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Chemotactic response of propagative and dispersal forms of the pinewood nematode *Bursaphelenchus xylophilus* to beetle and pine derived compounds

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Summary – A specialized life stage of the nematode *Bursaphelenchus xylophilus*, the JIV dispersal juvenile, is vectored by cerambycid beetles in the genus *Monochamus*. The propagative form of the nematode develops and reproduces in susceptible pine trees. The chemotactic response of JIVs and the mediation of JIV exit from beetle vectors are poorly understood. Experiments were conducted examining chemical attraction by nematodes across representatives of fatty acid, monoterpene and hydrocarbon groups. Chemical attraction between propagative and dispersal forms of the nematode was compared. The influence of chemical attraction on JIV exit from beetles was also examined. Propagative *B. xylophilus* were attracted to the fatty acids, linoleic acid and 1-monoolein, while JIVs were attracted to β-myrcene and toluene. The presence of neither fatty acids, monoterpenes nor hydrocarbons affected numbers of JIVs exiting beetles. It is suggested that other factors, possibly endogenous in nature, are also involved in JIV exit behavior. © Orstom/Elsevier, Paris

Résumé – ***Réponse chémotactique des formes de reproduction et de dispersion du nématode des pins,* Bursaphelenchus xylophilus, *à des composés dérivés de coléoptères et de pins*** – Un stade spécialisé du nématode des pins, *Bursaphelenchus xylophilus*, le quatrième stade juvénile de dispersion (JIV), est transporté par des coléoptères cérambycides du genre *Monochamus*. La réponse chémotactique du stade JIV et la médiation de la sortie de JIVs des coléoptères vecteurs ne sont pas bien comprises. Des expériences ont été conduites pour examiner l'attraction chimique des nématodes par des composés appartenant aux matières grasses, aux monoterpènes et aux hydrocarbures. L'attraction chimique entre les formes de reproduction et de dispersion du nématode a été comparée. L'influence de l'attraction chimique sur la sortie de JIV hors des coléoptères a été aussi examinée. La forme reproductive du nématode *Bursaphelenchus* est attirée par les matières grasses, l'acide linoléique et la 1-monooléine, alors que les JIVs sont attirés par la β-myrcène et le toluène. L'absence de matières grasses, de monoterpènes ou d'hydrocarbures a affecté le nombre de JIVs sortant des coléoptères. Cela permet de penser que d'autres facteurs, peut-être de nature endogène, peuvent affecter la sortie de JIVs hors des coléoptères. © Orstom/Elsevier, Paris

Keywords: *Bursaphelenchus xylophilus*, linoleic acid, 1-monoolein, *Monochamus carolinensis*, β-myrcene, α-pinene, pine wilt, pinewood nematode, toluene, vector.

A specialized life stage of the nematode *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, the JIV dispersal juvenile, is vectored to pine trees by cerambycid beetles in the genus *Monochamus* (Guér.) (Coleoptera: Cerambycidae). The propagative form of the nematode develops and reproduces in both healthy and dying pines (Mamiya, 1984). *Monochamus* beetles are attracted to dying trees where the beetles locate, mate and copulate. Females deposit eggs underneath the bark, and immature stages develop within the wood (Pershing & Linit, 1986). JIVs enter beetle tracheae prior to an adult beetle's emergence from the wood. The beetle vectors exhibit preferences for tree-derived terpenoid volatiles such as β-myrcene and α-pinene (Ikeda & Oda, 1980; Ikeda *et al.*, 1980*a*, *b*, 1981; Yamasaki *et al.*, 1989). Nematode exit from beetle vectors into new tree hosts may be mediated by these chemical attractants, as well.

Previous studies of chemical attraction of *B. xylophilus* have identified the most attractive chemicals present in pine, beetles, and beetle pupal chambers (Futai, 1979, 1980; Tominaga *et al.*, 1982, 1984; Watanabe, 1982 *in*: Tominaga *et al.*, 1984). Tominaga *et al.* (1984) and Watanabe (1982 *in*: Tominaga *et al.*, 1984) have characterized the chemical components of pine and, for propagative nematodes, β-myrcene appeared to be the most attractive of the volatile monoterpenes present in pine. α-pinene was also attractive to nematodes. Among the fatty acids and their derivatives, linoleic acid and 1-monoolein were the most attractive (Miyazaki *et al.*, 1977*a* *in*: Bolla *et al.*, 1989; Tominaga *et al.*, 1982; Bolla *et al.*, 1989). 1-monoolein is a glyceride of oleic acid, a common fatty acid in pine, and is closely related to linoleic acid. Linoleic acid is an essential fatty acid for animals and is one of the most common fatty acids in pine. Ninety-seven per cent of the fatty acids in the xylem

of Japanese red pine, *Pinus densiflora* Sieb. *et* Zucc., are oleic and linoleic acids (Mamiya, 1990). Linoleic acid has been found in the material lining *Monochamus* pupal chambers. Hydrocarbons from pentane washes of adult beetles have been characterized by HPLC. The chemicals found have been used in attraction studies. Among the hydrocarbons, toluene has been found to be attractive to propagative and dispersal nematodes (Shuto & Watanabe, 1987).

Previous studies of nematode attraction were not comprehensive in scope. They were conducted within groups of chemicals, not across different groups, *e.g.*, the terpenoids were compared with one another, not to fatty acid compounds. Also, these studies examined propagative nematodes almost exclusively. One study by Ishikawa *et al.* (1986) tested propagative juvenile and JIV dispersal juvenile attraction to terpenoids and found that both were attracted to β-myrcene. Shuto and Watanabe (1987) found toluene to have attracting activity for JIVs, but they did not test any other cuticular hydrocarbons or compare the activity of toluene to any other chemicals. Edwards (1989) conducted a preliminary experiment comparing JIV exit rates from beetles exposed either to pine chips or to a water control. Ten times as many nematodes exited when pine chips were present compared to the controls. However, the numbers exiting were very low overall, and the chemicals present in the pine were not characterized.

The following experiments attempt to bring together the above disparate experiments and expand on their scope; comparing chemical attraction across chemical groups and comparing chemical attraction between propagative and dispersal forms of *B. xylophilus*. These comparisons may shed some light on the control of nematode behavior as it relates to the beetle vector. Understanding the mechanisms that mediate the JIV-adult beetle association may provide opportunities for the development of novel methods to control pine wilt. The synchronization of beetle and nematode development within the wood of infested trees and the mechanism(s) controlling nematode exit from the vector are critical links in the pine wilt disease cycle.

Nematode chemical attraction was examined in two experiments. The objective of the first experiment was to determine the influence of several chemical compounds on JIV exit from *Monochamus carolinensis* (Olivier). The objective of the second experiment was to determine JIV and propagative nematode chemotactic response to the same chemical compounds in Petri dish arenas.

Materials and methods

Rearing of beetles and nematodes

M. carolinensis were collected from Scots pine, *Pinus sylvestris* L., grown near Ashland, MO, USA, and were reared in the laboratory as described by Linit (1985) with the following modifications to obtain nematode-infested beetles. Logs of jack pine, *Pinus banksiana* Lamb., *c.* 35 cm in length and *c.* 10-15 cm diam., were cut from the bole of a healthy tree. The fungus *Ophiostoma minus* (Hedgc.) H. *et* P. Sydow was grown in a Petri dish on malt agar, and a small amount of agar containing *O. minus* was placed in each of two 1.3 cm diam., 5 cm deep holes drilled through the bark and into the wood of each log. The holes were on opposite sides and ends of each log, *c.* 5-10 cm from each end (Warren *et al.*, 1995). The holes were sealed with styrofoam plugs and a thin layer of petroleum jelly.

B. xylophilus, pathotype US12 (Bolla & Boschert, 1993), isolated from *P. sylvestris* in Ashland, MO, were reared on *Botrytis cinerea* Pers. grown on potato dextrose agar and processed through a Baermann funnel to obtain nematodes in water (Southey, 1986). Seven days after *O. minus* inoculation into logs, two 1.3 cm holes were drilled into the opposite sides from the previous holes. Approximately 25 000 *B. xylophilus*, all life stages, in 2 ml of distilled water were pipetted into each hole and the holes sealed as above. Twenty-five inoculated logs, four at a time, were subject to beetle oviposition for 4 days in a large screen cage containing approximately 50 beetles. Logs were then placed in an incubator at 28°C, 70% RH, and adult *M. carolinensis* were collected upon emergence (about 8 weeks later).

A binary labelling technique using Liquid Paper® (Humphry & Linit, 1989) was used to identify individual beetles. Nematode-infested beetles were identified by examination of the first abdominal spiracle (Zhang *et al.*, 1995). Beetles carrying few or no nematodes were not used in the experiment. To obtain JIVs for the second experiment, nematode-infested beetles were macerated and processed in a Baermann funnel to obtain JIVs in water (Southey, 1986).

JIV exit

An experimental arena was designed to examine the effects of test compounds on JIV exit from beetles. The arena consisted of a 9 cm diameter plastic Petri dish with a raised screen wire floor. The screen floor was supported on three 1 cm high sections of plastic tubing. The lid was fitted with wire screen for ventilation. Several compounds were selected for assay: β-myrcene ($C_{10}H_{16}$, mw 136.24) and α-pinene ($C_{10}H_{16}$, mw 136.24), two component chemicals in volatiles released by pine; linoleic acid ($C_{18}H_{32}O_2$, mw 280.45) and 1-monoolein ($C_{21}H_{40}O_4$, mw

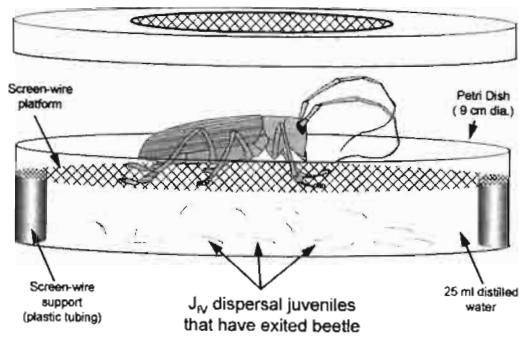

Fig. 1. *Design of the exit arena for JIV dispersal juveniles of* Bursaphelenchus xylophilus. *A beetle was placed on a raised screen-wire platform above 25 ml distilled water with 10^{-1} M of one of five chemicals or water only. JIVs were allowed to exit the beetle and drop off into the water for 24 h.*

356.55), two chemicals found in beetle pupal chambers; toluene (C_7H_8, mw 92.14) from pentane washes of adult beetles, and a distilled water control. Test compounds were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Test compound (1 ml of 0.1 M solution in hexane) was floated on the top of 25 ml distilled water in each arena. All tested compounds ranged from slightly soluble in water (toluene) to insoluble in water (β-myrcene) and all had specific gravities less than 1 (NB. water was used because we required the recovery of live JIVs for further experimentation not discussed in this paper). Individual, 14-day-old beetles infested with nematodes were placed in an arena and were kept at 24°C for 24 h. Fourteen-day-old beetles were used because nematode exit peaks 10 days after adult beetle emergence (Nakane, 1976; Hosoda & Kobayashi, 1977; Togashi, 1985; Linit, 1989). The beetles were then macerated, processed through a Baermann funnel, and the recovered nematodes were counted. The nematodes that had exited the beetles and dropped off into the distilled water of the arena were collected and counted using a stereomicroscope. Lack of continuity between beetles and water was not considered a deterrent to nematode exit because we have observed nematodes exiting in large numbers under similar circumstances (unpubl.). Each compound was tested five times.

The data were analysed as a balanced incomplete block design with six treatments in fifteen blocks, two beetles per block, for a total of 30 beetles, five per treatment (Cochran & Cox, 1957).

JIV AND PROPAGATIVE NEMATODE CHEMOTAXIS

A Petri dish arena, modified from Tominaga *et al.* (1982) was used to examine the chemotactic response of JIVs and propagative nematodes of all life stages to the test compounds. The following compounds were selected for the assay: β-myrcene, α-pinene, linoleic acid, 1-monoolein, toluene, and a hexane control. We did not include a pine chip wash as a treatment for a variety of reasons. The attractiveness of pine segments to the nematode is well documented (Futai, 1979, 1980). The objective of this experiment was to examine relative differences in attractiveness across groups of chemicals between two populations of nematodes. We were concerned that the addition of highly attractive pine chip washes to the experiment might mask subtle differences in attraction across chemicals tested. The difficulty in determining what constituted a representative and reproducible sample of pine material, in terms of tree age, health, sampling techniques, etc., also led us not to include pine in our experimental design.

A dose-response test was conducted to determine appropriate molar concentrations of the chemicals to be used in the chemotaxis study. Additionally, results from previous studies in the literature were examined (Futai, 1979, 1980; Tominaga *et al.*, 1982, 1984; Watanabe, 1982: *in* Tominaga *et al.*, 1984). Dose-response was monitored on an agar plate with 6 mm diameter filter paper disks placed equidistant around the perimeter. Each disk was dipped in a dilution (10^{-1} M to 10^{-5} M in hexane) of the chemical tested or in one of two control solutions. The control solutions consisted of distilled water and hexane. One thousand nematodes (all life stages) were placed in the centre on cotton and the plate was covered for 24 h at 21°C. The filter paper disks and the agar beneath and around them were removed with a cork borer (12 mm diameter) and the number of nematodes determined.

JIVs were not used in the dose-response test because of the limited availability of JIVs at the time of the experiment. Previous studies have shown JIVs and propagative nematodes respond to similar dose levels of chemicals (Ishikawa *et al.*, 1986; Shuto & Watanabe, 1987).

All compounds were assayed for chemotactic response at 10^{-4} M in hexane. Compounds were tested on an agar plate with equally spaced 6 mm diameter filter paper disks that had been dipped in each chemical or a hexane control, as in the dose response tests. Separate assays were carried out for propagative nematodes of all life stages obtained from fungal cultures and JIVs obtained from beetles. One thousand nematodes were placed in the center of the agar plate on cotton and the plate was covered for 24 h. The filter paper disks and the agar beneath and

around them were removed and the number of nematodes determined. This design and subsequent analysis followed that of Miyazaki *et al.* (1977*a in*: Tominaga *et al.*, 1982) and Tominaga *et al.* (1982).

The data were analysed as a repeated 6 × 6 Latin square design with six chemical treatments, six positions within a Petri dish and six Petri dishes. The procedure was repeated six times (no. replicates = 36) for the JIVs and three times (no. replicates = 18) for the propagative nematodes. Numbers of nematodes were converted to an 'attracting activity' index, $I_a = ((N_t\text{-}N_c)/N_c)*10$, where N_t = number of nematodes at a treatment disk, N_c = number of nematodes at the control disk (Ishikawa *et al.*, 1986). An I_a of 0 indicated no attraction, a negative I_a indicated repulsion and a positive I_a indicated attraction. Analysis of variance and planned orthogonal contrasts among chemicals tested were performed.

All statistical analyses were performed using SYSTAT for Windows Ver. 5 (SYSTAT, Inc. Evanston, Il, USA).

Results

JIV EXIT

Variation in exit rates was large for each of the chemical treatments and in a number of trials, no nematodes exited from beetles (Table 1). The number of JIVs exiting beetles did not differ among the test compounds ($F=1.0$; $df=5,10$; $P=0.485$).

Table 1. *Numbers (mean + SD) of JIV dispersal juveniles of* Bursaphelenchus xylophilus *exiting beetles placed in an exit arena for 24 h over five different compounds, all at a concentration of 0.1 M, and a no-chemical control*

Compound	N	No. of JIVs carried per beetle*	No. of exited JIVs
Distilled water	5	25259 ± 17198	12 ± 16 *a* (0-37)
β-myrcene	5	11230 ± 7587	1043 ± 2324 *a* (0-5200)
α-pinene	5	15382 ± 10876	11 ± 12 *a* (0-28)
Toluene	5	12475 ± 7949	19 ± 23 *a* (0-53)
Linoleic acid	5	24399 ± 15241	1929 ± 4289 *a* (0-9600)
1-monoolein	5	26009 ± 19714	663 ± 1912 *a* (0-5760)

*Sum of the exited JIVs and the JIVs recovered from a beetle following an assay.
Means followed by the same letter within a column did not differ significantly from one another at $P \leq 0.05$ according to Fisher's LSD test.

JIV AND PROPAGATIVE NEMATODE CHEMOTAXIS

In the dose-response studies, more nematodes were found at the 10^{-4} M concentration over the other concentrations for β-myrcene and linoleic acid only (Table 2). Although not significant, relatively high numbers of nematodes were attracted to the 10^{-4} M concentration of toluene and 1-monoolein. Nematodes did not exhibit a preference for any concentration of α-pinene. Consequently, taking into account dose-response studies in the literature (Tominaga *et al.*, 1982, 1984; Watanabe, 1982 *in*: Tominaga *et al.*, 1984), a 10^{-4} M concentration was used for all chemicals in the chemotaxis experiments.

In the chemotactic study, the number of propagative nematodes differed among the test compounds ($F=2.6$; $df=5,90$; $P=0.029$). The fatty acids, linoleic acid and 1-monoolein, had positive, significantly higher attracting activity indices than the other compounds (Table 3). The attracting activity among the remaining compounds did not differ.

The number of JIVs did not differ significantly among the test compounds in the analysis of variance ($F=1.3$; $df=5,195$; $P=0.275$), but toluene and β-myrcene had significantly higher attracting activity for JIVs than did the other tested compounds according to orthogonal contrasts (Table 4). The remaining compounds did not differ in their attractiveness to JIVs.

Discussion

JIV EXIT

The lack of a significant number of JIVs exiting beetles in response to β-myrcene or some other likely cue might occur because a chemical's range of influence may be quite small for *B. xylophilus* (Futai, 1980). Chemicals thought to be attractants may actually act more as aggregation stimulants than as attractants, with nematodes moving randomly until the desired chemical is reached, whereupon movement decreases or stops. If this is the case, chemical attractants may only play a role in JIV movement from beetles into pine once the nematodes have physically dropped onto the tree surface. Our results appear to contradict Edwards' (1989) results. He reported ten times as many nematodes exited beetles exposed to wood chips in water than exited beetles exposed to water alone (11.8 + 14.9 for pine chips and 0.9 + 2.5 for the control). Edwards' experiment was preliminary in nature because of the very low percentage of beetles with exiting nematodes; seventeen of the 24 beetles for pine chips and five of the 24 beetles for the control. Also the number of nematodes that exited was

Table 2. *Number (mean ± SD) of* Bursaphelenchus xylophilus *attracted to saturated filter paper disks with a concentration (dose) of each of five chemicals.*

Dose	No. of nematodes attracted to:				
	β-myrcene	α-pinene	Toluene	Linoleic acid	1-monoolein
Distilled water	4.7 ± 1.5 *b*	4.6 ± 1.3 *a*	38.6 ± 12.5 *a*	3.8 ± 1.6 *bc*	1.4 ± 0.4 *a*
Hexane	4.8 ± 1.4 *b*	9.0 ± 2.8 *a*	28.4 ± 4.4 *a*	1.5 ± 0.6 *c*	3.2 ± 2.5 *a*
10^{-1} M	4.7 ± 1.0 *b*	4.1 ± 1.6 *a*	28.2 ± 8.5 *a*	9.0 ± 3.5 *b*	0.8 ± 0.4 *a*
10^{-2} M	6.2 ± 1.7 *b*	5.1 ± 1.5 *a*	16.0 + 4.9 *a*	5.3 ± 1.7 *bc*	1.6 ± 1.4 *a*
10^{-3} M	7.7 ± 2.0 *b*	5.8 ± 2.2 *a*	23.8 ± 5.3 *a*	4.8 ± 2.2 *bc*	1.2 ± 0.8 *a*
10^{-4} M	18.4 ± 5.9 *a*	5.7 ± 1.7 *a*	35.0 + 8.5 *a*	16.8 ± 2.2 *a*	2.0 ± 0.8 *a*
10^{-5} M	7.7 ± 2.2 *b*	3.9 ± 1.3 *a*	17.4 ± 5.9 *a*	2.5 ± 1.3 *bc*	1.6 ± 0.9 *a*

Means followed by the same letter within a column did not differ significantly from one another at $P \leq 0.05$ according to Fisher's LSD test.

Table 3. *Attracting activity of tested compounds to propagative nematodes of* Bursaphelenchus xylophilus *in a Petri dish arena (All chemicals were tested at 10^{-4} M concentration).*

Chemical	Attracting activity*
β-myrcene	1.22 ± 0.34 *a*
α-pinene	-2.43 ± 0.70 *a*
Toluene	-0.54 ± 0.19 *a*
Linoleic acid	9.05 ± 2.43 *b*
1-monoolein	19.46 ± 6.91 *b*

*Attracting activity = $(N_t\text{-}N_c)/N_c$ **10* (N_t = number of nematodes at a treatment disk, N_c = number of nematodes at the control disk; mean ± standard error, n = 18).
Means followed by the same letter did not differ significantly from one another at $P \leq 0.05$ according to orthogonal contrasts.

Table 4. *Attracting activity of tested compounds to JIV dispersal juveniles of* Bursaphelenchus xylophilus *in a Petri dish arena (All chemicals were tested at 10^{-4} M concentration).*

Chemical	Attracting activity* (SE)
β-myrcene	1.54 ± 0.30 *a*
α-pinene	-2.82 ± 0.58 *b*
Toluene	5.00 ± 1.18 *a*
Linoleic acid	-0.19 ± 0.05 *b*
1-monoolein	-0.45 ± 0.09 *b*

*Attracting activity = $(N_t\text{-}N_c)/N_c$ **10* (N_t = number of nematodes at a treatment disk, N_c = number of nematodes at the control disk; mean ± standard error, n = 36).
Means followed by the same letter did not differ significantly from one another at $P \leq 0.05$ according to orthogonal contrasts.

low; the maximum number that exited from an individual beetle was only 53 nematodes, while the maximum observed in our study was 9600. Finally, Edwards' nematodes may have responded to the combination and/or ratio of chemicals emitted from wood chips, whereas those in the present study were exposed to a single chemical at a time. Differences in the volatility of the chemicals tested, the nature and size of the fatty acids making them less volatile than the terpenoids and the hydrocarbon, and the possible interactions between the hydrophobic compounds and the water may have played a role in JIV exit as well.

Alternatively, chemical cues may play a minor role in triggering nematode exit from vectors. Dispersal juvenile exit follows a temporal pattern, even in the absence of chemical cues; numbers of nematodes exiting are low in the first week following adult beetle emergence, the number peaks in the following 2 to 3 weeks, then gradually drops off over the remainder of the beetle's life (Enda, 1972; Nakane, 1976; Togashi, 1985; Linit, 1989). Some Japanese researchers claim exit is so discontinuous that generalization is not possible (Kobayashi *et al.*, 1984). These observations indicate chemical attraction may not be involved in initiating exit behaviour or, at least, chemical attraction may be modified by other unknown factors.

The discontinuous nature of nematode exit and a minor role for chemical cues would explain the very large standard deviations in exit numbers in our study. For example, 9600 of 19 800 resident nematodes exited from one beetle placed over linoleic acid, while no nematodes exited from another beetle containing 33 400 nematodes when placed over linoleic acid. Subsequent experiments with beetles in exit arenas indicates that the odds of improving the numbers in

the study are unlikely, and using exit from live beetles as an indicator of attraction is problematic at best.

JIV AND PROPAGATIVE NEMATODE CHEMOTAXIS

Previously reported dose-responses, as well as the dose-response studies presented here, suggested 10^{-4} M was an appropriate concentration at which to assay chemotactic response by *B. xylophilus* in Petri dish arenas. The concentration thresholds of attraction of many compounds have been examined in a variety of studies in Japan. Twenty-five terpenes were attractive to *B. xylophilus* at concentrations from 10^{-3} to 10^{-6} M and β-myrcene was most attractive at 10^{-4} M (Tominaga *et al.*, 1984). Tominaga *et al.* (1983) found the threshold value of activity for 1-monoolein, along with a variety of bitter and pungent substances, to be 10^{-4} M. Oleic acid has been found to be attractive to nematodes at the same concentrations as 1-monoolein (Tominaga *et al.*, 1982).

Of interest is the observation that only β-myrcene and linoleic acid produced a significant dose-response from the nematodes in the dose-response studies. A differential response to various concentrations would be expected if the compound in question had a physiological effect on the nematode and if concentration gradients were used in host location. The absence of a response to concentrations of α-pinene, toluene and 1-monoolein indicate these compounds may have little to do with host location. Tominaga *et al.* (1984) found α-pinene only weakly attractive to nematodes, as compared to β-myrcene.

Attraction of propagative nematodes to the fatty acids may have a facultative role in nematode location of beetles within the wood. Other components may include attraction to exhaled carbon dioxide or as yet uncharacterized beetle hormones (Bolla *et al.*, 1989). The wood surrounding beetle pupal chambers generally has a higher level of fatty acids than other wood tissue because the last larval instar of *M. carolinensis* lines the pupal chamber with its excretions (Giblin-Davis, 1993). JIII dispersal juveniles were reported to aggregate around beetle pupal chambers in response to insect-deposited fatty acids (Miyazaki *et al.*, 1977*b* *in*: Ishikawa *et al.*, 1986), however, Necibi (1996) reported an increase in JIII density in wood surrounding artificial galleries devoid of insects. Fatty acids are a rich food source and nematodes from fatty acid-supplemented cultures have a dark body color due to densely packed lipid droplets (Mamiya, 1990). Unsaturated fatty acids added to fungal cultures increased survivability and reproductive rates of *B. xylophilus* (Mamiya, 1990). Fatty acids may attract *B. xylophilus* juveniles to beetle pupal chambers and also may provide a rich source of storage lipid for the survival of dispersal JIII and JIVs.

The lack of a chemotactic response by propagative nematodes to α-pinene and β-myrcene may reflect the absence of these terpenes at the point in the nematode's life cycle when nematodes would be attracted to beetle pupal chambers, *i.e.* after the tree has died. Major changes in oleoresin composition occur when a tree is stressed and/or dying. In Scots pine, *P. sylvestris*, concentrations of α- and β-pinene decrease and unique compounds such as δ-cadiene and γ-terpineol appear starting 2 to 4 weeks after tree death (Bolla *et al.*, 1989). Because *Monochamus* spp. beetles develop only in dying trees and logs, it is unlikely that monoterpenes play a role in nematode attraction to beetle pupal chambers. This is further supported by the observation that the addition of α- and β-pinene to lipid extracts from *M. carolinensis* suppresses *B. xylophilus* attraction to the lipid extracts (Bolla *et al.*, 1989). Monoterpenes would more likely play a role in attraction during JIV exit from beetle vectors into pine trees and the migration of JIVs to resin canals prior to their development to adults.

Unlike the propagative form, JIV dispersals were attracted to the volatiles β-myrcene and toluene, compounds found in pine and beetles, respectively. Ishikawa *et al.* (1986), in comparing the seven most common terpenes of pine, found that JIVs were attracted to the same components of pine at the same concentrations as propagative nematodes. β-myrcene is considered a likely chemical cue for initiating JIV exit and has been documented to increase the rate of molting of JIVs to the adult stage (Hinode *et al.*, 1987; Giblin-Davis, 1993). β-myrcene may not be the only chemical involved in exit behavior. It may be the primary cue, with other minor constituents also involved in attraction of the JIV and in the JIV's exit behavior. The possibility of chemical blends as cues may also explain the lack of a significant increase in nematode exit from beetles in the presence of β-myrcene alone, as demonstrated in the first experiment.

Toluene, a beetle cuticular hydrocarbon attractive to JIVs in our experiments, is a likely component in a combination of cues that initiate JIV entrance into beetle tracheae prior to adult beetle emergence from pine (Shuto & Watanabe, 1987). This suite includes carbon dioxide and a hypothesized but as yet uncharacterized chemical unique to *Monochamus* spp. ecdysis (Ishibashi & Kondo, 1977; Shuto & Watanabe, 1987; Bolla *et al.*, 1989; Necibi, 1996).

The timing of JIV exit from beetles is an intriguing aspect of nematode behavior as it pertains to chemical cues. JIVs do not exit in any great number, or at all, the first 7-14 days of the adult beetle's life, even though the adult beetle is feeding on pine during this time and nematodes have ample opportunity to enter feeding wounds. Chemical cues such as β-myrcene are present, as well (Hosoda & Kobayashi, 1977,1978;

Togashi, 1985; Linit, 1989). JIVs exit in large numbers during weeks 2-4 of the adult beetle's life, and the numbers taper off after that for the remainder of the beetle's life (Togashi & Sekizuka, 1982). Why is JIV exit from the beetle delayed 2 weeks although conditions are apparently appropriate and likely exit cues are present?

Several possibilities exist for the control of JIV exit from beetles: *i*) exit is due to exogenous factors alone, *ii*) exit is due to endogenous factors alone, or *iii*) exit is due to a combination of exogenous and endogenous factors. The study presented here indicated that JIVs did not exit beetles in greater numbers in response to any of the tested chemicals, although they were attracted to β-myrcene and toluene in Petri dish arenas. The strong temporal pattern of nematode exit, even in the absence of pine volatiles, and the lack of significant increases in nematode exit in response to the tested chemicals in our first experiment may indicate timing of nematode exit has a significant and substantial endogenous component. Monitoring of internal states such as age or storage compounds used for energy reserves may provide the trigger for JIV exit from the beetle.

The combination of endogenous and exogenous control is another possibility. Chemical attraction of JIVs may be masked because the populations tested contain groups of individuals that differ in their response to external stimuli based on some internal cue(s). The free-living nematode *Caenorhabditis elegans*' response to a dauer-inducing pheromone and a food signal is modified by the age of the nematode (Golden & Riddle, 1982, 1984). Older nematodes are less responsive to the pheromone and/or more responsive to the food signal. If a similar situation exists for *B. xylophilus*, significant numbers of nematodes may not be associated with particular chemicals without first differentiating appropriate subpopulations of JIVs. An internal cue that changes over time may behaviorally differentiate JIVs and result in modified response to chemical cues. Young JIVs may be more attracted to beetle compounds such as toluene while older JIVs may be more attracted to pine compounds such as β-myrcene. These chemicals were significantly more attractive to JIVs in our assay. We are conducting further experiments to examine the role of intrinsic cues in the exit behavior of the nematode and the nematode's response to chemical cues. Additional work with other pathotypes of *B. xylophilus* is also warranted to confirm the attractiveness of the fatty acids to propagative forms and the attractiveness of β-myrcene and toluene to the JIV dispersal form and to paint a more complete picture of this nematode's chemotactic behavior.

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