

# Inoculation of seven pine species or hybrid seedlings with Korean isolates of pinewood nematode under greenhouse conditions

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## Abstract

- Seedlings of seven pine species or hybrids, *Pinus densiflora*, *Pinus thunbergii*, *Pinus virginiana*, *Pinus echinata*, *Pinus koraiensis*, *Pinus thunbergii* × *Pinus massoniana* and *Pinus* × *rigitaeda*, were inoculated in a greenhouse with 100, 1 000 and 10 000 pathogenic nematodes, *Bursaphelenchus xylophilus*, to compare initial symptom development and to evaluate susceptibility to pinewood nematodes.
- Genetic types were significantly different in the starting day of needle discoloration ( $\chi^2 = 43.48$ ;  $P < 0.001$ ) and the period from nematode inoculation to seedling death ( $\chi^2 = 32.85$ ;  $P < 0.001$ ), but treatment groups were not statistically different in the days from inoculation to seedling death in any of the pine species.
- There was no relationship between seedling mortality and the number of nematodes recovered from infected seedlings. Only eight of the nematode-inoculated seedlings remained alive by day 83 after inoculation, including four seedlings each of *P. echinata* and *P. × rigitaeda* grown from bulked seed collections.
- Seedlings from the bulk lots were likely more genetically diverse than those from the single-source lots. A broader genetic representation of pine species or hybrids is suggested for screening for planting stocks resistant to *B. xylophilus*.

## Résumé – Inoculation sous serre de semis de sept espèces ou hybrides de pin par des isolats coréens du nématode du pin.

- Des semis de sept espèces ou hybrides de pin, *Pinus densiflora*, *Pinus thunbergii*, *Pinus virginiana*, *Pinus echinata*, *Pinus koraiensis*, *Pinus thunbergii* × *massoniana*, et *Pinus* × *rigitaeda* ont été inoculés sous serre par 100, 1000 ou 10 000 nématodes pathogènes *Bursaphelenchus xylophilus*, pour comparer le développement des symptômes à leur début et évaluer la sensibilité de ces essences.
- Des différences significatives ont été trouvées entre types génétiques pour la date de début de décoloration des aiguilles ( $\chi^2 = 43.48$ ;  $P < 0.0001$ ), ainsi que pour le délai entre l'inoculation et la mort des plants ( $\chi^2 = 32.85$ ;  $P < 0.0001$ ), mais pas entre traitements, quelle que soit l'espèce.
- Aucune relation n'a été observée entre la mortalité des semis et le nombre de nématodes récupérés des semis infestés. Seulement huit plants inoculés étaient encore vivants après 83 jours, dont quatre de *P. echinata* et de *P. × rigitaeda* issus des collections de graines en vrac.
- Les semis des lots de vrac étaient probablement génétiquement plus variés que ceux des lots de source unique. Une plus large représentation des espèces et des hybrides serait nécessaire pour un screening des souches de plantations résistantes à *B. xylophilus*.

## 1. INTRODUCTION

Pine wilt disease, caused by *Bursaphelenchus xylophilus* (Steiner & Buhner) Nickle, the pinewood nematode (PWN), was first noticed in Korea in 1988 (Choi and Moon, 1989;

Enda, 1989; Yi et al., 1989). Since then the devastating disease has spread in a northward direction from the southern part of the Korean Peninsula. Pines occupy 23.5% of the total forested land in Korea and by 2006 pine wilt disease had damaged more than 7 800 ha of *Pinus thunbergii* Parl. and *Pinus densiflora* Sieb. et Zucc. (Shin and Han, 2006).

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The disease was also found in December 2006 in planted forests of a five-needle pine, *Pinus koraiensis* Sieb. et Zucc., in the middle of the Peninsula. The *B. xylophilus* recovered from *P. koraiensis* was alleged to have been transmitted by an insect vector, *Monochamus saltuarius* Gebler, a long-horned beetle (Cerambycidae), which infects healthy trees during maturation feeding on young twigs and shoots. A different species, *Monochamus alternatus* Hope, is the insect vector for *Pinus densiflora* and *Pinus thunbergii* (Kwon et al., 2006). In Japan, the PWN is also transmitted by two beetles, *M. alternatus* (in most areas) (Mamiya and Enda, 1972; Morimoto and Iwasaki, 1972) and *M. saltuarius* in the cooler regions (Sato et al., 1987).

Between 1988 and 2003, several studies were conducted in Korea on a limited number of test seedlings to examine the susceptibility of pine trees to the PWN (KFRI, 2002; Woo et al., 2007), but none were designed to screen for genetic resistance to the nematode using artificial inoculation. For example, seedlings of *P. densiflora* and *P. thunbergii* grown from seeds of natural forests were highly susceptible to nematodes isolated from an infected tree. However, *Abies holophylla* Maxim. and *Picea abies* (L.) Karst. were found to be resistant to the disease (KFRI, 2002).

The PWN is considered epidemic in Japan where native forests of *P. densiflora*, *P. thunbergii* and *P. luchuensis* have been heavily damaged (Mamiya, 1983). However, the pathogen is generally endemic in North America, and most North American pine species appear to be resistant to the disease (Dropkin, 1985). Developing hybrid species resistant to PWN is one strategy to prevent damage from the disease, but the resistance may not persist over time or across a variety of environmental conditions. The interspecific hybrid of *P. thunbergii* × *P. massoniana* was resistant to the PWN in Japan (Futai and Furuno, 1979; Mamiya, 1983). However, we confirmed that three-year-old open-pollinated families of *P. thunbergii*, *P. thunbergii* × *P. taiwanensis*, *P. thunbergii* × *P. massoniana* and *P. densiflora* were all susceptible to an isolate from *P. thunbergii* (Woo et al., 2007).

The degree of susceptibility of mature pine stands to PWN may vary even when stands are located near each other (Togashi et al., 1992). The density of the inoculum and the age of hosts also appear to influence susceptibility (Kishi, 1999). The objectives of this research were to: (1) determine variation in susceptibility of pine seedlings to pine wilt disease and (2) determine how densities of the nematode are related to the susceptibility of the pines to *B. xylophilus*.

## 2. MATERIALS AND METHODS

### 2.1. Plant materials

Three- to six-year-old seedlings of seven pine species or hybrids (Tab. I) were grown in nursery beds of the Department of Forest Genetic Resources, Korea Forest Research Institute (KFRI), Kyeonggi Province. The seedlings were transplanted into plastic pots and moved into a greenhouse in spring 2006. The soil used was a mixture of clay, sand, TKS2 Instant (Floragard, Oldenburg, Germany),

perlite and vermiculite (1:1:2.5:0.5:1 [v/v]). TKS2 Instant contains 100 to 400 mg of N per liter, 150 to 550 mg of potassium oxide per liter, and 100 to 400 mg of phosphorpentoxide per liter.

### 2.2. Culture of pinewood nematode

*Bursaphelenchus xylophilus* was isolated from naturally diseased *P. densiflora* in Jinju city. The diseased wood was cut into chips of about 1 cm in length and nematodes were isolated using the Baermann funnel technique for 24~48 h at room temperature (ca. 23–26 °C) (Southey, 1986). The nematodes were multiplied on a fungal mat of *Botrytis cinerea* Pers. growing on potato dextrose agar and incubated at 25 °C for about 10 days. They were stored at 4 °C until used.

### 2.3. Artificial inoculations

Twenty seedlings per seed source (14 seedlings for *P. thunbergii* × *P. massoniana*) were used in this study. On June 1, 2006, five seedlings of each source were assigned to each of three treatments and one control group. Seedlings in the treatment groups were inoculated with one of three densities of nematodes adjusted to 100, 1 000 or 10 000/100 µL sterilized distilled water. Sterilized water was applied to the control seedlings. A wound was made on the main stem of each seedling by removing an area of bark 2 cm long and 1 cm wide, and a piece of cotton was placed in the wound. The nematode suspension or sterilized water was pipetted onto the cotton, and covered with parafilm to prevent contamination and water loss. Minimum and maximum temperatures within the greenhouse ranged from 15 °C to 27 °C and from 23 °C to 42 °C for 83 days, respectively. Subsequent to the inoculation, the seedlings were watered, but no nutrients were supplied. Individual seedlings were examined for needle discoloration daily for the first three weeks after inoculation and then at weekly intervals until the end of the experiment. Pine seedlings were judged to be dead when all of the needles turned brown. The stems and branches of dead seedlings were cut into chips 5 mm thick and immersed in Baermann funnels to confirm the presence of *B. xylophilus*. The total number of extracted nematodes was counted for each seedling. The experiment was terminated when seedlings did not show any further disease development.

### 2.4. Data analysis

The starting day of needle discoloration and the period from nematode inoculation to seedling death were analyzed by seed source with the Kruskal-Wallis One-Way ANOVA (Chi-Square approximation) using SYSTAT 9 statistical software (SYSTAT Software Inc. 2004). The Mann-Whitney U-test was performed to determine whether nematode densities were related to these variables. Seedlings that survived beyond the experimental period were excluded from the analysis of the period between inoculation and seedling death. Species differences were analyzed by analysis of variance (ANOVA) using the SAS-PROC GLM statistical package and type III sums of squares (SAS Institute Inc. 1989). When differences among species were significant at  $P < 0.05$ , least squares means procedures were applied.

**Table I.** Age, number of seedlings and seed sources of seven pine species or hybrids inoculated with three densities of *Bursaphelenchus xylophilus*.

Species	Age (years)	No. of seedlings	Seed collections / places	No. of <i>B. xylophilus</i>
<i>P. densiflora</i>	3	5	Open-pollinated (a single clone) [Anmyon Seed Orchard]	0
		5		100
		5		1000
		5		10000
<i>P. thunbergii</i>	3	5	Open-pollinated (a parent tree) [Anmyon Experimental Forest]	0
		5		100
		5		1000
		5		10000
<i>P. virginiana</i>	6	5	Bulk [Kyeongbuk Forest Environment Research Center]	0
		5		100
		5		1000
		5		10000
<i>P. thunbergii</i> × <i>P. massoniana</i>	3	0	Open-pollinated (a parent tree) [Boseong Experimental Forest]	0
		5		100
		5		1000
		4		10000
<i>P. echinata</i>	5	5	Bulk [Kyeongbuk Forest Environment Research Center]	0
		5		100
		5		1000
		5		10000
<i>P. × rigitaeda</i>	5	5	Bulk [Hannam Experimental Forest]	0
		5		100
		5		1000
		5		10000
<i>P. koraiensis</i>	5	5	Open-pollinated (a single clone) [Chuncheon Seed Orchard]	0
		5		100
		5		1000
		5		10000

### 3. RESULTS

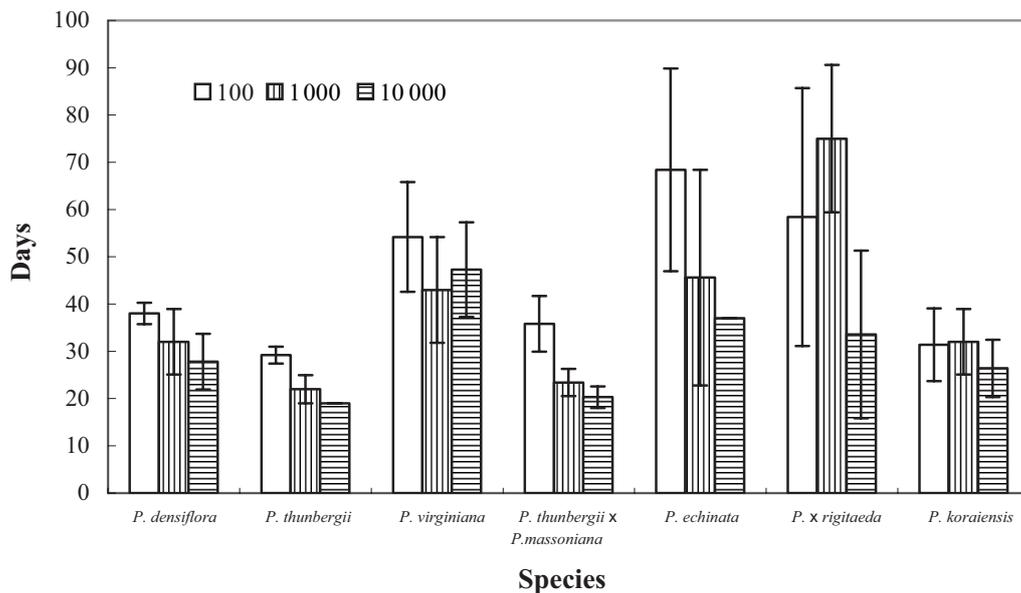
There was a statistically significant difference ( $\chi^2 = 43.48$ ;  $P < 0.001$ ) in the average starting day of needle discoloration among genetic sources and inoculation densities. We found statistical differences in the average starting day of needle discoloration between seedlings inoculated with 100 compared with 10 000 nematodes in four of the seven pine species or hybrids (Tab. II). Needles of *P. thunbergii* seedlings inoculated with 10 000 nematodes were the earliest to discolor ( $\bar{X} = 19$  days; Fig. 1); those of *P. echinata* inoculated with 100 nematodes were one of the most delayed ( $\bar{X} = 68$  days). None of the comparisons between inoculations of 1 000 compared with 10 000 nematodes was statistically significant. The period from inoculation to seedling death was significantly different ( $\chi^2 = 32.85$ ;  $P < 0.001$ ) among the seven pine species or hybrids, but individual comparisons using the Mann-Whitney U-test showed no statistically significant differences (Fig. 2).

All but four seedlings each of *P. echinata* and *P. × rigitaeda* of the nematode-inoculated seedlings were dead by 83 days after inoculation (Fig. 3). There was no relationship between age of seedlings and mortality. No water-inoculated control group seedlings had died by the end of the experiment (83 days).

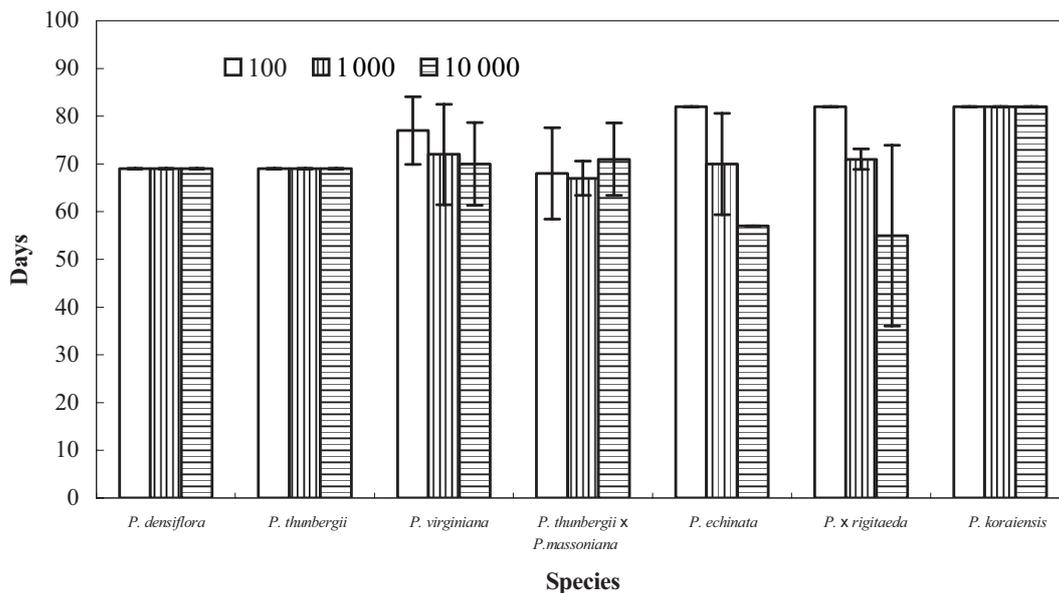
The average number of nematodes recovered from the three nematode treatments varied among the seven pine species or hybrids but not among treatments ( $P < 0.001$  and  $P = 0.45$ ,

**Table II.** The Mann-Whitney U-test for average starting day of needle discoloration between three densities of nematode in each of seven pine species or hybrids.

Species	Densities of <i>B. xylophilus</i>	U	P-value
<i>P. densiflora</i>	100 vs. 1000	6.0	0.111
	100 vs. 10000	2.0	0.016
	1000 vs. 10000	8.0	0.111
<i>P. thunbergii</i>	100 vs. 1000	0.5	0.01
	100 vs. 10000	0.0	0.004
	1000 vs. 10000	5.0	0.075
<i>P. virginiana</i>	100 vs. 1000	8.0	0.210
	100 vs. 10000	4.5	0.241
	1000 vs. 10000	9.0	> 0.50
<i>P. thunbergii</i> × <i>P. massoniana</i>	100 vs. 1000	1.0	0.008
	100 vs. 10000	0.0	0.018
	1000 vs. 10000	3.0	0.125
<i>P. echinata</i>	100 vs. 1000	4.5	0.062
	100 vs. 10000	0.0	0.004
	1000 vs. 10000	7.5	0.183
<i>P. × rigitaeda</i>	100 vs. 1000	9.5	> 0.50
	100 vs. 10000	6.5	0.133
	1000 vs. 10000	14	> 0.50
<i>P. koraiensis</i>	100 vs. 1000	13.5	> 0.50
	100 vs. 10000	8.5	0.242
	1000 vs. 10000	6.5	0.133



**Figure 1.** Average number of days from inoculation to beginning of needle discoloration of seven pine species or hybrids inoculated with three densities of *B. xylophilus*. Error bars are the mean ± SD of five replicates.

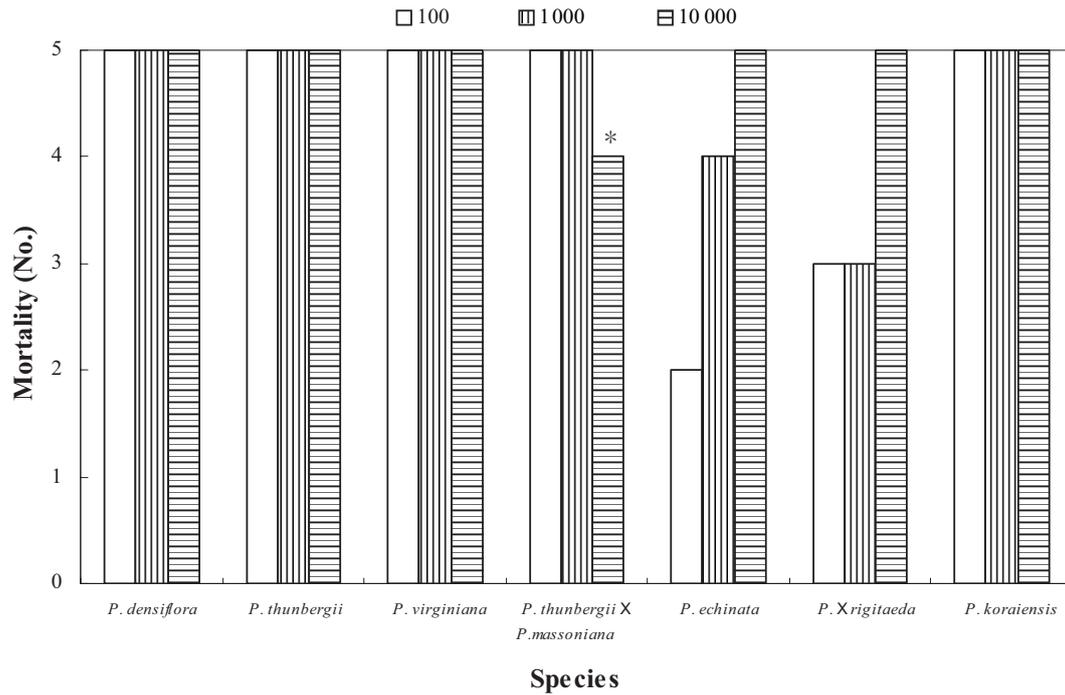


**Figure 2.** Average days from nematode inoculation to death of seven pine species or hybrid seedlings inoculated with three densities of *B. xylophilus*. The Mann-Whitney U-test showed that there were no significant differences for average days from nematode inoculation to seedling death between three densities of nematodes in each of seven pine species or hybrids. Error bars are the mean ± SD of five replicates.

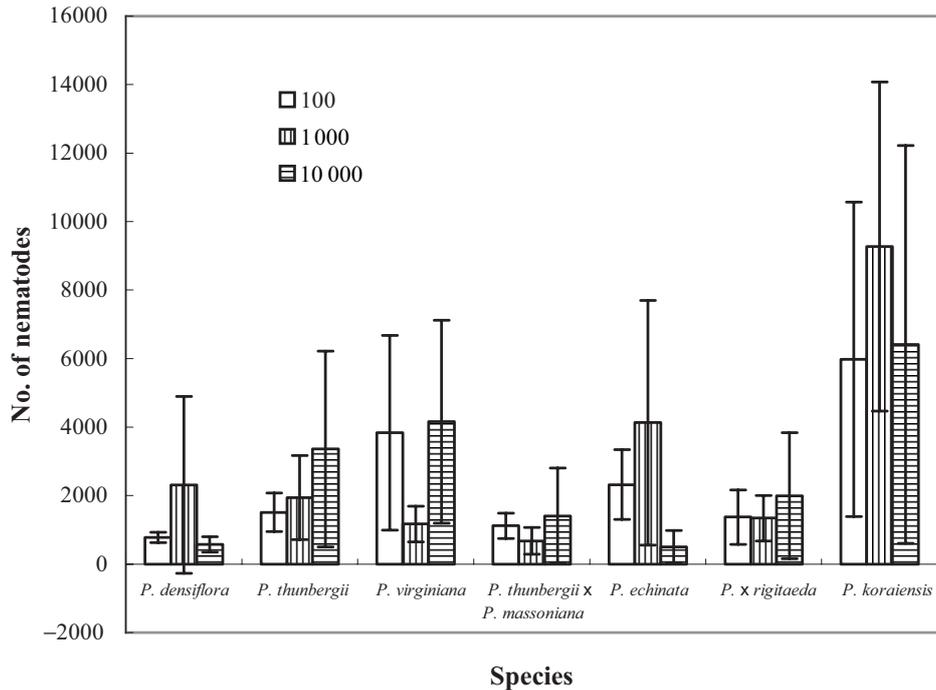
respectively, and Fig. 4). The least squares means test showed that differences were significant only between seedlings of *P. koraiensis* and those of other pines (data not shown). The greatest number of nematodes was isolated from the seedlings of *P. koraiensis* ( $\bar{X} = 7219$ ; range: 280–16100); the lowest was from the seedlings of *P. thunbergii* x *P. massoniana* ( $\bar{X} = 1043$ ; range: 80–3450) (Fig. 4). No nematodes were recovered from the control seedlings at the end of the experiment.

#### 4. DISCUSSION

The mechanism by which pine wood nematodes kill trees is unknown. Hypotheses include cell death, cavitation and embolism caused as a result of increased production of volatile terpenes (Kuroda, 1991; Ikeda and Kiyohara, 1995; Ichihara et al., 2000). Regardless of the mechanism, the results of the current study showed all seven pine species and hybrids to be susceptible to PWN. Although previous studies have reported



**Figure 3.** Mortality of seven pine species or hybrid seedlings 83 days after inoculation with three densities of *B. xylophilus*. Fifteen seedlings from each of the pine species were inoculated with *B. xylophilus*, except *P. thunbergii* × *P. massoniana*, in which fourteen seedlings were used for the nematode inoculation. None of the control seedlings died during the experiment. \*Only 4 seedlings inoculated.



**Figure 4.** Average number of *Bursaphelenchus xylophilus* recovered from dead seedlings of seven pine species or hybrids inoculated with three densities of nematodes. Error bars are the mean ± SD of five replicates.

pine species to vary in their susceptibility to PWN (Dropkin et al., 1981; Dwinell, 1985; Futai and Furuno, 1979; Yang and Wang, 1989), such discrepancies may be caused by variation in virulence among isolates of *B. xylophilus*, genetic variation in resistance among tested individuals, physiological differences resulting from variation in growth regimes or other environmental factors. For example, all of the six-year-old seedlings of *Pinus virginiana* in this study died from PWN, but the species was found to be resistant in a study of three-year-old seedlings inoculated in a greenhouse with three isolates of *B. xylophilus* from the United States (Dwinell, 1985), and Dropkin et al. (1981) reported that *P. virginiana* is moderately susceptible.

Previous studies have shown differences in susceptibility of mature pine species to infection by PWN related to inoculum densities (Akiba and Nakamura, 2005; Kishi, 1999). Mamiya (1983) reported that 30 nematodes per susceptible mature tree of *P. densiflora* and *P. thunbergii* could produce infection, but the level of disease development was slower than with 3 000 or 30 000 nematodes. In the present study, disease symptoms also appeared later in seedlings inoculated with a lower density of nematodes compared with those inoculated with large numbers (Fig. 1). The starting day of needle discoloration also varied with the combination of densities of nematodes and species (Fig. 1). In a previous test, we found no correlation between mortality of four pine species or hybrids and the densities of *B. xylophilus* at 3 000, 5 000 and 7 000 nematodes (Woo et al., 2007), suggesting even 3 000 nematodes per ml was beyond a critical threshold level of inoculum.

The dispersal fourth stage juveniles ( $J_{IV}$ ) of *B. xylophilus* are transmitted to host trees during maturation feeding of the *Monochamus* insect vector on young twigs and shoots (Linit, 1990; Mamiya, 1975; Wingfield and Blanchette, 1983), with the transmission rate depending on the age of insect vectors after emergence and number of nematodes they carry (Togashi, 1985). An insect vector carrying more than 10 000 nematodes transmitted a maximum of 750 nematodes per adult tree (Togashi, 1985). Thus, the inoculum densities of 1 000 and 10 000 nematodes per tree in the current study are within the natural range of densities occurring under field conditions. The lowest treatment level in the current study (100 nematodes/tree) may be low compared with field conditions, but inoculation with even this density of inoculum was sufficient to kill 30 of 35 treated seedlings.

Dwinell (1985), and Akiba and Nakamura (2005) found that there was a highly significant correlation between the percent dead and the number of nematodes recovered. In the current study, the number of nematodes extracted from dead seedlings was significantly different among the seven pine species or hybrids, with the fewest recovered from *P. thunbergii* × *P. massoniana* (Fig. 4). The relatively small number of nematodes recovered from the dead hybrids may suggest high susceptibility, a relatively unfavorable growth environment for the nematodes or both, but the number of nematodes or their effect was sufficient to kill the seedlings. However, since the 97 of 105 inoculated seedlings had died, there was no direct relationship between seedling mortality and the number of nematodes recovered.

The period from inoculation to death of 10-year-old *P. thunbergii* was significantly shorter than that of 20-year-old *P. armandii* var. *amamiana* inoculated with 1 000 nematodes, but the latter group did not differ significantly from seedlings of the same age and species inoculated with 100 000 nematodes (Akiba and Nakamura, 2005). In the current study, we found a significant difference in time until death among the seven pine species or hybrids, with *Pinus koraiensis* living the longest for all densities of nematodes. However, seedling age (which varied by only 2 or 3 years) was not a significant factor. The delayed symptom development in *P. koraiensis* may be associated with its chemical composition, as nematocidal compounds, methyl ferulate and 3-0-methyldihydroinosylvin, have been found, respectively, in heartwood and bark of *P. strobus*, another five-needle pine (Hanawa et al., 2001; Suga et al., 1993;).

Among the seven pine species or hybrids tested, the few seedlings that survived to the end of the trial came from bulk lots of *P. echinata* and *P. × rigitaeda*, suggesting the possibility that the bulk lots, with a generally higher level of genetic diversity than the single-source lots, included some useful genetic resistance to PWN. Based on this preliminary trial, we recommend further testing of susceptibility of host pine species to *B. xylophilus* using a broader genetic representation of the species of interest, different inoculum densities, tree ages, and duration of the experiment. Field tests are also recommended to further describe the relative susceptibilities of pine species to *B. xylophilus*. In Korea, a breeding project for resistance to pine wood nematodes has been initiated. Prior to establishing operational plantings with resistant pines, however, improved lots should be tested routinely by artificial inoculation with pine wood nematodes at the nurseries.

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