A novel rapid sampling method for pinewood nematode, *Bursaphelenchus xylophilus* (Nematoda: Parasitaphelenchidae)

Lilin Zhao, Wei Wei, Xinzhong Liu, Le Kang, and Jianghua Sun

Abstract: The pinewood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, is listed as a quarantine pest in the legislation of more than 40 countries. Rapid and accurate detection of the pinewood nematode in coniferous chips, sawn wood, and logs is critical in preventing the introduction of the nematode and forms the basis for quarantine regulations. The traditional but laborious Baermann-funnel sampling technique delays the detection of pinewood nematode. We applied the chemotactic response of pinewood nematode to its insect vector, *Monochamus alternatus* Hope, to develop a novel and rapid sampling method. A trap tube, baited with a blend of attractant terpenes (α-pinene, β-pinene, and longifolene, 1:2.7:1.1) is shown to effectively capture third-stage dispersal juveniles of pinewood nematode from infested wood under laboratory and field conditions. Nematodes were first isolated after a 2 h trapping period, and the number of nematodes recovered increased with the duration of trapping. This chemical attraction technique is simple, effective, and rapid and should assist greatly in the detection of pinewood nematode at both ports-of-entry and forest habitats.

Résumé : Le nématode du pin, *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, apparaît sur la liste des organismes soumis à la quarantaine dans la législation de plus de 40 pays. La détection rapide et exacte du nématode du pin dans les copeaux de conifère, le bois de sciage et les grumes est critique pour prévenir l'introduction du nématode et constitue la base des règlements sur la quarantaine. La technique traditionnelle mais laborieuse d'échantillonnage avec l'entonnoir de Baermann retarde la détection du nématode du pin. Nous avons eu recours à la réaction chimiotactique du nématode du pin à son insecte vecteur, *Monochamus alternatus* Hope, pour développer une méthode d'échantillonnage originale et rapide. Nous avons démontré qu'un piège constitué d'un tube appâté avec un mélange de terpènes (α-pinène, β-pinène et longifolène, 1:2.7:1.1) servant d'attractif capture efficacement le troisième stade larvaire de dispersion du nématode du pin dans le bois infesté en laboratoire et sur le terrain. Les premiers nématodes ont été isolés après une période de piégeage de deux heures et le nombre de nématodes capturés a augmenté avec la durée du piégeage. Cette technique d'attraction chimique est simple, efficace et rapide et devrait grandement aider dans la détection du nématode du pin tant aux ports d'entrée qu'en forêt.

[Traduit par la Rédaction]

Introduction

With globalization, effective and rapid quarantine of plant products is the most important measure available to prevent the dispersal of plant-parasitic nematodes. Any plant quarantine program requires accurate and rapid detection methods for the nematode species of concern. Many specific and sensitive biochemical or molecular techniques have been developed for the identification of species of plant-parasitic nematodes of regulatory concern (Boutsika et al. 2004; Powers 2004). Each is dependent on the ability to recover nematodes from infested plant materials. Traditionally the Baermann funnel technique has been used to extract nematodes for identification. The extraction process is not rapid (48 h) (Yang et al. 2003; Akbulut et al. 2006), is nonselective, in that all species of nematodes present in the wood are recovered, and in addition, the sampling is destructive. Therefore, there is an urgent need for a simple, effective, and rapid method to sample for the pinewood nematode (PWN; *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle).

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). The root knot nematode *Meloidogyne incognita* Chitwood, for example, is attracted by pea root tip exudates (Zhao et al. 2000). The animal-parasitic nematode *Heterorhabditis megidis* is attracted by (*E*)-β-caryophyllene released by damaged maize roots (Rasmann et al. 2005). Therefore, it may be possible to develop species-specific chemical attractants to sample and detect nematodes.

The PWN is the causal agent of the destructive pine wilt disease. It is native to North America and has been introduced to Japan, South Korea, Portugal, and China where it has caused irreparable damage to forest ecosystems (Kiyohara and Tokushige 1971; Dwinell and Nickle 1989;

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L. Zhao, W. Wei, L. Kang, and J. Sun.[1] State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing 100080, P. R. China.
X. Liu. Key Laboratory of Systematic Mycology and Lichenology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100080, P. R. China.

[1]Corresponding author (e-mail: sunjh@ioz.ac.cn).

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Mota et al. 1999; Mota and Vieira 2004; Sun 2005). More than 35×10^6 pines have already died from pine wilt disease in China (Yang et al. 2003). The direct economic losses caused by PWN in China are estimated at approximately $300 million with indirect economic losses exceeding $3 billion (Yang et al. 2003). Although there has been little damage to the forests of North America, the imposition of phytosanitary restrictions has resulted in serious economic consequences. Dwinell (1997) estimated that European phytosanitary restrictions resulted in an annual loss of $100 million in green lumber exports to Europe from the United States during the 1990s. The European Plant Protection Organization placed the pinewood nematode on the A1 list of quarantine pests in July 1985 (Smith 1985), and it has been listed as a quarantine pest in more than 40 countries (Rautapaa 1986; Dwinell 1997). However, such efforts have not been effective, because the nematode was recently found in Mexico and Portugal (Dwinell 1993; Mota et al. 1999).

There is no cure for pine wilt disease once a susceptible tree becomes infested with the pinewood nematode. Management of pine wilt disease is primarily limited to preventing the introduction and spread of the nematodes (Dwinell 1997). Any quarantine program that attempts to prevent the introduction or spread of pinewood nematode in wood packaging, coniferous chips, sawn wood, or logs requires accurate and rapid detection methods for the nematode (Evans et al. 1996; Dwinell 1997). Numerous methods, including polymerase chain reaction (PCR) and restriction fragment length polymorphisms (RFLPs) (Hoyer et al. 1998; Iwahori et al. 1998; Braasch et al. 1999; Burgermeister et al. 2005), random amplification of polymorphic DNA (RAPD) techniques (Braasch et al. 1995; Irdani et al. 1995), PCR-based diagnostics with species-specific primers (Kang et al. 2004; Matsunaga and Togashi 2004; Cao et al. 2005; Leal et al. 2005; and Takeuchi et al. 2005), have been developed for identification of pinewood nematode.

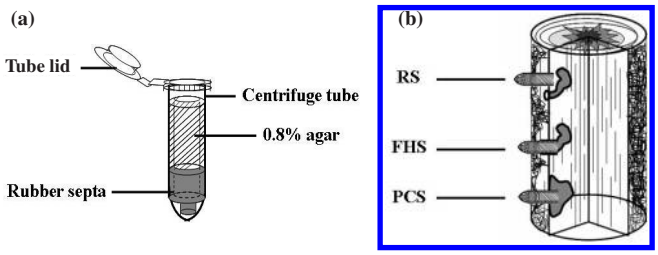
Third-stage dispersal juveniles of pinewood nematodes survive in dying pine trees and wood products, including sawn wood and logs, and aggregate in large numbers around the pupal chambers of *Monochamus* vectors (Mamiya 1972; Linit 1988). *Monochamus alternatus* Hope is the most important vector of *B. xylophilus* in Japan and China (Kobayashi et al. 1984; Sun 2005). In a previous study, we demonstrated that there was a specific ratio of α-pinene, β-pinene, and longifolene (1:2.7:1.1) in the larvae of *M. alternatus*. This terpene blend caused a very significant species-specific aggregation of third-stage dispersal juveniles (Zhao et al. 2007). The objective of this study was to develop a new rapid sampling and detection method for pinewood nematodes using these attractants.

Materials and methods

Artifical chambers and wild pines with third-stage dispersal juveniles of *B. xylophilus*

In the laboratory, third-stage dispersal juveniles were reared in artificial pupal chambers created in pine bolts (Aikawa and Togashi 1997; Jikumaru and Togashi 2003). A hole (2 cm deep and 1 cm diameter) was drilled in the centre of a cut end of healthy *Pinus massoniana* Lamb. bolts (5 cm long and 2.5 cm mean diameter), which were

Fig. 1. Sampling method design: (*a*) sampling tube and (*b*) different sampling methods in dying trees.



then set upright individually in polycarbonate containers (11 cm tall and 6.7 cm inside diameter) with quartz sand spread on the bottom. The bolts were autoclaved at 121 °C for 30 min, inoculated with the fungus, *Diplodia* sp., previously isolated from wilted *P. massoniana* from Ningbo, Zhejiang, that had been cultured on potato dextrose agar (PDA) medium at 25 °C in the dark for 1 week. Two weeks later when the mycelium covered the chambers, 500 propagative nematodes of *B. xylophilus* in 1 mL sterile water were inoculated into each bolt. The artificial pupal chambers were kept at 25 °C in the dark for 20, 30, or 40 days.

In the field, 50 dying trees (10–18 cm diameter at breast height (DBH)) and 50 logs, which were sawn 1 year previously (10–20 cm DBH), were both obtained from Ningbo, Zhejiang, in April 2006.

Terpenes, 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%) at a ratio of 1:2.7:1.1 dissolved in 40 μL *n*-hexane (Fisher Chemicals, 99%) at different concentrations.

Trap tubes consisted of a 2 mL centrifuge tube (Beijing Zohonice Science & Technology Development Co., Beijing, China) containing a rubber septum (Tongzhou Rubber Co., Beijing, China) with 0.8% agar (Beijing Shuangxuan Co., Beijing, China) solidified around the tube walls (Fig. 1*a*). Five hundred unbaited sampling tubes were covered and stored at –20 °C until needed. Before each experiment, 40 μL of *n*-hexane containing the concentrations of the attractant blend to be tested were 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.

Sampling in the artificial chamber

Experiment 1

Ten replicate trap tubes of each concentration of the attractant blend (0.0007, 0.007, 0.07, 0.7, or 7 mg) were inserted into the hole of artificial pupal chambers and left at 25 °C in the dark for either 24 or 48 h. The trap tubes were then removed from the pupal chambers, the agar was extracted into individual Petri dishes (5-cm diameter), and the inside wall of the tube was washed into the Petri dish with 2 mL distilled water to recover the nematodes. Nematodes were then counted microscopically.

Experiment 2

Ten chambers maintained for 20, 30, or 40 days after inoculation with PWN were sampled with the 7 mg lure for 24 h at 25 °C in the dark. The number of third-stage dispersal juveniles recovered per trap tube was determined as in experiment 1. The total number of third-stage dispersal juveniles in each chamber was determined by Baermann funnel technique.

Experiment 3

Pupal chambers inoculated 40 days previously were used to determine the effect of sampling duration on the recovery of PWN using trap tubes baited with the 7 mg lure. Ten replicates of each interval tested (0.5, 1, 1.5, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 h) were held at 25 °C in the dark. At the end of each time interval, the nematodes were recovered from the trap tubes and counted as noted above.

Sampling in dying trees

Three different sampling strategies were field tested to ascertain the most appropriate method (Fig. 1*b*). The mean density of third-stage dispersal juveniles was investigated around each pupal chamber (n = 10). The temperature ranged between 15 and 30 °C, and it rained occasionally during the 5 days that field bioassays were conducted.

Random sampling

In the dying trees, sampling holes (2 cm deep and 1-cm diameter) were drilled randomly around larval feeding holes of *M. alternatus* using an electric drill. During drilling, about 2 mL of water was used to cool the drill bit. Trap tubes with the 7 mg lures were inserted to sampling holes for 0.5, 1, 1.5, 2, 6, 12, 18, 24, 30, 36, or 42 h (n = 10 for each duration). Controls consisted of trap tubes treated with 40 μL *n*-hexane. Nematodes from the trap tubes were recovered and counted as noted above and then identified using real-time PCR (Cao et al. 2005).

Feeding hole sampling

Sampling holes (2 cm deep and 1-cm diam.) were drilled from sawyer larval feeding hole into the pupal chambers of *M. alternatus* in the xylem of dying trees using the technique noted above. The same experimental setup and evaluation techniques used for random sampling (RS) evaluation was used for feeding hole sampling (FHS).

Pupal chamber sampling

Pupal chamber sampling (PCS) was conducted by opening the U-shaped pupal chambers of *M. alternatus* in dying trees and placing the trap tubes directly into the chambers.

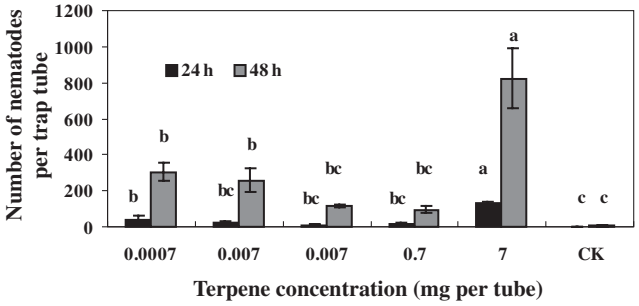
Sampling of sawn logs

Sampling holes (2 cm deep and 1-cm diameter) were drilled from sawyer larval feeding holes into the pupal chambers of *M. alternatus* in the xylem of logs cut 1 year previously. Ten replicate trap tubes baited with the 7 mg lures were inserted to sampling holes for 0.5, 1, 1.5, 2, 6, 12, 18, 24, 30, 36, or 42 h. The nematodes were recovered and identified as noted above.

Statistical analysis

Data analyses were carried out using the statistical software SPSS 11.0 for Windows. Differences among treatments with different concentrations were compared by ANOVA at $P \leq 0.05$. The correlation between number of nematodes recovered and the total third-stage dispersal juveniles present in the artificial chambers was tested by linear regression. The relationship between the number of nematodes recovered and the duration of sampling in the artificial chambers was determined by curvilinear regression.

Fig. 2. Attraction of pinewood nematodes to different lure concentrations in the artificial pupal chamber. Values are the means of 10 replicates for each treatment, and error bars are SEs. Bars with the same letters are not significantly different at $P > 0.05$.



Results

Sampling in the artificial chamber

Experiment 1

Third-stage dispersal juveniles were isolated from trap tubes across all lure concentrations tested (Fig. 2). There were significantly (5–10 times) more third-stage dispersal juveniles of *B. xylophilus* in trap tubes after 48 h than after 24 h. Significantly more dispersal juveniles were found in tubes with the highest terpene concentration (7 mg/tube). Because these results were highly repeatable, the 7 mg lure was used in all subsequent bioassays.

Experiment 2

To quantify the efficacy of trap tubes with 7 mg lures, we assessed the numbers of third-stage dispersal juveniles recovered from artificial pupal chambers containing a range of densities of PWN juveniles. Between 177 and 4195 dispersal juveniles were captured over the 24 h. The nematodes captured accounted for 7.14%–33.03% of the total nematodes present in the chambers. There was a strong correlation between the nematode densities in the artificial chambers and the numbers captured by the baited tubes with >90% of the variation explained by the regression equation (Fig. 3). Moreover, the regression was linear, indicating that the trap tube is effective across a wide range of nematode densities. However, no nematodes were captured in trap tubes when the initial number of nematodes in the chamber was <300.

Experiment 3

The relationship between the duration of sampling and the mean number of nematodes recovered per trap tube is shown in Fig. 4. Dispersal juveniles were found in the trap tubes infrequently (60%) after only 0.5 h and were recovered highly repeatedly after 2 h at 25 °C in the laboratory. The numbers captured increased rapidly after 16 h of sampling.

Fig. 3. Effect of pinewood nematode density in the artificial pupal chambers on trap tube captures of third-stage dispersal juveniles.

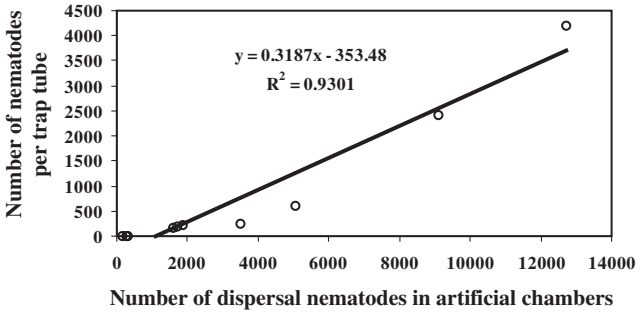
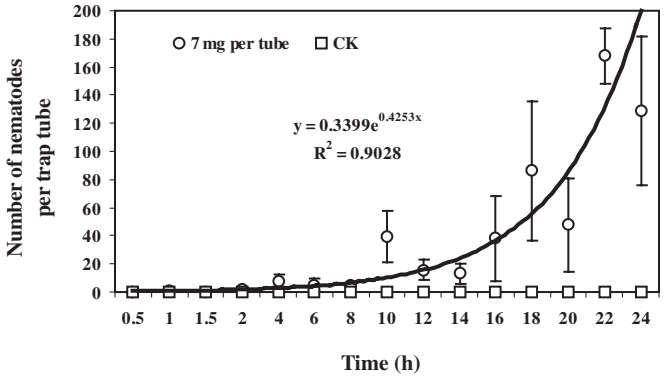


Fig. 4. Effect of trapping duration on captures of third-stage dispersal juveniles with trap tubes. Values are the means of 10 replicates for each treatment, and error bars are SEs.



More nematodes were captured with increasing duration of sampling.

Sampling in dying trees

The density of third-stage dispersal juveniles was 1073 ± 403/g wood (mean ± SE) around each pupal chamber (n = 10). The number of nematodes recovered from dying trees across increasing durations of sampling differed between sampling methods. There was no significant recovery of PWN when the trap tubes were placed randomly (RS) or in opened pupal chambers (PCS) (Fig. 5). There were only 5 ± 3 and 1 ± 1 dispersal juveniles attracted after 6 h by RS and 2 h by PCS, respectively. However, the number of nematodes per trap tube increased greatly when the sawyer larval feeding holes (FHS) were sampled. There were 11 ± 10 first dispersal juveniles attracted in 2 h and up to 276 ± 15 in 12 h. The proportion of sampled trees confirmed to be positive for PWN reached or exceeded 60% after 2 h of sampling with FHS and required 12 h with RS and 30 h with PCS, respectively. Nematodes were isolated with FHS from all 10 trees sampled within 12 h; RS required 36 h, of sampling to detect PWN in all trees, whereas nematodes were not recovered from four trees after 48 h of sampling with PCS.

Sampling of sawn logs

In logs, the first dispersal juveniles were caught after 6 h with FHS, and no PWN juveniles were recovered with the RS and PCS methods. Although the number of nematodes recovered with FHS was low and did not exceed 80 juveniles in any one sampling period across the full duration of sampling (Fig. 6), it succeeded in extracting nematodes from all samples after 12 h.

Fig. 5. Effect of trap tube placement in dying trees on the recovery of pinewood nematode juveniles: (*a*) nematodes per sampling tube for different sampling methods across time and (*b*) sampled ratios of different sampling methods. RS, random sampling; FHS, feeding hole sampling; PCS, pupal chamber sampling; CK, control. Values are the means of 10 replicates for each treatment, and error bars are SEs.

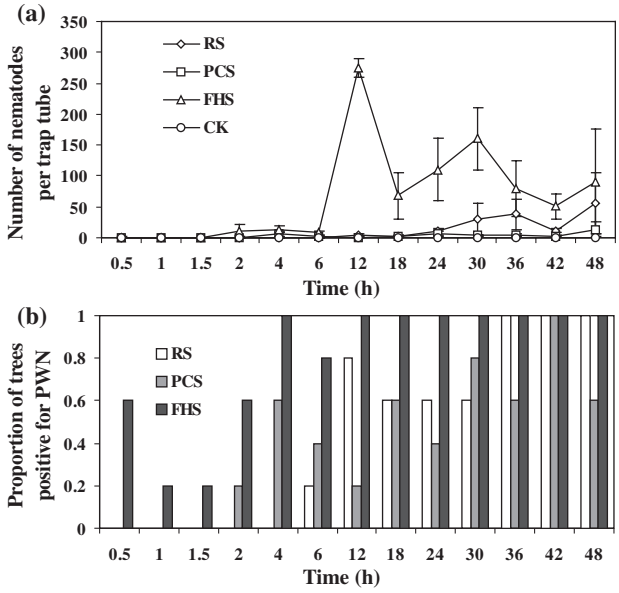
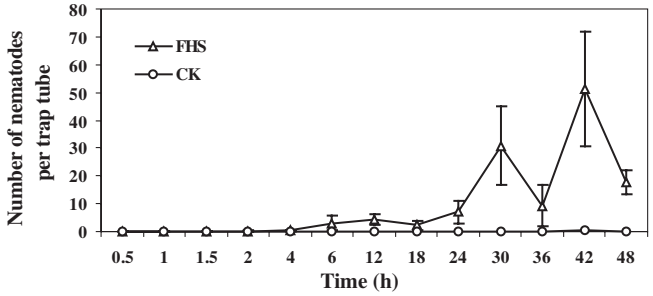


Fig. 6. Effect of trapping duration on the detection of PWN in 1-year-old logs. FHS, feeding hole sampling; CK, control. Values are the means of 10 replicates for each treatment, and error bars are SEs.



Discussion

Quarantine at ports-of-entry and field detection is believed to be both important strategies in integrated pest management. Pest sampling and population monitoring are the common basic techniques. Pheromones have been widely applied in sampling and population monitoring of insects (Weinzierl et al. 2005). Moreover, the numerous semiochemical-baited traps have been efficient for the mass trapping and even disruption of beetles and moths (Morewood et al. 2002; Weinzierl et al. 2005). However, there is no report of the application of attractants of plant-parasitic nematodes for sampling and population monitoring. We describe the first attractant-baited sampling trap for and

demonstrate the effective capture of PWN. The novel attractant-baited sampling technique may facilitate the rapid detection of plant-parasitic nematodes in infested wood for import quarantine and field monitoring as a part of integrated management of plant-parasitic nematodes.

The nonfeeding third larval dispersal stage of *B. xylophilus* is adapted to surviving unfavourable conditions prior to colonizing a new tree (Mamiya 1972, 1984). Large numbers of third-stage dispersal juveniles aggregate around the pupal chambers of the pine sawyer larvae in dying pine trees and wood products (Mamiya 1972). We utilized the ratio of terpenes present in pine sawyer larvae to develop a trap tube and demonstrate its effectiveness in detecting third-stage dispersal juveniles of pinewood nematodes within 2 h in the field.

During field sampling, both the numbers of nematodes captured and the number of trees confirmed to be positive increased with time, indicating that nematodes in the tissue surrounding the trap tube could continuously recognize chemical signals but move slowly to the trap tubes. The ability to quickly sample across the whole plant (tree bole, sawn log, or lumber), coupled with the chemotactic response of the nematode to the lure provides a more efficient method of sampling for PWN than the traditional Baermann-funnel technique, where there is an uneven distribution of nematode populations in the tissue. Detection of PWN in trees with this technique is dependent on the appropriate placement of the trap tubes (Fig. 5). Placement of trap tubes in association with larval feeding galleries (FHS) enhances capture. We suspect that the third-stage dispersal juveniles of *B. xylophilus* may move along the larvae tunnel to the trap tubes in response to a gradient of the trap volatiles. There is no advantage to placing the trap tube directly into pupal chambers (PCS), because fewer nematodes are captured. This may possibly be a function of a lack of a terpene gradient as a consequence of opening the pupal chamber. The success of FHS suggests that placement of baited trap tubes in sawyer beetle larval feeding galleries found in wood products, logs, or attacked trees will provide a useful tool for the detection for quarantine or field detection and dispersal monitoring of *B. xylophilus*. A minimum sampling time of 12 h was needed to isolate nematodes from all field samples. The highest concentration of attractants tested (7 mg/tube) provided the greatest efficacy for detecting PWN. It is also the maximum amount of terpenes that could be adsorbed into the rubber septum.

Multiple other species of saprophytic nematodes are generally isolated from the dying trees with the Baermann-funnel sampling techniques (Braasch et al. 2001). The trap tube methodology, however, is highly selective, with only *B. xylophilus* and a few *Bursaphelenchus mucronatus* Mamiya & Enda larvae recovered from the field samples. This selectivity eliminates the need to separate the *Bursaphelenchus* spp. from the wide array of saprophytic species traditionally recovered with Baermann funnels. This suggests that this sampling method could be useful in isolating those nematode species that require *M. alternatus* as a vector. Because *B. mucronatus* was also recovered in the field sampling, it is important that any nematodes recovered with this technique be definitively identified using morphological and preferably molecular techniques. Further testing of this sampling technique across different tree species, in sawn wood and wood packaging as well as in situations where different percentages of both *B. xylophilus* and *B. mucronatus* or different *Monochamus* vectors are present.

In addition, the sensitivity of this sampling method was limited by nematode population in wood products. The calibration curves and thresholds will have to be developed with the trapping tube for different species of pines, different states of decay, and different environmental conditions in the future for broad use.

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