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Rapid Diagnosis of the Infection of Pine Tree with Pine Wood Nematode (*Bursaphelenchus xylophilus*) by Use of Host-Tree Volatiles

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Supporting Information

ABSTRACT: Attraction of the *Bursaphelenchus xylophilus* nematode toward 18 volatiles of *Pinus* species was evaluated by a Petri-dish bioassay under laboratory conditions to develop a rapid diagnostic kit. Among these compounds, α-pinene, β-pinene, and camphor showed significantly higher attractiveness to *B. xylophilus* in both the reproductive and dispersal stages, whereas these compounds were not active against *Bursaphelenchus mucronatus*. A trap tube was developed as a diagnostic kit, which consisted of a tube filled with 0.8% agar and a matrix impregnated with an attractant: α-pinene, β-pinene, or camphor. All tested compounds attracted a significantly higher number of *B. xylophilus* than that in the control treatment. No significant difference was observed among attractants. The cotton-ball matrix was significantly more effective than the filter-paper matrix for attracting *B. xylophilus* in the artificial pupal chamber bioassay. In a bioassay with pine wood nematode (PWN)-infected pine tree logs, *B. xylophilus* was initially attracted after an 8 h trap period and the number of *B. xylophilus* increased with time. The trap tube using camphor and the cotton-ball matrix were most effective for attracting *B. xylophilus*. The semiochemical-based tube-trapping method is simple to use, requires minimal labor, and is economical and effective for detecting *B. xylophilus* living in host pine trees during field sampling.

KEYWORDS: *Bursaphelenchus xylophilus, pine wilt disease, trap tube, camphor*

INTRODUCTION

Pine wilt disease (PWD) is caused by the pine wood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle (Tylenchida: Aphenlenchoididae). This disease infects *Pinus* species, such as *Pinus densiflora*, *Pinus thunbergii*, *Pinus massoniana*, *Pinus luchuenses*, and *Pinus taiwanensis*.[1−4] PWD is native to North American countries[5,6] but afflicts serious damage to forest ecosystems in Korea, Japan, Portugal, and China.[7−11] It has caused irreparable damage to almost all of the pine forest ecosystems (642 million hectares) in Japan[4,7] and has damaged 1 million and 700 million hectares of pine forest in Korea and China, respectively.[12,13] The PWN has been listed as a quarantine pest in more than 40 countries.[9,14]

Until now, there has been no remediation technique for PWD once a susceptible pine tree becomes infected with PWN. Rapidly identifying pine trees infected by PWN is the most important issue to manage PWD. Helicopters and on-ground forecasting methods are currently used for monitoring PWN in Korea. After dying trees are located, they are cut and nematodes in the trees are extracted using the Baermann funnel method[15] to identify the *B. xylophilus* infection. The extracted nematodes are identified morphologically or by a molecular-biology-based method, such as polymerase chain reaction−restriction fragment length polymorphism.[16] Many causes, such as physiological disturbances, drought, forest fires, competition between plant species, and attacks by *Matsucoccus thunbergianae* Miller and Park (Homoptera: Margarodidae) may be responsible for the death of pine trees. However, it is quite difficult to determine the exact reason for the death of pine trees using the above-mentioned method, and field sampling and the Baermann funnel method take much time and labor to extract nematodes from dead pine tree chip samples. Additionally, these methods require special knowledge and techniques. Also, the reliability of the methods depends upon the ability to extract nematodes from infected pine trees.[17] Thus, a rapid *B. xylophilus* field sampling method is needed.

Semiochemicals and chemotaxis of *Bursaphelenchus* play an important role in their survival and dispersal. *Bursaphelenchus* should find out their insect vector using the semiochemical cue of the vector for moving out of dying and/or dead host trees. Once they have located and moved into the vector, they should move to a new host tree for survival. The third stage of dispersal juveniles *B. xylophilus* aggregating around the pupal chambers of the *Monochamus* vector (Coleoptera: Cerambycidae) was reported.[18] The PWN in the trachea of the vector insect is stimulated by β-myrcene from the pine tree and escape from the vector harboring *B. xylophilus*, which feeds on healthy pine trees.[19] The attractiveness of *Bursaphelenchus* species to different chemicals has been reported. *B. xylophilus* and *Bursaphelenchus mucronatus* are attracted to the sap of healthy host pines, although the active compounds have not been identified.[20] Several compounds containing an oleyl group and some terpenoids, such as farnesol, geraniol, myrcene, and phytol, are attractive to *B. xylophilus*.[21,22] *B. xylophilus* is attracted to the tree volatile mixture (α-pinene, β-pinene, and longifolene) of host pine trees and developed a rapid *B.*

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xylophilus sampling method using volatile components of *P. massoniana*.[17,22]

However, no reports on the attractiveness of volatiles from *P. densiflora* and *P. thunbergii* are available, which are predominant species in Asian countries and are very susceptible to *B. xylophilus*.[23] Therefore, we evaluated the attractiveness of 18 volatile components from *P. densiflora* and *P. thunbergii*, in addition to *P. massoniana*, for the reproductive (propagative) and dispersal types of the nematode. On the basis of the bioassay results on the attractiveness of those components, a rapid and simpler diagnosis kit was developed to be used in the pine tree forest for rapid testing of nematode infections in pine trees.

MATERIALS AND METHODS

Collection of PWNs. *B. xylophilus* and *B. mucronatus* colonies were obtained from Southern Forestry Research Center (SFRC; Jinju, Gyeongsangnamdo, Korea). They were maintained on a lawn of *Botrytis cinerea* cultured on potato dextrose agar (PDA) medium (Becton, Dickinson and Co., Sparks, MD) in the dark at 28 °C. The dispersal type of PWN was obtained by dissecting adult *Monochamus alternatus* beetles emerging from pine tree logs cut from *B. xylophilus*-infected dead pine trees. The nematodes were separated by the Baermann funnel method and used for a Petri-dish choice bioassay.

Chemicals. A total of 18 volatiles identified from *P. massoniana*, *P. densiflora*, and *P. thunbergii* were selected for the attractiveness test[17,24−28] and were purchased from Sigma-Aldrich (St. Louis, MO) (Table 1). A 0.1 mM solution of the compounds was prepared by serial dilutions in hexane (Sigma-Aldrich). The chemical concentrations were determined by referring to the results by Tominaga et al.[29]

Table 1. Volatiles from *Pinus* Species Used in This Study

compounds	purity (%)	*Pinus* species		
		P. massoniana	*P. densiflora*	*P. thunbergii*
α-pinene (**1**)	98	*a*	*a*	*a*
β-pinene (**2**)	99	*a*	*a*	*a*
camphene (**3**)	95	*a*	*a*	*a*
limonene (**4**)	97	*a*	*a*	*a*
myrcene (**5**)	95	*b*	*a*	*a*
3-carene (**6**)	90	*a*	*a*	*a*
terpinolene (**7**)	85	*b*	*a*	*a*
α-phellandrene (**8**)	95	*b*	*a*	*b*
α-terpinene (**9**)	95	*b*	*a*	*b*
γ-terpinene (**10**)	97	*b*	*a*	*b*
decane (**11**)	95	*a*	*b*	*b*
bornyl acetate (**12**)	97	*b*	*a*	*b*
α-terpineol (**13**)	96	*b*	*a*	*b*
borneol (**14**)	98	*b*	*a*	*b*
camphor (**15**)	96	*a*	*b*	*a*
thujone (**16**)	80	*b*	*a*	*b*
p-cymene (**17**)	99	*b*	*b*	*a*
α-humulene (**18**)	96	*b*	*a*	*b*

[a]Present in *Pinus* species. [b]Absent in *Pinus* species.

Attractiveness of Pine Tree Volatiles to PWN: Petri-Dish Choice Bioassay. *Experiment 1.* A Petri-dish choice bioassay was performed with both reproductive (propagative) and dispersal types of *B. xylophilus* to evaluate their attractiveness to each pine tree volatile. The 1 day starved and non-starved nematodes were also used to evaluate the effect of the nutrient condition. In the experiment, the starved nematodes were obtained by placing nematodes on a 0.5% water agar medium for 24 h before use. For the Petri-dish bioassay, three sets of Petri dishes were prepared. In each Petri dish, seven filter-paper discs (8 mm in diameter) (Toyo Roshi Kaisha, Tokyo, Japan) were placed on 0.5% water agar medium in the Petri dish (8 cm in diameter) (SPL Life Sciences, Pocheon, Korea) and one cotton ball (1.5 cm in diameter) was placed at the center of the medium. The 0.1 mM test compound solution was selected randomly and applied (10 μL) to the six filter-paper discs, and hexane (10 μL) was applied on a filter-paper disc as a control. Therefore, of the 18 volatile compounds, 6 compounds were randomly selected and tested on one Petri dish. A total of 1 mL of *B. xylophilus* culture solution, containing ca. 1000 reproductive-type *B. xylophilus* or ca. 200 dispersal-type *B. xylophilus*, was applied to the cotton ball. The Petri dish was covered with a lid, sealed with Parafilm to maintain humidity, and incubated at 24 °C for 24 or 48 h in the dark.[30] The filter-paper disc and the agar medium (1.2 cm in diameter) under the filter paper were detached from the associated agar medium and demounted in a Petri dish (60 mm in diameter) filled with distilled water to count the number of *B. xylophilus* attracted to each treatment. The filter-paper disc was washed 3−4 times with sterile water. The number of *B. xylophilus* separated from the filter-paper disc was counted under a dissection microscope. The experiment was replicated 3 times.

Experiment 2. On the basis of the results of experiment 1, α-pinene (**1**), β-pinene (**2**), and camphor (**15**) were selected to compare *B. xylophilus* and *B. mucronatus* attractiveness. Because of the coexistence of *B. xylophilus* in the dead pine tree, *B. mucronatus* was also used in this experiment. The same Petri-dish choice bioassay method was performed. Four filter-paper discs (8 mm in diameter) were placed on a 0.5% water agar medium in a Petri dish. α-Pinene, β-pinene, and camphor dissolved in hexane (10 μL at 0.1 mM) were applied to each filter-paper disc along with 10 μL of hexane as a control. The treatment positions were changed randomly. A total of 1 mL of *B. xylophilus* or *B. mucronatus* solution containing ca. 1000 nematodes was applied to a cotton ball and placed at the center of the medium. After 24 and 48 h of incubation, the number of attracted nematodes was counted as before. This experiment was performed 5 times.

Attractiveness of Pine Tree Volatiles to PWN in the Pine Tree Log. *Experiment 1: Artificial Pupal Chamber Bioassay (Non-choice Test).* An artificial pupal chamber assay was performed with reproductive-type *B. xylophilus* using a trap tube in pine tree log to evaluate the attractiveness of the compounds (**1**, **2**, and **15**) selected from the Petri-dish bioassay.

The trap tube consisted of a matrix prepared with the compound and 0.8% water agar in a 2 mL centrifuge tube (BioScience, Inc., Salt Lake City, UT). The agar was filled to 2 mm below the opening of the centrifuge tube, and half of the matrix was inserted into solidified agar. A cotton ball (0.5 cm in diameter) and a filter-paper strip (0.5 × 2.5 cm) were used as matrices to evaluate the matrix efficacy.

An artificial pupal chamber was prepared following the method by Aikawa and Togashi.[31] A hole (3−4 cm deep and 1 cm in diameter) was drilled at the center of a cut end of healthy *P. thunbergii* (5 cm height and 2.5 cm in diameter). The logs were autoclaved at 121 °C for 15 min and then incubated at 25 °C in the dark for 14 days after inoculation with *B. cinerea* as a food source for *B. xylophilus*. After 2 weeks, when the fungal mycelia covered the artificial pupal chamber, nematodes were inoculated (ca. 1000 reproductive *B. xylophilus* in 1 mL sterile water) in the chamber and the pine wood logs were again held at 25 °C in the dark until used in the experiment.

Before the trap tube was inserted into the artificial pupal chamber in the pine tree log, the attractant (**1**, **2**, and **15**) dissolved in hexane (10 μL at 0.1 mM) was applied to the cotton ball or filter paper in each trap tube. As a control, the cotton ball or filter paper was treated with 10 μL of hexane alone. After the trap tube was inserted, the pine tree logs were kept at 25 °C in the dark for 24 or 48 h. The trap tube was then removed from the pupal chamber, and the matrix and agar medium of the trap tube was extracted into a Petri dish (6 cm in diameter) and sliced into small pieces. The inside wall of the tube and the matrix were washed with distilled water 3 times into a Petri dish to recover the nematodes, which were counted microscopically. The whole experiment was conducted with three replications.

Table 2. AI of the Tested Compounds to *B. xylophilus* in the Petri-Dish Bioassay

	AI[a] (%; mean ± SD; n = 3[b])					
	reproductive type				dispersal type	
	starved		non-starved		non-starved	
compound	24 h	48 h	24 h	48 h	24 h	48 h
α-pinene (**1**)	41.21 ± 1.43 a	29.90 ± 1.26 bc	22.40 ± 3.98 abc	20.82 ± 1.70 cd	38.82 ± 2.46 ab	39.54 ± 2.55 abc
β-pinene (**2**)	44.86 ± 0.38 a	64.18 ± 0.60 a	29.70 ± 7.32 ab	43.80 ± 4.49 a	49.61 ± 2.49 ab	40.42 ± 0.57 ab
camphene (**3**)	0.22 ± 0.39 i	2.79 ± 0.45 defg	18.92 ± 2.08 abcd	4.53 ± 4.49 fgh	5.02 ± 4.22 bc	2.58 ± 1.57 de
limonene (**4**)	1.36 ± 0.88 hi	0.26 ± 0.26 g	6.54 ± 2.52 cdef	5.30 ± 0.51 efgh	2.19 ± 2.87 bc	3.45 ± 1.28 de
myrcene (**5**)	2.27 ± 1.41 ghi	0.63 ± 0.31 fg	11.47 ± 7.82 bcdef	4.37 ± 1.96 fgh	0.75 ± 1.30 abc	3.17 ± 2.16 de
3-carene (**6**)	1.38 ± 1.24 hi	0.89 ± 0.59 efg	4.06 ± 4.36 ef	9.06 ± 4.01 defgh	2.54 ± 0.63 bc	5.82 ± 2.27 bcde
terpinolene (**7**)	18.85 ± 1.03 bc	15.83 ± 7.26 bcd	22.78 ± 7.47 abc	23.19 ± 7.93 bcd	25.83 ± 22.41 abc	29.50 ± 20.45 abcd
α-phellandrene (**8**)	12.62 ± 2.15 bcde	33.77 ± 11.77 b	16.00 ± 4.22 abcde	15.31 ± 4.47 cdef	21.90 ± 2.71 abc	7.25 ± 6.34 cde
α-terpinene (**9**)	4.42 ± 1.51 efgh	11.24 ± 7.24 defg	19.94 ± 6.96 abcd	26.57 ± 3.43 abc	10.85 ± 9.72 abc	6.67 ± 11.55 de
γ-terpinene (**10**)	22.85 ± 5.69 b	6.50 ± 3.36 defg	15.41 ± 5.06 abcde	2.78 ± 2.84 h	15.63 ± 21.38 abc	14.97 ± 13.83 bcde
decane (**11**)	16.50 ± 1.81 bd	13.27 ± 4.84 bcde	6.41 ± 1.56 cdef	17.67 ± 4.14 cdef	7.83 ± 8.73 bc	22.53 ± 6.03 abcd
bornyl acetate (**12**)	9.17 ± 2.98 cdefg	3.89 ± 4.69 defg	11.38 ± 2.97 bcdef	2.86 ± 4.01 h	8.05 ± 13.94 bc	0.00 ± 0.00 e
α-terpineol (**13**)	20.71 ± 2.82 bc	0.90 ± 0.80 fg	18.43 ± 9.60 abcde	1.83 ± 1.34 h	18.18 ± 0.68 abc	15.09 ± 1.33 abcde
borneol (**14**)	1.35 ± 0.91 hi	2.17 ± 2.33 defg	1.95 ± 2.25 f	22.79 ± 1.94 bcd	18.01 ± 4.26 abc	17.45 ± 0.76 abcde
camphor (**15**)	51.31 ± 4.26 a	70.68 ± 5.83 a	33.74 ± 7.49 a	41.34 ± 2.66 ab	49.30 ± 9.81 a	54.03 ± 2.52 a
thujone (**16**)	9.52 ± 1.78 cdefg	4.47 ± 0.97 defg	20.21 ± 9.72 abcd	3.04 ± 2.61 gh	5.87 ± 2.71 c	2.99 ± 5.18 de
p-cymene (**17**)	9.83 ± 3.08 cdefg	13.29 ± 3.33 bcde	9.66 ± 10.49 cdef	5.56 ± 2.84 efgh	1.27 ± 2.19 c	2.3 ± 2.28 de
α-humulene (**18**)	3.22 ± 1.83 fghi	6.92 ± 2.53 defg	10.20 ± 5.95 bcdef	13.01 ± 5.04 cdefg	4.17 ± 5.29 bc	5.18 ± 2.40 cde
control	9.45 ± 5.31 def	6.14 ± 7.76 defg	6.93 ± 3.14 def	12.06 ± 2.44 def	4.73 ± 6.41 c	8.99 ± 14.18 de
statistics	$F_{[8,14]}$ = 45.12	$F_{[8,14]}$ = 26.94	$F_{[8,14]}$ = 7.04	$F_{[8,14]}$ = 22.80	$F_{[8,14]}$ = 5.43	$F_{[8,14]}$ = 7.02

[a]Means within a column followed by the same letters are not significantly different (p = 0.05; Tukey–Kramer HSD test). [b]Replicates of the control were 9.

Table 3. AI of the Reproductive-Type *Bursaphelenchus* Species

	AI[a] (%; mean ± SD; n = 5)			
	B. xylophilus		*B. mucronatus*	
compound	24 h	48 h	24 h	48 h
α-pinene	38.41 ± 5.37 a	39.42 ± 1.54 a	35.77 ± 18.20	32.40 ± 17.78
β-pinene	34.92 ± 4.34 a	29.30 ± 2.03 b	24.30 ± 9.76	25.59 ± 15.03
camphor	26.17 ± 3.69 b	31.28 ± 2.08 b	19.35 ± 11.95	18.45 ± 9.69
control	0.51 ± 0.69 c	0 c	20.59 ± 8.98	23.57 ± 12.62
statistics	$F_{[3,16]}$ = 155.78	$F_{[3,16]}$ = 1534.72	$F_{[3,16]}$ = 1.58	$F_{[3,16]}$ = 0.85
	p < 0.0001	p < 0.0001	p = 0.23	p = 0.49

[a]Means within a column followed by the same letters are not significantly different (p = 0.05; Tukey–Kramer HSD test).

Experiment 2: PWN-Infected Pine Tree Log Bioassay (Choice Test). The attractiveness test was performed on a died tree log to evaluate the trapping efficacy of each attractant and the trap tube on a *B. xylophilus*-infested pine tree. A dead *P. thunbergii* specimen, because of *B. xylophilus* infestation, was obtained from SFRC. Four holes (3–4 cm deep and 1 cm in diameter) were drilled around the perimeter at 10 cm intervals (8 cm in height and ca. 10 cm in diameter). A trap tube with a cotton ball as the matrix was used for this experiment. Attractant (**1**, **2**, and **15**) solution in hexane (15 μL at 0.1 mM) was applied to the cotton ball, and 15 μL of hexane was used as a control. The trap tubes with attractants and hexane (control) were inserted into the four holes of the pine tree log, and the log was then kept at 25 °C in the dark for 0.5, 1, 4, 8, 24, and 48 h. The position of the trap tubes was determined randomly for each replication. At each time, the trap tubes were removed from the pine tree logs, the number of attracted nematodes was counted as described above, and then the number of *B. xylophilus* was counted. This experiment was repeated 5 times.

Statistical Analysis. An attractiveness index (AI) was used to compare the attractiveness of each compound in the Petri-dish bioassay experiments. The values were calculated by dividing the number of attracted PWN to each compound by those attracted to all treatments in the Petri dish. The AI value was transformed to arcsine square root values for an analysis of variance (ANOVA). The means were compared and separated by the Tukey–Kramer honestly significant difference (HSD) test at p = 0.05. The effect of the compounds and nutritional condition was compared to a two-way ANOVA.

In the artificial pupal chamber bioassay and the pine tree log bioassay, the number of attracted *B. xylophilus* (χ) was transformed into log(χ + 1) and the means were compared and separated by the Tukey–Kramer HSD test at p = 0.05. The effect of compounds and matrices was compared to a two-way ANOVA. All statistical analyses were conducted using JMP, version 9.0.2 (SAS Institute, Inc., Cary, NC).

RESULTS

Attractiveness of Pine Tree Volatiles to PWN: Petri-Dish Choice Bioassay. *Experiment 1.* Of the 18 pine tree volatiles, camphor (**15**) had the highest AI value, regardless of the nutritional condition or type of *B. xylophilus* (Table 2). α-Pinene (**1**) and β-pinene (**2**) also showed relatively high attractiveness, but the values were not statistically different

from camphor, except for the non-starved reproductive-type *B. xylophilus* at 24 and 48 h of treatment. Terpinolene (**7**) showed only moderated attractiveness, whereas the other volatiles showed weak attractiveness, with no statistical difference from the control. From this result, *α*-pinene (**1**), *β*-pinene (**2**), and camphor (**15**) were selected as attractants for testing trap tube efficacy.

Significant main effects were observed for the compounds and nutrition and for the compound × nutrition interaction at 24 h. Non-starved nematodes were attracted more to the chemicals. At 48 h of treatment, the main effect for compounds and the compound × nutrition interaction was significant but the main effect for nutrition was not significant.

Experiment 2. *α*-Pinene (**1**), *β*-pinene (**2**), and camphor (**15**) had significantly higher attractiveness to *B. xylophilus* than that of the control in the Petri-dish bioassay for the attractiveness. However, *B. mucronatus* did not show any preference for the compounds tested (Table 3).

Attractiveness of Pine Tree Volatiles to *B. xylophilus* in the Pine Tree Log. *Experiment 1: Artificial Pupal Chamber Bioassay.* The three tested compounds (**1**, **2**, and **15**) attracted significantly more *B. xylophilus* than that of the control, regardless of the treatment time or matrix types (one-way ANOVA; filter-paper type, 24 h, $F_{[3,8]} = 5.54$, $p = 0.024$; 48 h, $F_{[3,8]} = 19.00$, $p < 0.001$; cotton-ball type, 24 h, $F_{[3,8]} = 15\,014.16$, $p < 0.0001$; 48 h, $F_{[3,8]} = 43.46$, $p < 0.0001$) (Figure 1).

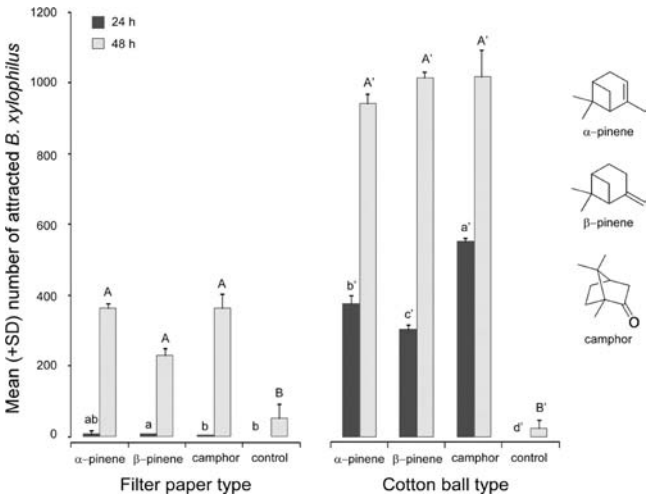

Figure 1. Mean (+SD; $n = 3$) numbers of reproductive *B. xylophilus* attracted to the trap tube impregnated each compound in filter-paper or cotton-ball matrix. Bars with the same letter are not significantly different (Tukey–Kramer HSD test at $p = 0.05$).

Different attractiveness occurred after 24 h of treatment, among the three tested compounds. However, the difference in the attractiveness was not shown after 48 h of treatment. Significant main effects were observed for compounds and matrices and the compound × matrices interaction at 24 and 48 h (two-way ANOVA). The cotton matrix was more effective for trapping.

Experiment 2: PWN-Infected Pine Tree Log Bioassay. Nematodes were trapped for the first time 8 h after inserting trap tubes in the PWN-infected pine tree log bioassay (Table 4). Camphor (**15**) showed significantly higher attractiveness among the tested compounds. The mean number of *B. xylophilus* caught in the camphor-baited trap was almost 10 times greater than that of the other compounds tested.

DISCUSSION

Nematodes are known to use semiochemical cues for finding their host for food, vector for movement, and conspecies for reproduction.[18,32,33] In this study, 18 host volatiles were tested for their attractiveness to two types of *B. xylophilus* (reproductive and dispersal). *α*-Pinene, *β*-pinene, and camphor were highly attractive to *B. xylophilus*, and the attractiveness of these three compounds was consistent throughout the experiments. However, the reported attractiveness of the tested compounds to *B. xylophilus* was inconsistent. *β*-Myrcene has been reported to be a strong attractant,[29,34] but it did not show any attractiveness in our experiments. *β*-Pinene has been reported as a weaker attractant[34] and as a repellent.[29] The mixture of *α*-pinene, *β*-pinene, and longifolene was attractive to *B. xylophilus*, whereas camphor was not attractive,[17] which was the most attractive compound in our experiments. These differences might be attributed to geographic differences among *B. xylophilus* populations or physiological differences. Different life stages of *B. xylophilus* (dispersal and propagative) are attracted to terpene mixtures in different ratios.[17]

The nutritional condition of the nematodes affects their chemotaxis.[35,36] In our Petri-dish bioassay, the AI values of the three active compounds (**1**, **2**, and **15**) tested with starved *B. xylophilus* were higher than those with non-starved *B. xylophilus* at 24 h of treatment, whereas the result was reversed at 48 h of treatment. A significant interaction was observed for attractiveness between the compounds and nutritional condition. The long starvation during the experiment probably changed the physiological status of *B. xylophilus* and, thus, affected their attraction to the compounds. However, the exact reason for the different attractiveness results according to the nutritional condition remains unclear.

α-Pinene and *β*-pinene are major volatile components of *Pinus*.[17,24−28] Camphor has been reported as a volatile component of *P. massoniana* and dying *P. thunbergii* and as a major component emitted from *M. alternatus* larvae.[17,28] In this

Table 4. Mean Numbers of *B. xylophilus* Attracted to the Trap Tube Baited with Each Compound in the Cotton-Ball Matrix in the PWN-Infected Pine Tree Log Bioassay

	mean (±SD; $n = 5$) number of trapped *B. xylophilus*[a]					
compound	0.5 h	1 h	4 h	8 h	24 h	48 h
α-pinene (**1**)	0	0	0	18.6 ± 6.3 b	149.8 ± 146.6 b	47.8 ± 44.4 b
β-pinene (**2**)	0	0	0	6.6 ± 5.5 c	136.2 ± 129.3 b	103.6 ± 119.2 b
camphor (**15**)	0	0	0	107.0 ± 25.0 a	1838.0 ± 452.7 a	4490.0 ± 2611.9 a
control	0	0	0	0 d	3.8 ± 4.15 c	2.2 ± 1.9 c
statistics				$F_{[6,16]} = 56.81$	$F_{[6,16]} = 36.39$	$F_{[6,16]} = 33.98$

[a]Means within a column followed by the same letters are not significantly different ($p = 0.05$; Tukey–Kramer HSD test).

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study, *B. xylophilus* was attracted to α-pinene, β-pinene, and camphor, whereas *B. mucronatus* was not. Different host preferences of *B. xylophilus* and *B. mucronatus* have been reported.[20] This suggests that *B. xylophilus* and *B. mucronatus* use different host volatiles for survival and dispersal.

Although α-pinene, β-pinene, and camphor were attractive to *B. xylophilus*, their attracting strength differed according to the bioassay method. α-Pinene showed the highest AI value in the Petri-dish bioassay. However, lower numbers of *B. xylophilus* were attracted to the α-pinene-baited trap tube than to the camphor-baited trap in the PWN-infected pine tree log bioassay. Although camphor showed the lowest AI value in the Petri-dish bioassay, the camphor-baited trap tube attracted the greatest number of *B. xylophilus* in the PWN-infected pine tree log bioassay. Large numbers of *B. xylophilus* were attracted to the three compounds in the artificial pupal chamber bioassay, but no significant difference was observed among the compounds. The different environmental circumstances of the bioassay (agar, autoclaved pine tree, and dead pine tree) might have resulted in the different diffusion results of the volatiles, and it would lead to different attracting strengths in the bioassay. The bioassay conditions of the PWN-infected pine tree log were closest to the natural condition. Therefore, camphor would be an effective attractant for *B. xylophilus* sampling.

A rubber septum is useful as a volatile matrix in a trap tube.[17,22] In this study, filter paper and cotton ball were tested as the volatile matrices. The results revealed that these two matrices were effective enough to detect *B. xylophilus* infection and that the cotton-ball matrix was more efficient. The interaction between the compound and matrix for attractiveness was significant and synergistic, suggesting that the matrix type was also one of the important factors when sampling *B. xylophilus*.

The first detection of *B. xylophilus* was 8 h after insertion of the trap tube in the PWN-infected pine tree log bioassay. Zhao et al.[22] reported that first detection of *B. xylophilus* in a dying tree was within 2 h when sampling was performed at a feeding hole, whereas 6 h was required for random sampling. Our result was similar to this random sampling result.[22] It could be expected that first detection might be hastened by sampling at a feeding hole. The PWN-infected pine tree log bioassay was performed in early March, while the *B. xylophilus* juvenile stage changed from reproductive type to dispersal type between February and May.[37] Therefore, *B. xylophilus* attracted to the trap tube may have been a mixture of the both reproductive and dispersal types.

As a part of PWD management, the identification of *B. xylophilus* has been intensively investigated.[16] However, only a few studies have been conducted on *B. xylophilus* sampling methods. The terpene-baited trap tube method[17,22] and loop-mediated isothermal amplification method[38] have been reported thus far. Although these sampling methods have been suggested as easy and time-saving, our newly developed diagnostic kit has the following advantages: it is easy to prepare, attracts any stage (reproductive or dispersal) of *B. xylophilus* with a single compound (e.g., camphor), is economical using a cheap cotton ball as a attractant matrix, and is time-saving compared to those of the traditional Baermann funnel method.

In conclusion, the diagnostic kit developed in this study showed good efficiency for detecting *B. xylophilus* under laboratory conditions. A field evaluation of the diagnostic kit remains to be conducted for an assessment of practical use.

■ ASSOCIATED CONTENT

Supporting Information

Results of a two-way ANOVA of the effect of compounds, nutritional condition (starved and non-starved), and their interaction on the attractiveness of the reproductive *B. xylophilus* (Table S1), results of a two-way ANOVA of the effect of compounds, matrices, and their interaction on trapping *B. xylophilus* (Table S2), and tube trap design and sampling method for the PWN-infected pine tree log bioassay (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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