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Variation in Susceptibility of Pine Species Seedlings with the Pine Wood Nematode, *Bursaphelenchus xylophilus*, in Greenhouse

Kwan-Soo Woo[1,*], Yeong-Sik Kim[1], Yeong-Bon Koo[1], Jin-Kie Yeo[1] and Yil-Seong Moon[2]

[1]*Department of Forest Genetic Resources, Korea Forest Research Institute, Suwon 441-350, Korea*

[2]*Southern Forest Research Center, Korea Forest Research Institute, Jinju 660-300, Korea*

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We conducted an inoculation test using nine open-pollinated families of pine trees to evaluate their susceptibility and mortality in different densities of pine wood nematode. Three-year-old nine open-pollinated pine families were inoculated with *Bursaphelenchus xylophilus* at levels of 3,000, 5,000, and 7,000 nematodes/seedling in greenhouse. There were no distinct patterns in latent period among three densities of *B. xylophilus* in all families. Most families showed the first disease symptoms of needle discoloration within 12-15 days after inoculation. However, open-pollinated progenies of *Pinus densiflora* showed the longest latent period because none of one-year-old needles were wilted until 14 days after inoculation with 5,000 and 7,000 nematodes. One-year-old needles were wilted earlier than current needles in all tested families with all densities of *B. xylophilus*. Current needles were not wilted until 14 days after inoculation in all seedlings. The mortality of all seedlings rapidly increased from 35 days to 49 days after inoculation, and all died within 80 days except two seedlings. A 3,000 nematodes/100 μL with sterilized distilled water are enough to screen 3-year-old pine seedlings for resistance to *B. xylophilus*.

Keywords **:** *Botrytis cinerea*, *Monochamus alternatus*, Pine wilt disease, *Pinus massoniana*, *Pinus taiwanensis*

Pine wilt disease caused by *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, the pinewood nematode (PWN), is a devastating disease and spreading in Korea since the first notice at Mt. Keumjeong in Pusan in 1988 (Yi et al., 1989; Enda, 1989; Choi and Moon, 1989). It has damaged about 7,811 ha of *Pinus densiflora* Sieb. et Zucc. and *P. thunbergii* Parl. until 2005 (Shin and Han, 2006). In 2006, the disease was found on a new host, *Pinus koraiensis* Sieb. et Zucc., artificially planted in Kwangju, Kyeonggi province, Korea. It has been known that the *B. xylophilus* in *P. densiflora* and *P. thunbergii* was spread by one native pine sawyer, *Monochamus alternatus*; however, *B. xylophilus* in *P. koraiensis* is supposed to be spread by the other native pine sawyer species, *Monochamus saltuarius* which is the most abundant in the middle to northern part of South Korea (Kwon et al., 2006). The epidemic of this devastating disease has not increased rapidly in Korea because the Korean government has made a lot of efforts on the management of the disease such as felling, burning, and fumigation of damaged trees. However, the damages increased gradually to the southern provinces, eastern coast, and middle of the peninsula (cities of Chuncheon, Kwangju, and Kyeonggi province) since the first report in 1988, implying that there is limit to control this disease.

Pines are important tree species in Korea and only a few pine species were tested for the susceptibility of the pinewood nematode in Korea (KFRI, 2002). There are a few reports on the pathogenicity of *B. xylophilus* to *P. massoniana*, *P. taiwanensis*, and *P. thunbergii* × *P. massoniana* (Futai and Furuno, 1979; Mamiya, 1983; Yang and Wang, 1989). *P. taiwanensis* was moderately susceptible to the pinewood nematode but *P. massoniana* was resistant (Yang and Wang, 1989). The interspecific hybrid of *P. thunbergii* × *P. massoniana* was resistant to the pinewood nematode (Futai and Furuno, 1979; Mamiya, 1983). However, no inoculation tests have been conducted to determine the susceptibility of open-pollinated progenies of *P. thunbergii* × *P. taiwanensis* and *P. thunbergii* × *P. massoniana* with a Korean isolate of the pinewood nematode.

A most promising method for controlling the pine wilt disease is a breeding of resistant trees. In the stems of seedlings from resistant families of *P. densiflora*, nematode reproduction was restricted more than those of susceptible families (Kuroda, 2004). The progenies of *P. densiflora* and *P. thunbergii* that were screened using grafts inoculated with *B. xylophilus* proved to be resistant to pine wilt disease (Toda and Kurinobu, 2002). In 1997, 52 seedlings of two resistant families (unknown sources) developed in Japan have been imported and planted at the Anmyon Experiment Forest, Korea Forest Research Institute. It is worthwhile to test whether the progenies of the families show resistance to Korean isolate of *B. xylophilus*.

Susceptibility of pine trees to pine wilt disease varies

*Corresponding author.
Phone) +82-31-290-1106, FAX) +82-31-292-4458
E-mail) woo9431@foa.go.kr

depending on tree ages and the population densities of *B. xylophilus*. Wilt occurred much more rapidly and severely in young trees than in old trees and in mature trees inoculated with 16,000 *B. xylophilus* than those with 125~500 (Kishi, 1999). Adult trees of *P. armandii* var. *amamiana* inoculated with 100,000 *B. xylophilus* showed initial symptoms 2 weeks after inoculation and all died within 29 weeks, however, the trees inoculated with 1,000 *B. xylophilus* all died within 33 weeks after inoculation (Akiba and Nakamura, 2005).

This study reported here was undertaken 1) to compare latent period of nine open-pollinated families of pine trees to *B. xylophilus*, 2) to compare the time of wilting between current- and one-year-old needles, 3) to compare variation in susceptibility and mortality of the pine trees to pine wilt disease, and 4) to clarify the relationship between nematode densities and susceptibility. The results from this study will be used for breeding resistant pine trees against to pine wilt disease caused by *B. xylophilus*.

Materials and Methods

Open-pollinated seeds of four individuals of *P. thunbergii*, two individuals of *P. thunbergii* × *P. taiwanensis*, two individuals of *P. thunbergii* × *P. massoniana*, and one individual of *P. densiflora* were sowed in a nursery bed of the Department of Forest Genetic Resources, Korea Forest Research Institute (KFRI) in spring 2003. In 2004, the seedlings were transplanted in plastic pots and moved in a greenhouse. Four mother trees of *P. thunbergii* were imported as seedlings from Japan and planted at the Anmyon Experimental Forest, Forest Seed Research Center, KFRI in Chungbuk province, Korea in 1997. The seedlings were supposed to be produced by the pine wilt disease resistance program in Japan. Four hybrid mother trees were planted at the Boseong Experimental Forest, Cheonnam province, Korea in 1985. One individual of the *P. densiflora* was planted at the Seonkamli Experimental Forest, Kyeonggi province, Korea.

A virulent isolate of *B. xylophilus*, originally from a dead *P. thunbergii* in Jinju city, was reared on a fungal mat of *Botrytis cinerea* Pers. growing on potato dextrose agar at 25°C for about 10 days. Three-year-old nine half-sib families were inoculated in a greenhouse with the nematode on July 6th, 2005. Twelve seedlings per family were inoculated with each of three densities adjusted to 3,000, 5,000, or 7,000 nematodes/100 μL with sterilized distilled water of *B. xylophilus* and six with sterilized water as control. The nematode suspension was pipetted on wound, which was made on the main stem by removing an area of bark 2 cm long and 1 cm wide. The inoculated wounds were covered with Parafilm to prevent drying of the inoculum.

Seedlings were examined weekly and observed for foliage discoloring, latent period, and mortality for 80 days after inoculation. *B. xylophilus* was re-isolated from the inoculated seedlings of four families, PT1-9, PT3-TW, PT12-MS, and PDN, using the Baermann funnel method (Southey, 1986) at 25°C for 24 hours.

Results and Discussion

Latent period of nine open-pollinated families was not

Table 1. Latent period of open-pollinated progenies of 9 individuals under three densities of *B. xylophilus*

Family[a]	Number of *B. xylophilus* inoculated		
	3,000	5,000	7,000
PT1-9	13~16 days[b](12/12)[c]	14~20 days (12/12)	13~20 days (10/12)
PT1-23	12~20 days (10/12)	14~20 days (12/12)	13~16 days (11/12)
PT2-3	13~20 days (11/12)	14~21 days (12/12)	14~20 days (10/12)
PT2-19	14~21 days (11/12)	14~21 days (9/12)	14~20 days (11/12)
PT3-TW	13~20 days (12/12)	15~20 days (12/12)	13~20 days (11/12)
PT5-TW	12~23 days (10/12)	14~21 days (10/12)	14~19 days (12/12)
PT9-MS	13~22 days (12/12)	12~21 days (7/12)	13~21 days (11/12)
PT12-MS	12~21 days (12/12)	12~17 days (12/12)	12~17 days (12/12)
PDN	14~23 days (12/12)	15~23 days (10/12)	17~21 days (9/12)

[a] PT1-9, PT1-23, PT2-3, and PT2-19: half-sib families from four mother trees of *P. thunbergii* which were imported as seedlings from Japan and planted at the Anmyon Experimental Forest, Forest Seed Research Center, KFRI in Chungbuk province, Korea in 1997; PT3-TW and PT5-TW: half-sib families from two hybrid mother trees, *P. thunbergii*3 × *P. taiwanensis* and *P. thunbergii*5 × *P. taiwanensis*; PT9-MS and PT12-MS: half-sib families from two hybrid mother trees, *P. thunbergii*9 × *P. massoniana* and *P. thunbergii*12 × *P. massoniana*. The mother trees of PT3-TW, PT5-TW, PT9-MS and PT12-MS were planted at the Boseong Experimental Forest, Cheonnam province, Korea in 1985; PDN: half-sib family from one mother tree of *P. densiflora* which was planted at the Seonkamli Experimental Forest, Kyeonggi province, Korea.

[b] The days from inoculation to initial appearance of needle discoloration.

[c] The number of seedlings showing needle symptoms/Total number of seedlings inoculated.

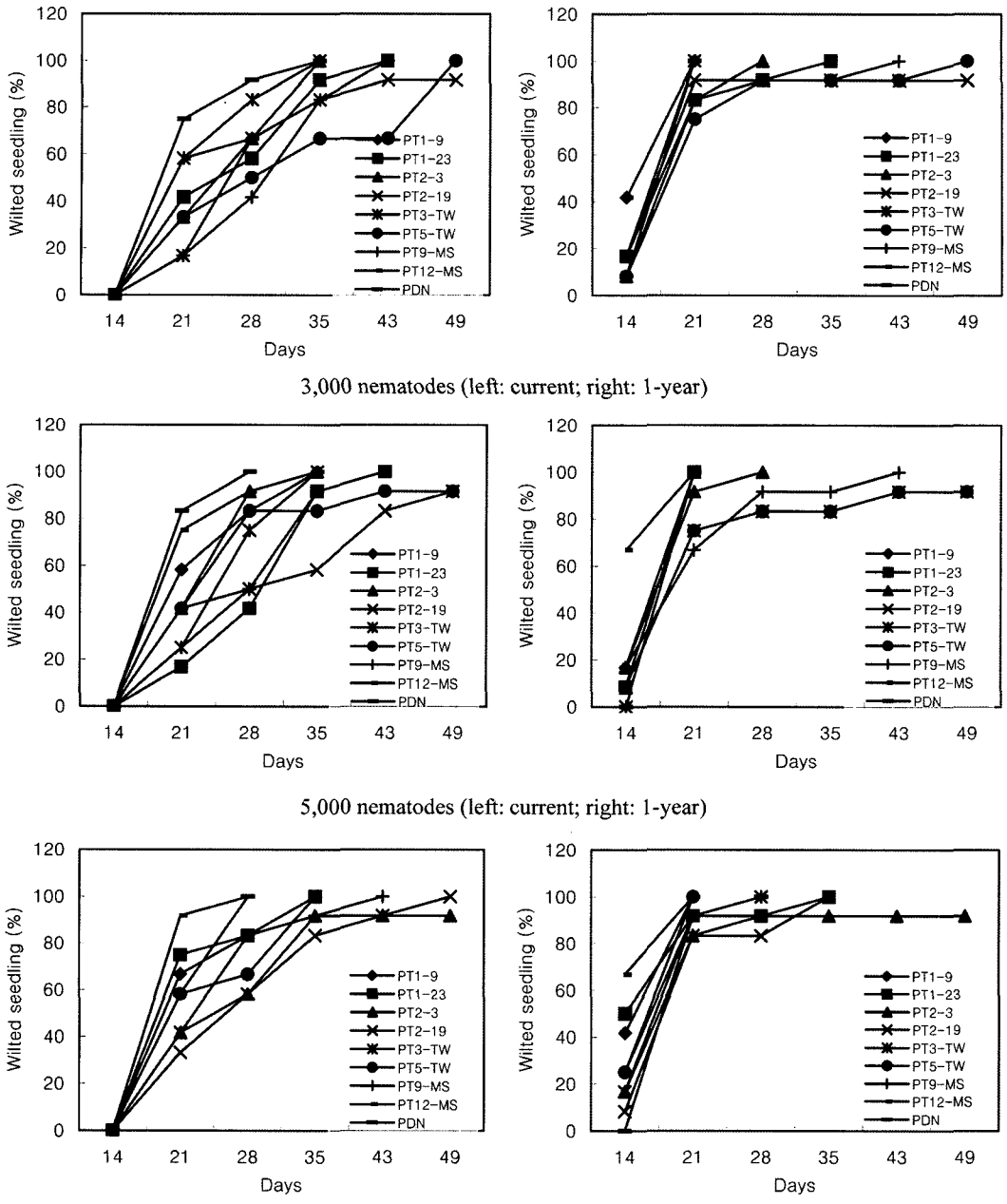


Fig. 1. Percentage of seedlings showing wilt in current- and one-year-old needles at three different densities of pine wood nematode.

different distinctively among three densities of *B. xylophilus* (Table 1). On species level, none of one-year-old needles on family PDN were wilted under 5,000 and 7,000 nematode until 14 days after inoculations (Fig. 1), indicating that *P. densiflora* has the longest latent period. The most number of seedlings (16.7%) of family PT9-MS showed no initial appearance of needle discoloration until 22 days after inoculation under three densities of nematode (Table 1). The difference of latent period appeared to be caused by genetic difference.

The first appearance of needle discoloration varied among families, but most of the families showed the symptom within 12~15 days after inoculation in all three densities of *B. xylophilus* (Table 1). An inoculation test of pine wood nematode by Togashi et al. (1997) showed that 3-year-old potted *P. thunbergii* seedlings varied in the time required from the initiation to completion of foliage discoloration. It ranged 0 to 12 weeks depending on their proximity to other tree species; however, the mean time required for complete foliage discoloration was not

statistically significant among treatments (Togashi et al., 1997). Five-year-old *P. thunbergii* seedlings inoculated with 10,000 nematodes showed that the color of one-year-old needles changed to brown by 5 weeks and all needles turned brown from about 9 weeks (Ikeda and Kiyohara, 1995). In this study, the completion of all needle discoloration was observed by 10 weeks except two seedlings survived through the experimental period.

One-year-old needles were wilted earlier than current needles in all tested families with all densities of *B. xylophilus* (Fig. 1). Current needles were not wilted until 2 weeks after inoculation in all seedlings and treatments. Malek and Appleby (1984) reported that 15- to 80-year-old Scotch pines (*P. sylvestris* L.) died in the spring by pine wood nematode usually showed the color change in the second- and third-year needles first.

Family PT12-MS showed the greatest percentage of one-year old needle wilt as 41.7%, 66.7%, and 66.7%, respectively when 3,000, 5,000, and 7,000 of *B. xylophilus* were inoculated (Fig. 1). All 36 seedlings appeared to be infected within 21 days. Four of nine families inoculated with 3,000 nematodes showed that one-year-old needles of all seedlings were wilted within 3 weeks after inoculation, however, five of nine families showed that current needles of all seedlings were wilted within 5 weeks after inoculation (Fig. 1). Five of nine families inoculated with 5,000 nematodes showed that one-year-old needles of all seedlings were wilted within 3 weeks after inoculation, but four of five families showed that current needles of all seedlings were wilted within 5 weeks after inoculation (Fig. 1). The 8.3% of seedlings of each of the three families, PT2-19 inoculated with 3,000 and 5,000 nematodes, PT5-TW inoculated with 5,000 nematodes, and PT2-3 inoculated with 7,000 nematodes, had both current- and one-year-old needles without any symptoms, until 49 days after inoculation (Fig. 1).

The susceptibility of pine species to infection by *B. xylophilus* varies depending on differences in inoculum densities, tree age and genotype, experimental length and environmental factors. For example, Yang and Wang (1989) tested the susceptibility of 2- to 5-year-old seedlings of *P. massoniana* and *P. taiwanensis* to pine wilt disease after inoculating with 7,000~8,000 nematodes each by injecting 5 ml of water into each seedlings, and found that *P. massoniana* was resistant and *P. taiwanensis* was moderately susceptible. Kishi (1999) reported that wilt occurred more rapidly and at higher levels in young *P. densiflora* and *P. thunbergii* than in old tree and in those trees inoculated with many *B. xylophilus* than others (Akiba and Nakamura, 2005) reported that adult trees of all *P. thunbergii* inoculated with 1,000 nematodes had died within 6 weeks after inoculation. In this study, the first mortality was shown on

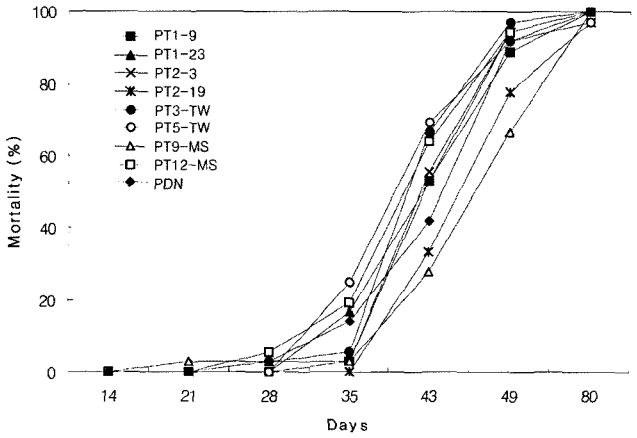
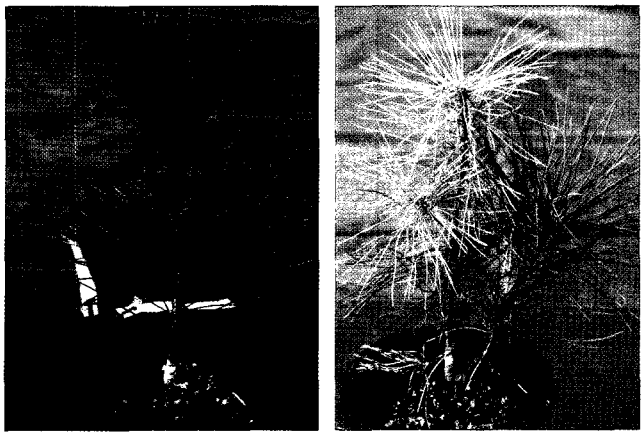


Fig. 2. Mortality of nine open-pollinated families within 80 days after artificial inoculation with *B. xylophilus*. PT1-9, PT1-23, PT2-3, and PT2-19: half-sib families from four mother trees of *P. thunbergii* which were imported as seedlings from Japan; PT3-TW and PT5-TW: half-sib families from two hybrid mother trees, *P. thunbergii*3 × *P. taiwanensis* and *P. thunbergii*5 × *P. taiwanensis*; PT9-MS and PT12-MS: half-sib families from two hybrid mother trees, *P. thunbergii*9 × *P. massoniana* and *P. thunbergii*12 × *P. massoniana*; PDN: half-sib family from one mother tree of *P. densiflora*.

21 days after inoculation with 3,000 nematodes for a seedling of family PT9-MS (Fig. 2). The same period of time was taken for mortality of seedlings of loblolly, pond, slash, and Virginia pines inoculated in a greenhouse at the rate of 4,000 nematodes in Southeastern United States (Dwinell, 1985). In our study, the mortality of seedlings of nine families rapidly increased from 35 days to 49 days after inoculation, and all died within 80 days except two seedlings (Fig. 3), one from family PT2-19 inoculated with 3,000 nematodes and the other from family PT5-TW inoculated with 5,000 nematodes. The two seedlings are supposed to be resistant genotypes. However, the latter showed that only inoculated branch was wilted until the end of the experiment, implying that the joint between branch and main stem might be effective as barrier to nematode dispersal as Kuroda reported (2004). No symptoms were observed in the control inoculations of all families.

Pine wilt disease has caused the most severe damage to forests of *P. thunbergii* in Japan (Mamiya, 1983). Ten-year-old five *P. thunbergii* inoculated with 1,000 nematodes in the field had all died 42 days after inoculation (Akiba and Nakamura, 2005). In this study, the open-pollinated progenies of four families, PT1-9, PT1-23, PT2-3, and PT2-19, continued to die from 35 days to 80 days after inoculation except one seedling of family PT2-19 (Fig. 2). Four families of the *P. thunbergii* were expected to show resistance to *B. xylophilus* because they were imported from Japan as resistance families. However, since the progenies



(A) (B)

Fig. 3. Two seedlings survived 80 days after artificial inoculation with *B. xylophilus*. (A) A seedling of family PT2-19 inoculated with 3,000 nematodes; (B) A seedling of family PT5-TW inoculated with 5,000 nematodes.

are half-sib family, we neither know genetic effects of pollen parent for resistance to *B. xylophilus* nor prove the virulence of *B. xylophilus* isolates from Japan and Korea. An 88.9% of the four families' seedlings were dead within 49 days after inoculation (Fig. 1) in which the time of mortality after inoculation was similar to the field test of Akiba and Nakamura (2005) even though the inoculum density, tree age, and environmental factors were different.

The seedlings of two families, PT3-TW and PT5-TW, start to die 28 days after inoculation and 93.1% of the seedlings died within 49 days. One seedling of family PT5-TW has survived until 80 days after inoculation. It has reported that the interspecific hybrid of *P. thunbergii* × *P. massoniana* is resistant to *B. xylophilus* (Futai and Furuno, 1979; Mamiya 1983). In this study, open-pollinated progenies (72 seedlings) of two families, PT9-MS and PT12-MS, were all dead within 80 days, implying that pollen parents may not contribute to resistance to pine wilt disease. The first mortality (1.4%) was shown 21 days after inoculation and 80.6% of the seedlings died within 49 days after inoculation. Family PDN tended to show delayed symptom development and mortality compared with other families. A seedling of family PDN died in 28 days after inoculation and 50% of all seedlings of the family died between 43 days and 49 days after inoculation.

We re-isolated *B. xylophilus* from nine seedlings of each of four families, PT1-9, PT3-TW, PT12-MS, and PDN, and the average number of nematodes per gram of dry weight recovered was ranged only from 9 to 182, appearing that they were poor host. In good hosts, much more *B. xylophilus* could be isolated. Dwinell (1985) reported that average number of pinewood nematodes per gram fresh weight recovered from stem base and inoculation site in 3-year-old seedlings of five pine species was ranged from 45 to 1,102 (average: 725). We could not find any correlation between mortality and the number of nematodes recovered and between mortality and the density of *B. xylophilus*. However, many inoculation tests demonstrated a positive relationship with rapidity and severity of the disease (Kishi, 1999, Akiba and Nakamura, 2005), implying that 3,000 to 7,000 nematodes do not make significant difference in the development of pine wilt disease. We confirmed that 3,000 nematodes/100 μl with sterilized distilled water are enough for screening 3-year-old pine seedlings for resistance to *B. xylophilus*.

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References

Akiba, M. and Nakamura, K. 2005. Susceptibility of adult trees of the endangered species *Pinus armandii* var. *amamiana* to pine wilt disease in the field. *J. For. Res.* 10:3-7.

Choi, Y. E. and Moon, Y. S. 1989. Survey on distribution of pine wood nematode (*Bursaphelenchus xylophilus*) and its pathogenicity to pine trees in Korea. *Korean J. Plant Pathol.* 5:277-286.

Dwinell, L. D. 1985. Relative susceptibilities of five pine species to three populations of the pinewood nematode. *Plant Dis.* 69:440-442.

Enda, N. 1989. The status of pine wilting disease caused by *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle and its control in Korea. *Jour. Korean For. Soc.* 78:248-253.

Futai, K. and Furuno, T. 1979. The variety of resistances among pine-species to pine wood nematode, *Bursaphelenchus lignicolus*. *Bull. Kyoto Univ. For.* 51:23-36.

Ikeda, T. and Kiyohara, T. 1995. Water relations, xylem embolism and histological features of *Pinus thunbergii* inoculated with virulent or avirulent pine wood nematode, *Bursaphelenchus xylophilus*. *J. Experi. Bot.* 46:441-449.

KFRI (Korea Forest Research Institute). 2002. Annual Report on Forest Disease and Insect Control. Korea Forest Research Institute, Seoul, pp. 518-576.

Kishi, Y. 1999. Influence of tree age on wilt and mortality of pines after inoculation with *Bursaphelenchus xylophilus*. *J. Jpn. For. Soc.* 81:330-333.

Kuroda, K. 2004. Inhibiting factors of symptom development in several Japanese red pine (*Pinus densiflora*) families selected as resistant to pine wilt. *J. For. Res.* 9:217-224.

Kwon, T. S., Lim, J. H., Sim, S. J., Kwon, Y. D., Son, S. K., Lee, K. Y., Kim, Y. T., Park, J. W., Shin, C. H., Ryu, S. B., Lee, C. K., Shin, S. C., Chung, Y. J. and Park, Y. S. 2006. Distribution patterns of *Monochamus alternatus* and *M. saltuarius* (Coleoptera: Cerambycidae) in Korea. *Jour. Korean For. Soc.*

95:543-550.

Malek, M. B. and Appleby, J. E. 1984. Epidemiology of pine wilt in Illinois. *Plant Dis.* 68:180-186.

Mamiya, Y. 1983. Pathology of the pine wilt disease caused by *Bursaphelenchus xylophilus*. *Ann. Rev. Phytopathol.* 21:201-220.

Shin, S. C. and Han, H. R. 2006. Current status on research and management of pine wilt disease in Korea. In: Proceedings of the international symposium on current status on research and management of pine wilt disease, Seoul, Korea, pp. 31-40.

Southey, J. F. 1986. Laboratory methods for work with plant and soil nematodes, 6th edn. HMSO, London.

Toda, T. and Kurinobu, S. 2002. Realized genetic gains observed in progeny tolerance of selected red pine (*Pinus densiflora*) and black pine (*P. thunbergii*) to pine wilt disease. *Silvae Genetica* 51:42-44.

Togashi, K., Aida, K., Nakamura, K., Horikoshi, T. and Takahashi, F. 1997. Different development of pine wilt disease in artificially infected *Pinus thunbergii* seedlings potted together with different tree species. *J. For. Res.* 2:39-43.

Yang, B. and Wang, Q. 1989. Distribution of the pinewood nematode in China and susceptibility of some Chinese and exotic pines to the nematode. *Can. J. For. Res.* 19:1527-1530.

Yi, C. K., Byun, B. H., Park, J. D., Yang, S. I. and Chang, K. H. 1989. First finding of the pine wood nematode, *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle and its insect vector in Korea. *Res. Rep. For. Res. Inst.* 38:141-149.