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HOST ATTRACTANTS FOR RED WEEVIL, *Rhynchophorus ferrugineus:* IDENTIFICATION, ELECTROPHYSIOLOGICAL ACTIVITY, AND LABORATORY BIOASSAY

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Abstract—A steam distillate from the freshly cut young bark of coconut palm *Cocos nucifera* was analyzed by gas chromatography, combined gas chromatography–electroantennographic detection (GC-EAD) and GC-MS to detect host attractants for the curculionid weevil *Rhynchophorus ferrugineus*, one of the major coconut pests in Sri Lanka. A twin FID peak consisting of a minor and a major component was shown to possess electrophysiological (EAG) activity. The minor peak was identified as γ-nonanoic lactone **1**, while the major peak was identified as 4-hydroxy-3-methoxystyrene **2**. In an EAG assay the synthetic racemic nonanoic lactone **1** did not elicit a considerable response in the antenna of *R. ferrugineus*, whereas the laboratory synthesized **2** showed activity. In a laboratory bioassay using a Y-type olfactometer, synthetic **1** and **2** elicited moderate attractant properties to *R. ferrugineus*, whereas a 1:1 mixture of the compounds showed increased attraction over that of the individual compounds.

Key Words—Host attractants, *Rhynchophorus ferrugineus*, coconut pest, γ-nonanoic lactone, 4-hydroxy-3-methoxystyrene.

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INTRODUCTION

Rhynchophorus ferrugineus F. (Coleoptera: Curculionidae), commonly known as the red weevil (Wattanapongsiri, 1966; Nirula, 1956) in Sri Lanka, is among five major pests of the coconut palm (Pinto, 1984). The female beetle is known to lay eggs in damaged or wounded tissue of palms. Its larvae feed by burrowing into the fresh tissue, finding their way into the bud region and into the heart of the crown, where they congregate and continue feeding for a period of two to four months. As a result, infested palms die (Rajapaksha and Kanagaratnam, 1988).

Difficulties have been encountered in the detection of infested coconut trees before they reach the stage of complete destruction (CRI, 1975). The present method of control recommended by the Coconut Research Institute in Lunuwila, Sri Lanka, is to drill holes into the soft bud region of the infested palm and to introduce insecticides (e.g., monocrotophos) (Advisory Leaflet No. 37) (CRI, 1976). This practice, however, is not satisfactory because only larvae coming into direct contact with the insecticide are killed and the plant can be saved only when the treatment is performed in an early stage of infestation. Another approach is the use of an electronic device for the detection of red weevils inside the coconut trunk (CRI, 1971). This method is not practical.

The sap oozing from wounded young coconut stem tissue is a widely known attractant for many species of palm weevils, and traps baited with coconut stem tissue have been used to reduce the weevil populations in India (Abraham and Kurian, 1975), the West Indies (Hagley, 1965) and Indonesia (Kalshoven, 1950), while in Sri Lanka they were used only in problem situations (CRI, 1976). Recently, palm tissues have been used to improve the efficiency of aggregation-pheromone-baited traps for *Rhynchophorus* species because the host volatiles were found to be synergistic for the respective aggregation pheromones of the species (Rochat et al., 1993). The introduction of host palm tissues into weevil traps increased the number of weevil catches with the aggregation pheromones of the palmetto weevil, *R. cruentatus* (Weissling et al., 1994), *R. palmarum* (Jaffé et al., 1993), *R. phoenicis* (Gries et al., 1994), and *R. ferrugineus* (Hallet et al., 1993). Traps baited with pheromone from host tissue have also been employed in the management of red ring disease (Griffith, 1987) in commercial oil palm. A reduction of red ring by 80% has been reported by the use of one trap per 5 ha (Chinchilla et al., 1993).

Our preliminary studies have shown that the steam volatiles of the coconut bark are highly attractive to both males and females of *R. ferrugineus* (Gunatilake and Gunawardena, 1986; Gunawardena and Gunatilake, 1993). Isolation of a host attractant for *R. ferrugineus* would allow the food component (coconut stem tissue) of the already used pheromone-food traps to be replaced (Hallet et al., 1993), thus simplifying the trap operations. This is because the species

aggregation pheromone, 4-methyl-5-nonanol (ferrugineol) lasts for more than two months in the field (Gunawardena and Bandarage, 1995), whereas the coconut stem tissue needs weekly replacement. We report for the first time the identification, electrophysiological activity and laboratory bioassay of two host-derived attractants for *R. ferrugineus*.

METHODS AND MATERIALS

Steam Distillation. Young coconut bark (3 kg) up to 10 cm depth from outside of the stem was cut into small pieces and steam distilled for 5 hr in an overall glass apparatus. The distillate (300 ml) was saturated with NaCl and subsequently extracted with diethyl ether (BDH, GPR Grade, 3 × 200 ml). The ether phase was dried over magnesium sulfate and concentrated to 1 ml in vacuo (concentration of **1** and **2** is 0.026 $\mu g/\mu l$ and 0.14 $\mu g/\mu l$, respectively, determined by gas chromatography). Due to the volatile nature of the attractants, the solvent was not completely removed from the extract, and this concentrated etheral solution was used in the laboratory bioassays.

Gas Chromatography. Gas chromatography (GC) was performed on: (1) a Hewlett Packard 5890 A chromatograph equipped with a spitless injector, flame ionization detector (FID), and a fused silica capillary column (25 m × 0.25 mm, SE-30), 4 min at 60°C, 60–260°C at 10°/min, hold, carrier gas N_2; (2) Hewlett Packard 5890 A chromatograph, fused silica column SP-2340 (30 m × 0.25 mm), 4 min at 60°C, 60–195°C at 3°/min, hold, N_2; and (3) Varian 3400 chromatograph, fused silica column SE-52 (25 m × 0.25 mm), 4 min at 60°C, 60–260°C at 6°/min, hold, carrier gas N_2.

Gas Chromatography–Mass Spectrometry (GC-MS). A Varian 3400 gas chromatograph, fitted with a split-splitless injector, coupled to a Finnigan MAT90 double focusing mass spectrometer was used. GC conditions were the same as above in method 3.

Chemicals. Chemical synthesis of 4-hydroxy-3-methoxystyrene **2** was achieved according to Reichstein (1932) by decarboxylation of ferulic acid with quinoline and copper powder. Racemic γ-nonanoic lactone **1** was purchased from Aldrich Chemical Co. Ltd., in Germany.

Electroantennography. Male and female *R. ferrugineus*, 1–5 days old, were air transported from Sri Lanka to Germany, maintained at a photoperiod of 10 hr light and 14 hr dark at 29°C ± 2°C and fed with apples and water. Each weevil (3–8 days old) was anesthetized with CO_2 and the antenna cut off as close as possible to its base. Following the methodology originally described by Schneider (1957), the antenna was fixed on two capillary Ag–AgCl electrodes filled with insect Ringer solution. The recording electrode was inserted into the antennal club on which the olfactory sensilla are located and the indifferent

electrode into the base of the antenna and sealed with vaseline. Olfactory stimuli were applied with an airstream towards the antenna at 3-min intervals passing aliquots on filter paper.

Coupled Gas Chromatography–Electroantennography. Coupled gas chromatography–electroantennographic detector (GC-EAD) (Struble and Arn, 1984) was performed according to GC conditions 2. The column effluent was split between the FID and the antenna and make-up gas (N_2, 20 ml/min) added to the electroantennographic detector (EAD) side to accelerate the GC effluent. The effluent was passed through a heated transfer line into a glass tube (200 mm length, 6 mm ID), the open end of which has been narrowed to 3 mm in order to direct the effluent effectively onto the antenna.

Insects. Larvae of *R. ferrugineus* obtained from infested coconut palms were introduced into a freshly cut young trunk, which was placed in a wooden cage fitted with a wire mesh for ventilation. Adults emerging from the trunk were transferred to a moistened container and fed with sugarcane and 10% sugar solution. Temperature was maintained at 29°C ± 2° and relative humidity at 80 ± 4%. Insects up to the age of two weeks only were used in the bioassays.

Behavioral Bioassay. A choice test was performed in a Y-shaped olfactometer (Gunawardena et al., 1989). The baits were prepared by introducing appropriate amounts of attractants from stock solutions (1 mg/ml ether) on filter paper and allowing the solvent to evaporate. In the case of the natural host attractant, 50 μl of the concentrated ether extract from the steam distillate was used. Blanks were prepared from 50 μl of diethyl ether. Baits were placed in one arm (e.g., A) of the Y tube, and blanks in the other arm (e.g., B), and this sequence was interchanged randomly in the subsequent replicates. A slow stream of air was passed from behind through both arms A and B, and the insect container containing the test insects was fitted into the open end of the third arm C, so that the beetles could move towards A and B against the airstream. After 2 min, the number of insects settled to each arm was counted, and their choice for the baited arm considered as the criterion for activity. Mean numbers of weevils in arms A and B were compared for each bait by the chi-square test. The activities of different baits were compared by ANOVA, and subsequently pairwise comparisons were made with Scheffe's test. The activities of **1**, **2**, and a 1:1 mixture of **1** and **2** were assayed over the dose range 31–750 μg. Subsequently a comparison of the activity of a 1:1 mixture of **1** and **2** (50 μg) was made with the natural host attractant (50 μl) and racemic 4-methyl-5-nonanol (50 μg). Possible host attractant–aggregation pheromone synergism was looked into by separately assaying 1:1 mixtures of the aggregation pheromone (50 μg) and the natural host, 1:2. All bioassays were conducted between 8:00 and 9:00 AM with 10 batches consisting of six weevils in each replicate, as far as possible with equal numbers of males and females.

RESULTS

To search for sensorially active constituents of the palm bark, the steam distillate of young coconut bark was separated by gas chromatography, the column effluent split in a ratio 1:1, and physiologically active compounds recorded with an insect antenna. Thirty-seven minutes after the injection, both female and male *R. ferrugineus* antennae showed a fairly prominent antennal response, the GC-EAD response profiles are depicted in Figure 1a (female) and Figure 1b (male), respectively. In a simultaneously recorded FID chromatogram, the most prominent peak in this elution range was **2** (Figure 1c), and just prior to it an accompanying peak **1** (ratio **1**:**2** = 1:8).

The mass spectrum of **1**, obtained from a subsequent GC-MS analysis, consisted of a base peak of *m/z* 85 and a parent peak *m/z* 138 and was considered to be that of γ-nonanoic lactone. From **2** a spectrum was obtained with parent/base peak of *m/z* 150, congruent with that of 4-hydroxy-3-methoxystyrene (Figure 2). Identical mass spectra as well as retention characteristics on three different GC columns (SE-30, SP-2340, and SE-52, GC conditions given in 1–3) in Methods and Materials) with those of authentic racemic γ-nonanoic lactone **1** and synthetic 4-hydroxy-3-methoxystyrene **2** confirmed the identity of these two palm bark components. Further constituents of the steam distillate were identified as diethylene glycol, triethylene glycol, acetic amide, hexanal, phenyl acetaldehyde, pentylfuran, dihydrobenzofuran and 2-pentanone.

In an electroantennogram (EAG) assay, single 1 sec stimuli of the steam distillate, synthetic **1** in a dose range from 0.1 to 100 μg, **1** as well as the additional identified compounds mentioned above did not elicit considerable electrophysiological responses compared with air controls. Stimuli of **2**, however, showed significant EAG amplitudes and revealed an increase of the electrophysiological activity at higher doses (Figure 3), characteristic for sensorially active compounds. The electrophysiological responses of **2** were even higher than those of equal amounts of the concentrated steam distillate of the natural host attractant.

Investigating the behavioral activity of bark distillate and constituents, the EAG studies were followed by olfactometer test. Searching movements of the snout coupled with rapid movements of the beetles towards the Y junction of the olfactometer and selection of the baited arm within 2 min were considered as positive responses. The results of the behavioral bioassay with the synthetic attractants in a dose range of 31–750 μg are shown in Figure 4. Except in its lowest concentration (31 μg), the γ-lactone **1** always revealed slightly higher activities than the styrene **2**. However, a 1:1-mixture of **1** and **2** attracted significantly higher numbers of red weevils than compounds **1** and **2** individually (e.g., 80.0% vs. 55.6% and 50.0% at 125 μg stimulus concentration). In a final

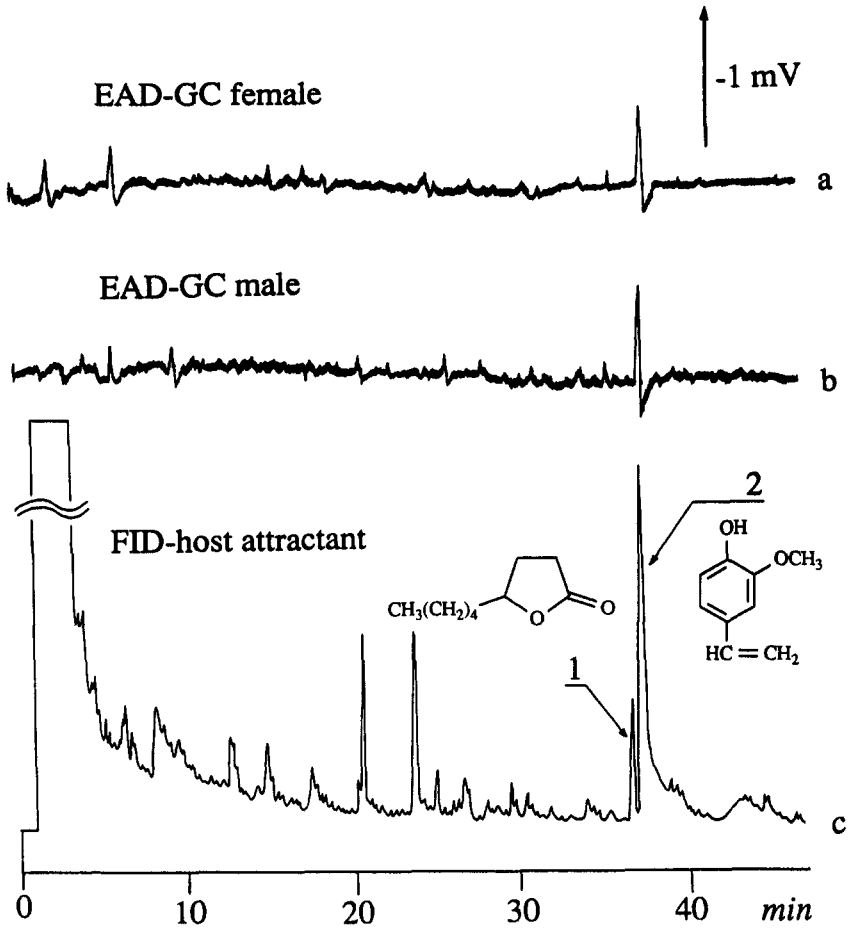
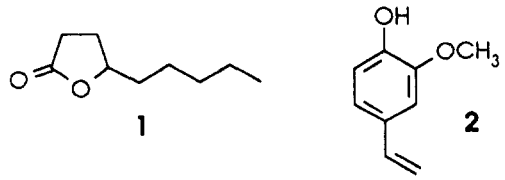


FIG. 1. Electroantennogram detector (EAD) responses of (a) female and (b) male antennae of *Rhynchophorus ferrugineus* and (c) FID chromatogram of a steam distillate of young bark of coconut palm *Cocos nucifera*. **1**, 4-nonalactone; **2**, 4-hydroxy-3-methoxystyrene.



1 2

FIG. 2. Structures of **1** and **2**.

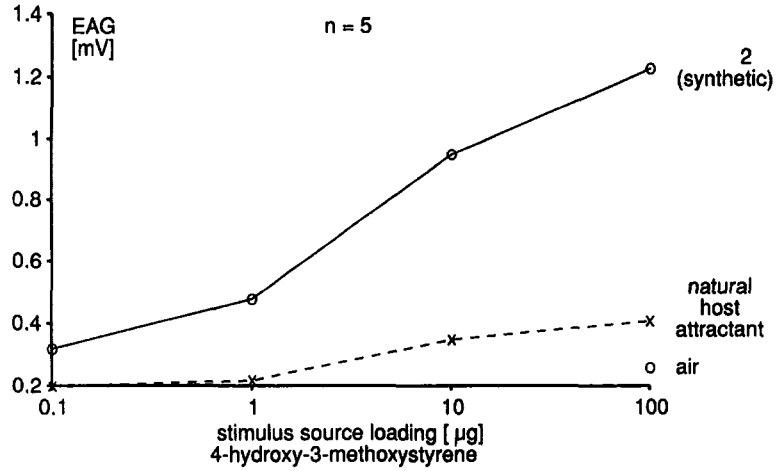


FIG. 3. Dose–response curves of relative electroantennogram response amplitudes of female *R. ferrugineus* to racemic 4-hydroxy-3-methoxystyrene **2** and natural host attractants from coconut bark.

test series, the attractivity of the synthetic mixture of **1** and **2** (50 µg) was compared with that of the concentrated solution of the palm distillate (50 µl) as well as with that of synthetic 4-methyl-5-nonanol (ferrugineol, 50 µg), which is the more attractive component of the aggregation pheromone of the red weevil

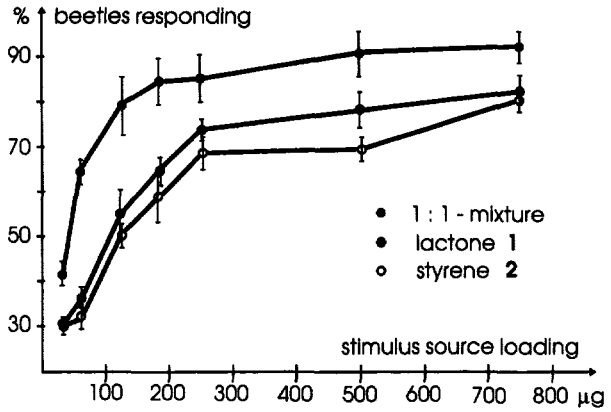


FIG. 4. Mean responses (percentage values) of an olfactometer bioassay with males and females of *R. ferrugineus* to 4-nonanolactone **1**, 4-hydroxy-3-methoxystyrene **2**, and a 1 : 1 mixture of both.

TABLE 1. OLFACTOMETER BIOASSAY RESULTS WITH ADULT *R. ferrugineus*[a]

Attractant (bait)	*N*	Responding (mean ± SEM)		Nonresponding (mean ± SEM)	t_{obs}
		Baited arm (A)	Nonbaited arm (B)	Arm C + insect tube	
Distilled water (50 μl)[b]	10	0.2 ± 0.42c	0.9 ± 0.73	4.9 ± 0.94	2.0
Natural host attractant (50 μl)	10	4.6 ± 0.31a	0.8 ± 0.42	0.6 ± 0.50	19.0
1:1 mixture of **1** and **2** (50 μg)	10	3.4 ± 0.57b	2.1 ± 0.56	0.5 ± 0.48	5.6
Ferrugineol (50 μg)	10	3.7 ± 0.48b	1.7 ± 0.48	0.6 ± 0.52	9.3
1:1 mixture of steam distillate (50 μl) + ferrugineol (50 μg)	10	3.8 ± 0.63b	1.7 ± 0.84	0.6 ± 0.57	6.6
1:1 mixture of **1** + **2** (50 μg) + ferrugineol (50 μg)	10	3.6 ± 0.52b	2.0 ± 0.51	0.4 ± 0.51	5.6

[a]Six insects were used in each experiment. Insects in each arm were counted 2 min after introducing the bait to arm A. Mean number of insects responding followed by similar letters are not significantly different ($P > 0.05$, ANOVA, Scheffe's test).

[b]Except in the case of distilled water, the mean number of insects in baited (A) and nonbaited arms (B) were significantly different ($P < 0.001$, chi-square test).

(Hallet et al., 1993). Distilled water was taken for control comparisons. The 1:1 blend revealed high attractivity similar to that elicited with the species-specific pheromone and also significantly different than the control. No activity increase was observed when aggregation pheromone was mixed 1:1 with the natural or synthetic host attractants (Table 1).

DISCUSSION

The present investigation demonstrates the potency of the 1:1 mixture of synthetic racemic nonanoic lactone **1** and 4-hydroxy-3-methoxystyrene **2** as a lure for the red weevil *Rhynchophorus ferrugineus*. In the choice test, both synthetic **1** and **2** showed moderate attractant properties, whereas the activity of the mixture was significantly higher than those of the single constituents (Figure 4) and almost reached that of the synthetic aggregation pheromone (Table 1). In the case of the control, redistilled water, more than 78% of the insects remained in the C arm of the Y tube and only 3% chose the baited arm. However, because synthetic **1** proved to be essential for the attractivity, it is difficult to understand why it did not evoke much EAG efficacy. A plausible

explanation for this could be that **1** evoked only minute EAG amplitudes that could not be detected under the test conditions used. Furthermore, it is likely that the number of olfactory receptors for **1** is much smaller than the number for **2**. As a consequence, the receptor responses of **1** might be undetectable.

Lactone **1** is chiral and its naturally occurring form could be optically active and exist in one preferred enantiomeric form only. The use of a lure with proper enantiomeric composition might enhance the attractivity of the bait. We plan to synthesize both stereoisomeric antipodes, (*R*)- and (*S*)-4-nonanoic lactone, in optically pure form to elucidate the composition of the naturally occurring attractant by GC on chiral columns.

γ-Substituted butyrolactones are described as defensive secretions from the pygidial gland of staphylinid beetles (Wheeler et al., 1972; Dettner and Schwinger, 1982), but also as a sex pheromone of the female Japanese beetle (Tumlinson et al., 1977), and can be obtained by microbial β-oxidation of suitable oxidized forms of C_{18} fatty acids (Cardillo et al., 1989). 4-Alkyl γ-butyrolactones are ubiquitous natural products and are found in fruits, flowers, tobacco, cooked meat and butter fat (Ravid et al., 1978). Nonanoic lactone **1** is a commercially available artificial coconut-odor component (Abricolin) for apricot and coco fragrances and used as a suntan lotion additive. 4-Hydroxy-3-methoxystyrene **2** is a derivative of ferulic acid (4-hydroxy-3-methoxycinnamic acid) and is formed with various reactions and pyrolytic and heating processes of lignin. Among others, it was found as an aroma component in coffee (Stoll et al., 1967; Gal et al., 1976), tomatoes (Viani et al., 1969), essential oil of Kudzu (Shibata et al., 1978), tar and smoke of hickory (Hruza et al., 1974; Fiddler et al., 1966), and smoke condensates of tobacco (Ishiguro et al., 1976). Generally, it is found in smoked, roasted, heated, and fermented products. More interestingly, alkoxy styrene derivatives were synthesized and tested for attractancy for fruit fly species, and the benzoate of **2** was found to be moderately attractive for male and female melon flies (Shaw et al., 1976).

γ-Nonanoic acid lactone **1** and 4-hydroxy-3-methoxystyrene **2** are new structures as curculionid host attractants, since only simple aromatic compounds such as benzaldehyde, aliphatic alcohols, and terpenes (Dickens, 1990; Müller and Haufe, 1991; Budenberg et al., 1993) are known so far as attractants for this coleopteran family. 3-Methylindol has been used as an artificial attractant in field trapping of *R. palmarum* rather successfully in the West Indies, although it was not confirmed as a host attractant (Hagley, 1965). Recently several simple and host-derived alcohols, acetates, aldehydes, and esters of carbon chain lengths varying from C_2 to C_6 were recognized as synergists to the respective aggregation pheromone of *Rhynchophorus* species in the field. For example, some synergists for the aggregation pheromones of three palm weevils are as follows: ethyl acetate or a mixture of ethyl alcohol, ethyl acetate, pentane, hexanal, isoamyl acetate, or isopentanol for *R. palmarum* (Jaffé et al., 1993); ethyl

propionate, ethyl butyrate, and ethyl isobutyrate for *R. phoenicis* (Gries et al., 1994), and ethyl acetate, ethyl lactate, ethyl butyrate, ethyl isobutyrate, and ethanol for *R. cruentatus* (Giblin-Davis et al., 1994). However, these compounds alone did not attract the weevils in the field. This led to the assumption that in these field attractant lures, viz. aggregation pheromone-host derived small molecules, the former acts as a long-range attractant, while the latter provides the short-range orientation cues to the bait (Jaffé et al., 1993; Weissling et al., 1994). The fact that palm tissues attract weevils from a distance in the absence of species's aggregation pheromone suggests that the host palms produce their own long-range attractants in addition to short-range attractants recognized above. The field observation that weevil catches for the host tissue–aggregation pheromone combination have often been higher than those of the host derived small molecule aggregation pheromone (Giblin-Davis et al., 1994; Gries et al., 1994) adds proof to this. The two host attractants **1** and **2** do not conform to the structural characteristics of short-range attractants known so far in the family Curculionidae in that they are of higher molecular weight, asymmetric, and one of them is chiral. Compounds **1** and **2** did not show synergism with the species aggregation pheromone, ferrugineol (Table 1). It is therefore likely that **1** and **2** are the host's long-range attractants. This, however, should be proved by a field bioassay. Literature evidence of long range attractants is rather scant in the family Curculionidae.

Young coconut palms are known to be more susceptible to red weevil attack than older ones. Since lignin as the possible precursor of **2** is found in many plants, however, it might be the physiological state of the young plant determines the content of the 4-hydroxy-3-methoxystyrene **2**. It is furthermore unlikely that the methoxystyrene alone is responsible for the host attraction. The synergism between the methoxystyrene and the lactone observed in the bioassay adds proof for this. Although the lactone **1** was shown to be electrophysiologically inactive compared with the methoxystyrene **2**, the responses by weevils in the behavioral assay appeared stronger to the former than to the latter (Figure 4). This suggests the possibility that the lactone **1** is an even more essential ingredient for the red weevil's attraction to the host volatiles. The possibility that there are still other important attractant components in the host volatiles can not be ruled out. Several coconut stem tissue-derived attractants already identified, i.e., ethanol, ethyl acetate, pentane, hexanal, isopentanol, and amyl acetate by headspace analysis (Jaffé et al., 1993) were not found in our steam distillates. In effect, our method of isolation, i.e., steam distillation, has yielded volatile attractants with higher molecular weight.

Identification of **1** and **2** in the host volatiles of the coconut bark is perhaps the first major breakthrough in understanding the host finding behavior of *R. ferrugineus*. This knowledge, coupled with the recently isolated aggregation pheromones of the species will lead to an efficient lure for the red weevil whose

detection has been difficult in the past. The fact that host attractants **1** and **2** are readily available compounds would make their use more economical and practical. Traps baited with **1** and **2** with and without ferrugineol would indicate whether they possess long-range attractant properties or they act more like short-range orientation cues for the weevils. Depending on the results, **1** and **2** may be combined with the species aggregation pheromone or potent short-range attractants in order to formulate an efficient lure for the red weevil.

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