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Review Paper

Advances in research of pathogenic mechanism of pine wilt disease

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Pine wilt disease, caused by the pinewood nematode, *Bursaphelenchus xylophilus*, is the most serious disease of pine tree with great economic losses. So far it is not clear why the pine trees turn wilting, though several hypotheses about the pathogenic mechanism of pine wilt disease have been presented, such as phytotoxins causing death of pine trees; cellulases hydrolyzing celluloses of pine tree; terpenoids causing cavitation and water column breakage of pine tree, etc. Recently, it was found that certain bacteria, symbiotically associated with the pinewood nematode, may play some roles in the pathogenicity of the disease. Since the pine wilt disease is a complex interrelationships among beetle, pine tree, fungi, bacterium and nematode, all the pathogenic factors are not mutually exclusive, which means a variety of factors make pine tree for death, rather than a single factor. Pinewood nematode and bacteria produce phytotoxins and cellulases, which cause the defense of pine tree and stimulate the production of terpenoids to form cavitation, break water columns and finally make pine trees wilting. Pinewood nematode is involved in the production of phytotoxins, cellulases and terpenoids; therefore it is a vital and indispensable factor for pine wilting disease.

Key words: Pinewood nematode, *Bursaphelenchus xylophilus*, pathogenic mechanism, terpenoid, cavitation, cellulase, phytotoxin, bacteria.

INTRODUCTION

Pine wilt is a disease of pine (*pinus* spp.) caused by the pinewood nematode (PWN), *Bursaphelenchus xylophilus*. PWN is native to North America and is not considered as a primary pathogen of native pines, but is the cause agent of pine wilt for some non-native pines. Although the first occurrence of pine wilt disease (PWD) was reported in 1905 in Nagasaki City, Japan (Yano, 1935), PWN was not identified as the causal agent of the disease until 1971 (Mamiya and Kiyohara, 1972). So far PWD has been reported from North America (Canada, the United States and Mexico), East Asia (Japan, Korea and China) and Europe (Portugal) (Yano, 1913; Cheng, 1983; Tzean and Jan, 1985; Guiran and Bruguir, 1989; Yi et al., 1989; Dwinell, 1993; Mota et al., 1999). It has become a world-wide threat to pine forests and forest ecosystem with great economic losses. In 2003, this disease had killed about 1,000,000 m^3 of pine trees in Japan (Forestry agency, 2004).

However, until now the pathogenic mechanism of PWD has not been clearly illustrated. For a long time, it was thought that the PWN was the only pathogenic agent causing the disease (Mamiya, 1975; Nickle et al., 1981; Nobuchi et al., 1984; Fukuda et al., 1992), to be exactly, phytotoxins, cellulases, which are produced by PWN, ethylene and terpenoids produced by pine trees which are stimulated by invasion of PWN. Recently, it was found that some bacteria are symbiotically associated with the PWNs and may play some roles in the pathogenicity of the disease (Oku et al., 1979; Kawazu, 1998; Han et al., 2003; Zhao et al., 2003; Zhao et al., 2005). In order to be sure of the real cause of pine wilt disease, some hypo-

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theses were introduced and discussed in this paper.

PHYTOTOXIN HYPOTHESIS

During the 70s and 80s of the 20th century, some scientists considered that phytotoxins which was isolated from infected pine trees or from *B. xylophilus* can directly cause wilt symptoms. Oku et al. (1979) found that the filtrate of pine leaf juice in which the pathogenic nematodes were infected could cause wilting of the seedlings, boiled extract of pine wood also contained toxin product. Subsequently, some chemical compound such as benzoic acid, catechol, dihydroconifery alcohol, 8-hydroxycarvotanacetone (carbone hydrate) and 10-dydroxyverbenone were isolated from infected PWN pine trees and could cause wilt symptoms of susceptible pine trees (Oku, 1979). Among them, 8-hydroxycarvotanacetone, dihydroconifery alcohol and 10-dydroxyverbenone could inhibit the reproduction of *B. xylophilus*. Shaheen (1984) also got a similar conclusion that the phytotoxins, which were lipid materials with low molecular weight and isolated from *B. xylophilus* infected scots pine, caused wilting of 45-day-old and 2-year-old pine seedlings in a certain dose.

However, Kozlowski (1962, 1968) held a contrary opinion that PWD kills tree by interfering with water translocation rather than immediate and direct toxic effects. Cao and Shen (1996) studied the toxicity of extraction of PWN, which was cultured on an artificial medium and found that nematode extraction was not toxic to 30-day-old seedlings of *Pinus thunbergii* and *Pinus massoniana*. They concluded that wilt toxins were not produced by PWN under artificial culture conditions. In addition, some scientists hold the alternate opinion that bacteria associated with the PWN produce toxin (Kawazu, 1998; Han et al., 2003; Zhao et al., 2003).

CELLULASE HYPOTHESIS

Cellulases were detected by the analysis of the homogenates and extracts of the nematode species in more than ten genera, including the genus *Bursaphelenchus* (Tracey, 1958; Krusberg, 1960; Dropkin et al., 1962; Morgan and Mcallan, 1962; Dropkin, 1963; Odanil et al., 1985). Cellulases were exuded outside of PWN and left in PWN migrating track which were detected by Yamanoto (1986). After that he collected 9.8×10^6 *B. xylophilus* in 10 ml of distilled water. And then 0.5 ml of the supernatant of this suspension was filtered and injected to pine seedlings. No complete necrosis of the needles was observed in the seedlings, but sporadic distribution of the necrotic needles was observed. Close observations of pine tissues infected with *B. xylophilus* indicated that the destruction of pine cells might be a result of cell wall degrading enzymes such as cellulase (Ishida et al., 1997; Ichihara et al., 2000).

Feeding of 0.5 ml 1% solution of cellulase Onozuka R-10 (Kinki Yakult) to 3-year-old *P. densiflora* seedlings resulted in a complete necrosis of living shoots less than 72 h. Other symptoms such as oleoresin leaks and partial blocking of xylem water conduction were also reproduced by feeding seedlings the crude or high molecular weight fractions of the extracts of the PWN. Therefore Odani et al. (1985) thought that cellulase produced by the PWN is responsible for the development of the early symptom and is a strong candidate of the pathogen. The glycosyl hydrolase family have been isolated from the *B. xylophilus* and characterized (Kikuchi, 2004). In 45 kinds of cellulases, Bx-ENG-1, 2 and 3 could be secreted through the nematode stylet into plant tissues and participate in the weakening of the cell walls, allowing nematodes to feed and migrate more easily in pine tissues. From total homogenates of *B. xylophilus*, Zhang (2006) found a high molecular weight cellulase antigen, which was able to hydrolyze carboxymethyl cellulose efficiently (155.65 U/mg) and had an approximate molecular mass of 58.9 kDa. The style of secreting cellulase from the stylet and using hydrolyze cellulase to facilitate the PWN entering host cells is consistent with other plant parasitical nematodes. Moreover, strong fluorescence signals from cellulase staining were observed in tracheid cells which were naturally infected by PWN, in addition to ray cells and the resin canal zone (Zhang, 2006).

These results support that the nematode–originated cellulase is one of the strong candidate of the pathogenic substances responsible for the development of the pine wilt disease. It is also reported that endoglucanases used by the nematode to degrade the cell walls of fungi on which it feeds as cellulose, as well as chitin and other polysaccharides, have been shown to be present in the walls of some of the fungi on which *B. xylophilus* feed (Cherif et al., 1993).

TERPENOID HYPOTHESIS

Kuroda et al. (1988) proposed that the ultimate death of pine tree is due to water deficit induced by extensive cavitation of sapwood. The pathway of water movement in a tree is via a bundle of capillary water columns. Once any breaks in the water column disrupt water flow, water conduction of xylem tracheids could be impeded due to embolism. Embolized tracheids are filled with air, resulting from cavitation produced by the breakage of water columns in xylem conduits (Ikeda, 1992). By using the acoustic emission technique, Ikeda approved that the occurrence of cavitation events in Japanese black pine growing under field conditions is comparatively rare, even in summer. Based on the results, it seemed that xylem cavitation is caused by pathogenic factor, not by cultivation condition. Cavitation of tracheids is a remarkable initial symptom of PWD caused by the PWN (Kiyohara,

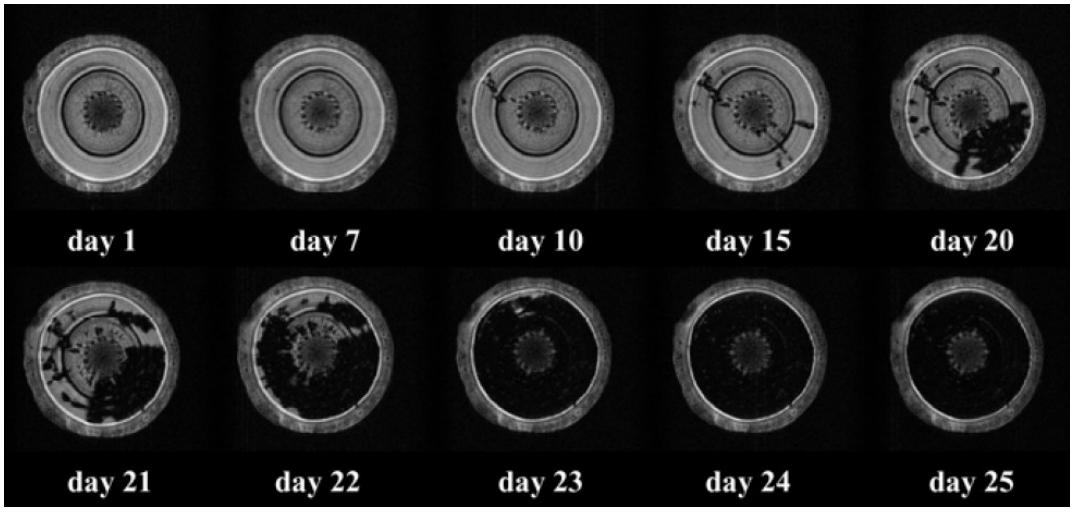

Figure 1. Spin–echo T1–weighted magnetic resonance images (transverse–slice) of inoculated seedling from 1 to 25 days after inoculation. Cavitated xylem appears as dark patches. Repetition time (TR) =500 ms, echo time (TE) = 22 ms, 256 × 256 pixels. Stem diameter 9 mm. Reproduced from Utsuzawa SK (2005).

1990). By observation Kuroda (1992) found that xylem water–blockage caused by cavitation started one week after nematode inoculation.

Kuroda (1989) found that parenchyma cells, which were injured by moving and feeding of nematode, synthesized terpenoids. Seven monoterpenes from infected pine trees: α–pinene, , camphene, β–pinene, myrcene, limonene, β–phellandrene, and p–cymene, were detected. Volatile terpenoids evaporated in tracheids under negative pressure and made bubble. Refilling of cavitated tracheids with water was prevented by hydrophobic effects of terpenoids, therefore, permanent cavitation enlarged gradually. Consequently, cavitated areas reached to cambium, water translation was broke, finally pine trees died due to the water deficit.

Ethylene acts as a signal transduction material to cause terpenoid produce in pine trees, experiments associated with ethylen have been done. Ethylene product was inoculated into seedlings which increased several times greater in the disease development which began a few days earlier than the water potential decrease and chlorosis in needles. Ethylene increase occurred synchronously with cambial death, and they were followed by water deficiency in leaves. After being injected with 0.1–1% ethrel (2–chlorophenylphosphonic acid) solution to seedlings, embolism in tracheids widely occurred and needles turned yellow quickly. A large embolized area was produced in xylem both above and below the injection site. Some days later it developed to almost the whole transverse area of the xylem. Furthermore, denaturation of xylem and cortex parenchyma cells were produced. Ethylene produced in xylem seems to trigger the cytological changes in xylem parenchymatous cell, embolism in tracheids and chlorosis of old needles (Fukuda, 1997).

Utsuzawa (2005) has observed the xylem cavitation caused by PWD by using the magnetic resonance for living trees, rather than by staining part of tree xylem which have to be sectioned. Through the nondestructive observation, he found that cavitaiton was limited to the inner xylem for about 10 days after infection, and the number of cavitated patches and the area of cavitation slowly increased. After 15 days, the cavitation area enlarged rapidly and reached the cambium, and at 21 days the relative area of cavitaion reached near 100%. Water conduction was completely dysfunction and the tree became wilting and dead (Figure 1). According to the symptoms, the development of pine wilt disease was divided into two stages: early and advanced stage (Fukuda, 1997). In the early stage, nematodes migrate through cortical and xylem resin canals in pine stems. They induce cavitation (breaking off the water column in tracheids), embolism (filling of the tracheids with gas) and occlusion of the tracheids with resin; in transverse sections of the stem, the affected tissues appear as dry patches. In the advanced stage, the nematodes multiply and destroy the cambium, which induces dysfunction of water conduction in the entire xylem and causes water potential, transpiration, and photosynthesis to rapidly decrease. Consequently, the needles wilt and the tree dies suddenly. The first stage is within 7 to 14 days and the advanced stage is during 15 to 30 days (Figure 2).

PWN AND BACTERIA HYPOTHESES

The bacteria carried by the PWN play an important role in pathogenicity of PWD. Oku et al. (1980) and Higgins et al. (1999) reported that bacteria were associated with

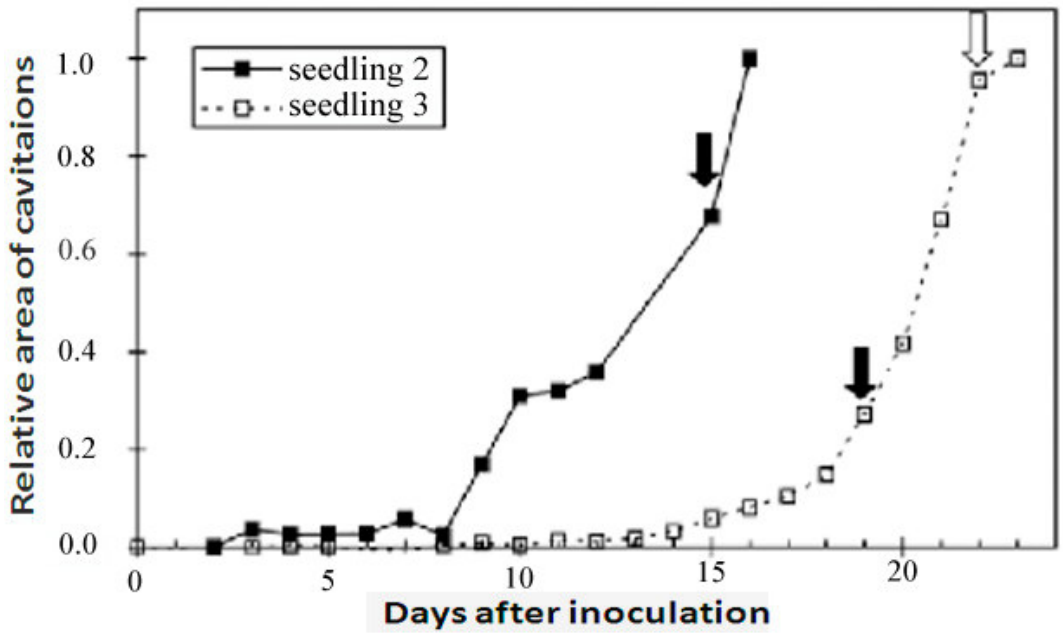

Figure 2. Cavitation development in seedlings 2 and 3 as revealed by magnetic resonance images (transverse–slice). The *y* axis refers to the proportion of the xylem that was cavitated. Image data are missing from seedling 2 on days 0, 1, 13, and 14. Solid and open arrows indicate the days when yellowing of old needles and wilting of current needles, respectively, were first observed. Reproduced from Utsuzawa SK (2005).

PWN. Bacteria adhered onto the body wall of PWN was observed by using electron microscopy and the average number of bacteria carried by one nematode isolated from infected pine tree was 2.9×10^2 (Zhao et al., 2000; Guo et al., 2002).

Some experiments indicated that aseptic PWN does not cause PWD of aseptic pine trees, while PWN associated with infecting bacterium causes wilting symptom. Oku et al. (1980) inoculated 3-year-old seedlings with a suspension of bacterium of the genus *Pseudomonas* isolated from pathogenic PWN. Three out of five of the treated seedlings subsequently wilted. Kawazu and Kaneko (1997) and Chi et al. (2006) reported aseptic *Pseudomonas densiflora* seedlings and 10-year-old *Pseudomonas thunbegii* trees did not wilt after being inoculated with aseptic PWN. Tan et al. (2004) reported that 1– or 2–year–old branches of *Pseudomonas massoniana* were inoculated with aseptic *B. xylophilus* and bacterium *Bacillus firmus* turned diseased. Therefore PWN associated with bacteria plays a significant role in the rapid wilting of pine trees.

Inoculating callus and aseptic black pine seedlings with aseptic PWN and the bacteria which isolated from PWN in the genus *Pseudomonas* showed severe symptoms, but only inoculating with aseptic PWN did not lead to browning (Han et al., 2003). In addition, the filtered liquid which bacteria were cultured in was directly applied to the callus of Japanese black pine induce browning. Han et al. (2003) and Zhao et al. (2003) concluded that wilting was due to toxins in the bacterial culture filtrate. Jiang et al. (2005) and Guo et al. (2007) isolated two chemical compounds, which showed obvious toxicity to both suspension cells and seedlings of *P. thunbergii*, from the culture of a strain of *Pseudomonas fluorescens* (*P. fluorescens GcM5–1A*) carried by PWN. The bacteria carried by PWN from isolated regions may be different. Such differences could explain why *Cedrus deodara* is sensitive to PWD in USA and Japan (Dropkin, 1981), while it is resistant in China (Zhao, 2003). It suggested that the disease was caused by co-infection of both PWN and bacteria and possible toxic effect of bacteria (Han, 2003).

Guo et al. (2006) reported that both the homogenates from live PWN and dead nematodes promoted the reproduction and pathogenicity of the bacterium by providing essential metabolites or nutrients, and that the promotion effect of living nematodal homogenates was stronger than that of dead ones. Furthermore, Zhao (2005) found that there is a mutualistic symbiotic relationship between PWN and 10 bacterial species in the genus *Pseudomonas*. The bacterial mutualistic symbionts are organized whole, which may have co-evolved with PWN rather than being accidentally associated. The finding provides that PWD is a complex process, induced by both PWN and associated phytotoxin-producing bacteria.

However, migration speed of PWN and bacteria is different, so how do the bacteria produce enough toxins to make pine tree wilting before its mass rearing need to

be researched.

DISCUSSION

Phytotoxins and cellulases produced by PWN, such as benzoic acid, catechol, dihydroconifery alcohol, 8-hydroxycarvotanacetone (carbone hydrate), 10–dydroxyverbenone and glycosyl hydrolase family, could cause dysfunction of pine trees in some degree and wilt symptoms of susceptible pine trees, even death. It needs a large number of PWN to produce enough quantity of phytotoxins and cellulases to cause wilt symptoms of pine trees. Consequently, the bigger pine trees should suffer more PWN and longer time to cause disease than smaller ones and the bigger pine trees survive longer time than smaller ones. However, infact the bigger pine trees show wilt symptoms earlier than small pine trees. Phytotoxin hypothesis and cellulase hypothesis could not explain the phenomenon adequately.

Terpenoid hypothesis explained how PWN causes dysfunction of water translocation of pine trees. Migrating of Nematodes through cortical and xylem resin canals in pine stems induces cavitation, embolism, and occlusion of the tracheids with resin. In the advanced stage, nematodes multiply and destroy the cambium, inducing dysfunction of water conduction and causing water potential, transpiration, and photosynthesis to rapidly decrease. Consequently, the needles wilt and the tree dies suddenly. Utsuzawa (2005) has observed the process of xylem cavitation forming caused by PWD by using the magnetic resonance for living trees, which was strong evidence to the terpenoid hypothesis.

PWN and bacteria hypothesis is an important complimentarily to pathogenic mechanism of PWD. The bacteria carried by PWN play a vital action to PWD; however, some scientists take a controversial standpoint about it.

CONCLUSION

In summary each hypothesis approves pathogenic factors exist and take effect to PWD. However, pathogenic factors are not mutually exclusive, which means a variety of factors make pine tree for death, rather than a single factor. PWN associated with bacteria diffuses from cortex resin canals to xylem resin canal, producing phytotoxins and cellulases, induce cytological changes in xylem ray and axial parenchyma cells as a defense reaction which produces ethylene. Subsequently, ethylene acts as a signal transduction material to cause the mass production of terpenoid in pine trees. Ethylene and terpenoid result in cavitation and embolism of tracheids, subsequent decrease in leaf water potential and photosynthesis. During the wilting process of pine tree, PWN is involved in the production of phytotoxins, cellulases, terpenoids; it therefore is a vital and indispensable factor for PWD.

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