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Supplemental Information

Hawkmoth Pollinators Decrease Seed Set

of a Low-Nectar Petunia axillaris Line

through Reduced Probing Time

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Supplemental Inventory

Figure S1. Illustrates the similarity between the introgression line F25 and the parental line *P. axillaris*.

 Table S1. Gives detailed information about the statistical output of the nested ANOVA.

Supplemental Experimental Procedures

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Figure S1. Low-Nectar Line F25 and P. axillaris in Comparison

Comparison of corolla colour, diameter and tube length between low-nectar introgression line (F25) and *P. axillaris* shows that F25 and the recurrent parent are indistinguishable in all measured morphological characters. For a detailed analysis of the differences in all measured parameters, please see Table 1 in the main text.

Table 51. Statistical Output of Nested ANOV

Source	df	SS	MS	F Ratio	p Value
Plant	2	93820.91	46910.5	1.9678	0.1422
Pollination mode[plant]	2	665919.11	332959.6	13.9672	<0.0001
Residual	229	5339875.0	23839		

Nested ANOVA analysing the difference in mean number of seeds produced per capsule in the categories *P. axillaris*, F25 and F25 supplemented with 15 µl nectar after pollination by hand and the first two treatments after pollination by moth. For a graphical display of the results, see Figure 2A.

Supplemental Experimental Procedures

1. Plant Material

Petunia axillaris axillaris N (here referred to as *P. axillaris*) (Solanaceae), is a hermaphroditic selfcompatible inbred line derived from a wild accession of *P. axillaris axillaris axillaris*. It was maintained in the greenhouses of the Institute of Plant Science (University of Bern) by self-fertilization. The flowers display typical characteristics of a hawkmoth pollination syndrome [S1]: showy white corollas, long, narrow floral tubes, emission of strong fragrance at night and large amounts of dilute nectar. Hawkmoth pollination has been observed repeatedly [22, S2]. Plants were kept under greenhouse conditions (minimum 14h light, supplementary light in winter months) at 18-25°C in peat-based soil in 15 cm diameter plastic pots and fertilized weekly.

2. Breeding of Low-Nectar Introgression Line (F25)

Petunia integrifolia ssp. inflata S6 (here referred to as *P. integrifolia*) was used as pollen donor in the breeding design to establish a low-nectar line of *P. axillaris*. Flowers of *P. integrifolia* contain low nectar amounts $(1.35 \pm 0.47 \ \mu$ l) and are cross-compatible with *P. axillaris* as the seed parent. A single F₁ progeny of an initial cross between *P. axillaris* and *P. integrifolia* was backcrossed (BC) three times with *P. axillaris* as recurrent parent. Note that this scheme selects for introgression of dominant *P. integrifolia* low-nectar loci. Both parents used in our breeding design were kindly provided by Dr. Ronald Koes, Vrije Universiteit Amsterdam.

We established a "*Petunia axillaris* similarity index" (PASI) to compare all phenotypic parameters of backcrossed individuals to *P. axillaris*. We used the PASI to select for a suitable low-nectar line (F25) featuring all phenotypic characteristics of *P. axillaris* except for nectar (see section 4).

3. Selection Process (PASI)

In a first step, a BC₁ population with 130 individuals using *Petunia axillaris* as recurrent parent was grown. We selected 24 plants with nectar volumes below 7 μ l. These 24 plants were crossed with *P. axillaris*, and 25 seedlings per plant were grown, resulting in a BC₂ population of 600 plants. 43 plants fulfilled the requirements to produce less than 7 μ l, and 5 BC₂ plants with the highest PASI were selected for further breeding, resulting in a BC₃ population of 280 plants.

The PASI is calculated by the relative proportion of numeric phenotypic traits such as tube length and corolla size of BC lines to *P. axillaris.* For the presence of a strong methylbenzoate-dominated floral scent and white colour we added 1 point, for the absence (pink corolla, no detectable scent) 0 points. Thus, the lines with the highest PASI index had the closest phenotypic similarity to *P. axillaris.* This method helped to select suitable *Petunia* lines that could be used for further backcrossing.

In the third backcross population we found one line (F25) with high similarity to *P. axillaris*, except for nectar volumes (Table 1). We used vegetative propagations of this line in behavioural assays.

4. Phenotypic Measurements

Phenotypic measurements included tube length, corolla diameter, nectar volume, nectar concentration, nectar sugar, UV reflectance, fragrance emission (floral, and vegetative tissue and nectar), ovary weight, ovule and pollen count and stigma surface area.

Tube length was divided into two sub-domains [S3]: D1 described the length of the tube from the base of the gynoecium until the point where the stamina separate from the tube. D2 described the length from this point until the corolla bulge. Corolla diameter was measured from the edge of the dorsal petals to the edge of the left ventral petal. Tube length and corolla diameter were measured with a measuring calliper, with an accuracy of 0.05 cm.

Corolla UV fluorescence patterns were measured on cut flowers with a minimal time delay (maximal 5 minutes). Flowers were placed in an agarose gel documentary system (Alphalmager[™]2200, Alpha Innotech) used to visualize DNA fluorescence in agarose gels. An image was taken during exposure to UV light. Plants that were UV-reflecting could be seen as bright white spots, while UV-absorbing plants were not visible. In addition, a photograph of cut flowers was taken with a regular camera (Nikon D 200), using a UV pass filter (B+W 403 UV pass filter) and a Nikkor AF 50 mm 1.8D lens. When a UV pass filter was applied, UV reflecting lines appeared pink on the image and UV absorbing lines red. Further processing in Adobe Photoshop CS (Adobe[®]) (using blue channel) produced a black and white picture where UV reflecting lines appeared white and UV absorbent lines dark grey. Odour of

both floral and vegetative tissue was measured with a proton transfer reaction coupled with mass spectrometry (PTR-MS) online volatile organic compound (VOC) detector (Ionicon®). During this reaction, a proton from protonated water is transferred to the odour molecule ($H_3O^+ +R \rightarrow RH^+ +H_2O$). Thereafter, the ionized odour molecule (RH+) can be measured by the highly sensitive volatile detector PTR-MS. For details on the PTR-MS, see [28]. All scent components (benzaldehyde, benzyl alcohol, benzene acetaldehyde, phenylethyl alcohol, benzyl acetate, methyl salicylate, (iso-) eugenol, vanillin, benzyl benzoate, benzyl salicylate, hexadecanoic acid methyl ester) were measured simultaneously, but in none of them, the difference was statistically significant except for methylbenzoate emission which was consistently higher in F25. Methylbenzoate (MB) has been shown to elicit a positive response in *Manduca sexta* [22 (main text)].

Nectar scents and vegetative tissue (whole plants without flowers) were measured similarly. We could not determine any scents emitted by nectar or vegetative tissue.

Nectar volume measurements were conducted by cutting the floral tube underneath the gynoecium and at D1. This part of the floral tube was placed in a small centrifugation tube (0.5 ml) with holes in the tip, acting as a sieve. This tube was placed in a regular centrifugation tube (1.5 ml) and centrifuged at 7000 rpm for 20 s. The two tubes were then separated and the nectar in the 1.5 ml centrifugation tube was measured with a calibrated pipette tip (1-10 µl) using an Eppendorf pipette (0.5-10 µl). Samples for nectar composition analysis were frozen at -80°C until analysis. Nectar components were separated on a Carbopac 100 PA column using high-performance anion-exchange chromatography (HPAE) (Dionex®). Nectar samples were diluted 1:1000. Eluents with a high pH (sodium hydroxide) were used to charge nectar carbohydrates (sucrose, glucose and fructose) and separated on the column. Peaks were identified by co-elution of standard glucose, fructose and sucrose solutions. The peak area was determined using Chromeleon software. Nectar sugar concentrations were measured using a pocket refractometer PAL-3 (0-93% Brix; ATAGO Co, Ltd[®]). For *P. integrifola*, 25 nectar samples were pooled and diluted 1:25. For low-nectar lines and P.axillaris axillaris N, 10 nectar samples were pooled and measured directly. This procedure was repeated three times. The values obtained by refractometer measurements (in % Brix) correspond to the total concentration of all soluble solids in the sample. Previous HPAE analysis had shown, that other than glucose, fructose and sucrose, no other sugars or amino acids could be detected in the samples (data not shown). The values in the table are therefore given as %w/v (g sugar/100 ml).

Ovules were counted on 6 opened ovaries that had been photographed with a scanning electron microscope (Hitachi S-3500N). Ovary weight was determined by a Kern ABT 120-5DM scale.

Anther dehiscence took place on the same day after floral opening in both F25 and *P. axillaris*. For the pollen count, all 5 anthers of one flower were harvested before dehiscence, and kept in an Eppendorf centrifuge vial until opening. The anthers were suspended in 50 µl distilled water. 5 µl of the pollen solution was applied on a microscope slide and photographed under a Keyence microscope. Pollen grains were counted on the photographs and multiplied by 10 to calculate the total number of pollen grains per flower.

Stigma surface was measured by photographing 10 stigmas of both F25 and *P. axillaris.* The surface was measured with ImageJ.

5. Pollinator Species Used in Behavioral Assays: Manduca sexta

Manduca sexta, the tobacco hornworm moth, is a specialized pollinator of solanaceous plants including *Petunia axillaris* [S3]. However, *P. axillaris* does not serve as a host plant for hawkmoth oviposition. For behavioral experiments, pupae of *M. sexta* were obtained from NCSU Insectary (Raleigh), USA and the MPI Jena (Germany). Animals were reared under standard laboratory conditions [S4]. Pupae were kept in BugDorm-3[®] insect tents at 24°C, with 60% air humidity and a 16/8 day/night cycle. Adult moths emerged 1-5 days before the trials, and were used unmated for experiments.

6. Details on the Setup of the Behavioral Experiments

The average number of flowers on the plants varied naturally. Flower number was not manipulated to avoid potential confounding effects of damaging the plant but in every trial, both plants had the same number of flowers (1-18 flowers per plant; average 3). The plants were used once per evening and after visitation all flowers were removed. Behavioral experiments with low-nectar lines took place from April 2007 to July 2007, experiments using F25 supplemented with nectar from July 2010 to July 2011.

Experiments were conducted in a flight arena (144cm height, 248x368 cm surface area), in the middle of a Petunia greenhouse. As a consequence, the flight arena was saturated with scent and a discrimination of two plants differing in scent might not have been possible, but remains to be tested. The arena had three different entrance sites for hawkmoth release, which were selected in a counterbalanced fashion to exclude potential side bias. We also counterbalanced plant positions within the arena. Prior to testing, hawkmoths were kept isolated in a bug-dorm[®] in the greenhouse. One pollination flight was done per moth. Moths were removed immediately after they had visited both plants, or after 300 s, if no visitation occurred. First choice was noted as the plant that hawkmoths first fed on. Probing duration was recorded to the nearest second from the moment the hawkmoth inserted the proboscis until its retraction. We only recorded the first probing event per flower, as flowers that had been visited were assumed to be empty. For each plant, we noted the number of flowers each hawkmoth probed. Revisits of flowers were not considered. If none of the plants were visited, the trial was annotated as no choice and omitted from statistical analysis. If only one of the two plants was visited, we included the data for "first choice" but exclude it for "number of visited flowers" and "probing duration".

All moth behaviors were recorded with a dictaphone. The experiments started at dusk (in summer 2100, in winter 1700 hrs). A 40 Watt incandescent lamp outside the arena was used to illuminate, however light intensity inside the arena was 0 μ mol m²s⁻¹ measured by a quantum light sensor model 36681 (Spectrum® Technologies, Inc). Therefore, the light had no marked effect on pollinator foraging behavior. Experiments terminated by 2300 hrs at the latest.

7. Seed Set Measurements

Seed set experiments were conducted from August to December 2007 and repeated from May to August 2011. For pollinator induced seed set, two plants with single flowers were placed in the flight arena. Naïve moths had to visit first a *P. axillaris* plant to collect pollen and then visit either another *P. axillaris*, an F25 or an F25 supplemented with 15 µl nectar (pollen recipient). Pollen recipients were emasculated prior to experiments. Pollen transfer therefore could only take place from *P. axillaris* to the pollen receiver plant. If a hawkmoth did not perform the required behaviors in the right order, the plants were excluded from the analysis. Each moth was used once. For hand pollination, the pollen receiver was emasculated and pollinated by the experimenter with *P. axillaris* pollen. Stigmas were loaded with pollen by smearing 2-3 anthers on the stigma. After pollination, flowers were bagged and labelled until seed maturation. Plants were allowed to ripen one seed capsule at a time; other flowers of the plant were not used for experiments simultaneously. After harvesting the seed capsule, plants were used again.

To measure seed set, 20 seeds from each capsule were weighed to calculate mean seed mass. The total seed mass was weighed and used to estimate the number of seeds per capsule. Additionally, we counted all seeds by hand. Analyzing the difference in number of seeds per capsule counted and weighed, we found no significant differences between the two types of seed measurement in *P. axillaris* (t-test, N=27, t=-1.417, p=0.168).

8. Seed Set and Probing Time Correlation

Data were collected in June/July 2011. Recipient emasculated flowers (*P. axillaris*, F25, or F25 supplemented with nectar) that have been probed by moths to deliver the pollen picked up previously on a donor *P. axillaris*, were labelled with the exact probing time. Seeds were counted after maturation. The probing time data set was then correlated to the number of seeds produced by this particular flower.

9. Statistical Analysis

Normality was tested using Shapiro-Wilk analysis. Values are given as median with interquartiles for data sets violating parametric assumptions. Mean feeding time per flower per moth and visitation rate (number of flowers per moth) were analyzed with Wilcoxon signed rank test for paired data sets. First approach was analyzed with χ^2 -test ("Goodness of Fit"; Vassar Stats). Seed set (=number of seeds per capsule) of the two plants *P. axillaris* and F25, and their pollination method (hand and moth-pollinated) was compared using analysis of variance (nested ANOVA) to account for interaction effects (plant and pollination method nested in line). No transformation of data was necessary as residuals were normally distributed and the variances homogenous. In a general linear model (GLM) we fitted the effect of the year on seed set before or after adding the other variables (plant/pollination mode) but could not find any effect of the year. Therefore we excluded it in further analyses. Seed set was the fixed factor. For the evaluation of the relation between seed set and probing time we calculated a linear regression.

For all non-parametrical statistical analysis and the linear regression, SPSS 15.0 for Windows (SPSS inc.) was used. For the nested ANOVA, the statistical package jmp 8 (JMP[®]) was used. We performed a post hoc Tukey HSD test to assess differences in seed set for each of the five treatments.

Supplemental References

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