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WHEN FLOWERS SMELL FERMENTED: THE CHEMISTRY AND ONTOGENY OF YEASTY FLORAL SCENT IN PAWPAW (*ASIMINA TRILOBA*: ANNONACEAE)

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Floral scent plays important roles in basal angiosperms such as the pantropical woody family Annonaceae. The North American genus *Asimina* (Adans.) (Annonaceae) includes eight species of shrubs and small trees, of which *Asimina triloba* has the broadest and northernmost distribution. We characterized the yeastlike fragrance of these flowers using gas chromatography–mass spectrometry in natural populations in South Carolina. The odors of *A. triloba* and baker's yeast *Saccharomyces cerevisiae* shared ethanol, ethyl acetate, acetic acid, and other compounds but differed in relative amounts of 3-methyl-1-butanol, 3-OH-2-butanone, and butane-2,3-diol. Immature green flowers of *A. triloba* produced sesquiterpenes common to the foliage of many plants. In contrast, sexually mature flowers emitted fermentation volatiles, with additional nitrogenous compounds (androgynoecium) and butanediols (outer corolla) emitted by male flowers. Some compounds were detected only when scent was sampled from at least 10 flowers. Chemical composition was more complex during day than night for immature and female flowers but not for males. Emission rates were fourfold greater in male than female flowers during the day but were comparable at night, perhaps because of overlapping gender expression. The yeasty odor of *A. triloba* is unusual in angiosperms and may serve to attract novel fly and beetle pollinators.

Keywords: acetoin, Diptera, floral signals, plant volatiles, pollination, protogyny.

Introduction

The woody pantropical family Annonaceae, like many early-radiating angiosperm lineages (Gottsberger 1988), is characterized by flowers that frequently are protogynous, strongly scented, thermogenic, and pollinated by beetles (Gottsberger 1989*a*, 1989*b*, 1999; Schatz 1990; Thien et al. 2000). Superficially, pawpaw flowers (*Asimina* Adans.) are typical of Annonaceae, with two whorls each of three petals that form a chamber in some species and feature protogynous floral ontogeny, the presence of a distinctive floral odor, and floral visitation by beetles and/or flies (Kral 1960; Norman and Clayton 1986; Norman et al. 1992). *Asimina* and *Deeringothamnus* are the only temperate genera of this otherwise tropical family, and *Asimina* is composed of eight species of shrubs and small trees distributed along the Atlantic seaboard from Florida to southern Canada and west to Oklahoma (Kral 1960; Brown and Kirkman 1990, pp. 138–139; Wunderlin 1998, pp. 305–307).

Asimina triloba (L.) Dunal, the most widespread species of pawpaw, is found throughout temperate eastern North America along river bottoms and floodplain forests (Willson and Schemske 1980). Its wine red flowers form an open chamber and, when fertilized, develop into banana-like pulpy berries that represent the largest edible fruit native to North America (Exell 1927). The floral phenotype of *A. triloba* changes markedly during floral ontogeny. Flowers open precociously, exhibiting green and apparently odorless immature flowers that enlarge and change color as they mature into the yeast-scented female and male phases, the latter with dehiscent stamens (Godfrey 1988; fig. 1). Other features of the floral biology of *A. triloba* indicate that its yeastlike floral odor and pollination biology in general merit closer attention. Willson and Schemske (1980) measured remarkably low fruit set (<1%) in parts of the range of *A. triloba*, presumably due to severe pollen limitation. *Drosophila*, other flies, and beetles have been identified as the primary floral visitors of *A. triloba* (Robertson 1928; Willson and Schemske 1980). We wish to understand how the unusual floral scent, shape, and color of *A. triloba* might affect pollinator attraction and (in)effectiveness.

This article represents the first stage of a long-term study designed to address this and similar questions concerning the evolution of floral phenotype in pawpaws. Here we characterize the yeasty odor of *A. triloba* flowers using complementary methods of floral headspace collection coupled with gas chromatography–mass spectrometry (GC-MS). First, we determined the chemical composition and detection threshold for floral scent components and compared the results with a "perceptual control" by analyzing the odor of baker's yeast (*Saccharomyces cerevisiae*). If flowers of *A. triloba* smell "yeasty," they should produce odors that are characteristic of yeast fermentation. Second, we collected odor from dissected flower parts and from flowers from different developmental stages in order to identify the source tissues for different odor compounds and track their changes as flowers matured. The presence of organ- or stage-specific odors in flowers of *A. triloba* might indicate specific behavioral functions to be

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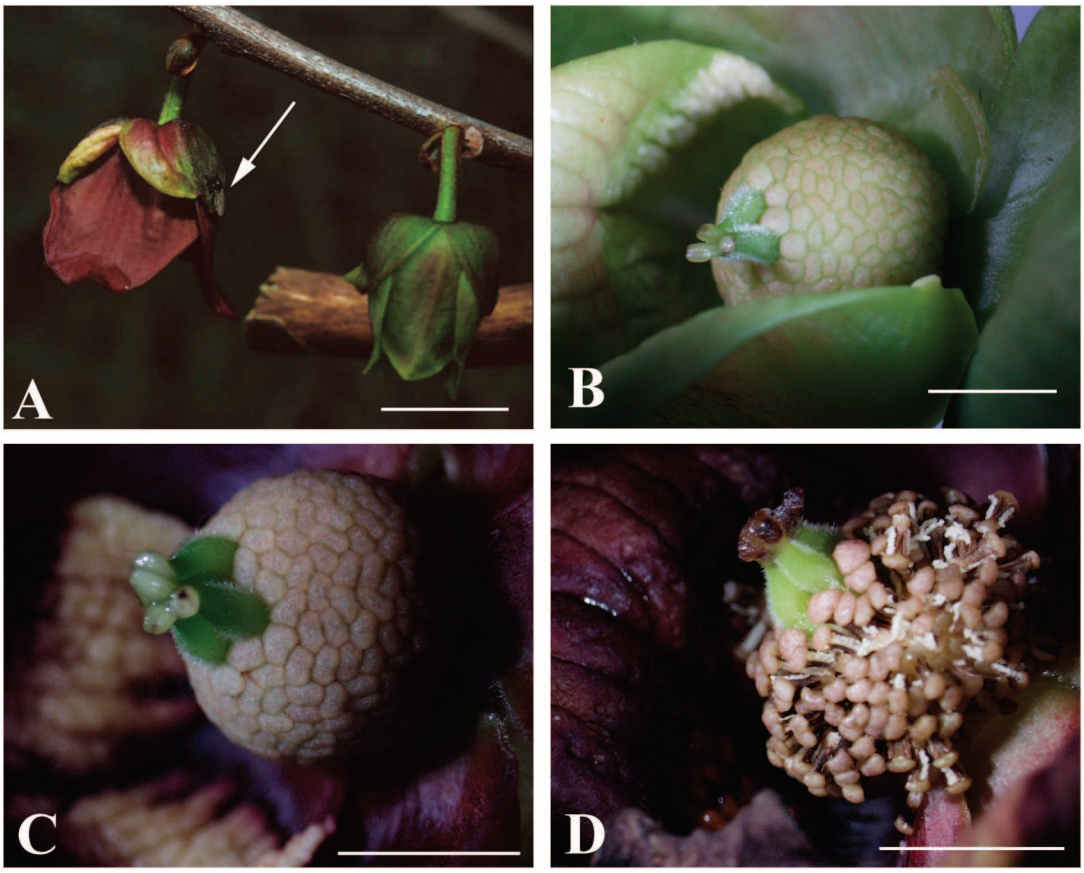

Fig. 1 Floral ontogeny in *Asimina triloba*. *A*, Mature (left) and immature (right) flowers; a small fly has landed on the calyx of the mature flower (arrow) before entering the corolla chamber. Androgynoecia and inner whorl petals are shown for (*B*) immature, (*C*) female-stage, and (*D*) male-stage flowers. Note the change in stigma color and dehiscence of pollen tetrads in *D*. Scale bar in *A* = 1 cm; all others = 0.5 cm.

tested in future pollinator assays (Raguso 2004*a*). Finally, we measured the chemical composition and emission rates of the complete odor blend during day and night in search of temporal patterns that might indicate windows of pollinator activity. This study is the first published analysis of floral odor from *A. triloba*, and it complements earlier investigations on the chemistry of edible pawpaw fruit (Peterson et al. 1982; Shiota 1991; McGrath and Karahadian 1994; Wood and Peterson 1999). The suitability of our methods and clarity of our results provide a template for pursuing more manipulative and comparative studies on reproductive ecology and floral evolution in this fascinating genus.

Material and Methods

Study Site and Floral Ontogeny

Flowering individuals of *Asimina triloba* were studied in late March and early April 2002 in a riparian gallery forest dominated by *Acer rubrum* L., *Celtis laevigata* Willd., *Liriodendron tulipifera* L., *Liquidambar styraciflua* L., *Platanus occidentalis* L., and *Ulmus americana* L. The forest is adjacent to the W. T. "Billy" Tolar boat ramp access on the west bank of the Wateree River along U.S. Route 76/378, 25 mi east of Columbia, Richland County, South Carolina. A second population of *A. triloba* growing in a similar gallery forest was studied for flower organ-specific odors in March 2004 along the west shore of the Congaree River, Columbia, South Carolina. Voucher specimens were deposited at the University of South Carolina's A. C. Moore Herbarium.

Floral ontogeny in *A. triloba* was studied by tagging 30 individual flowers spread across three trees spaced 10 m apart. Flowers were surveyed from April 7–12, 2003, for changes in color and sexual organ maturation. Immature flowers opened yellow green in color and then turned deep maroon as they matured. The female stage was defined by stigma receptivity, as characterized by bubble formation when bathed in 3% H_2O_2 solution (Kearns and Inouye 1993) and the absence of pollen. The male stage was defined by the shedding of pollen (fig. 1).

Fragrance Collection and Characterization

Qualitative analysis and detection threshold. To determine the number of flowers required to detect all scent components, branches with numerous flowers were cut and placed into glass bottles with 10% sucrose solution and transported directly to the University of South Carolina campus. In the lab, flowers of each stage were carefully excised from branches in groups of two, five, 10, and 20 and were sealed within headspace bags. Headspace bags were prepared

by cutting and resealing Reynolds (nylon resin) oven bags to 10×11-cm dimensions with an American International Electric impulse heat sealer, as described by Raguso et al. (2003). After 15 min of equilibration, we exposed a solid phase microextraction (SPME) fiber, coated either with polydimethylsiloxane (PDMS; 100-μm film thickness) or with PDMS-divinylbenzene (DVB; 65-μm film thickness) within this headspace for an additional 15 min. To distinguish volatile artifacts of cutting and wounding from the actual floral bouquet, we collected odor from living, attached flowers in the immature, female, and male stages in groups of two to three (the largest number of flowers logistically possible under natural conditions) in the field. To examine the role of different floral structures in odor emission, 10 flowers were dissected into outer petals, inner petals, and fused androgynoecium; then the structures were placed in respective headspace bags and assayed for floral organ-specific emissions. We tested for temporal patterns in chemical composition by repeating these analyses at ca. 0900–1100 hours (for diurnal emissions) and 1900–2400 hours (for nocturnal emissions). Detection threshold analyses revealed that 10 flower samples were sufficient to identify all volatiles from *A. triloba*, so we arbitrarily decided to standardize for flower number rather than total fresh mass. In this way, cell-autonomous emission of scent compounds from different floral tissues should be comparable in peak size and area to emission from whole flowers. However, it is possible that some trace-level compounds were missed because of insufficient mass of dissected floral tissues. SPME analysis of flower parts is still a relatively new technique, and protocols have not been standardized (Flamini et al. 2003).

Yeast Perceptual Control. In order to test the accuracy and utility of our perception that *A. triloba* flowers smell like yeast, we used SPME to analyze the odor of baker's yeast (*Saccharomyces cerevisiae*). We combined 4.5 g of baker's yeast (Fleischmann's, Fenton, MO) and 0.5 g sucrose with 100 mL of warm water in a glass beaker, covered the solution with aluminum foil, and placed it beneath a warm lamp for 10 min, until vigorous frothing was observed. The SPME fiber was exposed to the headspace for 5 min, injected directly into the injection port, and analyzed according to the same GC-MS parameters as were used for pawpaw flowers.

Emission Rates

Fragrance was collected from three replicates each of two to five intact, attached flowers of immature, female, and male stages using dynamic headspace methods (Raguso and Pellmyr 1998). Two slits were cut into headspace bags, one to admit fresh ambient air and the other to hold a cut Pasteur pipette filled with 100 mg of SuperQ adsorbent (80/100 mesh size, divinylbenzene/ethylvinylbenzene polymer; Levin et al. 2001). Fragrant air was drawn through the cartridge using a Supelco PAS-500 vacuum pump at a flow rate of 250 mL air/min over 4 h. Cartridges were wrapped in aluminum foil and transported in a plastic cooler to the lab, where they were eluted with 3 mL of high-purity hexane (Burdick and Jackson), concentrated to 75 μL with a flow of N_2 gas and supplemented with 16 ng of toluene as an internal standard. Total scent emission rates were calculated from pooled GC-MS total ion chromatogram peak areas for all compounds except ethanol and ethyl acetate, which were concealed beneath the solvent injection spike. Because of small sample sizes, no statistical comparisons were made.

GC-MS Analysis

SPME fibers were desorbed directly within the injection port of a Shimadzu GC17A with a Shimadzu QP5000 quadrupole, electron impact mass spectrometer (MS) as a detector. Single-microliter aliquots of concentrated solvent-eluted samples were injected directly using the Shimadzu AOC20i autoinjector. Analyses were made using splitless injections (at 240°C) on a polar GC column (0.25 mm i.d., length 30 m, film thickness 0.25 μm [EC-WAX, Alltech]), using He_2 as a carrier gas with a flow rate of 1 mL/min and a split ratio of 12 : 1. Oven temperature was held constant at 60°C for 3 min, then ramped up at 10°C per min until reaching 260°C, where it was held for 7 min. Details of the pressure program were described by Levin et al. (2001). Compounds were tentatively identified using Wiley and National Institute of Standards and Technology mass spectral libraries (with more than 120,000 mass spectra) and then were verified through coinjection of known standards. Once volatile compound peaks were identified, manual integration of the peaks was performed. In SPME samples, integrated peak areas were used to calculate relative percent composition for compounds within individual samples. In samples collected using dynamic headspace, integrated peaks were quantified by comparison with the internal standard.

Results

Floral Ontogeny

Flowers matured slowly; 14 of the 30 tagged flowers remained at the same stage (immature, female, or male) throughout the 6-d period of observation. Only two of the 10 flowers that were green (immature) on day 1 turned red after 6 d. The onset of stigma receptivity accompanied floral color change from green to red. Eight of the 10 flowers that were female on day 1 had transitioned to male phase by day 6. Stigmas remained receptive for at least 24 h (and sometimes longer) after anther dehiscence began in these flowers. Six of the 10 flowers that were male at the onset of the study abscised before the end of the observation period.

Characterization of Scent Chemistry

We identified a total of 66 volatile organic compounds from the headspace of baker's yeast and *Asimina triloba* flowers (appendix). Baker's yeast produced 18 volatile compounds, one-third of which were common to scents from male and female flowers of *A. triloba*; these included ethanol and 3-methyl-1-butanol, which represent 16.8% and 63.8% of yeast scent (by GC peak area), respectively. Of the remaining 12 yeast volatiles, six were fatty acids or aromatic compounds unique to baker's yeast, and the other six were aliphatic alcohols, ketones, or esters that were similar in class to those found in the scent of *A. triloba* (appendix). The floral scent of green, immature flowers of *A. triloba* was dominated by γ-terpinene (11.7%) and *E*-β-caryophyllene (49.5%), with several similar monoterpene and sesquiterpene hydrocarbons and the distinctive homoterpene, *E*-4,8-dimethylnona-1,3,7-triene, which also were present in green stems and

young leaves (appendix). In addition to these compounds, female-stage flowers produced distinctive fermentation volatiles (e.g., ethyl acetate, ethanol, acetic acid) and were dominated by 3-OH-2-butanone (also known as acetoin), which contributed 77.9% of the relative peak area in qualitative analysis. Male-stage floral scent added unique nitrogenous compounds (e.g., aldoximes and related nitrile and nitro derivatives) and two isomers of butanediol to the volatiles observed in female flowers (appendix). Female- and male-stage flowers emitted the same absolute amounts of acetoin (not shown), but this compound's relative contribution to male-stage odor (58.4%) was diminished by the addition of the butanediols and nitrogenous compounds. The comparison of cut and intact flowers and use of vegetative controls revealed that sesquiterpene hydrocarbons and their derivative homoterpene (*E*-4,8-dimethyl-1,3,7-nonatriene) were vegetative background odors that were enhanced in wounded tissues. Thus, the actual odor of mature *A. triloba* flowers consists of fermentation compounds, low levels of linalool, nitriles, and monoterpene hydrocarbons.

Threshold of Detection

Threshold effects were observed in scent compounds distinctive to both female and male stages in comparisons of two, five, 10, and 20 flower samples. In many cases, these effects were compound specific (appendix). For example, the mass spectral verification of acetoin is equivocal for fewer than five flowers, whereas *E*-4,8-dimethylnona-1,3,7-triene appeared to saturate the SPME fiber with two flowers (fig. 2). Furthermore, three out of four nitrogenous aldoximes were detected only when odor was sampled from 10 flowers (appendix; fig. 2). All compounds present in 20-flower samples also were detected in 10-flower samples. Scent collections for all subsequent comparisons were performed on 10-flower samples (or on flower parts dissected from 10 flowers) to make most efficient use of a limited number of available flowers.

Organ-Specific Scent Patterns

All tissues (outer petals, inner petals, androgynoecium) of sexually mature flowers produced odor blends with similar overall composition, including fermentation volatiles (fig. 3). Terpenoids present in all floral stages were emitted generally by most flower parts (appendix). Monoterpenes were nearly exclusively emitted by petals, and fermentation volatiles were largely absent from female-stage androgynoecia (fig. 3). Male-stage flowers with mature, dehiscing stamens and visible pollen grains emitted nitrogenous compounds from all tissues, but nitriles and fermentation volatiles were the dominant odors present in dissected androgynoecia (fig. 3). The butanediol compounds unique to male flowers comprised 56.7% of total scent production by outer corolla tissues but were not detected from inner corolla or androgynoecia. The lipoxygenase product *Z*-3-hexen-1-ol, typically associated with wounded foliage, was present at higher proportions in dissected flower parts (especially androgynoecia) than in intact flowers (appendix).

Temporal Patterns in Fragrance Composition

There were striking differences in the number and identity of scent compounds produced in green and female-stage flowers during day versus night. Green, immature flowers produced 31 scent compounds during the day that were absent at night. Eighteen of these day-specific compounds were sesquiterpenoids, and the remainder included four fermentation products that were emitted more abundantly from mature flowers (appendix). Similarly, female-stage flowers produced twice as many compounds during day (28) than night (14), but no one class of compounds was disproportionately represented. Male-stage flowers produced similar numbers of compounds during day (35) and night (36); the major differences were compound specific (appendix). Male-stage flowers emitted two- to sixfold more acetoin and butanediols during day than night, but surprisingly, *E*-β-caryophyllene emissions were eightfold greater at night.

Emission Rates

The mean emission rates for green, immature flowers were negligible (<15 ng/flower/h) during day and night, without obvious temporal rhythm (fig. 4). Mean scent emissions were eight- to 15-fold greater in female-stage flowers than in immature flowers during day and night, with moderately greater emissions at night (fig. 4). Male-stage floral scent emissions were dramatically greater and substantially more variable (mean ± SEM = 658.2 ± 425.8 ng/flower/h) than those of other ontogenetic stages during the day but were comparable to those of female-stage flowers at night (fig. 4).

Discussion

Characterization of a Novel Floral Odor

The floral odor of *Asimina triloba* is unusual among angiosperms. The wine red, spring-blooming flowers of *A. triloba* attracted the notice of early botanists because of their smell rather than their visually inconspicuous flowers. Delpino (1874) grouped the flowers of *A. triloba* among the "odori graveolenti" (stinking or unpleasant odors), with specific reference to fermentation, whereas Kerner von Marilaum (1895) placed *A. triloba* among the "indoloid" floral odors, putatively derived from decomposing albuminoid or nitrogenous substances. Kral (1960), the monographer of *Asimina*, compared the floral odor of *A. triloba* to that of decaying meat. Our chemical analyses confirm that the fragrance of *A. triloba*, at least in South Carolina, is composed of many of the same fermentation products emitted by baker's yeast (acetic acid, ethyl acetate, ethanol, 3-methyl-1-butanol) plus amino acid–derived aldoximes and nitriles. The latter nitrogen-bearing compounds are uncommon among flowering plants and often are associated with night-blooming, hawkmoth-pollinated flowers (Kaiser 1993; Knudsen and Tollsten 1993; Raguso et al. 2003). It is unclear what role (if any) these compounds play as male-stage-specific volatiles in *A. triloba*. However, we did not detect the dimethyl oligosulfides that characterize the microbial decomposition of meat and universally constitute the odors of carrion-mimicking flowers (Kite and Hetterschieid 1997; Stensmyr et al. 2002). We have identified these compounds in other flowers, fungi, and durian fruit using the same methods (Raguso 2004*b*; R. A. Raguso, unpublished data) and thus are confident that they were not present in *A. triloba*.

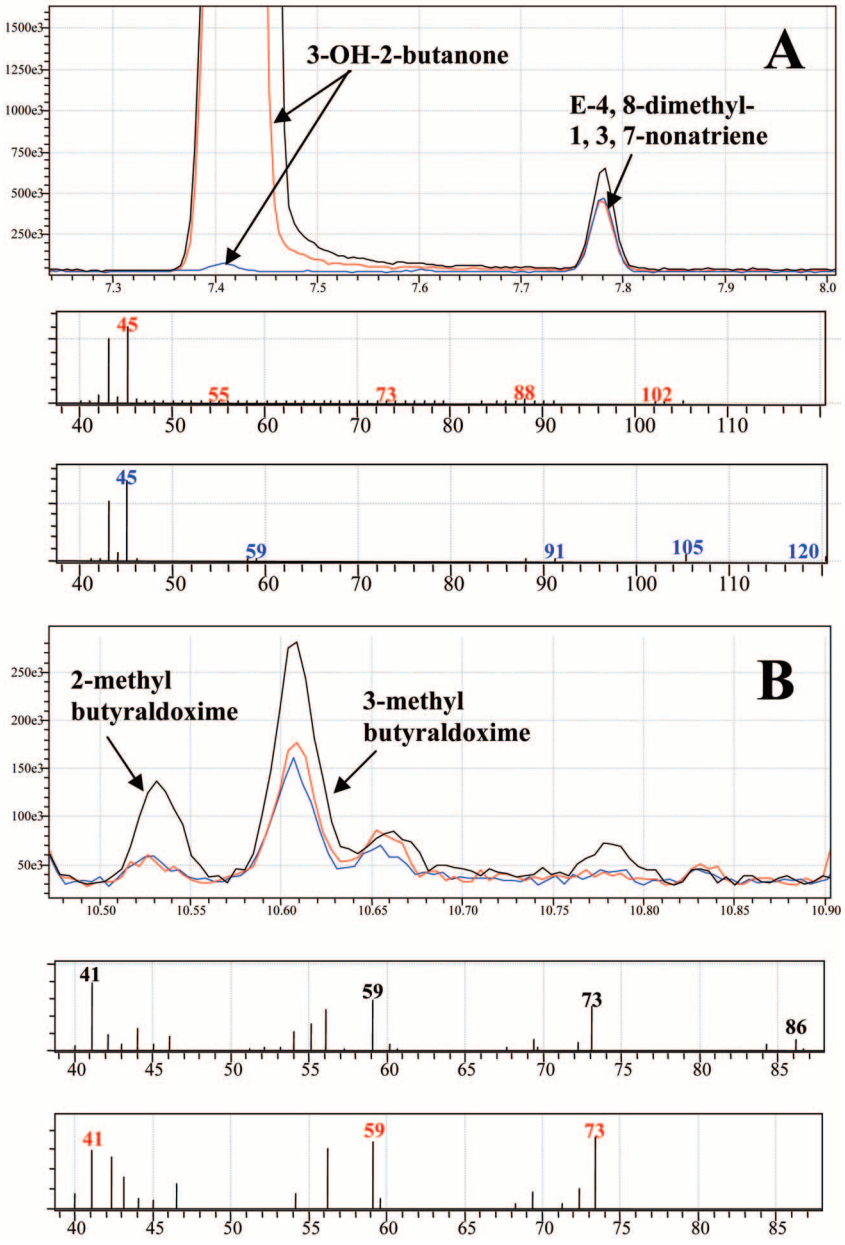

Fig. 2 Stacked solid phase microextraction–gas chromatography traces showing threshold effects for different floral volatiles. The vertical axes in traces and spectra represent abundance of sample. The horizontal axis is time (min) in the gas chromatography traces and mass per unit charge (m/z) for the mass spectra. In each chromatogram, the upper (black) trace is from 10 flowers, the middle (red) trace is from five flowers and the lower (blue) trace is from two flowers. In *A*, the mass spectra for 3-OH-2-butanone are identical for 10 and five flowers (upper spectrum), but critical information is lacking from the lower spectrum, taken from two flowers. *B* illustrates a similar result for 2-methyl butyraldoxime, in which the m/z 86 ion is missing from the lower spectrum (two and five flowers). No threshold was observed for *E*-4,8-dimethyl-1,3,7-nonatriene (*A*) or 3-methyl butyraldoxime (*B*).

The dioxygenated compounds that dominate the odor of mature *A. triloba* flowers (butane-2,3-diol and acetoin) also are rare in floral scents (Knudsen et al. 1993) and have been identified in very few flowers. Buttery et al. (1984) identified these compounds as minor components (2%–3%) of floral headspace from red clover (*Trifolium pratense*, Fabaceae), but they were present in the headspace of baker's yeast and are commonly encountered as microbial metabolites emitted by rotting fruit (Stensmyr et al. 2003*b*). Indeed, butane-2,3-diol and acetoin are attractive to *Drosophila melanogaster* in behavioral bioassays (Stensmyr et al. 2003*b*), and several species in the *D. melanogaster* and *Drosophila yakuba* species complexes have olfactory receptor neurons that are sensitively tuned to these compounds (Stensmyr et al. 2003*a*). Thus, it is possible that the primarily dipteran floral visitors to *A. triloba* are attracted via olfactory mimicry of rotting fruit. The effectiveness of such flies as pollinators in this system remains to be determined experimentally.

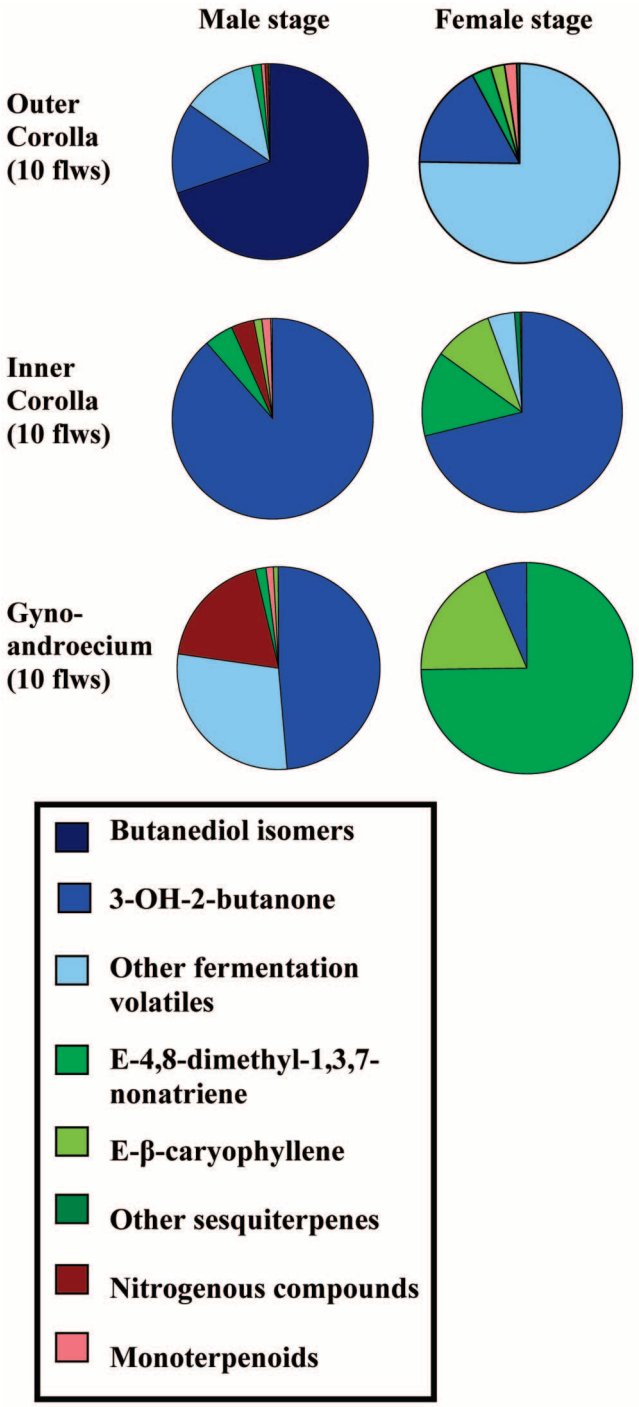

Fig. 3 Tissue-specific patterns of floral scent composition, expressed as relative proportions of total scent emitted from specific tissues and ontogenetic stages. Color-coded categories (see legend) represent prominent compounds (e.g., acetoin) or biosynthetic classes (e.g., sesquiterpenes); see appendix for details. Note that pie wedges represent relative, not absolute, amounts of scent. The emissions of female- and male-stage outer corollas were nearly identical, but the addition of large amounts of butanediols in males changes the relative proportions.

Dynamic Aspects of Floral Phenotype

The flowers of *A. triloba* are protogynous, with immature, female, and male stages that last several days each and overlap to some extent. This pattern contrasts with the nonoverlapping gender expression documented for the related *Asimina pygmaea* by Norman and Clayton (1986) and for several Neotropical *Annona* species by Gottsberger (1989*a*). Our observations of floral ontogeny in *A. triloba* concur with those of Willson and Schemske (1980), who showed that individual flowers remain open up to 2 wk, that stigmas remain shiny (receptive) for 4–6 d, and that pollen sometimes dehisces before stigmas turn brown. Future studies should test the extent to which variation in floral developmental rate is influenced by ambient temperature and, perhaps, tree size (as an indicator of potential resource allocation; Willson and Schemske 1980). One clear result of our study is that the floral scent of *A. triloba* varies temporally (with floral maturation) and spatially (across different floral organs). If scent had been collected from lumped samples of flowers at different stages of maturity, we would not have detected such dimensionality. Flowers of *A. triloba* emit background levels of terpenoids (particularly sesquiterpene hydrocarbons) throughout their development, but sexually mature flowers are more strongly scented than immature flowers, and their fermented odors are chemically more complex. Generally, only male stage flowers produce nitrogenous compounds, which are most abundant in the androgynoecium (fig. 3). Additional assays will be needed to test whether nitriles and aldoximes are pollen-specific odors in *A. triloba* and thus whether trace levels of these compounds in petal whorls are artifacts of pollen contamination.

Sexually mature flowers are dominated by acetoin, and male-stage flowers produce the structurally similar butane-2,3-diol, which is present only in the outer petal whorl (fig. 3; appendix). Acetoin and butanediol are metabolites of the same fermentation pathways in soil bacteria (Turinsky et al. 2000; Pasteris and de Saad 2005). Despite the small relative amounts of these compounds in our baker's yeast analysis (appendix), they were found in significant amounts in a study in which apple cider (rather than sucrose solution) was inoculated with baker's yeast (Valles et al. 2005). These patterns, combined with the rarity of fermentation odors in angiosperm flowers, raise the possibility that microbial symbionts might be responsible for the production of fermentation volatiles in flowers of *A. triloba*. The inner whorl petals have raised yellow corrugations on their adaxial surfaces, which produce liquid secretions in female- and male-stage flowers (fig. 5). Similar structures were described from *Asimina obovata* by Norman and Clayton (1986), who showed their composition to be at least 50% carbohydrate by dry mass and that their secretions are eaten by beetles. In *A. triloba*, corrugated tissues and their secretions may provide suitable domatia for floral yeasts or bacteria, suggesting that fermentation volatiles should be restricted to inner whorl petals. This prediction is not strictly upheld by our data, which indicate the presence of fermentation odors in all flower parts (fig. 3). However, ethanol, ethyl acetate, and 3-methyl-1-butanol comprise 60%–85% of the total odor emitted by inner whorl petals in female- and male-stage flowers (fig. 3), indicating that corrugated tissues and their secretions deserve

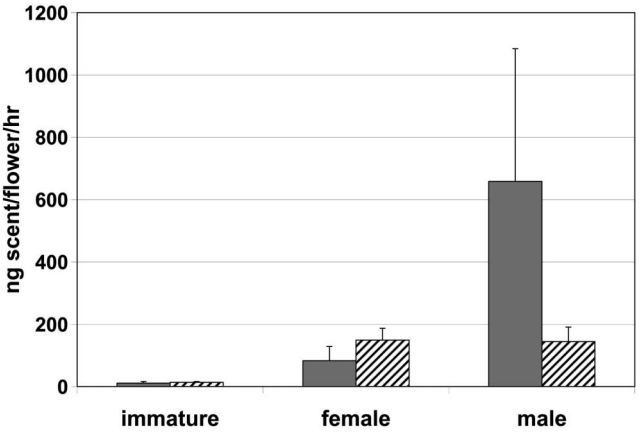

Fig. 4 Mean (±SE, $N = 3$) emission rates of total floral scent from different ontogenetic stages during day (solid bars) and night (hatched bars). Emission rates are expressed as nanograms of scent per flower per hour; collections were over 4-h periods. Note the unusually strong and variable diurnal emissions coinciding with pollen dehiscence in male flowers.

rigorous microbiological investigation. It is possible that odors released by inner whorl petals are absorbed and rereleased by the lipid-rich pollenkitt of dehiscing pollen grains in the adjacent androgynoecium, a phenomenon that was documented in other flowers by Dobson et al. (1990).

Finally, the marked day-night disparity in odor composition observed for immature and female-stage flowers points to several possible sources of variation. Although transitions in floral ontogeny (e.g., immature to female) were gradual during our study, such transitions could have occurred during our diurnal sampling of floral scent. This could explain the small amounts of 3-methyl-1-butanol, acetoin, and acetic acid detected in immature flowers, which typically do not have a fermented odor. Similarly, the unusual variation in odor emission rates of male-stage flowers during the day may indicate heterogeneity in flower age and senescence because this stage may persist for several days. Day-night differences in ambient temperature are unlikely to explain our results because all SPME analyses were performed under constant ambient conditions (20°C) in the laboratory. Furthermore, it is unlikely that the dramatic day dominance of sesquiterpenes in immature and female-stage flowers reflects a diurnal rhythm in their emissions because these compounds are equally abundant in male-stage flowers during day and night (appendix). SPME analyses performed on flowers whose absolute age is known would provide finer resolution of changes in scent chemistry associated with floral maturation in *A. triloba*.

The biological significance of spatial and temporal variation in floral odor is poorly understood because relatively few studies have addressed ontogenetic changes in scent chemistry from the standpoint of pollinator behavior (Miyake and Yafuso 2003). However, a recent study of *Sauromatum guttatum* (Araceae) revealed unexpected differences in fragrance chemistry in different parts of its protogynous trap inflorescences (Hadacek and Weber 2002). These authors and others (Terry et al. 2004) suggest that spatiotemporal variation in floral scent indicates multiple roles for odor in attracting pollinators and modulating their behavior once they arrive. Behavioral assays in which flower parts of different ontogenetic stages are exchanged to alter odor presentation (Patt et al. 1995) could be used to test the importance of dynamic odor phenotypes in the pollination of *A. triloba*.

Why Should Some Flowers Smell like Yeast?

Comprehensive surveys of floral scent chemistry across numerous angiosperm families (Knudsen et al. 1993), including several hundred species of orchids (Kaiser 1993), indicate that fermentation volatiles are unusual fragrance constituents and are more likely to be detected in rotting fruit or sap (Williams et al. 1981) than in flowers. Yeastlike odors are known to attract a wide variety of animals, including several orders of insects (Guerenstein et al. 1995; Landolt and Higbee 2002; Reed and Landolt 2002) and vertebrates (Levey 2003; Sanchez et al. 2004). One recent study reported the partial fermentation of strongly scented nectar in *Agave palmeri* (Agavaceae) (Raguso 2004*b*), which is pollinated by a number of animals, including bats (Slauson 2000). Otherwise, there are few published chemical analyses of flowers with yeastlike scents. Several examples of apparently yeast-scented flowers pollinated by nonflying mammals have been described from the Southern Hemisphere (Rourke and Wiens 1977; Carpenter 1978; Wiens and Rourke 1978; Johnson et al. 2001). The

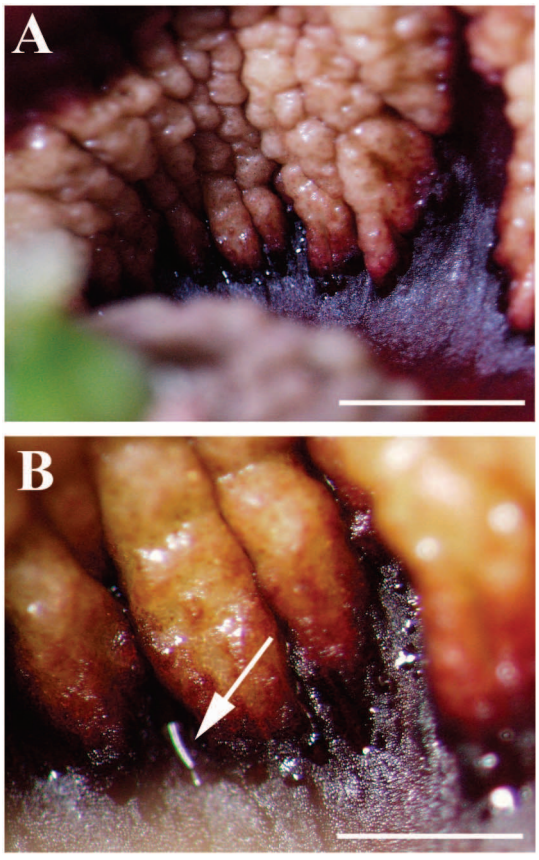

Fig. 5 Inner whorl corrugated tissues in sexually mature flowers, shown distal to the androgynoecium (*A*) and close up (*B*). Note the glistening secretions accumulating between the folds of corrugated tissue (arrow). Scale bars = 0.3 cm (*A*) or 0.1 cm (*B*).

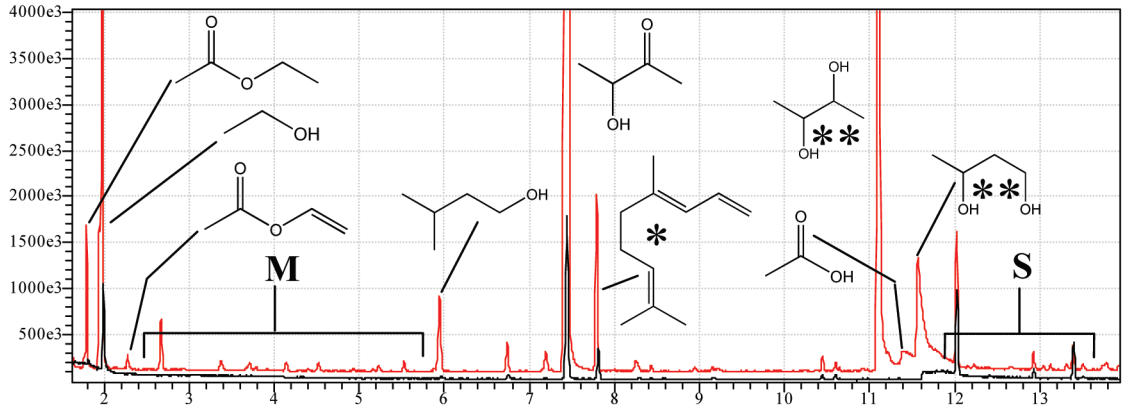

Fig. 6 Solid phase microextraction–gas chromatography traces of scent from 10 male-stage *Asimina triloba* flowers, contrasting the performances of the 65-μm polydimethylsiloxane-divinylbenzene (PDMS-DVB) fiber (upper, red) and more conventional 100-μm PDMS fiber (lower, black). Vertical axis is relative abundance; horizontal axis is time (min). The structures of major fermentation compounds and *E*-4,8-dimethyl-1,3,7-nonatriene (asterisk) are shown alongside their gas chromatography peaks, whereas those of monoterpenes (*M*) and sesquiterpenes (*S*) are too numerous to include here (see appendix). Note the poor representation of most fermentation volatiles (especially the butanediols; two asterisks) in headspace trapped by the 100-μm fiber (lower trace).

majority of these cases involve rodent pollination of Proteaceae shrubs, whose flowers secrete copious amounts of yeast-scented nectar. It is not clear whether these odors are innately attractive to these mammals, are easily learned in association with food, overlap with other dietary components, or mimic the odors of the animals themselves.

However, nonflying mammals are more likely to be involved with *A. triloba* fruit dispersal than with its pollination biology (Janzen and Martin 1982). It is important to consider odor in the appropriate context in which it is presented by flowers, including flower color and form, time of day and season, height above ground, and the presence of a nutritious reward (Raguso 2004*a*). For fruit flies and some beetles, fermenting odors elicit strong physiological and behavioral responses (Kim et al. 1998; Nout and Bartelt 1998; Cadieu et al. 1999; Stensmyr et al. 2003*b*). Trapping experiments using flowers concealed beneath white- or red-dyed cheesecloth suggest that both the odor and red color of *A. triloba* flowers are attractive to these insects (M. L. Zjhra, C. A. Ley, and R. A. Raguso, unpublished data). The combination of yeasty odor and wine red color that characterizes flowers of *A. triloba* contrasts markedly with the green yellow coloration and sweet fruity odor blends of aliphatic esters, benzenoid compounds, terpenoids, and naphthalene that characterize beetle-pollinated species from three Neotropical genera of Annonaceae (Jürgens et al. 2000). More intensive phylogenetic sampling is required to determine how frequently flowers with fermented odors have evolved in the Annonaceae.

Methodological Considerations

Novel odors present methodological challenges. The unusual composition and low emission rates of odor in *A. triloba* required methodological modifications that may be useful to botanists studying plants whose floral scents present similar challenges. Several compounds emitted by *A. triloba* flowers were not detected in samples numbering fewer than 10 flowers, whereas other compounds were present when two, five, or 10 flowers were sampled (appendix). Samples collected from 20 flowers did not differ in composition from those collected from 10 flowers (data not shown). Our results show that the relative proportions of specific compounds may change dramatically with floral mass, and therefore collections made from single flowers may underestimate fragrance complexity (appendix; fig. 2). Additionally, it is more difficult to identify unknown compounds when GC peaks are small and mass spectra are incomplete and may be masked by the baseline or ambient contaminants (fig. 2). These observations indicate the utility of dose-response studies in which scent is collected from increasing numbers of flowers until no new compounds are identified. Finally, our pilot data revealed that conventional dynamic headspace trapping and solvent elution (Raguso and Pellmyr 1998) were insufficient methods for the study of scent in *A. triloba* because the early GC peaks (ethanol, ethyl acetate) were concealed beneath the hexane solvent injection spike. SPME provided a sensitive, solvent-free alternative method for qualitative odor identification (Agelopoulos and Pickett 1998). We directly compared the standard 100-μm PDMS fiber with a 65-μm PDMS-DVB fiber recommended by the manufacturers (Supelco, Eighty Four, PA) for amine odor analysis. The deficiency of the standard PDMS fiber in trapping fermentation volatiles (fig. 6) suggests that the apparent rarity of these compounds in the floral scent literature may be a methodological artifact. We propose that the 65-μm PDMS-DVB fiber should be employed along with the PDMS fiber in all floral headspace analyses using SPME.

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Appendix

Floral Scent Chemical Data

Table A1

Floral Scent Chemical Data Presented for Different Ontogenetic Stages, Flower Parts, Time of Day, and Number of Flowers

		No. flowers needed for detection			Green % total peak area				Female % total peak area					Male % total peak area					
Compound name	EC-WAX (RT min)	Green	Female	Male	10 flowers (day)	10 flowers (night)	Total corolla	GA	10 flowers (day)	10 flowers (night)	Outer corolla	Inner corolla	GA	10 flowers (day)	10 flowers (night)	Outer corolla	Inner corolla	GA	Baker's yeast
Aliphatic compounds (20):																			
Ethyl acetate	1.80	na	5	2	…	…	…	…	0.65	0.27	0.42	3.14	2.35	0.66	1.04	1.12	23.79	5.02	1.27
Ethanol	1.98	na	5	2	…	…	…	…	13.80	8.97	14.26	57.78	…	5.75	1.11	5.37	46.60	5.79	16.75
Vinyl acetate	2.29	na	5	2	…	…	…	…	0.16	…	0.39	0.28	…	0.10	0.29	…	…	1.20	…
Isobutyl acetate	2.41	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	0.09
Ethyl butyrate	2.65	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	0.23
41(70), 45(100), 57(97), 73(8), 87(8), 102(22)	2.87	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	0.06
Isobutyl alcohol	3.74	na	5	5	…	…	…	…	0.55	0.18	0.49	1.64	…	0.09	…	0.26	1.74	11.09	8.75
Isoamyl acetate	4.26	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	2.07
3-methyl-1-butanol	5.96	10	2	2	0.28	…	1.11	10.20	1.37	1.58	1.40	3.13	3.84	1.93	3.06	7.84	15.39	21.85	63.83
Ethyl n-caproate	6.45	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	0.24
3-OH-2-butanone	7.45	10	2	2	0.35	…	1.60	…	77.86	83.04	75.14	4.25	…	58.42	49.79	12.12	0.29	29.30	0.82
Ethyl heptanoate	9.71	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	0.05
2,3-butanediol	11.10	na	na	2	…	…	…	…	…	…	…	…	…	21.94	6.37	56.73	…	…	…
Acetic acid	11.22	2	2	2	9.61	…	…	…	1.14	0.33	…	…	…	1.01	1.58	…	1.11	3.08	0.63
Butanediol isomer	11.57	na	na	2	…	…	…	…	…	…	…	…	…	3.69	2.06	12.58	…	…	…
Isobutyric acid	12.5	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	1.72
Butyric acid	13.35	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	0.21
2-methyl butyric acid	13.67	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	0.43
3-methyl butyric acid	13.73	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	0.9
Aromatic compounds (2):																			
2-phenyl ethyl acetate	14.5																		0.04
2-phenyl ethanol	15.5																		1.91
Lipoxygenase products (1):																			
Cis-3-hexen-1-ol (wound)	8.93	2	2	5	…	…	…	…	…	…	…	…	3.62	0.08	…	…	…	1.36	…
Monoterpenes (15):																			
***α*-pinene**	2.67	5	2	2	0.59	…	…	…	0.36	1.34	1.13	3.46	…	0.43	0.72	0.04	0.39	…	…
40(19), 41(37), 43(37), 53(14), 57(24), 72(10), 77(47), 91(70), 92(30), **93**(100), (136)	2.72	5	na	na	0.14	…	…	…	…	…	…	…	…	…	…	…	…	…	…

Table A1

(*Continued*)

Compound name	EC-WAX (RT min)	No. flowers needed for detection: Green	Female	Male	Green % total peak area: 10 flowers (day)	10 flowers (night)	Total corolla	GA	Female % total peak area: 10 flowers (day)	10 flowers (night)	Outer corolla	Inner corolla	GA	Male % total peak area: 10 flowers (day)	10 flowers (night)	Outer corolla	Inner corolla	GA	Baker's yeast
Camphene	3.34	5	5	2	0.94	1.96	3.96	…	0.01	…	0.10	1.14	…	0.11	0.28	…	0.15	…	…
β-pinene	4.14	5	2	2	0.09	…	…	…	0.06	0.30	0.19	1.04	…	0.05	0.26	…	…	…	…
δ-3-carene	4.93	5	10	5	0.72	0.57	…	…	0.01	…	0.16	…	…	0.01	0.09	…	…	…	…
β-myrcene	5.22	5	2	2	1.64	0.93	1.32	…	0.04	0.06	0.10	…	…	0.06	0.38	…	…	…	…
α-terpinene	5.53	5	2	2	3.37	2.22	1.40	17.69	0.04	…	0.04	0.29	…	0.09	0.94	…	…	…	…
DL-limonene	5.87	2	na	na	0.96	…	…	…	…	…	…	…	…	…	…	…	…	…	…
41(28), 44(26), 59(10), 77(46), 79(25), 91(65), 93(100), (136)	6.06	5	na	na	(trace)	…	2.88	…	…	…	…	…	…	…	…	…	…	…	…
γ-terpinene	6.74	2	2	2	11.65	8.50	7.99	8.86	0.18	0.12	0.30	1.32	…	0.42	1.95	…	0.07	…	…
Trans-β-ocimene	6.84	5	na	na	(trace)	…	…	…	…	…	…	…	…	…	…	…	…	…	…
Para-cymene	7.18	5	2	2	3.35	2.72	0.55	…	0.16	0.07	0.05	0.41	…	0.09	0.30	…	0.21	…	…
α-terpinolene	7.38	5	na	na	(trace)	…	…	…	…	…	…	…	…	…	…	…	…	…	…
40(33), 41(69), 43(12), 44(17), 46(11), 53(25), 55(44), 57(14), 59(17), 77(37), 79(30), 91(49), 93(100), 121(96), 136(14)	10.29	10	10	na	0.09	…	…	…	0.03	…	…	…	…	…	0.17	…	…	…	…
Linalool	11.22	5	2	2	1.49	…	…	…	0.06	…	0.09	…	…	0.72	1.23	0.43	0.32	0.77	…
Nitrogenous compounds (8):																			
2-methyl butane nitrile	3.79	na	na	2	…	…	…	…	…	…	…	…	…	0.05	0.05	0.04	0.67	3.45	…
3-methyl butane nitrile	4.53	na	na	2	…	…	…	…	…	…	…	…	…	0.22	0.07	0.14	0.79	15.35	…
Nitro-2-methyl butane	8.00	na	na	2	…	…	…	…	…	…	…	…	…	0.02	0.02	0.02	0.17	…	…
Nitro-3-methyl butane	8.25	na	na	2	…	…	…	…	…	…	…	…	…	0.11	0.05	…	1.26	0.82	…
2-methyl butyl aldoxime	10.52	na	na	2	…	…	…	…	…	…	…	…	…	0.11	…	…	0.13	…	…
3-methyl butyl aldoxime	10.60	na	na	10	…	…	…	…	…	…	…	…	…	0.17	…	0.51	0.39	…	…
2-methyl butyl aldoxime	10.77	na	na	10	…	…	…	…	…	…	…	…	…	0.06	…	0.72	…	…	…
3-methyl butyl aldoxime	11.10	na	na	10	…	…	…	…	…	…	…	…	…	…	…	…	0.27	…	…
Sesquiterpenes and derivatives (21):																			
Trans-4,8-dimethyl-nona-1,3,7-triene	7.79	10	2	2	1.33	…	…	…	0.84	0.48	2.94	12.81	72.00	0.88	0.92	1.93	4.77	…	…
α-cubebene	10.10	5	na	na	0.09	…	…	…	…	…	…	…	…	…	0.10	…	…	…	…
α-ylangene	10.46	5	na	na	0.09	…	…	…	…	…	…	…	…	…	…	…	…	…	…
α-copaene	10.60	5	2	10	0.53	…	…	…	(trace)	…	…	…	…	(trace)	0.41	…	…	…	…
β-bourbonene	10.97	5	5	10	0.16	…	…	…	0.02	…	…	…	…	0.02	0.38	…	…	…	…
Isocaryophyllene	11.72	5	na	na	0.31	…	…	…	…	…	…	…	…	…	0.48	…	…	…	…
β-elemene	11.90	5	na	na	0.08	…	…	…	…	…	…	…	…	…	…	…	…	…	…

41(27), 43(33), 44(12), 45(100), 53(11), 55(13), 77(12), 82(14), 91(19), 105(27), 161(35)	11.94	5	na	na	(trace)	…	…	…	…	…	…	…	…	…	…	…	…	…	…
Trans-β-caryophyllene	12.05	2	2	2	49.45	69.11	63.74	43.38	1.87	2.36	2.22	8.66	18.20	1.96	19.21	0.17	1.37	0.93	…
41(100), 53(18), 55(17), 67(22), 69(37), 77(20), 79(33), 80(12), 81(20), 91(36), 93(27), 105(19), 107(11), 120(46)	12.22	2	2	10	0.86	…	…	…	0.03	…	…	…	…	0.02	0.31	…	…	…	…
41(59), 42(17), 44(29), 53(13), 57(11), 69(51), 77(14), 81(66), 91(32), 105(100), 119(42), 161(98)	12.53	5	na	na	(trace)	…	…	…	…	…	…	…	…	…	0.06	…	…	…	…
40(17), 41(100), 42(25), 43(45), 51(21), 53(39), 55(37), 56(26), 65(12), 67(34), 69(19), 77(34), 78(10), 79(43), 80(19), 81(38), 91(52), 92(35), 93(40), 105(45), 107(19), 119(18), 133(10)	12.64	5	na	na	0.16	…	…	…	…	…	…	…	…	…	…	…	…	…	…
α-humulene	12.92	2	2	2	3.87	5.30	3.20	…	0.20	0.21	0.15	…	…	0.22	2.19	…	0.12	…	…
41(100), 42(14), 43(63), 53(23), 55(45), 56(14), 57(29), 59(12), 65(1), 67(22), 69(24), 71(11), 77(33), 79(47), 81(37), 91(53), 92(19), 93(53), 94(16), 95(11), 105(62), 106(12), 107(10), 119(41), 120(12), 133(17), 161(59) (204)	13.12	2	2	10	0.61	…	…	…	0.08	…	…	0.24	…	0.07	0.48	…	…	…	…
Germacrene-D	13.39	2	2	2	3.92	8.69	7.71	…	0.36	0.68	…	0.42	…	0.31	2.44	…	…	…	…
41(51), 45(23), 55(28), 60(94), 68(12), 73(21), 79(19), 81(45), 91(38), 93(10), 105(19), 107(15), 133(34), 134(19), 161(100), 204(11)	13.45	5	na	na	0.24	…	…	…	…	…	0.40	…	…	…	0.14	…	…	…	…

Table A1

(*Continued*)

Compound name	EC-WAX (RT min)	No. flowers needed for detection			Green % total peak area				Female % total peak area					Male % total peak area					Baker's yeast
		Green	Female	Male	10 flowers (day)	10 flowers (night)	Total corolla	GA	10 flowers (day)	10 flowers (night)	Outer corolla	Inner corolla	GA	10 flowers (day)	10 flowers (night)	Outer corolla	Inner corolla	GA	
41(100), 43(50), 65(17), 67(20), 69(24), 77(25), 79(45), 80(15), 81(21), 91(44), 93(37), 94(41), 96(19), 105(65), 106(17), 107(29), 120(32), 161(40)	13.52	5	na	na	0.56	…	…	…	…	…	…	…	…	…	…	…	…	…	…
41(100), 46(11), 53(20), 55(25), 67(26), 73(13), 77(29), 79(39), 81(27), 91(32), 93(67), 94(14), 105(28), 107(39), 119(10), 121(57) (133)	13.67	5	na	na	0.15	…	…	…	…	…	…	…	…	…	…	…	…	…	…
δ-cadinene	13.92	2	2	10	0.72	…	0.91	…	0.04	…	0.04	…	…	0.08	0.55	…	…	…	…
γ-cadinene	13.96	2	2	10	0.51	…	…	…	0.03	…	…	…	…	0.04	0.34	…	…	…	…
α-cadinene	14.33	5	na	na	(trace)	…	…	…	…	…	…	…	…	…	…	…	…	…	…
Total compounds = 66	…	…	…	…	40	9	13	5	28	14	20	16	4	35	36	16	21	12	18

Note. Scent compounds written in bold were verified using authentic standards; other names represent best fits (>90%) from mass spectral library searches (see "Material and Methods"). Unknowns are listed by ion fragments in ascending order of mass/unit charge, with relative abundance in parentheses (%) and the putative molecular ion in brackets. na = not present at given ontogenetic stage, GA = gynoandroecium.

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