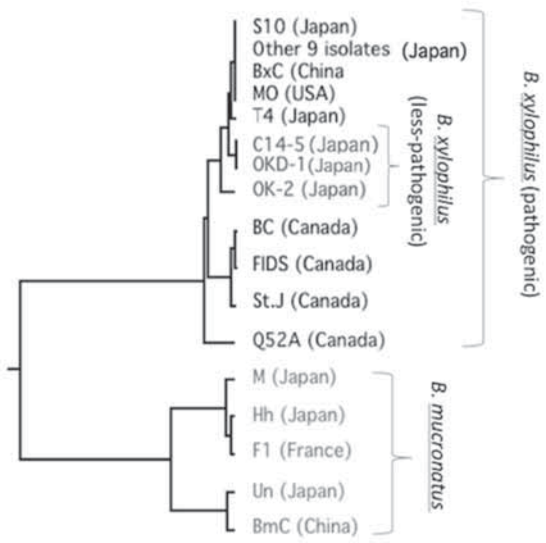

Fig. 72 The phylogenetic relationship among the different isolates of the pinewood nematode (Iwahori et al. 1998).

or between two closely related species. The ribosomal DNA sequences of each nematode isolate were amplified using the PCR method and used for the following phylogenetic analysis in our experiments: we estimated the phylogenetic relationship of the nematode isolates in question either by reading the entire nucleotide sequences of the region (sequencing method), or by cutting the DNA into small pieces with restriction enzymes, and then performed electrophoresis to compare the migration patterns (RFLP method).

In this way, we obtained a phylogenetic tree for 17 isolates of the pinewood nematode (Fig. 72). This phylogenetic tree gave some clear answers about the relationship between the pathogenic pinewood nematode *B. xylophilus* and the less-pathogenic pinewood nematode, *B. mucronatus*. One of them is that pathogenic pinewood nematodes and less-pathogenic pinewood nematodes are divided into two groups as different species. Among the pathogenic pinewood nematodes, 11 Japanese, one American, and one Chinese isolate had the same sequences for all the regions of the ribosomal DNA examined, strongly suggesting that they have a common origin. Apart from these 13 pathogenic strains, the three less-pathogenic strains in *B. xylophilus* formed a group, and the four Canadian strains formed an independent group at a further distance. This figure also shows that the less-pathogenic pinewood, nematode *B. mucronatus*, consists of at least two groups (Iwahori et al. 1998).

Where did the Japanese pinewood nematode come from?

The trade problems between the two continents contributed to the promotion of nematode molecular phylogeny. The less-pathogenic Japanese pinewood nematode, *B. mucronatus*, is regarded as a native species widely distributed in pine forests throughout Japan long before the pinewood nematode invaded the region. Pathogenic pinewood nematodes invaded Japan at the beginning of the 20th century, and the first record of their discovery was in a port city on Kyushu Island.

The widespread distribution of the pinewood nematode in North America, and the lack of pathogenicity of this nematode among many native pine species growing there, support the idea that this nematode originated in North America. The results of our molecular phylogenetic studies also confirmed this hypothesis.

Based on the results of the inoculation test, Furuno et al. (1993) classified the degree of resistance of each pine species against pine wilt disease into four classes, represented by different symbols, and plotted them on the respective distribution points of each pine species on the world map. From here, an exciting fact emerged about the North American pine species (Fig. 73).

Fig. 73 The distribution of pine species in North America, and their resistance against Pine wilt disease

Many pine species on the Pacific coast are more susceptible to pine wilt, but all of the pine species examined on the Atlantic coast are resistant. Dubos developed the concept that “Given enough time, a state of peaceful coexistence eventually becomes established between any host and parasite” (Ewald P.W.1983). If this is the case, it must have taken a long time from an evolutionary perspective to establish the peaceful relationship between the pinewood nematode and the pine species along Atlantic Coast.

It is easily assumed that the *Monochamus* beetles carrying many pinewood nematodes snuck into the pine woods harvested on the Atlantic coast and then exported to Japan at the time of the Russo-Japanese War. However, it was not possible to clarify whether these pine woods were imported from the far east Asian countries, as opposed to the closer Pacific coast of North America.

6. Development of subsequent research

Since pine wilt disease invaded Europe at the end of the 20th century, interest in this forest epidemic has risen again among scientists, and the research on this disease has advanced. Regarding the pathology of pine wilt disease, the studies at the level of the gene on the interaction between the pathogenic nematode and the host pine tissue after infection have progressed (Qiu et al. 2013, Shinya et al. 2012), and on the pathogenic nematode side, the body’s surface component (Shinya et al. 2010) and secretory enzymes (Shinya et al. 2013) have been studied. Research on the resistance of the host has also advanced at the gene level, and it may be possible to provide a theoretical basis for resistance breeding (Hirao et al.2012). In addition, the relationship between reactive oxygen species as a host resistance factor, and the nematode-side defense enzyme was also published (Li et al. 2016). The host response to the infection of the pinewood nematode has also been studied using transcriptome analysis, and several biological processes were found to be significantly enriched (Lee et al. 2019).

Research on pine wilt disease has dramatically facilitated the study on the taxonomy of *Bursaphelenchus* nematodes. At the time when *B.*

xylophilus was discovered as a pathogen for pine wilt disease, only 40 species of nematodes were reported as genus *Bursaphelenchus*, and now, there are more than 130 species of nematodes.

Pine wilt is an infectious disease that occurs in the forest. The development of a valid measure based on its spreading manner is crucial for the control of this forest disease. Forests are usually too vast and very difficult to investigate; mathematical model analysis is an excellent tool for overcoming this obstacle. So far, many mathematical biologists have attempted to tackle this problem (Togashi and Shigesada, 2006; Takasu, 2009; Robinet et al., 2011). Therefore, sufficient data of excellent quality is essential to obtain an appropriate model. However, the data obtained in previous studies on pine wilt disease are far from sufficient. Moreover, as revealed with the new viral infection, COVID-19, which is a great crisis to humankind, asymptomatic carriers play a significant role in the spread of damage in pine wilt. It is hard to say at this point that everything is well understood among experts, but the accumulation of such data in the field is of utmost importance.

EPILOGUE

The first edition of this book was published in Japanese in 2003; of course, for the Japanese public. Even though vast areas of pine forests in Japan had suffered devastating damage, the general public had almost no information about pine wilt disease. To compound the issue, many administrative officials involved in disease control, as well as contractors in charge of pest control did not have accurate knowledge about this forest epidemic. Therefore, inappropriate control practices were often observed, and thus the disease could not be contained. Furthermore, to establish an appropriate control method, it is necessary to have a full understanding of the multifaceted problems involved in this forest epidemic and to understand complicated biological relationships comprehensively. However, there were only a few people with such a comprehensive viewpoint, even among experts.

Later, the disease started to spread in East Asian countries such as China and South Korea, and even in Europe in 1999, so the problem became global and severe damage spread to the forests in those countries. I assumed that the experts in these countries would refer to the studies conducted in Japan and develop appropriate control methods for their countries. However, no country seems to have succeeded in calming the epidemic disease. Also, there was a wasteful repetition of studies that covered the problems from the outset, ones that had already been resolved in Japan. Many of the achievements obtained in Japan were published in Japanese and were not available in other countries. As a Japanese researcher of pine wilt, I felt it my duty to introduce how this disease had been studied and handled in Japan. Although now slightly outdated, the English version will introduce readers around the world to all the studies conducted in Japan on the deadly pine wilt disease.

Since pine wilt disease is a phenomenon that occurs in a situation of very complex biological relationships, it is essential to understand each of the organisms involved and their mutual relationships. The general reader need not be overwhelmed with such details, but the readers will surely realize that the relationships change with the season, and gain deeper understanding of the epidemic of pine wilt disease.

Pine wilt research has developed significantly since the first edition of this book was published in Japan. In particular, the progress in molecular biological techniques has helped to elucidate the genes that determine the pathogenicity of the pinewood nematode as well as those that influence host resistance. However, these advancements are not essential information for understanding the overall picture of this epidemic. The new developments in this field, are described in my review article (Futai 2013). Two textbooks published in Europe (Mota et al. 2008) and East Asia (Zhao et al. 2008) provide more detailed information on this forest epidemic. Considering pine wilt from the viewpoint of microbial ecology, I am again surprised that this forest epidemic depends on the various relationships of many organisms, and that the trend and course of the outbreak is determined by the delicate balance involved in that relationship. From the viewpoint of the association between organisms, attention should be paid to another point. When common tree species, such as red pine and black pine, become extinct in a wide area due to forest epidemics, many other accompanying species unique to these tree species will also be endangered. A well-known example in Japan is the precious matsutake mushroom (*Tricholoma matsutake* = syn. *Armillaria ponderosa*), the fruit body production of which has dramatically decreased with the decline of the red pine forests (*Pinus densiflora*).

Species diversity (the number of species and the number of individual members of each species) has been pointed out as an essential barometer of environmental health. From such an ecological point of view, the massive destruction of forests due to the sudden disaster caused by forest epidemics is a serious situation. Thus, pine wilt disease is a tremendous challenge cast back from nature to humankind. This problem cannot be solved simply by replacing pine trees with a different tree species but requires more sophisticated human intelligence and diligence.

I complete this book with the hope that researchers from each affected country will tackle the important and challenging issues to protect our pine forests from the pine wilt disease, and eventually overcome this forest epidemic.

Upon finishing this book, I would like to thank the many senior researchers for their support during my research. I also thank all the students who shared their interests and worked together with me in this essential field together. I am proud that many of them have become active and excellent scientists in their respective fields.

I express my heartfelt thanks to Ms. Tamara Stone and Dr. Sumiko Kaihara for their support and linguistic advice. My special thanks are due to my friend Dr. Zaki A. Siddiqui, professor of Aligarh Muslim University, for critical reading of the manuscript and valuable comments.

Last but not least, I dedicate this book to my deceased parents, who always showed their care and concern for me, and to my wife, Hiroko, who continually supports and encourages me so devotedly.

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