

**Determining changes in floral volatile composition
of *Ipomopsis aggregata* in response to nectar robbing
and its associated microorganisms**

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Abstract

Mutualisms involve complex relationships between multiple types of organisms. Traditionally, mutualisms like pollination have been studied using a pairwise perspective – only focusing on two individual species or groups of species with similar functions involved in the relationship. Including third-party organisms in pollination research allows for a greater understanding of how those mutualisms can persist or break down depending on the different types of interactions. Studying these dynamics are essential to maintaining the ecosystem services provided by pollination and understanding how these mutualisms are able to persist despite interference and exploitation. *Ipomopsis aggregata*, a hummingbird pollinated montane herb, experiences nectar robbing from native bumble bees, *Bombus occidentalis* and *Bombus mixtus*, which reduces hummingbird visitation, pollen transfer, and the reproductive success of *I. aggregata*. In this study, I examined how nectar robbers and the microbes they disperse alter the chemical phenotype of *Ipomopsis aggregata*. While volatiles of microbial origin were detected in a subset of microbe-inoculated samples, there were no detectable differences in volatile composition across a combination of robbed and unrobbed flowers with either a sterile sucrose solution, live yeast or bacteria, or dead yeast or bacteria. The lack of distinct volatile differences is likely due to confounding variables including chemical and microbial contamination and the high variability of *I. aggregata* floral volatiles, along with limited sample sizes. This research offers valuable insights into the difficulties of floral and microbial volatile analysis in the field and suggests that sampling single treated flowers from more controlled populations might reduce contamination and general phenotypic volatile variation.

Introduction

Complex mutualistic relationships, often involving organisms across trophic levels, have shaped the world we live in today.¹ While mutualisms have traditionally been – and still are – studied through a pairwise perspective,² a more comprehensive understanding of the organisms involved is needed to determine the conditions in which mutualisms can flourish or, alternatively, break down.

Pollination mutualisms are often studied with a sole focus on plant-pollinator relationships^{3,4} while excluding third-party interaction involving organisms like floral exploiters and plant-inhabiting microorganisms.⁵ Studying the influences of third-party organisms on pollination dynamics is essential to maintaining the ecosystem services provided by pollination and understanding how these mutualisms are able to persist despite interference and exploitation.

For example, *Ipomopsis aggregata*, a hummingbird-pollinated plant, experiences nectar robbing from the bumble bee *Bombus occidentalis* which allows for secondary nectar robbing and further resource depletion. The primary and secondary robbing of *I. aggregata* causes pollinator avoidance, leading to reduced pollen movement and reduced reproductive success.^{6,7,8} The exact mechanisms causing pollinator avoidance and secondary robber attraction remain

unknown, but past studies have indicated that changes in floral chemical cues in response to robbing and robber-introduced microbes might be influencing these interactions.^{6,9,10}

There are several different ways that the floral volatile profile can change in response to visitation by nectar robbers. Plants are capable of changing their volatile composition in response to different types of antagonists including pathogens and herbivores.¹¹ The tissue damage and presence of foreign microbes on the flower and in the nectar of *I. aggregata* could cause its volatile profile to change which might influence pollinator and robber attraction. Furthermore, the microbes introduced into the nectar from robbers could directly alter floral volatile composition by metabolizing floral volatiles or by creating their own volatile compounds.^{11,12,13}

I. aggregata flowers experience primary nectar robbing by the bumblebee *Bombus occidentalis* with an average of 66% of the flowers on a plant robbed during peak robbing activity.¹⁴ *B. occidentalis* cuts through the corolla of the flower using its mandibles and drinks the nectar without engaging in pollination. The opening allows for secondary robbers such as other bumblebees, wasps, ants, and flies to access the nectar as well.¹⁴ Although the act of nectar robbing does not harm the reproductive structures of *I. aggregata*, primary and secondary robbing decreases pollinator visitation and lowers reproductive success.^{7,8,14} Yeasts and bacteria introduced from pollinators and robbers are known to colonize the nectar of wildflowers in montane regions and impact nectar chemistry and floral volatile composition – possibly influencing pollinator and secondary robber attraction.⁹

In this study, I examined how nectar robbers and the microbes they disperse alter the chemical phenotype of *Ipomopsis aggregata*.

Methods

Study System - I studied *Ipomopsis aggregata*, a monocarpic herb with distinct red, trumpet-shaped flowers that is pollinated by broad-tailed and rufous hummingbirds (*Selasphorus platycercus* and *Selasphorus rufus*, respectively).⁸ Field work was conducted from mid June to early August across three populations of *I. aggregata* near Avery Mountain [38.979890, -106.999598] and the Kettle Ponds [38.946973, -106.976975] near the Rocky Mountain Biological Laboratory in Gothic, Colorado (Fig. 1). Prior to flowering, all *I. aggregata* individuals used in this study were placed in insect exclusion cages made out of fine aluminum mesh to limit pollinator and robber visitation.

Obtaining Microbes from Robbed Nectar - To obtain nectar microorganisms from robbed flowers, previously netted and cooled-to-torpor *B. occidentalis* and *B. mixtus* were placed in the insect exclusion cages surrounding *I. aggregata* individuals and at least 30 minutes were given to see if the bumblebees would primary rob. If no robbing was observed after that timeframe, the bumblebee was temporarily recaptured while incisions were made on the corolla of nectar containing flowers to mimic primary robbing. The bumblebee was then replaced in the pollinator exclusion cage and given an additional 45 minutes to engage in secondary robbing.

Once the bumblebee had robbed, or had been in the cage for the allotted time, the bumblebees were released and the insect exclusion cage was replaced. Robbed flowers were then collected using sterile tweezers after 24 h to allow for nectar replenishment and microbe inoculation. The nectar extracted from robbed flowers from the same treated inflorescence were mixed together and diluted in 50 μ L sterile water before being plated on yeast media (YM) agar supplemented with the antibacterial, chloramphenicol, and Luria-Bertani (LB) agar supplemented with the antifungal, cycloheximide. The most common individual colonies for both yeast and bacteria were then isolated on separate plates. These isolates were used to make stock cultures of at least 1,000 bacterial or yeast cells per microliter in a 20% sucrose solution supplemented with a total of 100 mM of amino acids found in *I. aggregata* nectar.¹⁵

Experimental Design Artificial Robbing and Inoculation - Inflorescences prior to flowering ($N_{total}=148$ inflorescences) were covered with an insect exclusion cage. *I. aggregata* individuals receiving the unrobbed and sterile nectar treatment had their nectar removed with microcapillary tubes and replaced with 2 μ L of sterile amino acid and 20% sucrose solution using 25 μ L Hamilton syringe and remain caged until scent sampling.¹⁶ For the manually robbed and sterile treatment, an incision was made in the side of the corolla using sterile dissecting scissors and the remaining nectar was removed through the robbing hole using a microcapillary tube. The nectar was then replaced with sterile amino acid and 20% sucrose solution using the same procedures as the unrobbed flowers to ensure similar flower handling.

For the microbial treatments, inoculation solutions were prepared (see *Obtaining Microbes from Robbed Nectar*). For the live microbe treatments, 2 μ L of yeast or bacteria inoculated sucrose solution (20% sucrose concentration) with amino acids were added after applying the robbed or unrobbed treatment outlined for the robbed and unrobbed sterile treatments. To see if microbes elicit a change in volatile organic compounds (VOCs) from the plant, heat-killed yeast and bacteria was used.¹⁷ Separate yeast and bacterial inoculated solutions were made from the existing solution to ensure similar cell count and were microwaved for 2 minutes prior to injecting them in the flower using the same methods as for the live microbes.

All treatments had the insect exclusion cages replaced and were left for one day before volatile sampling.

Collection of volatile organic compounds. - Floral and microbial volatiles were captured using methods modified from Campbell *et al.*, (2019).¹⁸ Solid-phase extraction (SPE) traps were made from standard, stainless steel Markes sampling tubes containing 40 mg of Tenax TA[®] (60–80 mesh) and 30 mg of Carboxen[®] 1003 (40–60 mesh; inlet and sorbents, Supelco, Bellefonte, PA, USA). The larger mesh Tenax was added first followed by a mesh screen and then the Carboxen. The two sorbents were then sealed in by another mesh screen and a thin layer of glass wool. Reynold's oven bags were heat-sealed to standardized dimensions (5 cm x 13 cm) and were placed around the portion of the inflorescence containing the treated flowers to capture the floral/microbial volatiles. Ambient air and leaf volatiles were also collected to determine the

volatiles produced by the flowers/microbes, and not other components of the environment or plant. The Reynold's oven bags were allowed to equilibrate for 15 minutes before scent sampling. The scent traps were connected to a micro air sampler (Supelco PAS-500) set to a flow rate of 100 mL min⁻¹ and were placed in the bags for 30 min to collect volatiles from the treatment flowers, leaf controls, and ambient air samples.

Single flower samples were also taken to reduce vegetative compounds. Treated flowers were removed just below the sepals and were placed in 5 mL vials with the damaged vegetative part in 200 µL of water. Similarly, single leaves were collected and placed in vials with the damaged end in 200 µL of water. The single flowers and single leaf samples were then transported back to the lab in a cooler and placed in glass pasteur pipettes with glass wool and activated charcoal sealing one end. Scent traps were then connected to the pasteur pipettes and were connected to a micro air sampler (Supelco PAS-500) set to a flow rate of 100 mL min⁻¹ and were allowed to sample for 45 minutes. Ambient air samples were also collected using a similar set-up without anything in the pasteur pipettes.

Volatile analysis was then conducted using thermal desorption gas chromatography-mass spectrometry (GC-MS) at RMBL. The scent traps were loaded into a Markes Ultra autoloading system connected to a Markes Unity-xr unit to undergo a two-stage thermal desorption before being processed by a Shimadzu GC-MS QP2020. The initial desorption was for 5 min at 250 °C, followed by trapping at a low temperature (-30 °C) on a Tenax-filled cold trap, then heating rapidly to 200 °C for 3 min. The GC oven cycle was held for 2 min at 40 °C to retrap volatiles, then ramped at 10 °C min⁻¹ to 250 °C and at 30 °C min⁻¹ to 275 °C, with a final hold for 3 min. Ultra-high purity helium was used as the carrier gas and an Rtx-5MS column (30 m × 0.25 mm internal diameter, 0.25 µm film thickness). Compounds were identified using the Shimadzu GCMS Postrun software to match volatile spectra to those from the NIST14 mass spectral reference library (National Institute of Standards and Technology). The compounds were then filtered with an 80% match and contaminants were removed using the R package, *bouquet*.^{19,20} The volatile composition for the ambient air and leaf controls were also compared to the floral volatiles using *bouquet* and those that appeared in similar quantities were excluded from the analysis.

Statistical Analyses - All analyses were done in R (4.2.1). PerMANOVA was used to compare the volatile blends for the different treatments, implemented using the *adonis* function in *vegan*,²¹ and their volatile composition was visualized using non-metric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarities. The mean of the ranked dissimilarities between treatment groups was then compared to the mean of ranked dissimilarities within groups through an analysis of similarities test (ANOSIM) using the *anosim* function in *vegan*.

Results

A total of 148 individual *I. aggregata* inflorescences were treated with at least 10 samples per treatment type for the whole inflorescence scent sampling method and from 2-6 samples per treatment type for the single flower sampling method.

The whole inflorescence sampling of *I. aggregata* yielded 63 compounds for statistical analysis after filtering out contaminants and compounds present in the vegetative and ambient controls (Fig. 2). Permutational multivariate ANOVA (PerMANOVA) was used to determine that there was no significant difference between floral volatile composition between all treatment groups or between all sterile, yeast, and bacterial treatment regardless of their viability ($P > 0.05$). The PerMANOVA detected a marginally significant difference between all robbed and unrobbed treatments ($P = 0.098$). An NMDS plot of all the different treatments (Fig. 3) and for the robbed and unrobbed treatments (Fig. 4) demonstrated little separation of treatment groups. An additional ANOSIM test for the robbed and unrobbed treatments ($R = 0.0076$) revealed that the variation of floral VOCs within the different treatment groups was greater than the variation between the groups.

For the single flower samples, a total of 56 compounds were identified and used for statistical analysis (Fig. 5). PerMANOVA did not detect any significant differences between all the treatment groups, between robbed and unrobbed groups, or between the sterile, yeast, and bacterial treatments ($P > 0.05$). The NMDS plots for all the treatments (Fig. 6) and for the robbed and unrobbed treatments (Fig. 7) supported that there is no distinguishable difference between those treatment groups. An ANOSIM test for the robbed and unrobbed treatments ($R = 0.0016$) also provided similar results indicating the variation in floral scent within the the groups was greater than the variation between treatments.

Discussion

While several common floral volatile compounds previously detected in *I. aggregata*, such as alpha-pinene and alpha-farnesene, were present in the whole inflorescence samples (Fig. 2), there were few detectable microbial compounds.¹⁸ The lack of significant differences in volatile composition between treatment groups is likely due to the scent traps not desorbing between samples completely, leading to contamination across almost all the samples (Fig. 8). It is possible that, even with the contamination, the robbed and unrobbed treatments were marginally different while the microbial treatments were not due to the significantly higher abundance of floral VOCs present in the samples compared to the microbial VOCs. Single flower samples were then taken with heat-conditioned scent traps to remove chemical residues left on the traps after initial desorption for GCMS analysis.

Similarly, common *I. aggregata* volatiles were found in the single flower samples (Fig. 5). However, there were also compounds known to be produced by nectar microbes that were detected such as ethyl acetate, 3-methyl-1-butanol, and 1-hexanol.²² This might indicate that the single flower sampling method was better at microbial VOC detection as this method limited the amount of floral and vegetative compounds sampled. Furthermore, with such a small sample size

for each of the treatments (2-6) it would be difficult to detect major differences between treatment types.

The large amount of variation in volatile composition even within treatment types for both types of treatment could have been due to other factors known to affect floral scent such as the presence of soil and other foreign microbes in the nectar and on the plant along with the different levels of moisture available to the plant.¹⁸

While the study of volatiles from nectar microbes remains a relatively unexplored field, this project offers insights into the variability of different scent sampling techniques and their different levels of volatile detection. Overall, single flower sampling seems to offer better detection of microbial VOCs, although further studies would benefit from larger sample sizes and a more controlled population to sample from to reduce some of the natural variation in *I. aggregata* volatiles. Next steps could include identifying VOCs produced from the nectar and robber associated microorganisms through collecting bulk nectar and scent sampling in the lab.

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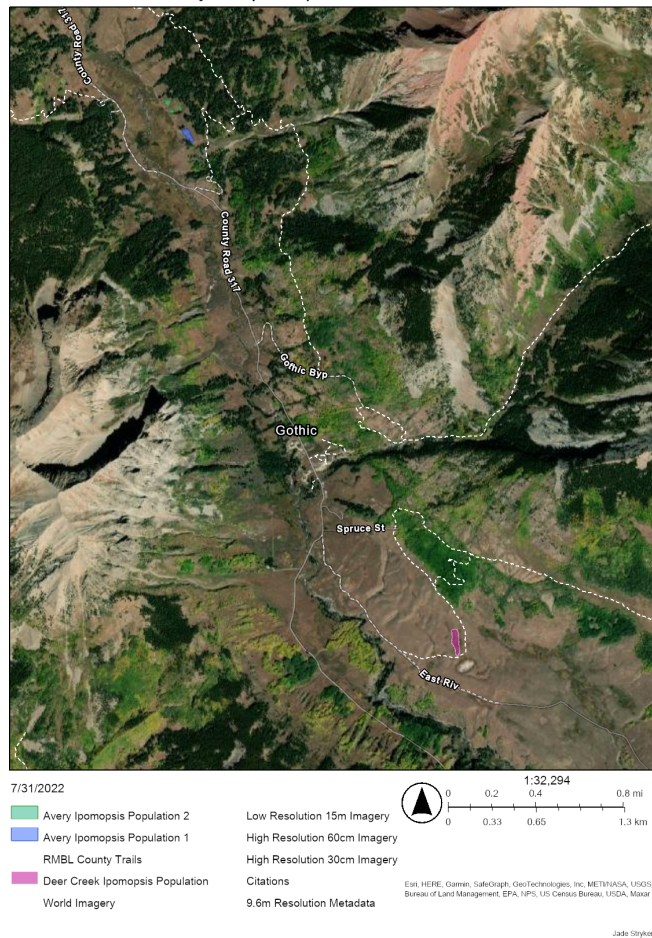


Figure 1: Map of field sites for *Ipomopsis aggregata* populations used in this research.

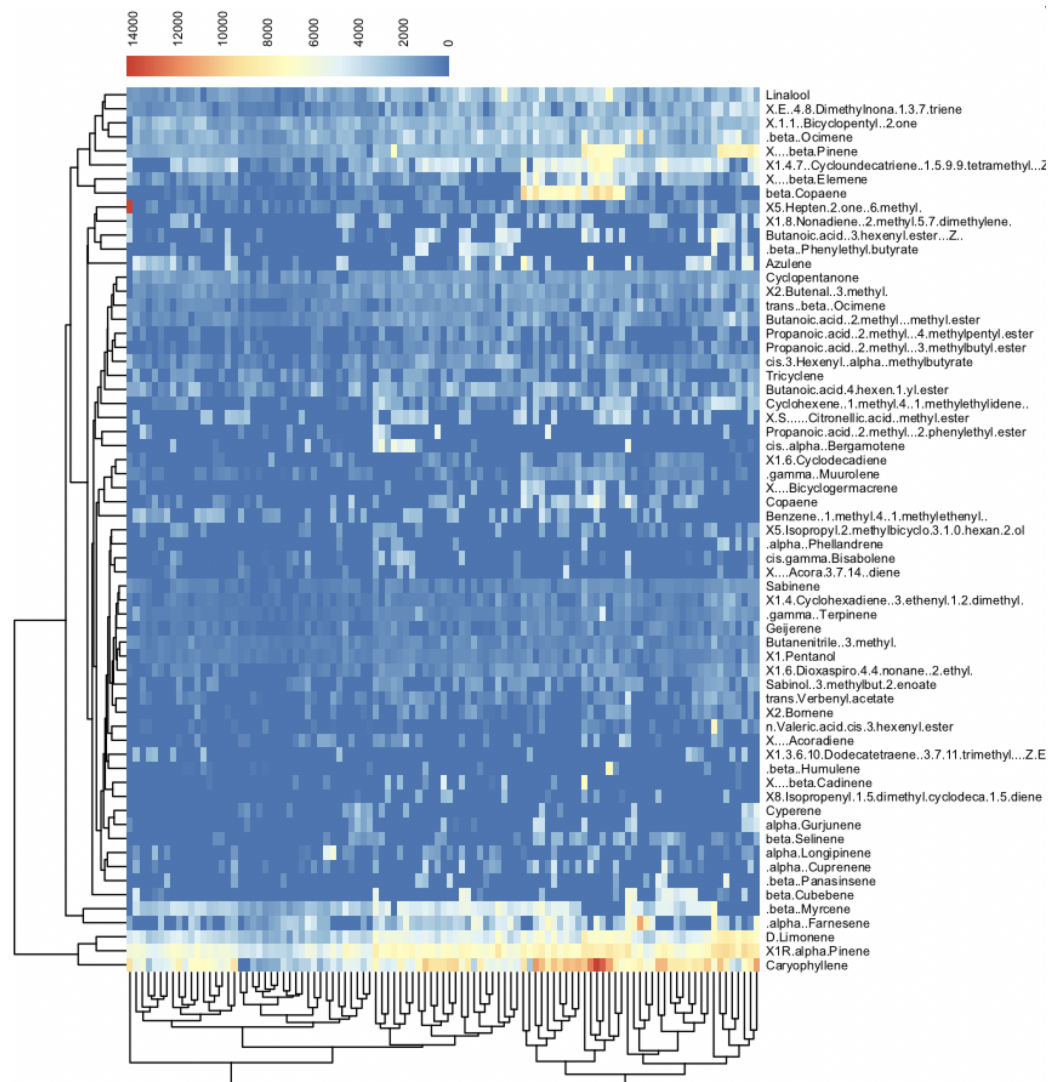


Figure 2: Heatmap of the compounds present in the whole inflorescence samples. Warmer colors indicate a higher amount of volatiles present in the sample.

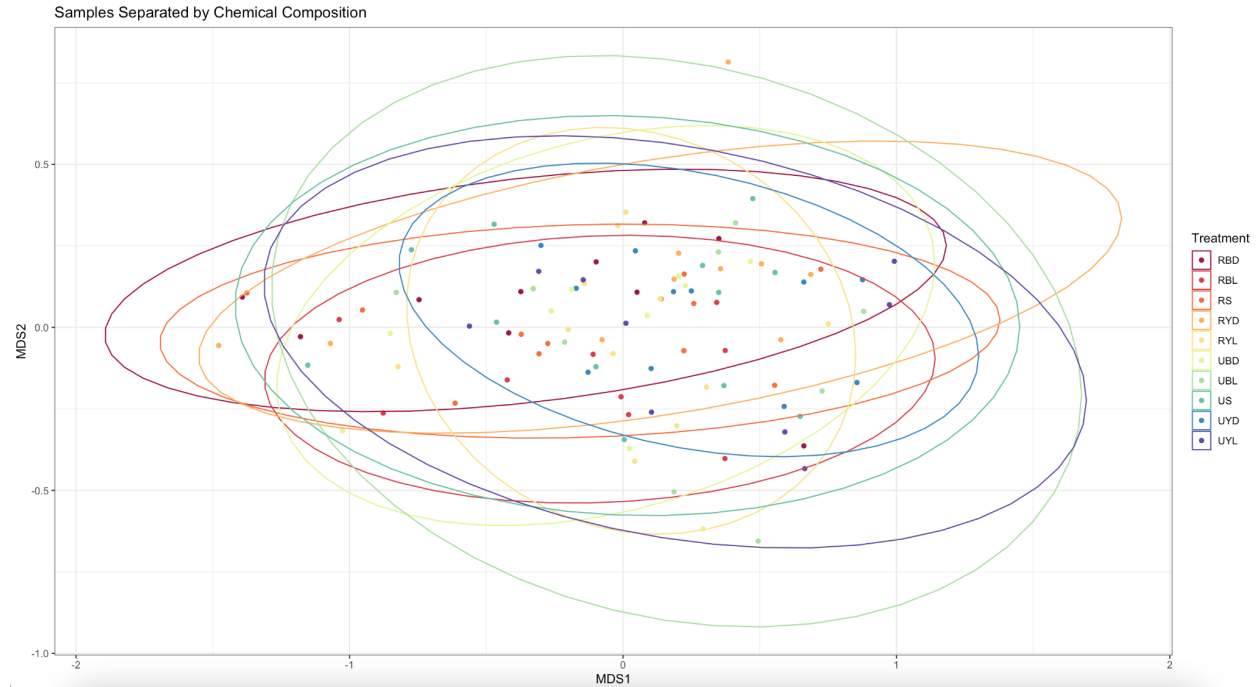


Figure 3: NMDS plot of all the whole inflorescence samples for the different treatments. (R = robbed, U = unrobbed, B = bacteria, Y = yeast, D = dead, L = live). Significant overlap of the ellipses indicate no major difference in volatile composition between the treatments.

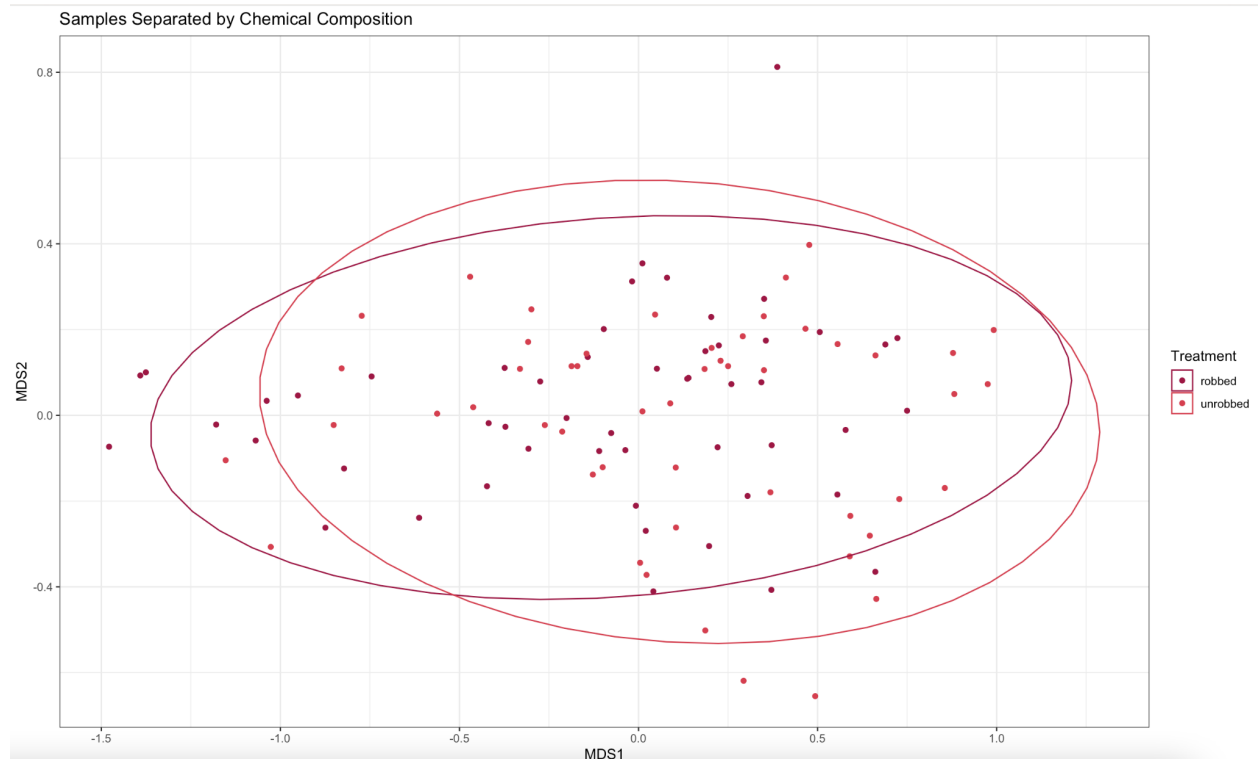


Figure 4: NMDS plot of all the whole inflorescence robbed and unrobbed samples. Significant overlap of the ellipses indicate no major difference in volatile composition between the treatments.

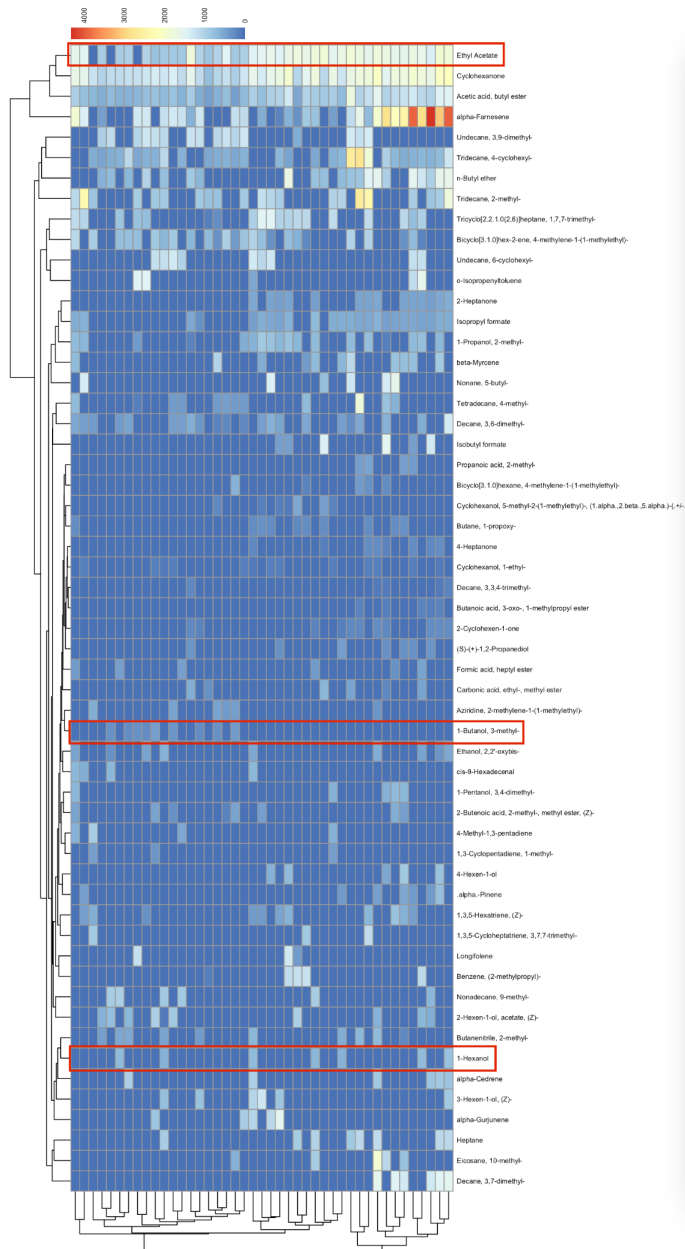


Figure 5: Heatmap of the compounds present in the single flower samples. Warmer colors indicate a higher amount of volatiles present in the sample. Compounds highlighted in red are volatiles known to be produced by nectar microorganisms.²²

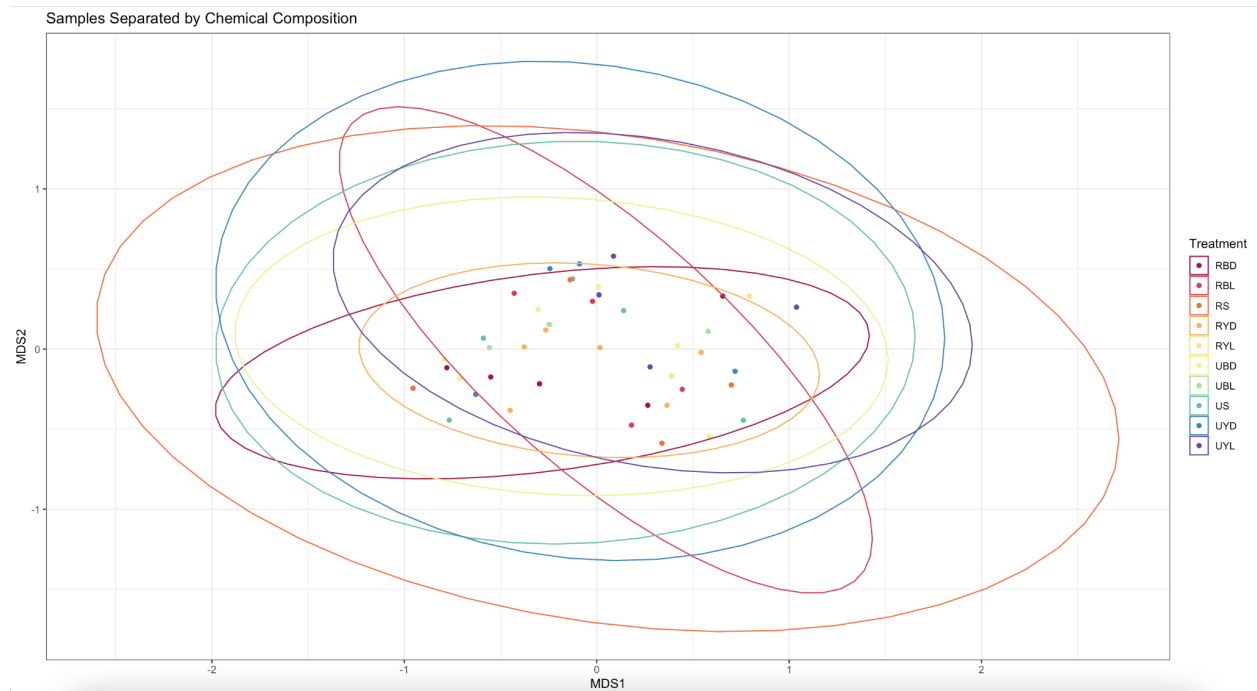


Figure 6: NMDS plot of all the single flower samples for the different treatments. (R = robbed, U = unrobbed, B = bacteria, Y = yeast, D = dead, L = live). Significant overlap of the ellipses indicate no major difference in volatile composition between the treatments.

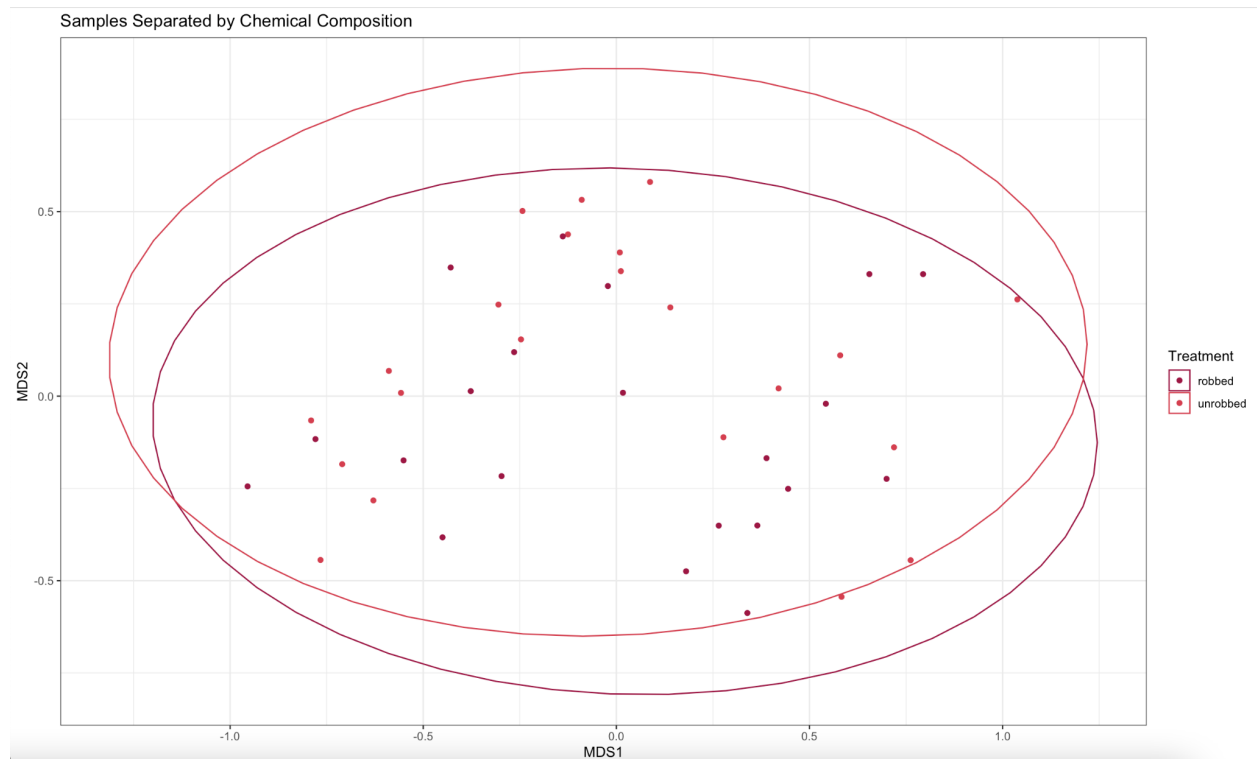


Figure 7: NMDS plot of the single flower sample robbed and unrobbed treatments. Significant overlap of the ellipses indicate no major difference in volatile composition between the treatments.



Figure 8: Contamination found in the whole inflorescence samples. The top chromatogram shows the compound peaks after an initial run through the GCMS and the bottom chromatogram shows the same scent trap run immediately after on the same method. Several compounds remained in the scent trap as indicated by the multiple peaks in the bottom chromatogram.