

# Inventory of leaf and flower odorants in plants associated with the life cycle of Japanese *Papilio* butterflies

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The odorants of eight Japanese mainland native species (*Citrus deliciosa*, *Zanthoxylum ailanthoides*, *Z. schinifolium*, *Z. piperitum*, *Phellodendron amurense*, *Orixa japonica*, *Skimmia japonica* and *Boeninghausenia albiflora*), one tropical species (*Euodia meliifolia*), and one invasive species (*Ruta graveolens*) of the Rutaceae family, and three Japanese mainland native species (*Angelica keiskei*, *Heracleum lanatum* and *Anthriscus sylvestris*) and one invasive species (*Foeniculum vulgare*) of the Apiaceae family were analyzed using gas chromatography-mass spectrometry with dynamic headspace and thermal desorption methods. These species are host plants to Japanese *Papilio* butterflies. In this study, these 14 plants were classified into six major groups

based on the odorant volatiles, which did not correspond to the current phylogenetic classification. Similarly, floral odorant analysis of the six plant species (*Clerodendrum trichotomum*, *Cayratia japonica*, *Robinia pseudoacacia*, *Lonicera japonica*, *C. deliciosa* and *Z. ailanthoides*) visited by *Papilio* butterflies for nectaring revealed the presence of linalool in all flowers. Floral volatiles in *C. deliciosa* and *Z. ailanthoides* exhibited a moderate resemblance to their respective leaf volatiles. Interestingly, the results obtained for *C. trichotomum* were not in complete agreement with those of previous reports, emphasizing the need for newer methods of extraction and analysis.

**Key Words:** *Papilio*; Plant volatile; Dynamic headspace; Thermal desorption; Gas chromatography; Mass spectrometry

## INTRODUCTION

During oviposition, female butterflies hit the leaf surface of the plant with their forelegs, in a behavior called “drumming” [1]. The leaf surface is injured by spines present on the ventral surface of the female foreleg tarsus [2] and leaf fluid is released from the wound [3]. Female butterflies sense the substances in the leaf fluid using their tarsal contact chemosensilla to determine whether the plant is a preferred host plant for oviposition. Ovipositional stimulants perceived by contact chemosensilla have been extensively examined in *Papilio troilus* [4], *P. polyxenes* Fabricius [5,6], *P. xuthus* [7,8], *P. maackii* [9], *P. bianor* [7,10,11], *P. polytes* [12], and *P. protenor* [7,13-15]. Oviposition-inhibiting substances have been identified for *P. xuthus*, *P. polytes*, and *P. protenor* [9,16,17]. Among these substances, phellamurin contained in *P. amurense* stimulates oviposition in *P. maackii*, but inhibits it in *P. protenor* [9]. Moreover, genes encoding the chemosensory and odorant-binding proteins have been identified in *P. xuthus* [18,19].

Although drumming behavior is commonly observed during oviposition, we often observe that *Papilio* butterflies oviposit without contacting the plant leaves with their forelegs; furthermore, butterflies sometimes oviposit their eggs on non-host plants or objects adjacent to host plants [20]. These behaviors could be attributed to plant volatile substances, which may excite the sensitive olfactory receptors of the butterflies. In fact, butterflies are known to locate the host plant habitats by following olfactory cues and consecutively identifying the host plant by taste cues. This has been demonstrated in *P. demoleus* [21], *P. polyxenes* [22-24], *P. machaon* in Japan [23], *P. troilus* [23], *P. cresphontes* [25], *P. glaucus*, and *P. canadensis* [26]. A recent study analyzed the volatile components released by host plants of *Papilio indra* occurring in the Rocky Mountains [27,28]. In addition, plant volatiles of Australasian *Rutaceae* plants have been analyzed in previous studies [29,30], and the relationship between plant volatiles and the oviposition behavior of two Japanese *Leptocircini* species has been previously examined [31,32]. In addition, several other studies have been conducted from a pharmacological, fungal defense, or pollination perspective [33-40]. However, a comprehensive study on *Papilio* butterflies and the odorants of their egg-laying plants in Japan has not been previously undertaken.

*Papilio* butterflies are also closely associated with many nectaring flowers. Although previous studies suggest that *Papilio* butterflies locate nectaring flowers using visual information [41,42], they sometimes procure nectar from flowers of inconspicuous plants, such as *C. japonica* and *Z. ailanthoides*. Thus, assuming that *Papilio* butterflies use olfactory information to locate nectaring flowers, we created an inventory of volatile substances of the flowers that are often visited by *Papilio* butterflies.

We hypothesized that host plant detection and flower-searching by Japanese *Papilio* species other than *P. machaon* could also be attributed to plant volatile substances. To prove this hypothesis, we attempted to identify and build an inventory of the volatile substances of the host plants and flowers visited by these butterflies.

## MATERIALS AND METHODS

Mature leaves of *C. deliciosa*, *Z. ailanthoides*, *Z. schinifolium*, *Z. piperitum*, *P. amurense*, *O. japonica*, *S. japonica*, *E. meliifolia*, *R. graveolens*, *F. vulgare*, *A. keiskei* and *H. lanatum*, were collected from plants cultured in the garden of Tsukuba, Ibaraki, Japan. *Citrus deliciosa*, *R. graveolens*, *F. vulgare* and *A. keiskei* leaves were procured from local gardening stores, *E. meliifolia* was replanted from Ishigaki Island of the Nansei Islands, and other plants originated from the forest where the butterflies for the experiments were collected. Selection criteria for these plants included the following: the plant is (1) distributed in most areas of mainland Japan (except *E. meliifolia*, which occurs only in Kyushu, Shikoku, and the south-end of Honshu), (2) abundant, and (3) the major host plant of at least one *Papilio* species occurring in mainland Japan. Although *R. graveolens* and *F. vulgare* are alien species in Japan, the former is used by many *Rutaceae*-using *Papilio* species, whereas the latter is used extensively by *P. machaon* in most of the Northern Hemisphere.

Flowers from *C. trichotomum* (Lamiaceae), *C. japonica* (Vitaceae), *R. pseudoacacia* (Fabaceae), *L. japonica* (Caprifoliaceae), *C. deliciosa* and *Z. ailanthoides* were collected from Tsukuba city in their respective flowering seasons. Plants were identified by Inoue TA according to several illustration books for plant identification published in Japan.

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For the identification of volatile substances in the leaves and flowers, all plant specimens were collected around noon, when the production of plant volatiles is expected to be maximal during the day. Samples were transferred to the Showa Denko Materials Techno Service laboratory and stored at -40°C until further analysis.

#### Analysis of plant volatiles (Method-1)

The leaf volatiles were subjected to Gas Chromatography-Mass Spectrometry (GC-MS) on an Agilent system consisting of a model 7890 gas chromatograph, a model 5977 mass-selective detector (EIMS, electron energy of 70 eV), and an Agilent ChemStation data system (Santa Clara, CA, USA). Volatiles in the leaf were trapped in an odorant-collecting cartridge (Tenax TA, GL Sciences, Tokyo, Japan) and subjected to GC using a newly developed non-solvent method known as dynamic headspace and thermal desorption system (Gerstel, Überhausen, Germany) with a COMPS2XLxt multipurpose sampler. The GC column was a DB-VRX column (Agilent, USA) with a film thickness of 1.44 µm, length of 60 m, and internal diameter of 0.25 mm. The carrier gas was helium, with a flow rate of 2.1 mL/min. The GC oven temperature was regulated as follows: 40°C initial temperature held for 3 min; increased at 5°C/min to 260°C and held for 8 min. Leaf samples (0.2 g) were placed in 20 mL vials and measured using the dynamic headspace technique. The mass-selective detector was set at 230°C. The volatiles were identified by comparing their MS fragmentation patterns to those in the MS library (NIST14 database).

#### Analysis of volatiles from *A. keiskei* (Method-2)

*Angelica keiskei* leaves were dried at 40°C, crushed to a powder, and the volatiles from 0.2 g of this powder were trapped at room temperature (22°C) for 24 h using an odorant-collecting cartridge (RCC18; GL Science). RCC18 was dipped in acetone (Wako, Fujifilm, Ōsaka, Japan) to extract volatiles that were then analyzed on a GC-MS QP2010 system (Shimadzu, Japan). The GC column was a DB-5MS column (Agilent, USA) with a film thickness of 0.25 µm, length of 30 m, and internal diameter of 0.25 mm. The carrier gas was helium, at a flow rate of 2.1 mL/min. The GC oven temperature was regulated as follows: 40°C initial temperature held for 2 min; increased at 5°C/min to 240°C and held for 5 min. The mass-selective detector temperature was set at 250°C. The volatiles were identified by comparing their MS fragmentation patterns to those in the MS library (NIST14 database). This analysis was performed as a part of Otani's bachelor's degree thesis.

## RESULTS

### Volatiles of the host plant leaves

The analysis of leaf volatiles of *Papilio* butterfly host plants using Method-1 is shown in Table 1. We detected a total of 62 volatiles in the 14 plant species studied. Based on the number and type of volatiles detected, these host plants were roughly divided into six groups. Group A consisted of *C. deliciosa*, *F. vulgare* and *A. keiskei*. The characteristic of this group was that the number of detected compounds was 11-18, and included mostly terpenes; alcohols, ketones, and aldehydes were not detected. Group B consisted of *B. albiflora*, *O. japonica*, *P. amurense* and *Z. ailanthisoides*. The number of compounds was 6-11 and included β-caryophyllene among sesquiterpenes (except for *Z. ailanthisoides*), along with other monoterpenes. Group C consisted of *Z. schinifolium*, *Z. piperitum* and *S. japonica*. The number of compounds was 10-14 and included predominantly monoterpenes containing toluene (except β-caryophyllene in *Z. schinifolium* and the sesquiterpene cubebene in *S. japonica*). Group D consisted of only *R. graveolens*. This species contained 18 different types of compounds containing furan. Group E consisted of *H. lanatum* and *A. sylvestris*. This group contained 10-12 compounds, and included predominantly monoterpenes, along with alcohols, ketones, and sesquiterpenes. Group F consisted of *E. meliifolia* that contained alcohols, ketones, aldehydes, and only β-caryophyllene among terpenes (Table 1).

A comparison of the volatiles of *A. keiskei* analyzed using Methods-1 and 2 are shown in Table 2. Although low-boiling point compounds were generally detected in both methods, Method-2 failed to detect high-boiling point compounds, such as isocaryophyllene, elixene, germacrene D or germacrene B (Table 2).

### Volatiles of the flowers

The analysis of volatile substances in the flowers of six species often visited

by *Papilio* butterflies is shown in Table 3. We detected a total of 45 volatiles. These plants were divided into two groups, G and H. The former consisted of *C. trichotomum*, *C. japonica*, *R. pseudoacacia* and *L. japonica*, whereas the latter consisted of *C. deliciosa* and *Z. ailanthisoides*. Group G species contained mainly aldehydes, ketones, and alcohols, whereas those of group H contained mainly monoterpenes, with the floral volatiles partially resembling the leaf volatiles. Although the flowers of *C. japonica* and *Z. ailanthisoides* appear physically similar, the volatiles did not correspond between the species. Linalool was detected in all plants. Unlike the leaves, no sesquiterpenes were detected in the flowers (Table 3).

The use of plant parts in the present study complies with international, national and/or institutional guidelines.

## DISCUSSION

The analysis of plant volatiles from the leaves revealed unexpected results, mainly because their detection did not correlate with either the phylogenetic classification or the host plant preference of *Papilio* species. Interestingly, β-myrcene was detected in 12 species (except *R. graveolens* and *E. meliifolia*), d-limonene in ten species, and β-caryophyllene in nine species. These three substances were detected in at least one of the 14 plants examined in this study, except *R. graveolens*; thus, these substances might be associated with the oviposition behavior of butterflies of the *Papilio* species. As *R. graveolens* is not indigenous to Japan, it may be necessary to consider the relationship between this species and Japanese *Papilio* species from a different perspective.

According to our observation in Tsukuba city, the grouping of *F. vulgare* with *C. deliciosa*, *O. japonica* and *S. japonica* was supported by the observation that female *P. machaon* sometimes oviposit on these plants (not published).

The criteria for host plant selection in female *P. xuthus* and *P. helenus*, both of which lay their eggs on *C. deliciosa*, *Z. ailanthisoides* and *E. meliifolia*, could not be explained by the composition of plant volatiles; namely, *E. meliifolia* shared no common volatiles with *C. deliciosa* and *Z. ailanthisoides*, except for β-caryophyllene that was not detected in *Z. ailanthisoides*. The odor of *E. meliifolia* leaves is similar to that of apple fruits; this is completely different from that of other Rutaceae plants in Japan. In fact, hexanal and 2-hexenal, detected in *E. meliifolia* but not in the other 12 species (only detected from *R. graveolens* other than *E. meliifolia*), have been detected in apples [43]. Thus, these substances might be also associated with the oviposition behavior of the *Papilio* species. Similarly, cubebene, detected in *Z. piperitum* and *S. japonica*, is found in *Piper cubeba* (Piperaceae) [44], but not in other plants examined in this study. In fact, in our observation, seeds and leaves of *Z. piperitum* are used as "Japanese pepper" and *S. japonica* has an odor similar to pepper (not published). These results suggest the existence of other key plant volatiles responsible for the oviposition behavior in *Papilio* species; however, these were not detected in this study. Mozuraitis et al. [45] also suggested that volatiles released from the foliar extract of host plants enhance the landing rates of gravid *Polygona calbuli* (Nymphalidae) females but do not stimulate oviposition. The host plant selection system of *Papilio* and other butterflies based on plant volatiles is more complicated than previously expected, and cannot be elucidated exclusively on the basis of scent components in plants. Further analysis of the components in leaves is warranted to address this.

Although the six nectaring plants were divided into two groups on the basis of their floral volatiles, linalool was detected in all species; thus, linalool may be a key substance inducing the nectaring behavior of *Papilio* butterflies. The result obtained for *C. trichotomum* have been previously reported [46] and the results from a previous study are summarized in Table 4 along with the results obtained in this study. This study revealed an important issue; in Miyake et al.'s [46] study, almost all volatiles with a comparatively low-boiling point were not detected. The authors, in their study methods, describe the following: "The compounds trapped by the absorption tube were eluted with 2 ml of diethyl ether for pesticide residue analysis. The eluate was concentrated to the limit by the passing of N<sub>2</sub> across its surface." Thus, in their study, low-boiling point volatiles were lost during concentration. In contrast, this study was unable to detect the four esters observed in their study. Therefore, those esters might have appeared during the concentration process used in the other study. Thus, this study emphasizes the need to reanalyze plant volatiles using the latest methods to identify novel candidates (Table 4).

TABLE 1

List of volatile substances detected from leaves examined in this study

RT	Plant Substance	<i>Citrus deliciosa</i>	<i>Foeniculum vulgare</i>	<i>Angelica keiskei</i>	<i>Boenninghausenia albiflora</i>	<i>Orixa japonica</i>	<i>Phellodendron amurense</i>	<i>Zanthoxylum ailanthoides</i>	<i>Zanthoxylum schinifolium</i>	<i>Zanthoxylum piperitum</i>	<i>Skimmia japonica</i>	<i>Ruta graveolens</i>	<i>Heracleum lanatum</i>	<i>Anthriscus sylvestris</i>	<i>Euodia meliifolia</i>
14.06	3-Methylbutanal														○
14.39	2-Methylbutanal														○
14.78	1-Penten-3-ol <sup>a</sup>														○
15.15	2-Pentanone												○		
15.72	2-Ethylfuran											○			○
15.91	3-Hydroxy-2-Butanone												○	○	
16.62	3-Methyl-1-Butanol													○	
18.79	Toluene <sup>a</sup>								○	○	○				○
19.47	Hexanal <sup>a</sup>											○			○
21.17	3-Hexen-1-ol								○			○			○
21.69	2-Hexenal											○			○
24.36	αThujene	○		○									○		
23.46	Stylene									○					
24.84	αPinene <sup>a</sup>	○	○				○	○	○	○	○		○	○	
26.16	Camphene?		○						○		○				
26.17	Sabinene?	○											○		
26.2	2-Octanone											○			
26.33	β Myrcene <sup>a</sup>	○	○	○	○	○	○	○	○	○	○		○	○	
26.38	β Pinene <sup>a</sup>	○	○											○	
26.52	Octanal				○										
26.78	HexenylAcetate?					○				○		○			
27.28	α Phellandrene <sup>a</sup>		○	○			○	○	○		○				
27.48	3 Carene?	○	○	○							○				
26.84	2 Carene?			○									○		
27.84	Zβ Ocimene	○	○		○	○	○	○	○		○				
28.04	pCymene <sup>a</sup>	○	○	○					○		○				
28.11	dLimonene <sup>a</sup>	○	○		○		○	○	○	○	○		○	○	
28.3	β Phellandrene		○	○			○	○		○	○			○	
28.44	Eβ Ocimene	○			○	○			○						

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28.88	$\gamma$ Terpinene?	O	O	O	O				O	O			O
29.78	2-Nonanone												O
30	Terpinolene?	O	O	O									
30.05	Linalool <sup>a</sup>							O		O		O	
30.14	Nonanal <sup>a</sup>												O
30.56	Phencone		O										
31.16	AlloOcimene		O		O				O				
31.91	3Methyl- 3,4DibynylCiclohexene?				O								
32.78	2-Decanone												O
33	OctylAcetate				O								
33.69	?												O
33.71	Estragole		O										
34.06	HexenylMethylbutyrate ?						O			O			
34.84	?											O	
34.89	LinalylAcetate <sup>a</sup>				O								
35.65	2-Undecanone												O
35.79	NonylAcetate												O
36	?												O
36.62	Anetol <sup>a</sup>		O										
38.91	?												O
39.24	Copaene	O	O										
39.45	?												O
39.52	Elemene?	O											
40.75	$\beta$ Caryophyllene <sup>a</sup>	O	O	O	O	O	O			O	O		O
40.96	?												O
41.66	$\alpha$ Caryophyllene	O			O								
41.7	$\alpha$ Farnesene												O
42.2	Cubebene										O		
42.01	$\gamma$ -Muurolene												O
42.93	3,7(11)-Selinadiene			O									
43.12	?												O
44.25	?												O
44.35	?												O

**Note:** a: Confirmed by comparing to purified reagents;  $\alpha$  Phellandrene was purchased from Tokyo Chemical Industry Co Ltd, others from Wako, Fujifilm; O: detected from this plant; ?: Not identified or estimated.

TABLE 2

Comparison of substances detected in *A. keiskei* using methods 1 and 2 in this study

Substance	Method 1	Method 2
$\alpha$ Thujene	O	
$\alpha$ Pinene		O
$\beta$ Myrcene	O	O
$\alpha$ Phellandrene?	O	
3 Carene?	O	O
2 Carene?	O	
p Cymene	O	O
$\beta$ Phellandrene	O	
$\gamma$ Terpinene?	O	O
Terpinolene?	O	
$\beta$ Caryophyllene	O	
3,7(11)-Selinadiene	O	
Isocaryophyllene		O
Elixene		O
Germacrene D		O
Germacrene B		O

Note: O: detected from this plant; ?: Not identified or estimated.

TABLE 3

List of volatile substances detected from flowers examined in this study

RT	Plant Substance	<i>Clerodendrum trichotomum</i>	<i>Cayratia japonica</i>	<i>Robinia pseudoacacia</i>	<i>Lonicera japonica</i>	<i>Citrus deliciosa</i>	<i>Zanthoxylum ailanthoides</i>
10.8	Methyl acetate	O					
12.49	2-Butanone	O					
14.34	3-Methyl-Butanal		O				
14.77	1-Penten-3-ol		O	O			
15.43	2-Pentanone	O	O	O	O		
15.7	3-Pentanone		O	O			
15.93	2-Ethylfuran			O			
10.8	3-Methyl-1-Butanol	O	O				
12.49	2-Methyl-1-Butanol	O					
17.73	1-Pentanol		O	O			
19.5	2-Hexanone		O				
19.47	Hexanal	O	O	O			
21.17	3-Hexen-1-ol	O	O	O	O		
21.69	2-Hexen-1-ol / 1-Hexanol	O	O	O	O	O	O
22.91	2-Heptanone		O		O		
24.29	$\alpha$ Thujene						O
24.77	$\alpha$ Pinene						O
25.9	1-Octene-3-ol	O					
26.11	2-3-Octanedione		O				
26.14	Sabinene						O
26.25	3-Octanone	O		O			
26.34	3-Octanol	O		O			
26.4	$\beta$ Myrcene						O
26.4	$\beta$ Pinene					O	
27.27	$\alpha$ Phellandrene						O
27.67	C10?						O
27.84	Z $\beta$ O cimene						O





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