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The Influence of Volatiles from the Hindgut of the Pine Sawyer, *Monochamus alternatus* (Coleoptera: Cerambycidae), on Its Oviposition Behavior

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Shui-Qing Li and Zhong-Ning Zhang (2007) The influence of volatiles from the hindgut of the pine sawyer, *Monochamus alternatus* (Coleoptera: Cerambycidae), on its oviposition behavior. *Zoological Studies* **46**(6): 726-733. The oviposition behavior and response of *Monochamus alternatus* females to bolts treated with hexane extracts of the hindgut contents of *M. alternatus* females and males were investigated in the laboratory. Females gnawed a significantly smaller number of oviposition scars on bolts i.e., cross-sections of trunks of *Pinus massoniana* treated with a hexane extract of the hindgut contents of *M. alternatus* females than on control bolts. The number of eggs deposited on bolts treated with the hexane extract of female hindgut contents was also significantly fewer than on control bolts. The number of scars made and eggs laid on bolts treated with the hexane extract of male hindgut contents did not significantly differ from those on control bolts. These results suggest the presence of a putative oviposition deterrent in the hindgut contents of *M. alternatus* females. Hexane extracts of the hindgut contents of both sexes were analyzed by gas chromatography-mass spectrometry; α-pinene, β-pinene, myrcene, 3-carene, limonene, terpinolene, and butylated hydroxytoluene were identified in the contents of both sexes, while *p*-vinylguaiacol was found only in females. Experiments with synthetic mixtures revealed that a mixture of *p*-vinylguaiacol and butylated hydroxytoluene exhibited oviposition-deterring activity. http://zoolstud.sinica.edu.tw/Journals/46.6/726.pdf

Key words: *Monochamus alternatus*, Oviposition deterrent, Hindgut, *Pinus massoniana*, Volatiles.

Adults of the pine sawyer, *Monochamus alternatus* Hope (Coleoptera: Cerambycidae), transmit the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle, which causes wilt disease of *Pinus densiflora* Sieb. et Zucc. and *P. thunbergii* Parl. in Japan (Kiyohara and Tokushige 1971, Mamiya and Enda 1972, Morimoto and Iwasaki 1972). The more nematodes an adult sawyer carries, the shorter its longevity becomes (Togashi and Sekizuka 1982). In China, the preferred host of *M. alternatus* is *P. massoniana* Lamb. Currently, in Anhui, Guangdong, Jiangsu, Zhejiang, and Shandong Provinces of China, *M. alternatus* and *B. xylophilus* are causing considerable losses of *P. massoniana*. Several control strategies for *M. alternatus*, including biological control (Shimazu 1994, Shimazu and Sato 2003), insecticide application (Togashi 1990), and the use of attractants (Ikeda et al. 1980, Sakai and Yamasaki 1990 1991), have been reported.

Oviposition behavior of many phytophagous and parasitic insects is often regulated by certain chemicals. These chemicals, emitted by many insect species as chemical messengers to deter gravid females from oviposition, may originate from conspecific eggs (Anbutsu and Togashi 1996 1997), larvae (Williams et al. 1986, Anbutsu and Togashi 1996), and larval frass (Dittrick et al. 1983, Anderson et al. 1993, Li and Ishikawa

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2004). Many insect species produce active semiochemicals in their guts; in Diptera for instance, semiochemicals may be produced in the midgut and released through orifices (Prokopy et al. 1982). *Ips pini* males synthesize the monoterpenoid pheromone component, ipsdienol, in their midgut tissue (Gregory et al. 2002). In the Coleoptera in contrast, the hindgut and possibly Malpighian tubules are important sources of semiochemicals (White et al. 1980). Hughes et al. (1976) identified the active chemicals, frontalin and exo-brevicomin, from the hindgut of *Dendroctonus adjunctus* Blandford. A sex pheromone of *Dendroctonus terebrans* Olivier was also identified as originating from the hindgut. *Dendroctonus terebrans* females produce and release frontalin, *trans*-verbenol, and myrtenol, while males produce *exo*-brevicomin, *trans*-verbenol, myrtenol, and traces of *endo*-brevicomin (Payne et al. 1987).

To date, there has been no report on the semiochemicals from the hindgut of *M. alternatus*. In this study, we investigated the influence of imaginal hindgut extracts of female and male *M. alternatus* on the female's oviposition behavior and also identified the active compounds of the semiochemicals from the female hindgut contents.

MATERIALS AND METHODS

Insect source

The experimental colony of *M. alternatus* was founded from insects collected on Jingting Mt., Xuancheng City, Anhui Province, China. Newly emerged adults were reared separately according to sex in iron-screened cages (50 x 40 x 30 cm) on 1 or 2-yr-old *P. massoniana* twigs at 25°C with a 12 h L: 12 h D photocycle regime. Two wk later, females and males were paired, and the adult females were allowed to oviposit before they were used for oviposition tests at 18-25 d after emergence.

Hexane extracts of hindgut contents

Adult females and males of *M. alternatus* were separately dissected, and the hindguts obtained were dipped in hexane (5 hindguts in 1 ml of hexane). After 24 h, the remains of the hindguts were removed from the hexane, and the extracts were stored at -20°C until needed for the oviposition tests. Another set of 5 females and 5 males were dissected, and the hindguts were dipped in 1 ml hexane; the extracts were slowly concentrated to 100 μl for content analysis under a constant N_2 stream, containing 1 ng/μl dodecane as an internal standard (dodecane 98%, Shanghai Chemical Reagent, Shanghai, China).

Choice oviposition tests

Ten 8-10-yr-old healthy *P. massoniana* trees were cut into 15-cm-long bolts without nodes. The cut ends of the pine bolts were sealed with paraffin liquid (with a melting point of 56-58°C) and stored in sealed black plastic bags at room temperature until needed for the oviposition tests. The bolts were 3.3-4.7 cm in diameter (mean ± SE, 3.8 ± 0.1 cm) and had a bark thickness of 1.1-1.5 mm (1.3 ± 0.1 mm).

Two pine bolts of similar diameter and bark thickness were selected for the oviposition tests. One milliliter of the hexane extract of hindgut contents of females (5 adult female hindgut equivalents, 5FE) or males (5ME) was applied to the bark surface of one of the 2 bolts using the tip of a calligraphy brush. The other bolt was treated with 1 ml hexane and served as the control. The treated bolt and control bolt were placed vertically in a transparent plastic container (20 x 20 x 20 cm) and were approximately 3.0 cm from the inner wall of the container. The distance between the treated and control bolts was about 14.0 cm, while two 1-2-yr-old *P. massoniana* twigs were placed in the center of a plastic container to serve as food. One gravid *M. alternatus* female was released onto the *P. massoniana* twigs at the beginning of the scotophase. Thirty minutes later, the position and behavior of each female were observed and recorded. The female was removed 48 h later, and the number of scars made and eggs laid were counted, after which the eggs were replaced into their original positions in the bolts. After egg incubation, the fertility was determined. The test was replicated 20 times with 20 different gravid females aged 18-25 d. The choice oviposition tests were designed to determine if gravid females had an oviposition preference between bolts treated with hindgut extracts of *M. alternatus* and control bolts.

No-choice oviposition tests

No-choice oviposition tests were designed to ascertain if gravid females oviposited when offered only bolts treated with hexane extracts of the hindgut contents of *M. alternatus*. Another reason for designing these tests was that the results of no-

choice oviposition tests are even more in accord with field results when an oviposition deterrent is applied in a forest. Bolts used for the no-choice oviposition tests were of the same length as in the choice oviposition tests with a 2.1-3.6 cm (mean ± SE = 2.9 ± 0.1 cm) diameter and 1.0-1.5 mm (1.3 ± 0.1 mm) bark thickness. There were 2 treatments in the no-choice oviposition tests: pine bolts treated with 1 ml hexane extract of *M. alternatus* female hindgut contents (5FE) and pine bolts treated with 1 ml hexane extract of male hindgut contents (5ME). Each treatment had a corresponding control of bolts treated with only hexane and was replicated 20 times. One treated bolt or a control bolt was placed vertically at the center of the plastic cage, and two 1-2-yr-old *P. massoniana* twigs were placed in the cage as food. One gravid *M. alternatus* female was released onto the *P. massoniana* twigs and 48 h later, the numbers of scars and eggs on the treated and control bolts were counted.

GC/MS-analysis of extracts from hindgut contents

Gas chromatography-mass spectrometry (GC-MS) was conducted on a Hewlett-Packard (HP) 6890 GC interfaced with a HP 5793 mass selective detector. A DB-5ms capillary column (60 m x 0.25 mm i.d., J&W Scientific, USA) was used and was maintained at 50°C for 2 min after injection and then programmed to rise to 200°C at 5°C/min, and held for 5 min. Nitrogen was used as the carrier gas at 1 ml/min linear velocity. The injector was operated in the split mode (split ratio 50: 1) with an injector temperature of 250°C. Compounds were identified by matching their mass spectra with those in the NIST Library and further verified by comparison of the diagnostic ions and GC retention times with those of respective authentic compounds. The quantity of each compound was calculated on the basis of the peak area and calibrated by comparing it with that of dodecane.

Determination of the deterrent activity of identified compounds

To confirm the deterrent effect of the compounds identified from the hindgut contents of females, synthetic chemicals were mixed in a similar ratio as found in the hindgut, dissolved in analytical pure hexane and used to treat healthy *P. massoniana* bolts in a choice oviposition test. The dimensions of the bolts were also 15.0 cm in length, 3.8-4.7 cm in diameter (mean ± SE = 4.2 ± 0.2 cm), and 1.2-2.3 mm bark thickness (1.7 ± 0.1 mm). Bolts treated with 1 ml of the mixture served as the treatment, while those treated with only hexane served as the control. Two bolts, 1 treatment and 1 control, were placed vertically in a transparent plastic container and insects were released as described earlier with 20 replicates. The number of scars made and eggs laid were recorded at 48 h when the choice tests ended.

Data analysis

Data from tests were subjected to statistical analysis using SPSS 11.0 for Windows software (SPSS, Chicago, IL, USA). Independent-sample *t*-test was used to compare the mean numbers of scars and eggs between the treatments and controls. All data are presented in the text as the mean ± the standard error of the mean (SE).

RESULTS

Influence of the hexane extract of the hindgut contents of *M. alternatus* females on adult oviposition

Thirty minutes after 20 adult females of *M. alternatus* were released onto the twigs, 3 were feeding on the twigs, 5 were found on the floor of the cage, 4 on the ceiling of the container, 1 on the inner wall, 1 on the treated bolt, and the other 4 on the control bolts. During the 48 h choice oviposition tests, the females excavated significantly fewer scars on treated bolts (2.15 ± 0.29) than on control bolts (4.90 ± 0.56) (t = 4.372, p < 0.001) (Fig. 1). Eggs deposited on treated bolts (2.10 ± 0.30) were also significantly fewer than those on control bolts (4.50 ± 0.57) (t = 3.736, p < 0.001) (Fig. 2). The results suggest that *M. alternatus* females preferred the control pine bolts to bolts treated with the hexane extract of female hindgut contents for oviposition. The total numbers of eggs deposited on the treated and control bolts were 42 and 90, respectively, and the fertility was 85.71% ± 1.77% (treated) and 97.78% ± 0.73% (control). It seemed that the female hindgut extract also had an influence on egg fertility (t = 6.296, p < 0.003).

Scars excavated by *M. alternatus* females on bolts treated with the hexane extract of female hindgut contents (3.35 ± 0.28) were also signifi-

cantly fewer than on control bolts (5.50 ± 0.48) (t = 3.833, p < 0.001) in the no-choice oviposition tests (Fig. 3). Similarly, the mean number of eggs deposited by each female on treated bolts (3.30 ± 0.28) was significantly fewer than that on control bolts (5.05 ± 0.52) (t = 8.873, p < 0.006) (Fig. 4).

The results of the choice and no-choice oviposition tests showed the existence of a hexane-soluble oviposition deterrent in the *M. alternatus* female hindgut contents.

Influence of the hexane extract of the *M. alternatus* male hindgut contents on adult oviposition

In the choice oviposition test, 4 of 20 females released at the center of the container for 30 min were walking on the treated bolts. Another 5 were walking on the control bolts, 4 were feeding on pine twigs, 6 were found on the floor of the cage, and 1 on the inner wall. The results of the choice oviposition tests are shown in figures 1 and 2.

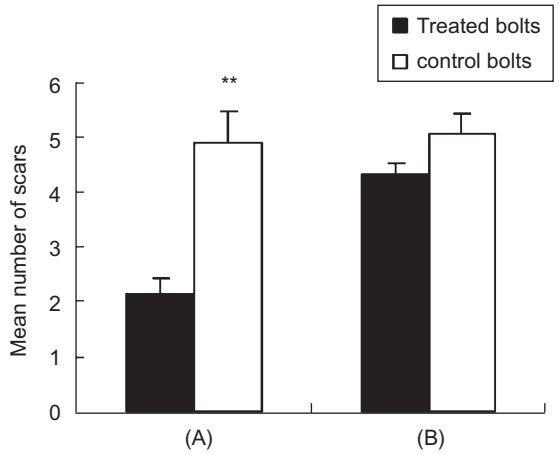

Fig. 1. Numbers of scars in the choice oviposition tests. The column shows the mean number of excavated oviposition scars. (A) Bolts treated with the female hindgut extract and the control; (B) bolts treated with the male hindgut extract and the control. Vertical bars are [+]SE, n = 20.

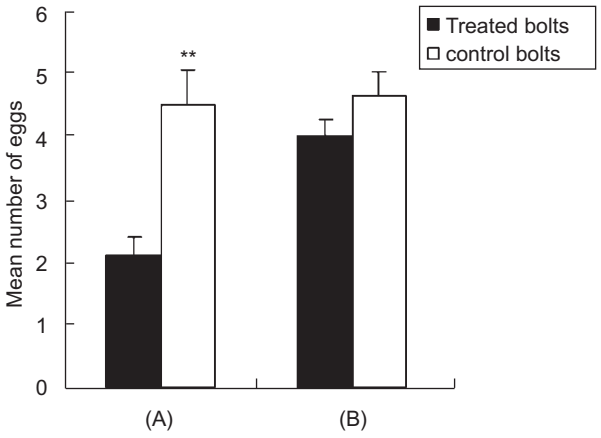

Fig. 2. Numbers of eggs in the choice oviposition tests. The column shows the mean number of eggs. (A) Bolts treated with the female hindgut extract and the control; (B) bolts treated with the male hindgut extract and the control. Vertical bars are [+]SE, n = 20.

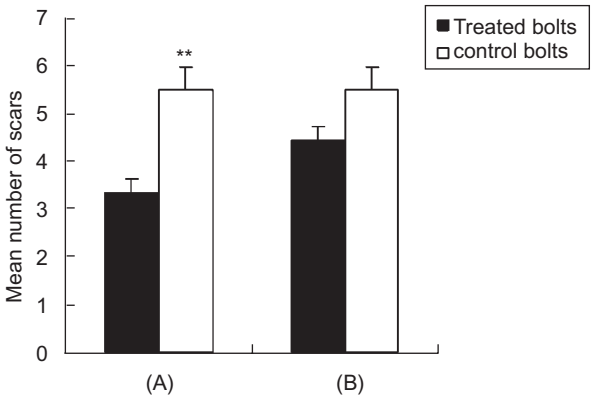

Fig. 3. Numbers of scars in the no-choice oviposition tests. The column shows the mean number of excavated oviposition scars. (A) Bolts treated with the female hindgut extract and the control; (B) bolts treated with the male hindgut extract and the control. Vertical bars are [+]SE, n = 20.

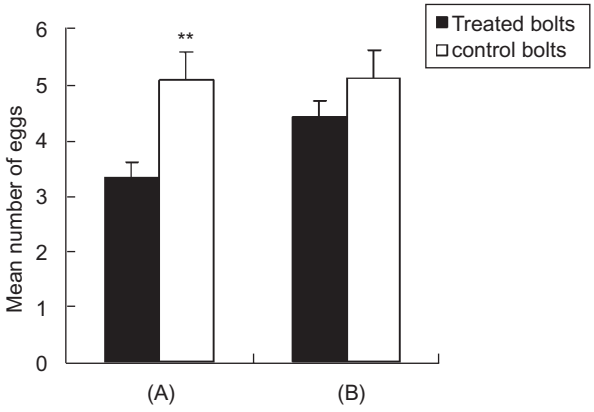

Fig. 4. Numbers of eggs in the no-choice oviposition tests. The column shows the mean number of eggs. (A) Bolts treated with the female hindgut extract and the control; (B) bolts treated with the male hindgut extract and the control. Vertical bars are [+]SE, n = 20.

Scars excavated on bolts treated with *M. alternatus* male hindgut extract (4.30 ± 0.24) did not significantly differ from those on control bolts (5.05 ± 0.37) (t = 1.709, p > 0.05). There was no significant difference between the numbers of eggs deposited on control bolts (4.65 ± 0.37) and on treated bolts (4.00 ± 0.28) (t = 1.395, p > 0.05). The total numbers of eggs deposited on treated and control bolts were 80 and 93, while the fertility rates were 96.25% ± 1.15% and 96.77% ± 0.84%, respectively. The male hindgut extract had no influence on the fertility of eggs (t = 0.366, p > 0.05).

The mean numbers of scars made and eggs laid on treated and control bolts in the no-choice tests are shown in figures 3 and 4. The mean number of scars on treated bolts (4.45 ± 0.27) did not significantly differ from that on control bolts (5.50 ± 0.46) (t = 1.989, p > 0.05). Similarly, there was no significant difference between the mean numbers of eggs deposited on treated bolts (4.40 ± 0.28) and on control bolts (5.10 ± 0.49) (t = 1.243, p > 0.05).

From the results, we arrived at a tentative conclusion that the *M. alternatus* male hindgut extract did not deter conspecific females from oviposition.

Chemical identification of volatiles from hindgut contents

Alpha-pinene, β-pinene, myrcene, 3-carene, limonene, terpinolene, and butylated hydroxytoluene were identified from the hexane extracts of hindgut contents of both sexes of *M. alternatus*, while *p*-vinylguaiacol was found only in that of females. These peaks appeared at 12.94, 14.44, 14.60, 15.39, 16.05, 17.86, 29.65, and 24.63 min, respectively. These results revealed sex-specific differences in metabolism by *M. alternatus*. The relative percentages of each compound identified from female hindgut contents are given in parentheses: α-pinene (5.41%), β-pinene (3.36%), myrcene (0.67%), 3-carene (0.72%), limonene (2.35%), terpinolene (0.77%), *p*-vinylguaiacol (21.99%), and butylated hydroxytoluene (64.72%). The amounts of each of these compounds in 1-ml hexane extracts were 0.73, 0.44, 0.12, 0.10, 0.35, 0.10, 1.94, and 5.75 μg, respectively.

Oviposition-deterring activity of the identified compounds

The mixture of synthetic compounds identified from female hindgut contents (SM1) exhibited significant deterrent activity against oviposition. Gravid females excavated and deposited only 37.9% of the number of scars and eggs on treated bolts compared to the control. Another mixture (SM5) was tested in which monoterpenes were excluded because of their high volatility. The result with SM5 showed that the remaining compounds (*p*-vinylguaiacol and butylated hydroxytoluene) still significantly deterred females from ovipositing. The females gnawed only 36.7% of the number of scars and deposited 34.7% of the number of eggs as on treated bolts. Other mixtures, all monoterpenes (SM2), only *p*-vinylguaiacol (SM3), or only butylated hydroxytoluene (SM4), showed no oviposition-deterring activity (Table 1).

DISCUSSION

Oviposition-deterring pheromones (ODPs) are very important in pest control. In many phytophagous insects, mature adult females recognize and avoid hosts already occupied by conspecific eggs or larvae (Anbutsu and Togashi 1996 1997). Such a behavioral strategy is helpful in spacing eggs and reducing mortality of early larval stages due to conspecific competition. The spatial distribution patterns of oviposition scars, eggs, larvae, larval mines, and emergence holes of *M. alternatus* are believed to be uniform (Shibata 1984). *Monochamus alternatus* females prefer dead pine trees, and pine trees felled by man or infested by the pinewood nematode, which releases a mixture of monoterpenes and ethanol to attract sexually mature females for oviposition (Ikeda et al. 1980). Before oviposition, an adult female searches for a suitable site on the bark of pinewood. After finding a suitable site, the female gnaws at the bark surface with her mandibles to make a wound and then inserts the ovipositor into the bark to deposit her eggs. The female then plugs the wound with a jellylike secretion and rubs the scar with the abdominal tip as soon as she withdraws her ovipositor. The jellylike secretion, which contains deterrent chemicals, originates from the spermathecal gland, and its methanol extracts or that of the female reproductive organ and larval frass have been reported to deter other adult females from oviposition (Anbutsu and Togashi 2001 2002, Li and Zhang 2006).

Our study showed that *M. alternatus* females were deterred from oviposition by the hexane extract of female hindgut contents. These results

indicate the existence of oviposition-deterring chemicals in the female hindgut. However, the hexane extract of *M. alternatus* male hindgut contents did not deter conspecific females from oviposition. From the GC-MS analysis, we identified α-pinene, β-pinene, myrcene, 3-carene, limonene, terpinolene, and butylated hydroxytoluene in both sexes, but *p*-vinylguaiacol only in females. The results of the chemical analysis of volatiles from hindgut contents of both sexes showed the existence of a sex-specific system of metabolism in *M. alternatus*. This sex-specific system of metabolism also exists in other species of beetles (Payne et al. 1987).

The oviposition deterrence of *M. alternatus* was elicited by a mixture of *p*-vinylguaiacol and butylated hydroxytoluene in a ratio similar to that found in female hindgut contents, as was demonstrated by the choice oviposition tests with the different synthetic mixtures (Table 1). The 2 compounds have a synergistic effect, because the removal of one of the compounds from SM5 resulted in loss of the oviposition deterrence. The origin of the oviposition-deterring compounds in female hindgut contents is unclear; a possibility is the host plant with subsequent concentration in the hindgut. Some investigators have found that a few deterrent compounds are unaltered plant constituents, and not metabolic by-products (Mitchell and Heath 1985).

When resource competition or cannibalism is likely, spacing mechanisms may contribute to avoiding conspecific competition. *Monochamus alternatus* females may produce and concentrate some semiochemicals in the hindgut and release them into the air or onto the host as a signal claiming pre-occupation of the host; this enables the offspring to maximize their utilization of limited food

Table 1. Oviposition response of *Monochamus alternatus* females to the test synthetic mixtures SM1-SM5. The amount used (μg/20 ml hexane) of each compound in each mixture, is listed after the compound

Compound[a]	Synthetic mixture no.				
	SM1	SM2	SM3[b]	SM4	SM5
Terpenes					
α-Pinene (14.6)	+	+			
β-Pinene (8.8)	+	+			
Myrcene (2.4)	+	+			
3-Carene (2.0)	+	+			
Limonene (7.0)	+	+			
Terpinolene (2.0)	+	+			
Hydroxybenzene					
p-Vinylguaiacol (38.8)	+		+		+
Butylated hydroxytoluene (115.0)	+			+	+
Deterrent activity					
Test (% of scar numbers)	37.9	48.9	50.3	46.2	36.7
Control (% of scar numbers)	62.1	51.1	49.7	53.8	63.3
n = total number of scars	161	231	175	171	158
Significance	*	ns	ns	ns	*
Test (% of egg numbers)	37.9	48.1	47.5	47.5	34.7
Control (% of egg numbers)	62.1	51.9	52.5	52.5	65.3
n = total number of eggs	145	212	160	158	144
Significance	*	ns	ns	ns	*

[a]Source and purity of compounds. α-Pinene (98%), limonene (92%), and butylated hydroxytoluene (≥ 99.8%), NJ, USA; β-pinene (> 95%), 3-carene (> 90%), and terpinolene (> 85%), Tokyo Kasei Kogyo, Toshima, Japan; myrcene (~90%), Sigma-Aldrich Chemie, Steinheim, Switzerland; *p*-vinylguaiacol (99%), Fujian Yongan Fengfan Fine Chemical, Yongan, China.

[b]A female died in the oviposition test, *n* = 19. *Significant difference. ns, not significant.

resources. Gravid females were found to deposit significantly fewer eggs on bolts containing the mixture of *p*-vinylguaiacol and butylated hydroxytoluene. This may have been because the mixture interferes with the insects' behavior preceding oviposition, namely, the arrival and length of stay on a host bolt. In our choice oviposition test, most females stayed on the pine twigs, the floor of the cage, or control bolts, and only a few stayed on the treated bolts. The influence of the mixture of *p*-vinylguaiacol and butylated hydroxytoluene on the behavior of *M. alternatus* males therefore, remains to be studied.

Interestingly, scars on the treated and control bolts in our oviposition tests differed from scars normally found on infested forest trees. Scars on bolts in our study were much smaller and not easily detected compared to those on infested forest trees. The cause remains unclear. In our oviposition tests, most scars contained only 1 egg, and a few scars had 2 eggs or none at all.

This study is important, especially in China, where wilt disease caused by the nematode transmitted by *M. alternatus* has destroyed about 6.7 million *P. massoniana* trees (Hu et al. 1997). In a pine forest infested with pine wilt disease, *M. alternatus* females were reported to have deposited their eggs on some dead pine trees. Adult beetles carrying nematodes emerged the following year, dispersed, and transmitted the nematodes to healthy trees, completing an intrusion cycle (Anbutsu and Togashi 2001). The management of this beetle, therefore, may be the most effective way of controlling the nematode. Oviposition deterrents may prevent *M. alternatus* females from ovipositing on pine trees killed by nematodes, resulting in a decrease in the population of beetles and reductions in the spread of destructive nematodes.

Further studies, especially in the field with application of these chemicals, are essential. They will not only elucidate the chemical and behavioral mechanisms, but also contribute to better management of field populations of beetles.

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