A new convenient assay method for nematode attractants was developed. Using this method, the attracting activity of volatile components in a pine, *Pinus densiflora*, was examined for the pine wood nematode, *Bursaphelenchus xylophilus*, which causes serious damage to pine trees in Japan. Among the volatile components, β-myrcene showed the strongest attracting potency. This compound was assumed to play an important role in the transmigration of the nematode from the sawyer to the pine tree and for the movement of the nematode inside the pine wood.

The pine wood nematode, *Bursaphelenchus xylophilus* Mamiya et Kiyohara,11 causes serious damage to Japanese red and black pines, *Pinus densiflora* and *P. thunbergii*. The life cycle of the nematode has been described previously in detail.2) Under favorable circumstances the nematode multiplies very rapidly and dispersal larva development occurs in response to starvation or low temperature. Of the developmental stages of the nematode, the dispersal 4th stage larva (LIV) is transmitted by the pine sawyer, *Monochamus alternatus* Hope,3,4) from dead to living pine trees, which is therefore responsible for the infection. It was proposed that specific substances present in pine trees might have an attracting potency for the nematode, so the nematode would transmigrate to pine trees from the sawyer.

As attractants of some species of nematodes, several compounds,5~14) such as carbon dioxide, amino acids, sugars, fatty acids, cyclic AMP and inorganic salts, have been reported. It is clear that nematodes respond to stimuli released by natural sources. However, whether they act as attractants or arrestants has not been clarified and species specific attractants for nematodes have not yet been identified. Although there are several assay methods for investigating nematode attractants,5,6,9~13) none of them seems to be suitable for testing volatile constituents. Since we assume that the attractants for the nematode are compounds which are to some extent volatile, it is necessary to devise a new testing method for such compounds.

In this paper, we describe a new convenient assay method, especially applicable for volatile nematode attractants. This paper also reports the results of an attracting test on volatile components of a pine tree for the nematode, *B. xylophilus*.

**MATERIALS AND METHODS**

**Nematode.** The nematode, *B. xylophilus*, was separated from a nematode-infected pine tree in Matsuyama city, Ehime Prefecture, and cultured on a fungal mat of *Botrytis cinerea* grown on a potato dextrose agar medium at 25°C after surface sterilization with merthiolate.15) The cultured nematodes were collected by the Baermann funnel technique60 from the medium. Dispersal 4th stage larvae of the nematode (LIV) were obtained from the tracheae of fresh sawyers within 1 day after emergence from the tree.

**Extraction of volatile components from pine wood.** New shoots (1 kg) of pine tree, *Pinus densiflora*, were subjected to steam-distillation, and an oily distillate (3.0 g) was obtained. Then, the volatile components (0.8 g) were separated by introducing nitrogen gas into the oily distillate at room temperature and trapped at −30°C.

**Identification of volatile components.** Each volatile component was identified by comparing its retention time on
GC with that of an authentic specimen and also by comparing its mass spectrum on GC-MS with that of an authentic specimen or the reported spectrum.\textsuperscript{17} GC was conducted with a Shimadzu 5-AP gas chromatograph equipped with a flame ionization detector and the analysis conditions were as follows: column, glass column (3 mm x 2 m) packed with 1.5\% Silicone GE SE-30 or 10\% PEG 20M-TPA (on Chromosorb W AW-DMCS); carrier gas, nitrogen (60 ml/min; inlet pressure, 3.0 kg/cm\(^2\)); column temperature, 65~150\(^\circ\)C (program rate: 3.0\(^\circ\)C/min); and injection temperature, 200\(^\circ\)C. GC-MS was performed with a Shimadzu LKB-9000 gas chromatograph-mass spectrometry and the conditions for GC were similar to those for GC. Peaks 6 and 7 were separated on a column of silica gel treated with 20\% AgNO\(_3\) (eluent: hexane containing 1\% ether) and then analyzed, because they showed close retention times on GC (Fig. 1). The main fragment ions of each peak in Fig. 1 were as follows: GC-MS \(m/z: 136(M^+), 93, 77\) for 1 (t\(_R\) 5.7 min); \(m/z: 136(M^+), 93, 69\) for 2 (t\(_R\) 6.2 min); \(m/z: 136(M^+), 93, 69\) for 3 (t\(_R\) 7.6 min); \(m/z: 136(M^+), 93, 69\) for 4 (t\(_R\) 8.6 min); \(m/z: 136(M^+), 93, 79\) for 5 (t\(_R\) 9.4 min); \(m/z: 136(M^+), 93, 68\) for 6 (t\(_R\) 10.0 min); and \(m/z: 136(M^+), 93, 77\) for 7 (t\(_R\) 10.3 min).

**Assay procedure.** The assay apparatus consisted of T-glass tubing containing 9 ml of 1.5\% agar, as shown in Fig. 2, and a siphon that created a constant air stream in the T-tubing through three needles of different lengths, 6 cm, 3 cm and 1 cm, with which it was possible to produce a vapor concentration gradient of a sample. A test compound was absorbed with a small boiling stone, to allow gradual vaporization of the sample during the test. The stone with the absorbed sample was placed on one side of the agar in the T-tubing (sample side). After pre-airing for 20 min, the nematodes (ca. 10,000 nematodes in a minimum volume of water) were placed in the middle of the T-tubing and excess water was removed with a small cotton ball. After airing for 3 hr in a dark chamber at 26\(^\circ\)C, the nematodes, which had moved away from the center to either side of the T-tubing, were collected individually from each side and counted under a microscope (\(\times 40\)). The attracting activity was determined with the following equation:

\[
\text{Attracting activity} = \frac{A - B}{B} \times 10
\]

where \(A\) is the number of nematodes on the sample side and \(B\) that on the control side.

**RESULTS AND DISCUSSION**

Several techniques have been developed for measuring the movement of nematodes in response to chemical stimuli. Most of them are orienting methods and are not suitable for volatile attractants or repellents.\textsuperscript{14} We de-

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**Fig. 1.** GC Spectrum of Volatile Components of \(P.\ densiflora\) on 1.5\% Silicone GE SE-30.

**Fig. 2.** Apparatus for the Nematode Attracting Test.
1, test compound; 2, needle; 3, silicon rubber stopper; 4, 1.5\% agar; 5, nematodes.
β-Myrcene, as a Nematode Attractant

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Fig. 3. Attracting Activity of Monoterpenes for Cultured B. xylophilus.

○, β-myrcene; ●, 3-carene; △, α-pinene; ×, β-pinene; ▲, l-limonene.

veloped a new convenient assay method which was especially applicable for volatile compounds. Using this method, we investigated attractants for the pine wood nematode, B. xylophilus.

The volatile mixture obtained from shoots of the pine, Pinus densiflora, considerably attracted the cultured nematode. The volatile constituents were analyzed by GC and GC-MS. Consequently, 7 kinds of monoterpenes were identified as main components, i.e. α-pinene (1), camphene (2), β-pinene (3), β-myrcene (4), 3-carene (5), l-limonene (6) and β-phellandrene (7) (Fig. 1). These compounds were almost the same as those reported in the previous paper, except for their smaller amounts. Ikeda et al. reported the attractiveness of such monoterpenes for the pine sawyer. However, there was no attempt to examine these compounds as attractants for the nematode.

A nematode attracting test was carried out with each authentic specimen of the compounds identified, except for β-phellandrene.

In Fig. 3, the attracting activity is plotted as a function of the sample volume on a semi-logarithmic scale. Of the tested compounds, β-myrcene showed the strongest activity; the highest activity was observed at a dose of 2 μl, however, it decreased significantly with a volume of more than 3 μl. This dose-response pattern suggested that the most appropriate vapor concentration might occur in nature to attract the nematode. 3-Carene, α-pinene and β-pinene seemed to have weak activity, and l-limonene and camphene were not attractive at all. The activity of β-phellandrene was examined using the material isolated from the pine, P. rigida, because an authentic specimen was not available. It probably has a weak or no attracting effect. Several natural and synthetic monoterpenic derivatives, such as alcohols, aldehydes and amines, were tested similarly. But, although citronellol had a considerable effect, all of them were less attractive than β-myrcene. Citronellal acted rather as a strong repellent for the nematode. Considering the structure-activity relationship, the chain structure might play an important role in the activity.

Table I. Attracting Activity of Monoterpenes for B. xylophilus

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>Compound</th>
<th>Propagative form (All stages)</th>
<th>Dispersal form (L₁V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>α-Pinene</td>
<td>16</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>Camphene</td>
<td>—1</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>β-Pinene</td>
<td>5</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>β-Myrcene</td>
<td>33</td>
<td>67</td>
</tr>
<tr>
<td>5</td>
<td>3-Carene</td>
<td>10</td>
<td>17</td>
</tr>
<tr>
<td>6</td>
<td>l-Limonene</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>β-Phellandrene</td>
<td>6</td>
<td>—</td>
</tr>
</tbody>
</table>

* Peak numbers in this table correspond to the numbers in Fig. 1.
* Tested at 3 μl.
* Separated from the pine tree, P. densiflora.
* As camphene was a solid, the test was carried out with 5 mg.
* Not tested.
In nature, the nematode larval form which participates in the transmigration from the sawyer to a pine tree is the dispersal 4th stage larva.\textsuperscript{4)} Dispersal 4th stage larvae obtained from the tracheae of fresh sawyers were used for the attracting test. Although the 4th larva is morphologically different from all other developmental stages of the nematode, the results obtained were consistent with those for the cultured nematode. Since the dispersal 4th stage larvae showed little mobility during the attracting test, the attraction values were great for all the samples, as compared with in the case of the cultured nematode (Table I).

During the life cycle of the nematode, unsaturated fatty acids secreted by the larvae of the sawyer make the nematodes (dispersal 3rd stage larvae) aggregate around the pupal chamber of the sawyer in wilted pine wood.\textsuperscript{20)} Then, the molting to dispersal 4th stage larvae might be induced by the non-residual matter secreted by the pupating insect.\textsuperscript{21)} Although the dispersal 4th stage larva is the infecting stage for a living pine tree, the host-finding requires the aid of the sawyer.\textsuperscript{3,4)} Carbon dioxide\textsuperscript{6)} and/or other volatile compounds exhaled by the sawyer just after emergence might cause these larvae to transmigrate onto the body of the sawyer. During the post feeding period of the sawyer, the larvae of the nematode would transmigrate to the healthy pine tree. At that time, $\beta$-myrcene would act as an attractant. Probably, this is the first description of a specific compound responsible for the attraction of the nematode. After the transmigration to a pine tree, $\beta$-myrcene might also play an important role in the movement of the nematode in the pine wood, because propagative nematodes were attracted by the compound. The role of $\beta$-myrcene in the life cycle of the nematode, \textit{B. xylophilus}, is very interesting and it may be important for elucidation of the mechanism involved in pine wilting.

**REFERENCES**