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Chapter 10

Quantitative Assessment of Nectar Microbe-Produced Volatiles

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Nectar microbe-produced volatiles can contribute to floral blends and modify pollinator preference for flowers. Identifying and describing the compounds that may underlie this effect is a key goal. Semi-quantitative or quantitative data is often critical for chemical ecology investigations, given that biotic responses (i.e., insects or plants) can fluctuate with concentration, relative ratios, or composition of the emitted volatile profiles. However, field-based, *in situ* microbial volatile detection and quantification is difficult due to the small scale of the nectar microhabitat. Laboratory-based inoculations of bulk synthetic nectars are useful for screening weakly-abundant and difficult to analyze volatiles, which may be crucial in eliciting responses. Despite the limitations of this approach, it allows rapid identification of target molecules for further validation with bioassays. Targeted analytical methods to identify or quantify semiochemicals may then be developed and validated for field tests. Here, we report the first quantitative assessment of microbe volatile emission in laboratory-based tests with synthetic nectar. We also review briefly solid-phase microextraction collection and gas-chromatography data interpretation. Nectar microbe interactions with plants and insects offer opportunities for agricultural improvement, and a selection of potential uses are highlighted.

Introduction

Visitor-mediated pollination is necessary or beneficial for the majority of the world's food crops, yet reports indicate populations of managed bees are declining worldwide (1, 2). Rapidly deployable and flexible solutions to enhance pollination despite limited pollinator abundance and poor pollinator health are needed. Recent studies have suggested the importance of the nectar microbiome in plant-pollinator interactions (3–6). Tritrophic (plant-insect-microbe) interactions promise opportunities for agricultural improvement and a few potential applications are highlighted, though this chapter is not the first to outline their potential utility (7). Here, we offer a chemo-centric discussion of the interactions between nectar-inhabiting microbes, plants and pollinators, with a focus on the methods required to quantify volatile chemicals.

Materials and Methods

Microbes and Inoculated Solutions

Four microorganisms isolated from California wildflowers were selected for study and include the specialist yeast, *Metschnikowia reukaufii* and generalists, *Aureobasidium pullans*, a fungus, and the bacteria *Neokomagataea* sp. and *Asaia astilbes* (Genbank IDs MF319536, MF325803, MF340296, and KC677740, respectively). Sterile synthetic nectar (0.3% w/v sucrose; 0.6% w/v each: glucose and fructose; 0.1 mM each: glycine, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline, L-serine), designed to mimic floral nectar of bee-pollinated flowers was inoculated with cells from actively growing subcultures (8, 9). Cells were grown on selective plate media (R2A agar with 100 mg L⁻¹ cycloheximide for bacteria, YM agar with 100 mg L⁻¹ chloramphenicol for fungi) for 4 d at 29 °C, then suspended in sterile synthetic nectar to create microbial stock solutions. Cell density in stock solutions was determined via UV absorbance (600 nm) for bacteria and a cell counter (Bio-Rad Laboratories, Inc, Hercules, CA) for yeast. Appropriate dilutions for stock solutions were delivered such that final cell densities in 20 mL synthetic nectar were 10³ cells μL⁻¹ (corresponding to 0.4 A_{UV600} for bacteria). Samples and sterile controls were incubated at 29 °C in sealed 118 mL Mason jars (n=3 replicates of each microbial species, *M. reukaufii*, *A. pullans*, *Neokomagataea*, and *Asaia* and n=4 sterile controls). Lids were modified to accommodate two sampling ports by fitting gas chromatograph septa into pre-drilled holes.

Volatile Quantitation

Mixed stock solutions of identified compounds were prepared in acetonitrile and used to prepare calibration standards under identical sterile conditions as microorganism inoculation samples: Stock solutions was spiked into 20 mL sterile synthetic nectar that had been previously incubated at 29 °C and analyzed

as described below. Calibration standards, prepared immediately before analysis, contained less than 0.1% v/v acetonitrile (carrier solvent) and spanned eight orders of magnitude (0.1 ng mL⁻¹ – 10 mg mL⁻¹).

Analysis by GC-MS

Headspace volatiles from inoculated microbes and prepared calibration standards were collected onto solid-phase microextraction fibers (SPME; Supelco, Bellefonte, PA; 50/30 μ m, 2 cm, divinylbenzene/ carboxen/ polydimethylsiloxane). Inoculated synthetic nectars were sampled at intervals of 0, 48 and 96 hr ($\pm$ 0.4 h). Standards were prepared immediately before analysis. Jar lids were vented under sterile conditions immediately prior to volatile collection, to allow dissipation of volatiles accumulated between sampling intervals and then sealed and returned to the incubator, set to 29 °C. Volatile collections used the following fiber parameters: accumulation of volatiles in the newly closed container, 15 min; exposure of fiber to adsorb volatiles, 15 min; storage time of volatiles on fiber, $\leq$ 1 min; and, thermal desorption of volatiles in gas chromatograph inlet, 6 min. The adsorbed volatiles were thermally desorbed in splitless mode onto an Agilent 7890A GC coupled to a quadrupole 5975C MSD in electron ionization mode (Palo Alto, CA) outfitted with a J&W Scientific (Folsom, CA) 60 m DB-Wax column. Volatiles were analyzed using parameters identical to those previously described except flow was set to 3 mL min⁻¹ (10). Retention indices were calculated using a homologous series of n-alkanes and were used to assist with initial identification. Identities were further confirmed by comparison to retention times and fragmentation patterns of standards. Compound identities not verified with a commercial standard were marked as tentatively identified and are not discussed here. Peaks identified as background from the containers, fibers, column, and synthetic nectar that were found in sterile controls and blanks were not considered.

Results

Microorganism volatile composition differed between species (Table 1). Volatiles were detectable immediately after inoculation (day 0, 30 min after inoculation into synthetic nectar with 15 min each permeation and exposure time), further diverging after 2 and 4 days of growth (11). Alcohols, esters, and ketones were more abundant in fungal solutions, while the volatile 2,5-dimethylfuran was characteristic of bacteria. All four microbes produced *n*-hexanol. The specialist nectar yeast *M. reukaufii* was characterized by esters, including ethyl butyrate, 2-methylpropyl acetate and 3-methylbutyl acetate, the alcohols 2-butanol and 3-ethoxy-1-propanol, and a relatively greater abundance of ethyl acetate, and the alcohols 3-methyl-1-butanol, 2-methyl-1-butanol, 3-methyl-3-buten-1-ol, ethanol and 2-phenylethanol.

Table 1. Dissolved concentration of volatile compounds identified in single strain inoculations of synthetic nectar after 96 h growth, sorted by *M. reukaufii* concentration.

<i>Compound</i>	<i>M. reukaufii</i> (μ M)	<i>A. pullulans</i> (μ M)	<i>A. astilbes</i> (μ M)	<i>Neokomagataea sp.</i> (μ M)
ethanol	197,155 ($\pm$ 9,983) ^a	22,461 ($\pm$ 4,991) ^a	150 ($\pm$ 50)	105 ($\pm$ 22)
2-methyl-1-butanol and 3-methyl-1-butanol ^b	511 ($\pm$ 9) ^a	16 ($\pm$ 1)	0.28 ($\pm$ 0.02)	0.31 ($\pm$ 0.01)
isobutanol	148 ($\pm$ 1) ^a	177 ($\pm$ 7) ^a	0.11 ($\pm$ 0.05)	0.15 ($\pm$ 0.01)
3-hydroxy-2-butanone (acetoin)	49.0 ($\pm$ 0.8) ^a	22 ($\pm$ 2) ^a	18 ($\pm$ 1) ^a	15.9 ($\pm$ 0.2) ^a
2-phenethyl alcohol	33 ($\pm$ 2)	9.0 ($\pm$ 0.8)	0.34 ($\pm$ 0.07)	0.33 ($\pm$ 0.03)
1-propanol	20 ($\pm$ 3)	101 ($\pm$ 5)	0	0
3-ethoxy-1-propanol	2.4 ($\pm$ 0.5)	0	0	0
2-butanol	1.52 ($\pm$ 0.03)	0	0	0
ethyl acetate	1.1 ($\pm$ 0.1)	0.16 ($\pm$ 0.02)	0	0
4-penten-1-ol	0.44 ($\pm$ 0.03)	0.44 ($\pm$ 0.02)	0	0
3-methyl-3-buten-1-ol	0.35 ($\pm$ 0.01)	0.23 ($\pm$ 0.01)	0	0
1-hexanol	0.06 ($\pm$ 0.01)	0.06 ($\pm$ 0.01)	0.059 ($\pm$ 0.001)	0.0489 ($\pm$ 0.0003)
2-methylpropyl acetate	0.0439 ($\pm$ 0.0009)	0	0	0
3-methylbutyl acetate	0.0323 ($\pm$ 0.0008)	0	0	0
2-ethyl-1-hexanol	0.025 ($\pm$ 0.003)	0.2 ($\pm$ 0.2)	0.13 ($\pm$ 0.02)	0.090 ($\pm$ 0.003)
ethyl butyrate	0.022 ($\pm$ 0.002)	0	0	0

<i>Compound</i>	<i>M. reukaufii</i> (μM)	<i>A. pullulans</i> (μM)	<i>A. astilbes</i> (μM)	<i>Neokomagataea sp.</i> (μM)
2-nonanone	0	9 (± 1)	0	0
4-methyl-2-pentanone	0	0.4 (± 0.4)	0	0
2,5-dimethylfuran	0	0	0.03 (± 0.03)	0.031 (± 0.001)

^a Data is semi-quantitative due to poor linearity and/or saturation of MS signal. ^b 2-methyl-1-butanol and 3-methyl-1-butanol could not be independently quantified due to co-elution and common fragmentation pattern.

Data Interpretation and the Need for Calibration

Vapor-phase concentrations (Figure 1, C_g) of volatile abundance in the sealed jars are not reported or inferred by this approach. Instead, the correlation between dissolved concentration is correlated with the combined analytical factors: compound ionization efficiency in the GC-MS electron ionization source, chromatographic quality (i.e., co-elution, background interference), compound affinity for a given fiber type (Figure 1, K_d), partitioning between air and nectar, (Figure 1, K_H) and aqueous concentration (Figure 1, C_{aq}). Equilibrium based air-water partitioning (Figure 1, K_H) depends on a compound's water solubility limit and vapor pressure at a given temperature (12). The polar, low molecular weight volatiles produced by nectar microorganisms in this study have high aqueous solubility and volatility and so are well distributed between condensed and gas phases.

Figure 1. Partitioning behavior of volatiles in sealed jars.

Quantitative Method Evaluation

Analytical column choice can affect peak shape and detection limits. In this study, samples were quantified on a polar analytical GC column because of improved retention and separation of analytes, relative to a non-polar column (data not shown).

The co-eluting isomers 2-methyl-1-butanol and 3-methyl-1-butanol could not be independently quantified due to common fragmentation patterns (no unique ions for extraction ion analysis; Table 2). These analytes were quantified with a 1:1 mixture of both compounds and so concentrations reported here are only estimates of total 2- and 3-methyl-1-butanol content. Isomerism is known to impact bioactivity. Future chromatographic methods should be modified to incorporate independent quantitation of these volatiles.

Table 2. Volatile calibration details.

<i>Compound</i>	<i>Calibration description</i>	<i>R² value^a (calibration level, where applicable)</i>	<i>Calibration standard concentration range (ng mL⁻¹)</i>
ethanol	TIC, 8 standards, 2 curves	0.941 (high); 0.966 (low)	1E6-1E7 (high); 1,000-10,000 (low)
2- and 3-methyl-1- butanol	TIC, 7 standards, 2 curves	0.716 (high); 0.999 (low)	2,000-10,000 (high); 10-2,500 (low)
isobutanol	TIC, 6 standards, 2 curves	0.920 (high); 0.991 (low)	500-25,000 (high); 25-100 (low)
3-hydroxy-2- butanone (acetoin)	TIC, 4 standards	0.871	2,500-10,000
2-phenethyl alcohol	TIC, 7 standards	0.997	10-1,000
1-propanol	TIC, 4 standards	0.991	1,000-10,000
3-ethoxy-1- propanol	EIC (59 <i>m/z</i>), 4 standards	0.969	50-1,000
2-butanol	EIC (45 <i>m/z</i>), 6 standards	0.993	1-1,000
ethyl acetate	TIC, 8 standards	0.988	1-100
4-penten-1-ol	TIC, 7 standards	0.993	10-1,000
3-methyl-3-buten- 1-ol	TIC, 5 standards	0.981	10-100
1-hexanol	TIC, 7 standards	0.991	1-100
2-methylpropyl acetate	TIC, 6 standards	0.998	1-1,000
3-methylbutyl acetate	TIC, 4 standards	0.998	0.1-100
2-ethyl-1-hexanol	TIC, 4 standards, blanks subtracted	>0.999	0.1-1,000
ethyl butyrate	TIC, 4 standards	0.999	1-100
4-methyl-2- pentanone	EIC (100 <i>m/z</i>), 4 standards	>0.999	0.1-100
2,5-dimethylfuran	EIC (96 <i>m/z</i>), 4 standards	0.994	0.1-100
2-nonanone	TIC, 4 standards, blanks subtracted	0.999	5-1,000

^a Coefficient of determination. TIC: total ion chromatogram, EIC: extracted ion chromatogram.

Table 3. *M. reukaufii* analyte concentration at 96 h growth vs. uncalibrated peak areas, sorted by mean dissolved concentration.

Compound	<i>M. reukaufii</i> concentration at 96 h (mean $\pm$ SE, n=3)	
	μM	Peak Area ($\times 10^5$)
ethanol	197,155 ($\pm 9,983$)	6,800 (± 200)
2-methyl-1-butanol and 3-methyl-1-butanol	511 (± 9)	6,990 (± 80)
isobutanol	148 (± 1)	614 (± 3)
3-hydroxy-2-butanone (acetoin)	49.0 (± 0.8)	53 (± 0.9)
2-phenethyl alcohol	33 (± 2)	260 (± 20)
1-propanol	20 (± 3)	30 (± 2)
3-ethoxy-1-propanol	2.4 (± 0.5)	1.8 (± 0.4)
2-butanol	1.52 (± 0.03)	10 (± 1)
ethyl acetate	1.1 (± 0.1)	130 (± 10)
4-penten-1-ol	0.44 (± 0.03)	8.9 (± 0.6)
3-methyl-3-buten-1-ol	0.35 (± 0.01)	5.5 (± 2)
1-hexanol	0.06 (± 0.01)	6 (± 2)
2-methylpropyl acetate	0.0439 (± 0.0009)	5.3 (± 0.6)
3-methylbutyl acetate	0.0323 (± 0.0008)	41 (± 2)
2-ethyl-1-hexanol	0.025 (± 0.003)	29 (± 2)
ethyl butyrate	0.022 (± 0.002)	6 (± 1)

Analytes in microbial volatile headspaces collected at 96 h spanned seven orders of magnitude (1×10^{-8} -0.1 M). To quantify this wide range of compounds, two calibration curves were sometimes necessary. Despite these efforts, poor linearity was observed for several compounds, particularly those produced at high concentrations (i.e. ethanol, 2- and 3-methyl-1-butanol, 3-hydroxy-2-butanone, Table 2). At 48 and 96 h sampling intervals, these volatiles saturated mass spectrometer response, as indicated by individual extracted ion chromatograms (EIC) traces. Semi-quantitative estimates (with poor percent accuracy; i.e. percent error $>30\%$) of these compounds dissolved in nectar are presented in Table 1. Superficial method modifications, like shorter SPME fiber permeation and exposure times, would prevent saturation.

Several compounds detected within microorganism replicate headspace were also present in sterile controls, at lower abundance. These compounds were quantified by subtracting mean sterile control peak area from all sample and calibration peak areas (Table 2, calibration description). Other compounds co-eluted near background peaks and in these cases unique fragmentation ions were used for calibration purposes (EIC, Table 2).

Care should be taken when interpreting peak area data of instruments with non-universal response, like mass spectrometry. Structural chemical diversity results in differing ionization efficiencies between compounds, particularly those with widely-variant structures. Therefore, relative peak areas do not always correspond directly to volatile abundance in the headspace. Furthermore, GC-MS peak areas do not correspond to dissolved concentrations (Table 3). For example, peak areas are similar between ethanol and 2- and 3-methyl-1-butanol, but dissolved ethanol concentrations are actually three orders of magnitude higher. Even if universal detection techniques are adopted, collected volatiles may not be representative of gas-phase volatile abundance due to differing sampling recoveries.

The adoption of calibration curves prepared using identical conditions correlates the previously described variability directly to calibration standard concentration, but cannot directly describe volatile abundance in the sealed containers. In this case, dissolved concentration is still useful; informing solutions for field- and laboratory-based bioassays.

Discussion

Nectar Microorganisms: Opportunities for Agriculture

The presence of microbes in floral nectar has been known for decades, yet their distribution and effects in agriculture are not well understood, with the exception of globally-distributed floral pathogens like *Erwinia amylovora*, the causative agent in fire blight (13, 14). When flowers first open, nectar is often free of culturable microorganisms, but with successive visitation, cell densities increase, with some long-blooming floral nectars containing cell densities greater than 3.5×10^6 cells μL^{-1} (15, 16). Though fungi and bacteria are common inhabitants of floral nectar, a given nectar sample typically hosts few species (1-2 species/flower) (5, 15, 17–19). Certain species like the yeast, *Metschnikowia reukaufii*, are frequently observed and widely distributed (5, 18–20).

All evidence suggests agricultural floral nectar regularly contains floral microorganisms (13, 21, 22), but their effects on plant reproduction and pollination are rarely considered. Reports in the literature are mixed: in wild herb populations, the presence of yeasts increased pollen transfer in one study, but reduced seed set in another (3, 6). We envision that with thoughtful application and stewardship of selected mutualist microorganisms in flowering crops, there may be the potential to affect yields through altered pollinator behavior, and thus reduce production costs and agrochemical application.

Manipulation of Floral Visitation via Microbial Inoculation

Floral volatiles may simultaneously attract and deter various visitors in what has been described as a “filtering” effect (23). Nectar microorganisms may also filter floral visitor species by enhancing or reducing affinity to nectar (24). Certain microbial species increase floral visitation and consumption of nectar, while others deter visitors (4, 5, 25, 26). The need for pollination services in agriculture is

outpacing supply (27). The ability to direct pollination services toward poorly attractive crops via inoculation of attractant species could alleviate consequences for supply limitations.

The use of deterrent microbes may be useful in directing insect distribution in plants not dependent on visitor-mediated pollination. Floral microbiome manipulation could be directed toward repulsion of deleterious floral visitors, which consume or infect flowers. Alternatively, inoculation could protect beneficial or sensitive native insects from toxic crops. Co-application of deterrent microorganisms with insecticides could reduce pollinator exposure via ingestion and contact with treated flowers, reducing risk of toxicity (28). Volatiles identified to be generally insect-deterrent could be incorporated into pesticide blends, at concentrations informed by quantitative emission data.

Environmental Remediation of the Nectar Microhabitat for Improved Pollinator Health

Plant-produced compounds may be toxic or deterrent to pollinators or may impact bee learning, memory and behavior (29–32). Nectar secondary metabolites can also reduce disease incidence in bees but in other cases, may exacerbate infection (33–35). Application of beneficial nectar microbes capable of selectively degrading harmful compounds from nectar could influence pollinator health and increase pollination services.

Nectar Microbes for Floral Pathogen Protection

Priority effects, where early-arriving nectar microbes suppress the growth of later-arrivers, have been demonstrated in nectar communities and may be exploited for floral pathogen protection (36, 37). Rapid nectar resource depletion, particularly for amino acids, is hypothesized to drive competition between species (38). Intentional rapid establishment of selected microorganisms in crops may prevent subsequent colonization by pathogens. Biocontrol agents are already used for floral pathogen control in agriculture, but new, more effective strains are still sought (39, 40). Moreover, understanding the mechanisms by which biocontrol agents suppress pathogens (resource competition, antibiotic production, etc.) may be informed by a quantitative chemical approach.

Quantitative Unbiased Chemical Assessment: The Need and Unattainable Goal

Any assessment of a metabolome strives for a complete, unbiased, quantitative snapshot of all metabolites relevant to a particular organism, tissue or process with a single extraction procedure and instrumental analysis. This goal is unachievable using current technologies. Iterative metabolite sampling and detection strategies may be prohibitively expensive and will eventually yield diminishing returns in terms of new chemical data. Even routine volatile measurement using GC-MS has considerable instrument acquisition, operation, maintenance and technical support costs, putting it out

of reach of many non-chemistry specialists. Additionally, there are costs for purchasing or synthesizing large collections of pure chemicals needed to confirm chemical identity and prepare calibration curves (commonly >\$100 for sub-gram quantities).

A key tenet in chemical ecology is the dose-response relationship between semiochemicals and induced interactions: the magnitude and direction of an effect (i.e. attraction vs. deterrence) often depends on semiochemical concentration. Low abundance compounds may play crucial roles in biological responses and so exhaustive analysis of volatiles is needed. Volatile quantification is particularly necessary to assess microorganism contribution to floral scent *in planta*, since many microbial volatiles are also emitted by plants.

The nectar metabolome poses unique analytical challenges. Nectar collection can be an arduous process, since many plants produce small (fractions of microliters) and intermittent nectar crops. The relevant biomolecules present in nectar samples are susceptible to abiotic degradation post-collection even when frozen, according to some reports (41, 42). The small scale of nectar also poses a challenge for detection. Volatile analysis performed on collected *ex situ* floral nectar has relied on pooling nectar to overcome analytical detection limits (43). Nectar chemistry can be highly variable even within nectaries of the same flower and so sample pooling may mask influential fluctuations between flowers and ideally should be avoided, where possible (17, 44).

The physicochemical properties of some nectar volatiles also complicates detection. Microbial volatiles are typically polar, low molecular weight, high vapor pressure compounds which are poorly retained on many adsorbent materials. The range of concentration encountered in nectar samples spans many orders of magnitude, likely requiring sample preparation (dilution and concentration) of nectar samples for quantitative detection.

Laboratory-based studies overcome many of these challenges by allowing essentially unlimited quantities of “nectar” to be uniformly prepared and analyzed. Specific nectar-solute effects can be rapidly reproduced, revealing important new chemistry. Laboratory experiments also are not restricted by limited and variable nectar plant production. Here, we describe an approach for assessing microbial volatiles in laboratory cultures. Additional details, including honey bee acceptance and electrophysiological tests related to this data is available (11).

Reports of microbial-produced volatiles in nectar are very rare; however, several studies have inferred the contribution of microbe-produced volatiles to floral headspace. An early tentative detection was reported in a study contrasting volatile production in floral tissue and nectar of angiosperms (45). Headspace volatiles from excised flowers or nectar (5-10 μ L) were analyzed by SPME-GC-MS. Nectar samples of two species contained unique compounds, including fermentation volatiles, which were tentatively ascribed to microbial contamination of nectar. The authors note that these plants are likely to host microorganisms, due to their extended flowering phase, large, stable nectar crop and frequent, diverse animal visitors.

In other studies, the influence of the entire above-ground microbiome of a plant (i.e. phyllosphere and anthosphere) on floral aroma was investigated. Floral terpene abundance was reduced after whole-plant antibiotic fumigation (46). In

a complimentary approach, different volatile profiles were observed between *Brassica rapa* plants grown under sterile conditions and those inoculated with field-isolated bacteria (47). In both reports, the difficulty of parsing plant- vs. microbe-derived variations in floral volatile blends is discussed.

The first direct measurement of microbe-produced volatiles in floral nectar was described in the weakly scented, insect-pollinated flower, *Silene caroliniana* (43). Nectar (2 μ L) was collected in sterilized autosampler vials from open flowers and transported back to the laboratory, where volatiles were allowed to accumulate for 24 h prior to analysis and then screened for yeasts. From the resulting cultures, four frequently observed nectar yeasts, including two *M. reukaufii* strains, were selected for further study in synthetic nectar under analogous conditions to real nectar samples. Due to the study of common nectar microbes and the use of the same SPME fiber type and analytical technique, data between these analyses can be compared to some extent.

During method development, a dose-response approach to unknown volatile detection is advised, wherein increasingly large samples are pooled and analyzed, and the sample size at which no (or very few) new compounds are discovered is established. Golonka and colleagues adopted this approach to synthetic nectar inoculations: they compared volatile production in 2 and 200 μ L synthetic nectar and found similar volatile emissions in both cases and so selected the more relevant 2 μ L nectar volume (43).

Many volatiles, including fermentation volatiles like ethanol, isobutanol, and 2- and 3-methyl-1-butanol, were detected in both floral and bulk and small-volume synthetic nectar analyses. In total, large-volume inoculated synthetic nectar revealed a broader suite of volatiles, probably due to higher cell counts. These volatiles had the largest relative peak areas and saturated detector response in bulk nectar (Table 1). Differential volatile emission could also be explained by genetic variation between the naturally-occurring *M. reukaufii* strains selected for study or could arise as a consequence of differing synthetic nectar chemistry.

Different volatile blends were identified in collected *S. caroliniana* nectar and inoculated synthetic nectar prepared by Golonka and colleagues, an observation which demonstrates the need to validate *ex situ* floral nectar and synthetic nectar data with *in planta* studies. Particularly in the case of synthetic nectar-based investigations, the interactions of nectar microorganisms with their plant hosts is entirely excluded.

Plant-specific factors like nectary structure and genetics largely define nectar excretion and composition. It is well understood that microbial metabolites will vary depending on the nutritional quality of their growth substrate. However, a suite of environmental factors also have demonstrated effects, including: light quality, soil nutrients and moisture, herbivory, atmospheric carbon dioxide levels, humidity and temperature (48–53). Other epiphytic microorganisms can influence floral scent emissions (47). Even nectar removal by floral visitors may change nectar composition and excretion over time (54). Plants may also respond to nectar contamination by inducing defense strategies, or perhaps, supporting microbial mutualist colonization (55). Synthetic and *ex situ* studies of nectar microbe volatiles must be validated with *in situ* experiments, to parse these interconnected and cascading plant, microbe and environmental factors.

Conclusions

Nectar microbes are an un-tapped resource in agriculture, and may lend themselves to a variety of applications. Here, we provide the first quantitative report of nectar microbe volatile emission in synthetic nectar. Quantitative chemical data should be validated with bioassays. Pollinator response between microorganism-inoculated and sterile, volatile-laced nectars could indicate whether key semiochemicals were detected and accurately quantified. Differing acceptance of these solutions may also point to non-volatile mediated attraction or deterrence.

Bulk synthetic nectar inoculation proved to identify new, previously unknown volatiles that may mediate pollinator response. Unsurprisingly, some compounds were poorly quantified by this rather crude approach. In this case, saturation of some analytes was accepted in order to screen for low abundance volatiles.

Parallel comparisons of synthetic and real nectars, like the experiment undertaken by Golonka and colleagues, may offer a compelling template for further investigations to evaluate the suitability of synthetic nectar-based screening of microbial semiochemicals (43).

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