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Odor-Mediated Push-Pull Pollination in Cycads

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Odor-Mediated Push-pull Pollination in Cycads

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Supporting Online Materials

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Materials and Methods

Fig. S1

Movie S1

Y- tube olfactometer behavioral tests with cycad cone sporophylls

These sporophyll tests were performed to investigate whether cone volatiles alone (without other cues from cones) could affect thrips behavior. Cones with thrips were excised from male *Macrozamia lucida* plants near Brisbane, Queensland. Excised cones and sporophylls continue their typical thermogenic behavior (S1, S2). Test cones were kept at 23°C:15°C (day (0630-2000hr): night) resulting in thermogenic temperature peaks at ~1100-1300 hr. Thrips were aspirated into vials and tested within two hours. Olfactometer tests were conducted in a darkened cage (24 ±0.8°C), with a fluorescent light at the end of each Y-tube arm (light levels, 10 µmol $m^{-2}s^{-1}$), and completed within 10 min.

Each day's olfactometer experiments with sporophylls involved placing 9g (avg.) of sporophylls plus some cone axis from one male cone into a glass vessel connected to one Y-tube arm. An empty control vessel was attached to the other arm. Charcoal-filtered room air was pulled through both vessels by withdrawing air (60 ml min^{-1}) from the Y-tube base. Although the extracted sporophylls heated slightly (with the same general timing as their source cone), air flow rates were sufficient to keep the air temperatures entering both arms within 0.3°C. Calibration tests indicated no left-right bias, but to ensure further against bias, the Y-tube treatment and control arm positions were alternated frequently. Clean Y-tubes and vessels were introduced for different chemical treatments and hourly during sporophyll tests. Each replicate involved testing a group of 10-15 thrips of one sex, and tests were conducted between ~0700 and 1800 hrs to include the complete cone thermogenic cycle (pre-, during, and post-thermogenesis).

To analyze the data, we first examined the percent thrips response to sporophyll volatiles over two hour periods, from early, 0700-0900, to mid-day, 1100-1300 to late, 1500-1700, which showed changes from neutral /slight attraction (62%, ± 0.04 SE, n=13), to repellency (29%, ± 0.04 SE, n=42), to attraction (79%, ± 0.02 SE, n=60), respectively for the four cones tested over eight days. To test the time of day and cone effects more specifically, we used a general linear model (GLM) regressing the % response to volatiles on time of day with cones as

a factor. When all cone days were included in the model, there was a significant cone day ($F_{7,194}$= 114, P<0.0001) and time of day effect (P<0.0001 for linear $F_{1,194}$= 43.5 and quadratic $F_{1,194}$ =56.5 terms of the model), as well as significant interactions between cone days and the time of day coefficients describing the linear and quadratic terms of the equation (P<0.0001, $F_{7,194}$ = 108 and 91, respectively). (These cone day effects result from the inclusion of very young and old cones in the analysis because thrips responses to very young, "green", pre-dehiscent cones, for example, are predominantly neutral to attractive.) The temporal dynamics of the complete attraction, repulsion and attraction phases are most clearly illustrated by mid-pollination stage (~10-90% dehiscence) cones (Fig. 1A, where each point represents a single experiment). A GLM (with all mid- stage cones) showed a significant time of day effect (P<0.001 for both linear, $F_{1, 119}$ = 101, and quadratic, $F_{1,119}$ = 120, terms), but there was no significant cone effect ($F_{3,119}$, P= 0.51) and no significant interactions between cone and time of day (P>0.45 for interactions with both linear $F_{3,119}$=0.75 and $F_{3,119}$= 0.89 terms) (model, % thrips attracted to sporophylls = 4.1 – 15.9 x (time of day) + 15.8 x (time of day)^2, with r^2= 0.62**).**

Electrophysiological tests

To identify the specific cone volatile components that potentially cause the repulsion and attraction, we tested for *Cycadothrips'* electrophysiological responses to cone volatiles with gas-chromatography electroantennographic detection (GC-EAD). For each GC-EAD experiment, a 1µL sample of cone volatile or standard chemical was injected, splitless mode, into the HP 5890 GC (HP Ultra-2 column, 30m x 0.25mm x 0.25 µm, programmed from 40^o-260^{o}C at 10^{o}C /min). Further details of the linkage between the GC and EAG are found in S3. For each experiment, the body of the thrips was secured in Blu-Tack®, the reference electrode (glass/saline) was placed over one antennal tip and the ground electrode pierced the thorax of the intact thrips. Each thrips preparation was tested for antennal activity with hexanol, to which *Cycadothrips* responded, both before and after the GC-EAD. A 400 µL headspace sample from a vial of hexanol was extracted with a gas-tight syringe, and was then manually injected into the air stream 10 cm away from the delivery point to the antenna.

Six cone volatile samples from both male and female cones, collected by headspace techniques (S4), were tested on at least one thrips of each sex. With these cone samples, male and female thrips showed responses only to β-myrcene (Fig. 1B). Of other cone volatile components, the (E)-β-ocimene had three characteristics that motivated us to test it further. It is a significant fraction of total emissions (after accounting for β-myrcene), absolute levels dramatically change during thermogenesis in *M. lucida* (see section on chemical standards) and in other species (S4), and it is found consistently in other *Cycadothrips*-pollinated *Macrozamia* species (S4). Thus, we separately tested the chemical standard of ocimenes (see section on chemical standards) on one thrips of each sex. Thrips responded to two of the three isomers in the standard, (E)-β-ocimene and (allo-)ocimene (Fig. 1 C).

The above GC-EAD responses to β-myrcene and ocimenes were confirmed by electroantennogram (EAG) tests with standard chemicals. The headspace volatiles (400 µL) of each standard were extracted with a gas- tight syringe and injected as described above for the GC-EADs. Two male and female thrips were tested in this manner, and they responded to the ocimene mixture. For β-myrcene, two male and female thrips were tested also. In addition, one female thrips was tested over a range of headspace volatiles from 400 µL to 5µL, and the thrips responded to all levels,. but linalool, a monoterpene found in *Macrozamia* species not pollinated

by thrips, did not produce a response. Additional EAG experiments showed that thrips do not respond to CO_2 (400 µL of headspace from a flask filled with CO_2).

Y-tube olfactometer tests with standard chemicals

Because thrips responded physiologically to β-myrcene and the two ocimene isomers, chemical standards of these compounds were tested for their effects on thrips behavior. The standards used were β-myrcene (Sigma, 90% purity) and ocimenes - a blend of three ocimene isomers derived from a natural source (S3) [(Z)-β, (E)-β- and (allo-) isomers] - which is 85% (E)-β-ocimene. The identities of these ocimene isomers were confirmed by GC retention times and GC-mass spectral (GC-MS) methods. The (allo-)ocimene isomer was previously listed as "unknown" from *M. lucida* cone volatiles at GC retention time at 11.35 min (see Table 1 in S4) but has now been confirmed by GC-MS.

To test each standard, we placed a measured amount into three drops of castor oil in a weigh boat for insertion into the Y-tube arm. Groups of 10-15 thrips of each sex were tested in a single experiment, and at least three such groups were tested per standard chemical concentration. The control arm vessel had three drops of castor oil. (No significant difference was observed between castor oil and filtered air controls; paired *t*-test with nine olfactometer experiments, t_8=0.28, P=0.79.) Thrips were attracted to the ocimene standard at levels of 2, 6 and 10 µL of standard (Fig. S1, averages of 74 (±0.05 SE), 63 (±0.05) and 58 (±0.05) attraction, respectively) with no significant gender effect or interaction between gender and the level of ocimene (GLM model results, sex $F_{1,24}$= 0.02, P= 0.8, level of ocimene, $F_{2,21}$, P=0.13; sex X level, $F_{2,24}$ = 3.12, P=0.06). In a GLM model examining the effects of sex and level of β-myrcene tested on the % of thrips responding to the β-myrcene standard, there was no significant sex effect ($F_{1,52}$=0.12, P=0.73) or sex X level interaction ($F_{8,52}$= 1.01, P=0.45). However there was a significant effect of β-myrcene level ($F_{8,52}$= 26.9, P<0.0001). The 0.01 µL and 10 µL levels were not significantly different from 50% attraction (averages of 51 (6.1 SE) and 58 (4.5)%, respectively); however, levels of 0.1, 0.2, 0.5, and 1 µL were significantly greater than 50% (averages of 61 (±3 SE), 59.4 (±4 SE), 59 (±4.1 SE) and 60 (±2.9)%, respectively), and levels at 40 and 100µL were significantly less than 50% (averages of 13.6 (±4.9 SE) and 11.7 (±3.3)%, respectively) (Fig. 1, A). (Because there were no significant gender effects on the response to these standard chemicals, the results of male and female tests were combined in the sporophyll tests.)

To determine whether levels of standard chemicals used in Y-tube tests were ecologically relevant (i.e., compared with levels emitted by sporophylls), we sampled air from the base of the Y-tube for 30min during Y-tube tests of sporophylls and of β-myrcene (see headspace methods in S4). During Y-tube sporophyll tests, we sampled on several days at different time periods that included repellent, attraction and neutral periods. Levels of β-myrcene standard chemicals tested in the olfactometer experiments were within ranges of β-myrcene emitted by the sporophylls (total integrated GC areas of β-myrcene peaks of the standard at 0.1 µL to 100 µL ranged from 210 to 11, 963 pA and that of sporophylls from 267 to 15,364 pA). In both sporophyll and standard chemicals, the highest levels of β-myrcene were repellent to thrips and the lower levels were slightly attractive or neutral. Estimates of ocimenes are inferred from levels of the ocimene standard injected directly into the GC, where 1 µL produced areas of 60 pA and 18 pA for (E)-β-ocimene and (allo-)ocimene, respectively. Sporophyll samples produced from undetectable levels to 329 pA for (E)-β-ocimene and undetectable to 18 pA for (allo-)ocimene. The 2, 6 and

10 µL levels of ocimene standard used in Y-tube tests, which were mixed in castor oil, likely yielded much lower quantities than a direct injection.

Supplemental References

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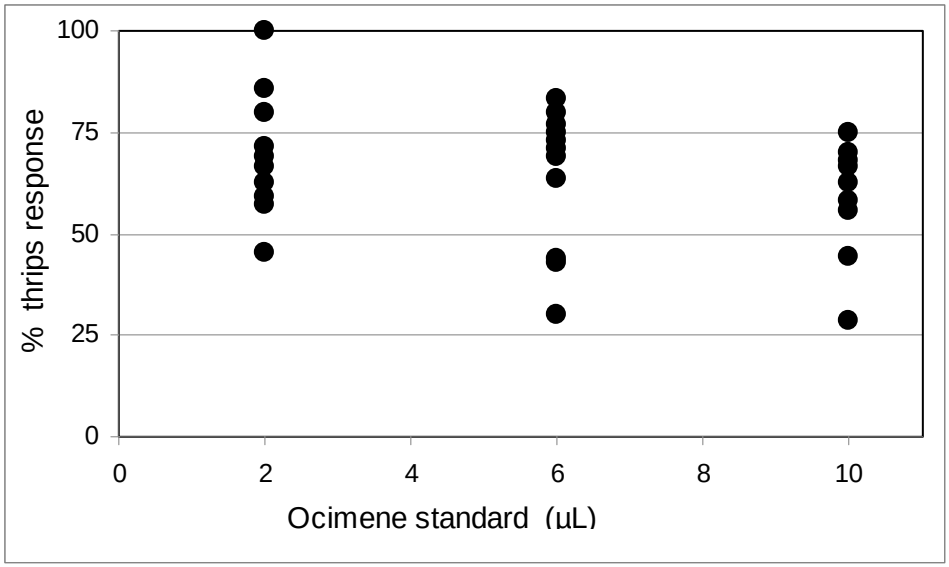

Fig. S1. Response of *Cycadothrips* to different levels of ocimenes in Y-tube olfactometer tests that gave thrips a choice between the ocimene standard in castor oil and an oil control. Each point represents one olfactometer experiment, which is one group of 10-15 thrips.