Seed odor mediates an obligate ant–plant mutualism in Amazonian rainforests

Elsa Youngsteadt*, Satoshi Nojima*, Christopher Häberlein†, Stefan Schulz‡, and Coby Schall*

*Department of Entomology and W.M. Keck Center for Behavioral Biology, North Carolina State University, Box 7613, Raleigh, NC 27695; and †Institut für Organische Chemie, Technische Universität Braunschweig, Hagenring 30, 38106 Braunschweig, Germany

Seed dispersal mutualisms are essential for the survival of diverse plant species and communities worldwide. Among invertebrates, only ants have a major role in seed dispersal, and thousands of plant species produce seeds specialized for ant dispersal in “diffuse” multispecies interactions. An outstanding but poorly understood ant–seed mutualism occurs in the Amazonian rainforest, where arboreal ants collect seeds of several epiphyte species and cultivate them in nutrient-rich nests, forming abundant and conspicuous hanging gardens known as ant-gardens (AGs). AG ants and plants are dominant members of lowland Amazonian ecosystems, and their interaction is both specific and obligate, but the means by which ants locate, recognize, and accept their mutualist seeds while rejecting other seeds is unknown. Here we address the chemical and behavioral basis of the AG interaction. We show that workers of the AG ant Camponotus femoratus are attracted to odorants emanating from seeds of the AG plant Peperomia macrostachya, and that chemical cues also elicit seed-carrying behavior. We identify five compounds from P. macrostachya seeds that, as a blend, attract C. femoratus workers. This report of attractive odorants from ant-dispersed seeds illustrates the intimacy and complexity of the AG mutualism and begins to illuminate the chemical basis of this important and enigmatic interaction.

seed dispersal | ant-garden | myrmecochory | Camponotus femoratus | Peperomia macrostachya

Seed dispersal mutualisms play an essential role in community regeneration and species survival (1–3). Myrmecochory, or seed dispersal by ants, occurs in some 3,000 plant species in over 80 families worldwide, and it is generally a diffuse multispecies interaction mediated by seed-borne nutritional rewards called elaiosomes that are rich in proteins and lipids (4). Ants carry these seeds to their nests, consume the elaiosomes, and abandon the seeds with enhanced prospects for survival and germination (4). Behavioral assays and chemical analyses indicate that ant preference for elaiosomes is mediated by characteristic nonvolatile lipids, especially 1,2-diolein, that are more typical of insect prey than of seeds (5, 6). Myrmecochory is best described in temperate mesic forests and fire-dominated ecosystems, where it can be vital to community organization (1, 4).

Tropical ant–seed interactions, on the other hand, are poorly understood, despite the fact that ants are the most common animals in tropical moist forests (7, 8), where they play important roles in seed dispersal and viability (9–11). In the tropics, ant-dispersed seeds may lack discrete nutritional rewards, or be collected independently of them (11–13). Such seeds are best known from the Neotropical ant-gardens (AGs), an ant–plant mutualism that occurs throughout lowland Amazonia. At least two ant species are obligate gardeners that retrieve seeds of AG epiphytes (but not other seeds), embed them in arboreal carton nests, and depend on the resulting plants for nest integrity (Fig. 1) (11–13). Ten epiphyte species in seven families are obligate AG inhabitants and benefit from seed dispersal, nutrients, and defense provided by the ants (13–15). Where they occur, AG ants can be the most abundant arboreal arthropods, their territories occupying nearly 40% of forest area and their nests providing the single most important substrate for vascular epiphytes (7, 13, 16). Despite its important role in the structure of Amazonian ecosystems, the behavioral basis of this mutualism is unknown, as are the specific cues that guide ants to retrieve certain seeds while ignoring others.

Previous observations suggested that nonnutritive chemical cues mediate ant recognition of AG seeds. AG ants do not consume the seeds themselves, but appear to use them as construction material in the nest walls (ref 13 and E.Y., unpublished observation). Although some AG seeds have elaiosomes or adhering fruit pulp that could act as nutritional rewards, ant preference for seeds does not reflect the value of these rewards, and seeds are still retrieved after rewards are removed, either by hand or by passage through a vertebrate digestive system (12, 13). This observation is in contrast to typical myrmecochory, which is absolutely dependent on the elaiosome (4). In a search for chemical cues from AG seeds, essential oils of 10 AG seed species were found to comprise blends of related phenolic volatile compounds, including methyl 2-hydroxy-6-methylbenzoate [methyl 6-methylsalicylate (6-MMS)] in nine species (17). Nonetheless, behavioral activity of the extracted oils was not tested, and behavioral assays with synthetic compounds identified from the oils were ambiguous (18).

Here, to test the hypothesis that chemical cues mediate the interaction between AG ants and seeds, we used behavioral assays with the ant Camponotus femoratus Fabricius and the plant Peperomia macrostachya (Vahl). These are the dominant AG species in southeast Peru, and they occupy more than 90% of AGs at the study site (ref. 13 and E.Y., unpublished observation). In a seed-carrying behavioral assay, we applied organic solvent extracts of P. macrostachya to other seeds that ants typically ignore (Piper laevigatum Kunth) and presented these test seeds, paired with solvent-treated controls, to foraging C. femoratus. To isolate the role of seed odor from contact chemical cues, we used a spatially controlled two-choice olfactometer assay. Finally, we used a behavior- and physiology-guided chemical analysis to pinpoint candidate compounds, and we tested their behavioral activity in the olfactometer and seed-carrying assays.

Results

Hexane extracts of P. macrostachya seeds elicited seed-carrying behavior in the AG ant C. femoratus. During a total of 54 20-min trials with five ant colonies, ants retrieved 83% of Piper laevigatum seeds that had been treated with 1 seed equivalent of P. macrostachya extract, but retrieved only 6% of control seeds treated only with hexane (Fisher’s exact test, P < 0.001). In

Author contributions: E.Y. and C.S. designed research; E.Y., S.N., C.H., and S.S. performed research; E.Y. analyzed data; and E.Y. and C.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

To whom correspondence should be addressed. E-mail: coby.schal@ncsu.edu.

© 2008 by The National Academy of Sciences of the USA

www.pnas.org/cgi/doi/10.1073/pnas.0708643105

PNAS | March 25, 2008 | vol. 105 | no. 12 | 4571–4575
separate trials, retrieval rate increased with the dose of AG seed extract applied (Fig. 2).

In the seed-carrying assay, ants could have used both olfactory and contact chemical cues to find and accept seeds. The two-choice olfactometer assay (Fig. 3a) tested the attractiveness of seed odor alone. In this assay, control (grass) seeds were neither attractive nor repellant, but *C. femoratus* workers chose the aroma of *P. macrostachya* seeds significantly more often than that of control seeds (Fig. 3b). In a second experiment, filter paper treated with *P. macrostachya* extract was more attractive than paper treated with solvent only (Fig. 3b). During these experiments, glass wool barriers prevented ants from touching odorant sources, so ants responded to volatile compounds alone. Finally, we removed the glass wool barriers, and ants entered the sample chambers and retrieved AG seeds. After ants began to carry seeds out of the olfactometer, subsequent ants showed enhanced preference for the AG arm of the olfactometer (Fig. 3b; mean percentage of choices for AG seeds ± SEM = 59.1 ± 2.08% when only odor was available and 71.1 ± 2.88% when contact was also allowed; *t* test, *P* < 0.01). Thus olfactory preference for seeds can be reinforced after direct contact.

To characterize chemical cues in the complex *P. macrostachya* extract, we fractionated the behaviorally active crude extract by using column chromatography and tested each fraction in the seed-carrying assay. Some behavioral activity was retained in each fraction, but only one fraction (5% ethyl acetate in hexane) was as active as the crude extract (ants retrieved both in 12 of 15 trials with three ant colonies, while ignoring all solvent-treated controls).

Compounds involved in olfactory attraction are likely to be physiologically perceived by *C. femoratus* antennae. To identify candidate compounds, we analyzed the active fraction by using gas chromatography–electroantennographic detection (GC-EAD) (19). Eight peaks in the 5% ethyl acetate fraction elicited a consistent response from *Camponotus* antennae over 19 trials, including three sample concentrations processed on polar and nonpolar columns (Fig. 4). The polarity of the antenna response...
These were detected in the active fraction at concentrations of anisate, methyl 3,5-dimethoxybenzoate, and geranyl linalool. 

Columns at doses as low as 0.04 seed equivalent. The active compounds are as highlighted GC peaks were active in 19 analyses on polar and nonpolar extract, separated on a nonpolar column. FID, flame ionization detector. Youngsteadt et al. 

Authentic standards: 3,5-dimethoxytoluene, 6-MMS, methyl 3,5-dimethoxybenzoate; tetradecadienal; unknown sesquiterpene alcohol; (2E,4Z)-2,4-tetradecadienal; (2E,4E)-2,4-tetradecadienal; coeluting compounds whose activity could not be consistently correlated to a specific compound in analyses on different columns; geranyl linalool.

Eight compounds elicited consistent electrophysiological responses from antennal neurons—remains to be elucidated. Eight compounds elicited consistent electrophysiological responses in ant antennae. Five of these compounds were identified by their mass spectra and coionization of authentic standards: 3,5-dimethoxytoluene, 6-MMS, methyl o-anisate, methyl 3,5-dimethoxybenzoate, and geranyl linalool. These were detected in the active fraction at concentrations of 45 ± 6.7, 7 ± 1.4, 25 ± 3.9, 10 ± 4.0, and 221 ± 55.8 ng per seed (mean ± SD, based on three analyses), respectively. Ratios and absolute amounts of these odorants varied greatly, but within an order of magnitude, among extracts. The mass spectrum of compound 5 suggests a sesquiterpene alcohol but differs substantially from published spectra; identification awaits isolation, further analysis, and synthesis. Compounds 6 and 7 both have a molecular weight of 208 and mass spectra (base peak at m/z 81) characteristic of 2,4-dienals. They were identified as (2E,4E)-2,4-tetradecadienal and (2E,4Z)-2,4-tetradecadienal by comparison with synthetic materials, which were available only after the conclusion of behavioral trials.

To determine whether the five identified compounds that elicited electrophysiological responses were also behaviorally relevant to C. femoratus workers, we combined them in proportions mimicking the active fraction and tested them by the olfactometer assay. C. femoratus workers preferred the five-component blend over solvent-treated control papers, and over papers treated with pure geranyl linalool, the most abundant component in the blend (Fig. 5). Ant response did not differ between the two blend concentrations, nor between the blends and the crude extract (mean percentage of choices for blend ± SEM = 58.3 ± 2.89% for 1× blend, 61.7 ± 2.83% for 10× blend, 66.0 ± 3.89% for extract; ANOVA, P = 0.27).

Although attractive in the olfactometer, the same five-component blend did not elicit retrieval in the seed-carrying assay. During a total of 20 20-min trials with four ant colonies, ants retrieved no P. laevigatum seeds that had been treated with 1 seed equivalent of the blend, and only 15% of those treated with 10 seed equivalents of the blend. In these trials, a single colony was responsible for all retrieval, including three treated seeds and one solvent blank; ants from the other three colonies investigated seeds but retrieved none. Seed removal was independent of treatment (Freeman–Halton extension of Fisher’s exact test, P = 0.31).

Discussion

Our results show that chemical cues alone, rather than visual or tactile characteristics of AG seeds, are sufficient to attract AG ants and elicit the seed-collecting behavior that underlies the complex AG mutualism (Fig. 2). We also identify a blend of five volatile compounds from P. macrostachya seeds that attract the AG ant C. femoratus in the olfactometer but do not elicit seed-carrying behavior. To our knowledge, this is the first identification and behavioral confirmation of attractive odorants from ant-dispersed seeds.

Although AG seed chemistry has been examined previously, candidate compounds were identified based upon co-occurrence in multiple seed species, rather than occurrence in behaviorally active extracts (17). When those compounds were tested in a seed-carrying assay, results were highly variable and ambiguous (18). The consistent positive response of C. femoratus to P. macrostachya extract in two assays is, therefore, the strongest available evidence that chemical cues mediate the AG interaction. By focusing on compounds in behaviorally active fractions, and using two behavioral assays, we quantified specific aspects of ant behavior and demonstrated attraction that would have been ambiguous or undetectable in a seed-carrying assay alone.

It is noteworthy that, despite the differences in approach, both our study and ref. 18 identified 6-MMS as a potentially important seed recognition cue. Also outstanding for its unusual pattern of occurrence in nature, this compound has never been reported from plants other than AG seeds. It is, however, a common feature of P. macrostachya.
metabolite of fungi and insects, particularly as a semiochemical of ants (21–24). It is a trail pheromone in two myrmicine species, elicits alarm or stinging behavior in two ponerine species, and is a component of queen sex pheromone in the formicine Polyrhachis rufescens. It is widespread in the mandibular glands of male Camponotus, and its release coordinates mating flights in Camponotus herculeanus. In C. femoratus, however, 6-MMS occurs only as a minor component of male mandibular glands. C. femoratus workers may thus be predisposed to respond to this component (17, 24).

The other four components of the attractive blend are common plant secondary metabolites, frequently reported from essential oils and floral scents. There is circumstantial evidence that, as components of floral scents, 3,5-dimethoxytoluene and methyl o-anisate guide foraging behavior in flower-feeding insects (25–27), whereas methyl o-anisate and methyl 3,5-dimethoxybenzoate are reported as antifeedants in pine weevil (28).

Geranyl linalool, the most abundant component of the active blend, is frequently encountered in both plants and insects. We have found this chemical in every extract of nine species of AG seeds that we have analyzed, but not in five non-AG congeners (data not shown). It is produced as a marking signal in some bumblebees, is collected by orchid bees, and is a defensive secretion in Reticulitermes termite soldiers (29–31). In light of its defensive function, Lemaire et al. (32) investigated the toxicity of geranyl linalool to several species of European ants, which varied widely in their ability to withstand treatment. LD50 values ranged from 1.8 to 20,200 ng per individual, and Lemaire et al. hypothesized that geranyl linalool interferes with neurotransmitters of susceptible insects. The amount on a single P. macrostachya seed, 〜220 mg, might therefore be deterrent to some species even though it is accepted by C. femoratus.

The active blend thus includes semiochemicals (i.e., insect pheromones and floral scents) that also have toxic and/or repellent properties for some insects. These characteristics are reasonable for a blend emitted by seeds that are dispersed by just a few ant species. In a study that will be detailed elsewhere, we compared the community of ants attracted to general food baits and AG seed baits along forest transects. We collected a total of 〜70 ant species, but of these, only 3 species other than C. femoratus were observed to carry P. macrostachya seeds, and 85% of the observed dispersal was attributable to C. femoratus. Thus, in addition to attracting mutualist ants, P. macrostachya seed compounds might also avert inappropriate dispersers and seed predators with toxic and/or repellent properties.

The result that the five-component, electrophysiologically active blend was preferred over its major component, geranyl linalool, suggests that either behavioral property is restricted to one or more of the phenolic minor components, or that the complete blend is necessary for attraction. Unfortunately, we were unable to conduct olfactometer assays to test all five compounds individually and in various combinations, so we cannot say how the components interact to elicit ant response. Future studies should examine the activity of each component individually and identify minimal and optimal blends.

Ant response to the blend we tested did not differ significantly from ant response to crude extract. The slightly lower activity of the blend at the natural concentration suggests a possible role for the unknowns in blend activity. Isolation, structural elucidation, and synthesis of these components is not yet accomplished. Stereochemistry of geranyl linalool should also be further investigated in optimizing the blend. In the present study it was tested as a racemic mixture; chirality of the naturally occurring compound is unknown, and previous attempts to separate R and S enantiomers of geranyl linalool from natural samples were unsuccessful (33). Nevertheless, it is noteworthy that the tested blend of just five components is as attractive, or nearly as attractive, as the crude seed extract, which contains more than 150 compounds. A blend including additional or purified components would be expected to elicit an only slightly stronger ant response.

In contrast to the crude extract, however, the attractive five-component blend did not elicit seed-carrying behavior in C. femoratus. We therefore suggest that seed collecting is mediated by a series of different chemical cues. First, volatile compounds attract ants to an AG seed resource. We have identified and behaviorally confirmed components that are active in this step. Second, ants handle the seeds and detect contact chemical cues that elicit carrying behavior. We have not identified these contact cues but hypothesize that they exist, because seed extracts elicit the complete behavioral sequence whereas synthetic attractants alone do not. We also found that some chromatographic fractions of P. macrostachya extract that are preferred in the seed-carrying assay are less preferred in the olfactometer, and vice versa (data not shown). This pattern supports the interpretation that different chemicals mediate attraction and carrying. It is perhaps not surprising that we did not pinpoint contact cues by using the GC-EAD bioassay, which targets volatile compounds perceived by olfaction. Third, when one or a few ants begin to carry the seeds, response among other ants is enhanced. This response, detected in the olfactometer assay when ants were allowed to retrieve seeds and exit the olfactometer with them, is likely mediated by ant recruitment cues, but may also involve increased diffusion of volatile compounds when seeds are mobilized, and/or direct interactions between seed-carrying ants and other foragers. Confirmation of this putative behavioral sequence depends on the identification and behavioral testing of volatile unknowns and further analysis of nonvolatile seed compounds.

Nonetheless, the confirmed role of olfaction in the AG system sets it apart from other described ant–seed interactions. Elaiosomes of typical myrmecochorous seeds elicit seed carrying with one or more nonvolatile lipids, and there is evidence that olfaction does not play a role in these systems (34). The presence of an additional signaling dimension in the AG mutualism makes it similar to other insect communication systems for mate finding, host plant location, and pollination, in which volatile cues bring an insect to the general location of its target, whereas contact chemical cues are necessary for completion of the behavioral sequence (35–38). The complexity of the AG communication system relative to other ant–seed mutualisms may reflect its increased specificity and reciprocally obligate nature.

Materials and Methods

Study Area. All field work was conducted from October to December in 2004, 2005, and 2006, in Madre de Dios, Perú, at the Centro de Investigación y Capacitación Río Los Amigos (12°34’S, 70°6’W) in moist forest on both terra firme and seasonally inundated terrain.

Seed-Carrying Assay. Hexane extracts of P. macrostachya seeds (see Chemical Analyses) were applied to other seeds that ants typically ignore (Piper laveigatum). Extract-treated seeds were paired with solvent-treated controls and presented within 5 cm of foraging trails of the AG ant C. femoratus. Each pair of seeds was observed for 20 min and scored as carried or not carried. The synthetic blend tested included 3,5-dimethoxytoluene, 6-MMS, methyl o-anisate, methyl 3,5-dimethoxybenzoate, and (E,E)-geranyl linalool at a weight ratio of 2:1:2:1:10 for a total of 160 ng material per seed equivalent. The blend was presented at 1 and 10 seed equivalents per test seed. Tests were conducted as described for extract-treated seeds except that three, not two, seeds were presented in each trial: one of each concentration and one blank.

Olfactometer Assay. The olfactometer was a Y-shaped glass tube with a 2.6-cm diameter and each of the three arms 10 cm in length. Air flow of 750 ml/min was generated with an air pump (MiDan) that directed ambient air through an air filter, a coiled Teflon tube, a balloon (to dampen air vibrations), and a charcoal filter before entering a Y-shaped Teflon tube that split air flow evenly between the two arms of the olfactometer. Odorant sources (60 P. macrostachya seeds or the chemical equivalent, to mimic a typical AG seed
resource, that is, a fallen P. macrostachya seed spike) were placed in separate Teflon sample tubes inserted in the air stream and separated from the Y-tube proper by loose glass wool plugs that prevented ants from contacting the samples. The apparatus was placed near foraging trails of C. femoratus such that ants entered one arm of the olfactometer and then chose between two odorant sources by turning right or left. A single trial was a pool of 30 decisions by different ants; a “decision” was counted when an ant reached the end of the basal arm and proceeded at least 5 cm down the right or left arm of the olfactometer. Trials were conducted in pairs in which the orientation of the odorant sources was reversed to control for spatial effects. The Y-tube was washed or replaced with a clean tube after each trial. The synthetic blend was the same as that described for the seed-carrying assay above. When geranyl linalool alone was tested against the blend, it was presented at the same concentration in which it occurred in the blend.

Chemical Analyses. Behaviorally active extracts were obtained by collecting mature seeds directly from P. macrostachya plants (the most abundant and fecund of the AG epiphytes at the study site) and soaking them in groups of 100 seeds per 3 ml of GC-grade n-hexane for 30 min. Crude extract (50 seed equivalents) was concentrated under a gentle stream of air, applied to 200 mg of silica gel, and eluted with 3 ml each of the following solvents: hexane; 5%, 25%, and 60% ethyl acetate in hexane; ethyl acetate; and methanol. Chromatographic procedures conducted at the field site in Peru were constrained by availability of appropriate gases (hence air instead of an inert gas) and solvents (high-purity ether and dichloromethane could not be imported or obtained locally).

The 5% ethyl acetate fraction was further analyzed on an HP 5890 gas chromatograph equipped with flame-ionization and electroantennographic detectors (FID and EAD) interfaced with HP ChemStation software (A.09.03). Antennae of C. femoratus or Camponotus pennsylvaniaicus were mounted in Camponotus saline (39) in capillary gold electrodes interfaced with a custom-made DC amplifier (40). Analyses were conducted on two GC columns at three sample concentrations (1/2, 1/5, or 1/25 seed equivalents per analysis), for a total of 19 analyses. For the nonpolar column (DB-5, 30 m x 0.25 μm x 0.25 μm) the oven temperature was programmed from 80°C (2-min hold) to 300°C (20-min hold) at 10°C/min – 1. The splitless inlet and FID were held at 320°C. For the polar column (EC-WAX, 30 m x 0.25 μm x 0.25 μm) the oven temperature was programmed from 80°C (1-min hold) to 260°C (20-min hold) at 10°C/min – 1. The inlet and FID were held at 280°C. The carrier gas (He) was set at a head pressure of 135 kPa and flow rate of 2.0 ml/min – 1.

Chemical structures of electrophysiologically active compounds were elucidated by using an Agilent 6890 gas chromatograph coupled to an Agilent 5975 mass selective detector interfaced with Agilent Productivity ChemStation. Analyses were run in both EI (electron ionization) and CI (chemical ionization) modes, using comparable columns and programs as used for GC-EAD. The carrier gas was He at a flow of 1.2 ml min – 1. Chemical profiles were matched to the GC-EAD/FID runs, and physiologically active components were identified by comparison with reference spectra in the Wiley 275 mass spectra database and by coinjection of authentic standards on both polar and nonpolar columns.

Chemicals. 6-MMS (methyl 2-hydroxy-6-methylenzoate, 92%) was synthesized and purified as previously reported (17). 3,5-Dimethoxytoluene (1,3,5-dimethoxy-5-methylenzoene, 99%), methyl 3,5-dimethoxynitrobenzoate (99%), and methyl o-anisate (methyl 2-methoxybenzoate, 99%) were obtained from Aldrich. Geranyl linalool (66,105-3,7,11,15-tetramethylenyl-1,6,10,14-hexadeca-3,6,9,12-tetraen-3-ol, 63%) was purified from Acros technical grade material by using silver nitrate chromatography and later obtained at 95% from Fluka.

ACKNOWLEDGMENTS. We thank Silvia Castro, Scott Chilton, Dan Comins, Dinah Davidson, Patricia Guerra, Phil Harris, Rick Santangelo, and Erick Yabar for their assistance. This work was supported by a National Science Foundation predoctoral fellowship, a U.S. Department of Education Graduate Assistance in Areas of National Need fellowship, an Amazon Conservation Association graduate research grant, a Sigma Xi Grant in Aid of Research, a North Carolina Entomological Society travel grant (E.Y.), the North Carolina State University Office of International Affairs, and the Blanton J. Whitmire endowment (C.S.). Permission to work in the Los Amigos conservation concession was granted by the Instituto Nacional de Recursos Naturales de Peru.