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# Maize plants sprayed with either jasmonic acid or its precursor, methyl linolenate, attract armyworm parasitoids, but the composition of attractants differs

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**Key words:** herbivore-induced plant volatile, *Cotesia kariyai*, Hymenoptera, Braconidae, *Mythimna separata*, Lepidoptera, Noctuidae, parasitic wasp, caterpillar, *Zea mays*

## Abstract

Treatment of both uninfested and armyworm-infested maize plants with jasmonic acid (JA) is known to attract the parasitic wasp, *Cotesia kariyai* Watanabe (Hymenoptera: Braconidae). Here, we show that treatment with a methyl ester of a JA precursor, methyl linolenate (MeLin), also causes maize plants to attract this wasp, yet does not cause elevated levels of endogenous JA. The volatile chemicals emitted from either infested or uninfested maize plants treated with MeLin were qualitatively and quantitatively different from those emitted from JA-treated plants. Among compounds emitted from MeLin-treated plants,  $\alpha$ -pinene and menthol attracted wasps in pure form in a two-choice test using a choice chamber. A mixture of methyl salicylate,  $\alpha$ -copaene, and  $\beta$ -myrcene also attracted wasps. In contrast, (*Z*)-3-hexenyl acetate was among the main attractants for *C. kariyai* in JA-treated plants. These data show that in addition to JA, MeLin also has the potential to increase the host-finding ability of *C. kariyai*, but that the composition of attractants they induce differs.

## Introduction

Plants emit herbivore-induced plant volatiles (HIPV) in response to damage caused by herbivorous insects. Emission of HIPV benefits the plant in that it triggers attraction of carnivorous natural enemies of the plant's enemies (Takabayashi & Dicke, 1996; Paré & Tumlinson, 1999; Sabelis et al., 1999; Dicke, 2000). HIPVs are specific for a plant and herbivore species (e.g., Takabayashi et al., 1991; Takabayashi & Dicke, 1996; de Moraes et al., 1999), and several carnivorous arthropods show specific responses to a herbivore species-specific blend of HIPV (e.g., Takabayashi & Dicke, 1996; Guerrieri et al., 1999; de Moraes et al., 1999). Studies on how plants emit a herbivore-specific blend of volatiles have revealed both herbivore-associated and plant-associated factors. Herbivore-associated factors (so-called elicitors) have been found in the regurgitant of lepidopteran larvae. Different classes of elicitors have been identified, including

fatty acid–amino acid conjugates (e.g., volicitin) in the regurgitant of moth larvae, for example, *Spodoptera exigua* (Hübner),  $\beta$ -glucosidase in cabbage butterfly larvae, *Pieris brassicae* (L.), and a disulfide-bridged peptide, inceptin, in oral secretions of *Spodoptera frugiperda* (Smith) larvae (Alborn et al., 1997; Mattiacci et al., 1995; Schmelz et al., 2006). Inceptin triggers an increase in the defense-related phytohormones, salicylic acid and jasmonic acid (JA). Volicitin also induces accumulation of JA (Schmelz et al., 2003a).

Several plant-associated factors have been reported to be responsible for the specific production of HIPV, including JA, ethylene, and salicylic acid (Ozawa et al., 2000; Horiuchi et al., 2001; van Poecke & Dicke, 2002; Schmelz et al., 2003b; Matsushima et al., 2006). Jasmonic acid is an important plant hormone that triggers a signaling pathway involved in defense against herbivores and pathogens (Creelman & Mullet, 1997). Hopke et al. (1994) first reported that treating Lima bean leaves with JA increased the emission of volatiles similar to HIPV from leaves infested with spider mites, *Tetranychus urticae* Koch, or with *S. exigua* larvae. Subsequently, several studies on the role of JA in HIPV production have been published

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(e.g., Koch et al., 1999; Ozawa et al., 2000; Arimura et al., 2002; Schmelz et al., 2003b; Matsushima et al., 2006). Furthermore, many studies have attempted to use JA to manage herbivorous insects (Dicke et al., 1999; Gols et al., 1999; Thaler, 1999, 2002; Shimoda et al., 2002; Ozawa et al., 2004). For example, Thaler (1999) reported that jasmonate treatment of tomato plants in the field increased the number of parasitized caterpillars near the treated plant. We recently reported that treating maize plants with JA increased attraction for the parasitic wasp, *Cotesia kariyai* Watanabe (Hymenoptera: Braconidae), which was useful for locating and controlling the oriental armyworm *Mythimna separata* (Walker) (Lepidoptera: Noctuidae) under laboratory conditions (Ozawa et al., 2004).

In this study, we asked whether plant treatment with precursors of JA can also elicit parasitoid attraction and trigger the emission of the same volatile attractants. JA is synthesized via the octadecanoid pathway (Vick & Zimmerman, 1984). In the initial steps of this pathway, lipase releases  $\alpha$ -linolenic acid (Lin) from plasma membrane lipids of cells either undamaged or damaged by herbivores (Conconi et al., 1996). It is in fact the conjugate of Lin and glutamine that induces JA accumulation and emission of volatiles (e.g., linalool) in tobacco plants, similar to those known as HIPV (Halitschke et al., 2001). Koch et al. (1999) also reported that treating Lima bean plants with Lin induced the production of two volatiles known to be induced by JA: (*E*)-4,8-dimethyl-1,3,7-nonatriene and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. Thus, we hypothesized that exogenous treatment of plants with JA precursors (i.e., Lin and its derivatives) may increase JA levels in plants, thereby increasing the production of volatiles known from blends of HIPV and promoting the response of parasitoids. Furthermore, we reported that a previous treatment of maize plants with JA makes the plants more attractive to *C. kariyai* than untreated maize plants when damaged by armyworms (Ozawa et al., 2004). We thus further hypothesized here that treatment of maize plants with methyl linolenate (MeLin) would make them more attractive to *C. kariyai* than untreated plants when subsequently damaged by armyworms.

The objective of this study was to test the above two hypotheses in a tritrophic system consisting of maize plants, the oriental armyworm, *M. separata*, and its specialist parasitoid, *C. kariyai*, under laboratory conditions. We assessed the quantity of endogenous JA in uninfested MeLin-treated and untreated maize plants. We then measured the chemical composition of headspace volatiles of maize plants treated with either MeLin or JA. We also compared the response of parasitoids to maize plants treated with MeLin, relative to untreated plants. Finally, volatile compounds that were released at elevated con-

centrations following these treatments were tested for their ability to attract *C. kariyai*.

## Materials and methods

### Insects and plants

In 2001, *M. separata* was transferred to our laboratory from a culture maintained at the National Institute of Sericultural and Entomological Science in Tsukuba, Ibaraki, Japan. The insects were reared on artificial diet (Insecta LF; Nihon Nousan Kogyo, Yokohama, Japan) at  $25 \pm 2$  °C, L16:D8, and 50–70% r.h.

*Cotesia kariyai* is a gregarious endoparasitoid of second to early sixth instars of *M. separata* caterpillars. In 2001, a sample of this species was obtained from a stock culture at the Institute of Agriculture and Forestry, University of Tsukuba, Japan. To rear the wasps in our laboratory, third- to fourth-instar *M. separata* were offered to female wasps for oviposition. Wasp cocoons spun soon after emergence of wasp larvae from their hosts were placed in clusters in a glass tube (22 mm diameter, 200 mm long). Each tube was provided with a drop of honey as an energy source for the adult wasps emerging from the cocoons. Mating occurred right after emergence of the wasps. Adult wasps were maintained in the laboratory at  $18 \pm 2$  °C and 50–70% r.h., under continuous darkness until experimentation, which occurred within 7 days of emergence. Oviposition-inexperienced females were used to test innate responses to volatiles.

Maize plants [*Zea mays* L. cv. Royal Dent (Poaceae)] (three plants per pot) were grown from seeds in potted soil in a growth chamber ( $25 \pm 2$  °C, L16:D8). The plants were used for experiments about 10 days after they had been sown.

### Treatments on maize plants

*Treatment 1: methyl linolenate, a precursor of jasmonic acid.* To assess how spraying MeLin on maize plants affects the behavior of parasitoids and the emission of volatiles from maize plants, we conducted the following experiments. Six pots of plants (three plants per pot) were sprayed with 1.5 ml of a MeLin (ICN Pharmaceuticals, Aurora, OH, USA) solution (1 mM in 0.1% aqueous ethanol), 10 h after the onset of the light period. As a control, we prepared plants sprayed with 0.1% aqueous solution of ethanol. On day 1 after the spraying, three pots of plants (either treatment or control) were subject to infestation by third-instar *M. separata* in a plant growth chamber ( $25 \pm 2$  °C, L16:D8). Ten larvae were placed on three plants with each treatment (MeLin or control) in a

pot. The infestation was initiated at 10 h after the onset of the light period. After 18 h, the larvae and their feces were removed, leaving a damaged area covering ca. 10% of the total leaf surface. The damaged areas of leaves made by armyworms in MeLin-treated and control plants were roughly the same. Three pots of the uninfested plants sprayed with MeLin and the uninfested control plants were maintained in a growth chamber ( $25 \pm 2$  °C, L16:D8) until the plants were used for the experiments. On day 2 after the spraying, the infested plants and the uninfested plants were used for two-choice tests with wasps and the collection of volatiles.

To test whether effects of MeLin on responses of parasitoids to the maize plants lasted, we prepared maize plants in the same manner as above, except for the timing of infestation. On day 9 after spraying, treated plants and control plants (three pots each) were subjected to infestation by third-instar *M. separata* for 18 h as described above. The damaged areas of leaves in MeLin-treated and control plants made by the armyworms were again roughly the same (ca. 10%). On day 10 after the spraying, these infested and uninfested plants were then used for the wasp choice test.

*Treatment 2: jasmonic acid.* To test the effects of JA on volatile emission, we prepared maize plants that were sprayed with 1.5 ml of an aqueous solution of JA (1 mM; Sigma-Aldrich, St. Louis, MO, USA) instead of MeLin as described in treatment 1. As a control, plants were sprayed with distilled water. On day 1 after the spraying, plants received either no herbivore damage or they were infested by 10 third-instar *M. separata* for a period of 18 h. Next, the larvae and their feces were removed, leaving a damaged area equal to approximately 10% of the total leaf surface. The damaged areas of leaves in the JA-treated and control plants made by the armyworms were roughly the same. On day 2 after the spraying, the infested and uninfested plants were used for the collection of headspace volatiles.

#### Measurements of endogenous jasmonic acid

We predicted that plants sprayed with MeLin would use the chemical as a precursor for the production of endogenous JA. If so, we would be able to detect higher amounts of JA in MeLin-treated plants than in control plants. To clarify the effects of MeLin on the production of JA, we measured the amount of endogenous JA in maize plants at 0, 30, and 60 min after the MeLin spraying. We measured the amounts of endogenous JA for up to 60 min after MeLin treatment, since it has been reported that endogenous JA production occurs rapidly after elicitor treatment (Engelberth et al., 2001). JA was extracted from 4–6 leaves of three MeLin-treated plants according to the

method of Weber et al. (1997), with slight modifications. We used (trimethylsilyl) diazomethane to methylate extracted JA, and dihydrojasmonic acid [(2-butyl-3-oxocyclopentyl)-acetic acid] as an internal standard. The amounts of JA and dihydrojasmonic acid were determined by gas chromatography and mass spectrometry (GC-MS). GC was performed on an Agilent 6890 chromatograph with an HP-5MS capillary column (Agilent Technologies, Santa Clara, CA, USA) of 0.25-mm inner diameter, 30 m long, and, 0.25- $\mu$ m film thickness; injection temperature was 250 °C. Mass spectrometry was performed on an Agilent 5973 mass selective detector, with ionization at 70 eV. The temperature gradient was 60 °C for 1 min, 60–120 °C at 20 °C/min, 120–180 °C at 3 °C/min, and 180–300 °C at 30 °C/min. The JA was quantified using calibration curves for JA and dihydrojasmonic acid.

#### Chemical analysis

If our prediction was correct that sprayed MeLin was used as a precursor of endogenous JA, the volatiles induced by MeLin treatment and those induced by JA treatment should be similar. Thus, we analyzed the induced volatiles emitted by spraying either MeLin or JA on maize plants. Collections of headspace volatiles were performed 4–9 h after onset of the light period. Six plants of each treatment were placed in a 2-l glass bottle that had two nozzles. One nozzle was connected to an air cylinder and the other to a glass tube packed with Tenax TA adsorbent (100 mg, mesh 20/35; GL science, Tokyo, Japan). Pure air gas from the cylinder was drawn into the glass bottle, and volatile compounds from the headspace of the bottle were collected with Tenax TA for 1 h at a flow rate of 100 ml/min. We collected volatiles from uninfested plants, MeLin-treated uninfested plants, *M. separata*-infested plants and MeLin-treated *M. separata*-infested plants. In a separate series, volatiles were collected from JA-treated plants and uninfested plants, prepared as described above.

The volatile compounds collected were analyzed by GC-MS [GC: Agilent 6890 with an HP-5MS capillary column of 30 m long, 0.25 mm inner diameter, and 0.25- $\mu$ m film thickness; MS: Agilent 5973 mass selective detector, 70 eV, equipped with a thermal desorption cold trap injector (TCT) (CP4010; Chrompack, Bergen op Zoom, The Netherlands)]. Headspace volatiles collected on Tenax TA were released from the adsorbent by heating in the TCT at 220 °C for 8 min within a flow of helium gas. The desorbed compounds were collected in the TCT cold trap unit (SIL5CB-coated fused silica capillary) at –130 °C. Flash heating of the cold trap unit injected the compounds into the capillary column of the gas chromatograph to which the cold trap unit was connected. The oven temperature of

the GC was programmed to rise from 40 °C (5-min hold) to 280 °C at 15 °C/min. The headspace volatiles were identified by comparing their mass spectra to those of the database (Wiley7N and Wiley275) and by comparing their retention times to those of authentic compounds. For some volatiles, no authentic compounds were available, marked as (MS) in the text; their identification should be considered tentative. To compare the ion intensities of a compound detected in two plants with different treatment, data were analyzed with a Mann–Whitney U-test using StatView-J 5.0 (Hulinks, Tokyo, Japan).

#### Flight responses of parasitoids to methyl linolenate-treated maize plants

We tested the response of the parasitoid wasp *C. kariyai* to MeLin-treated plants in different conditions compared to control plants, since the changes in headspace volatiles of MeLin-treated plants might affect behavior of the wasp. Choice tests were performed 4–9 h after the onset of the light. The two pots of maize plants, each receiving a different treatment (MeLin treatment or 0.1% aqueous ethanol treatment), were positioned in an acrylic cage (25 × 35 × 30 cm), with three windows covered by nylon gauze and one acrylic door for introducing plants and wasps (Shiojiri et al., 2000). The cage was placed in a climate-controlled room (25 ± 2 °C, 50–70% r.h.). As a light source, a fluorescent lamp was positioned above the cage. The intensity of light in the cage was ca. 4000 lux. There was no detectable airflow in the cage. To exclude directional biases, the positions of the two pots in the cage were exchanged halfway through an experiment. Five to 10 wasps at a time were released halfway between the two pots. The pots with plants in the cage were replaced with new ones for every replicate experiment. The first plant that a wasp landed on was recorded as its choice. Once a wasp landed on a plant, it was immediately removed from the cage with an insect aspirator. If the wasp did not land on any of the plants within 30 min, it was scored as a no-choice result. Two or three replicates were performed. The statistical significance of preferences in two-choice tests was analyzed using a replicated G-test (Sokal & Rohlf, 1995) under the null hypothesis that wasps would have a 1:1 distribution over the two plant groups. The number of no-choice results was excluded from the analyses. In earlier experiments, we found that releasing 5–10 wasps simultaneously did not significantly affect the choice of individual wasps (K Shiojiri, unpubl.). Under these conditions, we reported that infested plants were more attractive to *C. kariyai* than uninfested plants (Ozawa et al., 2004).

To rule out the possibility that MeLin itself is attractive to the wasps, we tested the preference of the wasps for

**Table 1** Concentrations of synthetic chemicals used to assay the flight response of *Cotesia kariyai*

Synthetic chemical	Content (μg)
MeLin-induced chemicals in uninfested plants	
Menthol	0.01
β-Myrcene	0.5
Methyl salicylate	25
α-Copaene	1
MeLin-induced chemicals in infested plants	
α-Pinene	5
β-Myrcene	1
Jasmonic acid (JA)-induced chemicals in uninfested plants	
(Z)-3-Hexenyl acetate	10
JA-induced chemicals in infested plants	
(E)-2-Hexenal	5
(Z)-3-Hexenol	10
(Z)-3-Hexenyl acetate	30

MeLin. A piece of cotton wool (2 × 2 cm) containing 1.5 ml of MeLin solution (1 mm in 0.1% aqueous ethanol) was placed on a plastic Petri dish (3 cm diameter). As a control, 1.5 ml of 0.1% aqueous ethanol was used. The Petri dish containing the MeLin or the control piece of cotton was placed on the soil in a pot with three uninfested plants. It was tested whether the wasps preferred to fly toward plants next to dishes with MeLin when plants next to control dishes were the alternative.

#### Flight responses of parasitoids to synthetic chemicals

To assess which compounds from treated maize plants are involved in attracting the parasitoids, we tested flight responses of parasitoids to individual (or a mixture of) volatile compounds shown to be induced by either MeLin or JA treatment. We impregnated a cellulose sponge (0.5 × 2.5 × 2.5 cm) with 10 μl of a hexane solution of synthetic chemicals. After evaporation of the solvent for 1 min, the sponge was double bagged into sealed polyethylene film plastic (7 × 5 cm; 0.4-mm film thickness) to facilitate a low volatilization rate. The concentration of each chemical is shown in Table 1. We checked the amount of each chemical emitted from the bag with Tenax-trapping in combination with GC-MS, and found that the amount was roughly the same as that emitted from MeLin-treated plants or JA-treated plants. As we were unable to trap menthol emitted from a bag containing cellulose impregnated with a high dose (0.1 mg) of menthol, we could not compare the amount emitted from the bag to that emitted from the plants. Therefore, instead of measuring the amount of menthol emitted from a bag, we

used 0.01  $\mu\text{g}$  as the initial value, an amount that is lower than the other compounds in Table 1. As we observed the positive responses of wasps to the bag with menthol at this dose, we did not test higher or lower concentrations. As a control, we used 10  $\mu\text{l}$  of pure hexane. The two bags containing the sample and the control sponge were placed on the soil in two pots with three uninfested plants each.

## Results

### Endogenous jasmonic acid levels in methyl linolenate-treated plants

The amounts of JA in MeLin-treated plants after 30 min and 1 h were  $24.5 \pm 3.2$  (mean  $\pm$  SE) ng/g fresh weight (FW) ( $n = 6$ ) and  $19.0 \pm 4.7$  ng/g FW ( $n = 4$ ), respectively. In uninfested control plants, the amount of JA was  $19.9 \pm 4.2$  ng/g FW ( $n = 4$ ). There was no significant difference in endogenous JA levels across these treatments (uninfested, 30 min, and 1 h after MeLin treatments;

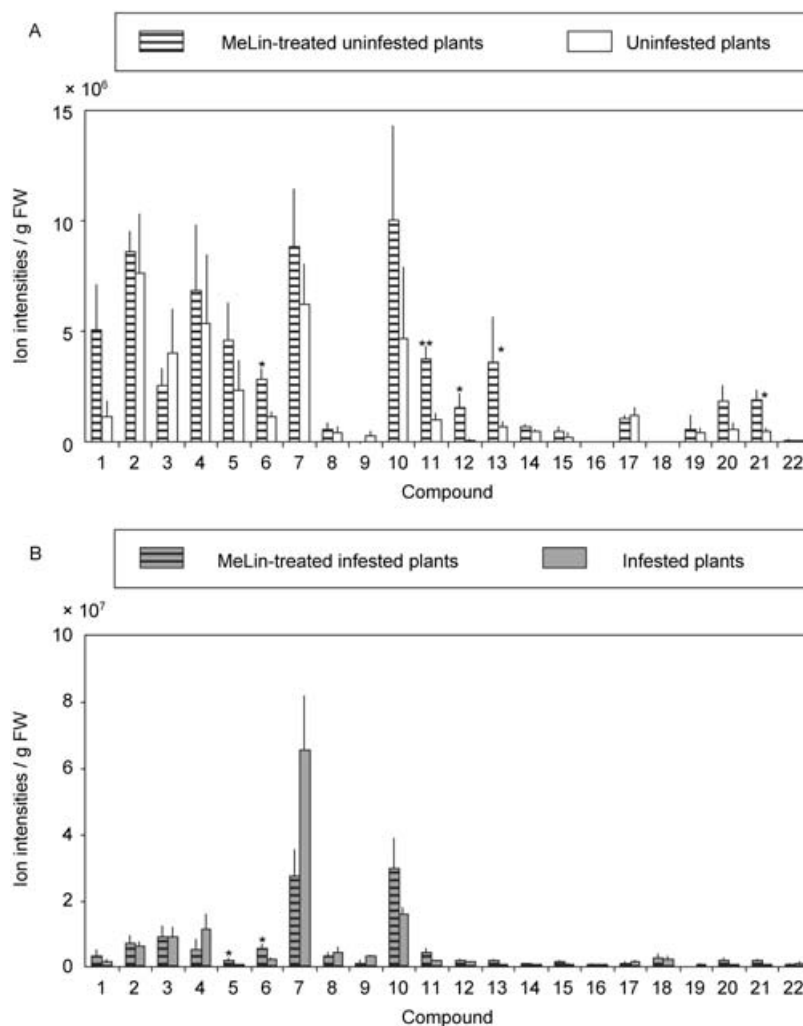
ANOVA:  $P = 0.33$ ). The results suggest that the sprayed MeLin was not used as a precursor for endogenous production of JA in the treated plants.

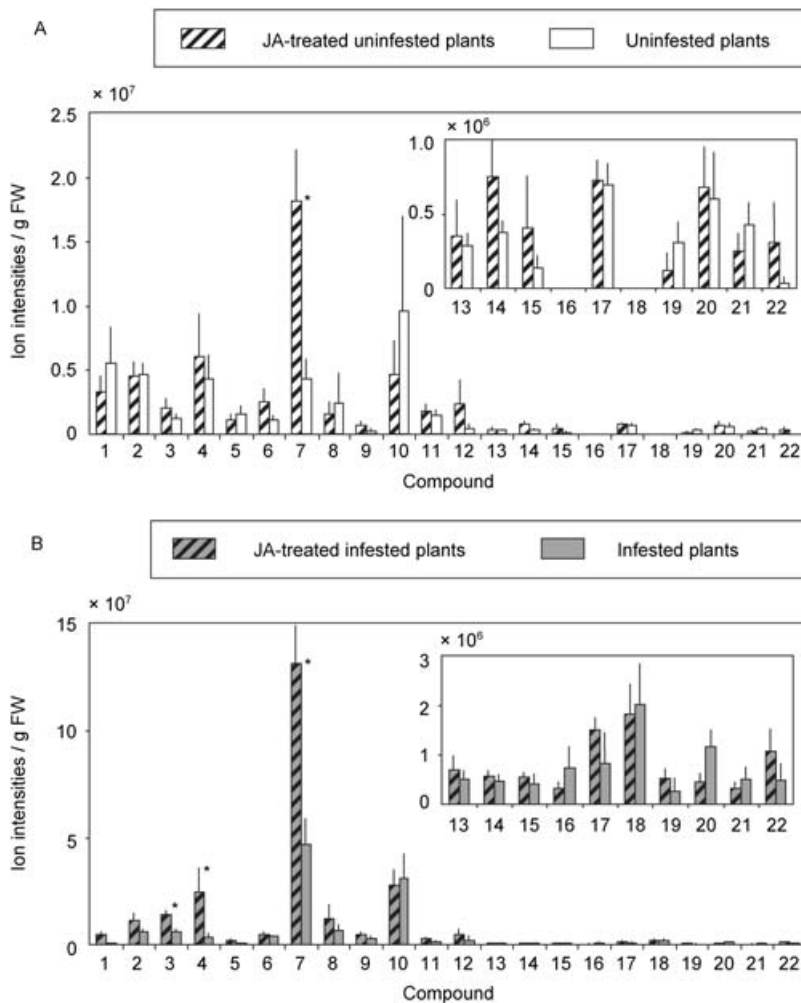
### Chemical analysis

Significantly higher amounts of  $\beta$ -myrcene, menthol, methyl salicylate,  $\alpha$ -copaene, and  $\gamma$ -cadinene (MS) were found in the headspace of MeLin-treated uninfested plants than in uninfested plants (Mann–Whitney U-test:  $P < 0.05$ ; Figure 1A). Conversely, significantly higher amounts of  $\alpha$ -pinene and  $\beta$ -myrcene were found in MeLin-treated infested plants than in control-infested plants ( $P < 0.05$ ; Figure 1B).

We also analyzed the volatiles induced by JA spraying. A significantly higher amount of (*Z*)-3-hexenyl acetate was found in JA-treated uninfested plants than in uninfested plants (Mann–Whitney U-test:  $P < 0.05$ ; Figure 2A). In addition, significantly higher amounts of (*E*)-2-hexenal,

**Figure 1** Amounts of compounds found in the headspace of maize plants. (A) Uninfested plants or (B) infested plants were treated with methyl linolenate (MeLin) or 0.1% aqueous ethanol, and headspace volatiles were collected 2 days after spraying. Error bars represent standard error ( $n = 4-5$ ). Mann–Whitney U-test: \* $P < 0.05$ , \*\* $P < 0.01$ . Compound names: 1, (*Z*)-3-hexenal; 2, *n*-hexanal; 3, (*E*)-2-hexenal; 4, (*Z*)-3-hexenol; 5,  $\alpha$ -pinene; 6,  $\beta$ -myrcene; 7, (*Z*)-3-hexenyl acetate; 8, (*E*)- $\beta$ -ocimene; 9, linalool; 10, (*E*)-4,8-dimethyl-1,3,7-nonatriene; 11, menthol; 12, methyl salicylate; 13,  $\alpha$ -copaene; 14, longiforlene; 15,  $\beta$ -caryophyllene; 16, unknown 1 [mass spectrometry (MS) similar to that of bergamotene]; 17, geranyl acetone; 18, (*E*)- $\beta$ -farnesene; 19, unknown 2 (MS similar to that of  $\alpha$ -amorphone); 20,  $\alpha$ -muurolene (MS); 21,  $\gamma$ -cadinene (MS); and 22, (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene.





**Figure 2** Amounts of induced compounds found in the headspace of maize plants. (A) Uninfested plants or (B) infested plants were treated with jasmonic acid (JA) or distilled water, and headspace volatiles were collected 2 days after treatment. The inserts show the enlarged views of compounds between no. 13 and no. 22. Error bars represent standard error ( $n = 4-6$ ). Compound names are the same as in Figure 1. Mann-Whitney U-test: \* $P < 0.05$ .

(*Z*)-3-hexenol, and (*Z*)-3-hexenyl acetate were found in JA-treated infested plants than in control infested plants ( $P < 0.05$ ; Figure 2B). Thus, the volatile compounds induced by MeLin spraying of maize plants were different from those by induced JA spraying.

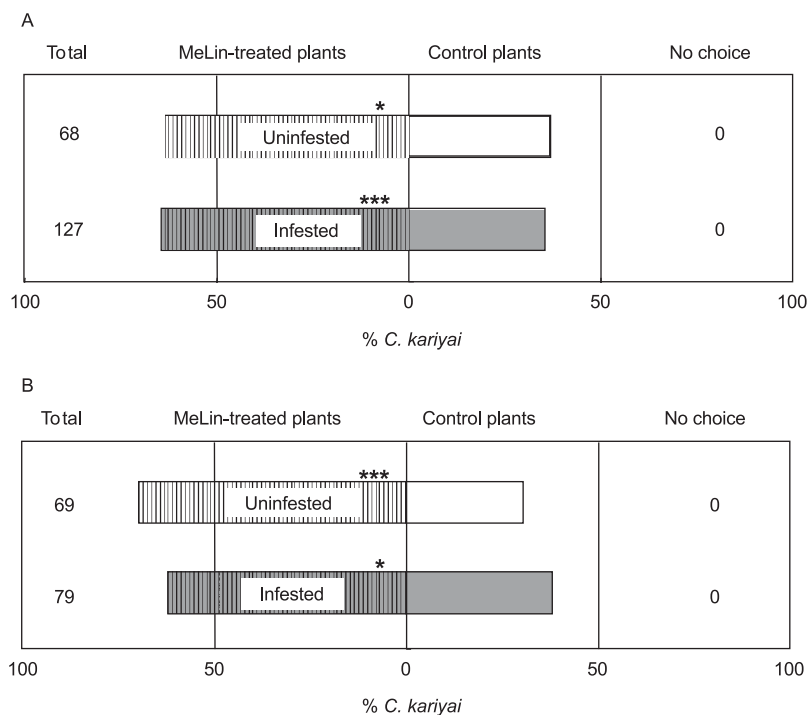
#### Flight responses of parasitoids to methyl linolenate-treated maize plants

In treatment 1, there was no significant heterogeneity among replicates in any of the flight response experiments (non-significant  $G$ -values for heterogeneity;  $G_H$ ). Given this, we evaluated the results of data pooled over all replicate experiments (Figure 3). Wasps preferred MeLin-treated uninfested plants (day 2) to uninfested plants (day 2) (serving as a negative control) ( $G_p = 4.82$ ,  $P = 0.03$ ;  $G_H = 0.01$ ,  $P > 0.05$ ; Figure 3). Significantly more wasps also preferred MeLin-treated infested plants (day 2) to infested plants (day 2) (serving as a positive control) ( $G_p = 10.9$ ,

$P < 0.001$ ;  $G_H = 0.13$ ,  $P > 0.05$ ; Figure 3). Furthermore, there was no significant difference in wasp preference for uninfested plants next to dishes with MeLin, when uninfested plants next to Petri dishes with 0.1% ethanol were provided as the alternative. Twenty-four and 23 wasps selected maize plants next to MeLin and control uninfested plants, respectively (no choice: 13;  $G_p = 0.02$ ,  $P > 0.05$ ;  $G_H = 0.71$ ,  $P > 0.05$ ). Thus, the effect of treating maize plants with MeLin on wasp flight responses was not due to the attraction of MeLin alone.

Similar results were obtained for MeLin-treated uninfested compared to uninfested plants (day 10) (serving as a negative control) ( $G_p = 10.85$ ,  $P < 0.001$ ;  $G_H = 0.36$ ,  $P > 0.05$ ; Figure 3) and MeLin-treated infested compared to infested plants (day 10) (serving as a positive control) ( $G_p = 4.61$ ,  $P = 0.02$ ;  $G_H = 0.71$ ,  $P > 0.05$ ; Figure 3). Thus, the effects of MeLin on the attraction of wasps lasted at least 10 days.

**Figure 3** Preference of *Cotesia kariyai* to land on one of two alternatively treated maize plants in a small choice chamber ( $30 \times 35 \times 25$  cm) after treatment with methyl linolenate (MeLin). Plants were left uninfested or infested with armyworms (gray vs. white columns), untreated or treated with MeLin (columns with or without stripes) or any combination thereof. (A) Two days after the treatment. (B) Ten days after the treatment. Plants treated with 0.1% aqueous ethanol were used as a control. Each experiment was replicated two times. Pooled G-value in a replicated G-test: \* $P < 0.05$ , \*\*\* $P < 0.001$ .



#### Flight responses of parasitoids to synthetic chemicals

We tested compounds that were induced by MeLin or JA sprays of maize plants. No significant heterogeneity was observed among replicates for all flight assays in response to chemicals (non-significant  $G_H$  values). Given this, we evaluated the results of data pooled over all replicate experiments (Figures 4 and 5). Significantly more wasps were attracted to uninfested maize plants with menthol than to uninfested (control) plants ( $G_p = 3.99$ ,  $P = 0.046$ ;  $G_H = 0.02$ ,  $P > 0.05$ ; Figure 4A), but not to plants with  $\alpha$ -copaene, methyl salicylate, and  $\beta$ -myrcene offered at the same concentrations as those emitted from MeLin-treated uninfested maize plants (Figure 4A). However, a mixture of  $\alpha$ -copaene, methyl salicylate, and  $\beta$ -myrcene did significantly attract wasps ( $G_p = 3.99$ ,  $P = 0.046$ ;  $G_H = 1.54$ ,  $P > 0.05$ ; Figure 4A). We also tested compounds emitted at higher concentrations from MeLin-treated infested than control-infested plants. Significantly more wasps were attracted to uninfested maize plants with  $\alpha$ -pinene than to uninfested control plants ( $G_p = 4.47$ ,  $P = 0.034$ ;  $G_H = 3.66$ ,  $P > 0.05$ ; Figure 4B). The wasps distributed equally between uninfested plants with a high dose of  $\beta$ -myrcene (equivalent to MeLin-treated infested plants) and uninfested control plants.

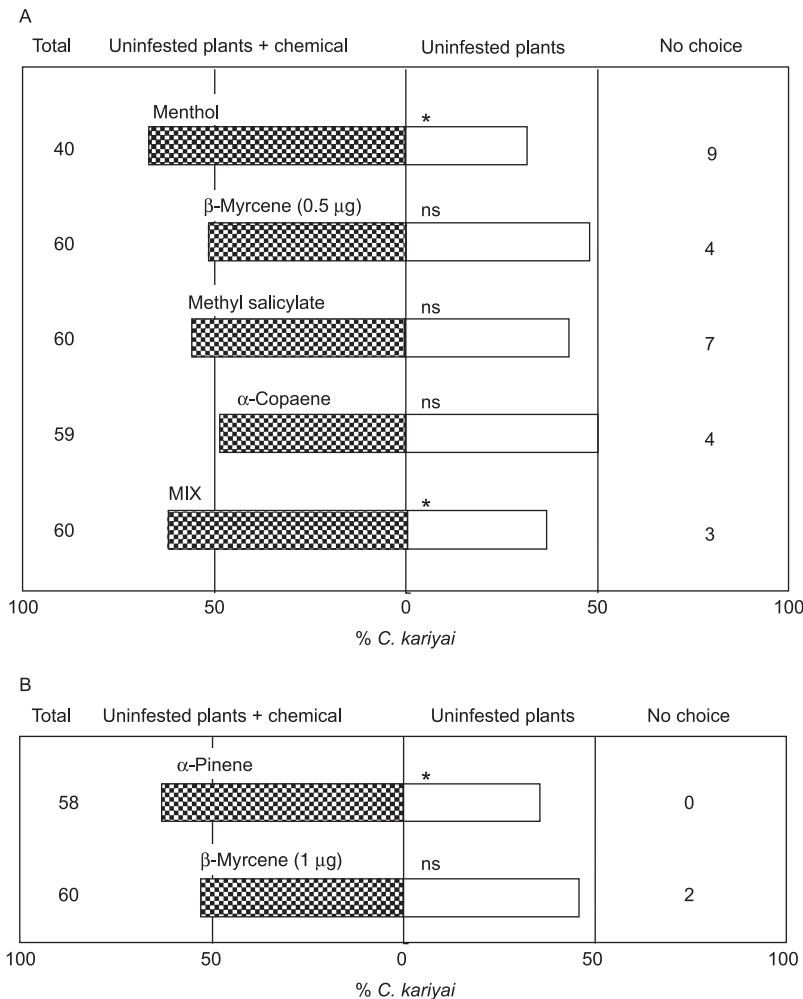
Finally, we tested (*Z*)-3-hexenyl acetate, the emission of which was significantly increased in JA-treated uninfested maize plants and JA-treated infested maize plants

compared to distilled water-treated controls. Significantly more *C. kariyai* females were attracted to uninfested maize plants treated with (*Z*)-3-hexenyl acetate at a high dose (equivalent to JA-treated infested plants) and low dose (equivalent to JA-treated uninfested plants) than to control plants (significant pooled G-value;  $G_p = 7.04$ ,  $P = 0.008$ ;  $G_H = 8.52$ ,  $P > 0.05$  for low dose;  $G_p = 7.92$ ,  $P = 0.005$ ;  $G_H = 0.83$ ,  $P > 0.05$  for high dose; Figure 5). Uninfested plants treated with (*E*)-2-hexenal or (*Z*)-3-hexenol did not attract wasps ( $G_p = 0.83$ ,  $P > 0.05$ ;  $G_H = 1.04$ ,  $P > 0.05$  or  $G_p = 0.07$ ,  $P > 0.05$ ;  $G_H = 1.94$ ,  $P > 0.05$ , respectively; Figure 5B).

#### Discussion

As previously reported (Ozawa et al., 2004), treating maize plants with JA increases their attractiveness to *C. kariyai*. In the present study, treatment of maize plants with MeLin resulted in similar attraction of the parasitoid *C. kariyai*. This result prompted us to hypothesize that JA and its precursor trigger maize plants to produce similar blends of volatiles. Indeed, Koch et al. (1999) reported that treating Lima bean plants with Lin resulted in the production of two homoterpene compounds known to be induced by JA and by herbivorous arthropods: (*E*)-4,8-dimethyl-1,3,7-nonatriene and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. These authors also showed that Lin and



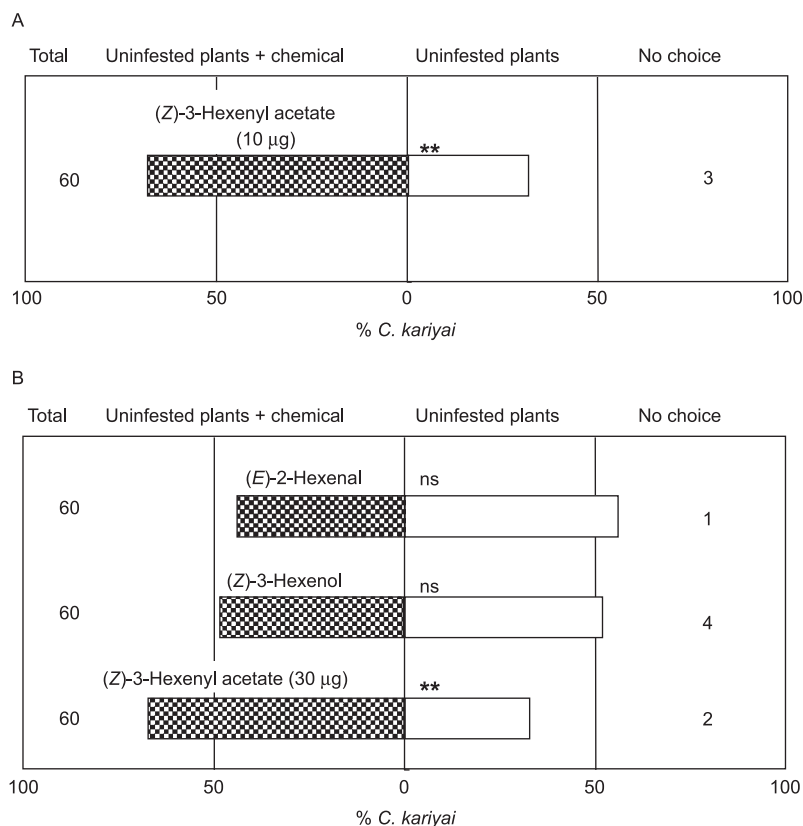


**Figure 4** Preference of *Cotesia kariyai* to land on uninfested maize plants with chemicals vs. uninfested maize plants without chemicals in a small choice chamber. The chemicals were emitted in significantly higher amounts from methyl linolenate (MeLin)-treated uninfested plants (A) or MeLin-treated infested plants (B) than respective control plants (see Figure 1). The concentration of each chemical treatment is shown in Table 1. Each experiment was replicated three times. Pooled G-value in a replicated G-test: \* $P < 0.05$ , ns, not significantly different.

two amino acid conjugates of Lin, Lin-Gln and Lin-Ile, induced the same pattern of HIPV in Lima bean plants, and discussed the possibility that Lin conjugates could undergo amide hydrolysis, allowing free Lin to enter the octadecanoid signaling pathway (Koch et al., 1999). Thus, we initially hypothesized that exogenous treatment of maize plants with MeLin, the JA precursor, would increase the amount of endogenous JA, thereby inducing volatiles that attract carnivores. However, our hypothesis did not hold for the case of maize plants, because endogenous JA levels did not significantly differ between MeLin-treated plants and control plants, and because the blends of volatile chemicals emanating from JA- and MeLin-treated maize plants (either infested or uninfested) highly differed. Some green leaf volatiles [e.g., (*Z*)-3-hexenyl acetate] were induced in JA-treated infested as well as JA-treated uninfested maize plants, yet not in MeLin-treated infested and MeLin-treated uninfested plants. This suggests that

exogenous JA, but not MeLin, affects the octadecanoid pathway. Hopke et al. (1994) reported that maize plants emitted (*E*)-4,8-dimethyl nonatriene, bergamotene,  $\beta$ -farnesene, nerolidol, and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene after JA treatment. However, in our experiments none of these volatiles were found, possibly because we used a different maize cultivar.

The effects of JA and MeLin on maize plants appeared to differ in the way these compounds interacted with infestation by herbivorous insects. When sprayed with JA, maize plants, subsequently infested by armyworm larvae, emitted three times more (*Z*)-3-hexenyl acetate and were more attractive to *C. kariyai* parasitoids than infested maize plants that received no prior JA spray. In contrast, when treated with MeLin, maize plants, subsequently infested by armyworm larvae, did not emit more volatiles, yet were more attractive to wasps than infested plants that received no prior MeLin treatment.



**Figure 5** Preference of *Cotesia kariyai* to land on uninfested maize plants with chemicals vs. uninfested maize plants without chemicals in a small choice chamber. The chemicals were emitted in significantly higher amounts from jasmonic acid (JA)-treated uninfested plants (A) or JA-treated infested plants (B) than respective control plants (see Figure 2). The concentration of each chemical treatment is shown in Table 1. Each experiment was replicated three times. Pooled G-value in a replicated G-test: \*\* $P < 0.01$ , ns, not significantly different.

Whereas JA and MeLin treatments both induced maize plants to emit volatiles attracting parasitoids, the key attractants differed. Flight response experiments with *C. kariyai* parasitoids showed that (Z)-3-hexenyl acetate in pure form partially explained their response to JA-treated maize plants, whereas  $\alpha$ -pinene and menthol explained their response to MeLin-treated maize plants. A mixture of  $\alpha$ -copaene, methyl salicylate, and  $\beta$ -myrcene that were induced by MeLin treatment attracted *C. kariyai* as well. This might indicate an additive or a synergistic effect of compounds on the response of wasps. Earlier (Z)-3-hexenol (at higher concentration than used here) was reported to be an attractant of *C. kariyai*, whereas (Z)-3-hexenyl acetate (at lower concentration than used here) was reported to be not attractive (Takabayashi et al., 1991). These results differ from those reported here, probably due to the dose-dependence of parasitoid attraction. Another reason for the difference may be that the synthetic volatiles were offered in bags placed next to uninfested maize plants, thereby potentially allowing these volatiles to trigger emission of volatiles from plants (Choh et al., 2004; Engelberth et al., 2004). However, the uninfested plants next to the bag with any of the synthetic

compound tested were not attractive to the parasitoids (R Ozawa, unpubl.).

Thus, the innate parasitoid responses to MeLin-treated and JA-treated maize plants are similar, but the key attractants differ. This is somewhat surprising because the MeLin-induced maize plant volatiles may not occur under natural conditions. However, it is known that *M. separata* larvae feed on many different grass species that emit different blends of HIPV. Possibly, the parasitoid *C. kariyai* responds innately to a much wider range of volatiles than those emanating from armyworm-infested maize plants. Thus, even when parasitoids show similar behavioral responses to odors from a single plant species/cultivar subjected to different phytohormone treatments, the volatile chemicals triggering these responses are not necessarily the same.

Jasmonic acid and MeLin effects on maize plants share the property that they last for a period of at least 10 days (Figure 3 and Ozawa et al., 2004). Such a long-lasting plant response needs further study to elucidate the underlying biochemical mechanisms, as well as the impact on the indirect defense against not only armyworms but also other herbivorous insects.

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