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Volatile microbial semiochemicals and insect perception at flowers

Amber Crowley-Gall[1,3], Caitlin C Rering[2,3], Arthur B Rudolph[2], Rachel L Vannette[1] and John J Beck[2,4]

Many plant-associated microbial communities produce volatile signals that influence insect responses, yet the impact of floral microorganisms has received less attention than other plant microbiomes. Floral microorganisms alter plant and floral odors by adding their own emissions or modifying plant volatiles. These contextual and microbe species-specific changes in floral signaling are detectable by insects and can modify their behavior. Opportunities for future work in floral systems include identifying specific microbial semiochemicals that underlie insect behavioral responses and examining if insect species vary in their responses to microbial volatiles. Examining if documented patterns are consistent across diverse plant–microbe–insect interactions and in realistic plant-based studies will improve our understanding of how microbes mediate pollination interactions in complex system.

Addresses
[1] Department of Entomology and Nematology, University of California Davis, 43 Briggs Hall, Davis, CA 95616, USA

[2] Chemistry Research Unit, Center for Medical, Agricultural, and Veterinary Entomology, Agricultural Research Service, United States Department of Agriculture, Gainesville, FL 32608, USA

Corresponding author: Crowley-Gall, Amber (acrowleygall@ucdavis.edu)
[3] Shared first authors.
[4] Contributing author: John J Beck (john.beck@usda.gov).

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Introduction

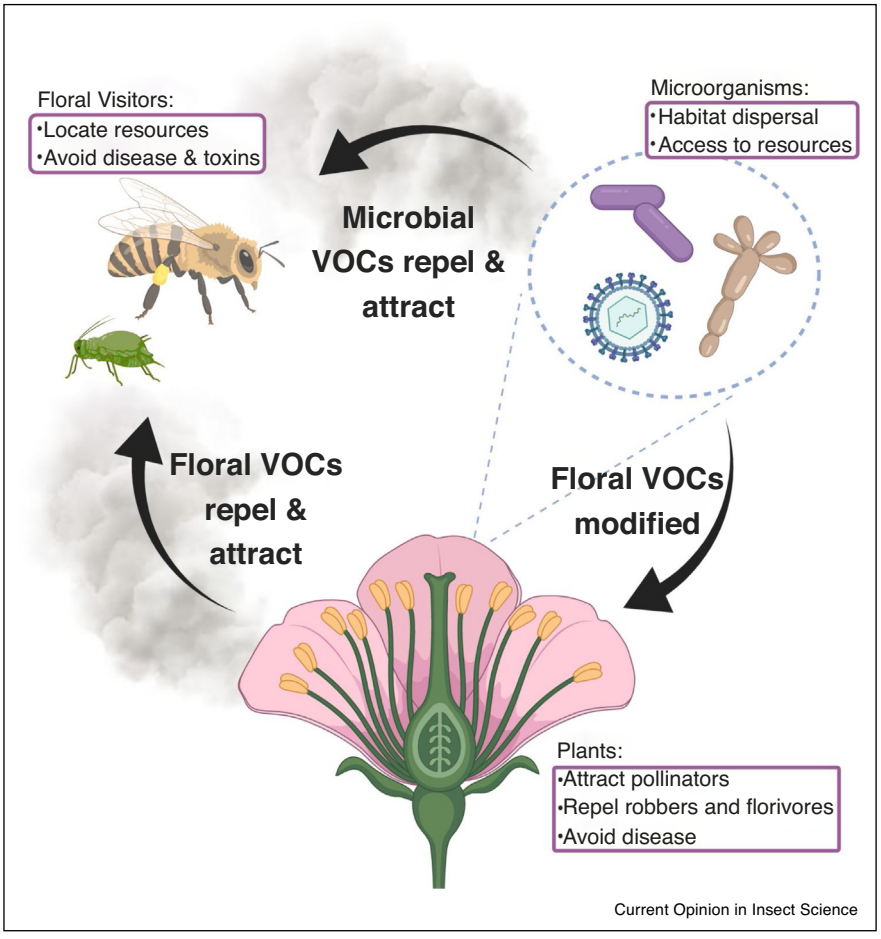
Semiochemicals are metabolites that facilitate communication between organisms. Volatile organic compounds (VOCs) are airborne metabolites that can mediate interactions at great distances, making them of particular importance for sessile species such as plants [1]. Indeed, volatile chemicals produced by plants mediate insect perception and host selection [2]. Plant-associated microbial communities (reviewed in Ref. [3]) modify plant volatile blends, thereby affecting interactions with insects [4–6,7••]. Although plant–insect interactions mediated by microbes and their volatiles (mVOCs) have been a key topic of research for years [4,5,8], more recently the impacts of floral microbial communities have been recognized. Recent reviews have explored the role of floral microbes in microbial community assembly, floral evolution, and plant–animal interactions [9•,10••,11••]. However, volatile signaling of floral microorganisms and their role in insects' selection of flowers has received much less attention. In this review, we highlight what is known about flower–microbe–visitor interactions as mediated through volatile semiochemicals (Figure 1). We summarize the floral microbiome, current methods used to identify the mVOCs most commonly produced, and insect perception and detection of these compounds. Throughout, we highlight our suggestions for future work in this rapidly growing field.

Distribution and diversity of floral microbes

The floral microbiome (including microbes isolated from nectar, pollen, stigmas, petals, etc.) is largely distinct from those in other plant-associated communities [12,13] (but see Ref. [14]). Microbes are introduced to flowers by wind, rain, or through interactions with floral visitors (e.g. buzz pollination, nectar foraging, defecation and florivory) [15–20]. The environmental conditions of specific floral tissues or environments, such as high sugar content and/or sometimes low pH in nectar, combined with rapid growth rates of nectar-inhabiting microbes determine which microbes successfully colonize different floral tissues and rewards [21–23], and often result in distinct microbial communities associated with each floral microhabitat [24].

Among floral microhabitats, the microbial communities of nectar have been investigated the most extensively, but see [25] for a review of pathogenic fungi. Yeasts from the genera *Metschnikowia* and *Starmerella* [11••], and *Proteobacteria* are commonly isolated from nectar [26,27•], although floral microbiomes of many plant species remain to be described. Nectar microbe diversity in a single flower is typically low, but the density of nectar yeast or bacteria can be very high [28,29] and early colonizers prevent ingress by other microbes [30]. In some cases higher microbial diversity is found in other floral microhabitats, including pollen, petals, and stigmas [10••,31]. Microbes in nectar [32••,33] or on petals [34••] can impact

Figure 1



A subset of volatile-mediated interactions between floral microbes, flower-visiting insects and flowers, and the focus of this review. Purple boxes represent hypothesized mechanisms for volatile effects on fitness of each interacting group of species. Not to scale.

flower–insect interactions and mVOCs may mediate these effects, as we outline below.

Floral microbes affect insect behavior

Insects use a complex array of visual, and chemical (scent and gustatory) cues to find food sources [2]. Some chemical cues may be innately attractive to insects, while others might be attractive as part of complex blends of compounds, or detected and learned as cues of particular resources [35]. Insect responses to flower-inhabiting microbes are impacted by microbial chemical cues and likely fall into multiple categories (Table 1). Floral microbes can alter inherent preferences for nectar or nectar-containing flowers [6,7••,32••,33,36–38,39••,40•,41]. For example, *Clematis akebioides* flowers that were supplemented with synthetic nectar containing *Metschnikowia reukaufii* experienced higher visitation from *Bombus friseanus* foragers than control flowers [33]. On the other hand, learned behavioral responses have been associated with floral microbe presence in laboratory-based [41] and field-based trials [34••,42]. For example, bacteria on flowers were innately less preferred by *B. impatiens* foragers, but over time foraging workers learned to associate bacterial cues with floral rewards [32••]. In addition, flower-visiting insects can distinguish among floral microbial species [32••,34••,37,40•] and in some cases, differentiate microbes isolated from flowers over those from other plant tissues [43]. Interestingly, microbes eliciting varying behavioral responses also emitted distinct volatile profiles [32••,39••,41] supporting the importance of microbial volatile cues on insect behavior.

Among microbes and insects characterized to date, nectar-specialist yeasts appear to be more attractive to pollinators than yeast or bacteria isolated from other environments [32••,37,39••,41]. However, it is unclear if this is due to the limited number of microbe and insect species studied so far, or a general pattern. Moreover, the context of experiments assessing microbial effects on insect

Table 1

Studies examining the effect of floral microbes on insect visitor behavior

Insect species	Microbe species	Microbe kingdom	Microbial culture media	Experimental assay	ehavioral response	VOC analysis	Ref.
Aphidius colemani	40 *Bacillus* strains (five from floral nectars)	Bacteria	Standard lab culture media	Y-tube	Significant effect of bacterial clade on response	Yes	[67]
Apis mellifera	*Asaia astilbes*, *Metschnikowia reukaufii*, mixture	Bacteria, fungi	Synthetic nectar	Artificial flowers-consumption	Preference for control over inoculated nectars	Yes	[44•]
Apis mellifera	*Erwinia amylovora*	Bacteria	Inoculated apple plants	Foraging assay (trained bees)	Preference for control flowers	Yes	[7••]
				Blind choice test (naïve bees)	Higher visitation to feeding solution associated with control branches		
Bombus impatiens	Mixture of *Leuconostoc fructosum*, *Lactobacillus micherneri*, *M. gruessi*, *Candida rancensis*	Bacteria, fungi	Microbial suspension spread on flower corolla	Artificial flowers-visitation	Ability to learn with and without microbes, but learn faster without microbes	No	[34••]
				Live flowers-visitation	Ability to learn in presence of natural microbial community		
			Microbial supernatant on flower corolla		Ability to learn based on microbial chemical cues, suggests discrimination at a distance		
	Leuconostoc fructosum, *Lactobacillus micherneri*, versus *M.* gruessi, *C. rancensis*	Bacteria versus fungi	Microbial suspension spread on flower corolla		Preference for flowers without supplemental bacteria communities		
Bombus impatiens	*A. astilbes*, *M. reukaufii*	Bacteria, fungi	Synthetic nectar	Y-tube	No significant effect on first choice, more time in *A. astilbes* arm of Y-tube	Yes	[40•]
				No-choice feeding	Consumed more *M. reukaufii* nectar		
Aphidius ervi	*M. reukaufii*	Fungi	Synthetic nectar	Y-tube (naïve parasitoids)	Preference for arm containing yeast	Yes	[41]
	Sporobolomyces roseus				Preference for control arm		
	Hanseniaspora uvarum				No effect		
	M. reukaufii, *S. roseus*, *H. uvarum*			Y-tube-conditioned and tested on same yeast	Preference for arm containing yeast for all yeasts for at least 24 hour, and 48 hour for *M. reukaufii*		
	M. reukaufii			Y-tube-conditioned with one yeast and tested with others (for all tests responses replicated naïve parasitoids after 24 hour)	Strongly attracted to *H. uvarum* and repulsed by *S. roseus* after two hour		
	S. roseus				Strongly attracted to *H. uvarum* and *M. reukaufii* after two hour		
	H. uvarum				Strongly attracted to *M. reukaufii* and no effect of *S. roseus* after two hour		
Bombus friseanus	*M. reukaufii*	Fungi	Synthetic nectar	Live flowers supplemented with synthetic nectar-	Higher visitation to yeast containing flowers	Yes	[33]
Apis mellifers	*M. reukaufii*, *Aureobasidium pullulans*, *Asaia*, *Neokomagatea*	Bacteria, fungi	Synthetic nectar	Proboscis extension response	Reduced responses to *A. pullulans*, *Asaia* and *Neokomagatea*	Yes	[32••]
Aphidius ervi	*M.* gruessi	Fungi	Synthetic nectar	Y-tube	Attractive	Yes	[39••]
	M. reukaufii				Attractive (strongest)		

Table 1 (*Continued*)

Insect species	Microbe species	Microbe kingdom	Microbial culture media	Experimental assay	ehavioral response	VOC analysis	Ref.
	A. pullulans				Attractive		
	H. uvarum				No effect		
	S. roseus				Repellent		
	M. gruessi, *M. reukaufii*, *A. pullulans*, *H. uvarum*, *S. roseus*			Capillary feeder-no choice	Consumed less *S. roseus* and *A. pullulans*		
				Longevity	Reduced longevity with *A. pullulans*, *S. roseus*, and *H. uvarum*		
				Survival	Reduced survival with *A. pullulans*, *S. roseus*, and *H. uvarum*		
Bombus impatiens	*M. reukaufii*	Fungi	Synthetic nectar	Artificial flowers (trained bees)	Preferred yeast-inoculated flowers	No	[42]
				Artificial flowers (naïve bees)	Higher proportion of early visits to flowers with yeast, no effect across all visits. Longer foraging duration on flowers with yeast		
Apis mellifera	*Asaia astilbes*, *M. reukaufii*, *Erwinia tasmaniensis*, *Lactobaccillus kunkeei*	Bacteria, fungi	Synthetic nectar	Artificial flowers-consumption	Reduction in nectar removal across bacterial species	No	[37]
Bombus terrestris	22 bacterial strains isolated from *Lamium maculatum* (leaves, flowers and nectar) and *Achillea millefolium* (flowers)	Bacteria	Glucose solution	Proboscis extension response	Reduced responses relative to control (density-dependent). Responses varied based on isolation source.	No	[43]
Bombus appositus and *B. flavifrons*	*M. reukaufii*	Fungi	Synthetic nectar	Live flowers supplemented with synthetic nectar	Higher proportion of visits and more probing at flowers with yeast. Significant species effect (*B. appositus*)	No	[38]
Captive *Bombus terrestris*	*M. reukaufii*	Fungi	Synthetic nectar	Artificial flowers-consumption	Preference for yeast-containing flowers	No	[36]
	M. gruessi				No effect		
	M. reukaufii, *M.* gruessi, and *C. bombi* (all trials)				Preference for yeast-containing flowers		
Wild bumbebees	*M. reukaufii*			Live flowers supplemented with synthetic nectar	Preference for yeast-containing flowers		
Acalymma vittatum	*E. tracheiphila*	Bacteria	Cucurbit plants naturally infected	Recruitment in field	Preference for symptomatic branches but healthy flowers	Yes	[6]
	Zucchini yellow mosaic virus	Virus			Preference for healthy flowers		
	E. tracheiphila	Bacteria	Cucurbit plants inoculated in lab	Y-tube	Preference for volatiles of infected foliage but healthy flowers		
	Zucchini yellow mosaic virus	Virus			Preference for volatiles of healthy flowers, no effect of foliage volatiles		

behavior are important. For example, insect responses to *M. reukaufii*, the most extensively studied floral nectar microbe, vary across studies and insect species. Examples of *M. reukaufii* variability include: eliciting no effect on foraging or feeding in honey bees or parasitoids [32••,37,39••], increased preference to bumble bees or parasitoids [33,36,38,39••,40•,41,42] and decreased preference in some honey bees and bumble bees [40•,44•]. Some of this variation may be due to the behavioral assay employed. For example, *M. reukaufii* elicited differential effects in Y-tube (scent only) and consumption-based assays, where gustatory cues were available [39••,40•]. The ability of insects to learn and distinguish between microbial species introduces the possibility of context-dependent variation in behavior, due to previous exposure to microbial communities in nature and hives. Moreover, as described below, the substrate for microbial growth can affect volatile production [6,44•].

The majority of recent studies have used synthetic nectar and/or artificial flowers to jointly characterize metabolites and insect behavior (ex. [32••,40•,44•]). Synthetic systems are a useful first step in examining the effects of floral microbes on floral visitor interactions as they provide insight into the impacts of microbes alone on behavior in a controlled environment. However, there are several limitations associated with synthetic systems that call for a move towards in plant experiments when possible: first, some flower-isolated microbial species do not grow in artificial nectar sources; second, volatile composition can vary qualitatively and quantitatively with media type or substrate composition, discussed below; and third, contributions of plant–microbe interactions cannot be accounted for in synthetic systems and therefore cannot fully represents the complex interactions that occur in nature. Ultimately a combination of synthetic and plant-based approaches is required to parse out the contributions of microbe, plant and plant–microbe interactions on floral visitor behavior.

Finally, microbial effects on insect behavior may change over time, not only due to insect learning, but the interplay between microbial growth and plant physiological responses to microbial colonization. The extent to which flower physiology changes after microbial colonization is largely unknown, but studies in other plant-response-based studies have provided evidence that mVOCs play a dynamic role in communication between organisms [6,7••,33]. For example, inoculation of cucurbit plants and apple blossoms with bacterial pathogens in both field-based and lab-based experiments revealed that after an initial exposure, insects prefer healthy flowers over those that are infected [6,7••]. This suggests a potentially volatile-mediated shift in preference induced upon infection, which is thought to contribute to secondary spread of the pathogen. Such an approach, where communities are experimentally manipulated within plants (also see Refs. [33,36,38]) likely offer the next advances in this area.

The examples presented above show that the presence and identity of flower inhabiting microbes can affect insect foraging on flowers and floral nectar, and many of these studies implicate volatile chemicals. But which compounds are produced and how may they mediate insect behavioral responses?

Floral microbial volatiles: production and ecology

From the current literature, the volatile profiles of only 14 microbial species isolated from flower habitats have been characterized. These studies report production of mVOCs that span many chemical classes (Tables 2 and 3), and reflect the chemical diversity generally observed among microorganisms [45–47]. Aldehydes, alcohols, and esters are most frequently detected. Relative to other plant-associated microorganisms, we note few detections of terpenoids from nectar microbes grown in nectar or a synthetic nectar analog (Table 2). In real flowers, microbial addition or removal can modify floral terpenoid emission (among other VOCs, Table 3; [6,7••,48]), suggesting microbial modification of plant VOC emission rather than de novo synthesis of terpenoids by microbes.

Given the composition of mVOCs of nectar microbes, what is the ecological significance of these volatiles in pollination systems? Microbe volatiles may cue the presence or quality of nectar rewards acting as an honest signal [49]. This could increase visitation by mobile pollinators and facilitate microbial dispersal to new flowers. Alternatively, low VOC production may reduce detection by flower-visiting insects, thereby improving the dispersal potential of microbes that produce deterrent mVOCs. Floral fungi produce a greater diversity and abundance of volatiles than bacteria [32••,44•]. By virtue of their abundance, fungal VOCs may be more prevalent and detectable to insect vectors than bacterial VOCs, and may therefore also be more likely to act as honest signals, though this requires further study.

Commonly detected mVOCs include metabolites that are produced by microorganisms and macroorganisms, such as products formed upon catabolism of amino acids and those produced via sugar fermentation (Tables 2 and 3) [45,46]. The presence of broadly conserved fermentation volatiles as cues for sugar-rich resources has been proposed to mediate insect responses in other systems, such as the attraction of *Drosophila* to fermented fruits [50]. In support of this hypothesis, the majority of mVOC components are produced by many bacteria and fungi including those isolated from diverse environments. Indeed, Sobhy *et al.* [39••] compared mVOC emission between nectar-inhabiting yeast and the baker's yeast *Saccharomyces cerevisiae*, reporting similar VOCs among the species, albeit higher emission for the cultivated *S. cerevisiae*. This finding suggests that the major volatile components of nectar mVOC blends are not necessarily specific to nectar

Table 2

Microbial volatiles (VOCs) identified in synthetic floral systems

Microbe species	Microbe kingdom	Collection method	Detection method	VOC classes detected	Major VOC components	No. of VOCs	Ref.
AA	Bacteria	SPME, passive	GC–MS	Alcohols, aldehydes, alkanes, esters, furanoids, ketones, S-containing	2-Ethyl-1-hexanol, 2-furanmethanol, acetoin	30	[44•]
AA	Bacteria	SPME, passive	GC–MS	Alcohols, aldehydes, isoprenoids, ketones, furanoids	2-Ethyl-1-hexanol, 2-methyl-1-butanol, 3-methyl-1-butanol, ethanol	12	[32••]
AP	Fungi	SPME, passive	GC–MS	Alcohols, aldehydes, esters, ketones	Ethanol, 2-methyl-1-butanol, 3-methyl-1-butanol, isobutanol	17	[32••]
AP	Fungi	Direct headspace injection	GC–FID	Alcohols, aldehydes, esters, ketones, S-containing	Acetaldehyde, isobutanol, 3-methyl-1-butanol	7	[39••]
HU	Fungi	Direct headspace injection	GC–FID	Aldehydes, esters, S-containing	Acetaldehyde, dimethyl disulfide, ethyl acetate	9	[41]
HU	Fungi	Direct headspace injection	GC–FID	Alcohols, aldehydes, esters, ketones, S-containing	2-Phenylethanol, acetaldehyde, 3-methyl-1-butanol	7	[39••]
MG	Fungi	Direct headspace injection	GC–FID	Alcohols, aldehydes, esters, ketones, S-containing	2-Phenylethanol, 3-methyl-1-butanol, acetaldehyde	6	[39••]
MK (2 strains)	Fungi	SPME, passive	GC–MS	Alcohols, aldehydes, alkanes, carboxylic acids, esters, ketones	Ethanol, isobutanol, 2-methyl-1-butanol, 3-methyl-1-butanol	9, 12	[53]
MR	Fungi	SPME, passive	GC–MS	Alcohols, aldehydes, alkanes, esters, furanoids, ketones, S-containing	2-Methyl-1-butanol, 3-methyl-1-butanol, ethanol, 2-ethyl-1-hexanol	34	[44•]
MR	Fungi	SPME, passive	GC–MS	Alcohols, aldehydes, esters, ketones	2-Methyl-1-butanol, 3-methyl-1-butanol, ethanol, isobutanol	19	[32••]
MR	Fungi	SPME, passive	GC–MS	Alcohols, carboxylic acids, ketones	Ethanol, 2-methyl-1-butanol, isobutanol	5	[33]
MR	Fungi	Direct headspace injection	GC–FID	Aldehydes, esters, S-containing	Acetaldehyde, ethyl acetate, dimethyl disulfide	9	[41]
MR	Fungi	Direct headspace injection	GC–FID	Alcohols, aldehydes, esters, ketones, S-containing	3-Methyl-1-butanol, acetaldehyde, isobutanol	7	[39••]
MR (2 strains)	Fungi	SPME, passive	GC–MS	Alcohols, carboxylic acids	Ethanol, isobutanol, 2-methyl-1-butanol, 3-methyl-1-butanol	6[a]	[53]
N	Bacteria	SPME, passive	GC–MS	Alcohols, aldehydes, isoprenoids, ketones, furanoids	2-Ethyl-1-hexanol, 2-methyl-1-butanol, 3-methyl-1-butanol, ethanol	11	[32••]
SR	Fungi	Direct headspace injection	GC–FID	Aldehydes, esters, S-containing	Dimethyl disulfide, ethyl acetate, ethyl butyrate	6	[41]
SR	Fungi	Direct headspace injection	GC–FID	Alcohols, aldehydes, esters, ketones, S-containing	2-Phenylethanol, 3-methyl-1-butanol, isobutanol	8	[39••]

AA = *Asaia astilbes*, AP = *Aureobasidium pullulans*, HU = *Hanseniaspora uvarum*, MG = *Metschnikowia gruessii*, MK = *M. koreensis*, MR = *M. reukaufii*, N = *Neokomagataea* spp., SR = *Sporobolomyces roseus*.

[a] In both strains.

Table 3

Microbe-induced changes to volatile (VOC) emission identified in real floral systems

Plant species	Manipulation	Microbe species	Microbe kingdom	Collection method	Detection method	VOCs		Ref.
						Increased	Decreased	
Pollenizer apple, *Malus* × *domestica* Borkh.	Spray inoculated open flowers	*Erwinia amylovora*	Bacteria	SPME, passive	GC–MS	1-Penten-3-ol, 3-(*E*)-hexen-1-ol		[7••]
Pollenizer apple, *Malus* × *domestica* Borkh.	Spray inoculated open flowers	*Erwinia amylovora*	Bacteria	Closed loop stripping analysis, active	GC–MS	[a](*Z*,*E*)-α-farnesene, (*E*,*E*)-α-farnesene, (*E*)-β-farnesene, curcumene, copaene, ledene, methyl salicylate, 2-phenylethyl acetate	[a]Benzaldehyde, phenylacetonitrile, benzyl alcohol, nonanal, decanal, (*E*)-ocimene, linalool, (*Z*)-jasmone, (*E*)-4,8-dimethyl-1,3,7-nonatriene	[7••]
Various wildflowers	Unmanipulated open flowers	Unspecified	Fungi	Twister bar, passive	GC–MS	2-Nonanone, 2-ethyl-1-hexanol, 1-hexanol		[32••]
Brassica rapa	Inoculated buds and leaves	*Staphylococcus*, *Bacillus*, and *Sphingomonas*	Bacteria	Tenax/Carbotrap B, active sampling	GC–MS	Acetoin, 2,3-butanediol, one unknown	1,2-Propanediol, 2,3-dimethylpentanol, longifolene, two unknowns	[68]
Sambucus nigra	Fumigation via antibiotics	Unspecified	Bacteria	Tenax/Unicarb, active sampling	GC–MS		*trans*-β-Ocimene, linalool, epoxylinalool, linalool oxide	[48]
Silene caroliniana	Pollinator exclusion	Yeast and potentially bacteria	Fungi & bacteria	SPME, passive	GC–MS	Acetone, isobutanol, 1-methoxy-2-propoxy-ethane, 3-methyl-1-butanol, 4-methyl-octane, 2,2-dimethyl-1,3-propanediol, 2-ethyl-1-hexanol	Vinyl acetate, heptane, 1-methoxy-2-propanone	[53]
Cucurbita pepo ssp. texana	Identified as infected	*Erwinia tracheiphila*	Bacteria	SuperQ, active sampling	GC–MS & FID		Linalool, 1,4-methoxybenzene, nonatriene	[6]
Cucurbita pepo ssp. texana	Identified as infected	Zucchini yellow mosaic virus	Virus	SuperQ, active sampling	GC–MS & FID		1,4-Methoxybenzene	[6]

[a] Partial least squares discriminant analysis determined floral VOC emission differed between inoculated and control flowers, but the exact compounds underlying this difference were inferred via ordination and not further investigated.

or flower-dwelling yeasts. Instead, flower-visiting insects may respond to VOCs common to many sugar-fermenting microbes, rather than the emission of a key blend of attractive mVOCs (or the reduction of repulsive and/or induction of attractive floral VOCs). However, with limited insect–microbe pairs and few *in-situ* studies performed, this hypothesis requires further study.

Floral mVOCs: considerations for sampling methods and analytical approaches

Sampling methods

As mentioned above, both synthetic and in plant-based assessments of floral mVOCs have been used. The pros and cons associated with these sampling systems are commonly noted across disciplines. For example, synthetic approaches allow larger sample sizes and volumes, which can help overcome analytical constraints related to the relatively small populations of microbes within flowers. To date, synthetic systems have facilitated the detection of a greater number of mVOCs (Table 2) than plant-based studies (Table 3). This may be due to greater microbial (and therefore volatile) abundance allowed by synthetic systems, facilitating detection of usually low-abundance microbe-produced compounds. Synthetic approaches also offer more controlled experimental conditions, which is of particular relevance in floral mVOC studies given that floral volatile emission profiles are highly abundant and variable relative to other tissues [51]. Floral VOC emission can vary on a diurnal cycle [51] and across flower phenology [52], generating a more complex background on which to detect mVOCs.

Despite their experimental and analytical tractability, synthetic approaches are simplifications of natural systems that exclude biological interactions between plants and floral microbes. For example, plant metabolites or responses to infection may be required as substrates or elicitors for certain mVOCs. In contrast, plants may change emission of floral VOCs in response to microbial presence. In addition, artificial media likely lack flower-specific nutrient sources for microbes which may serve as substrates for ecologically important semiochemicals and floral mVOC emission fluctuates depending on the growth substrate [6,44•]. Furthermore, the growth and physiology of floral microorganisms likely vary among habitats, which could affect the emission rate or composition of mVOCs. These effects may be crucial to understanding VOC-mediated communication at the floral interface and can only be definitively understood in plant-based studies.

Floral mVOC studies rarely simultaneously incorporate both synthetic and plant-based approaches (but see Ref. [53]), yet a dual approach is beneficial [54], providing greater clarity on flower–microbe–insect interactions as well as partitioning candidate volatiles into groups: (1) those directly produced by floral microorganisms, and thus likely to occur in a variety of flowers; (2) those produced as a result of plant response to colonization (induction or reduction of antimicrobial or other floral VOCs); and, (3) microbial transformation of plant metabolites, detectable only when the full suite of chemical complexity is available to microbes, including oils, waxes, pollen grains, vitamins, minerals, and other plant metabolites.

Analytical approaches

VOCs described above (Tables 2 and 3) are most frequently detected and characterized using gas chromatography–mass spectrometry (GC–MS) for in-plant and *in-vitro* approaches. GC–MS is often used because of its sensitivity, suitability to the detection of broad chemical classes, and capacity for identifying unknown compounds.

In order to analyze VOCs, most studies adopt a pre-concentration step to collect VOCs onto a porous polymer (or sorbent matrix) from sample headspace before analysis. VOCs are then released via chemical or thermal desorption. Collection methods can be classified as either passive or active. Active collection techniques use a pump, external gas flow, or both to direct sample headspace through a sorbent, while passive collection relies on equilibrium-based partitioning of molecules from a defined volume headspace to the sorbent. Compared to studies of floral VOCs [55], studies of floral microbes more frequently rely on passive collection methods like solid-phase microextraction (SPME) to concentrate volatiles before analysis, rather than active sampling techniques (Tables 2 and 3). The prevalence of SPME in floral mVOC studies may be explained by the small molecular mass and high polarity of many major metabolites comprising mVOC blends. Small, polar metabolites are often either poorly retained or irreversibly bound to sorbent matrices frequently used in active sampling techniques of plants such as Tenax®, Poropak^{TM} Q, or graphitized carbon (Rering, unpublished data), whether emitted from microorganisms or macroorganisms. At present, insufficient data exists to definitively advise on what techniques are best able to capture microbe-induced changes to floral aroma and we recommend further study on this subject. Given current understanding, we offer the following suggestions:

1) Choose a collection technique-based on the goals of the study. If semi-quantitative data is suitable for a research question (e.g. if a study compares VOCs between related species/samples, or as a first step to characterizing VOCs), SPME is a good choice, especially when limited specialized instrumentation is available. If fully quantitative data is needed (e.g. for the development of specific trap or lure formulations or dose-response bioactivity tests), active sampling techniques should be considered. This is not to

say that quantitative data cannot be obtained from SPME, but because of its equilibrium-based adsorption, the logistics for calibration are substantially more intensive.
2) When possible, thermal desorption should be used. In chemical desorption, the solvent peak obscures many early eluting mVOCs. If chemical desorption is selected, researchers should consider additionally analyzing a subset of their samples with a solventless technique.
3) When possible, select mixtures of sorbent polymers for SPME or active sampling filters, to capture a wide variety of chemicals.
4) Acknowledge limitations of any approach. In practice no chemical analysis method can measure all molecules simultaneously (e.g. challenging analytes like small polar metabolites and inorganic gases are often excluded or poorly detected). Metabolite limits of detection, and classes of compounds that can be detected should be mentioned when results are reported.

Insect chemoreceptivity: testing mechanisms and identifying bioactive compounds

Although the specific volatiles emitted by microbes are beginning to be characterized, understanding of insect detection of mVOCs remains limited. We propose that increased attention to insect detection may reveal which mVOCs mediate ecological interactions.

Insects are able to detect chemical cues from the environment (e.g. mVOCs), through the use of chemosensory systems. For an in-depth examination of the mechanisms underlying insect olfactory systems, we direct readers to several recent reviews and book chapters [2,56–59]. The majority of VOCs in the environment are detected by insect olfactory receptors (ORs), which can be identified through sequencing and/or transcriptomics, and characterized using electrophysiological techniques (reviewed in Refs. [56,60]). Candidate insect chemosensory genes are being discovered at a rapid rate; however, variation in the number of ORs across species, large number of environmental VOCs, and difficulty present in finding a universal expression system hinders the ability for large scale electrophysiological characterization of individual ORs across insect species [56]. Therefore, electroantennograms (EAGs) and electropalpograms from populations of ORs on the surface of insect olfactory organs [61], are a useful first step in examining an insect's ability to detect ecologically relevant compounds.

Studies to date suggest that insects are able to detect a subset of floral mVOCs. Studies using EAG found that honey and bumble bee antennae are able to detect VOCs emitted from floral microbes. Antennae of both species were able to detect approximately 20% of compounds at a concentration that provided consistent antennal response for bee pheromones [32••,40•] and honey bees were further able to detect approximately 70% of VOCs when concentrations were increased 100-fold [32••]. However, EAG analyses screen only for the detectability of specific VOCs via ORs and cannot be directly correlated with behavioral responses as there is more downstream processing of olfactory signals after initial detection. In order to identify which components of microbial volatile blends are contributing to insect behavioral responses, or whether mixtures have synergistic or antagonist effects, behavior trials with individual odorants and mixtures identified through electrophysiological assays are needed.

Conclusion

The integration of flower-dwelling microorganisms in the study of plant–insect interactions is relatively new. Among such studies, few have directly examined volatile communication between floral microbes and insects. Fewer still have incorporated plants in these studies. Future studies should:

1 **Explore more diverse study systems:** Most studies focus on nectar (particularly nectar-specialist yeast and monocultures) and examine responses in generalist pollinators, including only a few plant species. However, other systems likely differ and require study. Systematic studies across microbial guilds, ideally in a phylogenetic context, may provide more insight into plant–floral microbe–insect interactions and reveal conserved or novel mVOCs with semiochemical activity.
2 **Integrate more plant-based approaches:** Though bottom-up approaches are helpful for highly complex systems such as this, holistic understanding is still needed. For example, it is likely that many behavioral shifts observed in nature are mediated by microbe modification of repulsive/attractive floral volatiles and not specifically via mVOCs.
3 **Adopt an interdisciplinary approach:** Chemical ecology, an inherently interdisciplinary field requiring expertise from diverse scientific backgrounds (e.g. behavioral ecology, chemistry, entomology), has revealed cues that mediate interspecies interactions. Continuing to use a combination of techniques in the study of plant–insect–microbe interactions should identify novel interactions and help identify semiochemicals responsible for mediating them.
4 **Integration of volatile databases:** In the past microbes were not necessarily considered as contributors to floral volatile emissions; however, understanding the role of volatiles cues in plant–microbe–insect interactions requires an understanding of where these cues originate from (i.e. flower, microbe or microbe induced floral VOC). Therefore, integration across floral [62–64] and microbial [47,65,66] volatile databases may improve our

understanding of the volatile landscape in which plants, microbes and floral visitors interact.

Although microbes and mVOCs can mediate insect behavior, the exact changes to volatile emission perceived by insects and mediating certain behavioral responses have not been identified and likely vary across floral microbe–insect–plant interactions. Discovery of key volatile semiochemicals underlying interactions occurring between diverse plants, insects, and floral microbes in nature will be facilitated by collaborative studies that systematically expand biological and chemical complexity.

Conflict of interest statement

Nothing declared.

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