Stimulating Effects of β -Myrcene on Molting and Multiplication of the Pine Wood Nematode, *Bursaphelenchus xylophilus*

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The stimulating effects of monoterpenes in pine trees on the molting and the multiplication of the pine wood nematode were investigated. The results indicated that the molting of the dispersal 4th stage larva (L_{IV}) to the adult nematode was stimulated by these monoterpenes, especially β -myrcene. The population of the nematode cultured on fungal mats of *B. cinerea*, *A. kikuchiana* and *C. miyabeanus* increased considerably in the presence of a small amount of β -myrcene. This compound seemed to play a hormone-like role for both the molting of L_{IV} and the multiplication of the nematode.

Biological and ecological investigations concerning the pine wood nematode, *Bursaphelenchus xylophilus*, which causes serious damage to pine trees in Japan, have been extensive.^{1~8)} In the life cycle of the nematode, the dispersal 4th stage larvae (L_{IV} s) carried by the sawyer are responsible for the infection of living pine trees.^{3,4)} Since the L_{IV} s are dauer,⁹⁾ which is a form specialized for survival and dispersal, they have a special morphological structure and lack the feeding function.³⁾ So, molting of L_{IV} to adult is necessary to resume multiplication after transmigration to a living pine tree. It was considered that some chemical stimuli contained in pine trees would promote the molting of L_{IV} s to adult nematodes and subsequent multiplication.

Previously, we reported that volatile monoterpenes in pine trees, especially β -myrcene, attract the nematodes.⁸⁾ Furthermore, we recognized that the resistance of the tree to the nematode infection was related to the amount of β -myrcene.¹⁰⁾ This clear relationship might be attributed to the physiological roles of β -myrcene for the molting and/or the multiplication of the nematode, since both processes

were essential for the infection as well as transmigration to the pine tree.

In this paper, we describe the stimulating effects of monoterpenes, especially β -myrcene, on the molting and the multiplication of the pine wood nematode.

MATERIALS AND METHODS

Chemicals. All chemicals used were of the highest quality commercially available.

Microorganism. The cultured nematodes and the L_{IV} s were obtained by our method.⁸⁾ The fungi, *Botrytis cinerea*, *Alternaria kikuchiana* and *Cochliobolus miyabeanus*, were generous gifts from the Laboratory of Plant Pathology of Ehime University.

Assay for molting of L_{IV} . About 1,000 L_{IV} s were put on 1 ml of 1.5% agar in a glass vessel (18 mm i.d. $\times$ 40 mm). Each test sample (3 μ l) was absorbed on a piece of filter paper (7 $\times$ 15 mm) attached to the stopper of the vessel. It was left in a dark chamber at 23°C. After 48 hr, 50 nematodes were taken out randomly from the vessel and their structure was observed to distinguish adults from L_{IV} s by the structural differences between stages³⁾ under a microscope ($\times$ 150). The results are indicated as a percentage of the molted adults.

Assay for multiplication. A fungus was cultured on a

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Fig. 1. Apparatus for Testing Effects of Volatile Compounds on the Growth of Pine Wood Nematodes.

1, plastic box (W30 × L25 × H35 cm); 2, vinyl tube (4 mm i.d.); 3, glass tube (3 mm i.d.); 4, beaker (10 ml); 5, sample; 6, slant; 7, testing tube stand.

potato dextrose agar medium in testing tubes (18 mm i.d. × 180 mm) for 10 days at 20°C. Then, about 200 nematodes were inoculated on the fungal mats of *B. cinerea* and *A. kikuchiana*. In the case of *C. miyabeanus*, about 400 nematodes were inoculated. The nematodes were incubated for multiplication in a rectangular plastic desiccator (1 in Fig. 1), in which a constant air stream (3.5 l/hr) was introduced through tubes (2 and 3 in Fig. 1) to vaporize a sample (5 in Fig. 1). The desiccator was left at 23°C with 95% humidity in a dark growth cabinet. In the case of β -myrcene, one liter of air contained about 10 mg of the compound. To separate the nematodes from each testing tube, water was added and left at 23°C. After 1 hr the nematodes in the water were collected. This procedure was repeated 3 more times to collect the nematodes that had crept into agar. The number of the nematodes collected was counted under a microscope ($\times 40$). The result was expressed as the average number of the nematodes of 5 testing tubes.

Mycelial dry weight. On the tenth day, 9 testing tubes were taken out from the desiccator used for examining the multiplication of the nematodes on *B. cinerea*. A fungal mat was harvested from each tube by boiling with water. The residual mycelium was dried at 110°C for 5 hr and the hyphal weight was measured.

RESULTS AND DISCUSSION

During the developmental stages of the pine wood nematode, *B. xylophilus*, the dispersal 4th stage larva (L_{IV}) participates in infection of living pine trees through the medium of the pine sawyer.^{3,4} Therefore, L_{IV} is the dauer, which is a nonfeeding form specialized for survival and dispersal.⁹ When such larvae get on a living pine tree, they molt to adult

TABLE I. EFFECTS OF MONOTERPENES ON MOLTING OF L_{IV}

Compound	Molting rate ^a (%)
Control	9.6 ± 3.8
α -Pinene	21.5 ± 9.6
β -Pinene	13.3 ± 10.1
3-Carene	18.4 ± 7.5
β -Myrcene	40.4 ± 5.2
<i>l</i> -Limonene	23.3 ± 14.7
Resin ^b	47.9

^a The value represents the mean of 4 trials and standard deviation.

^b Separated from the pine tree, *P. densiflora*.

nematodes and resume multiplication. The molting of L_{IV} to adult could be observed easily at low magnification by the differences in their morphological structures, such as differences in shapes of the head and tail and disappearance of the stylet, esophagus and esophageal gland in L_{IV} .³ The effects of monoterpenes on the molting are shown in Table I. About 10% of L_{IV} s developed to adult nematodes without chemical stimulation. The molting was accelerated in the presence of β -myrcene vapor (40.4%). Other monoterpenes also stimulated the molting, but their activity was lower than that of β -myrcene. A similar tendency was observed in the attracting test for the nematode described previously.⁸ Considering the above results, it might be concluded that monoterpenes, especially β -myrcene, contained in a pine tree would be effective for the molting of L_{IV} s after transmigration from the sawyer to pine trees.

After developing to the adult stage from L_{IV} , the nematode starts to multiply rapidly under favorable circumstances. It is known that the nematode feeds on parenchymatous cells of pine wood¹¹) and fungi grown in the cells.¹²) Dozono¹³) examined the suitability of various kinds of fungi for the multiplication of the nematode. Three kinds of these fungi were used as the diet for the nematode to assay for the multiplication. *B. cinerea* was found to be the most suitable media for growth of the nematode, while *C. miyabeanus* was not. *A.*

FIG. 2. Effects of Monoterpenes on the Growth of Pine Wood Nematodes on *B. cinerea* (A), *A. kikuchiana* (B) and *C. miyabeanus* (C).
 ○, control; ●, β-myrcene; △, α-pinene; □, β-pinene; ■, 3-carene; ▲, l-limonene.

kikuchiana was moderately suitable. When *B. cinerea* and *A. kikuchiana* were used with β-myrcene, the nematode could multiply rapidly showing a maximum number of about 150,000 and 60,000, respectively (A and B in Fig. 2). The nematode could multiply even on the medium of *C. miyabeanus* with β-myrcene and its population level reached about 30,000 per tube (C in Fig. 2). In the presence of the other monoterpenes, the multiplication did not dif-

TABLE II. EFFECTS OF MONOTERPENES ON THE GROWTH OF *Botrytis cinerea*

Sample	Mycelial weight ^a (mg)
Control	84.7
α-Pinene	85.6
β-Pinene	56.7
β-Myrcene	75.9
l-Limonene	44.0
3-Carene	81.1

^a Mean of 9 testing tubes.

FIG. 3. Effects of β-Myrcene on Pine Wood Nematodes on *B. cinerea*.

○, control; ●, β-myrcene; →, start of sample supply.

fer much from the control. The multiplication was observed to be depressed a little by l-limonene. However, this depression might be due to growth inhibition of *B. cinerea* by l-limonene (Table II). While, β-myrcene did not change the hyphal weight, but increased the nematode population. After culturing for about 2 weeks on *B. cinerea*, the number of nematodes decreased gradually and many dead nematodes were observed. However, when β-myrcene vapor was supplied about 2 days before reaching the maximum population level of the nematode, the dead nematodes evidently decreased (Fig. 3). In a recent investigation, we found a growth factor in *B. cinerea* corresponding to β-myrcene in pine trees. Probably, when the dead nematodes were observed, this substance disappeared. β-Myrcene may replace this substance as a growth factor. From these results, β-myrcene was expected to play a hormone-like role in the growth of the nematode.

Considering the attracting activity⁸⁾ and the present hormone-like activity of β -myrcene, a small difference of β -myrcene content in pine trees might affect the resistance or susceptibility of the tree when infected by the nematode. Details of the resistance to the nematode, *B. xylophilus*, depending on β -myrcene content will be described elsewhere.

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