Removal of floral microbiota reduces floral terpene emissions

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The emission of floral terpenes plays a key role in pollination in many plant species. We hypothesized that the floral phyllospheric microbiota could significantly influence these floral terpene emissions because microorganisms also produce and emit terpenes. We tested this hypothesis by analyzing the effect of removing the microbiota from flowers. We fumigated Sambucus nigra L. plants, including their flowers, with a combination of three broad-spectrum antibiotics and measured the floral emissions and tissular concentrations in both antibiotic-fumigated and non-fumigated plants. Floral terpene emissions decreased by ca. two thirds after fumigation. The concentration of terpenes in floral tissues did not decrease, and floral respiration rates did not change, indicating an absence of damage to the floral tissues. The suppression of the phyllospheric microbial communities also changed the composition and proportion of terpenes in the volatile blend. One week after fumigation, the flowers were not emitting β-ocimene, linalool, epoxylinalool, and linalool oxide. These results show a key role of the floral phyllospheric microbiota in the quantity and quality of floral terpene emissions and therefore a possible key role in pollination.

Proficient performance in plants is strongly associated with distinct microbial communities that live in and on the organs. These communities are especially important in roots1,2. The microbiotas of the phyllosphere (in above-ground plant tissues and on above-ground plant surfaces) are abundant and are assumed to play critical roles in protecting plants from diseases and in promoting growth by various mechanisms. They may also offer indirect protection against pathogens13 and contribute to plant communication with different types and quantities of biogenic volatile organic compounds (BVOCs)14,15. Microbiotas, however, have generally not been well characterized, and little is known about their actual physiological and ecological roles13–15. The composition and physiological and ecological roles are much less well known for the microbiotas in and on flowers than for those in and on leaves. Microorganisms produce and emit many BVOCs including several terpenes16, so we hypothesized that floral phyllospheric microbiotas could significantly contribute to the emission of BVOCs, including terpenes, that play a key role in attracting pollinators17,18. Here we tested this hypothesis by studying the floral emissions of Sambucus nigra L. flowers before and after removal of their floral microbiota with a combination of three broad-spectrum antibiotics: streptomycin, oxytetracycline, and chloramphenicol.

Results
Reduced diversity and rates of emission of floral terpenes. The total floral emissions of terpenes decreased after antibiotic fumigation by nearly two thirds (Fig. 1a). The flowers of S. nigra emitted a terpene mixture dominated by linalool, with lower emission rates of (Z)-β-ocimene and two oxygenated terpenes derived from linalool, epoxylinalool and linalool oxide (Fig. 2a). The composition of the emissions significantly changed after fumigation (pseudo-F = 6.66, P = 0.05) (Fig. 2a). The percentage of trans-β-ocimene decreased from 7 to 0.4% (F = 10.05, P < 0.05) by day 2. By day 8, trans-β-ocimene, linalool, epoxylinalool, and linalool oxide were not emitted by the fumigated flowers (Fig. 2a).

Unaltered floral terpene contents. In contrast with terpene emissions, the terpene concentrations of floral extracts did not change in the fumigated plants (Fig. 1b). Floral respiration rates were also not altered by fumigation (Fig. 1c), indicating an absence of plant damage.

Even though the emission rates of floral terpenes were high, ranging between 50 and 250 μg gDW⁻¹ h⁻¹ (Fig. 1a), the terpene concentrations of floral extracts ranged from 0.5 to 2.5 μg gDW⁻¹ (Fig. 1b), indicating...
Discussion

The decrease in emissions was thus likely due to the effect of the antibiotics on the floral phyllospheric microbiota. Bacteria and fungi emit volatile organic compounds from de novo biosynthesis21–22 and biotransformation23–25, including linalool and other terpenes26,27,28. Terpene biosynthesis is well known in microbial metabolism, even though only a few bacterial and fungal genes encoding terpene synthases have yet been reported, likely due to the low amino-acid-sequence identities with homologous enzymes in eukaryotes29. β-ocimene and linalool are emitted by yeasts from the genera Debaryomyces, Kluyveromyces, and Pichia30, which are commonly found in the nectar of flowers31.

The emitted bouquet of S. nigra was dominated by linalool (3,7-dimethyl-1,6-octadien-3-ol), an acyclic monoterpene with a sweet, pleasant fragrance common in floral scents32. The dominant volatile in the floral extract, however, was epoxylinalool. De-epoxidation to linalool is favored at moderately low pH33, so the frequent presence of phyllospheric microorganisms producing extracellular acidic compounds34,35, along with the likely action of microbial epoxide hydrolases36, may have favored the emission of linalool.

Other possible effects of the antibiotic treatment, however, cannot be discarded. For example, the presence of certain phyllospheric microbes can induce an emission of defensive terpenes from flowers to control microbial communities37. We could thus hypothesize that the removal of phyllospheric microorganisms could have temporarily released the plants from the need to maintain this defensive response, thus reducing the emissions. Direct interference of antibiotics with plant terpene synthesis, their reactions with terpenes, or the release of hydroxyl radicals from dying bacteria by bactericidal antibiotics cannot be fully discarded either.

Flowering plants use diverse, multifunctional biosynthetic pathways to produce a broad spectrum of BVOCs that collectively confer characteristic fragrances to flowers38. The results of this study highlight the mostly neglected role of phyllospheric microbiota in these emissions. The attractiveness of floral emissions to a wide range of pollinators, herbivores, and parasitoids and thus the key role emissions play in reproduction and defense may ultimately be due to the direct or indirect action of phyllospheric microbiota.

Methods

Plant material and experimental setup. We used twenty flowering four-year-old potted S. nigra plants grown in a nursery (Tres Turons S.C.P., Castellar del Vallès, Catalonia, Spain) outdoors under ambient Mediterranean conditions. They were grown in 15-L pots with a substrate of peat and sand (2:1) and received regular irrigation, ensuring that the substrate was held at field capacity throughout the experiment. Ten plants were fumigated with antibiotics. The plants were fumigated with 1600 ppm streptomycin, 400 ppm oxytetracycline, and 200 ppm chloramphenicol in 50 ml of H2O with 1% glycerol to ensure the elimination of floral phyllospheric microbiota. These antibiotics are used in agriculture mainly in prophylactic treatments39. The other ten plants served as controls and were fumigated with 50 ml H2O with 1% glycerol but without antibiotics. The terpenes in both floral emissions and contents of the control and fumigated plants were measured at day 2 (pre-treatment, two days before fumigation) and at days 2 and 8 (post-treatment) with a dynamic headspace technique.

Measurements of CO2 and BVOC exchange. Floral CO2 and H2O exchanges were measured with the LCpro + Photosynthesis System (ADC BioScientific Ltd., Herts, England) at standard conditions of temperature (30°C) and light (PAR = 1000 μmol m−2 s−1). Several flowers from one inflorescence were enclosed in the chamber (175 cm2) without detaching the flowers from the plant. In order to determine and quantify BVOC exchange, flow meters were used to monitor the air entering and exiting the floral chamber and system blanks were sampled previous and after each sampling. The air exiting the chamber was then analyzed by proton transfer reaction–mass spectrometry (PTR-MS; Ionicon Analytik, Innsbruck, Austria) to calculate monoterpene emission rates. Every 15 minutes, the output air flowing from the leaf chamber was also sampled for 10 additional minutes using stainless steel tubes filled with VOC adsorbents. Thereafter, the adsorbed terpenes were analyzed by thermal desorption and gas chromatography–mass spectrometry (GC-MS) to characterize the relative concentration of each single terpene. The floral terpene emissions were calculated from the difference between the concentration of terpenes from chambers exposed to flowers and the concentration from chambers with no flowers and adjusted with the flow rates. A Teflon tube connected the chamber to the PTR-MS system (50 cm long and 2 mm internal diameter). The system used was identical for all measurements. The flowers measured in each sample replicate were collected each
sampling day, after finishing the measurements, and dried into an oven at 70°C until constant weight to get the dry weight of the floral emitting sample.

PTR-MS. PTR-MS is based on chemical ionization, specifically non-dissociative proton transfer from H$_3$O$^+$ ions to most of the common BVOCs, and has been fully described elsewhere. In our experiment, the PTR-MS drift tube was operated at 2.1 mbar and 50°C, with an E/N (electric field/molecule number density) of approximately 130 Td (townsend) (1 Td = 10$^{-17}$ V cm$^2$). The primary ion signal (H$_3$O$^+$) was maintained at approximately 6 x 10$^6$ counts per second. The instrument was calibrated using an aromatic mixed-gas standard (TO-14A, Restek, Bellefonte, PA, USA) and a monoterpene gas standard (Abello Linde SA, Barcelona). Masses 155, 137 and 81 were continuously monitored to calculate monoterpene emission rates.

Terpene sampling and analysis by GC-MS. Exhaust air from the chambers was pumped through a stainless steel tube (8 cm long and 0.3 cm internal diameter) (Markes International Inc, Wilmington, USA) filled manually with the VOC adsorbents (115 mg of Tenax$^R$ and 230 mg of Unicarb$^R$, Markes International Inc. Wilmington, USA) separated by a metallic grid. Samples were collected using a Q-MAX air-sampling pump (Supelco Inc., Bellefonte, PA, USA). For more details, see

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**Figure 2 | Effects of antibiotic fumigation on the composition and contents of floral terpene emissions.** Time course of terpene composition of floral terpene scents (a) and floral terpene contents (b) of control and antibiotic-fumigated Sambucus nigra plants. The antibiotics were applied to treated plants on day 0. * indicates a significant difference ($P < 0.05$) between control and antibiotic-fumigated flowers (ANOVA).
Terpenes were trapped in the tubes using an automatic sample processor (TD Autosampler, Series 2 Ultra, Markes International Inc. Wilmington, USA). The tubes were processed with a mass spectrometer (5975C Inert MSD with Triple-Axis Detector, Agilent Technologies). The terpenes were transferred to an injector (Unity, Series 2, Markes International Inc. Wilmington, USA) into a 30 m × 0.25 mm × 0.25 μm film capillary column (HP-5ms, Agilent Technologies INC). The chromatographic program used for the identification and quantification of the terpenes is detailed in Peñuelas et al. For pre-desorption and desorption, the flow was 50 ml min⁻¹, the split 10 ml min⁻¹, and the desorption temperature 330°C.

Data analysis: The changes in the composition of the floral terpene emissions and concentrations were analysed using PERMANOVA with Euclidean distances. The PERMANOVA analyses were conducted with R software using the adonis function of the vegan package. Statistica v8.0 (StatSoft) was used to perform the ANOVAs. Percentages were transformed to the arcsine of the square root previous to the ANOVA analyses comparing control and antibiotic-fumigated flowers.

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