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

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Abstract

The odorants of eight Japanese mainland native species (*Citrus* x *deliciosa*, *Zanthoxylum ailanthoides* Siebold & Zucc, *Z. schinifolium* Sieb. et Zucc., *Z. piperitum* L., *Phellodendron amurense* Ruprecht, *Orixa japonica* Thunb., *Skimmia japonica* Thunb., and *Boenninghausenia albiflora* (Hook.) Rchb. ex Meisn.), one tropical species (*Euodia meliifolia* (Hance) Benth.), and one invasive species (*Ruta graveolens*) of the Rutaceae family and three Japanese mainland native species (*Angelica keiskei* (Miq.) Koidz., *Heracleum lanatum* W. Bartram, *Anthriscus sylvestris* (Blume) DC), and one invasive species (*Foeniculum vulgare* Mill.) of the Apiaceae family were analyzed using gas chromatography–mass spectrometry with dynamic–headspace and thermal–desorption methods. These plants are hostplants to Japanese *Papilio* butterflies. Herein, 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* Thunb., *Cayratia japonica* (Thunb.) Gagnep., *Robinia pseudoacacia* L., *Lonicera japonica* Thunb., *C. deliciosa*, *Z. ailanthoides*) visited by *Papilio* butterflies for nectaring, revealed the presence of linalool in all the flowers. Floral volatiles in *C. deliciosa* and *Z. ailanthoides* exhibited moderate resemblance to their respective leaf volatiles. Interestingly, our results in *C. trichotomum* was not in complete agreement with previous reports, emphasizing the need for newer methods of extraction and analysis.

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Introduction

During oviposition, female butterflies hit the leaf surface of the plant with their forelegs, in a behavior called “drumming” (Ilse, 1937). The leaf surface is injured by spines present on the ventral surface of the female foreleg tarsus (Inoue, 2006) and leaf fluid is released from the wound. Roessingh et al (1991) first showed the female butterflies sense the substances in the leaf fluid using their tarsal contact chemosensilla to determine whether the plant is a preferred hostplant for oviposition. Ovipositional stimulants perceived by contact chemosensilla have been extensively examined in *Papilio troilus* L. (Carter et al, 1999), *P. polyxenes* Fabricius (Feeny et al, 1988; Carteret al, 1998), *P. xuthus* L. (Abe et al, 1981; Ohsugi et al, 1991), *P. maackii* Ménétriés (Honda et al, 2011), *P. bianor* Cramer (Abe et al, 1981; Ono et al, 2000a; Ono et al, 2000b), *P. polytes* L. (Nakayama et al, 2003), and *P. protenor* Cramer (Abe et al, 1981; Ichinosé and Honda, 1978; Honda, 1986, 1990). Oviposition–inhibiting substances have been identified for *P. xuthus*, *P. polytes*, and *P. protenor* (Honda et al, 2011; Nishida et al, 1990; Nakayama and Honda, 2004). Among those substances, phellamurin contained in *P. amurense* stimulates the oviposition in *P. maackii*, but inhibits it in *P. protenor* (Honda et al, 2011). Moreover, genes encoding the chemosensory and odorant–binding proteins are identified in *P. xuthus* (Ozaki et al, 2008; Ozaki et al, 2011).

Although drumming behavior is commonly observed during oviposition, we have often observed that *Papilio* butterflies oviposit without contacting the plant leaves with their forelegs, furthermore, butterflies sometimes oviposit their eggs on non–hostplants or objects adjacent to hostplants (Yokohari et al, 2017). 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 hostplant habitats by following olfactory cues and consecutively identifying the hostplant by taste cues. This has been demonstrated in *P. demoleus* L. (Saxena and Goyal, 1978), *P. polyxenes* (Feeny et al, 1989; Baur and Feeny, 1994; Baur et al, 1993), *P. machaon* L. in Japan, *P. troilus* (Baur and Feeny, 1994), *P. cresphontes* Fabricius (Fadamiro et al, 2010), *P. glaucus* L, and *P. canadensis* Rothschild & Jordan (Mercadar et al, 2008). The volatile components released by hostplants of *Papilio indra* Reakirt occurring in Rocky Mountains were recently analyzed (Beauchamp et al, 2009; Dev et al, 2010). Beside of them, plant volatiles of Australian Rutaceae plants were analyzed by Ghisalberti (1998), and the relationship between plant volatiles and oviposition behavior of Japanese Papiloninae species was already examined by Li et al (2010).

Papilio butterflies are also closely associated with many nectaring flowers. While studies have suggested that *Papilio* butterflies search nectaring flowers using visual information (Kakiuchi et al, 1986; Kelber and Pfaff, 1999), 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 prepared an inventory of volatile substances from the flowers that are often visited by *Papilio* butterflies.

We hypothesized that the hostplant detection and flower–searching of Japanese *Papilio* species other than *P. machaon* could also be attributed to plant volatile substances. To prove this, we attempted to identify and prepare an inventory of volatile substances of their hostplants and flowers.

Materials And Methods

Materials

Mature leaves of *C.* x *deliciosa*, *Z. ailanthoides*, *Z. schinifolium*., *Z. piperitium*, *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. *C. 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 our experiments were collected. Selection criteria of 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 south–end of Honshu), (2) abundant, and (3) the major hostplant 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.

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 the maximal in the day. Samples were transferred to the Showa Denko Materials Techno Service laboratory and stored at -40 ºC 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 into 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 COMPS2XLxt multi–purpose 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 ºC initial temperature held for 3 min; increased at 5 ºC/min to 260 ºC and held for 8 min. Leaf samples (0.2 g) were placed into 20 mL vials and measured by the dynamic–headspace technique. Mass–selective detector was set at 230 ºC. 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)

A. keiskei leaves were dried at 40 ºC, 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, Ôsaka, 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. The GC oven temperature was regulated as follows: 40 ºC initial temperature held for 2 min; increased at 5 ºC/min to 240 ºC and held for 5 min. Mass–selective detector temperature was set at 250 ºC. The volatiles were identified by comparing their MS fragmentation patterns to those in the MS library (NIST14 database). This analysis was performed as bachelor thesis of Otani.

Results

Volatiles in the host plants

The analysis of leaf volatiles of *Papilio* butterfly hostplants using Method–1 are shown in Table 1. We detected a total of 62 volatiles in the 14 plant species studied. Based on the number and types of volatiles detected, these hostplants 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, mostly terpenes, and alcohols, ketones, and aldehydes are not detected. Group B consisted of *B. albiflora*, *O. japonica*, *P. amurense*, and *Z. ailanthoides*. The number of compounds was 6–11, and included β–caryophyllene among sesquiterpenes (except for *Z. ailanthoides*), along with other monoterpenes. Group C consisted of *Z. schinifolium*, *Z. piperitum*, and *S. japonica*. The number of compounds was 10–14, 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, 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.

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

Volatiles of the flowers

The analysis of volatile substances in the flowers of six species often visited by *Papilio* butterflies are 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. ailanthoides*. Group G species contained mainly aldehydes, ketones, and alcohols, whereas those of group H species contained mainly monoterpenes, with the floral volatiles partially resembling the leaf volatiles. Although the flowers of *C. japonica* and *Z. ailanthoides* appear physically similar, the volatiles did not correspond between the species. Linalool was detected in all the plants. Unlike leaves, no sesquiterpenes were detected in flowers. The result of *C. trichotomum* have been reported before (Miyake et al, 1998) and are summarized in Table 4 together with our results this time.

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 hostplant–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 are contained at least in one of the 14 plants examined this time, except for *R. graveolens*, thus, these substances might be associated with the oviposition behavior of the *Papilio* species. Since *R. graveolens* is not an indigenous plant in Japan, it may be necessary to consider the relationship between this plant and Japanese *Papilio* species from a different perspective.

According to our observation in Tsukuba city, the grouping of *F. vulgare* together 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 hostplant selection in female *P. xuthus* and *P. helenus*, both of which lay their eggs on *C. deliciosa*, *Z. ailanthoides*, 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. ailanthoides*, except for β–caryophyllene that was not detected in *Z. ailanthoides*. The odor of *E. meliifolia* leaves is similar to that of apples 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*), are detected from apples (Ohta et al, 1966). 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), 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 oviposition behavior in *Papilio* species, but were not detected in our current study. Mozūraitis et al (2016) also suggested that volatiles released from the foliar extract of host plants enhance the landing rates of gravid *Polygonia c–album* (Nymphalidae) females, but do not stimulate oviposition. The hostplant 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 the species; thus, linalool may be a key substance inducing nectaring behavior of *Papilio* butterflies. Our current study revealed an important issue, as shown in Table 4. This table compares the results of a previous study (Miyake et al, 1998) with ours. In the previous study, almost all volatiles with a comparatively low–boiling point were not detected. The authors in their study methods describe, “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 N2 across its surface.” Thus, in their study, low–boiling point volatiles were lost during concentration. In contrast, our study was unable to detect the four esters observed in their study. Therefore, those esters might have appeared during concentration in that study. Thus, our current study emphasizes the need to reanalyze plant volatiles using latest methods to identify novel candidates.

Similarly, benzaldehyde was not detected in the flowers of all the six species we examined; however, Miyake et al (1998) detected it in *C. trichotomum*. Benzaldehyde is known to induce a proboscis extensional reaction in six Japanese Nymphalinae species (Ômura and Honda, 2009). Among these six butterflies, we have earlier confirmed this behavior in *Vanessa indica* (Yokohari et al, 2017). Thus, Nymphalinae and Papilioninae butterflies may respond to same substances differently. Additionally, it was interesting to note the difference in volatiles determined by Method–1 and –2 in the same plant (*A. keiskei*). Hence, our study highlights that different method of extraction and analysis may be necessary to obtain a comprehensive profile of the plant volatiles involved in oviposition behavior of butterflies. We already obtained the results the relationship between some of these plant volatiles and reaction of *Papilio* butterflies to these volatiles, we will show these in our another article.

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Tables

Table 1 List of volatile substances detected from leaves examined in this study

RT	Substance / Plant	*Citrus deliciosa*	*Foeniculum vulgare*	*Angelica keiskei*	*Boenninghausenia albiflora*	*Orixa japonica*	*Phellodendron amurense*	*Zanthoxylum ailanthoides*	*Zanthoxylum schinifolium*	*Zanthoxylum piperitum*	*Skimmia japonica*	*Rut gra*
14.06	3-Methylbutanal											
14.39	2-Methylbutanal											
14.78	1-Penten-3-ol [a]											
15.15	2-Pentanone											
15.72	2-Ethylfuran											
15.91	3-Hydoroxy-2-Butanone											
16.62	3-Methyl-1-Butanol											
18.79	Toluene [a]								O	O	O	
19.47	Hexanal [a]											
21.17	3-Hexen-1-ol								O			
21.69	2-Hexenal											
24.36	αThujene	O		O								
23.46	Stylene									O		
24.84	αPinene [a]	O	O				O	O	O	O	O	
26.16	Camphene?		O						O		O	
26.17	Sabinene?	O										
26.20	2-Octanone											
26.33	βMyrcene [a]	O	O	O	O	O	O	O	O	O	O	
26.38	βPinene [a]	O	O									
26.52	Octanal				O							
26.78	HexenylAcetate?					O				O		
27.28	αPhellandrene [a]		O	O			O	O	O		O	
27.48	3 Carene?	O	O	O							O	
26.84	2 Carene?			O								
27.84	*Z*βOcimene	O	O		O	O	O	O	O		O	
28.04	*p*Cymene [a]	O	O	O					O		O	
28.11	*d*Limonene [a]	O	O		O		O	O	O	O	O	
28.30	βPhellandrene		O	O			O	O		O	O	
28.44	*E*βOcimene	O			O	O			O			
28.88	γTerpinene ?	O	O	O	O				O	O		
29.78	2-Nonanone											
30.00	Terpinolene ?	O	O	O								
30.05	Linalool [a]					O			O		O	
30.14	Nonanal [a]											
30.56	-Phencone		O									
31.16	AlloOcimene		O		O				O			
31.91	3Methyl-3,4DibynylCiclohexene?				O							
32.78	2-Decanone											
33.00	OctylAcetate				O							
33.69	?											
33.71	Estragole		O									
34.06	HexenylMetylbutyrate ?						O			O		
34.84	?										O	
34.89	LinalylAcetate [a]					O						
35.65	2-Undecanone											
35.79	NonylAcetate											
36.00	?											
36.62	Anetol [a]		O									
38.91	?											
39.24	Copaene	O	O									
39.45	?											
39.52	Elemene?	O										
40.75	βCaryophyllene [a]	O	O	O	O	O	O			O	O	
40.96	?											
41.66	αCaryophyllene	O			O							
41.70	αFarnesene											
42.20	Cubebene										O	
42.01	γ-Muurolene											
42.93	3,7(11)-Selinadiene			O								
43.12	?											
44.25	?											
44.35	?											

[a]: Confirmed by comparing to purified reagents; αPhellandrene was purchased from Tokyo Chemical Industry Co Ltd, others from Wako, Fujifilm.

Table 2. Comparison of detected substances from *A. keiskei* between Method– 1 and Methode– 2 in this study

Substance	Method 1	Method 2
αThujene	O	
αPinene		O
βMyrcene	O	O
αPhellandrene?	O	
3 Carene?	O	O
2 Carene?	O	
*p*Cymene	O	O
βPhellandrene	O	
γTerpinene ?	O	O
Terpinolene ?	O	
βCaryophyllene	O	
3,7(11)-Selinadiene	O	
Isocaryophyllene		O
Elixene		O
Germacrene D		O
Germacrene B		O

Table 3. List of volatile substances detected from flowers examined in this study

Γ	Plant Substance	*Clerodendrum trichotomum*	*Cayratia japonica*	*Robinia pseudoacacia*	*Lonicera japonica*	*Citrus deliciosa*	*Zanthoxylum ailanthoides*
80	Methyl acetate	O					
49	2-Butanone	O					
34	3-Methyl-Butanal		O				
77	1-Penten-3-ol		O	O			
43	2-Pentanone	O	O	O	O		
70	3-Pentanone		O	O			
93	2-Ethylfuran			O			
80	3-Methyl-1-Butanol	O	O				
49	2-Methyl-1-Butanol	O					
73	1-Pentanol		O	O			
50	2-Hexanone		O				
47	Hexanal	O	O	O			
17	3-Hexen-1-ol	O	O	O	O		
69	2-Hexen-1-ol / 1-Hexanol	O	O	O	O	O	O
91	2-Heptanone		O		O		
29	αThujene						O
77	αPinene						O
90	1-Octene-3-ol	O					
11	2-3-Octanedione		O				
14	Sabinene						O
25	3-Octanone	O		O			
34	3-Octanol	O		O			
40	βMyrcene						O
40	βPinene					O	
27	αPhellandrene						O
67	C10?						O
84	*Z*βOcimene						O
04	*p*Cymene				O		
11	*d*Limonene					O	O
28	Cyneol						O
44	*E*βOcimene					O	O
04	γTerpinene ?					O	O
46	αThujenol						O
00	Terpinolene ?					O	O
05	Linalool	O	O	O	O	O	O
35	Cymene?					O	
20	AlloOcimene						O
86	Sabinol						O
93	LimoneneMonooxide or Citroneral					O	
19	Verbenol						O
54	Terpineol						O
57	Terpineol				O	O	O
17	Azulene			O			
75	Verbenone						O
07	?						O

Table 4. Comparison of detected substances from *C. trichotomum* between Miyake et al (1998) and our current study

Results Substance	Miyake et al (1998)	Our results
Methyl acetate		O
2-Butanone		O
2-Pentanone		O
3-Methyl-1-Butanol		O
2-Methyl-1-Butanol		O
Hexanal		O
3-Hexen-1-ol		O
2-Hexen-1-ol / 1-Hexanol		O
1-Octene-3-ol		O
BenzAldehyde	O	
3-Octanone		O
3-Octanol		O
Linalool	O	O
Benzyl Acetate	O	
Methyl Benzoate	O	o
Methyl Salicylate	O	o