

Volatiles from Apple Trees Infested with Light Brown Apple Moth Larvae Attract the Parasitoid *Dolichogenidia tasmanica*

D. M. Suckling,* A. M. Twidle, A. R. Gibb,[†] L. M. Manning, V. J. Mitchell, T. E. S. Sullivan, S. L. Wee,[‡] and A. M. El-Sayed

The New Zealand Institute Plant and Food Research Limited, Post Office Box 4704, Christchurch, New Zealand

ABSTRACT: The volatile compounds emitted from uninfested apple seedlings, cv. Royal Gala, and apple seedlings infested with generalist herbivore *Epiphyas postvittana* larvae were sampled using headspace collection and analyzed by gas chromatography–mass spectrometry. Nine additional compounds were only detected in infested apple seedlings [including benzyl alcohol, (*E*)- β -ocimene, benzyl cyanide, indole, (*E*)-nerolidol, and four unidentified compounds]. Infested apple seedlings produced larger amounts of (*Z*)-3-hexenyl acetate, linalool, 4,8-dimethyl-1,3(*E*),7-nonatriene, methyl salicylate, β -caryophyllene, germacrene D, (*E,E*)- α -farnesene, and (*Z*)-3-hexenyl benzoate than uninfested plants. Female parasitoids flew exclusively upwind to infested and not to uninfested apple seedlings in wind tunnel choice tests and preferred infested leaflets in still air, even after the removal of larvae. The attraction of a parasitoid to infested apple seedlings in the laboratory and in the field to apple and many other plants in at least six families supports considerable generality of the tritrophic signaling process.

KEYWORDS: *Malus*, tritrophic interactions, induced plant volatiles, wind tunnel, parasitism, parasitoid

■ INTRODUCTION

The role of induced plant volatile emissions [or host-induced plant volatiles (HIPVs)] in attracting natural enemies to herbivore-damaged plants has been well-studied, particularly for a number of economically important plant species, including corn¹ and other crops.^{2,3} The herbivore feeding on plant tissue typically induces a response in the plant, whereby there is an enzymatically controlled change in the volatile profile emitted from the plant. Natural enemies, such as parasitoids, are attracted to this changed odor profile¹ and, as a consequence, are able to find and parasitize their hosts. In some plants, the induced response can also be initiated by mechanical damage in addition to herbivory,⁴ while in other plants, the induced response results only from herbivory. Herbivore oral secretions are known to induce a change in the volatile profile of the plant,^{5–7} and the capacity for this phenomenon is widespread in 19 families of Lepidoptera,⁸ including *Epiphyas postvittana* (Lepidoptera: Tortricidae).

The light brown apple moth (LBAM, *E. postvittana*) is a leafroller with a particularly wide host range of ~500 plant species.⁹ It is a pest of apples (*Malus domestica* Borkh.) (Rosaceae) and other horticultural crops in New Zealand and its native Australia, and it is now present in California and Europe.¹⁰ Because of their economic importance, apple fruit volatiles have been some of the most widely examined,¹¹ particularly in the context of insect behavior, including codling moth,^{12–16} apple maggot,¹⁷ and apple sawfly.¹⁸ Concentrations of volatiles from mature apple trees have also been seasonally collected in studies of codling moth and apple fruit attack.^{19,20} However, there has been less enquiry into volatiles from apple foliage, although terpene-mediated parasitoid host location behavior to leafminer attack was documented on transgenic and classically bred apple genotypes.¹⁶ Apple fruit are used by *E. postvittana* only incidentally;^{21,22} therefore, it is more likely that

attraction to apple trees by adult female LBAM (and potentially parasitoids) will be mediated by foliage volatiles.²²

Apart from the economic interest in the adventive pest and its biological control, of interest here is the novel combination of an Eurasian perennial tree (*M. domestica*) being attacked by an Australian herbivore (Tortricidae) and a parasitoid responding to the plant signaling from the Eurasian plant and probably many other landscape plants in New Zealand.²³ We chose to examine the apple–leafroller system with the solitary wasp *Dolichogenidea tasmanica* (Hymenoptera: Braconidae), a larval endoparasitoid of first and second instar leafroller larvae, because this parasitoid can locate and attack at least three species of economically important leafrollers on apples from Australia and New Zealand.²³ The solitary wasp can achieve parasitism rates of up to 60% in the field, although parasitism varies with plant and tortricid host species.^{23,24} The mechanism by which a range of natural enemies contribute to biological control of *E. postvittana* and more primitive New Zealand native leafrollers (e.g., *Planotortrix octo*) on a range of host plants in New Zealand is unclear.

It has been proposed on the basis of current trends in convergent areas that, if the volatiles produced from infested plants could be characterized sufficiently, the parallel development of electronic nose technology could enable field detection of insect-infested trees.^{25,26} Early identification of these infected trees or parts of trees or orchards could provide the basis for more targeted control tactics, avoiding reliance on broadcast insecticides. In a step toward this goal, we investigated the chemistry underlying the attraction of female *D. tasmanica* to apple seedlings infested with larvae of *E. postvittana* and the

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induced changes in the apple plant volatile chemical profile resulting from *E. postvittana* larval feeding.

MATERIALS AND METHODS

Insects and Plants. Egg batches of *E. postvittana* were obtained from a laboratory-reared colony. Apple seedlings (var. Royal Gala) were infested with ca. 30 larvae per seedling and left for 24 h in the laboratory to enable larvae to settle. Upon making contact with the adaxial leaf surface, larvae orientated to a position on the abaxial surface where they webbed up along the midrib and began to feed on epidermal tissue. Visible signs of larval feeding were evident after 24 h. Infested apple seedlings were placed individually in an apple orchard for 7 days to allow for parasitism to take place.²³ The larvae were then harvested from the apple leaves and reared in individual artificial diet tubes²⁷ until emergence of parasitoids. Female parasitoids were pre-exposed individually for 4 h (0930–1330 h) to first instar *E. postvittana* larvae webbed up on the leaf of an apple seedling placed in a Petri dish before being used for wind tunnel or still air choice experiments. Infested and uninfested apple seedlings were also used to provide volatile odor profiles by air entrainment and in wind tunnel and field experiments.

Air Entrainment of Volatiles Emitted by Apple Seedlings. Infested and uninfested apple seedlings (ca. 30 cm high) were enclosed for 48 h in individual 14 × 50 cm glass chambers (five replicates). Each chamber inlet was fitted with an air filter consisting of 1.5 mm granular activated charcoal (Merck, Darmstadt, Germany), contained in a 14 × 3 cm two-piece glass cylinder, which could be readily opened. Filtered air (1 L min⁻¹) was drawn into the sampling chamber with a CF-MP1 sampling pump (Brey, Memmingen, Germany) and upward and over the leaves of the seedlings. A volatile entrainment filter, comprising a glass tube containing 100 mg of Tenax-GR 35/60 (Grace, Deerfield, IL) and fitted with glass wool plugs, was attached to the sampling chamber outlet. For qualitative and quantitative analyses, the Tenax entrainment filters were washed with 1 mL of *n*-hexane (BDH Laboratory Supplies, Poole, U.K.), containing 500 ng of dimethyl salicylate as the internal standard. This standard was chosen because it was not seen in the extracts yet is similar in structure and retention times to some of the volatiles. The resulting eluate was stored at -80 °C until analysis by gas chromatography–mass spectrometry (GC–MS). Used sampling filters were cleaned by flushing with nitrogen (50 mL min⁻¹) in an oven heated to 200 °C for 16 h. After each sampling occasion, all glassware were disassembled and glass and charcoal were heated to 140 °C in an oven for 16 h before reuse. The total leaf area (cm²) was determined for apple seedlings using a LI-3000 portable area meter (Lambda Instruments Corp., Lincoln, NE).

Analysis of Air-Entrainment Samples by GC–MS. We analyzed air-entrainment samples with GC–MS (Varian 3800 GC coupled to a Varian 2200 MS). Helium was used as the carrier gas (1 mL min⁻¹), and injections were splitless for 0.6 min. Transfer line and ion trap temperatures were 250 and 180 °C, respectively. The GC injector temperature was set at 220 °C, and the oven ramp was 40 °C for 2 min, 4 °C min⁻¹ to 240 °C, hold for 10 min, and then 15 °C min⁻¹ to 260 °C, using a VF-5 ms column (30 m × 0.25 mm inner diameter × 0.25 μm film; Varian, Inc., Walnut Creek, CA). A 1 μL aliquot was injected after first concentrating 100 μL of each sample to ca. 10 μL with a gentle stream of argon. Kováts retention indexes²⁸ (KI) were calculated for the compounds (Table 1). Structural assignments of the compounds were made by comparing their mass spectra with the MS library (NIST 2002), as well as by comparison to Kováts retention indices published in the literature.²⁹ Identification of volatiles was confirmed by comparison to authentic samples.

Chemicals. Sources (Sigma-Aldrich-Fluka, unless otherwise indicated) and chemical purity of the standards used to identify the compounds in apple seedling headspace are as follows: (*Z*)-3-hexenyl acetate (98%), benzyl alcohol (99%), β-ocimene [mix of (*E*)- and (*Z*)-β-ocimene; mix of 70% (*Z*)-β-ocimene and 25% limonene], linalool [95% (±)-linalool], (*E*)-nerolidol (85%), benzyl cyanide (99%), methyl salicylate (98%), indole (99%), β-caryophyllene (97%), geranyl

Table 1. Release Rate (pg h⁻¹ cm⁻² of Leaf Area, ±SEM) of Volatile Organic Compounds from Apple Seedlings with or without Infestation by *E. postvittana* (Lepidoptera: Tortricidae) Larvae

compound ^a	compound class	uninfested	infested	Kováts ^b
(<i>Z</i>)-3-hexenyl acetate	aliphatic ester	8.5 ± 3.0	22.1 ± 10.2	1007
benzyl alcohol	aromatic alcohol		0.3 ± 0.2	1037
(<i>E</i>)-β-ocimene	monoterpene hydrocarbon		13.6 ± 4.7	1048
(±)-linalool	monoterpene alcohol	0.1 ± 0.0	2.9 ± 1.4	1102
4,8-dimethyl-1,3(<i>E</i>),7-nonatriene	monoterpene hydrocarbon	0.1 ± 0.1	5.1 ± 2.9	1115
benzyl cyanide	aromatic nitrile		6.8 ± 3.9	1143
methyl salicylate	aromatic alcohol, ester	11.7 ± 7.2	16.7 ± 6.7	1197
unidentified compound 1			2.6 ± 1.5	1221
unidentified compound 2			0.9 ± 0.4	1252
unidentified compound 3			0.3 ± 0.1	1273
indole	aromatic amine		0.2 ± 0.2	1298
β-caryophyllene	sesquiterpene hydrocarbon	0.2 ± 0.2	5.8 ± 3.8	1425
geranyl acetone	sesquiterpene ketone	0.4 ± 0.2	0.2 ± 0.1	1450
germacrene D	sesquiterpene hydrocarbon	0.1 ± 0.1	15.2 ± 7.5	1486
unidentified compound 4			0.5 ± 0.2	1501
(<i>E,E</i>)-α-farnesene	sesquiterpene hydrocarbon	3.0 ± 2.0	62.8 ± 18.7	1506
(<i>E</i>)-nerolidol	sesquiterpene alcohol		0.9 ± 0.4	1564
(<i>Z</i>)-3-hexenyl benzoate	aromatic ester	0.3 ± 0.2	9.7 ± 5.3	1577

^aCompounds were identified by mass spectra analysis, Kováts index, and comparison to synthetic standards. ^bKováts index on a VF-5 ms column.

acetone (96%), (*Z*)-3-hexenyl benzoate (97%), and dimethyl salicylate (99%). 4,8-Dimethyl-1,3(*E*),7-nonatriene (99%) was synthesized.³⁰ Germacrene D was a crude extract.³¹ (*E,E*)-α-Farnesene (98%) was isolated from apples.³²

Trapping with Sticky Infested Leaves. We modified the method of Suckling et al.²³ to confirm that *D. tasmanica* would be attracted to foliage infested with *E. postvittana* in the field by adding a sticky surface to leaves. A total of 28 potted apple trees were infested with 20–30 first instar larvae for 24 h (ensuring good establishment). The leaves were treated with a thin layer of Tanglefoot (Contech Enterprises, Inc., Victoria, British Columbia) on both sides and placed in local unsprayed apple orchards for a week from April 16, 2008. Larvae were reared to adult emergence of moth or parasitoid. Parasitoids trapped on sticky leaves were counted, and female *D. tasmanica* were identified. A total of 28 uninfested trees with sticky leaves were used as a control.

Wind Tunnel Experiments. Single potted apple trees either infested or uninfested were placed at the upwind end of a plastic flight tunnel (50 × 50 × 150 cm). The flight tunnel was operating at a wind speed of 10 cm/s under fluorescent light. Light intensity was 340 lx (measured in the middle of the tunnel), and temperature was maintained at 25 ± 2 °C. Air from the room was drawn into the tunnel system through a charcoal filter before passing through a grill, which helped to maintain an even airflow. Female parasitoids were then placed individually in a wind tunnel and offered the choice of flight to

the uninfested or infested plant for 10 min, and the choice (or non-response) was recorded. Assays were conducted between 1415 and 1600 h, when wasps were normally active in the field, and the order of infested or uninfested plants was reversed every five insects, with a total of 9–10 insects tested per day, with the results pooled for the tests across seven dates (73 different insects to each treatment). Low response levels led to the development of the still air assay.

Still Air Bioassay. Female parasitoids were placed in a plastic box (20 × 20 cm) with side ventilation. Single leaves from potted apple trees were tested as (a) uninfested versus infested, (b) uninfested versus uninfested, and (c) uninfested versus previously infested (infested with larvae removed just before the test). Each parasitoid was tested 4 times with the bioassay treatments rotated, and the scores were pooled, so that each parasitoid could achieve a maximum score of 4 by always choosing the infested leaves (15 replicates).

Statistical Analysis. Parasitoid choice scores were compared by analysis of variance and χ^2 tests using Minitab Statistical Software.³³ Larval counts were log-transformed to stabilize the variance and compared by analysis of variance or Student's *t* tests.

RESULTS

Volatiles Emitted by Uninfested and Infested Apple Seedlings. Whereas only 9 compounds were detected in the headspace of uninfested apple seedlings, a total of 18 compounds were detected in infested apple seedling headspace (Table 1). Benzyl alcohol, (*E*)- β -ocimene, benzyl cyanide, indole, and (*E*)-nerolidol were present only in infested apple seedling volatile emissions, concomitant with four unidentified compounds. In addition, there were large quantitative differences in emissions between uninfested and infested apple seedlings (Table 1), with infested apple seedlings producing larger amounts over time of (*Z*)-3-hexenyl acetate, linalool, 4,8-dimethyl-1,3(*E*),7-nonatriene, methyl salicylate, β -caryophyllene, germacrene D, (*E,E*)- α -farnesene, and (*Z*)-3-hexenyl benzoate.

Trapping with Sticky Infested Leaves. A total of 234 moth larvae were recovered and reared, with an average of 12.9 larvae per tree, with 17% parasitism accumulated in a week by *D. tasmanica*. A total of 43 *D. tasmanica* were trapped on the infested sticky apple leaves on 28 potted trees [mean \pm standard error of the mean (SEM): 1.54 \pm 0.43 per tree, with a range of 0–10] compared to 12 *D. tasmanica* trapped on uninfested leaves (mean \pm SEM: 0.43 \pm 0.16, with a range of 0–2), with the infested leaves proving attractive ($p = 0.001$) to *D. tasmanica*. The infested trees also had a higher frequency of trees (61%), with one or more *D. tasmanica* caught, compared to uninfested trees (25% had one or more *D. tasmanica*).

Wind Tunnel Choice Experiments. A total of 13 pre-exposed *D. tasmanica* parasitoids flew to *E. postvittana*-infested seedlings in the choice test in the wind tunnel of 73 attempts with different individuals, while none flew to uninfested seedlings. Although the response rate was comparatively low (mean \pm SEM: 21.9 \pm 5%), the behavioral response to infested plants was unequivocal.

Still Air Bioassay. The parasitoids showed significant attraction to infested over uninfested leaves in a still air choice test (Figure 1) ($F_{2,42} = 14.04$; $p < 0.001$). Leaves that had been disinfested just prior to the experiment were also attractive to the parasitoid. In the presence of infested leaves, 15% of insects made no choice, while in the presence of uninfested leaves, 43.3% of insects made no choice, and previously infested foliage showed an intermediate 21.7% of insects with no choice. Differences between parasitoid choices for infested and uninfested foliage were highly significant ($\chi^2 = 38.21$; $p < 0.001$). Differences in parasitoid choices between previously

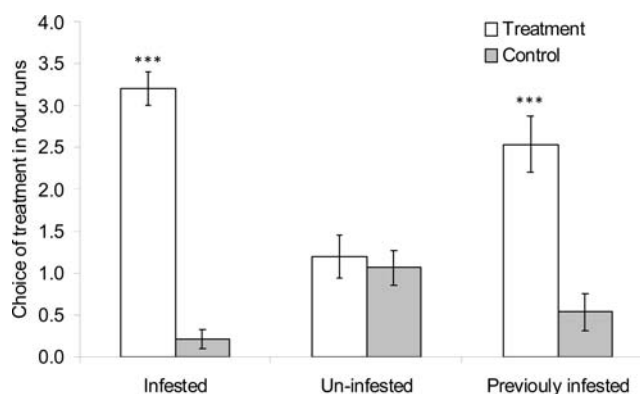


Figure 1. Still air choice bioassay of parasitic *D. tasmanica* for single uninfested apple leaves or leaves infested with early instar of LBAM, *E. postvittana* larvae. The control was an uninfested leaf in each case. Bars indicate one standard error.

infested and uninfested foliage were also highly significant ($\chi^2 = 19.56$; $p < 0.001$), but there was no difference between choices of uninfested–uninfested, as expected ($\chi^2 = 0.12$; $p > 0.05$).

DISCUSSION

The parasitoids consistently chose infested over uninfested apple seedlings in the two bioassays, indicating a highly general and presumably very adaptable process, because the combination is recent and novel rather than co-evolved as in the majority of cases reported.^{1–7} In this case, the tritrophic system involves a Eurasian plant (apple) signaling through HIPVs the presence of feeding from an Australian herbivore to its parasitoid. This evolutionary bridge between higher trophic levels that had not previously encountered the plant in evolutionary time points strongly to generality in the signal in both transmitter and receiver. From the plant perspective, as many parasitoids as possible should be alerted upon attack and as many plants as possible should be detected by the correct parasitoid for the herbivore to be attacked. The fact that the caterpillar has such a wide plant host range (121 families) suggests that there could be an evolutionary advantage for the parasitoid in tracking its prey on so many plants. Certainly, the caterpillar has successfully expanded to attack new plant hosts upon encountering them.⁹

In addition to Rosaceae [apple and briar, *Rosa rubiginosa* (L.)], the range of plants potentially showing this type of signaling and reception by this parasitoid and its caterpillar host include Fabaceae [white clover (*Trifolium repens* L.)], broom [*Cytisus scoparius* (L.) Link.], and poplar [*Populus nigra* (L.)].^{9,23,24} The parasitoid was also reared from *E. postvittana* and two native New Zealand tortricid larvae from a wider range of plant and tree species in the Fabaceae [gorse, *Ulex europaeus* (L.)]; lucerne, *Medicago sativa* (L.)], Betulaceae [alder, *Alnus glutinosa* (L.) Gaertner], Salicaceae [crack willow, *Salix fragilis* (L.)], Polygonaceae [broad-leaved dock, *Rumex obtusifolius* (L.)], Ulmaceae [elm, *Ulmus glabra* (L.)], Juglandaceae [walnut, *Juglans regia* (L.)], and Plantaginaceae [narrow leaf plantain, *Plantago lanceolata* (L.)].²⁴

Parasitoids also chose the apple foliage with the host insects no longer present, indicating the attractive effect was from feeding rather than the larvae. This suggests a degree of specificity of signal reception at the parasitoid level. A few parasitoids were trapped on uninfested plants in the field, which

could suggest the role of learning for minor compounds present in both infested and uninfested plants or could simply be the result of appetitive exploration of the environment rather than attraction. Typically, complex blends are produced by different biosynthetic pathways and are sensitive to external triggers.³⁴

Chemically, the changes in volatiles from foliage of young potted apple trees showing responses to herbivory by *E. postvittana* followed the predictions from other plant and insect species combinations quite closely.³⁴ Previous work on apple leaf volatiles of both uninfested¹³ and infested³⁵ leaves have also shown similar results to those obtained in this study. A notable exception to this is the detection of benzyl cyanide, which, to the best of our knowledge, has not been seen in apple volatiles before. Benzyl cyanide has been reported from *Brassica rapa*³⁶ and is also known as a parasitoid attractant.^{37,38} There could be potential for this compound to be used as an indicator of *E. postvittana* infestation in apple orchards, especially because the compound appears to be rare without infestation. However, further investigation will be required along with technological advances in real-time detection for volatile organic compounds. Some additional compounds, with Kováts index numbers smaller or larger than those indicated, were occasionally but not consistently present.

In the field, a low catch of *D. tasmanica* on sticky leaves was recorded in comparison to a high visitation rate per tree (61% of infested trees), which suggests that the parasitoid was adept at avoiding the glue but effective at patch discovery, as seen previously.²³ It is quite probable that the glue reduced the parasitism rate. The significant increase in *D. tasmanica* suggests specific volatile cues from the infested trees to which *D. tasmanica* responds, supporting the laboratory studies with two types of bioassays. The two bioassays and field results were complementary in indicating both longer range and close range searching behaviors.

When we also consider the very wide host plant range of the caterpillar and the ability of the parasitoid to locate its caterpillar prey on so many different types of plants and trees from clover and other understorey species to woody weeds and a range of deciduous trees,^{23,24} it raises the probability of a generic role of plant signaling involving the second and third trophic levels of this system. However, there appear to be some differences in the efficiency of the discovery process by parasitoids between plants.^{23,24} Whether some or all of these systems are induced from the plant biochemical response to the oral secretions of *E. postvittana* caterpillar containing linolenoyl glutamic acid⁸ is yet to be proven.

AUTHOR INFORMATION

Corresponding Author

*Telephone: +64-3-977344. E-mail: max.suckling@plantandfood.co.nz.

Author Contributions

[†]46 Hay Street, Bromley 8062, Christchurch, New Zealand.

Author Contributions

[‡]School of Environmental and Natural Resource Sciences, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi 43600, Selangor, Malaysia.

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