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Original Article

Inhibitory substances contained in calcium carbonate wettable powder on the oviposition of the peach fruit moth, *Carposina sasakii*

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Spraying a calcium carbonate suspension "White Coat" on the fruit of apples significantly suppresses the oviposition of the peach fruit moth, *Carposina sasakii*. In gas chromatography (GC) with an electroantennographic detector analysis, adult female antennae showed responses to three compounds that were identified as 2,2,4-trimethyl-1,3-pentanediol diisobutyrate (TXIB) and its two mono-hydrolyzed analogs, texanols (1- and 3-isobutyrates), all added as a plasticizer to the agents. An oviposition-choice test using adult moths revealed that TXIB has clear deterrent properties when applied to young apple fruits. Video recording analysis showed that female moths spent longer on self-grooming and searching around TXIB-treated fruits. In the same assay, pure calcium carbonate treatment prevented the moths from climbing up or landing on the fruits, while such was not the case with White Coat-treated fruits. TXIB, an adjuvant aimed to provide rain/wind resistance, weakened the slipperiness of the calcium carbonate coating but, coincidentally, maintained the oviposition inhibitory activity of the White Coat by its deterrent odorant.

Keywords: Txib, peach fruit moth, oviposition, calcium carbonate, pest control, electron microscope.

Electronic supplementary materials: The online version of this article contains supplementary materials (Supplemental Figs. S1 and S2), which are available at http://www.jstage.jst.go.jp/browse/jpestics/.

Introduction

The peach fruit moth, *Carposina sasakii* Matsumura,[1)] is a serious insect pest of apples, *Malus domestica* Borkh., and other rosaceous fruits in Japan.[2)] Their habitat is within Northeast Asia including Korea and China,[3)] and their infestation is regarded as a matter for international quarantine.[4)] Neonate larvae burrow into the fruits and spend their entire larval phase as internal fruit feeders, which renders insecticides ineffective during this phase. Adult moths generally emerge from spring to summer over several months.[5)] Since the seasonal pattern of adult emergence is very unpredictable from year to year,[6)] timely spraying of insecticides targeting adults is also difficult. As a result, management of this pest relies on the constant spraying of insecticides. Mating disruption using synthetic sex pheromones is currently practiced, but it is not sufficiently effective.[7)] Overall, more efficacious management is urgently required.

Preventing oviposition could be one of the most effective methods of controlling *C. sasakii*. This species oviposits on the hairy surface of the apple stalk cavity and calyx end, while no eggs are laid on smooth surfaces.[2,8)] In the case of peach fruits, which are thoroughly covered with pubescence, the insects lay eggs all over the fruits.[2)] The hairy texture of the fruit surface is considered a physical stimulus for oviposition.[9)] In addition to physical stimuli, chemical cues are also thought to be important in oviposition.[10)] For example, despite pubescence growing on the entire surface of apple leaves, this insect never lays eggs there.

During World War II, lime (CaO) solution was sprayed in Japan as a deterrent, with the goal of reducing the number of eggs laid on the fruit of *C. sasakii*.[6,11–13)] This suppression mechanism has been explained by the destruction of the hair structure due to the adhesion of lime.[10,11)] Another explanation is that microcrystals of calcium carbonate may injure antennae of the adult female, making it impossible to orient to oviposition targets.[14)] Recently, lime solution has been revived in a better (commercial) form, named "White Coat" (Shiraishi Calcium, Osaka,

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Japan), which consists primarily of calcium carbonate and contains a spreading agent as a minor constituent so that rain will not easily wash off the calcium carbonate micro-powder. In an orchard test, spraying apple fruits with White Coat significantly reduced the number of eggs laid by *C. sasakii*.[15] This pest control agent has also been reported to effectively protect mandarin orange fruits from yellow tea thrips, *Scirtothrips dorsalis* Hood.[16] This mechanism has been explained by the fact that the color change (whitening) of the fruits caused by calcium carbonate micro-powder makes the pests unable to spot the fruits.[17] However, in the case of the peach fruit moth, oviposition activity is observed only after dark, and the moth relies on chemical detection rather than optical detection. Therefore, the whitening hypothesis may not be appropriate in this case. Sequential bioassays suggested that oviposition was invoked by at least two steps: detecting volatile chemical cues from a distance during exploration and receiving physical contact stimuli on the fruit surface to choose the oviposition site.[15] Treating the fruit surface with calcium carbonate had no adverse effects on the mechanical function of pubescence, and there was no evidence that calcium carbonate repels or inhibits the ovipositional activity of females.[15]

The purpose of this study was to elucidate the mechanism by which White Coat causes oviposition suppression. Expecting that the treatment may affect apple volatile emissions, either by absorbing attractive odors or by adding repellent odors, GC-MS and GC-EAD analyses were conducted with young apple fruits with/without treatment. Consequently, candidate repellent compounds were examined for inhibitory effects using closed cages. The speculated mechanisms of action were further analyzed using video recordings of bioassays and using scanning electron microscopy (SEM).

Materials and Methods

1. Chemicals

White Coat was purchased from Shiraishi Calcium. Calcium carbonate, dodecane, pentadecane, hexadecane, heptadecane, nonyl acetate, dichloromethane, tetramethylsilane, and deuterated chloroform were obtained from Wako Pure Chemicals (Osaka, Japan). Other standards were obtained as follows: tridecane and tetradecane (Tokyo Chemical Industry, Tokyo, Japan), farnesene (mixture of isomers) and hexyl 2-methylbutanoate (Sigma-Aldrich, St. Louis, MO, USA), TXIB (Matrix Scientific, Columbia, SC, USA), texanol (Santa Cruz Biotechnology, Dallas, TX, USA).

2. Insects and plants

Larvae were obtained from a laboratory colony that originated in an abandoned orchard in Hirosaki, Aomori Prefecture, in 1999. The offspring of the source individuals were reared on immature apples for successive generations at 23°C and with a 16 hr/8 hr (light/dark) photoperiod in the laboratory at Aomori Prefectural Industrial Technology Research Center (AITC; Aomori, Japan). Subsequent generations of this laboratory colony were used for all experiments.

Immature apple fruits ('Fuji'/*Malus prunifolia* rootstock) were harvested in July 2016 and 2017 in the orchards at AITC and stored at 4±5°C under dark conditions. In all orchards, conventional disease control was practiced, but no insecticide was applied.

3. Volatile collection and analyses

In 2015, an experiment was conducted to compare the volatile components of White Coat-sprayed fruits and unsprayed fruits in the orchard at AITC. Immature apple fruits on the tree were covered with a PVDF bag (Omi Odor Air Service, Shiga, Japan) and connected to an air pump attached to a pre-filter to remove contamination. The air that passed through the bag was collected in another filter trap packed with 30 mg HayeSep Q (80/100 MESH, Hayes Separations, Bandera, TX, USA). Collection in the field began after dawn and was carried out for 12 hr. Volatiles were collected from fruits treated with White Coat and intact fruits and leaves (N=3–4). After collection, the filter was extracted with 100 μL of hexane: dichloromethane=1:1. Nonyl acetate (400 ng) was added as an internal standard. Then, 1.0 μL of each sample was analyzed by gas chromatography-mass spectrometry (GC-MS) (Agilent 6890 N gas chromatograph with an Agilent HP-5MS capillary column 30 m×0.250 mm i.d., 0.25 μm thick film, interfaced to an Agilent Technologies 5975 mass spectrometer). The temperature of the injection port was 240°C. The column temperature was held at 60°C for 2 min after injection and then programmed at 10°C/min until 290°C and held for 5 min. Chromatograms were analyzed using Agilent 5975 Inert/N MSD ChemStation. The leaf volatile samples were diluted tenfold before the analysis.

In addition, the volatile component of the treated/untreated fruits was also collected in the laboratory using solid-phase microextraction (SPME) (Supelco, Bellefonte, PA, USA). The fibers used were coated with Carboxen/Polydimethylsiloxane (CAR/PDMS, 1 cm long, 85 μm thick). The SPME fiber was exposed to the headspace air for 2 hr in a 500 mL glass beaker containing three immature picked apples. Apples collected included both untreated fruits and fruits treated with White Coat (N=5). Fruits were treated by soaking the entire fruit in White Coat aqueous solution (250 g/L) and completely drying it. Untreated fruits were immersed in tap water and air-dried. The GC-MS analysis conditions were the same as described above.

Identification of compounds was achieved using the Wiley7 NIST05 mass spectral database and then by comparing the mass spectra and retention time of the chromatographic peaks with those of authentic samples.

4. GC-EAD analyses

This experiment was conducted in 2016 to investigate the responses of moth antennae to volatiles from young apple fruits treated with White Coat. Analyses were carried out using a GC with an electroantennographic detector (EAD) and a flame ionization detector (FID) (n=11). Volatiles were collected from SPME fibers as described above. Fibers were held in a beaker

for 2.5 hr. The Agilent Technologies 6890 N gas chromatographs were equipped with Agilent HP-5MS (30 m×0.250 mm i.d., 0.320 μm thick film; Agilent Technologies). At the end of the column, the flow path was split using a Y-shaped quartz splitter (Agilent Technologies), and the sample was divided equally and introduced into the FID and EAD. The temperatures of the injection port and FID port were 240°C and 290°C, respectively. Oven temperatures were programmed at 60°C for 1 min, then 20°C/min up to 290°C, and then kept for 2.5 min. An antenna including a basic segment was gently excised from an anesthetized moth head with tweezers under a stereoscopic microscope and was placed between recording/indifferent metallic electrodes with SPECTRA® 360 electrode gel (Parker Laboratories, Fairfield, NJ, USA). Under the same condition, *n*-hexane solution of TXIB (20 ng in 1 μL) was tested to evaluate the antennal responses. Mated females and males (1–2 days after emergence) were tested in this assay. The obtained electrical potential changes were drawn using Igor Pro (Hulinks, Tokyo, Japan).

5. Isolation and identification

One hundred grams of White Coat was extracted with dichloromethane, concentrated, and then separated with silica gel column chromatographic purification using a mixture of *n*-hexane and ethyl acetate (9 : 1). The compound was identified by using NMR. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were measured with a Bruker AV-400 III Spectrometer (400 MHz) (Bruker, Billerica, MA, USA) using tetramethylsilane as an internal standard and deuterated chloroform as a solvent.

6. Oviposition assays

This experiment was carried out in 2017 to examine preferences in ovipositional targets. The assay system is shown in Supplemental Fig. S1 (n=14–29). White Coat was used in the same concentration as that used in the volatile collection using SPME. The concentration of the pure calcium carbonate treatment was set to be equivalent to that of White Coat (250 g/L). In both treatments, fruits were soaked in aqueous solution and air-dried. TXIB did not dissolve in the aqueous solution, and the organic solvents tested so far were corrosive to the fruits to some extent. In this assay, TXIB was dissolved in rapeseed oil (400 μg/mL, Healthy Lisetta; Nisshin OilliO, Tokyo, Japan), and two droplets were applied to a fruit and spread over the entire surface with KimWipes. The concentration was determined by analyzing the volatile from the treated fruits, so the amount of emitted TXIB was equal to that of the fruits treated with White Coat. Two analogs of TXIB, which were obtained during the extraction of White Coat, were also tested, as was the case with TXIB. The eggs laid in each treatment group after a one-night assay were counted. Insect behaviors were also recorded with a camera (HDR-PJ680; Sony, Tokyo, Japan), under a small LED light covered with a red cellophane sheet. In each case, two singly treated fruits and 10 pairs of insects were placed in the same cage. The behaviors were classified as searching (walking around/on the fruits waving antennae), abdomen-bending, and self-grooming. The total time of each behavior in the first 2 hr of the assay was measured and compared among the treatments. All tests were conducted under the same conditions as the breeding environment.

7. Scanning electron microscope photograph

In order to check any changes in the antennae or tarsus/hook of adult females after contact with treated fruits, the microstructures of these parts from mature females were observed under a scanning electron microscope (VE-8800; Keyence, Osaka, Japan) (n=4–6). Fruits were placed in the bottom of a small cage (4 cm×8 cm i.d.), and an adult female (mated, 0 days after emergence) was released to walk on the fruit for two minutes. The tip of the foreleg of the moth, anesthetized by CO_2, was detached with a tweezer and placed under the microscope. The results were compared among females who made contact with intact fruits, calcium carbonate-treated fruits, and White Coat-treated fruits.

8. Statistical analyses

All data in this paper were statistically analyzed using BellCurve for Excel software (Social Survey Research Information, Tokyo, Japan).

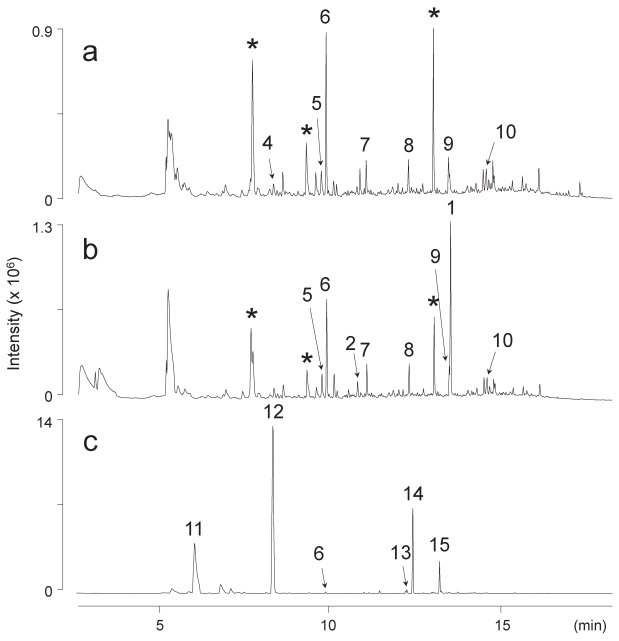

Fig. 1. Total ion chromatogram (TIC) of GC-MS analysis of volatiles collected from intact apple fruits (a), fruits treated with White Coat (b), and foliage (tenfold dilution) (c). 2,2,4-Trimethyl-1,3-pentanediol diisobutyrate (TXIB) (1); 2,2,4-trimethyl-1,3-pentanediol 3-isobutyrate (texanol) (2); dodecane (4); tridecane (5); nonyl acetate (I.S.) (6); tetradecane (7); pentadecane (8); hexadecane (9); heptadecane (10); β-ocimene (11); methyl salicylate (12); (*Z*,*E*)-α-farnesene (13); (*E*,*E*)-α-farnesene (14); and (*Z*)-3-hexenyl benzoate (15). The asterisks indicate unidentified compounds.

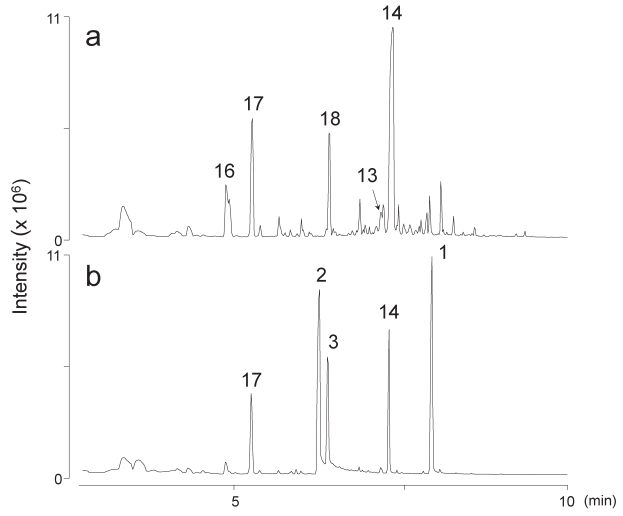

Fig. 2. TIC of GC-MS analysis of volatiles collected from intact fruits (a) and processed fruits treated with White Coat (b) using SPME. 2,2,4-Trimethyl-1,3-pentanediol diisobutyrate (TXIB) (1); 2,2,4-trimethyl-1,3-pentanediol 3-isobutyrate (texanol) (2); 2,2,4-trimethyl-1,3-pentanediol 1-isobutyrate (texanol) (3); (*Z*,*E*)-*α*-farnesene (13); (*E*,*E*)-*α*-farnesene (14); butyl caproate (16); hexyl 2-methylbutanoate (17); and hexyl caproate (18).

Results

1. Structural determination of volatile compounds

The analytical results of volatile components collected from intact apples, apples treated with White Coat solution, and leaves in the orchard, using the HayeSep Q filter are shown in Fig. 1. The chromatogram of volatiles from White Coat-treated fruits (Fig. 1b) was basically the same as that of intact fruits (Fig. 1a) except for two additional peaks (compounds 1 and 2). Figure 2 shows volatile compounds from picked fruits collected using SPME. The two compounds specific to White Coat-treated fruits were found again (Fig. 2b, compounds 1 and 2), as was another compound that was also analogous (Fig. 2b, compound 3).

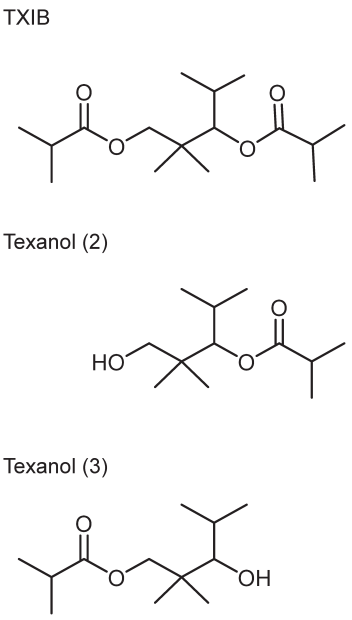

Fig. 3. Chemical structures of TXIB and its analogs, texanol(s).

The three compounds obtained from White Coat extracts and purified using a silica gel column were analyzed with NMR. From the NMR signal analysis and comparison with the standard, these compounds were identified as 2,2,4-trimethyl-1,3-pentanediol diisobutyrate (TXIB, compound 1) and its analogs, 2,2,4-trimethyl-1,3-pentanediol 3-isobutyrate and 2,2,4-trimethyl-1,3-pentanediol 1-isobutyrate (compounds 2 and 3, respectively, both named texanol) (Fig. 3). 2,2,4-Trimethyl-1,3-pentanediol diisobutyrate (TXIB): ^{1}H-NMR (CD_3OD, 400 MHz) δ0.90 (3H, d, J=6.8 Hz), 0.97 (3H, d, J=6.9 Hz), 0.97 (3H, s), 0.99 (3H, s), 1.18 (3H, d, J=6.9 Hz), 1.18 (3H, d, J=7.0 Hz), 1.20 (3H, d, J=6.9 Hz), 1.20 (3H, d, J=7.0 Hz), 2.04 (1H, dsep, J=3.1, 6.9 Hz), 2.57 (1H, sep, J=7.0 Hz), 2.60 (1H, sep, J=7.0 Hz), 3.80 (1H, d, J=11.0 Hz), 3.88 (1H, d, J=11.0 Hz), 4.78 (1H, d, J=3.1 Hz). ^{13}C-NMR (CD_3OD, 100 MHz) δ 18.05, 19.16, 19.21, 19.40, 19.47, 21.55, 21.92, 23.34, 28.50, 34.35, 34.65, 39.09, 70.05, 79.75, 176.77, 177.21. 2,2,4-Trimethyl-1,3-pentanediol 3-isobutyrate: ^{1}H-NMR (CD_3OD, 400 MHz) δ0.86 (3H, s), 0.94 (3H, d, J=6.9 Hz), 0.97 (3H, d, J=6.8 Hz), 1.02 (3H, s), 1.22 (6H, $(CH_3)_2CHCOO$–, d, J=7.0 Hz), 2.07 (1H, dsep, J=2.8, 6.8 Hz), 2.65 (1H, sep, J=7.0 Hz), 3.77 (1H, d, J=10.9 Hz), 4.12 (1H, d, J=10.9 Hz), 4.76 (1H, d, J=2.7 Hz). ^{13}C-NMR (CD_3OD, 100 MHz) δ 17.71, 19.27, 19.30, 19.59, 22.31, 22.96, 28.29, 34.59, 40.01, 69.98, 79.24, 178.41. 2,2,4-Trimethyl-1,3-pentanediol 1-isobutyrate: ^{1}H-NMR (CD_3OD, 400 MHz) δ0.93 (3H, d, J=6.8 Hz), 0.96 (3H, s), 0.97 (3H, s), 1.01 (3H, d, J=6.9 Hz), 1.19 (3H, d, J=7.0 Hz), 1.20 (3H, d, J=7.0 Hz), 1.92 (1H, dsep, J=2.4, 6.8 Hz), 2.58 (1H, sep, J=7.0 Hz), 3.77 (1H, d, J=10.9 Hz), 4.12 (1H, d, J=11.0 Hz), 4.77 (1H, d, J=3.12 Hz). ^{13}C-NMR (CD_3OD, 100 MHz) δ 16.71, 19.03, 19.08, 20.47, 22.07, 23.57, 28.67, 34.21, 39.33, 71.43, 79.47, 177.30.

The following describes the compounds from the apple fruits and foliage. Comparing the retention time and mass spectrum with authentic samples, we identified nine compounds in total (Fig. 1: dodecane (4), tridecane (5), tetradecane (7), pentadecane (8), hexadecane (9), heptadecane (10), (*Z*,*E*)-*α*-farnesene (13), (*E*,*E*)-*α*-farnesene (14), and hexyl 2-methylbutanoate (16)). Five of them were saturated hydrocarbons. Five compounds (Fig. 1, *β*-ocimene (11), methyl salicylate (12), (*Z*)-3-hexenyl benzoate (15), butyl caproate (16), and hexyl caproate (18)) were tentatively identified using the mass spectral database search and reference data from previous research.[18–22] The fruit and leaf samples contained no common volatile compounds.

While only trace amounts of (*E*,*E*)-*α*-farnesene were emitted from on-the-tree fruits (Fig. 1a), it was a dominant component in picked fruits (Fig. 2a). In order to see whether such a difference might be associated with the collection methods, we checked the volatiles from picked fruits using the HayeSep Q filter and found that the compound was still dominant in picked fruits.

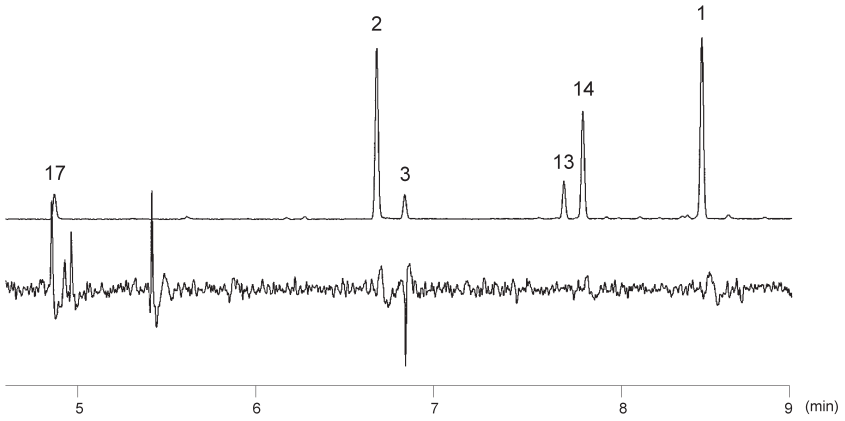

Fig. 4. GC analysis of volatile compounds from fruits treated with White Coat. Detection was simultaneously carried out by FID (upper) and EAD with a mated female antenna (lower). The active components were 2,2,4-trimethyl-1,3-pentanediol diisobutyrate (TXIB) (1); 2,2,4-trimethyl-1,3-pentanediol 3-isobutyrate (texanol) (2); 2,2,4-trimethyl-1,3-pentanediol 1-isobutyrate (texanol) (3); (*E*,*E*)-α-farnesene (14); and hexyl 2-methylbutanoate (17).

2. GC-EAD analysis of collected volatiles and TXIB standard

GC-EAD analysis was conducted to investigate the antennal responses to volatiles from apples treated with White Coat solution (Fig. 4). Antennae of female moths responded to the compounds at retention times of 4.8 min, 6.7 min, 6.8 min, 7.8 min, and 8.5 min (2,2,4-trimethyl-1,3-pentanediol diisobutyrate (1), 2,2,4-trimethyl-1,3-pentanediol 3-isobutyrate (2), 2,2,4-trimethyl-1,3-pentanediol 1-isobutyrate (3), (*E*,*E*)-α-farnesene (14), and hexyl 2-metylbutanoate (17)) (n=11). Injection of commercially available TXIB in hexane solution also gave a clear electroantennographic response in GC-EAD (Supplemental Fig. S2) (n=25). Male antennae also responded to the authentic TXIB (n=21), and there was no difference in the electrophysiological responses of males and females.

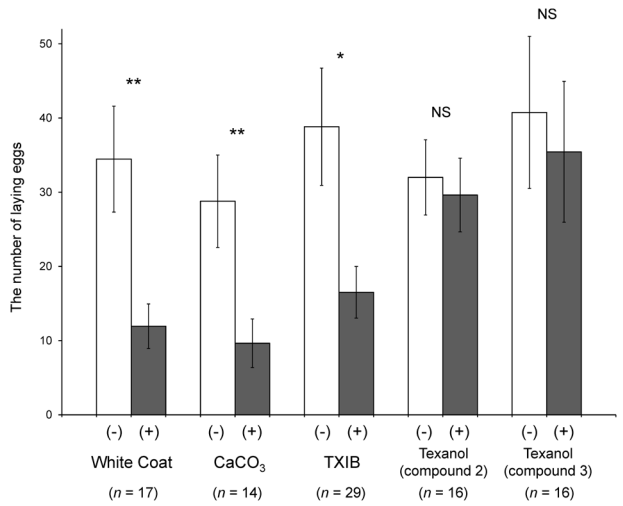

Fig. 5. The number of eggs laid on intact, White Coat-treated, calcium carbonate-treated, TXIB-treated, and TXIB's analog-treated apple fruits (n=14–29, mean±S.E.). *p<0.05, ** p<0.01; NS, Not significant (Mann–Whitney *U*-test).

3. Oviposition assay and behavioral observation

The numbers of eggs laid in the oviposition assay are shown in Fig. 5. In this assay, clear oviposition inhibition was observed in White Coat-treated fruits as compared with intact fruits. In the oviposition test of TXIB, the number of eggs laid was also significantly smaller in the treated fruits (+) than in the control fruits (−, rapeseed oil only). The control fruits treated with rapeseed oil did not show any difference in the number of eggs laid as compared to intact fruits (white bars of Fig. 5; one-way ANOVA, $F_{4,87}$=0.34, p=0.851). In the analogs of TXIB, there was no decrease in the number of eggs in the treated plots. Calcium carbonate-treated fruits also showed an inhibitory effect.

Behavioral analysis of the assay was conducted using a video recorder, and the results are shown in Fig. 6. Female moths typically start by searching, walking, or landing on or around fruits, waving the antennae, and then climbing up the fruits and bending the abdomen to lay eggs around the stalk cavity and calyx end of the fruits. The time searching for fruit on the surface (Fig. 6a) was significantly shorter in all treated groups than in the untreated group. The same tendency was also observed at the time of abdomen bending (Fig. 6b). Interestingly, the time for searching/wandering around fruits (Fig. 6c) was longer on TXIB-treated fruits than on other fruits. In addition to antenna waving, frequent movement of labial palps was observed. Furthermore, the time spent on self-grooming of the antennae (Fig. 6d) was especially long on TXIB-treated fruits. On the other hand, on calcium carbonate-treated fruits, we observed many individuals failing to climb up and sliding down from the fruit surface when landing.

4. Microstructural observation of moth antennae and forelegs

Scanning electron microscope photographs showed that the White Coat particles did not adhere to the antennae (Fig. 7a). In contrast, there was adhesion of calcium carbonate particles to the antennae, but no obvious damage, as expected (Fig. 7b).[14] The particles observed on the antennae were identical to those observed on the surface of fruits treated with pure calcium car-

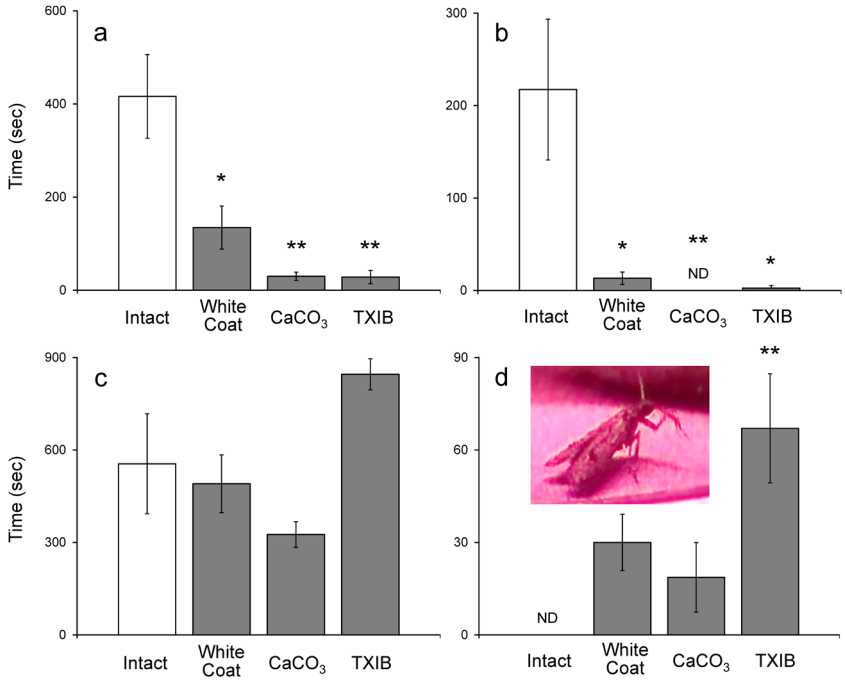

Fig. 6. Behaviors of mated female moths up to oviposition toward intact calcium carbonate treatment, White Coat treatment, and TXIB treatment in apple fruits (n=3, mean±S.E.). Each behavior indicated searching on fruit surfaces (a), abdomen-bending for oviposition (b), searching/wandering the surroundings of fruits (c), and antenna cleaning (d). ND, Not detected. *p<0.05, ** p<0.01 (Dunnett's test).

bonate (Fig. 7c). Nothing was attached to the tarsus of moths that walked on the intact fruit (Fig. 8a). Calcium carbonate fine particles also stuck to the hooks of the foreleg tarsus, in both cases of fruits treated with White Coat and pure calcium carbonate (Fig. 8b, c). However, the adhesion of particles to the arolium, an organ for walking and hanging, was observed only in the case of treatment with pure calcium carbonate (Fig. 8b).

Discussion

Calcium carbonate wettable powder, White Coat, is a relatively newly registered agricultural reagent for protecting fruits from pest insects. Its efficacy was reported in an apple orchard against the peach fruit moth, while the mechanism of the effect was unclear.[15] To evaluate whether there is a chemical effect, we collected volatile compounds from young apple fruits treated with White Coat and analyzed them using GC-EAD. GC-EAD analysis is a common technique used to search for physiologically active substances. We repeatedly observed clear electrophysiological responses to (*E*,*E*)-α-farnesene, which was a minor volatile from on-the-tree fruits but a dominant volatile from picked fruits in our experiments ('Fuji'), and also was reported as a dominant volatile produced by cut branches with unripe apple fruits ('Discovery').[21] Although there are currently no reports that this compound has any effect on peach fruits moths, it is well known as an ovipositional stimulant of codling moths.[23] Similarly, antenna responses were also obtained toward three chemicals' characteristics of White Coat-treated fruits. One of the compounds with the strongest signal was identified as TXIB, which is added in small amounts to White Coat as a spreading agent, probably to prevent the calcium carbonate powder from being easily washed off by rain.[24,25] The other two compounds were mono-hydrolyzed analogs of TXIB (diisobutyrate), named texanol(s), which also have an effect as a spreading agent. It is unclear whether these were added for some reason or were de-

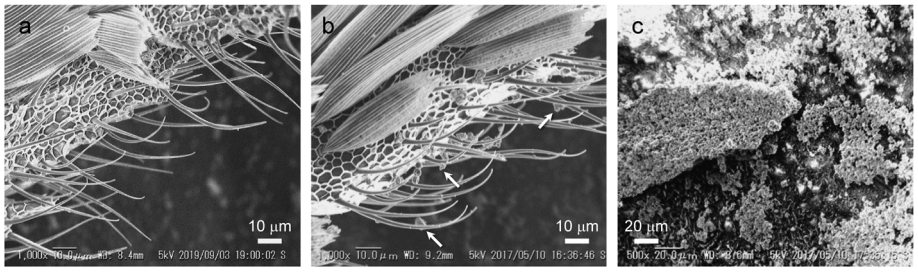

Fig. 7. An antenna of a mated female moth contacted with White Coat-treated fruit (a), an antenna contacted with calcium carbonate-treated fruit; arrows indicate calcium carbonate particles (b), the surface of calcium carbonate-treated fruit (c).

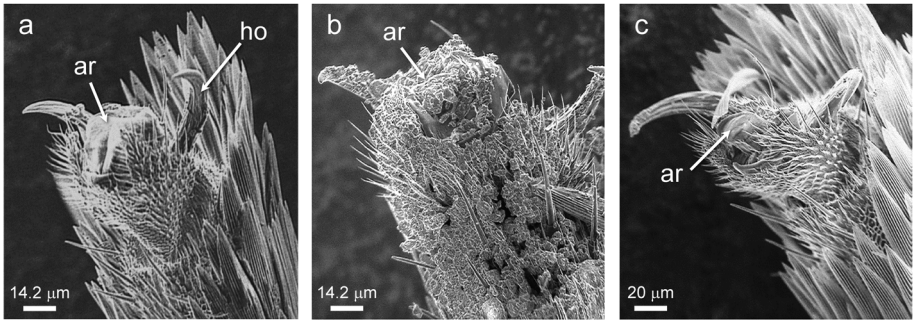

Fig. 8. The fifth tarsomere of the foreleg of an adult female. An individual brought into contact with intact fruit (a), an individual contacted with calcium carbonate-treated fruit (b), and an individual contacted with White Coat-treated fruit (c). Ho, hook; ar, arolium.

gradants/by-products of TXIB.[26] The TXIB used in the test is racemate; the activity of the stereoisomers is currently under investigation.

The oviposition choice tests using female moths showed that TXIB treatment on fruits had a clear oviposition-inhibitory effect equivalent to that caused by White Coat treatment. Interestingly, in the same assay, two TXIB analogs (texanol) were not found to have such an inhibitory effect (Fig. 5), while both monoisobutyrate analogs were electrophysiologically active (Fig. 4). Although the mechanism of how moths distinguish the chemical structure is unknown, the compound structure of diisobutyrate may be necessary for inhibitory activity. More importantly, pure calcium carbonate, a main component of White Coat, was also comparably active in this assay. These two active components, TXIB and calcium carbonate, apparently have different mechanisms of inhibitory activity against insect oviposition. First, the behavior of female moths during the assay was distinctly different among the treatments. In the case of TXIB-treated fruits, females took a particularly long time to search around and performed antennation as well as the moving of labial palps. Oviposition behavior was suppressed, probably as a result of obtaining some information by the chemoreception of TXIB. Another remarkable feature of the insect reaction to TXIB-treated fruits was the high frequency of antennal self-grooming (Fig. 6d). This may be associated with the antennal detection of TXIB volatilized from the fruit surface. In the case of pure calcium carbonate treatment, many individuals slipped down from the fruits, suggesting the decrease in the number of eggs laid was due to landing failure. Thus, TXIB acts chemically and calcium carbonate acts physically to exert their inhibitory effects. Electron microscope analysis revealed that direct contact with calcium carbonate particles could clog the moth tarsus. As shown in Fig. 8b, the particles stuck to the arolium, a flexible pad of the tarsus tip, which is important for gripping smooth surfaces. This was probably the cause of the insects' difficulty in climbing the fruit surfaces. Interestingly, such severe adhesion was not observed with White Coat treatment, except for a minor attachment of the particles on hooks (Fig. 8c). Such a minor attachment may not hinder insects' walking ability, and, actually, not many individuals were observed to slip on White Coat-treated fruits in the behavioral assay. Considering all of these results together, we conclude that TXIB is responsible for a large part of the oviposition-inhibitory effects of White Coat against the peach fruit moth on apple fruits.

A spreading agent that enhances permeability, rain resistance, ultraviolet resistance, and stability is called an adjuvant, a functional spreader.[27] TXIB is a synthetic plasticizer generally used as an adjuvant in agricultural chemicals, paints, and adhesives. The reason TXIB is added to White Coat is, presumably, to help calcium carbonate powder attach to fruit surfaces and, consequently, enhance rain/wind tolerance. This, ironically, weakens the slipperiness that pure calcium carbonate powder originally had, as shown in our oviposition bioassay. In our experiment, female moths seldom failed to climb fruits treated with White Coat, in contrast to the pure calcium carbonate treatment. Coincidentally, TXIB exhibited an inhibitory effect toward moth oviposition in the assay. GC-EAD results and the moth behavior (self-grooming) suggest that it functioned as an active substance in chemoreception. It was not obvious whether the White Coat treatment also functioned as a repellent in our assay. The time for self-grooming (Fig. 6d) with White Coat-treated fruits was longer than that in intact fruits, but there was no significant difference with calcium carbonate-treated fruits. Electron micrograph (Fig. 7b) showed calcium carbonate particles attached to the moth's antennae, which may cause the insects to self-groom. Such an attachment was not obvious in the case of White Coat-treated fruits (Fig. 7a). This suggests that self-grooming in response to White Coat-treated fruits (Fig. 6d) may be caused by TXIB contained in White Coat. The reason the moth's behavior toward White Coat-treated fruits and TXIB-treated fruits was not identical (Fig. 6a, c, d) can be partly explained by our assay. We could not test the effects of TXIB under the same conditions as those of White Coat. The concentration of TXIB in the air at a given time was set to be equal in both White Coat treatments and TXIB treatments, but the solvents were different. This could affect the release rate of TXIB, which may directly or indirectly influence the moth's behavior. At this point, we have no clear answer whether TXIB has completely replaced calcium carbonate as the main active substance in White Coat. Further evaluation is necessary, not only in the lab but also in orchards.

TXIB has been widely accepted as a spreading agent and is already used in agrichemicals. The reason *C. sasakii* moths avoid this compound is still unclear. We have not yet checked whether other agrichemicals containing TXIB also exhibit the same effects. Our study brings TXIB into the spotlight as a useful approach to controlling this pest. Considering the popular use of TXIB in agrichemicals, possible risks of environmental pollution or toxicity have already been assessed to set safety standards,[28] which is an advantage as compared to completely new synthetic pesticides.

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