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# Within-tree distribution and attractant sampling of propagative pinewood nematode, *Bursaphelenchus xylophilus*: An early diagnosis approach

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ABSTRACT

Early detection is of primary importance to enable rapid actions to prevent the spread and introduction of invasive species. The pinewood nematode (PWN), *Bursaphelenchus xylophilus*, a serious invasive and destructive species, is listed as a quarantine pest in the legislation of more than 40 countries. However, Baermann funnel extractions of wood from discs cut from trees at breast-height often do not detect the presence of PWN in infested trees. A serious consequence of such false negatives is the loss of the best window for implementation of eradication or quarantine measures to prevent establishment of incipient PWN infestations. Here we document the within-tree horizontal and vertical distribution of PWN in infested stands in China, using a newly developed kairomonal trapping technique. Our results provide a simple, effective, rapid and non-destructive sampling method that takes into account the changes of PWN within-tree distribution in relation to pine wilt disease (PWD) symptom development. When 60–80% of the foliage has become pale green, PWN is recovered from larger diameter branches. As disease symptoms progress, PWN moves into and down the trunk. As the needles turn yellow, PWN was recovered from the trunk at 1–2 m above the ground. The correlation between the within-tree distribution of PWN and the expression of symptoms indicated a strong association between the distribution of PWN and physiological and pathological changes that develop in attacked pines through the interaction between PWN and tree. This systematic sampling technique takes into account the within-tree distribution of the nematode and should greatly enhance early detection of PWN in field surveys, monitoring and phytosanitary inspections.



## 1. Introduction

With globalization, effective and rapid early detection is the most important measure available to prevent the invasion and dispersal of plant–parasitic nematodes. The pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle (Tylenchida: Aphenlenchoididae), the causal agent of the destructive pine wilt disease (PWD), is an invasive species of global significance that has caused irreparable damage to forested ecosystems (Kiyohara and Tokushige, 1971; Mota et al., 1999; Sun, 2005). Effective early detection is a prerequisite for the implementation of surveillance and eradication programs, as well as for the establishment of standards for pest risk analysis and the determination of pest-free areas (Dwinell, 1997; Schrader and Unger, 2003).

The first visible symptom of PWD in infested trees is a general wilting of the needles. As the disease progresses, the wilted needles redden or turn brown, an indicator of tree death (Mamiya, 1984). These symptoms can easily be confused with those caused by other agents (e.g. drought, attack by several species of bark beetles, or *Fomes annosus* root rot). PWD can only be diagnosed if PWN can be recovered from diseased pines. However, in a newly infested tree or stand, the population of PWN is very low (Cheng et al., 2009), and PWN is difficult to detect using traditional Baermann funnel extractions of wood from discs cut from trees at breast-height (Yang et al., 2003). These initial infections are often not detected. One consequence of such false negatives is an increase in PWN density, thereby increasing the risk and prevalence of pine infestation and as a consequence, making eradication or quarantine efforts much more extensive (Mamiya, 1984; Shen et al., 2001; Yang et al., 2003; Robinet et al., 2009).

The development of any sampling technique for PWN must focus on the life cycle of nematode, its horizontal and vertical distribution within the tree, and its interactions with the host plants and environment (Cochran, 1977). After initial infection in

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the spring, populations of propagative PWN colonize the host tissues and expand throughout the tree. As this phase encompases two thirds of the annual life history of the nematode (April through November), standardized techniques to detect the propagative stage of PWN are needed to enable early detection of incipient populations of the nematode. However, little is known about the distribution of propagative PWN in host pines across time or space. Additionally, the occurrence of asymptomatic host pines, which, in at least one instance, have harbored PWN for 6 years, could confound the detection of PWN during stand surveys (Bergdahl and Halik, 1999; Takeuchi and Futai, 2007).

Chemotaxis plays an important role in host, food, and mate location of nematodes during certain phases of their life cycle (Zuckerman and Jansson, 1984; Perry, 1996; Rasmann et al., 2005). In a previous study, we demonstrated that there was a specific ratio of α-pinene, β-pinene and longifolene (1:0.1:0.01) present in the healthy xylem of *Pinus massoniana* Lambert that induced a very significant aggregation of propagative juveniles of PWN (Zhao et al., 2007a). That discovery provided the foundation for kairomonal evaluations of the within-tree distribution of PWN in infested stands. In this study, we describe the radial and axial distribution of PWN in infested pines at different stages in the development of pine wilt disease and provides the basis for effective, rapid sampling for and detection of propagative PWN.

## 2. Materials and methods

### 2.1. Attractants and trap tubes

The attractant blend consisted of α-pinene (Acros Organics, 98%), β-pinene (Fluka Chemie AG, 80%) and longifolene (Acros Organics, 99%) in a ratio of 1:0.1:0.01 dissolved in 40 μL n-hexane (Fisher Chemicals, 99%). Several concentrations of the attractant blend (0.01, 0.1, 1.3, 13 or 130 mg) were used to be tested.

Trap tubes consisted of a 2 ml centrifuge tube (Beijing Zohonice Science & Technology Development Co., Beijing, China) containing a rubber septum (Tongzhou Shunyi Rubber Co., Beijing, China) with 0.8% agar (Beijing Shuangxuan Co., Beijing, China) solidified around the tube walls (Zhao et al., 2007b). Before each experiment, 40 μL of n-hexane containing the attractant blend was transferred onto the rubber septa of each trap tube and the solvent allowed to volatilize before use. The septa of the controls in each experiment were treated with 40 μL n-hexane alone.

### 2.2. Optimizing attractant blend in artificial culture blocks

Propagative nematodes were reared in artificial culture blocks created in pine bolts in the laboratory (Jikumaru and Togashi, 2003). A hole (2 cm deep and 1 cm diameter) was drilled in the centre of one cut-end of healthy *P. massoniana* Lambert bolts (5 cm long and 2.5 cm mean diam). The bolts were autoclaved at 121 °C for 30 min, then, the drilled chamber was inoculated with a fungus, *Diplodia* sp., as a food source for PWN, and held at 25 °C in the dark for 14 days. Two weeks later, when the fungal mycelia covered the chambers, 500 propagative nematodes of PWN in 1 ml sterile water were inoculated into each bolt. The artificial culture blocks were then held at 25 °C in the dark for 10 days.

In order to determine the optimal concentration of the attractant blend and to test the efficacy of the trap tubes, we first conducted 2 trap tube sampling experiments with artificial culture blocks.

*Experiment 1*: In order to find the best concentration of the attractant blend, ten replicate trap tubes for each concentration of the attractant blend tested (0.01, 0.1, 1.3, 13 or 130 mg) were inserted into the hole of the artificial culture blocks and held at 25 °C in the dark for either 24 or 48 h. The trap tubes were then removed from the blocks, the agar extracted into individual Petri dishes (5 cm diam) and the inside wall of the tube washed into a Petri dish with 2 ml distilled water to recover the nematodes. Nematodes were then counted microscopically. Differences in sampled nematode abundance between 24 and 48 h were compared by *t*-test (using SPSS 11.0 for Windows). Differences among treatments with different concentrations were compared by ANOVA at $P \leq 0.05$ (using SPSS 11.0 for Windows).

*Experiment 2*: In order to quantify the efficacy of trap tubes with 130 mg lures (the most effective concentration in Experiment 1), we assessed the numbers of propagative nematodes recovered from artificial culture blocks containing a range of densities of PWN. Ten blocks maintained for 10, 20 or 30 days after inoculation with PWN were sampled with the 130 mg lure for 48 h at 25 °C in the dark. The number of propagative nematodes recovered per trap tube was determined as in Experiment 1. The total number of propagative nematodes in each chamber was determined by chipping each culture block and extracting nematodes from the chips using Baermann funnels. The correlation between number of nematodes recovered and the total propagative nematodes present in the artificial culture blocks was determined by linear regression (using SPSS 11.0 for Windows).

### 2.3. Optimizing sampling duration in infested pine stands

Field trapping experiments were conducted in masson pine (*P. massoniana* Lambert) and Japanese black pine (*Pinus thunbergii* Parlatore) stands in the southern provinces of Zhejiang, Anhui, Guangdong and Hubei. There were twenty trees (DBH 10–18 cm) expressing symptoms of PWD sampled in each of these provinces between 2006 and 2008 (Table 1).

In order to determine the optimal sampling date for propagative PWN, thirty sampling holes (3–5 cm deep and 1 cm diam) were drilled at 1.5 m height in ten masson pines and ten Japanese black pines exhibiting PWD Class III symptoms. During drilling, about 2 ml of water was used to cool the drill bit. Five replicate trap tubes baited with the 130 mg lures were inserted into the drilled holes for 2, 4, 6, 12, 18 or 24 h. The temperature ranged from 15 to 30 °C and it rained occasionally during the days that field bioassays were conducted. Nematodes from the trap tubes were recovered and counted as noted above and then identified both microscopically and with real-time polymerase chain reaction (PCR) (Leal et al., 2007). The relationship between the number of nematodes recovered and the duration of sampling in the artificial culture

**Table 1**
Sampling sites in China.

| Infestation class | Site | Location | Year PWN detected | Date sampled |
|---|---|---|---|---|
| Uncertain | Yichang, Hubei | 18°42′E 111°30′N | 2006 | Sep, 2006 |
| Newly infested | Chonghua, Guangdong | 33°34′E 113°23′N | 2006 | Sep, 2008 |
| Moderately infested I | Enshi, Hubei | 30°16′ 109°29′ N | 2000 | Sep, 2006 |
| Moderately infested II | Huangpu, Guangdong | 23°10′E 113°44′N | 2001 | Sep, 2008 |
| Old infestation I | Zhapu, Zhejiang | 30°42′E 121°01′N | 2000 | Sep, 2006 |
| Old infestation II | Xuancheng, Anhui Chinese Alligator Nature Reserve | 44°57′E 118°31′N | 1999 | May, 2007 |

blocks was determined by curvilinear regression (using SPSS 11.0 for Windows).

### 2.4. Radial distribution of PWN in infested trees

Twenty discs of varying diameters (6–20 cm DBH) cut at breast-height from infected *P. massoniana* were divided into 3 sections across the radius of the stem and the density of propagative nematodes present in each section (inner third including the pith; central third and the outer third of the stem) was determined by extracting the chipped sections with Baermann funnels. The chips were dried in drying oven at 100 °C to a constant weight. One-way ANOVA was used to analyze differences among the number of nematodes per gram of dry chips from different radial sections across the stem (using SPSS 11.0 for Windows).

The effect of orientation on PWN density was determined by sampling the northern and southern faces of infested stems of *P. massoniana* exhibiting PWD Class III symptoms. Sampling holes (3–5 cm deep and 1 cm diam) were drilled in each face of dying pine stems at breast-height. Trap tubes baited with the 130 mg lures were inserted into the sampling holes for 24 h ($n = 10$). The nematodes were recovered and identified as noted above. Differences in sampled nematode abundance between northern and southern faces were compared by *t*-test (using SPSS 11.0 for Windows).

### 2.5. Axial distribution of PWN in infested trees

In each stand the trees sampled for PWN were classified into 6 categories based on the symptoms of PWD they expressed (Table 2). Five trees of each species (*P. massoniana* and *P. thunbergii*) from each PWD symptom category were cut for sampling. Sampling holes (3–5 cm depth and 1 cm diam) were drilled on the south face of the stem at 1 m intervals for the first 10 m. Five replicate trap tubes baited with the 130 mg lures were inserted into sampling holes for 24 h. The nematodes were recovered and identified as noted above. Differences among treatments with different sampling positions were compared by ANOVA at $P \leq 0.05$.

## 3. Results

### 3.1. Sampling in the artificial culture blocks

*Experiment 1*: Propagative nematodes were isolated from trap tubes across all lure concentrations tested (Fig. 1a). Significantly more propagative nematodes were found in tubes baited with the highest terpene concentration (130 mg per tube) ($P = 0.001$). There were significantly (5–10 times) more propagative *B. xylophilus* in trap tubes after 48 h than after 24 h ($P = 0.001$). The 130 mg lure was used in all subsequent bioassays.

*Experiment 2*: Between 77 and 1092 propagative juveniles were captured per tube over 24 h. The nematodes captured accounted for 4.72–15.41% of the total nematodes present in the blocks. There was a strong correlation between the nematode densities in the artificial culture blocks and the numbers captured by the baited tubes with >90% of the variation explained by the regression equation ($R^2 = 0.9219$) (Fig. 1b). Moreover, the regression was linear, indicating that the trap tube was proportionally effective across a wide range of nematode densities. However, no nematodes were captured in trap tubes when the initial number of nematodes in the blocks was <900.

### 3.2. The relationship between the duration of sampling and the mean number of nematodes recovered per trap tube

Propagative nematodes were found in 80% of the trap tubes after only 2 h and, by 12 h, 34.55 ± 5.48 nematodes were recovered per tube (Fig. 1c). More nematodes were captured as the duration of sampling increased, with 488.90 ± 84.70 nematodes recovered after 24 h. There was a strong correlation between the sampling duration and the number of nematodes captured by the baited tubes with >90% of the variation explained by the regression equation in *P. massoniana* and *P. thunbergii* ($R^2 = 0.9870$ and 0.9430 respectively) (Fig. 1c and d).

### 3.3. Radial distribution of PWN in infested trees

The radial distribution of nematodes is related to the diameter of pine trees (Fig. 2a). In smaller diameter pines (6–11 cm DBH) the most nematodes (mean ± SE = 99.62 ± 26.86) were recovered from the inner third of the stem ($P = 0.024$), while in larger diameter trees (11–20 cm DBH), the most nematodes were recovered from the centre third of the stem (91.77 ± 17.09) ($P = 0.033$). The fewest number of nematodes were recovered from the outer third of the stem in both diameter classes (22.29 ± 11.77 and 28.98 ± 11.33, respectively). The number of nematodes recovered differed significantly between aspects sampled (Fig. 2b); more nematodes were recovered from the southern face of sampled stems ($P = 0.003$).

### 3.4. Axial distribution of PWN in infested trees

The relationship between sampling height and nematode abundance differed between stages in the development of PWD. Table 2 summarizes the axial sampling position appropriate for each stage in the development of PWD symptoms for masson pine and Japanese black pine. The distribution of PWN changed with the developmental stage of the disease. The sampling position for PWN was similar in both *P. massoniana* and *P. thunbergii* at similar stages of development of PWD (Fig. 3). When a healthy pine is first infested, low numbers of PWN were recovered. As nematode

**Table 2**
Prescribed sampling position for propagative PWN using trap tubes.

| PWD class | Symptom | Sampling position |
|---|---|---|
| 0 | Healthy green foliage | Trunk, 2 m above ground. Tree is healthy if trap tube fills with resin |
| I | Early symptom: 60–80 percent of needles becomes pale green, maturation feeding evident on twigs, no oviposition scars of sawyer, no bark beetles or termites | Trunk adjacent to branches with adult *Monochamus* feeding scars, 6–7 m above ground |
| II | 99% needles become pale green or reddish brown, no *Monochamus* oviposition scars evident, no bark beetles or termites present | Trunk, 2 m above ground |
| III | Needles reddish brown in the upper crown, yellow brown in the lower crown, many *Monochamus* oviposition scars, sawyer frass evident, many bark beetles and termites present | Trunk, 1.5 m above ground |
| IV | Symptoms evident throughout the entire crown, many *Monochamus* oviposition scars and many bark beetle and termites present | Trunk, 1 m above ground |
| V | Asymptomatic host pines, harbored PWN for 2 years, no symptoms in year 1, symptoms as III in year 2 | Trunk, 2 m above ground in year 1[a], 1.5 m above ground in year 2 |

[a] See comment for PWD Class 0 above.

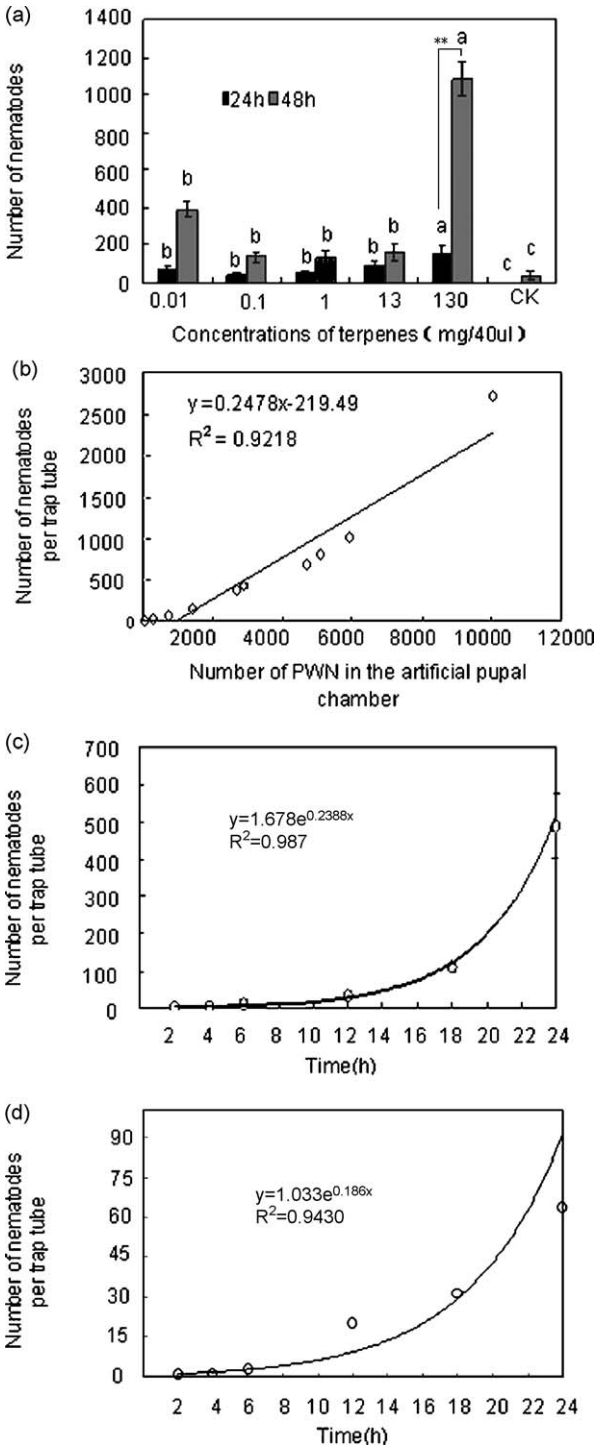


**Fig. 1.** PWN recovery from the artificial culture blocks and pine trees. (a) Attraction of PWN to different lure concentrations in the artificial culture blocks; (b) effect of PWN density in the artificial culture blocks on trap tube captures of propagative juveniles. (c) Effect of trapping duration on captures of propagative juveniles with trap tubes in masson pine (*P. massoniana*). Values represent the means and standard errors of ten replicates for each treatment. (d) Effect of trapping duration on captures of propagative juveniles with trap tubes in Japanese black pine (*P. thunbergii*). Values represent the means and standard errors of ten replicates for each treatment. Bars with the same letters are not significantly different among treatments with different concentrations at $P > 0.05$. Asterisks indicate a significant difference in sampled nematode abundance between 24 and 48 h ($^{**}P < 0.01$).

populations increased and moved downwards in the tree, early symptoms of PWD become apparent. When 60–80% of the foliage turns pale green (Stage I), most PWN were recovered from larger diameter branches, with the highest abundance of nematodes

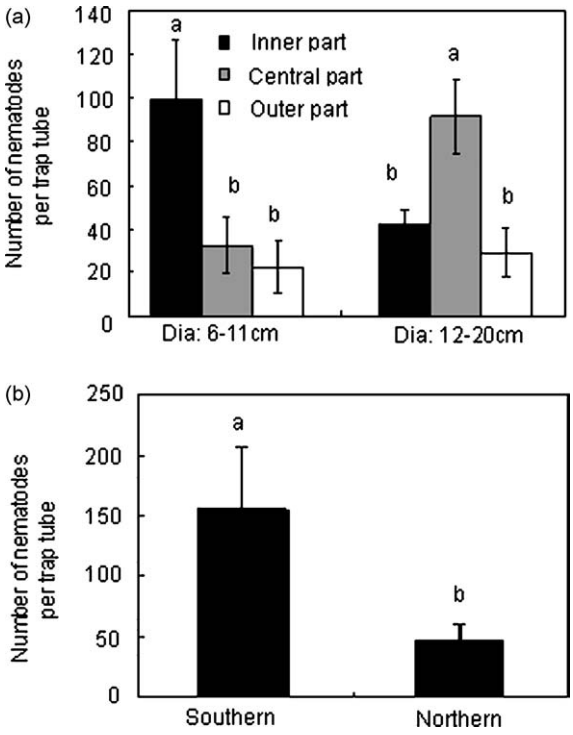


**Fig. 2.** Radial distribution of PWN in stem cross-sections. (a) Effect of radial sampling position on the recovery of nematodes. Inner part: inner third of the stem; central part: centre third of the stem; outer part: outer third of the stem. (b) Effect of aspect sampled on captures of propagative juveniles with trap tubes. Values represent the means and standard errors of twenty replicates for each treatment. Bars with the same letters are not significantly different at $P > 0.05$.

nearest the trunk ($1077.4 \pm 119.48$ and $337.4 \pm 117.91$, respectively), and no nematodes were recovered from the trunk near the ground (Fig. 3). As the needles turn yellow (Stage III), PWN was recovered most frequently in the trunk at 1–2 m above the ground; however nematode numbers were lower than at Stage I ($203.52 \pm 25.69$ and $106.4 \pm 14.75$, respectively) (Fig. 3).

## 4. Discussion

Early detection of new incursions of plant–parasitic nematodes in forest stands is of primary importance to the implementation of management and/or eradication strategies against these invasive alien species (Schrader and Unger, 2003). The efficiency of the sampling technique used directly affects the result of survey. While the Baermann funnel technique has traditionally been used to extract nematodes for identification, the extraction process is neither rapid (48 h) (Yang et al., 2003; Akbulut et al., 2006), nor selective, in that all species of nematodes present in the wood are recovered. Considerable time and taxonomic expertise is required to separate the *Bursaphelenchus* spp. from the large number of plant parasitic taxa (e.g. Bowers et al. (1992) recovered 46 additional genera of Nematoda during stand surveys for PWN). In addition, the traditional sampling technique is destructive as it entails cutting a disc from the tree trunk. There has long been a need for a simple, effective and rapid method to sample for PWN, in order to enhance the speed and accuracy of surveys (Shen et al., 2001).

We first reported the attractant-baited sampling trap for nematodes and demonstrated its effectiveness in capturing third-stage dispersal juveniles of PWN, which aggregate around the chambers of larval *Monochamus* before being dispersed to new healthy pine trees by the beetles (Zhao et al., 2007b). In this paper, we applied this newly developed and rapid trapping technique to document variation in the spatial distribution of propagative PWN within its host as disease symptoms are expressed.

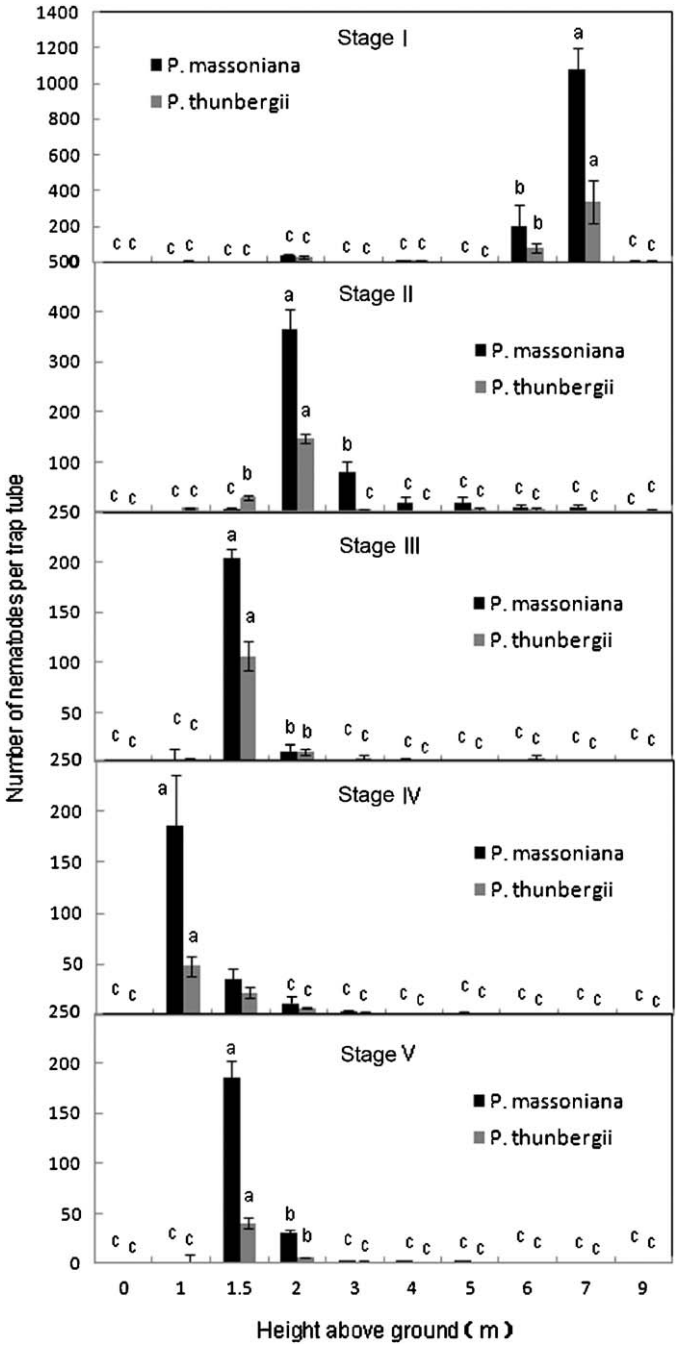


**Fig. 3.** Axial distribution of PWN in infested trees by PWD symptom class in masson pine (*P. massoniana*) and Japanese black pine (*P. thunbergii*). Values represent the means and standard errors of ten replicates for each treatment. Bars with the same letters are not significantly different at $P > 0.05$ within-tree species.

Our results provide a simple, effective, rapid and non-destructive sampling technique that takes into account the changes of PWN within-tree distribution in relation to PWD symptom development. The correlation between the within-tree distribution of PWN and the expression of PWD symptoms indicates that the physical properties, and physiological and pathological changes of host pines that develop through the interaction between the nematode and tree (Yamada, 2008) influences the within-tree distribution of the nematode. PWN enters the resin canals and proliferate there, rapidly producing many more nematodes whose secretions (e.g. cellulase) result in the death of parenchymatous tissues (Ishida and Hogetsu, 2001). As nematode populations increase, water translocation is reduced, resin secretion declines and the foliage turns pale green (Ichihara et al., 2001). At the highest densities of PWN sap flow throughout the whole tree stops (Stage I, Table 2, Fig. 3). As additional nutrients and energy are not available to stems and branches, host defense responses are overwhelmed, the foliage becomes yellow-brown (Stages II and III, Table 2, Fig. 3), and PWN migrates down the stem, and declines in abundance. Finally, the tree wilts from the accumulation of secondary metabolites when the population density of PWN in the stem is at its lowest (Stage IV, Table 2, Fig. 3) (Yamada, 2008).

We demonstrate the effectiveness of the technique for rapidly detecting propagative PWN, with the first recoveries occurring after only 2 h of sampling. As the number of PWN recovered in trap tubes increased with the time and was correlated to initial number of nematodes present (Figs. 1b and 3), which indicate that the nematodes in the tissue surrounding the trap tube continuously recognized chemical signals but only moved slowly into the trap tubes. In addition, our results indicate that at least 88 propagative nematodes per dry weight of tissue (Fig. 2b) are needed to isolate nematodes from field samples.

The chemotactic response of the nematode to the lure provides a non-destructive, rapid and more efficient method of sampling for propagative PWN than the traditional disc and Baermann funnel techniques even when nematodes are distributed unevenly within the host tissue. This characteristic makes attractant sampling more effective in detecting PWN infested areas when populations of adult nematodes are very low. At Yichang (near the Three Gorges Dam), Hubei, where numerous dead pines were present and only *Bursaphelenchus mucronatus* Mamya and Enda had previously been recovered from dead trees using the traditional sampling technique, we detected the presence of PWN using trap tubes. The rapid detection of infested trees at Yichang enabled them to be removed in time to prevent the development of a new PWN infestation. This simple, selective, rapid and portable sampling technique is an especially valuable sampling tool for individual high value trees in protected natural reserves as it allows non-destructive sampling of such trees. For example, a protected pine that exhibited symptoms of PWN in the Chinese Alligator Nature Reserve in Anhui province was sampled and found to be free of PWN. Not surprisingly, after three months, the pine tree recovered as it had not been infected with PWN.

The detection of PWN with this technique is dependent on the appropriate positioning of the trap tubes. Sampling locations are related to the distribution of PWN which changes with the stage of development of PWD (Table 2). Even asymptomatic infections (Stage V, Table 2) do not influence the relationship between PWN distribution in the tree and expression of PWD symptoms (Fig. 3). Sampling holes should preferentially be drilled in the south side of pine trees to 3–5 cm depth between Apr. and Nov. (Fig. 2). The sampling position for PWN was similar for both *P. thunbergii* and *P. massoniana* at similar stages of development of PWD, but the number PWN recovered from *P. thunbergii* was lower than that of *P. massoniana* (Fig. 3), possibly as a consequence of differences in the movement of PWN in the tissues of the more rigid *P. thunbergii*.

The only other species of Nematoda collected during our systematic survey was the native species, *B. mucronatus* Mamya and Enda (unpublished data). It was usually detected at a height of 2–3 m in trees exhibiting early symptoms of PWD, while PWN was recovered higher in the stem (at 7 m) and was significantly less abundant than PWN. Our observatins are in agreement with those of Cheng et al. (2009), who reported that PWN has a higher fecundity and stronger competitive ability than *B. mucronatus*.

Generally, systematic attractant sampling gives more reliable results than random sampling. It should assist greatly in the early detection of PWN in field surveys, monitoring and phytosanitary inspections.

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## References

Akbulut, S., Vieira, P., Ryss., A., Yuksel, B., Keten, A., Mota, M., Valadas, V., 2006. Preliminary survey of the pinewood nematode in Turkey. Bull. OEPP/EPPO Bull. 36, 538–542.

Bergdahl, D.R., Halik, S., 1999. Inoculated *Pinus sylvestris* serve as long-term hosts for *Bursaphelenchus xylophilus*. In: Futai, K., Togashi, K., Ikeda, T. (Eds.), Sustainability of Pine Forests in Relation to Pine Wilt and Decline, Proceedings of International Symposium. Nakanishi Printing, Tokyo, pp. 73–78.

Bowers, W.W., Hudak, J., Raske, A.G., 1992. Host and vector surveys for the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle (Nematoda: Aphelenchoididae) in Canada. In: Forestry Canada Information Report N-X-285. Labrador Region, Newfoundland, pp. 1–55.

Cochran, W.G., 1977. Sampling Techniques, 3rd ed. Wiley, New York, USA.

Cheng, X.Y., Xie, P.Z., Cheng, F.X., Xu, R.M., Xie, B.Y., 2009. Competitive displacement of the native species *Bursaphelenchus mucronatus* by an alien species *Bursaphelenchus xylophilus* (Nematoda: Aphelenchida: *Aphelenchoididae*): a case of successful invasion. Biol. Invasions 11, 205–213.

Dwinell, L.D., 1997. The pinewood nematode: regulation and mitigation. Ann. Rev. Phytopathol. 35, 153–166.

Ichihara, Y., Fukuda, A.Y., Suzuki, K., 2001. Suppression of ectomycorrhizal development in young *Pinus thunbergii* trees inoculated with *Bursaphelenchus xylophilus*. Forest Pathol. 31, 141–147.

Ishida, K., Hogetsu, T., 2001. Pathogenicity of the pine wood nematode at different developmental stages. J. Forest Res. 6, 253–256.

Jikumaru, S., Togashi, K., 2003. Boarding abilities of *Bursaphelenchus mucronatus* and *B. xylophilus* (Nematoda: Aphelenchoididae) on *Monochamus alternatus* (Coleoptera: Cerambycidae). Nematology 5, 843–849.

Kiyohara, T., Tokushige, Y., 1971. Inoculation experiments of a nematode, *Bursaphelenchus* sp., onto pine trees. J. Jpn. Forest Soc. 53, 210–218.

Leal, I., Green, M., Allen, E., Humble, L., Rott, M., 2007. Detection of pinewood nematode, *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) using a real-time PCR assay in wood samples from lodgepole pine. Nematology 9, 351–362.

Mamiya, Y., 1984. The pine wood nematode. In: Nickle, W.R. (Ed.), Plant and Insect Nematodes. Marcel Dekker, New York, pp. 589–626.

Mota, M.M., Braasch, H., Bravo, M.A., Penas, A.C., Burgermeister, W., Metge, K., Sousa, E., 1999. First report of *Bursaphelenchus xylophilus* in Portugal and in Europe. Nematology 1, 727–734.

Perry, R.N., 1996. Chemoreception in plant parasitic nematodes. Ann. Rev. Phytopathol. 34, 181–199.

Rasmann, S., Köllner, T.G., Degenhardt, J., Hiltpold, I., Toepfer, S., Kuhlmann, U., Gershenzon, J., Turlings, T.C.J., 2005. Recruitment of entomopathogenic nematodes by insect-damaged maize roots. Nature 434, 732–737.

Robinet, C., Roques, A., Pan, H.Y., Fang, G.F., Ye, J.R., Zhang, Y.Z., Sun, J.H., 2009. Role of human-mediated dispersal in the spread of the pinewood nematode in China. PloS ONE 4, e4646, doi:10.1371/journal.pone.0004646.

Schrader, G., Unger, J.G., 2003. Plant quarantine as a measure against invasive alien species: the framework of the International Plant Protection Convention and the plant health regulations in the European Union. Biol. Invasions 5, 357–364.

Shen, J., Chen, H., Ying, S.K., 2001. Distribution and fluctuation of natural infected pines by *Bursaphelenchus xylophilus*. Zhejiang Forest Sci. Technol. 21, 10–11.

Sun, J.H., 2005. Forest invasive species: Country report—P.R. China. In: Philip, M., Chris, B., Sun, J.H., Wu, J. (Eds.), The Unwelcome Guests. Proceedings of the Asia-Pacific Forest Invasive Species Conference. FAO Reg. Office for Asia and the Pacific, Bangkok, Thailand, pp. 80–86.

Takeuchi, Y., Futai, K., 2007. Asymptomatic carrier trees in pine stands naturally infected with *Bursaphelenchus xylophilus*. Nematology 9, 243–250.

Yamada, T., 2008. Biochemical responses in pine trees affected by pine wilt disease. In: Zhao, B.G., Futai, K., Sutherland, J.R., Takeuchi, Y. (Eds.), Pine Wilt Disease, Part IV. Springer, Japan, pp. 223–234.

Yang, B.J., Pan, H.Y., Tang, J., Wang, Y.Y., Wang, L.F., Wang, Q., 2003. *Bursaphelenchus xylophilus*. Chinese Forestry Press, Beijing, pp. 6–143.

Zhao, L.L., Wei, W., Kang, L., Sun, J.H., 2007a. Chemotaxis of the pinewood nematode, *Bursaphelenchus xylophilus*, to volatiles associated with host pine, *Pinus massoniana*, and its vector *Monochamus alternatus*. J. Chem. Ecol. 33, 1207–1216.

Zhao, L.L., Wei, W., Liu, X.Z., Sun, J.H., 2007b. A novel rapid sampling method for pinewood nematode, *Bursaphelenchus xylophilus* (Nematoda: Parasitaphelenchidae). Can. J. Forest Res. 37, 1867–1872.

Zuckerman, B.M., Jansson, H.B., 1984. Nematode chemotaxis and possible mechanisms of host/prey recognition. Ann. Rev. Phytopathol. 22, 95–113.