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Characterization of volatiles in strawberry varieties 'Elsanta' and 'Sonata' and their effect on bumblebee flower visiting

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Abstract The bumblebee *Bombus terrestris* is a general pollinator in Europe and also highly suitable as greenhouse pollinator of various agricultural and horticultural crops such as tomato, sweet pepper or strawberry. Here, we highlight the importance of volatile emissions for the attractiveness and pollination efficiency of strawberry varieties, starting from the unique observation that two related strawberry varieties are pollinated to a different extent when cultivated together. In a flight cage *Fragaria × ananassa* var. 'Sonata' was pollinated more frequently (>2 times) and with longer-duration visits (11.2 vs. 6.7 s) by *B. terrestris* as compared to var. 'Elsanta.' To investigate whether this visitation difference could be attributed to differential production of flower volatile compounds, we quantified and analyzed the floral emissions of both varieties. Samples of var. 'Elsanta' contained more green leaf volatiles such as E-2-hexenal (0.53 vs. 0 ng/3 flowers), Z-3-hexenol (2.26 vs. 0.20 ng/3 flowers) and Z-3-hexenyl acetate (2.15 vs. 0.46 ng/3 flowers) which are known to play a role in plant defense. In a third series of experiments, we determined olfactory responses of *B. terrestris* to some similar individual synthetic green leaf volatiles presented in a Y-tube olfactometer. *B. terrestris* workers responded in an aversive manner to these volatile compounds compared to purified air. Since the floral bouquet of var. 'Elsanta' contains more green leaf volatiles, bumblebees will exhibit a preference for var. 'Sonata.' Our observations suggest that the pollination preference for 'Sonata' is due to being 'less repellent' instead of 'more attractive' than 'Elsanta,' with variety-specific flower emissions lying at the basis.

Keywords Strawberry · Sonata · Elsanta · Flower visiting · Green leaf volatiles

Introduction

Bombus terrestris or the buff-tailed bumblebee is an important pollinator of several horticultural and agricultural crops. Particularly, in greenhouse crops such as sweet pepper, tomato and strawberry, it plays a key role in the establishment of successful fruit setting (Velthuis and Van Doorn 2006; Goulson 2010; Morse et al. 2012). A higher level of cross-pollination often leads to an increase in fruit set and a reduction in the percentage of malformed fruits. For these reasons, bumblebees are commercially reared for their pollination practices (Zebrowska 1998; Velthuis and Van Doorn 2006; Abrol 2012; Goulson 2010; Zaitoun et al. 2006).

The most common way plants lure pollinators to visit their flowers is by providing nutritional rewards such as nectar and pollen (Goulson 2010). Bumblebees will consequently learn to visit these rewarding flowers to maintain the colony. Multiple floral traits such as flower color (Peitsch et al. 1992), flower accessibility (Gegear and Laverty 2005), inflorescence architecture (Ishii et al. 2008), floral fragrances (Dudareva and Pichersky 2006; Raguso

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2009), and pollen and nectar content (Rasmont et al. 2005) determine the choices of pollinating insects and their foraging behavior (Goulson 2010; Wright and Schiestl 2009). This enables the colony to find and collect food with maximum efficiency. Depending on the pollinator's preferences, differences in the perceptual qualities of floral traits can have an influence on the plant's reproductive output (Raguso 2009; Dudareva and Pichersky 2006).

Besides being unique in morphological characteristics and providing sufficient quantity and quality of the rewarding food resources, plants keep up their likeliness to pollinators by fragrances. These biogenic volatile organic compounds (BVOCs) play a crucial role in the discrimination and recognition of a plant species, and flowers of different species can be distinguished even more by their smells than by their morphological flower characteristics (Kunze and Gumbert 2001; Abrol 2012; Wright and Schiestl 2009; Parachnowitsch et al. 2012). Olfactory systems of bumblebees are particularly adapted to process complex odor mixtures and to perceive subtle differences in the perceptual qualities of floral scents (Laska et al. 1999). Such floral volatiles can be grouped into a range of different chemical classes including terpenoids, phenylpropanoids/benzenoids, and fatty and amino acid derivatives. These compounds are derived from the plant's secondary metabolism through anabolic and catabolic processes (Dudareva and Pichersky 2006; Knudsen et al. 2006). The possible combinations of such BVOCs are numerous, differing quantitatively and/or qualitatively, resulting in a unique blend of volatiles for each plant species (Kessler and Halitschke 2009; Dudareva and Pichersky 2006). Fragrances emitted by floral organs function mainly as attractants for pollinators, but some compounds might function as repellents. Indeed, absence or presence of certain compounds can affect the attractiveness of a scent and consequently influence the pollinator's foraging pattern (Raguso 2009; Schiestl et al. 2010). Vegetative plant parts mainly emit so-called green leaf volatiles (GLVs), which act as an indirect defense against herbivorous insects and even posses antifungal or antimicrobial properties (Friedman et al. 2002). Furthermore, BVOCs can function as inter-plant signals that activate defense pathways in neighboring plants (Heil and Ton 2008) or as an indirect defensive strategy by recruiting natural enemies of herbivores (Kessler and Halitschke 2009; Arimura et al. 2005). Hence, the unique blend that is emitted will have an effect on the plant's visitors.

Bumblebee pollination plays a key role in the quality and quantity of marketable strawberry fruits (Zaitoun et al. 2006; Velthuis and Van Doorn 2006; Klatt et al. 2013). Despite the high efficiency of *B. terrestris* as pollinator in strawberry cultivation (Abrol 2012; Zaitoun et al. 2006), differences in flower preference between closely related varieties can cause major problems for fruit set (Klatt et al. 2013). Strawberry growers are challenged with difficulties in pollination when the varieties 'Elsanta' and 'Sonata' are cultivated together with *B. terrestris* implemented as pollinator in the greenhouse: var. 'Sonata' will be overpollinated while var. 'Elsanta' will be underpollinated. This results in significant fruit malformations for both varieties (Abrol 2012).

This study investigates the floral scent characteristics of strawberry and their effect on bumblebee pollination by (1) characterizing the foraging preferences of bumblebees on the greenhouse strawberry varieties 'Elsanta' and 'Sonata' in a flight cage, (2) identifying and analyzing the floral odor bouquets of both varieties using a GC/MS setup, and finally (3) testing the reaction of *B. terrestris* to some individual GLV organic compounds by means of a Y-tube olfactometer.

Materials and methods

Test organisms

Two commercially produced *B. terrestris* colonies from Biobest (Westerlo, Belgium) containing 1 queen and 40 workers each, were used and fed with commercial sugar water (60 % Biogluc, Biobest) and pollen (EPC pollen mix, Biobest). Before the start of the experiments, *B. terrestris* workers were only fed with sugar water during 3 days to stimulate foraging.

Strawberry plants of var. 'Elsanta' and var. 'Sonata' were obtained from Proefcentrum Hoogstraten (Meerle, Belgium) and grown in a growth chamber (19 °C, 16 h of photoperiod with artificial lights, and water and fertilizer supplied on a weekly basis).

Bumblebee preference test toward two strawberry varieties

Twelve randomly chosen bumblebee foragers entered individually a flight cage of 60 cm × 60 cm × 60 cm containing two plants of *Fragaria × ananassa* var. 'Elsanta' and var. 'Sonata' with five flowers each during each repeat. In each repeat, new flowers were used to avoid the effect of scent marks (Stout et al. 1998). Each bumblebee was able to fly and forage freely and return to the hive at the end of each foraging trip. The workers were marked with opalith plates and color markings to avoid using the same worker multiple times. A flower was considered as visited if a worker spent more than 3 s on it. The number of flower visits was counted, and significant differences were tested (depending on normality of the data) using a Mann–Whitney U test. In addition, the time that each bee spent

during each single flower visit was recorded. To assess the effect of strawberry variety on the visiting time, a linear mixed-effects model was built where the number of flower visits was incorporated as repeated measures by specifying bee as a random factor.

BVOC sampling

Three flowers of the same plant were placed inside a cuvette made out of nalophan (Foodpack, Harderwijk, The Netherlands) using a slightly modified design of Joo et al. (2010). A membrane pump (N035AN.18, KNF Neuberger GmbH, Freiburg i.Br., Germany) with a dust filter (2 μm pore size Zefluor PTFE Membrane Filter, Pall, MI) provided a continuous air stream of 300 mL min^{-1} (GF40, Brooks Instruments, Hatfield, CA). The incoming air was purified of pollutants and ozone by two active carbon filters (Airpel 10, Desotec, Roeselare, Belgium) and an ozone filter (ETO342FC002A, Ansyco, Karlsruhe, Germany), respectively.

Multi-adsorbent tubes (Markes international Ltd., Llantrisant, UK) were used to collect the BVOCs. Each tube contained 210 mg Tenax TA (35 m^2/g, Markes International, Llantrisant, UK) and Carbotrap (100 m^2/g, 20/40 mesh, Markes International) with a volumetric ratio of 50:50. Before sampling, each tube was conditioned for 1 h at 300 °C under a He flow (±50 mL min^{-1}); toluene-D8 was chosen as internal standard, for the quantification of the BVOCs using a closed two-phase system (Demeestere et al. 2008). Sampling of the air in the cuvettes was carried out using a Flec air pump (1001, Markes International); 50 mL was drawn out of each cuvette at a rate of 100 mL min^{-1}. Background VOCs were checked by sampling an empty cuvette.

BVOC analysis

After sampling, the adsorbed BVOCs were thermally desorbed (Ultra 50-50-UNITY, Markes International) and analyzed with a GC Trace 2000 gas chromatograph (ThermoFinnigan, Milan, Italy) connected to an MS Trace DSQ WE-250 mass spectrometer (ThermoFinnigan, Austin, TX). The entire system was controlled by Unity 1.2.0 (Markes International) and XCalibur 1.3 (ThermoFinnigan, Austin, TX) software. Thermal desorption was performed at 260 °C for 10 min with He as the carrier gas. Compounds were transferred to the GC with a flow of 10 mL min^{-1} at 130 °C using splitless mode. To separate the compounds, a VF-1 ms column (Varian, Sint-Katelijne-Waver, Belgium; 30 m × 0.25 mm × 1 mm) was used. The temperature program started at 30 °C for 10 min and ramped to 60 °C with 2 °C min^{-1}, then ramped to 170 °C at 8 °C min^{-1} and further ramped to the final temperature of 240 °C at 15 °C min^{-1}.

This analysis was repeated six times on recently emerged, but fully developed flowers (2 days old) from healthy plants that had not been pollinated. As floral scent is highly variable depending on flower age, pollination status and circadian rhythm (Rodriguez-Saona et al. 2011), we sampled flowers under the same conditions. Sampling was conducted during the peak of emission intensity, i.e., between 11:00 a.m. and 4:00 p.m. Significant differences were tested using an independent samples t test.

Preference test for synthetic GLVs

To determine bumblebee responses to individual synthetic GLVs, an experimental design was used as described in Giner et al. (2013). This was achieved by using a standardized glass Y-tube whereby the entrance channel and the arms had a length of 10 cm and 7.5 cm, respectively. The arms were separated by 60° and had a diameter of 1 cm. Responses to some general GLVs (Sigma-Aldrich, Bornem, Belgium) were tested: Z-2-hexen-1-ol and 2-hexanol (alcohols, aliphatic fatty acid derivatives), and Z-3-hexenyl acetate and E-2-hexenyl acetate (carbonic acids, aliphatic fatty acid derivatives). Acetophenone (benzenoids) was used as a negative control as it is known for its repellent properties (Suchet et al. 2010; Klatt et al. 2013; Knudsen et al. 2006). Each volatile was presented as a 10 μL of a 5 % (v/v VOC/distilled water) solution in a small recipient at the end of the Y-tube, against fresh air at the other end.

Bumblebee foragers were placed at the entrance of the Y-tube individually, and the choice they made between the two ends of the Y-tube was recorded. Individual bumblebees were never used twice during the same binary trial; in total 51 bumblebees from two different nests were used per binary choice experiment. The Y-tube was cleaned for each trial with a 70 % ethanol solution to avoid any markings left by previous bumblebees. Differences in number of choices were tested for its significance using a Chi-square test. Additionally, the time needed to make a choice in the Y-tube was recorded.

Results

Preference test with two strawberry varieties

Bumblebees significantly (Mann–Whitney U test: $U = 37.5$, $N = 12$, $p = 0.049$) preferred the flowers of 'Sonata,' with 11.9 ± 3.2 visits, over the 'Elsanta' flowers, with only 5.8 ± 1.5 visits (mean $\pm$ SE). When the foragers entered the cage, in 75 % of the cases, a 'Sonata' flower was chosen first. In 66.7 % of the cases, the first and second visit was recorded on a 'Sonata' flower, and in 41.7 %

of the cases, the first three visits were spent on a 'Sonata' flower.

Bumblebees spent significantly (linear mixed model: $\chi^2 = 11.45$, $p < 0.001$) more time on 'Sonata' flowers compared to flowers of 'Elsanta' in all repeats: the mean visit time on 'Sonata' was 11.9 ± 0.7 s, while on 'Elsanta' this was only 8.1 ± 0.6 s (mean $\pm$ SE). Additionally, visual observation of the bumblebees showed more destructive behavior such as biting, on the flowers of var. 'Sonata.' Such behavior can be attributed to collect more pollen.

GC–MS analysis of floral volatiles

Clear qualitative as quantitative differences in floral volatile emissions for certain compounds were observed between the varieties (Fig. 1). Variety 'Elsanta' produced more GLVs like E-2-hexenal (0.53 ± 0.10 vs. 0 ± 0 ng/3 flowers) (independent samples t test: $t(9) = 5.56$, $p < 0.001$), Z-3-hexenol (2.26 ± 0.87 vs. 0.20 ± 0.11 ng/3 flowers) (independent samples t test: $t(10) = 2.34$, $p = 0.041$) and Z-3-hexenyl acetate (2.15 ± 0.64 vs. 0.46 ± 0.16 ng/3 flowers) (independent samples t test: $t(9) = 2.78$, $p = 0.022$) in comparison with var. 'Sonata' (mean $\pm$ SE). Also a higher (independent samples t test: $t(10) = 2.29$, $p = 0.045$) share of the monoterpenoid alpha-pinene was found in the floral emissions with 0.13 ± 0.05 ng/3 flowers in 'Elsanta' while only 0.02 ± 0.009 ng/3 flowers in 'Sonata' (mean $\pm$ SE). The other BVOCs such as 2-tridecanone, 2-heptanone, 6-methyl-5-hepten-2-one, beta-pinene, limonene, linalool and alpha-farnesene differed only marginally between the varieties. Limonene was present in much higher proportions than the other volatiles (4.68 ± 1.66 and 4.14 ± 1.98 ng/3 flowers) in both varieties; emissions of beta-pinene on the contrary were more than 10 times smaller (0.0044 ± 0.002 and 0.0042 ± 0.002 ng/3 flowers) than the other components (mean $\pm$ SE).

Preference test for synthetic VOCs

In the binary choice experiments, bumblebee workers preferred the end of the Y-tube that contained no volatile at all (Fig. 2), indicating a repellent effect of the tested monomolecular synthetic GLVs. This was the case for the GLVs 2-hexanol (Chi square test: $\chi^2 = 4.42$, $p = 0.038$) and Z-2-hexen-1-ol (Chi-square test: $\chi^2 = 5.66$, $p = 0.018$). Also, for the remaining volatiles E-2-hexenyl acetate and Z-3-hexenyl acetate, a repellent trend was noticeable, although not significant (p values of 0.126 and 0.209, respectively) (Fig. 1). The control treatment with acetophenone had a significant repulsive effect (Chi-square test: $\chi^2 = 39.71$, $p < 0.001$). Finally, there was no significant difference in time needed to make a choice between the two arms of the Y-tube in each of the binary

choice experiments (Mann–Whitney U tests: $p > 0.05$ for all experiments). Also, the different choice experiments did not differ in time compared to each other (Kruskal–Wallis test: $p > 0.05$ for all experiments).

Discussion

Bumblebees are widely used as pollinators in order to enhance fruit yield and quality in greenhouse crops (Velthuis and Van Doorn 2006; Zaitoun et al. 2006). However, the preferences of bumblebees for certain varieties can lead to different visitation rates, affecting the intended pollination services and thus fruit yield and quality (Dötterl and Vereecken 2010). Here, we investigate the importance of subtle fragrance differences mediating plant–pollinator interactions in strawberry varieties. More specifically, our results indicate a significant preference of *B. terrestris* workers for flowers of the variety 'Sonata' over 'Elsanta.' As a consequence, when bumblebee densities in the greenhouse are too high for the number of flowers present, there is a danger for overpollination with subsequent loss of quality due to malformed fruits (Klatt et al. 2013; Dötterl and Vereecken 2010). Hence, 'Elsanta' will experience a yield loss as well but due to underpollination.

The preference for var. 'Sonata' can be explained by a distinct floral emission profile, as significantly higher proportions of the GLVs E-2-hexenal, Z-3-hexenol and Z-3-hexenyl acetate were present in the scent profile of 'Elsanta' (Fig. 1). As demonstrated in experiments with the Y-tube olfactometer, GLVs evoke a repellent response, in agreement with the known role of these volatiles in the plant's defense mechanisms. E-2-hexenal has antimicrobial and antifungal characteristics and is effective against certain pathogens (Kishimoto et al. 2008). Z-3-hexenol is an herbivore-induced plant volatile, known as one of the most important signals in repelling phytophagous insects (Wei and Kang 2011). It also plays a role in the attraction of parasites and predators against these herbivores, and in the communication between neighboring plants (Wei and Kang 2011). Z-3-hexenyl acetate is also known to be emitted after leaf injury and herbivory, and as a signal in inter-plant communication (Kost and Heil 2006). It is as well one of the most frequently emitted flower volatile compounds (Klatt et al. 2013; Knudsen et al. 2006) and presumably emitted from ovaries and styles like Rodriguez-Saona et al. (2011) found out in *Vaccinium*. Because of the higher share of such GLVs in the floral scent of 'Elsanta,' it is plausible that they also evoke a repellent response to pollinating insects, thereby explaining the observed aversion of the tested bumblebees against this variety. As bumblebees use olfactory cues to gather information about the condition of the plant (reward
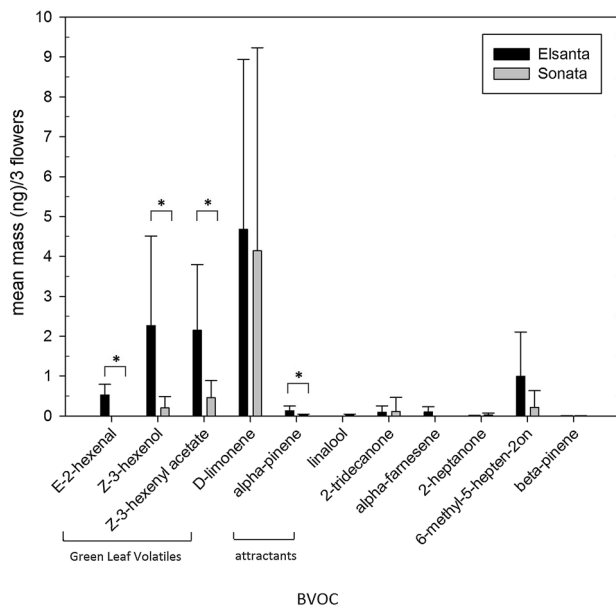
Fig. 1 Quantitative analysis of the volatile spectra of the cultivars *Fragaria × ananassa* var. 'Elsanta' and var. 'Sonata' with the use of TD-GC/MS technique. The variety 'Elsanta' produces more of the green leaf volatiles (GLVs) E-2-hexenal, Z-3-hexenol and Z-3-hexenyl acetate. In addition, 'Elsanta' had also a higher emission rate for alpha-pinene. Significance levels (independent *t* test) are indicated with asterisks (*$p < 0.05$), bars denote 95 % confidence intervals for visualizing statistical differences. Data were based on the BVOC analysis of three flowers per plant with six replicates per variety
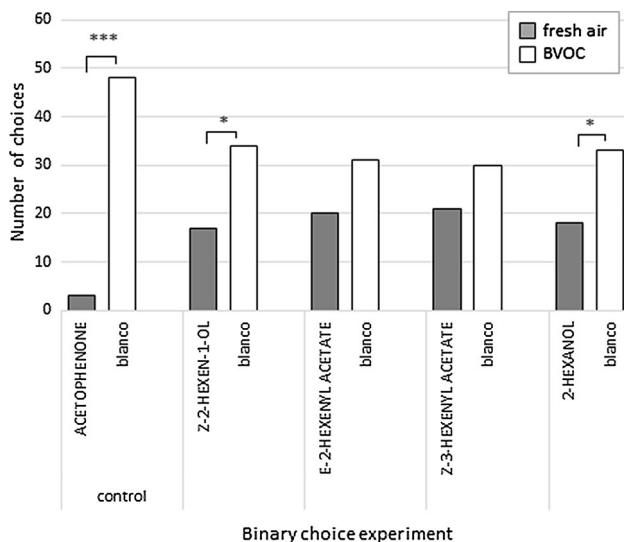
Fig. 2 Binary choice experiments with the Y-tube olfactometer for testing response to some green leaf volatiles (GLVs) versus fresh air (denoted as 'blanco'). Tested volatiles are Z-2-hexen-1-ol, E-2-hexenyl acetate, Z-3-hexenyl acetate and 2-hexanol. Acetophenone was used as control treatment. In general, the individual GLVs evoked a repellent response. Statistical significance (Chi-square test) between samples is indicated by asterisks (*$p < 0.05$; ***$p < 0.001$)

quality, predation risk, etc.) (Kessler and Halitschke 2009), they could presuppose that the 'Elsanta' variety is a less good choice than 'Sonata.' In this regard, the preference for

'Sonata' is the best choice in the context of the optimal foraging strategy and foraging efficiency (Heinrich 2004; Gegear and Laverty 2005). Similar quantitative differences in BVOC emissions between strawberry varieties were described previously by Klatt et al. (2013). For instance, Z-3-hexenyl acetate was present in the bouquets of varieties 'Honeoye,' 'Darselect' and 'Sonata,' but each with different amounts. These differences were reflected in the perceptual qualities of the scent which rely on the ratio and the relative intensity of the volatile compounds (Wright and Smith 2004; Wright and Schiestl 2009). Absence or presence of certain compounds can change the attractiveness of a scent, and consequently influence the pollinator's foraging pattern. It is the plant's challenge of attracting pollinators while evading herbivores (Raguso 2009; Schiestl et al. 2010; Kessler and Halitschke 2009).

Furthermore, a difference for the BVOC alpha-pinene, a frequently occurring volatile in allelopathic plant emissions (Knudsen et al. 2006; Singh et al. 2006), was observed between the two cultivars, with 'Elsanta' having a higher emission rate. Interestingly, it is also recognized as a chemical compound of the bumblebee's recruitment pheromone (Granero et al. 2005), and therefore indicating a plausible attractive nature. However, the share of alpha-pinene seems not to compensate for the repellency evoked by the GLVs present in the 'Elsanta' bouquet. Relative concentrations of repellents and attractants are important, like Galen et al. (2010) showed in *Polemonium viscosum,* but the interaction of the different components can be of even more importance in the perceptive qualities of a floral scent. Naturally, odorants will never be emitted individually in a plant, but are always associated with other compounds in the volatile mixture. Two compounds can exert an inhibitory effect on each other or even compete for the same neuronal receptors (Getz and Akers 1995; Wright and Smith 2004), while others can act synergistically to increase their respective intensity (Abrol 2012; Dudareva and Pichersky 2006), and therefore elicit a specific neuronal response to pollinators.

The phenomenon of the observed variety preferences not only occurs in strawberry, but also in tomato (Jackson 1993), sweet pepper (Abrol 2012), blueberry (Rodriguez-Saona et al. 2011) and other crops. For example, Suchet et al. (2010) observed that *B. terrestris* workers were capable of discriminating subtle differences in floral volatile emissions between two subspecies of snapdragon *Antirrhinium majus.* The more attractive subspecies had a higher share of attractive monoterpenes, and the repellent effect of acetophenone was stronger in the less attractive one. Similar observations concerning differences in floral attractiveness due to fragrance were reported by Rodriguez-Saona et al. (2011) in a comparison with three varieties of blueberry *Vaccinium corymbosum*. Because of this,

Mackenzie et al. (1997) recommended that commercial plantings consist of solid blocks of single cultivars so that differences in floral attraction cannot interfere with the intended pollination practices.

A difference in quality and/or quantity of food resources can be an additional reason why var. 'Sonata' is preferred. In natural conditions, floral scent is always accompanied by other flower characteristics, such as a food reward, which suggests an associative basis for olfactory perception (Laloi et al. 1999; Chandra and Smith 1998). Different sensory cues, such as visual, olfactory and reward, are integrated into one multimodal combined stimulus on which *B. terrestris* depends for its foraging choices, as Kunze and Gumbert (2001) and Kulahci et al. (2008) suggested. However, our results designate olfactory cues as the primary determining factor for the observed preference since the first visit was mostly directed to a 'Sonata' flower without probing other flowers in advance. In addition, bumblebees spent more time on 'Sonata' flowers designating a better food reward, as the presence of more nectar and/or pollen in a single flower requires more time to collect compared to a less rewarding one. Future studies should focus on the pollen quantity and quality which might vary among cultivars, as is the case with blueberries (MacKenzie 1997), since it can be an additional factor in flower preference.

In conclusion, we have shown that *B. terrestris* workers prefer the flowers of the strawberry variety 'Sonata' over those of 'Elsanta' when co-cultivated. This preference can be attributed to a higher share of GLVs in the floral bouquet of 'Elsanta,' volatiles that evoke a repellent response to bumblebees. Since pollination differences are undesirable for commercially important fruit crops, strawberry breeding programs should also draw more attention to volatile-mediated pollinator attraction in order to increase crop productivity and fruit quality.

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