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Early Disease Development and Stem and Leaf Water Content in the Seedlings of *Pinus koraiensis* Inoculated with Pinewood Nematodes in a Greenhouse

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Korean pine (*Pinus koraiensis* Sieb. et Zucc.), a five-needle pine, has recently been suffering pine wilt disease caused by non-native pinewood nematode, *Bursaphelenchus xylophilus*. Three-year-old Korean pine seedlings were inoculated with 10,000 pathogenic nematodes in a greenhouse to investigate disease development, water content and the density of nematodes in stems. Needle dehydration, xylem drying and pith browning started 20 days after inoculation (DAI). There were significant differences between seedlings inoculated with nematodes and control seedlings in the relative water content of stems and leaves at 20 and 30 DAI. At 60 DAI, all remaining seedlings inoculated with nematodes had died, but control seedlings all remained alive. The average number of nematodes recovered from stems of Korean pine dramatically increased from 10 to 20 DAI, and then decreased at the end of the experiment at 60 days. This study suggests that the relative water content of stems and leaves in current-year branches could be used as a useful physiological indicator for early diagnosis of pine wilt disease.

Keywords : *Bursaphelenchus xylophilus*, pathogenic nematodes, *Pinus koraiensis*, water content, xylem drying

Korean pine (*Pinus koraiensis* Sieb. et Zucc.), a five-needle pine, is distributed throughout Korea, eastern Manchuria into southern Siberia, and Honshu and Shikoku in Japan (Critchfield and Little, 1966). Korean pine has a relatively narrow range of natural distribution on the Korean Peninsula, mainly in the northeastern high elevation areas from 600 to 1,200 meters (Lim, 1989). Korean pine is one of the most ecologically and economically valuable tree species in terms of planted forests. It is widely planted in Korea due to its high value for both timber and edible nuts (Hyun, 1969). As of December 2006, Korean pine accounted for 231,000 ha (8.6%) out of 2,695,000 ha of national softwood area in Korea (Korea Forest Service, 2007). Korean pine is generally known as a disease- and insect-tolerant and is shade-tolerant at young stages.

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The non-native pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, has been a serious threat to native two-needle pine forests of Japanese red pine (*Pinus densiflora* Sieb. et Zucc.) and Japanese black pine (*P. thunbergii* Parl.) since the first occurrence in 1988 in Korea (Enda, 1989; Shin and Han, 2006). Pine wilt disease caused by the nematode was first found in a planted five-needle pine forests of Korean pine in December 2006 in Kwangju, Kyeonggi province (Woo et al., 2007), and the disease has also been found in planted forests of Korean pine in Chuncheon and Wonju, Kangwon province, and Namyangju, Kyeonggi province in 2007 (unpublished observation). However, there has been no report for damage in the natural forests of *P. koraiensis* caused by PWN. The *B. xylophilus* isolated from Korean pine was alleged to have been transmitted by a native pine sawyer, *Monochamus saltuarius*, which is putatively the sole insect vector of PWN for Korean pine in South Korea (Kwon et al., 2006).

Introduction studies of *B. xylophilus* into stems of a susceptible pine seedlings resulted in quick appearance of disease symptoms like cessation of resin exudation and tree mortality within two or three months (Ikeda and Kiyohara, 1995; Mamiya, 1972; Mamiya, 1980; Togashi et al., 1997). On small seedlings, it takes no more than a month from nematode infection by artificial inoculation to symptom development in a greenhouse under optimum temperature (25 °C) (unpublished observation). Early diagnosis of the pine wilt disease is indispensable to prevent the disease dispersion. Some biochemical and physiological reactions of the host trees at an early stage of disease development have been suggested as an effective early diagnosis (Kuroda, 2004; Tan and Ye, 2003; Wang et al., 2001). Korean pine is susceptible to PWN, but little is known of the water content and disease development at an early stage after inoculation. This study was conducted to clarify the disease development at an early stage after inoculation and to examine the changes of stem and leaf relative water contents of three-year-old seedlings of *P. koraiensis* inoculated with virulent PWN.

Materials and Methods

Culture of pinewood nematode. The PWN was isolated from naturally diseased Japanese red pine in city of Jinju, Korea. The wood was cut into chips about 1 cm in length. Nematodes were isolated using the Baermann funnel method (Southey, 1986) for 24-48 hrs at 23 °C to 26 °C. The nematodes were cultured on *Botrytis cinerea* Pers. grown on potato dextrose agar and incubated at 25 °C in the dark for about 10 days.

Plant materials. Three-year-old seedlings of Korean pine had been grown from open-pollinated seed in nursery beds of the Department of Forest Resources Development, Korea Forest Research Institute (KFRI), Kyeonggi Province, Korea. The seedlings were transplanted into round plastic pots (20.1 and 13.0 cm of upper and lower diameters, respectively and 19.1 cm deep) in spring 2006, and 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. Seedlings were watered equally as needed, but no supplemental nutrients were supplied. Seedlings were initially placed outdoors, but were moved into a vinyl tunnel-house (3 × 5 × 2 m) enclosed in a greenhouse three weeks before inoculation. No supplemental light was applied in the greenhouse, and temperatures within the tunnel-house ranged from 17 °C to 38 °C for 60 days of experiment. Seedlings were inoculated in September 2007.

Artificial inoculations. Fifteen seedlings were inoculated with 50 μl of 10,000 nematode suspensions and fifteen with sterile water as a control. A wound was made on the main stem by removing an area of bark 2 cm long and 0.8 cm wide and a piece of cotton was placed into the wound. The nematode suspension (or sterile water for the controls) was pipetted onto the cotton, and it was covered with parafilm to prevent contamination and water loss. Subsequent to the inoculation, the seedlings were watered, but no nutrients were supplied. Individual seedlings were examined for wilting of needles, resin (R) and xylem (X) drying and pith browning (P) symptoms at 5, 10, 20, 30, and 60 days after inoculation (DAI). Internal symptoms (R, X, and P) were observed on two cross sections at 3 cm-above and -below the inoculation site of the stem of each seedling. Resin and xylem drying on the surfaces of the cross sections was observed by applying constant pressure to the cross sections. Three seedlings from both the inoculated and controlled treatments were destructively sampled at each of the five time periods. The stems of dead seedlings were cut into chips 5 mm thick and immersed in a Baermann funnels to count the number of *B. xylophilus* per gram of stem dry weight under a dissecting microscope.

Three seedlings from each of inoculated and control seedlings were harvested to determine stem and leaf relative water contents at 5, 10, 20, and 30 DAI. About 0.2 g of stem and 0.2 g of leaf from a randomly selected current branch were collected and dried at 80°C for 48 hrs on each harvest date. Water content was calculated using equations from Tan et al. (2005).

Data analysis. Averages of water content and the number of nematodes were compared with the two-sample t-test at the 5% level using SYSTAT 9 statistical software (SYSTAT Software Inc. 2004).

Results and Discussion

Symptom development. Early symptom development of PWN infected seedlings of pine species varies depending on temperature, tree age, and soil moisture. Sixteen-month-old seedlings of *P. thunbergii* inoculated with PWN showed more rapid symptom development than older seedlings (Kuroda et al., 2007). In an early study, we found that discolored needles of *P. koraiensis* seedlings inoculated with 10,000 nematodes showed earlier than those of *P. virginiana* and *P. densiflora* seedlings, but later than those of *P. thunbergii* seedlings (Woo et al., 2008). In this study, all three of the nematode inoculated seedlings were dead while three control seedlings were all alive at 60 DAI (the final sampling time) (Fig. 1). Symptoms of resin and xylem drying and pith browning were very distinct between seedlings inoculated with PWN and its control. No internal disease symptoms were observed on any of the seedlings inoculated with sterile water (Fig. 1). In the current study, no external and internal symptoms had developed on any seedlings at 5 and 10 DAI (Fig. 2). Needle dehydration, an external symptom, was first observed on seedlings at the 20 days post-inoculation inspection, and subsequently, the dehydrated needles changed the color to yellow and finally wilted. The percentage of symptomatic needles at 60 DAI was 100% on two of the three seedlings inoculated with nematodes, and the third seedling had 97% symptomatic needles (Fig. 2). External disease symptoms were not observed on the control seedlings throughout the experiment.

Internal symptoms, such as xylem drying and pith browning, caused by the pathogenic nematode in the seedlings of *P. thunbergii* began to occur about 15 DAI in the area below the inoculation site on the main stem (Tan et al., 2005). In this study using Korean pine, xylem drying and pith browning began to occur on cross sections of the main stems of

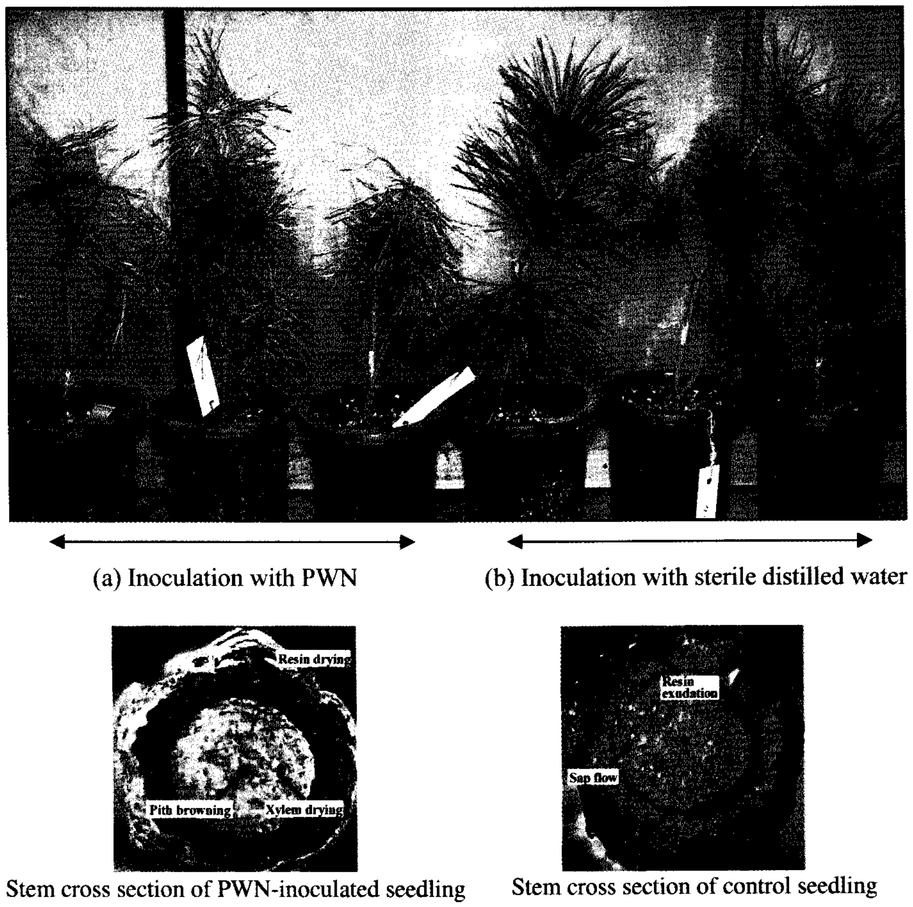

Fig. 1. Disease development of seedlings of Korean pine at 60 days after inoculation with (a) PWN and (b) sterile distilled water. Resin and xylem drying and pith browning symptoms occurred in PWN-inoculated seeding. No resin and xylem drying and pith browning symptoms occurred in control seedling.

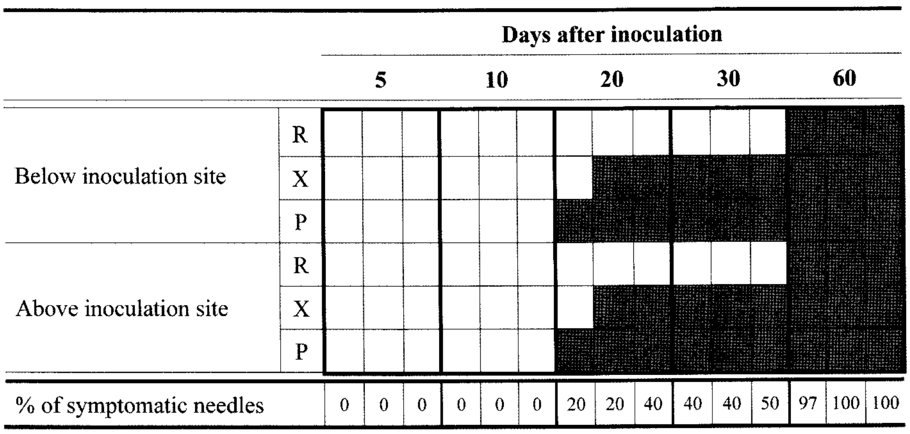

Fig. 2. Disease development of Korean pine seedlings inoculated with a virulent isolate of PWN. R: resin drying; X: xylem drying; P: pith browning. White block: symptom absent; grey block: symptom present. Each of the 15 columns represents one of the 15 seedlings treated with PWN during the time period in which it was sampled.

the seedlings by 20 DAI (Fig. 2). These symptoms occurred in all three seedlings per sample time at 20, 30, and 60 DAI, but xylem drying was observed on only two of three seedlings at 20 DAI. Complete resin drying was only observed on samples at 60 DAI (Fig. 2).

Stem and leaf water content. One of the early physiological changes occurring in pine trees after PWN infection

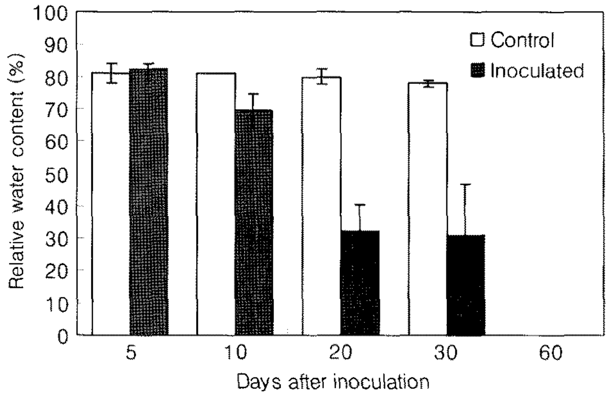

Fig. 3. Stem relative water content in current-year branches of Korean pine inoculated with *Bursaphelenchus xylophilus*. Error bars are the mean±SD of three replicates.

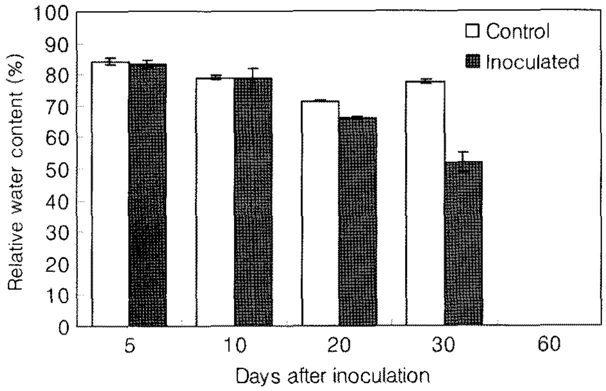

Fig. 4. Leaf relative water content in current-year branches of Korean pine inoculated with *Bursaphelenchus xylophilus*. Error bars are the mean±SD of three replicates.

partially interrupted water movement in sapwood, which starts in two weeks after nematode infection (Tamura et al., 1987). When the needles of *P. thunbergii* saplings start to discolor at about three weeks after nematode inoculation, water content in the xylem decreased dramatically (Kuroda and Ito, 1992). Moreover, high population densities of the PWN resulted in a decline of needle water potential of one-year-old seedlings of *Pinus sylvestris* within three days post-inoculation, and host physiological processes are preceded by an effect on water relations (Melakeberhan et al., 1991). Ikeda and Suzaki (1984) reported that the basic xylem pressure potential, which is an indicator of tree water status, decreased in the seedlings of *Pinus thunbergii* from 10 to 20 DAI, and it decreased drastically from 21 to 29 DAI.

In the current study, the relative water content of stems was statistically different between inoculated and control seedlings at 20 and 30 DAI ($p<0.05$) (Fig. 3). Significant differences were also found between inoculated and control seedlings in the relative water content of leaves at 20 and 30 DAI ($p<0.01$) (Fig. 4). We found that the relative water content of stems and leaves in current-year branches of inoculated seedlings decreased continuously from five DAI to the end of the experiment, which is consistent with a previous report (Ikeda and Suzaki, 1984), with the highest rate of decrease 37.2% in stems between 10 and 20 DAI (Fig. 3) and 13.9% in leaves between 20 and 30 DAI (Fig. 4), respectively. We could not measure the relative water content of stems and leaves in current-year branches at 60 DAI because all three inoculated seedlings had died by the measurement periods. A previous study suggested that a pine species responds to the PWN infection by producing volatile substances, which evaporate to cause tracheid cavitation or embolism and interrupt the translocation of water (Kuroda, 1991).

Nematode extraction. In a previous test, we found that the greatest number of nematodes was isolated from dead seedlings of *P. koraiensis* inoculated with three densities of nematodes compared to those from other pine species (Woo et al., 2008), indicating that *P. koraiensis* is a relatively good host for the nematodes. It has been reported that the PWN population in *P. thunbergii* stems at 2-3 weeks after inoculation is still low (Kuroda, 2008), but the population of PWN in the tree tissue is dramatically increased when the needles start to discolor at three weeks after inoculation (Kuroda and Ito, 1992). The number of nematodes decreases as a host becomes severely diseased or dead (Mamiya, 1972; Asai and Futai, 2005). The decreases may be caused by the lack of the food for the nematodes. In the current study, the average number of *B. xylophilus* extracted from stems of Korean pine increased rapidly from 10 to 20 DAI and then decreased at the end of the experiment at 60 days (Fig. 5). The greatest numbers of nematodes were those recovered at 20 DAI ($\bar{X}$=4,593; range: 1,279-7,695 nematodes g^{-1}); the fewest were observed in the sample

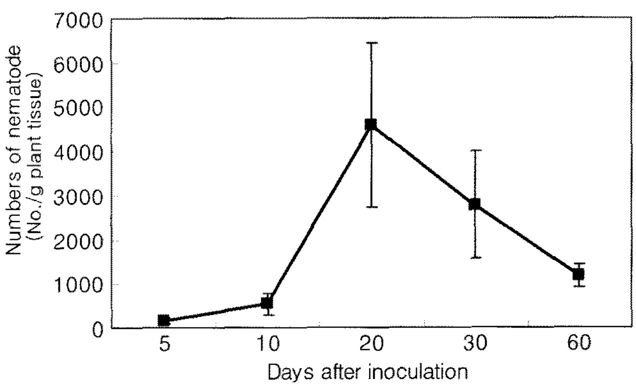

Fig. 5. Average numbers of *Bursaphelenchus xylophilus* recovered from one gram of Korean pine stem. Error bars are the mean±SD of three replicates.

collected at five DAI ($\bar{X}$=168; range: 59-231 nematodes g^{-1}). No *B. xylophilus* was recovered from the control seedlings at any of the measurement periods of the experiment.

In a previous inoculation study (Woo et al., 2008), there was a significant difference in time until death among seven pine species or hybrids, with 5-year-old *P. Koraiensis* living the longest (83 days) for three different densities of nematodes. Natural five-needle pines distributed mainly at higher elevations seem to be free from the PWD, but a planted five-needle pine stands are very susceptible to the disease (Futai and Furuno, 1979; Futai, 2003; Woo et al., 2007).

From this study, we confirmed that Korean pine seedlings are very susceptible to PWN in a greenhouse environment. This study suggests that the relative water content of stems and leaves in current-year branches could be used as a useful physiological indicator for early diagnosis for PWD. Additional inoculation tests are also required to clarify resistance mechanisms, especially those that interrupt the translocation of water in tracheids.

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