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Bioprospecting for improved floral fragrance in wild sunflowers

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Abstract: Cultivated sunflower (*Helianthus annuus*) is not typically considered to have a pleasant floral fragrance. In field production, seed yield is often limited by pollination services, particularly in the production of hybrid seed. Improved floral fragrance, as determined by volatile organic compounds, may be a route to improving pollinator attraction for oilseed and confectionary production and could also add value to ornamental sunflowers. Wild relatives of *H. annuus* have a long history of being used to breed improved traits into cultivated varieties, yet it is unknown whether favorable scents are present in wild *Helianthus* species and thus an available resource for fragrance breeding. In order to assess the diversity of floral fragrance available in crop wild relatives, 30 diverse accessions of wild *Helianthus* as well as seven varieties of *H. annuus* spanning a domestication gradient were grown in greenhouse experiments and variation in floral volatiles was analyzed by solid phase microextraction-gas chromatography-mass spectrometry. While alpha-pinene made up a significant portion of the volatiles emitted for most taxa, there was substantial diversity present across the genus as well as within *H. annuus*. Most volatiles emitted were monoterpenoids with a significant share of sesquiterpenoids. The diversity identified here will inform further targeted study of which compounds affect pollinator attraction and health. Several wild accessions such as *Helianthus debilis* subsp. *tardiflorus* and *Helianthus praecox* subsp. *praecox* as well as open-pollinated domesticated accessions of *H. annuus* show promise for breeding for improved floral fragrance due to high volatile abundance and likely favorable compound compositions.

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1 Introduction

The genus *Helianthus* contains approximately 50 species found throughout North America (Heiser et al. 1969). The range of habitats that wild sunflowers have colonized has led to a remarkable amount of diversity (Kane et al. 2013). However, crop sunflower, a commodity worth over $30 billion USD annually worldwide, has lost some of its genetic variability through recurring genetic bottlenecks arising from founder effects and artificial selection during the process of domestication and improvement (Kane et al. 2013; Pickersky and Dudavera 2007; Seiler et al. 2017). Like many other crops, cultivated *Helianthus annuus* has been found to have a marked decrease in allelic diversity compared to the wild populations of *H. annuus* from which cultivated sunflower was domesticated (Arias and Riesberg 1995). Wild sunflower relatives are a known resource for the introduction of preferable traits into cultivated varieties (e.g., insect and disease resistance), and the use of wild sunflower as a trait donor contributes well over $250 million USD annually to the world economy (Kantar et al. 2015; Seiler et al. 2017).

Floral fragrances could be desirable traits in sunflower for both ornamental purposes and for the potential to attract greater amounts of pollinators for both oilseed and confectionary sunflower production. Ornamental flowers destined for the cut-flower industry are typically not bred for fragrance but rather other more economically marketable traits like color and post-harvest longevity (Pichersky and Dudareva 2007), though consumer research indicates that fragrance is actually a top consumer preference that has long been ignored (Clark et al. 2013). In self-compatible crops where insect pollination is not required for fruit or seed set, selection for high productivity may result in reduced plant investment in energetically expensive traits like floral fragrance. This has led to many domesticated species losing much of their fragrance (Pichersky and Dudareva 2007), with a few noted exceptions being those ornamentals where floral fragrance was a focal trait. While cultivated sunflowers can self-pollinate to a large extent, most varieties will produce higher seed yields when pollinated by either honeybees or native bees (Chabert et al. 2022; Greenleaf and Kremen 2006; Parker 1981). Research demonstrates that native bees likely provide the bulk of pollination services in North America, with managed honeybees often providing supplementary pollination in the central United States - though honeybees may have higher importance in other regions (Mallinger and Prasifka 2017; Portlas et al. 2018). To produce elite F1 hybrid varieties, pollination is required

(Greenleaf and Kremen 2006), and for self-compatible oilseed production insect pollination has been shown to increase both total seed count and total seed mass per head by around 50 % (Chabert et al. 2022). Research to date on the drivers of pollinator visitation rate in sunflower has demonstrated that both wild bees and managed honeybees prefer shorter disc florets (which provide easier access to nectar by proboscis) as well as higher amounts of nectar sugars (Mallinger and Prasifka 2017; Portlas et al. 2018), though different genera of wild bees have been shown to have different preferences as well as have preferences that change when foraging alongside one another (Ferguson et al. 2021). Further, wild bees have been shown to have a preference for abundant pollen rewards, while domesticated honeybees disfavor abundant pollen (Mallinger and Prasifka 2017). Despite these important insights into sunflower handling and reward traits that drive pollinator preferences, there exists much less research on variation in cultivated sunflower floral fragrance or its role in pollinator preference (Bahmani et al. 2023; Pham-Delegue et al. 1986, 1989, 1990).

In wild angiosperms, flowering traits are suggested to have evolved convergently due to selection from pollinators, resulting in flowers that have attractive characteristics (Van der Niet et al. 2014). Pollinators often are attracted to scent, and domestication has changed many scent producing biochemical pathways in ways that can disrupt natural pollinator responses (Pichersky and Dudavera 2007). In wild sunflowers, more diversity in morphology and pigmentation exists among species than seems likely to have arisen by genetic drift alone (Mason et al. 2017), suggesting the potential for floral trait diversification under natural selection by native pollinators. However, floral fragrance has yet to be assessed in wild *Helianthus*, and we thus know little about its evolution across the genus or comparison to domesticated cultivated sunflower varieties. Crop sunflower has very little noticeable floral fragrance besides the woody scents of pinene and sabinene (Bahmani et al. 2023; Bertoli et al. 2011). Despite being one of the few crops domesticated in North America, growers in the United States often rent managed hives of the non-native European honeybee *Apis mellifera* to pollinate field crop sunflower (especially in hybrid seed production) rather than rely on pollination by native wild bees (Greenleaf and Kremen 2006). However, the pollination efficiency of managed honeybees has been demonstrated to be enhanced by over five-fold in the presence of wild bees, and direct pollination by wild bees steadily increases with inter-annual continuity of sunflower cultivation (Greenleaf and Kremen 2006). Given these findings, improved attraction of both managed honeybees and wild bees to field crop sunflower is a route to improved pollinator services, in ways that increase yields or lower the input costs of managed non-native pollinators. Since bees are in part attracted to floral scent (Borghi et al. 2017; Pham-Delegue et al. 1990; Posey et al. 1986), plant breeders may be able to influence pollinator attraction by manipulating floral fragrance.

Floral fragrance is determined by a complex mixture of volatile organic compounds. Volatile organic compounds are secondary metabolites synthesized in specific plant organs for various physiological purposes and are actively released based on developmental events or biotic interactions (Cheng et al. 2007; Lucas-Barbosa et al. 2016). These mixtures are primarily made up of terpenoid, benzenoid, phenylpropanoid, and fatty-acid compounds (Borghi et al. 2017, Pichersky and Dudareva 2007). Among these volatiles, monoterpenes and sesquiterpenes are thought to be the floral volatiles that attract most pollinators (Pichersky and Raguso 2018; Zhou and Pichersky 2020). Additionally, some fatty-acid derived compounds have been found to mimic insect pheromones, thus increasing pollination (Borghi et al. 2017). Cultivated sunflower has been found to contain as many as 60 different volatile compounds, primarily terpenes but with fatty-acid derived compounds making up a significant portion as well (Bahmani et al. 2023; Bertoli et al. 2011; Schuh et al. 1997). Honeybees have been demonstrated to respond to a specific volatile fraction containing 15 compounds found in domesticated *H. annuus* (Pham-Delegue et al. 1990).

It is now possible to identify genes that regulate the biosynthetic pathways that synthesize desirable floral fragrances. Efforts have already been made to change the volatile compounds emitted by plants (Pichersky and Dudareva 2007). A key first step in breeding desirable floral fragrances into crop sunflower varieties is to identify and describe variation in the volatile organic compounds present across domesticated and wild sunflower, where shared biosynthetic pathways are likely producing different fragrance profiles. Until recently, the volatile organic compounds contained in *Helianthus* flowers were largely unknown other than reports for two commercial varieties (Bertoli et al. 2011; Pham-Delegue et al. 1990). Recent description of a cross-section of cultivated germplasm indicates limited floral volatile variation despite wide genetic diversity (Bahmani et al. 2023). Given the much broader phytochemical diversity noted within leaves and petals across the genus (Bahmani et al. 2022; Mason et al. 2016), wild *Helianthus* may contain a plethora of volatile compounds that could be leveraged to increase pollination in field crop sunflower or even for ornamental cut-flower purposes. Furthermore, examination of the variation in floral volatile compounds can illustrate what effects both domestication and subsequent artificial selection during crop improvement have had on cultivated sunflower floral fragrance. This study examines floral volatile profiles expressed in a diverse set of *Helianthus* species to understand the scope of floral fragrance variation and provide a base for further research into improving floral fragrance in crop sunflower.

2 Methods

2.1 Plant growth and sample collection

Thirty-seven accessions of species spanning the genus *Helianthus* were grown from seed during the 2021 growing season (Table S1). Seeds obtained from the USDA National Plant Germplasm System were scarified and germinated in moistened petri dishes, and transferred into sterilized sand in seedling trays in a growth chamber. When seedlings developed true leaves, they were transferred to the UCF Transgenic Greenhouse and planted in 16-inch azalea pots filled with a 2:1 mixture of sand and potting soil. Pots were inoculated with arbuscular mycorrhizal fungi (*Rhizophagus intraradices*, Mykos, Xtreme Gardening, Gilroy, CA), and fertilized with Osmocote Plus slow-release fertilizer (15-9-12, 5–6 month formulation, Scotts, Maryville, OH). Plants were watered to field capacity twice per week, staked if necessary to provide additional support, and treated with permethrin to suppress whiteflies (Bonide Eight, Oriskany, NY). Poorly developed seedlings were replaced with regerminated plants during the first month. Plants began to flower in August 2021, and sampling commenced once inflorescences (composite heads) with open disc florets were present. Disc florets were selected for sampling to assess floral fragrance profiles because of their far higher numerical abundance relative to ray florets within the composite head, as well as previous research indicating that in cultivated sunflower disc florets produce larger quantities of volatiles than petals (Bahmani et al. 2023). Whole composite heads were collected with subtending peduncles or stems in the manner of cut flowers, with the stem placed in water to ensure freshness and transported to the laboratory. Disc florets were then extracted from the head with ethanol-cleansed forceps, weighed for fresh mass, and approximately 0.5 g of disc floret tissue was placed into a glass headspace vial for subsequent analysis by gas chromatography-mass spectrometry. Each sample contained either disc floret tissue from one unique head, or for small-flowered species, disc floret tissue from multiple heads from the same plant. Tissue from the same head was never placed into multiple vials to ensure vials represented independent biological replicates.

Sampling of fresh inflorescences was repeated once per week until flowering ended, and most plants were sampled. Additionally, flowers from *H. annuus* varieties spanning a gradient of domestication and improvement were sampled from a parallel experiment in the same greenhouse, grown under similar conditions. These plants were grown from commercial seed sources, including two sources for wild *H. annuus* (Great Basin Seed, Inc. and Everwilde Farms, Inc.), two Native American landraces (Hopi and Seneca; Southern Exposure Seed Exchange, Inc.), two open-pollinated heirloom varieties (Mammoth and Peredovik; Southern Exposure Seed Exchange, Inc.), and one elite F1 hybrid variety (Hornet; NuSeed, Inc.). For these cultivated *H. annuus* varieties, rather than removing entire inflorescences in the manner of cut flowers, disc florets were directly removed from the head and placed in vials on ice for analysis alongside the wild species described above. Overall, across the two experiments an average of 6.5 (±4.1 SD) biological replicates were able to be included, with specific replicate counts per accession presented in Table S1.

2.2 Chemical analysis

Fresh disc floret samples were analyzed using solid-phase microextraction gas chromatography mass spectrometry (SPME GC-MS) using a single quadrupole QP2020 (Shimadzu, Inc.). Two methods were used during the study. The first method had a solvent cut time of 5.5 min and started mass spectrometry analysis at 5.8 min, with a total program time of 25.42 min. This method was used for the first five weeks of sampling and reflects about half of the data collected, and was designed based on literature protocols and pilot data. The method was then updated as two peaks (oxime and alpha-pinene) were found under preliminary analysis to be partially or fully cut off for some samples, preventing semiquantitative analysis of peak area for those two compounds. The updated method had a solvent cut time of 4.5 min and started mass spectrometry analysis at 4.8 min to address this. Other than the timing of the start of data collection, all other conditions were identical between the two methods. The conditioning temperature was 270 °C, with a pre-conditioning time of 60 min. Incubation lasted 60 min, with an incubation temperature of 40 °C and an agitator speed of 280 rpm. Sample extraction time was 10 min, followed by an analysis time of 30 min. Splitless injection mode was used, with an injection temperature of 225 °C. The column oven temperature was 35.0 °C, with a sampling time of 4.00 min. The raw GC-MS data generated was cross-referenced against the NIST17s, TERP1, NIST17-1 and NIST17-2 libraries for identification of compound identity for each peak (Lemmon et al. 2017). Integrated peak areas were divided by the initial sample mass placed into the headspace vial, in order to mass-normalize relative peak areas across samples.

2.3 Statistical analysis

GC-MS data was compiled and converted into a matrix using the *tidyverse* package within the R programming language, implemented in RStudio (R Core Team 2021; Rstudio Team 2020; Wickham et al. 2019). Compound retention times were manually verified to be consistent across the data set. Compound identities produced by cross-reference to NIST libraries were further checked against PubChem and NIST databases to align compound synonyms across the four libraries used (Kim et al. 2019; Lemmon et al. 2017). Trivial names were preferred when available for ease of interpretation against known terpenoid biosynthetic pathways (Kanehisa and Goto 2000). Mass-normalized peak areas were considered to reflect semiquantitative relative compound abundance across floral samples. Accession-level means were calculated for mass-normalized peak areas for each individual compound across biological replicates. To account for any missing peaks in samples analyzed with the first analytical method (5.5 min solvent cut time), accession means for compound peak areas were calculated with zeros excluded. In order to ensure data validity for cross-genus comparisons, accessions that were not able to be re-sampled after the second GC-MS method was implemented were not included in subsequent statistical analysis; only accessions analyzed with both methods or with the second method alone were included. For this reason, *Helianthus microcephalus*, *Helianthus divaricatus* (4N), and *Helianthus debilis* subsp. *debilis* were excluded, though volatile profile data for these accessions is provided in the supplement (Dataset S1, Dataset S2).

To examine patterns of covariation among the more common volatile compounds, principal components analysis was performed on a reduced dataset containing compounds present in at least half of the accessions. Using accession-level means for mass-normalized peak areas, the PCA function in Rstudio was used to generate principal components and the *factoextra* package was used to extract and visualize PC1 and PC2 in two panels (Kassambara and Mundt 2020; R Core Team 2021, RStudio Team 2020). Spearman's correlation coefficients showing pairwise relationships among all 237 compounds were obtained using the *corr* function on accession means of mass-normalized peak areas in Rstudio (Kolde 2019; R Core Team 2021; Rstudio Team 2020). Favorable scents in the context of human aesthetics, defined here as having odor described as floral, fruity, sweet, citrus, minty, or herbal, were identified using The Good Scents Company Information System web database of volatile compounds sponsored by the flavor, fragrance, food, and cosmetics industry (The Good Scents Company, Inc.) that combines organoleptic information from multiple literature and industry sources and has been used for research in scent engineering (Rodrigues et al. 2021).

3 Results

3.1 Variation in floral volatiles

Overall, 237 distinct compounds were found across all accessions sampled, but only 60 made up more than one percent of the total floral volatile abundance in any accession. Volatile compound richness varied greatly among wild accessions (Table S2). The highest number of compounds detected was 86 in *Helianthus atrorubens*, while the lowest number of compounds detected was 9 in *Helianthus salicifolius*. Within *H. annuus*, the number of compounds ranged from 25 to 57, with no strong distinction between wild *H. annuus* and cultivated accessions in compound diversity. Most accessions had comparable numbers of monoterpenoids and sesquiterpenoids present, and markedly few non-terpenoid compounds present.

Total volatile abundance was highly variable among species. The highest total volatile abundance was observed in *H. debilis* subsp. *tardiflorus* with nearly twice the abundance of the next highest accession. Other species with high volatile abundance included several basal rosette perennials (*Helianthus occidentalis, Helianthus longifolius, Helianthus heterophyllus*), as well as several annuals (*H. annuus, Helianthus argophyllus, Helianthus winteri*) (Table S3). Within *H. annuus*, the open pollinated heirlooms Mammoth and Peredovik had the highest floral volatile abundance. In general, floral volatile abundance was dominated by monoterpenoids (Figure 1), but variation in relative monoterpenoid-sesquiterpenoid balance was observed across the genus. In annual species, floral volatile abundances contained a minimum of 90 % monoterpenoids (Figure 1), including all *H. annuus* accessions. While many sesquiterpenoid compounds were present in *Helianthus* flowers, they were typically present at lower relative abundance than monoterpenoids. The highest relative abundance of sesquiterpenoids (~25 %) was observed in two tetraploid erect perennials (*Helianthus decapetalus* 4N and *Helianthus hirsutus* 4N). Other accessions that had high proportions of sesquiterpenes (>10 %) were the tetraploid *Helianthus smithii*, and the diploids *H. decapetalus* (2N) and *Helianthus mollis* (Figure 1). The monoterpene alpha-pinene made up a significant portion (at least 40 %) of floral volatile abundance in all accessions except for *Helianthus heterophyllus* (Figure 1). *H. heterophyllus* was instead dominated by the nonterpenoid 1-undecene and was the only accession whose volatiles were not primarily composed of monoterpenoids (Figure 1). Within *H. annuus*, the open-pollinated varieties Mammoth and Peredovik

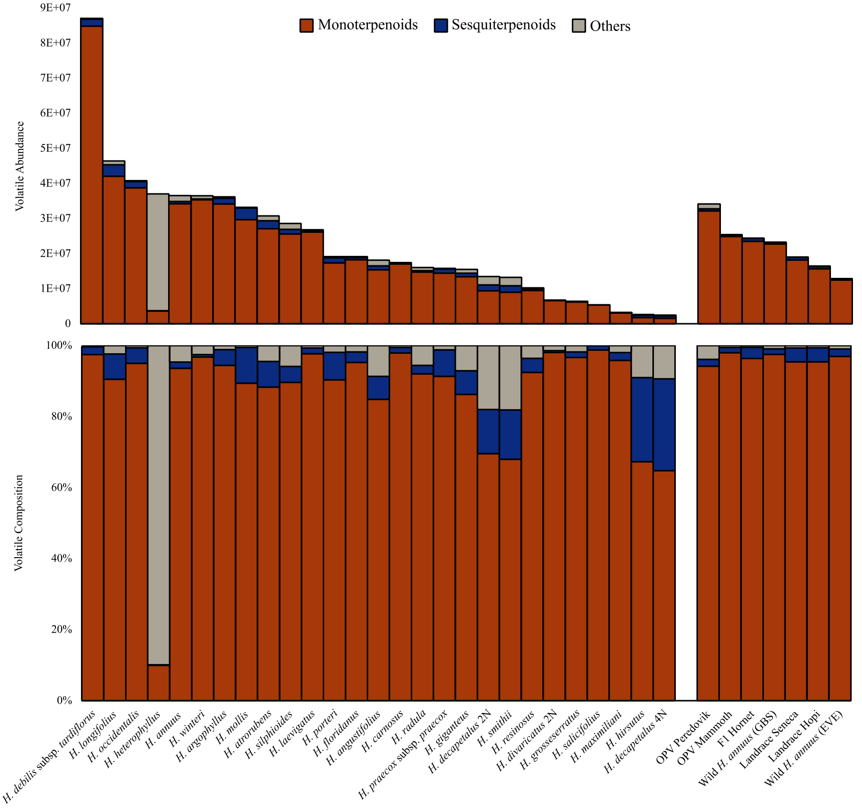

Figure 1: Bar plots showing total floral volatile abundance (top) and proportional composition (bottom) by mass-normalized peak area from GC-MS, coded by chemical class. Accessions are sorted by decreasing total volatile abundance within each experiment (left: wild *Helianthus* species, right: *H. annuus* domestication gradient). Information on accession sources and replicate counts are presented in Table S1. Full data on compound abundance and proportional composition is provided in Dataset S1 and Dataset S2.

had the smallest proportions of alpha-pinene and the greatest proportions of other monoterpenoids (Figure 1). Overall, the domesticated accessions of *H. annuus* contained 6–20 % sabinene, while the wild accessions contained 1 % or less.

Out of the 60 compounds that comprised at least one percent of total volatiles in any accession, 13 were identified as favorable fragrance compounds in the context of human aesthetics (Table 1). D-limonene, a citrus-scented monoterpene, was present in all accessions except *H. heterophyllus* and exhibited the highest relative proportion (~10 %) in *H. occidentalis*, *H. debilis* subsp. *tardiflorus*, and *Helianthus*

Table 1: Thirteen favorable compounds that made up at least 1 % of any accession. Favorable compounds are those with odors described as floral, fruity, sweet, citrus, minty, or herbal in The Good Scents Company Information System web database of organoleptic properties. For each compound, the number of accessions in which the compound was detected is listed, along with the maximum percentage found in any accession (minimum percentage was always 0 %), and the odor description category.

Favorable compound	Number of accessions	Maximum percentage	Odor description
beta-Phellandrene	19	16 %	Minty
D-Limonene	33	11 %	Citrus
trans-beta-Ocimene	2	10 %	Floral
psi-Limonene	3	7 %	Citrus
3-Carene	4	3 %	Citrus
beta-Cubebene	21	3 %	Citrus
cis-beta-Ocimene	12	2 %	Floral
1-Undecanol	1	2 %	Floral
alpha-Phellandrene	20	1 %	Citrus
beta-Elemene	11	1 %	Herbal
Eucalyptol	1	1 %	Herbal
allo-Ocimene	1	1 %	Floral
Farnesol	1	1 %	Floral

resinosus. A related citrus-scented monoterpene, psi-limonene (also known as pseudolimonene), was present in both *H. mollis* and *Helianthus praecox* subsp. *praecox* at 7 % and 4 % of total volatile abundance, respectively. The herbal-scented monoterpene *trans*-beta-ocimene was present in *H. hirsutus* and tetraploid *H. decapetalus* at 6 % and 10 % of volatile abundance, respectively. The minty-scented monoterpene beta-phellandrene was present in *Helianthus silphioides* and diploid *H. decapetalus,* at 8 % of total volatile abundance in both (Figure 3). Within the *H. annuus* accessions, cultivated varieties generally had higher proportions of favorable volatile compounds, including D-limonene, eucalyptol, and beta-phellandrene. The open-pollinated heirloom varieties (Mammoth and Peredovik) had the highest proportions of beta-phellandrene, at 8 % and 16 % respectively, as well as the highest total proportions of favorable compounds.

3.2 Chemical relationships

Among the volatile compounds detected, there appear to be very few negative correlations among mass-normalized abundances (Figure S1). Notably, even the most abundant compound (alpha-pinene) has few negative correlations with other compounds. This suggests that even though it is often the dominant volatile organic compound, increased production of alpha-pinene does not inhibit the production of other volatiles. The few strong positive correlations observed among compounds are mainly between closely related compounds that likely share biosynthetic pathways, for example the monoterpenoids pinocarvone and several related derivatives, the three sesquiterpenoids isocomene, modephene, and silphinene, and the alkenes 1-undecene, 1-decene, and 1-tridecene.

Principal components analysis of compound abundance shows that monoterpenoid abundance and sesquiterpenoid abundance are largely independent, with the spread of eigenvectors for compounds in these two terpenoid subclasses roughly orthogonal (Figure 2). The first two principal components axes together explain 58 % of total variation among species in the 60 major compounds included (Figure 2). The first principal component (PC1) loads positively with all compounds, such that accessions with higher values of PC1 have higher abundance of most volatile compounds. The second principal component (PC2) loads positively with most sesquiterpenoids and negatively with most monoterpenoids, distinguishing taxa with

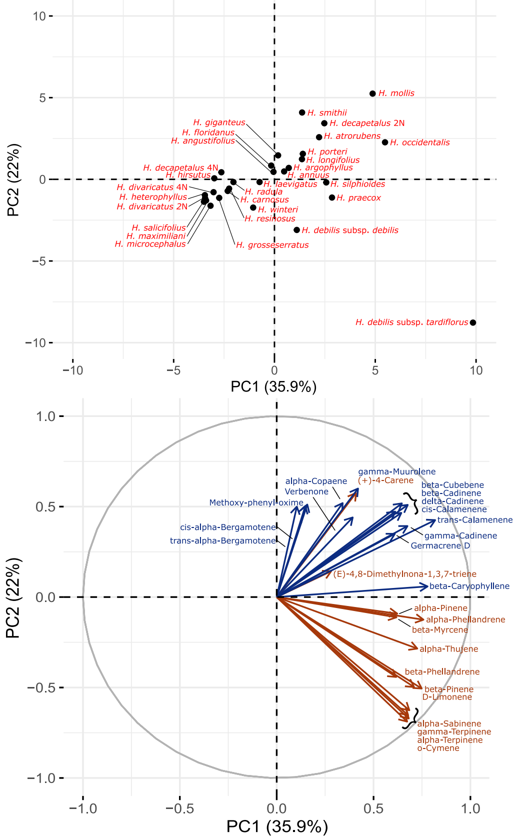

Figure 2: Principal components analysis of volatile abundance for the 27 compounds found in at least half of accessions of wild *Helianthus* species, showing species separation in principle component space (top), and volatile compound loadings (bottom). Compound loadings are color coded by chemical subclass (monoterpenoids in orange and sesquiterpenoids in blue). The first two principal components capture approximately 58 % of observed variation (PC1: 35.9 %, PC2: 22 %). Full data on compound abundance by accession is provided in Dataset S1.

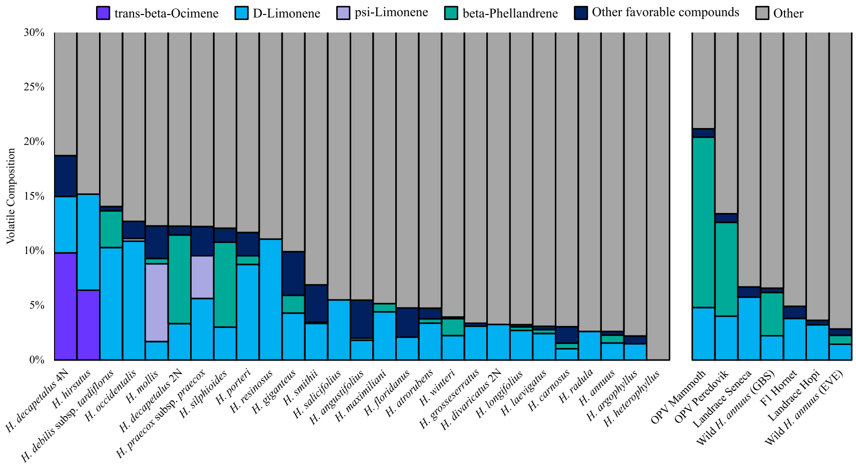

Figure 3: Bar plot showing the proportion of favorable compounds across accessions based on mass-normalized peak area. The four favorable compounds present at >5 % of the volatile profile for any accession are labeled separately, while all others are summed as "other favorable compounds". Accessions are sorted by decreasing proportion of favorable compounds within each experiment (left: wild *Helianthus* species, right: *H. annuus* domestication gradient). Information on accession sources and replicate counts are presented in Table S1, while full data on compound proportional composition is provided in Dataset S2.

higher absolute abundance of one terpenoid subclass versus the other. Eigenvectors indicate that individual volatile compound abundance tends to move in concert with other compounds within the same chemical subclass, likely due to fluxes through the same biosynthetic pathways affecting multiple compounds. Principal components analysis also indicates that some closely related species are distinguishable by variation in the abundance of monoterpenoids (the annuals *H. praecox* and the two subspecies of *H. debilis*), while other sets of closely-related species are distinguishable by variation in the abundance of sesquiterpenoids (the perennials *H. mollis* and *H. occidentalis*, or *H. atrorubens* and *H. silphioides*).

4 Discussion

4.1 Notable variation in fragrance among sunflower germplasm

When considering traits for breeding into domesticated *H. annuus*, one must examine both the nature of the desired trait and the ease of movement from the source germplasm. Wild sunflower species are categorized into primary, secondary, and tertiary germplasm based on the ease of genetic introduction into domesticated *H. annuus* (Kantar et al. 2015). Wild *H. annuus* and *H. winteri* are primary germplasm species and have no reproductive barriers to breeding through direct introgression, but from our results these taxa have little to offer as they do not have especially high volatile abundance or a high proportion of favorable volatiles. Much wider variation in total volatile abundance and proportion of favorable compounds was observed in the secondary germplasm (other annual *Helianthus*, like *H. praecox* and *H. debilis*) and tertiary germplasm (perennial *Helianthus*, like *H. decapetalus*, *H. hirsutus, H. longifolius, H. mollis*, and *H. occidentalis*). It is interesting to note that in taxa with variable ploidy levels among accessions (cytotypes of *H. decapetalus* and *H. divaricatus/H. hirsutus*), polyploidy did not appear to increase floral volatile abundance or the number of detected compounds – but may have influenced the proportional composition of volatiles. While only two instances of contrasting cytotypes spanning a polyploidization event were able to be examined here, the two accessions with the highest proportion of sesquiterpenoid compounds were tetraploid *H. decapetalus* and *H. hirsutus,* which suggests that further study of volatile abundance in other polyploid accessions is warranted to identify increased floral volatiles and improved fragrance. However, our limited contrasts here do not permit us to conclude much about the general effects of polyploidy on floral fragrance, as the effects of polyploidy on secondary metabolites are known to be highly variable and unpredictable across species, often causing both increases and decreases in target compounds depending on conditions during and after polyploidization (Gaynor et al. 2020; Iannicelli et al. 2020). More broadly, the diversity in disk floret floral fragrance observed in this study mirrors that observed in leaves and petals across the genus *Helianthus* in earlier work (Bahmani et al. 2022), including variation in volatile abundance, composition, and the apparent independence of sesquiterpene and monoterpene production as observed by PCA.

Within *H. annuus*, we observed higher diversity in floral fragrance than expected. Overall, the examined cultivated and wild accessions were similar in volatile abundance and subclass composition, and there did not seem to be a consistent reduction from wild to domesticated accessions as predicted. The open-pollinated heirloom varieties Mammoth and Peredovik had slightly higher volatile abundance, and substantially higher proportions of favorable volatiles (especially D-limonene and beta-phellandrene) than any of the other cultivated varieties, or indeed than the wild *H. annuus* accessions assessed. This is somewhat surprising, given that the open-pollinated heirlooms represent a germplasm pool that was ultimately derived from the Native American landraces, and from which the modern elite breeding pools for oilseed and confectionary germplasm were developed in the late 1900s (Park and Burke 2020). As named varieties, Mammoth and Peredovik have been in existence in some form since sometime between the late 1800s and 1960s, respectively (Jocic et al. 2015). Given the known pollinator limitation in seed production in cultivated sunflower (Chabert et al. 2022; Greenleaf and Kremen 2006; Parker 1981), it is possible that open-pollinated varieties have faced inadvertent selection for higher floral fragrance output or higher relative abundance of favorable volatiles over many decades, if mutants with these differences generated disproportionately higher seed set and were therefore overrepresented in subsequent generations. Given that both Mammoth and Peredovik were both initially developed in eastern Europe, the native pollinator context was also likely very different than that experienced by the wild *H. annuus* and Native American landrace progenitors in North America before the introduction of the Eurasian honeybee. The one modern elite F1 hybrid (Hornet) assessed here is more similar in floral volatile composition to the wild *H. annuus* and landrace varieties than to the open-pollinated varieties, and a recent assessment of disc floret volatile composition in a cross-section of inbred oilseed and confectionary breeding lines found volatile compositions similar to the landraces and modern hybrid assessed here – including a lack of detectable beta-phellandrene (Bahmani et al. 2023).

Regardless of the specific mechanism generating the volatile profiles observed in these open-pollinated heirlooms, our results indicate that further screening of so-called ‘exotic’ and ‘ornamental’ cultivated germplasm has value (Park and Burke 2020). The sort of decentralized mass selection that generates open-pollinated heirloom varieties is likely to generate some degree of local adaptation to the pollinator communities present in a given geographic region, such that heirlooms from different continents and climates may hold further floral fragrance diversity. Given the ease of breeding using only cultivated *H. annuus*, this suggests screening for floral fragrance diversity across heirlooms is a low-risk, high-reward strategy for moving toward the goal of improved floral fragrance in ornamental, oilseed, and confectionary sunflower. Furthermore, the lack of negative correlations seen among floral volatile compounds suggests that the upregulation of the abundance of a target compound (e.g., a single monoterpene) would be unlikely to substantially decrease the production of other terpenoid compounds that may play important roles in sunflower heads, such as insect or pathogen defense (Prasifka et al. 2015).

4.2 The potential for improved pollination

Floral volatiles are known to play a role in pollinator attraction and health. A previous study of two cultivated *H. annuus* varieties identified 15 volatile organic compounds that were attractive to honeybees (Pham-Delegue et al. 1990). However, only a small portion of these compounds were identified as common across the wild and cultivated accessions sampled here. This indicates that further study is needed to more completely describe key volatile compounds that function to attract pollinators in both wild *Helianthus* and cultivated sunflower. Many volatile compounds were detected in wild species that were not present in domesticated sunflower, suggesting that these compounds may play some role in plant-pollinator interactions with native pollinators in the diverse habitats *Helianthus* species occupy across North America. Alternatively, variation in these volatile terpenoids may be related to pest and pathogen resistance, as has been shown for nonvolatile terpenoids and phenolics (Prasifka et al. 2015; Prats et al. 2007). Among compounds that were abundant across *Helianthus* species, the individual citrus-scented compounds of D-limonene and psi-limonene have been shown in prior work to attract bees (Detzel and Wink 1993). This suggests that these monoterpenes may be important for pollinator attraction across both wild and cultivated sunflower. Given the moderate abundance of D-limonene, and the variation observed within cultivated accessions, increasing the production of D-limonene could be a straightforward breeding target for improved pollinator attraction. In addition, the high abundance of beta-phellandrene in heirloom varieties indicates that further study is needed on the role of this compound in pollinator attraction or plant defense.

There is growing evidence that plant secondary metabolites can play an important role in pollinator health, with a wide range of compounds present in nectar or pollen influencing bee immunity to viruses and parasites (Fitch et al. 2022; Palmer et al. 2017; Stevenson et al. 2017). Further, evidence suggests that bees which consume a diversity of secondary metabolites have longer lifespans and superior resistance to pathogens (Erler et al. 2014; Tihelka 2018). In largely monoculture agroecosystems, both wild and managed bees visiting a single cultivar of a single crop type may have reduced colony health relative to visiting a wild plant community (Cohen et al. 2021; Egan et al. 2018). One potential strategy to improve pollinator health could be to ensure that crop nectar and pollen rewards contain a wide diversity of multifunctional secondary metabolites. Cultivated sunflower pollen itself has been demonstrated to reduce pathogen and parasite loads in both bumblebees and honeybees, while increasing colony health, though the exact mechanisms are unknown (Giacomini et al. 2018). Further examination of sunflower floral chemistry may be a useful route to deploying cultivars that can improve pollinator health and persistence in production systems, with economic benefits given the direct relationship between bee abundance, visitation, and economic yield (Abbasi et al. 2021; Chabert et al. 2022; Greenleaf and Kremen 2006; Parker 1981).

Wild species across the genus *Helianthus* have broad diversity in quantity and composition of floral volatile emissions, suggesting that these crop-wild relatives are a deep resource for cultivar fragrance improvement beyond the standard cultivated breeding pools. However, further research is needed to identify which volatile compounds within *Helianthus* attract pollinators, either individually or in combination, and their roles in pollinator health when consumed. Among the germplasm assessed here, specific accessions with lower barriers to breeding, high volatile abundance, and high proportion of favorable compounds such as *H. debilis* and *H. praecox* as well as open pollinated heirloom domesticated varieties are good places to begin in support of breeding efforts. We encourage other academic, governmental, and industry research groups to expand screening of germplasm for strong and variable floral fragrance profiles, as well as include floral fragrance in assessments of sunflower pollination.

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