



Pollen quantity, but not grain size, is correlated with floret size in cultivated sunflower, *Helianthus annuus* L.

Jarrad R. Prasifka , Zoe M. Portlas  and Brent S. Hulke 

Edward T. Schafer Agricultural Research Center, USDA-ARS, 1605 Albrecht Boulevard North, Fargo, ND 58102-2765, USA

Research Article

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Corresponding author:

Jarrad R. Prasifka;
Email: jarrad.prasifka@ars.usda.gov

Abstract

Cultivated sunflower (*Helianthus annuus* L.) pollen helps attract wild and managed bees needed to produce hybrid seed. Pollen quantity and grain size (\approx quality) are affected by the environment, but are also heritable traits of interest for breeding. Florets from public inbred B-lines (maintainer) and R-lines (restorer) were used to evaluate pollen quantity and quality, test for trait correlations and determine if line development has changed pollen traits. Pollen quantity (\approx 25,000–67,000 grains per floret) and diameter (\approx 30–37 μ m) were similar to previous reports and values of each parameter were correlated across years. Pollen quantity per floret was positively correlated with floret size (area; mm^2) but floret sizes and pollen quantity were unrelated to pollen grain size. Groups of lines released relatively early (1968–1986) or late (1988–2006) did not differ in pollen quantity or size, and male (R-line) parents did not produce larger grains. The strong, positive correlation between floret size and pollen quantity reveals a possible trade-off because wild bees generally prefer sunflowers with shallower florets. The apparent lack of change in pollen quantity or pollen grain size over time (and lack of increased pollen size in R-lines relative to B-lines) suggests that the quantity and quality of pollen may not be limiting factors in the success of inbred lines or resulting hybrids. Though sunflower lines with larger florets contain more pollen, additional variation in pollen visible on sunflower heads may relate to the timing or completeness of pollen extrusion from anther tubes.

Introduction

Pollen is a key resource for the function of agricultural and wild communities. The most obvious way pollen is important is in acting as the source of male gametes for flowering plants, and pollen receipt during flowering determines quantity and quality of seed and some associated reproductive tissues (Burd, 1994). Additionally, pollen is recognized as a primary food resource for bees, whose pollination of various crops supports human nutrition (Eilers *et al.*, 2011). Of course, pollen is consumed by many other (non-bee) groups of animals and its importance is not limited to plant reproduction or fitness of pollen-feeders, as pollen contributes to interactions between multiple species and trophic groups (Wäckers *et al.*, 2007).

Many different factors can affect pollen quantity and quality. The quantity of pollen plants produce is heritable, and recent work in model systems has begun to identify genes responsible for variation in pollen production (Kakui *et al.*, 2022). Pollen quality is arguably less simple to assess, but pollen grain diameter is heritable and positively associated with viability in sunflower (Bonciu, 2013), pollen tube growth in maize (Sari-Gorla *et al.*, 1995) and generally contributes to reproductive success (discussed by Ejsmond *et al.*, 2015), making pollen diameter useful as a simple proxy for quality. Among various environmental factors, deficiency of nutrients can decrease both the quantity and quality of pollen produced (Young and Stanton, 1990; Lau *et al.*, 1995). Other variables including temperature (Hedhly, 2011), water-stress (Descamps *et al.*, 2018) and ultraviolet radiation (Demchik and Day, 1996) influence pollen quantity or quality but may also have inconsistent effects within plant species because of genotype \times environment interactions (Hedhly *et al.*, 2005).

Cultivated sunflower, *Helianthus annuus* L., is well-known for producing large amounts of pollen. The evolutionary cause of high pollen production in *Helianthus* spp. is certainly genetic self-incompatibility, a trait that is common in the genus (Heiser *et al.*, 1969); copious pollen helps ensure cross-pollination by bees and other insects. Sunflower breeding and genetics research have produced self-compatible sunflowers (Gandhi *et al.*, 2005), but because the crop uses a three-line system to create hybrid seed (Fick and Miller, 1997), pollen must still be moved by bees from male-fertile to male-sterile parents. As a result, the quantity and quality of pollen remains a concern for cultivated sunflower. Examining inbred lines and their hybrids, Vear *et al.* (1990) found heritable variation in pollen quantity, with typical values



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of 25,000–40,000 grains per floret. More recent research by Astiz and Hernandez (2014) with two landraces found a somewhat broader range of 20,000–50,000 grains per floret, but also attributed variation, in part, to differences in the size of the anther tube (length or width) and location of florets collected relative to the centre of the capitulum. With regards to pollen quality, Bonciu (2013) found a significant, positive correlation between the mean diameter of pollen grains and their viability across 21 sunflower hybrids. It also appears that temperature and humidity during reproductive development affect pollen quantity in sunflowers (Astiz and Hernandez, 2013).

As part of recent efforts to understand sunflower–pollinator interactions and the potential to enhance pollination through breeding (Prasifka *et al.*, 2018), floret samples were collected from a variety of public inbred lines. Specific objectives were to: (i) examine possible relationships between floret size measures and pollen quantity (grains per floret) and quality (grain size), and (ii) assess whether selection of sunflower inbred lines has modified pollen quantity or quality over the first four decades of hybrid sunflower breeding. These objectives are important to the productivity and profitability of cultivated sunflowers, but also address the food resources available to wild and managed bees that forage on sunflower pollen.

Materials and methods

Plant materials comprised inbred lines released by the USDA-ARS breeding programme between 1968 and 2006. Sunflower hybrids are created by crossing a male-sterile A-line with a male-fertile R-line. The A-lines are functionally female due to cytoplasmic male sterility (cms), while the R-lines possess a fertility restoration gene that ensures the progeny, planted and grown by farmers, are self-fertile. Because A-lines lack pollen, near-isogenic B-lines are also needed to create a supply of A-line seed (Fick and Miller, 1997). Accordingly, to assess pollen from both male and female heterotic groups, uniquely numbered R-lines (RHA) and B-lines (HA) were used and A-lines (cms HA) excluded.

Field sampling

A large sunflower association mapping panel (the ‘core set’ in Mandel *et al.*, 2011) was planted in the USDA-ARS breeding nursery near Glyndon, MN on 27 May 2016. Inbred B- and R-lines were grown in single row plots, 6 m long, with 0.76 m between rows. After seedling emergence, plants were thinned to 20–25 plants per plot. Florets were originally collected and used to map quantitative trait loci that affect floret size (Reinert *et al.*, 2020), a trait known to influence pollinator preference in cultivated sunflower (Portlas *et al.*, 2018). In brief, a small wedge was cut from each of five plants per plot when they reached the first or second day of anthesis (stage R5.1–5.2; Schneiter and Miller, 1981). Labelled samples were stored in a freezer (−20°C) until further processing. To assess variation between years, a subset of 30 B-lines sampled in 2016 were planted in a research plot near Casselton, MN on 7 June 2017. Other details of plots and plant sampling were similar between 2016 and 2017.

Sample processing and analysis

After removal from storage, frozen sunflower wedges were slightly thawed so that forceps could be used to remove five pre-anthesis

(=unopened) florets from each wedge immediately adjacent to florets opened on the day of collection. This method of selecting florets provided samples without loss of pollen by wind or insects that were located near the periphery of the head (because of positional effects on pollen quantity noted by Astiz and Hernandez, 2014). A flatbed scanner was used to obtain high-resolution images (TIFF, 600 dpi) of florets from each plant. ImageJ (Schneider *et al.*, 2012) was used to measure the dimensions (length, width, area [\approx lengthwise or longitudinal cross-section]) for the 25 individual florets (5 plants \times 5 florets) collected from each inbred line with a user-generated macro (included as Supplementary File S1).

After measurements of floret size were made, inbred lines were selected for pollen quantification. In 2016, this included 30 B-lines and 30 R-lines; the same 30 B-lines were evaluated again for 2017. For each five-floret sample, the basal end of each floret was cut off (to reduce debris from hairs) and florets were sliced lengthwise towards the closed end to avoid losing pollen. Cut florets were then added to microcentrifuge tubes. A prepared agar solution (500 μ l of 35 mg agar in 50 ml water) in which pollen grains are neutrally buoyant was added to each tube, after which the tubes were agitated using a vortex mixer. A cell counter (Cellometer Auto T4; Nexcelom Bioscience LLC, Lawrence, MA, USA) was used to estimate the number of pollen grains in samples and average diameter (μ m) of pollen grains (including spines as in Vaissière and Vinson, 1994) based on a small (20 μ l) subsample pipetted into a counting chamber on a disposable slide. Images in Cellometer software (Auto Counter version 3.3.9.5, Nexcelom Bioscience LLC) were checked and adjusted to exclude any debris that was accidentally counted or include pollen cells that were uncounted because they were too close to adjacent grains. Observations of anthesis suggest that not all of the pollen produced may be extruded and made available to foraging bees, but this distinction (between pollen produced and extruded) will be addressed in future research.

Data analysis

Other than summary statistics (means, standard deviations), data on floret sizes, pollen counts and pollen diameters were analysed using SAS (SAS Institute Inc., 2016). To evaluate consistency of measurements on pollen samples across two sample years (environments), Pearson’s correlations for the number and diameter of grains were calculated for B-lines ($n = 30$) grown in both 2016 and 2017. To assess floret size measures as potential predictors, floret length, width and cross-sectional area (mm^2) were tested for significant correlations with the number and diameter of pollen grains in all inbred lines and years ($n = 90$). The best significant predictor was used to create regressions specific to each of the three groups of samples (i.e. 2016 B-lines, 2016 R-lines, 2017 B-lines) separately. Tests of whether the relationships between grains per floret and floret size differed between heterotic group (2016 B-lines versus 2016 R-lines) and year (2016 B-lines versus 2017 B-lines) were made by comparing 95% confidence intervals of regression slopes. To test whether inbred lines released relatively early (1968–1986; $n = 37$) or late (1988–2006; $n = 23$) produced greater than expected quantities of pollen, residuals from regressions on 2016 data were compared using a Welch’s *t*-test (adjusting for unequal variance). Tests of whether pollen size differed between early and late releases or between heterotic groups were also made via Welch’s *t*-tests.

Results

Inbred lines sampled in 2016–2017 varied with pollen quantities of $\approx 25,000$ – $67,000$ grains per floret, and pollen diameters ≈ 30 – $37 \mu\text{m}$; relative to means, estimates of pollen grains per floret showed greater sample-to-sample (\approx plant-to-plant) variation than pollen diameter (Supplementary Table S2). For the 30 lines evaluated in both years (i.e. the B-lines), pollen quantity showed a moderate correlation between years ($r = 0.59$, $n = 30$, $P < 0.001$) and diameter a slightly stronger correlation ($r = 0.67$, $n = 30$, $P < 0.001$; Fig. 1).

Across all inbred lines and both years, pollen quantity was most closely correlated with floret area, though significant ($P < 0.05$) correlations with floret length and width were also evident. The diameter of pollen for inbred lines was not associated with any measures of floret size (Table 1). When pollen quantity was regressed onto floret area separately for each heterotic group and sample year combination, the relationship appeared to differ for B-lines in 2017 compared to either group in 2016 (Fig. 2); however, confidence intervals (95%) of regression slopes for B-lines [2041–3932] and R-lines [2318–4362] in 2016 overlapped with each other, as well as B-lines in 2017 [738–2690]. Comparisons of residuals between inbred lines released relatively early (1968–1986) or late (1988–2006) did not show increases in pollen quantity over time after accounting for floret size ($t = -1.35$, $df = 46.4$, $P = 0.18$). Using data from 2016, pollen grain diameters did not differ between relatively early and late releases ($t = 0.14$, $df = 38.2$, $P = 0.89$), but pollen diameters of B-lines were $\approx 1 \mu\text{m}$ larger than samples from R-lines ($t = 2.94$, $df = 52.1$, $P < 0.01$). Mean values for inbred lines (floret size, pollen diameter, grains per floret and residuals from pollen quantity predictions) are included in Supplementary Table S3.

Discussion

The quantity and quality (grain diameters) of pollen from florets collected in 2016–2017 were generally similar to previous research with cultivated sunflowers (Vear *et al.*, 1990; Astiz and Hernandez, 2013, 2014; Bonciu, 2013), but the inbred lines evaluated were a more representative sample of germplasm (e.g. included more lines, both heterotic groups, confection and oilseed market classes) than prior studies. The positive correlation of

pollen quantity with floret size in sunflower seems logical, while the apparent lack of correlation between floret size and pollen grain size is counterintuitive. However, correlations among these and related floral traits in other plants are varied. Patterns of floral traits in wild radish (*Raphanus sativus* L.), in which floral size (corolla width) and pollen production per flower are positively correlated but neither is related to pollen size (Young *et al.*, 1994), appear most similar to observations in sunflower. However, selection for increased pollen size in mustard, *Brassica rapa* L., resulted in greater flower size but lower pollen quantity per flower (Sarkissian and Harder, 2001). Directional selection on pollen grain size in *Mimulus guttatus* Fischer ex DC showed no consistent correlation with floral characters (including pollen grains per flower and corolla width and length) across three populations (Lamborn *et al.*, 2005). Populations of *M. guttatus* and *Mimulus micranthus* showed no correlations between flower size (corolla width) and pollen quantity or size and inconsistent correlations between pollen quantity and size (Fenster and Carr, 2008).

The strong, positive correlation between floret size and pollen quantity suggests a potential trade-off for pollinators. Prior work with wild bees shows small- or medium-sized sunflower specialists (e.g. *Andrena helianthi* Robertson, *Melissodes trinodis* Robertson and *Melissodes agilis* Cresson; noted as oligoleges primarily associated with *Helianthus* by Hurd *et al.*, 1980) prefer sunflower lines with shorter florets (Portlas *et al.*, 2018), ostensibly because nectar at the base of florets is accessible or closely matched to their proboscis length for efficient foraging. Available floral rewards (pollen and nectar) affect the size of individual bees (Burkle and Irwin, 2009) and bee populations (Holzschuh *et al.*, 2013), but the degree to which very large (or very small) florets could be detrimental to sunflower specialists is unclear and probably context-dependent. However, the idea that more pollen (via larger florets) could cause reduced preference or nectar accessibility probably does not apply to larger bees (e.g. *Bombus* spp.) that can easily reach nectar in and may prefer sunflowers with larger florets (Ferguson *et al.*, 2021). For honey bees, *Apis mellifera* L., there may be times at which nectar or pollen is considered the key resource (i.e. pollen in spring and nectar during summer), but honey bees are generalists that collect nectar and pollen from different locations (Couvillon *et al.*, 2015).

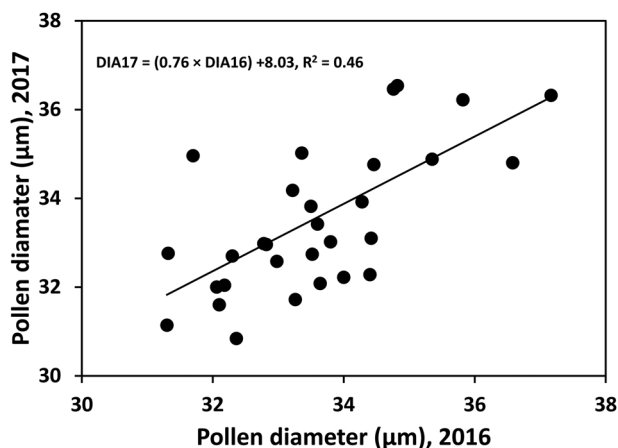


Figure 1. Regression of mean pollen diameter (μm) from 2017 onto 2016 data for $n = 30$ inbred maintainer (HA) lines. For each year, pollen diameters were measured from five plants (replicates) from which five florets were collected 1 d before anthesis.

Table 1. Correlations (Pearson's r) between mean pollen quantity (grains per floret [GPF]), quality (diameter [μm]) and measures of floret size for inbred lines of *H. annuus* L. grown in 2016 ($n = 60$) and 2017 ($n = 30$)

	Pollen diameter (μm)	Floret area (mm^2)	Floret length (mm)	Floret width (mm)
Pollen grains/floret	-0.06	0.76*	0.52*	0.70*
Pollen diameter (μm)		-0.06	0.04	-0.05
Floret area (mm^2)			0.86*	0.89*
Floret length (mm)				0.64*

*Asterisks indicate statistically significant ($P < 0.05$) correlations.

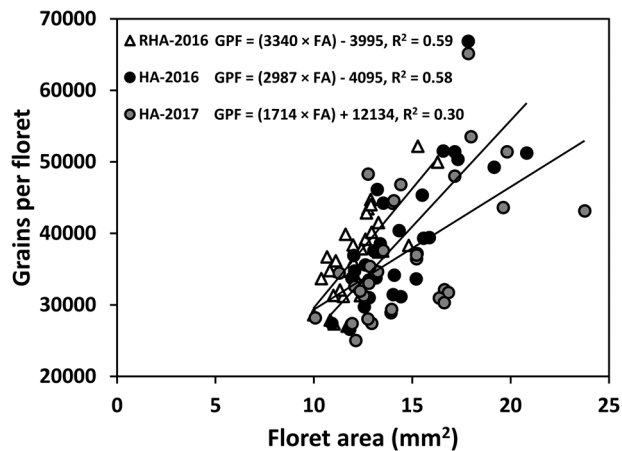


Figure 2. Regression of mean pollen quantity (grains per floret) onto floret area. Each regression includes $n = 30$ inbred maintainer (HA) or restorer (RHA) lines in 2016 or 2017. Each pollen quantity value includes measures from five plants (replicates) from which five florets were collected 1 d before anthesis.

Of course, the value of sunflower for honey bees in particular is a source of debate; though low protein content of sunflower pollen may reduce its value (Nicolson *et al.*, 2018), it is an abundant source of food in resource-poor agricultural landscapes and may also provide benefits to bees in mitigating the effects of parasites and pathogens (Palmer-Young *et al.*, 2023). Further, in some instances, sunflower pollen may apparently result in reduced visitation by honey bees (Mallinger and Prasifka, 2017), though the attractiveness of sunflower pollen likely depends on colony nutritional status and available alternative sources of pollen.

Relative to sunflower inbred line development, the apparent lack of change in pollen quantity (adjusted for floret area) or pollen grain size over time (and lack of increased pollen size in pollen donor R-lines relative to B-lines) between early (1968–1986) and more recently (1988–2006) released lines is surprising. Though there has been no direct selection for increased pollen quantity or pollen grain size in USDA's inbred line development, both traits seem to have value in a hybrid production system and in promoting seed development in self-fertile hybrids (Lamborn *et al.*, 2005; Bonciu, 2013). The lack of change over time suggests that the quantity and quality of pollen may not be limiting factors in the success of released inbred lines or resulting hybrids. However, recently developed genetic markers for other sunflower floral traits, including floret size (Reinert *et al.*, 2020) and nectar quantity (Barstow *et al.*, 2022) imply the process of selecting for changes to pollen-specific traits may be relatively straightforward if they were considered important goals, given moderate-to-high heritability.

Though data clearly show sunflower lines with larger florets contain more pollen, observable differences among inbred lines for the amount of pollen on heads during anthesis (when pollinators are excluded) suggest the simple explanation that larger florets contain more pollen is incomplete. Recent research on the mechanics of pollen extrusion (or 'presentation') may provide some context: pollen extrusion depends on the elongation of the anther filaments and style, but these parts 'are differentially sensitive to temperature' (Creux *et al.*, 2021). Perhaps differences in pollen extruded (not explained by floret size) are transient and represent differences among inbreds in the rate of style elongation. Alternatively, observations on some lines that appear to be low pollen producers show that some lines may simply fail

to extrude pollen contained in the anther tube as well as others. Regardless of the precise cause, understanding variation in pollen quantity and presentation remains important to cultivated sunflower as a crop and to the various species that depend on this floral resource.

Supplementary material. The supplementary material for this article can be found at <https://doi.org/10.1017/S1479262123000709>

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