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RAPID COMMUNICATION

Evidence for Behavioral Attractiveness of Methoxylated Aromatics in a Dynastid Scarab Beetle-Pollinated Araceae

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Abstract Many plants attract their pollinators with floral scents, and these olfactory signals are especially important at night, when visual signals become inefficient. Dynastid scarab beetles are a speciose group of night-active pollinators, and several plants pollinated by these insects have methoxylated aromatic compounds in their scents. However, there is a large gap in our knowledge regarding the compounds responsible for beetle attraction. We used chemical analytical analyses to determine temporal patterns of scent emission and the composition of scent released from inflorescences of *Philodendron selloum*. The attractiveness of the main components in the scent to the dynastid scarab beetle *Erioscelis emarginata*, the exclusive pollinator of this plant, was assessed in field biotests. The amount of scent increased rapidly in the evening, and large amounts of scent were released during the activity time of the beetle pollinators. Inflorescences emitted a high number of compounds of different biosynthetic origin, among them both uncommon and also widespread flower scents. Methoxylated aromatic compounds dominated the scent, and 4-methoxystyrene, the most abundant compound, attracted *E. emarginata* beetles. Other compounds, such as (*Z*)-jasmone and possibly also the methoxylated aromatic compound 3,4-dimethoxystyrene increased the attractiveness of 4-methoxystyrene. Methoxylated aromatics, which are known from other dynastid pollinated plants as well, are important signals in many scarab beetles in a different context (e.g., pheromones), thus suggesting that these plants exploit pre-existing preferences of the beetles for attracting this group of insects as pollinators.

Keywords Araceae · Dynamic headspace · Electronic sensor and gas chromatography-mass spectrometry · Flower scent · Plant-pollinator interactions · Scarabaeidae

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Introduction

Flower scents are important for pollinator attraction in many pollination systems. Plants pollinated by a specific group or guild of animals, such as bats, moths, or dung/carcass flies, often converge in their scent composition (Dobson, 2006). Such convergence also has been found in plants pollinated by neotropical dynastid beetles. Some species, though not closely related, have methoxylated aromatics in their scent (Dobson, 2006), pointing towards a function of these compounds in attracting the beetle pollinators. However, the ability of these compounds to attract neotropical dynastid beetles has not yet been tested.

The inflorescence of *Philodendron selloum* C. Koch (Araceae) is highly attractive to *Erioscelis emarginata* (Mannerheim) (Scarabaeidae, Dynastinae), the only pollinator of this plant (Gottsberger and Silberbauer-Gottsberger, 1991). The flowers of Araceae are arranged in a spike inflorescence that consists of a spadix (main axis) and a spathe (large bract).

Pistillate flowers are situated in the basal area of the spadix, which is surrounded by the spathe to form a chamber (kettle). Fertile staminate flowers are on the distal part of the spadix, and between the pistillate and fertile staminate flowers is a zone of sterile staminate flowers. The inflorescence of *P. selloum* emits a strong scent at dusk, following the opening of the inflorescence, at which time the pistillate flowers are receptive. The scent has been shown to be essential for long distance attraction of *E. emarginata* (Gottsberger and Silberbauer-Gottsberger, 1991). The pollinator beetles typically arrive within less than 30 min, and the number attracted to a single inflorescence can be high (up to 200). The volatilization of scent is favored by an extraordinary strong increase in temperature of the spadix at dusk, with temperatures of 46 °C (more than 30 °C higher than ambient temperatures) being reached. If attracted beetles carry pollen from another inflorescence, they serve as effective pollinators. The beetles stay in the kettle until the evening of the next day, when the fertile staminate flowers release their pollen; during this staminate stage, the scent emitted is weak and does not attract additional beetles. The beetles present feed on pollen before they leave the inflorescence, and then are attracted by a pistillate stage inflorescence.

Given the scent and temperature changes of the inflorescence in relation to the behavior of the dynastid beetles, which serve as exclusive pollinators, we asked three main questions in relation to the role of scent in beetle attraction: 1) what is the temporal pattern of scent emission in the evening of a pistillate stage inflorescence, i.e., are highest amounts of scent emitted at dusk when the temperature of the spadix is high and beetles are attracted? 2) do *Philodendron* inflorescences emit methoxylated aromatic compounds in their scent, as has been found in other species pollinated by dynastid beetles? and 3) are abundant compounds in the inflorescence scent capable of attracting *E. emarginata* in the field ?

Methods and Materials

Plant Material, Volatile Collection, and Chemical Analyses To learn more abut the temporal pattern of scent emission, the scent emission from 3 different inflorescences from a plant cultivated in the Botanical Garden at the University of Ulm was followed in the time course of 18:05 – 21:30 (10 min resolution) on-line by a zNose 4200 (Electronic Sensor Technology, Newbury Park, CA, USA) as described by Kunert et al. (2002). The zNose is a portable gas chromatographic system, containing all necessary equipment for volatile collection, desorption, and separation (column: 1 m, DB 5, film thickness 0.25 μm, ID 0.25 mm; carrier gas: Helium at 3 ml min^{-1}). The analytical cycles were repeated automatically every 10 min and were programmed as follows: SAW detector temperature: 60 °C; sampling time: 30 sec, flow rate: 30 ml min^{-1}. Sampling of volatiles was achieved by directing a needle connected to the inlet of the instrument directly to the opening of an inflorescence. The analysis was started by trapping the emitted volatiles on a porous polymer (tenax-trap) for 30 sec. Next, the trap was rapidly (mseconds) heated to 280 °C, while the desorbed volatiles were directly transferred by the carrier gas onto the separation column. Temperature-program for elution of volatiles: 40 °C to 180 °C with an increase of 5 degrees C per sec, followed by a cleaning period of the SAW detector at 125 °C for 30 s. To visualize the data, the retention times and peak areas of the eluting compounds were exported to Microsoft Excel.

To learn more about the composition of scent emitted from pistillate stage inflorescences, we collected scent in the field (Botucatu city, State of São Paulo, Brazil). In 2007, scent samples from 5 different plants (one inflorescence per plant) were collected for 2.5 to 3.5 h between 16:30 and 20:20. All collections started before the activity time of the beetles (arrival c. 19:15-19:45) and ended shortly after their arrival. Individual inflorescences were covered with a polyester ovenbag (Bratschlauch, Melitta GmbH, Germany) for scent accumulation. With a battery-operated membrane pump (G12/01 EB, Rietschle Thomas, Puchheim, Germany) and a flow rate of 200 mlmin^{-1}, the air from within the bag was sucked through a glass tube. Volatiles were captured by a 1:1 (vol.) absorbent mixture (c. 200 mg) of Tenax TA (80/100 mesh, Macherey-Nagel, Germany) and Carbopack X (20/40 mesh, Supelco, Germany). The mixture was fixed in the tubes using glass wool. Absorbed scent was eluted with 1 ml of high grade acetone (Merck, Germany) and kept frozen (-20 °C) in 2 ml silanized glass vials (Supelco, Germany) until analysis.

In 2008, scent was collected from two unbagged inflorescences for only 2 min at 19:15 and 19:45 (time of beetle arrival), respectively. The procedures differed from those in 2007 in that the scent samples were trapped in smaller tubes (ChromatoProbe quartz microvials, closed side was cut; length: 15 mm; inner diam.: 2 mm; Varian Inc., Palo Alto, CA, USA) that were filled with a mixture of 1.5 mg Tenax-TA (mesh 60-80; Supelco, Germany) and 1.5 mg Carbotrap B (mesh 20-40, Supelco, Germany) and placed close to the kettle entrance of an inflorescence. In both years, the surrounding air was collected to distinguish between plant volatiles and ambient contaminants.

To determine the amount of scent released from paper inflorescences used in the behavioral assays (see below), volatiles were collected in the lab from such a filter paper to which a mixture of 4-methoxystyrene, 3,4-dimethoxystyrene, and (*Z*)-jasmone (100 μl each) was applied. The scent collection protocol was similar to that used for unbagged inflorescences in 2008.

For identification of trapped volatiles, headspace samples were analyzed on a Varian Saturn 2000 mass spectrometer

coupled to a Varian 3800 gas chromatograph equipped with a 1079 injector (Varian Inc., Palo Alto, CA, USA), which had been fitted with the ChromatoProbe kit. Before filling 1 μl of the samples collected in 2007 in a quartz vial and placing this into the injector port by means of the ChromatoProbe, 1 μg of nonadecane was added as internal standard. Samples collected in 2008 using the small tubes were directly inserted into the injector by means of the ChromatoProbe and analysed by thermal desorption. For all samples, the injector split vent was opened, and the injector was heated to 40 °C to flush any air from the system. The split vent was closed after 2 min, and the injector was heated at a rate of 200 °C/min to 200 °C, then held at 200 °C for 4.2 min, after which the split vent was opened and the injector cooled down. Separations were achieved with a fused silica column ZB-5 (5 % phenyl polysiloxane; 60 m long, inner diam. 0.25 mm, film thickness 0.25 μm, Phenomenex). Electronic flow control was used to maintain a constant helium carrier gas flow of 1.0 ml min^{-1}. The GC oven temperature was held for 7 min at 40 °C, then increased by 6 °C per min to 250 °C, and held for 1 min. The MS interface worked at 260 °C, and the ion trap at 175 °C. Mass spectra were taken at 70 eV (in EI mode) with a scanning speed of 1 scan sec^{-1} from *m/z* 30 to 350. The GC-MS data were processed using the Saturn Software package 5.2.1.

Identification of compounds was carried out using the NIST 08, Wiley 7, and Adams 2007 mass spectral data bases, or the data base provided in MassFinder 3, and confirmed by comparison of retention times with published data. (*E*)-8(9)-Dehydro-4(5)-dihydrotheaspirone was identified by Roman Kaiser (Uster, Switzerland). Structure assignment of individual components was confirmed by comparison of both mass spectra and GC retention times with those of authentic standards. To quantify the absolute amount of scent present in the samples collected in 2007, the total peak area (sum of all compounds) was compared with the peak area of the internal standard. To determine the amount of scent trapped on the small filters in 2008, known amounts of monoterpenes, aliphatics, and aromatics were injected into the GC-MS system; mean peak areas of these compounds were used to determine the total amount of scent.

Biotests with Abundant Synthetic Floral Scent Compounds For field biotests, paper model inflorescences (cone) with or without (negative control) inflorescence scent(s) of *P. selloum* were used. These artificial inflorescences, which superficially imitated the form and size of the spathe (length: c. 25 cm), were installed close (within 5 m) to live flowering plants. The distances between each cone varied, but were always about the same in each experiment, either 0.8 or 2 m. Each scent cone received one, two, or three scent compounds (c. 100 μl per compound; purchased by Sigma Aldrich, highest purity available) just before the beginning of the flight activity period of *E. emarginata*. The scent compounds were the three most abundant constituents of the scent emitted from pistillate stage inflorescences, i.e., 4-methoxystyrene, 3,4-dimethoxystyrene, and (*Z*)-jasmone. Four different choice experiments were performed on four different evenings. In the first experiment (A; 31.10.2008, 19:35-20:00, 5-choice test), a control was tested against the three single compounds and a blend of all three compounds. In the second (B, 28.10.2009, 19:40-19:50, 4-choice test), a control was tested against the three possible two-component blends. In the third (C, 25.11.2009, 19:45-20:00, 4-choice test), a control was tested against 4-methoxystyrene and the two binary blends containing 4-methoxystyrene. In the fourth (D, 17.11.2009, 19:45-20:00, 2-choice test), the control was tested against 4-methoxystyrene. All beetles that were inside the cone at the end of the experiment were counted. Differences in attractiveness of the control and treatments were assessed using randomization tests of goodness-of-fit using 10000 replicates (http://udel.edu/~mcdonald/statrand.xls). When these global tests (experiments A-C) had a significant outcome, it also was determined which of the single treatments differed in their attractiveness from the control (exact binomial test, http://udel.edu/~mcdonald/statexactbin.xls). In experiment B, we compared additionally the attractiveness of 4-methoxystyrene+3,4-dimethoxystyrene and of 4-methoxystyrene+(*Z*)-jasmone using an exact binomial test.

Results and Discussion

In *P. selloum*, a strong increase in the amount of scent released from pistillate stage inflorescences can be detected by the human nose in the evening shortly before beetle arrival (Gottsberger and Silberbauer-Gottsberger, 1991). This finding was supported by data on the temporal pattern of scent emission. The amount of scent increased rapidly and reached its maximum (19:40) within an hour, remaining there only about 10 min, after which the amount started to decrease (Fig. 1a). In the field, beetles are attracted to pistillate stage inflorescences shortly after or during the time of maximal scent discharge (as perceived by the human nose; Gottsberger and Silberbauer-Gottsberger, 1991).

The total amount of scent trapped from the bagged inflorescences (2007) varied from 2-35 μg/h, and the scent trapped during the 2-min-collections from unbagged inflorescences (2008) was 16 and 216 μg of scent (equivalent to 469 and 6475 μg converted to a per h basis). The amount of scent trapped (per h), therefore, was higher in 2008 compared to 2007, which can be explained by methodological issues, mainly temporal parameters. In 2008, scent was collected only during the time when both beetles were

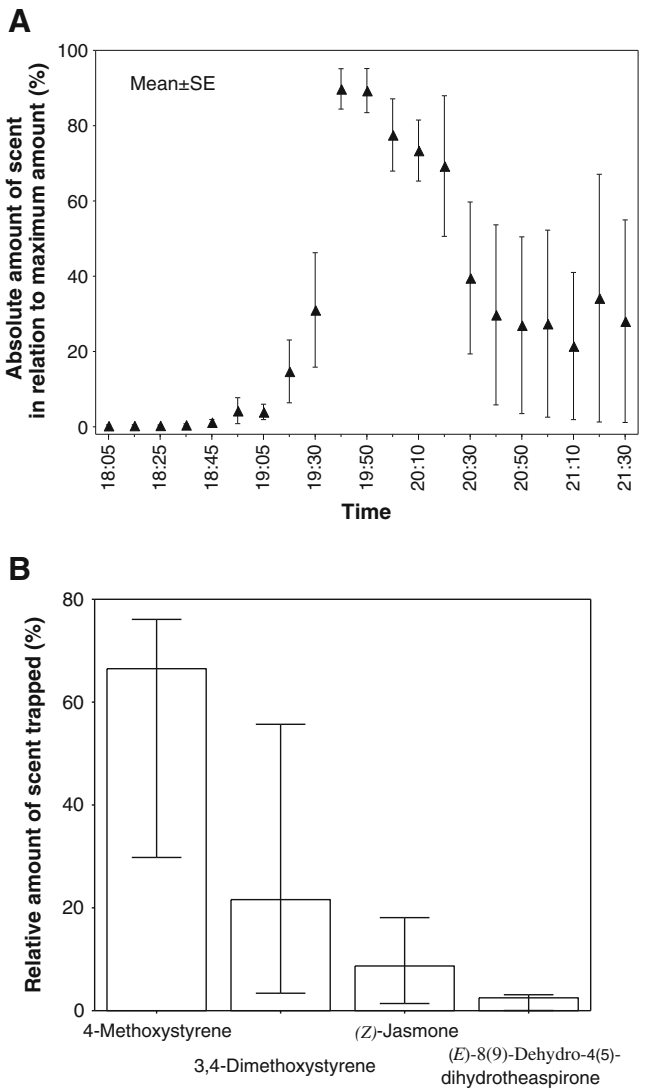

Fig. 1 Scent emitted from pistillate stage inflorescences of *Philodendron selloum*. **a** Temporal pattern of scent release over the course of an evening as determined by zNose measurements (N=3); **b** Relative amount of each scent compound (median, Min-Max) contributing in the median at least 1 % of the total scent released, as determined by dynamic headspace and GC-MS analyses (N=5)

attracted and scent emission was at or close to its maximum, whereas in 2007, scent collections started before the activity period of the beetles, when no or only low amounts of scent were emitted from the inflorescences (compare Fig. 1a).

The total amounts of scent trapped during peak of emission are extraordinarily high, and this is especially evident when considering that in 2008 scent was collected from unbagged inflorescences, and thus only a small amount of the actual scent emitted was trapped. However, flowers/inflorescences from dynastid pollinated plants are often strongly scented, and indeed, scent quantities comparable to those of the present study have been found in other plants pollinated by these beetles (e.g., Gottsberger et al., 2012). The high amount of scent in dynastid pollinated plants is likely due to three factors: i) the large size of the inflorescences/flowers, ii) the occurrence of floral thermogenesis, which favors synthesis/volatilization of compounds produced, and iii) a possible preference of dynastid beetles for strongly scented plants (untested). Interestingly, although we used pure substances in our biotests (see below), emission rates from artificial flowers (total scent discharges: 94-113 μg per h; thereof 93 % 4-methoxystyrene, 2 % 3,4-dimethoxystyrene, 5 % (*Z*)-jasmone) were lower as the natural ones found during the time beetles are attracted demonstrating again that the scent amount emitted from inflorescences is tremendous.

A total of 68 compounds were detected in the scent samples (Supplemental Material Resource 1), of which most occurred in relatively small amounts; only four compounds contributed in the median at least 1.0 % to the total scent. Samples were dominated strongly by the two aromatic compounds 4-methoxystyrene (66.5 %) and 3,4-dimethoxystyrene (21.6 %), followed by (*Z*)-jasmone (8.7 %) and (*E*)-8(9)-dehydro-4(5)-dihydrotheaspirone (2.5 %) (Fig. 1b). This scent composition differs strikingly from the scent of the dynastid pollinated *P. acutatum*, the only other *Philodendron* species with a known scent composition, which is dominated by terpenoid and aliphatic compounds (Maia et al., 2010). Of the four main compounds of *P. selloum*, 4-methoxystyrene and (*Z*)-jasmone are widespread in flower scents, known (as minor constituents) from plants of several families with various pollination strategies (Knudsen et al., 2006), including dynastid beetle pollination

Table 1 Number of *Erioscelis emarginata* beetles that were attracted to the three most abundant compounds from pistillate stage inflorescences of *Philodendron selloum* when tested singly or as mixtures in four different two-/multiple-choice experiments (A-D)

Scent compound(s)	Experiment			
	A	B	C	D
none (control)	0	0	0	0
1	0	-	4	**33**
2	1	-	-	-
3	0	-	-	-
1+2	-	**25**	4	-
1+3	-	**18**	**14**	-
2+3	-	0	-	-
1+2+3	**10**	-	-	-
chi^2 =	34.9	45.3	19.5	33.0
P <	0.001	0.001	0.001	0.001

Compounds tested: 1: 4-methoxystyrene, 2: 3,4-dimethoxystyrene, 3: (*Z*)-jasmone. Compounds were offered on artificial inflorescences made of filter paper; an empty filter paper was used as a control (negative). The test outcomes of (global) randomization tests of goodness-of-fit for each experiment are also given. Single compounds and compound mixtures that attracted significantly more beetles compared to the controls are in bold. -=not tested

(Dobson, 2006; Maia et al., 2010). The other two compounds, however, are uncommon in floral scents. To the best of our knowledge, 3,4-dimethoxystyrene has been described only as a trace constituent in floral scent of *Muscari muscarimi* (Liliaceae; Kürkcüoglu and Baser, 2010), and (E)-8(9)-dehydro-4(5)-dihydrotheaspirone is known only from *Reseda odorata* (Resedaceae; Surburg et al., 1993; R. Kaiser, personal communication). The four main compounds have never been found together in another plant, and might serve as a specific, reliable signal for *E. emarginata* dynastid beetles, which are the only pollinators.

Among the four main components in the floral scent of *P. selloum,* the three most abundant ones were available for behavioral experiments in the field (Table 1). The results from the biotests indicate that: i) a mixture of the three compounds, but not any single compound, was preferred by the beetles compared to the control (experiment A), ii) the two binary mixtures containing 4-methoxystyrene, but not the binary mixture with 3,4-dimethoxystyrene+(*Z*)-jasmone, attracted more beetles than a control (experiment B), iii) 4-methoxystyrene+(*Z*)-jasmone, but not 4-methoxystyrene alone or combined with 3,4-dimethoxystyrene, was more attractive than a control (experiment C), and iv) beetles preferred 4-methoxystyrene when tested against a control (experiment D). These experiments demonstrate that 4-methoxystyrene alone has the capability to attract free-flying *E. emarginata.* In addition, (*Z*)-jasmone, a compound already known to attract flower visiting scarab beetles (Rutelinae, *Popillia japonica*; Loughrin et al., 1998) in non-tropical regions outside the natural habitats of *P. selloum* and *E. emarginata*, increases the attractiveness of 4-methoxystyrene, as might 3,4-dimethoxystyrene as well, given that the mixture of 3,4-dimethoxystyrene and 4-methoxystyrene had the same attractiveness as 4-methoxystyrene+(*Z*)-jasmone (see experiment B, P=0.36). It should be kept in mind that the importance of both 3,4-dimethoxystyrene and (*Z*)-jasmone in attracting beetle pollinators may have been underestimated in the present study, considering that the amount (relative and absolute) of these compounds was higher in volatile samples collected from natural inflorescences than from the artificial paper flowers (see before).

Aromatic compounds also are known from other dynastid pollinated plants (Dobson, 2006; Schiestl and Dötterl, 2012). Our data demonstrate that they are involved in host plant finding of these beetles. During the biotests, no insects other than *E. emarginata* were attracted to our artificial flowers, which is consistent with the finding that *P. selloum* is pollinated only by *E. emarginata* (Gottsberger and Silberbauer-Gottsberger, 1991) and indicates a high specificity in the chemical signals used by *P. selloum* to attract *E. emarginata*. Our finding that beetles in the biotests were attracted only to artificial flowers if no pistillate stage inflorescence was flowering in the neighborhood (data not shown) suggests that other compounds released from inflorescences, such as (*E*)-8(9)-dehydro-4(5)-dihydrotheaspirone, the fourth most abundant volatile in the floral scent, also elicits behavioral responses in beetles, or that beetles preferred the natural inflorescences because they emitted higher amounts of scent. No data have been gathered on whether thermogenesis in the inflorescences of *P. selloum* contributes to the signal for the beetles. Methoxylated aromatics are important signals (e.g., pheromones) also in other more basal scarabs, suggesting that plants emitting methoxylated aromatics and pollinated by dynastid beetles exploit preexisting preferences of the beetles for attracting this group of insects (Schiestl and Dötterl, 2012).

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