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Cover Photograph: (Top left) *Metschnikowia reukaufii*, strain 2 from SREC plant 7, 4 days old, grown on PDA/Y. (Bottom left) Unvisited *Silene caroliniana* flower collected from SREC in 4 mL SPME vial. (Top right) SPME fiber adsorbing VOCs from nectar. (Bottom right) *S. caroliniana* flowers at FAR. (Middle) Bumble bee collecting nectar from *S. caroliniana* flowers at SREC.

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Impact of Nectarivorous Yeasts on *Silene caroliniana*'s Scent

Annette M. Golonka[1,*], Bettie Obi Johnson[1], Jonathan Freeman[1,2], and Daniel W. Hinson[1,3]

Abstract - *Silene caroliniana* is considered a scentless flower, but is insect-pollinated and produces a nectar reward. This plant is host to nectar-associated *Metschnikowia* yeast species. In this study, the scent profile of *S. caroliniana* was determined, and the contribution of nectar inhabiting yeasts to its scent was evaluated using solid phase micro-extraction and gas chromatography-mass spectrometry (SPME-GC-MS). We identified the scent compounds produced by nectar isolated *Metschnikowia* species and determined their impact on the flower's scent. Analyses of the scent profiles of unvisited nectar, unvisited flowers, and visited nectar confirmed that this plant produced few scented compounds unless microbial organisms were present in the nectar. *Metschnikowia* species contributed aliphatic alcohols, including ethanol, 2-methyl-1-propanol, 3-methyl-1-butanol, and 2-methyl-1-butanol to *S. caroliniana*'s scent.

Introduction

Floral color, scent, and morphology are important features in attracting pollinators to flowers (Andersson 2006, Chittka and Raine 2006, Dobson 2006, Farré-Armengol et al. 2013, Fenster et al. 2004, Kunze and Gumbert 2001, Smith et al. 2006, Wright and Schiestl 2009). The fragrances emitted by flowers serve a wide range of purposes including the attraction of nectar-feeding pollinators and the repulsion of predatory visitors (Armbruster 1997, Cunningham et al. 2004, Dötterl et al. 2006, Farré-Armengol et al. 2013, Junker and Bluthgen 2010, Raguso 2008). Floral scent may be produced by a number of floral structures, including petals, sepals, anthers, stigmas, and nectaries (Dötterl and Jürgens 2005, Effmert et al. 2006, Farré-Armengol et al. 2013, Goodrich et al. 2006, Jetter 2006). Floral scent compounds comprise a wide variety of volatile organic compounds (VOCs) that vary qualitatively and quantitatively between plant species, and include benzenoids (e.g., benzaldehyde, phenyl acetaldehyde, methyl salicylate), terpenoids (e.g., lilac aldehydes, α-pinene), and fatty acid derivatives (e.g., cis-3-hexenyl acetate, cis-3-hexenol) (Dötterl et al. 2005, Farré-Armengol et al. 2013, Knudsen et al. 2006). The biosynthetic pathways involved in the production of floral odors along with their spatial and temporal regulation and pattern have been well studied for a large number of plant species (Dudareva and Pichersky 2006).

Floral scent is used by some pollinators, such as bees, to locate flowers and cue them in to a food source such as nectar (Chittka and Raine 2006, Heinrich 1979, Wright and Schiestl 2009). Diurnal pollinators such as butterflies and hawkmoths utilize color as the predominant mechanism to locate flowers with scent enhancing

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the learning process and increasing floral constancy (Andersson 2006, Kelber et al. 2003). Nectar is the most common floral reward a plant produces to attract pollinators (Simpson and Neff 1983), and attracting nectar-feeding pollinators is important for the reproductive success of many plant species (Majetic et al. 2009, Raguso 2004). Nectar is used by pollinators as an energy source (Carpenter 1983, Heinrich 1983) because it is high in sugars and amino acids and may also contain smaller concentrations of proteins, lipids, essential oils, polysaccharides, antioxidants, alkaloids, and vitamins (Baker and Baker 1983, Dafni 1992). It is also a potential habitat for microorganisms, such as yeasts (Belisle et al. 2012; Brysch-Herzberg 2004; de Vega et al. 2009; Eisikowitch et al. 1990; Golonka and Vilgalys 2013; Grüess 1917; Hautmann 1924; Herrera et al. 2008, 2009; Lund 1954; Nadson and Krassilnikov 1927; Phaff 1978). Recent studies suggest that nectarivorous yeasts, and potentially other microbes, may alter nectar quality by altering sugar composition (Canto et al. 2007, 2008; de Vega et al. 2009; Herrera et al. 2008), floral odor (Goodrich et al. 2006), floral temperature (Herrera and Pozo 2010), and potentially flower attractiveness to pollinators (Kevan et al. 1988).

In the Caryophyllaceae family of angiosperms, floral scent has been well characterized in several species (Knudsen et al. 2006). For example, *Silene latifolia* Poiret has a strong floral scent comprised predominately of the terpenoids *trans*-β-ocimene and lilac aldehyde isomers (Dötterl and Jürgens 2005, Dötterl et al. 2005, Waelti et al. 2008). This flower's scent has been found to attract moths such as *Hadena bicruris* Hufnagel, who use the plant for nectar drinking and oviposition (Dötterl et al. 2006). *Silene caroliniana* Walter, a diurnal hermaphroditic angiosperm native to eastern North America, has intermediate-sized, tubular pink flowers that are considered scentless (Fenster et al. 2004, Reynolds et al. 2009). *Silene caroliniana* is pollinated predominately by large bees and diurnal clearwing hawkmoths (Reynolds et al. 2009, Reynolds and Fenster 2008). Although this plant is considered scentless, the authors noticed a light fragrance associated with the flowers while conducting a yeast diversity study on *S. caroliniana*. In addition, yeast samples extracted from the flower nectar and grown in the laboratory emit an odor similar to the scent detected in the field. These observations led the authors to investigate the floral scent associated with this plant.

High concentrations of yeasts (up to 10^5 cells per μL of nectar) have regularly been found to occur in the floral nectar of many plant species with yeast concentrations correlating mostly with bumble bee visitations (Herrera et al. 2009). Specific yeast species isolated from *Silene latifolia* included *Metschnikowia* spp. Grimm, *Microbotryum violaceum* (Persoon) Deml & Oberwinkler, and *Aureobasidium pullulans* (de Bary) Arnaud (Golonka and Vilgalys 2013). Nectarivorous yeasts are known to decrease sugar concentration and distribution in floral nectars (Canto and Herrera 2012, de Vega et al. 2009) and potentially degrade the quality of nectar (Herrera et al. 2008). The presence of yeasts in nectar has also been shown to increase pollinator visitation time (Golonka 2002). The reason for this has not been determined but it may be the result of microbes changing the scent or quality of the nectar as has been found in other plant species (Pozo et al. 2009).

In this study, we isolated and identified the most common species of yeasts inhabiting the nectar of *S. caroliniana* flowers. We sampled two populations of *S. caroliniana* in South Carolina. We determined the volatile organic compounds produced by these yeasts using static solid phase microextraction sampling (SPME) with gas chromatography-mass spectrometry detection (GC-MS). The SPME-GC-MS technique has been well established as an effective method for the analysis of volatiles emitted by flowers and flower parts (Flamini et al. 2003, Goodrich et al. 2006, Goodrich and Raguso 2009). The objectives of this study were to 1) determine the floral scent of *S. caroliniana,* which has not previously been characterized, 2) identify the VOCs associated with nectar and flower samples taken from unvisited (unopened) flowers, 3) identify the VOCs produced by the common nectar inhabiting yeast species of *S. caroliniana*, *Metschnikowia reukaufii* Pitt & Miller and *M. koreensis* Hong, Chun, Oh & Bae, and 4) compare the VOCs produced by yeasts to the VOCs found in visited and unvisited nectar and flowers.

Methods

Study sites

We collected flowers of *Silene caroliniana* (wild pink, Caryophyllaceae) during March and April in 2012 from 2 different populations approximately 67 km apart. The first population was in the Sandhills Research and Education Center (SREC) which is run by Clemson University in Columbia, SC), 34°08.147'N, 080°52.395'W. The second population was in the Forty Acre Rock Nature Preserve (FAR) which is run by the Department of Natural Resources in Kershaw, SC. We sampled several subpopulations along a main trail FAR2 (34°40.187'N, W 080°31.500'W), FAR3 (34°40.022'N, 080°31.468'W), and FAR7 (34°39.971'N, 080°31.478'W).

Sample collection and processing

Initially, we collected 4 types of samples from *Silene caroliniana*: 1) Visited Nectar (VN), nectar extracted from open flowers in the field, 2) Unvisited Nectar (UN), nectar extracted from flowers 48 h after unopened flowers collected in the field opened in the lab under ambient conditions of light and temperature, 3) Unvisited Flowers (UF), intact flowers analyzed 48 h after unopened flowers collected in the field had opened in the lab, and 4) Visited Flowers (VF), intact flowers collected from the field analyzed 24 h after collection. This last sample category is not included in the data set because only a few samples could be collected due to small plant population size and flower abundance. Samples that were collected had either no VOCs present or only acetone with low peak area (n = 2). During flower collection, we noted that sticky hairs along the stems and on the sepals might pose a sterility issue, potentially impacting both unvisited flower and nectar samples. In addition to collecting nectar and flower samples, we collected control samples for each sample type in appropriate vials (either 4 mL or 2 mL) to control for scent of the environment (i.e., background noise) and gas-off compounds from the vials.

Visited nectar collection. We used sterile microcapillary tubes (1 µL) to collect nectar from flowers located in SREC and FAR. We sampled flowers during March and April between 09:00 and 12:30. The flowers of *Silene caroliniana* are protandrous and remain open for approximately 3–5 d. Flowers were considered "visited" by pollinators if flowers were open, anthers were dehisced or beginning to dehisce, and stigmas were not completely extended (i.e., flowers were ~24–48 h old). Sample status as "visited" was confirmed by the presence of yeast by plating the nectar on media following VOC analyses (see *Yeast Isolation* below), past research has indicated that unvisited flowers do not contain yeast while visited flowers do (Golonka, 2002). Flowers from individually numbered plants were removed by cutting the stem just below the sepals and petals, flower petals were pulled back to expose nectaries, and nectar was extracted until ~ 2 µL of nectar was collected from each plant whenever possible. These samples were labeled visited nectar. The volume of nectar extracted per flower varied between 0.02 and 1.0 µL of nectar. Flowers at FAR had significantly lower nectar volumes (mean = 0.1 µL) than flowers at SREC (mean = 0.5 µL, t = 2.0, df = 8, P = 0.04). This difference in nectar volume among flowers meant that we had to combine extracted nectar from more flowers at FAR to acquire enough nectar for sample analysis. At SREC, we extracted nectar from a total of 23 flowers from 5 plants (~4 flowers per plant) to produce 6 samples. At FAR, we extracted nectar from a total of 13 flowers from 6 plants to produce 4 samples for analyses. After nectar was collected, we recorded the length of nectar in each microcapillary and used a bulb to blow the contents into sterile 2 mL GC vials with Teflon/red rubber septa (National Scientific, Rockwood, TN, part #C4000-80). We sterilized all glass SPME and GC vials used for sampling, we did not sterilize the caps, because they emit VOCs if they are autoclaved. We used controls to confirm the sterility of caps, vials, and microcapillary tubes used to sample flowers and nectar. Microbes were allowed to grow for 24 h after collection of controls and then the samples were tested for the presence of VOCs using headspace SPME-GC-MS.

Unvisited nectar collection. We collected unopened flowers from plants in SREC and FAR during March and April 2012. We selected unopened flowers with petals visible and extended but with the corolla still tightly curled. We cut flower stems just below the sepals and petals, and immediately placed flowers in sterile 4 mL SPME vials with PTFE/silicone septa (Supelco, part #27136) containing 0.5 mL sterile distilled water. Flowers were allowed to open in the lab under ambient light and temperature similar to the field conditions. Unvisited nectar was extracted 48 h after each flower was collected to mimic the visited nectar samples taken from flowers already 24 h old. We also used the same target volume of nectar, 2 µL, as used with visited nectar. We placed nectar in sterile 2 mL GC vials and conducted headspace SPME-GC-MS analysis 48 h after collection (see *Headspace SPME-GC-MS analysis* below). From SREC, we collected 5 unvisited nectar samples by sampling flowers from 3 plants and all but 1 of the samples had 2 µL of nectar. The unvisited flowers from SREC had greater nectar stores than the flowers from FAR. As a result, we needed to combine extracted nectar from more flowers from FAR

to obtain a single unvisited nectar sample for analysis. This sample was left out of statistical analyses.

Unvisited flower collection. We collected unopened flowers as described above from SREC and FAR. We treated flowers for unvisited flower collection the same way we treated flowers for unvisited nectar collection and analyzed flowers via headspace SPME-GC-MS. At each site, we collected flowers from 4 plants. We collected 5 unopened flowers from SREC and 4 unopened flowers from FAR.

Yeast isolation and identification by molecular techniques

Yeast isolation. After we analyzed samples using SPME-GC-MS, we serially diluted visited and unvisited nectar samples by adding sterile distilled water for final dilutions of 10^{-2} and 10^{-3}, based on nectar volume initially placed in the SPME vial. These dilutions were then vortexed for 1 min, and for each dilution, we spread 2 aliquots of 50 µL for 10^{-2} dilutions and 100 µL for 10^{-3} dilutions onto 2 plates of potato dextrose agar (PDA) with 0.1 % yeast extract. We incubated plates at ambient lab temperature (21–25 °C) for 2–4 days. This dilution procedure occurred 48 h after initial collection of visited nectar samples and 48 h after initial collection of unvisited nectar from flowers allowed to bloom in the lab (i.e., 72–96 h after initial field collection of unopened flowers).

Identification of yeast species. Once yeast colonies were visible on the serial dilution plates, we counted colonies and separated them into 4 morphospecies labeled types 1a, 1b, 2, and 3. Morphospecies were identified based on the following characteristics: colony color (e.g., pigmentation, lack of pigmentation), colony shape (e.g., amorphous, circular), colony margin (e.g., entire, undulating, filamentous), colony surface (e.g., shiny, dull, smooth), colony texture (e.g., mucoid, viscous), colony elevation (e.g., flat, convex, raised), cell shape (e.g., ovoidal [oval], ellipsoidal, cylindrical [rod], elongate [long and narrow], triangular, globose [spherical]), cell size (tiny cells [< 1.0 µm], small cells [1.0–2.0 µm], medium cells [2.0–3.5 µm], and large cells [> 3.5 µm]), filamentation (e.g., pseudohyphae or hyphae), and vegetative reproduction method (budding, fission, conidia formation). This terminology and categorization was taken from Kurtzman and Fell (1998). Sixteen strains were used for molecular identification. We used 4 of these strains for further analyses with pseudonectar (see below). Because we extracted chromosomal DNA from the majority of isolates, we temporarily maintained only representative strains.

We used molecular systematic techniques to identify the morphospecies. This method is used extensively in identification of both culturable and unculturable strains of fungi (Arnold et al. 2000, Brysch-Herzberg 2004, Golonka and Vilgalys 2013, Head et al. 1998, Herzberg et al. 2002, Hong et al. 2003, Kurtzman and Blanz 1998, Kurtzman and Fell 1998, Lachance et al. 2003, Pozo et al. 2011, Sugita et al. 1999). We extracted nuclear DNA according to the method of Xu et al. (2000) utilizing a lysing and protoplasting buffer. We identified yeast by PCR amplification and sequencing approximately a 1.2 kb section of the internal transcribed spacers (ITS1 and ITS2) and the D1/D2 region of the large subunit nrDNA following the methods of Kurtzman and Robnett (1997) and Fell et al. (2000). We used the

primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3'). We performed PCR for 35 cycles with denaturation at 95 °C for 1 min, annealing at 62 °C for 1 min, and extension at 72 °C for 1 min. Cycle sequencing was conducted by Engencore (University of South Carolina, Columbia, SC) with ITS1 and NL4 primers. Sequences were aligned and trimmed using Geneious software (Drummond et al. 2010).

We submitted the D1/D2 region of the large subunit to GenBank BLAST searches and recorded the most probable taxonomic match for each sequence following Kurtzman and Robnett (1997), Fell et al. (2000), and Scorzetti et al. (2002). If nucleotide substitutions occurred in less than 2% of the D1/D2 region when compared to BLAST search results (modified from Kurtzman and Robnett 1997, Peterson and Kurtzman 1991), we identified isolates to the closest species. When possible, a section of DNA containing ITS1, 5.8S, and ITS2 was secondarily submitted to GenBank BLAST searches as support for taxonomic identification.

Pseudonectar experiments

Pseudonectar. Pseudonectar (49.9% sugars w:v) was made by dissolving sucrose, glucose, and fructose in sterile water with final concentrations of 0.5%, 43%, and 57% (w:v) respectively (concentrations based on Baker and Baker 1983, Jürgens et al. 2002, and field-collected nectar from SREC, 44.1% sugar, J. Freeman, unpubl. data). We selected this sugar concentration and composition to mimic a generic high hexose/low sucrose nectar as found in other *Silene* species (Witt et al. 1999), with a sucrose concentration similar to *Silene latifolia,* a species studied by Golonka that is known to contain *Metschnikowia* yeast species (Golonka and Vilgalys 2013). We filter-sterilized (0.20 μm, Fisherbrand part #09-719C) pseudonectar and then used a refractometer (Abbe ThermoSpectronic, Rochester, NY) to confirm fructose and glucose concentrations. We created a standard curve for %fructose to calculate %glucose because glucose and fructose have similar refractive index values. We plated a sample of pseudonectar to confirm sterility of the solution and ran SPME-GC-MS analysis on a sample as a control.

Yeast grown in pseudonectar. We grew 4 strains of yeast (*M. reukaufii:* 3FAR2 and 27SREC3, *M. koreensis:* strains 7FAR3 and 3FAR3) for 24 h on PDA with 0.1% yeast extract (w:v). We transferred cells to sterile pseudonectar, vortexed for 1 min, and then counted 5 medium squares on 5 hemacytometer chambers to determine cell count of each solution. For each strain of yeast, we adjusted cell concentration to ~6000 cells/μL (N_o) and transferred 2.0 μL of this solution to sterile 2 mL-GC vials for VOC analysis using headspace SPME-GC-MS after 24 h, 48 h, or 72 h of cell growth. Preliminary testing comparing 2 μL and 200 μL samples determined that the sample volume did not affect the number of VOCs present in the headspace nor the relative percentage of each VOC. We used 2 μL of solution because it is closer to the volume used with field-collected samples. At the same time, we set up samples containing 200 μL of yeast/pseudonectar to determine yeast growth rate from cell concentration (cells/μL) at the end of each growth period: 24 h, 48 h, and 72 h (N_f). We incubated samples at room temperature and light levels similar to field

conditions (12 h on, 12 h off). For each growth period, we set up 3 samples of yeast grown in pseudonectar for strains *M. reukaufii* 27SREC3 and *M. koreensis* 3FAR3 and 4 samples for strains *M. reukaufii* 3FAR2 and *M. koreensis* 7FAR3 (n = 7 for each yeast species). For determination of cell concentration, we opened the larger volume vials after 24 h, 48 h, or 72 h (t_f), vortexed each vial and then counted cells on 5 medium squares of 5 hemacytometer grids. These samples were not used for VOC analysis. We used the exponential growth equation $N_f = N_o e^{rt}$ to calculate r, the intrinsic growth rate (min^{-1}), where N_0 = initial population size (6000 cells/µL, the initial inoculum), N_f = population size at time t_f (based on cell counts in solutions for each growth period), and t = time from t_0 to t_f (min). We calculated the intrinsic growth rate for each sample using the equation $r = (\ln N_f - \ln N_o)/t$. We used controls to confirm sterility of caps, vials, and pseudonectar.

Analysis of VOCs using Headspace SPME-GC-MS

Samples. We analyzed 6 categories of samples by headspace SPME-GC-MS: 1) visited nectar (VN), 2) unvisited nectar (UN), 3) unvisited flower (UF), 4) pseudonectar inoculated with yeast (PN), 5) air and vial controls at each field site and lab where nectar was collected, and 6) pseudonectar control samples (2 µL) without yeast present to determine a background VOC profile. The appropriate control samples were collected and run every time samples were collected.

Headspace SPME-GC-MS analysis. Volatile compounds emitted from the samples described above were collected by headspace solid phase micro-extraction (SPME) using a manual SPME fiber holder (Supelco, part #57347-U) and SPME fiber coated with divinylbenzene/carboxen/polydimethylsiloxane (50/30 µm DVB/CAR/PDM, Supelco part #57348-U). This SPME fiber was chosen because it provides excellent adsorption and desorption with minimal carryover between injections. A time-dependent extraction study showed the extraction reached equilibrium within 12 minutes, therefore, a 15 minute extraction time was used for all analyses. We thermally conditioned fibers at 275 °C for 1 h and ran a blank fiber injection each day to verify no contaminants would bleed from the fiber during sample runs.

To analyze volatile compounds, we used a Shimadzu QP 2010S GC-MS system (Columbia, MD) with ultra-high-purity grade helium (99.9995% pure, Airgas National Welders, part #325541) as the carrier gas and a 30-meter-5% phenyl methyl silicone column (SHR5XLB, 30 m × 0.25 mm × 0.25 µm). We used a low-volume inlet liner (Shimadzu part #220-94769-00) and low-bleed thermo-green septum (Shimadzu part #221-35507-02) for the SPME injection. The SPME fiber was thermally desorbed at 275 °C in splitless injection mode for 0.5 min at a sampling depth of 4.5 mm. The GC–MS oven temperature was maintained at 31 °C for 5 min, increased to 200 °C at a rate of 20 °C per minute, held at 200 °C for 1.05 min, increased to 250 °C at a rate of 20 °C per minute, then held at 250 °C for 3.0 min for a total run time of 20.0 min. The mass spectrometer scanned over a mass range of 30.00 to 300.00 m/z units for the entire 20 minute run time. The fiber was left in the injection port for the entire run to ensure complete removal of volatile compounds from the fiber to prevent carryover between injections. We tested the

effectiveness of this cleansing method for the SPME fiber by injecting blank fibers after sample runs. The blank fibers produced little or no detectable compounds.

Compounds were tentatively identified using Wiley and National Institute of Standards and Technology mass spectral libraries (with more than 120,000 mass spectra), and then verified by co-injection of standard compounds found in the samples. We performed semi-quantitative analysis by integrating all chromatographic peaks, removing peaks present in the control samples (< 10:1 sample:control peak area ratio), and calculating relative percent areas (abundances) for each remaining peak by dividing the peak area of each compound by the sum of all peaks for a given sample. We determined Kovats retention indices for each peak using a standard mixture of alkanes (C7-C40), pure hexane (C6), pentane (C5), and butane (C4) standards.

Data analyses

Univariate analyses (PROC UNIVARIATE, SAS Institute Inc., 2012) indicated data were non-normally distributed. Therefore, we used multivariate analyses, MDS, Cluster, and PERMANOVAs (Clarke 1993), to compare the overall scent profile of each sample and non-parametric tests Wilcoxon, and Kruskal-Wallis (NPAR1WAY, SAS Institute Inc. 2012) to compare relative percent areas of each compound and total peak area of VOCs across sample types. All analyses were conducted in either PRIMER v6 with the PERMANOVA+ add-on package (Clarke and Gorley 2006) or SAS (SAS Institute Inc. 2012). We used relative peak areas to conduct non-metric multidimensional scaling (MDS) analyses (Clarke and Warwick 2001, Majetic et al. 2014). Relative peak areas were used to calculate a similarity matrix between each sample type (unvisited nectar, visited nectar, etc.) using the Bray-Curtis similarity index. An iterative process was then applied to create a best-fit set of axes to represent scent profile similarity between sample types with close proximity in space indicating greater similarity in scent profiles between samples and greater distances representing greater dissimilarity between samples (Clarke and Gorley 2006, Jürgens et al. 2002, Majetic et al. 2014). We performed a cluster analysis on the similarity indices to determine the level at which sample types clustered together and superimposed the results on the MDS plots using PRIMER v6 (Clarke and Gorley 2006). One unvisited nectar sample from SREC did not contain any VOCs and had to be removed from the MDS in order to fit the MDS plot (Fig. 1); however, the sample was included in all PERMANOVAs. Because the data were not normal and did not fit the assumptions of a multivariate ANOVA (MANOVA), we used PERMANOVA, an analogous analysis that is distribution independent, to analyze the scent profiles of samples (Anderson 2001, Majetic et al. 2014). We used the Bray-Curtis similarity indices, to perform a series of PERMANOVAs on different data sets. PERMANOVAs were used on similarity/dissimilarity resemblance indices to calculate pseudo-*F* and permutation-based *P*-values to test for the response of variables (VOCs) to one or more factors (i.e., sample type, population, and *Metschnikowia* strain). We performed the following PERMANOVAs on the data: 1) a 2-way PERMANOVA to test for differences

between sample type and population (FAR or SREC), and the interaction between sample type and population, 2) one-way PERMANOVAs to test for differences within a sample type, within a strain, within a species, or between sample types where population was not an issue, and 3) a nested PERMANOVA to determine whether there were differences in culture age nested within strain nested within *Metschnikowia* species. When PERMANOVA results indicated significant differences in scent profiles, we conducted post hoc pairwise tests in PERMANOVA. Spearman's rank correlations (PROC CORR, SAS Institute Inc. 2012) were used to test for an association between the VOCs present in the headspace of yeast species grown in pseudonectar.

We used initial (N_0) and final cell counts (N_f) to calculate the intrinsic growth rate, $r = (\ln N_f - \ln N_o)/t$, of each yeast strain in the pseudonectar for each growth period (24 h, 48 h, or 72 h). For each growth period, we used 7 replicates of each yeast species (*M. reukaufii* $n = 3$ for 27SREC3, $n = 4$ for 3FAR2, *M. koreensis* $n = 3$ for 3FAR3, $n = 4$ for 7FAR3). Univariate analyses (PROC UNIVARIATE, SAS Institute Inc., 2012) indicated these data were non-normally distributed. Therefore, we used non-parametric Wilcoxon and Kruskal-Wallis tests (NPAR1WAY, SAS Institute Inc., 2012) to compare growth rates and final colony forming units (CFU) for each of the strains and across species. Because strains within each *Metschnikowia* species were not significantly different in terms of growth rate or final CFU, we pooled the strains when comparing across *Metschnikowia* species.

Results

Yeast Species Isolated from *Silene caroliniana*

A number of yeast strains were isolated from the nectar samples collected at FAR and SREC in 2011 (Table 1, A. Golonka unpubl. data). Using molecular systematic techniques, the 16 molecular sequences yielded approximately 4 distinct operational taxonomic units (OTU). Type 1 strains were pink pigmented species (1a: *Aureobasidium pullulans*—identified by pseudohyphae on plate, 1b: *Rhodotorula* spp.). Because Type 1 species were rare in the nectar samples (A. Golonka unpubl. data), they were not a focus of this study. We identified the type 2 strain as *Metschnikowia reukaufii*, and type 3 as *Metschnikowia koreensis*. These 2 species were the predominant inhabitants of the nectar and were the focus of the pseudonectar experiments. Four strains were used, 2 each of the *Metschnikowia* species: *M. reukaufii* (3FAR2 and 27SREC3) and *M. koreensis* (7FAR3 and 3FAR3).

Scent of *Silene caroliniana*

Unvisited flowers. We identified a total of 25 VOCs from the headspace of unvisited *Silene caroliniana* flowers, and 13 of them were unique to these samples (Table 2). Typical VOCs present in other *Silene* species, such as terpenoids and lilac aldehyde isomers (Dötterl et al. 2005, Dötterl and Jürgens 2005, Waelti et al. 2008), were not detected. The scent compounds found only in unvisited flowers included on average: 32% ethyl acetate, 14% 4-methyl-1-pentanol, 11%

2-ethoxy-2-methyl-propane, 5.2% hexyl acetate, 0.6% isopentyl acetate, 0.4% methyl acetate, 0.4% octane, 0.3% 2-pentanol, 0.01% hydroxymethylacetate, and various unidentified compounds (Table 2). Unvisited flower samples also contained compounds produced by yeast species or compounds present in visited nectar samples including, on average: 13% 3-methyl-1-butanol, 7.7% 2-methyl-1-propanol, 3.0% 2-methyl-1-butanol, 2.6% 2,2,-dimethyl-1,3-propanediol, 0.5% ethanol, 0.3% acetone, 0.2% heptane, 0.2% acetic acid, 0.1% isobutyl acetate, 0.08% vinyl acetate, and 0.07% 2-methyl-2-butanol.

PERMANOVA indicated a significant effect of population on overall scent of unvisited flower samples (Pseudo-$F = 4.38$, df = 1, $P = 0.02$); however, a comparison of the relative percent areas for compounds detected in the unvisited flower samples from FAR and SREC were not significantly different from each other, except for 2 compounds: 2-ethoxy-2-methyl-propane ($\chi^2 = 6.6$, df = 1, $P = 0.01$), and an unidentified compound with retention index 914 ($\chi^2 = 4.8$, df = 1, $P = 0.03$) which predominantly occurred in FAR samples and not in any other sample group (Table 2). Spatial analysis of the data using MDS and clustering supported these findings visually as all the unvisited flower samples clustered together at the 5% similarity level regardless of population (Fig. 1A).

Unvisited nectar. We obtained only 1 sample for population FAR, therefore, we only used data from SREC ($n = 5$) for this analysis. We detected a total of 5 VOCs from the headspace of unvisited nectar samples from SREC (Table 2). Spatial analysis of the data using MDS and cluster analysis indicated these samples cluster together and have a scent different than unvisited flower and visited nectar samples (Fig. 1A). Of the 5 VOCs present, 1 is associated with yeast-inhabited pseudonectar (19% ethanol), 2 are associated with unvisited flowers (25% heptane and 20% vinyl acetate), and 2 were only found in the unvisited nectar samples (0.19% 1-methoxy-2-propanone and 15% of an unknown compound with retention index 1521).

Unvisited flowers versus unvisited nectar. Statistical analysis using PERMANOVA found a significant effect of sample type on scent (Pseudo-$F = 3.43$, df = 1, $P = 0.001$). A spatial analysis of the data using MDS supported these findings and suggested substantial differences in the scent of unvisited flowers and unvisited nectar samples (Fig. 1A). A PERMANOVA within unvisited flower samples

Table 1. Yeast species submitted to GenBank (isolated in 2011). Results are GenBank BLAST search results from 5-16-13 with base pair matches for D1/D2 of 26S and ITS1-ITS2. *P*-values < $4e^{-134}$. N.S. means the region was not sequenced by other researchers.

Morphotype (strain)	Closest species identification	GenBank accession #	D1/D2	ITS1-2
1a (7SREC1)	*Aureobasidium pullulans*	KF059238	580/581	382/382
1b (26SREC1)	*Rhodotorula* spp.	KF059239	592/596	465/466
2 (27SREC3)	*Metschnikowia reukaufii*	KF059240	498/505	274/278
2 (3FAR2)	*Metschnikowia reukaufii*	KF059241	513/524	293/299
3 (3FAR3)	*Metschnikowia koreensis*	KF059236	506/514	N.S.
3 (7FAR3)	*Metschnikowia koreensis*	KF059237	506/514	N.S.

Table 2. Relative average percent area of each volatile organic compound detected in unvisited flowers (UF), unvisited nectar (UN), visited nectar (VN), and pseudonectar (PN) with cells of *M. reukaufii* or *M. koreensis*. Populations and strains are listed below sample type along with the number (*n*) of samples. Compounds are listed by order of their Kovats Index (KI) and retention time in minutes (RT). Italicized compounds are produced entirely by microbes or have higher relative percentages in visited samples. All scent compounds, except those with an * by their name, were verified using authentic standards. Those compounds not verified were identified by obtaining >90% matches with library spectra. For the unidentified compounds, mass spectral ion fragments are listed in descending order of ion abundances with relative abundance in parentheses, the top 10 are fragments listed. Relative % values may not total to 100% due to the absence of scent compounds in some samples. (Contd.)

		UF		UN	VN		PN—*M. reukaufii*		PN—*M. koreensis*	
Identified compounds:	**SHR5XLB KI (RT)**	**FAR *n* = 4**	**SREC *n* = 5**	**SREC *n* = 5**	**FAR *n* = 4**	**SREC *n* = 6**	**27SREC3 *n* = 10**	**3FAR2 *n* = 14**	**3FAR3 *n* = 10**	**7FAR3 *n* = 14**
Acetaldehyde	439 (1.00)									4.8
Ethanol	488 (1.09)		0.9	19.0	18.0	18.0	91.0	88.0	91.0	86.0
Methyl acetate*	523 (1.19)		0.9							
Acetone	526 (1.20)		0.6		3.4	9.4			0.6	0.5
2-Methyl-2-propanol	559 (1.34)						0.2	0.2		
*Butanal**	571 (1.41)									0.0
Vinyl acetate*	604 (1.64)	0.2		20.0						0.2
2-Ethoxy-2-methyl-propane	614 (1.71)	22.0	0.3							
Ethyl acetate	619 (1.74)	0.3	63.0							
2-Methyl-1-propanol	633 (1.85)	15.0	0.4		12.0	3.2	5.3	8.4	3.1	3.7
2-Methyl-2-butanol	647 (1.98)	0.1						0.0	0.1	0.2
Acetic acid	659 (2.10)		0.4				0.2		3.3	
*1-Methoxy-2-propoxy-ethane**	692 (2.50)				23.0					
Heptane	702 (2.66)	0.4		25.0		1.4				
1-Methoxy-2-propanone	709 (2.78)			0.2						
2-Pentanol	714 (2.87)	0.5								
Hydroxymethylacetate	733 (3.28)		0.0							
3-Methyl-1-butanol	751 (3.75)	25.0	0.2		4.3		2.3	3.1	1.5	1.2
2-Methyl-1-butanol	754 (3.85)	5.4	0.6				0.6	0.7	0.5	0.4
*2,3-Dimethyl-hexane**	766 (4.20)									0.4
Isobutyl acetate*	790 (5.10)	0.1	0.1						0.1	
Octane	801 (5.56)	0.5	0.2							
4-Methyl-1-pentanol	860 (6.70)	5.5	23.0							
*4-Methyl-octane**	873 (6.99)					0.4				
*2-Butoxy ethanol**	923 (7.91)									2.3
Isopentyl acetate	930 (8.00)	0.4	0.8							

Table 2 (contd.)

		UF		UN	VN		PN—*M. reukaufii*		PN—*M. koreensis*	
Identified compounds:	**SHR5XLB KI (RT)**	**FAR** *n* = 4	**SREC** *n* = 5	**SREC** *n* = 5	**FAR** *n* = 4	**SREC** *n* = 6	**27SREC3** *n* = 10	**3FAR2** *n* = 14	**3FAR3** *n* = 10	**7FAR3** *n* = 14
2,2-Dimethyl-1,3-propanediol	970 (8.50)	3.5	1.6		0.6	0.2				
Hexyl acetate	1028 (9.17)	3.0	7.3							
2-Ethyl-1-hexanol	1044 (9.32)				16.0	27.0				
Unidentified compounds [m//z (rel abund)]:										
44(100), 39(68), 42(67), 41(60), 43(58), 40(39), 58(27)	675 (2.28)	0.1								
41(100), 39(61), 43(56), 44(38), 57(38), 58(31)	684 (2.39)	0.1								
C8 alkane: 43(100), 41(38), 85(29), 57(26), 71(16), 39(14), 42(11), 55(10), 56(9), 70(9)	829 (6.08)				1.3	12.0				
41(100), 56(65), 43(34), 31(24), 42(24), 69(11)	846 (6.40)	1.5								
133(100), 151(68), 77(39), 45(33), 135(27), 68(23), 75(11), 134(10), 152(5), 47(4)	914 (7.80)	15.0			11.0					
41(100), 45(93), 57(70), 42(50), 31(35), 43(19), 56(18), 39(17)	959 (8.36)								0.1	
43(100), 41(23), 71(21), 57(14), 87(9), 39(8), 59(7), 42(4)	988 (8.74)									0.0
43(100), 57(61), 71(53), 41(51)	1021 (9.10)					0.3				
43(100), 57(77), 41(59), 71(29), 85(15)	1055 (9.43)					3.2				
59(100), 43(71), 55(41), 41(41), 67(32), 31(31), 39(25), 94(23), 68(22), 93(22)	1088 (10.13)					2.6				
41(100), 43(58), 57(57), 44(39), 39(38), 55(32), 56(20), 42(13), 68(11)	1130 (10.13)				0.8					
91(100), 92(20), 39(16), 65(15)	1145 (10.26)				1.1					
43(100), 59(99), 68(62), 39(29), 67(28), 94(26), 41(24), 55(11), 53(10)	1193 (10.67)				2.1	1.1				
55(100), 31(80), 43(77), 41(51), 93(48), 67(44), 111(44), 39(34), 71(19), 53(18)	1241 (11.03)				2.2	21				
41(100), 7935), 69(34), 91(31), 39(25), 67(22), 93(17), 77(16), 53(15), 55(15)	1459 (12.59)	1.9								
41(100), 93(34), 69(32), 55(30), 77(26)	1521 (13.00)			15.0						
41(100), 120(89), 43(79), 57(62), 39(54), 55(50), 138(48), 65(48), 121(47), 70(42)	1850 (15.37)				3.0					
41(100), 69(82), 39(51), 109(50), 55(46), 67(43), 65(34), 120(32), 138(31), 43(28)	1923 (15.90)				2.3					
Sample % with VOCs absent				20.0						

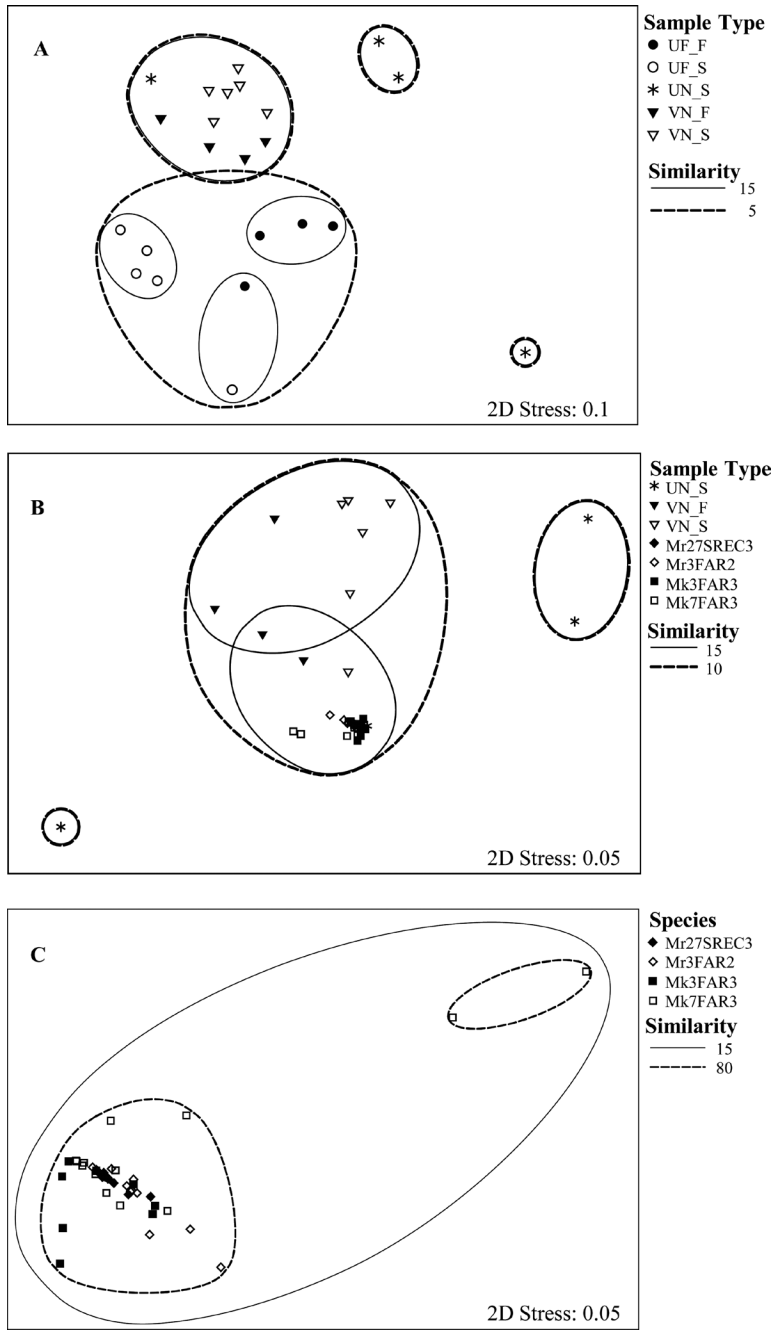

Figure 1. A. All sample types except pseudonectar-*Metschnikowia* samples. B. All nectar samples including visited, unvisited, and pseudonectar-*Metschnikowia* samples. At 10% similarity all VN samples cluster together with PN samples. C. Only pseudonectar-*Metschnikowia* samples, 24-h and 48-h samples as in Table 2. Two-dimensional MDS analysis based on Bray-Curtis similarity indices calculated from relative areas of each compound across sample types listed in Table 2 with superimposed group-averaged clustering from Bray-Curtis similarities. Solid-lined clusters indicate 15% similarity level, dash-lined clusters indicate different levels of similarity based on samples types as indicated on figure. UF_F = unvisited flowers from FAR, UF_S = unvisited flowers from SREC, UN_S = unvisited nectar from SREC, VN_F = visited nectar from FAR, VN_S = visited nectar from SREC, Mr27SREC3 = pseudonectar with *M. reukaufii* strain 27SREC3, Mr3FAR2 = pseudonectar with *M. reukaufii* strain 3FAR2, Mk3FAR3 = pseudonectar with *M.* koreensis strain 3FAR3, Mk7FAR3 = pseudonectar with *M. koreensis* strain 7FAR3.

indicated a significant effect of population; however, nonparametric analyses indicated there were no significant differences among SREC and FAR populations in relative percent of each VOC for unvisited flowers except for 2-ethoxy-2-methyl propane and an unidentified compound with retention index 914 (compounds not found in unvisited nectar samples). To compare the relatively few compounds present in unvisited nectar samples to unvisited flower samples, we pooled values for both populations of unvisited flower samples (n = 9) and used the means to determine whether there were significant differences between unvisited flower and unvisited nectar (n = 5) samples. Of the 5 compounds present in the unvisited nectar-samples, 3 were also present in unvisited flower samples (ethanol, vinyl acetate, and heptane), but relative abundances of these compounds were not significantly different among unvisited samples. However, the following 8 compounds were found in relatively high abundances (>2% relative peak area) in the unvisited flowers, but were not found in the unvisited nectar samples: 32% ethyl acetate, 14% 4-methyl-1-pentanol, 13% 3-methyl-1-butanol, 11% 2-ethoxy-2-methyl-propane, 7.7% 2-methyl-1-propanol, 5.2% hexyl acetate, 3.1% 2-methyl-1-butanol, and 2.6% 2,2-dimethyl-1,3-propanediol (Table 2).

Characterization of yeast-*Silene caroliniana* scent composition

Visited nectar. We confirmed the presence of yeasts in all visited nectar samples. SREC had a lower mean cell count (± SE) than FAR (SREC: 607 ± 328, FAR: 4350 ± 2840), but the cell counts were not significantly different from each other ($P > 0.14$). The total ion chromatogram peak areas for visited nectar samples from FAR and SREC were not significantly different from each other (Table 3). In the headspace of visited nectar samples from SREC and FAR, a total of 20 VOCs were detected, 11 of which were not identified (Table 2). Statistical analysis with PERMANOVA indicated a significant effect of population for visited nectar samples (Pseudo-F = 2.86, df = 1, P = 0.02). A visual analysis of the data using

Table 3. Mean total ion chromatogram peak area ± SE for volatile organic compounds found in samples. An * indicates significant differences within sample type. Population or strain refers to the population from which the sample was taken, either (FAR) or (SREC), and strain indicates the strain of *Metschnikowia* grown in pseudonectar (PN). Values for PN samples are based on an average of the 24-h and 48-h samples.

Sample type	Population or strain	*n*	Average total peak area (±SE)
Unvisited Flowers	FAR	4	$1.74 \times 10^6 (\pm 1.06 \times 10^6)$
	SREC	5	$3.86 \times 10^6 (\pm 2.76 \times 10^6)$
Unvisited nectar	SREC	10	$1.38 \times 10^4 (\pm 1.25 \times 10^4)$
Visited nectar	FAR	4	$6.18 \times 10^5 (\pm 6.67 \times 10^4)$
	SREC	6	$4.60 \times 10^5 (\pm 1.20 \times 10^5)$
PN with *M. reukaufii* (*P = 0.014)	27SREC3	10	$6.44 \times 10^6 (\pm 1.80 \times 10^6)$
	3FAR2	14	$2.54 \times 10^6 (\pm 5.67 \times 10^5)$
PN with *M. koreensis*	3FAR3	10	$1.88 \times 10^6 (\pm 3.91 \times 10^5)$
	7FAR3	14	$2.91 \times 10^6 (\pm 7.34 \times 10^5)$

MDS suggested no significant difference between the overall scents of visited nectar samples from FAR and SREC at the 10% similarity level (Fig. 1B); however, within the visited nectar samples SREC did contain a higher average relative percent of two compounds compared to FAR samples (acetone: $\chi^2 = 3.7$, df = 1, $P = 0.05$; retention index 829: $\chi^2 = 4.7$, df = 1, $P = 0.03$), while FAR samples had a higher relative percent area of the unidentified compound with retention index 914 ($\chi^2 = 5.6$, df = 1, $P = 0.02$). Visited nectar samples cluster together with pseudonectar samples containing *Metschnikowia* species at the 10% similarity level (Fig. 1B). Of the 20 compounds detected in visited nectar samples, 4 were also found in pseudonectar samples containing the 2 isolated *Metschnikowia* yeast species: 18% ethanol, 7.6% 2-methyl-1-propanol, 6.4% acetone, and 2.2% 3-methyl-1-butanol. One of these 4 compounds, 2-methyl-1-propanol, is known to be produced by *Metschnikowia* species and is also found in unvisited flower samples. This compound may be present in unvisited flowers due to microbes on the sticky hairs. Of the remaining identified compounds, 0.7% heptane was found in both unvisited flower- and unvisited nectar samples, and 0.4% 2,2-dimethyl-1,3-propanediol was found only in unvisited flower samples. Other known compounds found exclusively in visited nectar included: 22% 2-ethyl-1-hexanol, 12% 1-methoxy-2-propoxy-ethane, and 0.2% 4-methyl-octane.

Visited nectar versus unvisited nectar. The results of the MDS analysis suggested that the scent of visited nectar samples were substantially different from unvisited nectar samples (Figs. 1A and 1B). Statistical analysis using PERMANOVA also indicated a significant difference between unvisited nectar and visited nectar samples for overall scent (Pseudo-$F = 4.13$, df = 1, $P = 0.001$). As indicated earlier, PERMANOVA results suggest a population effect on the overall scent profile of visited nectar samples; however, except for acetone and 2 unidentified compounds with retention indices of 829 and 914, the total ion chromatogram peak areas and relative percent areas of each VOC for visited nectar samples from FAR and SREC were not significantly different from each other using nonparametric analyses. Therefore, samples from both populations were pooled and the means were used to determine whether there were significant differences between visited nectar samples ($n = 10$) and unvisited nectar samples based only on SREC ($n = 5$). The mean total peak area of visited nectar samples was significantly lower than the total peak area produced by unvisited nectar samples ($\chi^2 = 9.4$, df = 1, $P = 0.002$, Table 3); however, 20 VOCs were detected in the headspace of visited nectar samples while only 5 VOCs were detected in unvisited nectar samples (Table 2). Spatial analysis of the data using MDS indicated visited nectar samples were more similar to the scent of the pseudonectar-*Metschnikowia* samples than unvisited nectar samples (Fig. 1B). The additional VOCs in visited nectar samples included 4 *Metschnikowia*-associated compounds: ethanol, acetone, 2-methyl-1-propanol, 3-methyl-1-butanol. Two of these compounds were significantly higher in visited nectar samples than in unvisited nectar samples: acetone ($\chi^2 = 6.7$, df = 1, $P = 0.01$) and 2-methyl-1-propanol ($\chi^2 = 4.3$, df = 1, $P = 0.04$). Visited nectar samples contained more *Metschnikowia*-associated compounds than unvisited nectar

samples (4 compounds versus 2). Unique compounds were only detected in visited nectar samples that were not present in unvisited nectar samples, unvisited flower samples, or in the pseudonectar samples containing the 2 *Metschnikowia* species tested. These included: 1-methoxy-2-propoxy-ethane, 4-methyl-octane, 2-ethyl-1-hexanol, and various unidentified compounds (Table 2).

Characterization of yeast-specific scent chemistry and growth

We compared the relative abundances of VOC compounds for pseudonectar (49.9% sugars in sterile water) inoculated with 2 species of *Metschnikowia* isolated from the visited FAR and SREC nectar samples. A visual analysis of the MDS space indicated that both species cluster together at the 15% similarity level (Fig. 1C). A nested PERMANOVA indicated there was no significant effect of species on overall scent profile (Pseudo-F = 2.09, df = 1, P = 0.13); however, there were significant effects of strain nested within species (Pseudo-F = 3.16, df = 2, P = 0.02) and culture age nested within strain nested within species (Pseudo-F = 2.04, df = 8, P = 0.02). Post hoc pairwise tests indicated there were significant differences among strains within *M. reukaufii* (t = 2.15, df = 1, P = 0.023), but there were no significant differences among strains within *M. koreensis* (t = 1.29, df = 1, P = 0.17). Post hoc pairwise tests within each species and strain indicated there were no significant differences between 24-h and 48-h samples ($P > 0.24$ for all strains). Therefore, we pooled results for samples of pseudonectar incubated for 24 h and 48 h after yeast inoculation (n = 24 for each yeast species) for comparison with field-collected nectar samples (Tables 2 and 3). The statistics below reflect this pooling.

Pseudonectar containing M. reukaufii. Strain 27SREC3 produced a significantly higher peak area for the total ion chromatogram than did strain 3FAR2 (χ^2 = 6.1, df = 1, P = 0.01, Table 3); however, the relative percent area for compounds identified in the headspace of pseudonectar containing strains 27SREC3 and 3FAR2 were not significantly different from each other based on nonparametric analyses. A total of 7 VOCs were identified in the headspace of these samples, most of which were aliphatic alcohols including: 90% ethanol, 6.9% 2-methyl-1-propanol, 2.7% 3-methyl-1-butanol, 0.6% 2-methyl-1-butanol, 0.2% 2-methyl-2-propanol, and 0.01% 2-methyl-2-butanol. Additionally, acetic acid (0.2%) was produced by strain 27SREC3, and 2-methyl-2-butanol (0.02%) was produced by strain 3FAR2 (Table 2).

Pseudonectar containing M. koreensis. The peak areas of the total ion chromatogram of *M. koreensis* strains were not significantly different from each other (Table 3). In addition, the relative percent area for compounds detected in the headspace of strains 3FAR3 and 7FAR3 grown in pseudonectar were not significantly different from each other as determined by PERMANOVA and nonparametric analyses. A total of 15 VOCs were detected, most of which were aliphatic alcohols including: 89% ethanol, 3.4% 2-methyl-1-propanol, 1.3% 3-methyl-1-butanol, 0.5% 2-methyl-1-butanol, and 0.1% 2-methyl-2-butanol. The other compounds present were: 2.4% acetaldehyde, 1.7% acetic acid, 1.1% 2-butoxy-ethanol, 0.5% acetone, 0.2% 2,3-dimethyl-hexane, 0.09% isobutyl acetate, 0.09% vinyl acetate, 0.02%

butanal, and 2 unidentified trace level compounds (Table 2). Strain 7FAR3 produced 12 compounds while strain 3FAR3 produced only 9 compounds with slight differences in the compounds produced. For example, 7FAR3 produced acetaldehyde (4.8%) whereas 3FAR3 produced acetic acid (3.3%).

Comparison of Metschnikowia species. We detected at least twice as many VOCs in the headspace of *M. koreensis* (15) compared to *M. reukaufii* (7) with 6 VOCs common to both species (Tables 2 and 4). Within each species, there were no significant differences between strains in the amounts of the 6 common VOCs. Therefore, for each species, we pooled the data for the 2 strains. The appropriateness of pooling was confirmed by spatial analysis of these data using MDS and clustering (Fig. 1C). We then used the means for each species (data pooled for both strains and from 24-h and 48-h samples) to determine whether there were significant differences in the quantities of the most common VOCs found in samples of *M. reukaufii* and *M. koreensis* (n = 24 for each species, Table 4). Of the 6 VOCs common to both species, *M. reukaufii* produced significantly higher relative abundances than *M. koreensis* for 3 of the VOCs: 3-methyl-1-butanol, 2-methyl-1-butanol, and 2-methyl-1-propanol (P values in Table 4). However, PERMANOVA (P = 0.13) and spatial analyses of overall scent profile using MDS and clustering indicated the two species of *Metschnikowia* produced a similar overall scent and clustered together at the 15% similarity level, regardless of strain type or culture age (Fig. 1C).

Temporal differences in compound production. For the 6 VOCs identified in both species, there was a significant temporal difference across growth periods for 1 VOC in *M. reukaufii* but none in *M. koreensis* (Table 5). The relative abundance of 2-methyl-1-butanol in *M. reukaufii* strains comprised a larger proportion of the headspace for 72-h samples (χ^2 = 6.3, df = 1, P = 0.04) compared to 24-h and 48-h samples. Although there were no significant differences in the relative percent areas of the other shared VOCs, we detected a correlation between ethanol and the secondary alcohol products in the headspace (Table 5). For *M. reukaufii*, Spearman correlation coefficients indicated a negative correlation between ethanol and each of the following alcohols: 3-methyl-1butanol (ρ = -0.80, n = 36, P < 0.0001), 2-methyl-1-butanol (ρ = -0.72, n = 36, P < 0.0001), and 2-methyl-1-propanol (ρ = -0.95, n = 36, P < 0.0001). There were significantly positive correlations

Table 4. Average relative percent ± SD of the major volatile organic compounds produced by *Metschnikowia reukaufii* or *M. koreensis* grown in pseudonectar for 24-h and 48-h growth periods. Sample means are based on 2 strains for each species, n = 24.

Compound	*M. **reukaufii***	*M. **koreensis***
Ethanol	89.0 (± 7.0)	88.0 (± 13.0)
2-methyl-2-propanol	0.2 (± 0.7)	0.3 (± 1.5)
2-methyl-1-propanol (P = 0.002)	7.1 (± 4.3)	3.5 (± 3.6)
Acetic acid	0.1 (± 0.4)	1.4 (± 4.7)
3-methyl-1-butanol (P = 0.005)	2.7 (± 2.1)	1.3 (± 2.0)
2-methyl-1-butanol (P = 0.038)	0.6 (± 0.8)	0.5 (± 0.1)

Table 5. Average relative percent ± SD of the major volatile organic compounds produced by *Metschnikowia reukaufii* or *M. koreensis* grown in pseudonectar, for 24-h, 48-h, and 72-h growth periods. Also presented are mean ± SD of final cell concentration (CFU/μL) and mean growth rate (CFU/min) for each species and each growth period. For each species and culture age $n = 12$ for relative percentages of each VOC, $n = 7$ for cell counts and growth rate.

Compound	*M. reukaufii*			*M. koreensis*		
	24 h	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
Ethanol	91.0±3.8	88.0±8.4	75.0±28	91.0±6.2	85.0±17.0	84.0±16.0
2-methyl-2-propanol	0.4±1.0	0	0	0.6±2.1	0	0
2-methyl-1-propanol	6.2±2.0	8.1±5.7	12.0±11.0	2.4±2.2	4.5±4.5	6.4±6.3
Acetic acid	0	0.2±0.6	0.3±0.6	1.1±3.9	1.6±5.6	0
3-methyl-1-butanol	2.4±1.3	3.1±2.7	8.8±11.0	1.2±1.4	1.4±2.6	5.3±6.7
2-methyl-1-butanol	0.5±0.5	0.8±1.1	3.5±6.6	0.3±0.5	0.7±1.3	2.1±3.8
Final CFU/μL ($x10^3$)	5.6±1.2	6.3±1.7	6.2±1.2	7.1±0.9	8.0±1.2	9.0±1.9
Growth rate ($x10^{-5}$)	-6.0±16.0	0.4±10.0	0.4±4.8	11.0±8.6	9.5±5.2	9.1±4.8

between each of these 3 alcohols (all P values < 0.0001). For *M. koreensis*, there was a negative correlation between ethanol and the same 3 alcohols: 3-methyl-1-butanol ($\rho = -0.38$, $n = 36$, $P = 0.02$), 2-methyl-1-butanol ($\rho = -0.48$, $n = 36$, $P = 0.003$), and 2-methyl-1-propanol ($\rho = -0.73$, $n = 36$, $P < 0.0001$). As with *M. reukaufii*, there were positive correlations between these 3 alcohols (P values < 0.001). PERMANOVA analysis indicated an effect of culture age, and post hoc pairwise tests indicated a significant effect of age for 24-h and 72-h samples of *M. reukaufii* 3FAR2 ($t = 2.06$, df = 1, $P = 0.04$) and *M. koreensis* 7FAR3 ($t = 2.01$, df = 1, $P = 0.03$).

Growth rates. Culture age did not have a significant effect on final cell counts for *M. reukaufii* ($\chi^2 = 0.92$, df = 2, $P = 0.63$, Table 5); however, culture age did have a significant effect for *M. koreensis*, with older cultures having higher cell counts ($\chi^2 = 6.3$, df = 2, $P = 0.042$, Table 5). A comparison across *Metschnikowia* species for the 48-h growth period indicated that *M. koreensis* (8000 CFU/μL) had a significantly higher final cell number than *M. reukaufii* (6300 CFU/μL, $\chi^2 = 3.9$, df = 1, $P = 0.048$, Table 5). At 48 h, differences in the growth rates for these 2 species approach significance ($\chi^2 = 3.0$, df = 1, $P = 0.085$), with *M. koreensis* having a higher growth rate than *M. reukaufii* (Table 5).

Comparison of yeast-specific scent chemistry to visited nectar

To compare scent chemistry of pseudonectar samples to visited nectar samples, we pooled values for 24-h and 48-h samples to produce the means in Tables 2 and 3. The statistics below reflect this pooling. The 2 species of *Metschnikowia* grown in pseudonectar produced higher total peak areas than those grown in visited nectar samples (*M. reukaufii:* $\chi^2 = 17$, df = 1, $P < 0.0001$, *M. koreensis:* $\chi^2 = 15$, df = 1, $P = 0.0001$, Table 3). Most of the visited nectar samples clustered separately from the pseudonectar-*Metschnikowia* samples at the 15% similarity level, but

visited nectar samples clustered together with the pseudonectar-*Metschnikowia* samples at the 10% similarity level (Fig. 1B). Separate PERMANOVA analyses indicated that the overall scent profile of VN was significantly different from pseudonectar-*M. reukaufii* (Pseudo-$F = 45.9$, df = 1, $P = 0.0001$) and *M. koreensis* (Pseudo-$F = 39.1$, df = 1, $P = 0.0001$). We identified a total of 20 VOCs from the headspace of VN samples from SREC and FAR, which is more diverse than the 7 VOCs detected in pseudonectar-*M. reukaufii* samples and the 15 VOCs in *M. koreensis* samples. Examining the 6 VOCs identified in both pseudonectar-*Metschnikowia* species (Table 4), both strains of *M. reukaufii* produced significantly higher relative abundance of VOCs than visited nectar samples for the following compounds: ethanol ($\chi^2 = 21$, df = 1, $P < 0.0001$), 3-methyl-1-butanol ($\chi^2 = 5.5$, df = 1, $P = 0.02$), and 2-methyl-1-butanol ($\chi^2 = 12$, df = 1, $P = 0.0006$). However, there was no significant difference in VOC production for 2-methyl-2-propanol, 2-methyl-1-propanol, and acetic acid (Table 2). Both strains of *M. koreensis* also produced significantly higher relative abundances compared to visited nectar samples for ethanol ($\chi^2 = 20$, df = 1, $P < 0.0001$), but there were no significant differences for 3-methyl-1-butanol, 2-methyl-1-butanol, 2-methyl-1-propanol, 2-methyl-2-propanol, or acetic acid (Table 2).

Discussion

Scent of *Silene caroliniana*

Overall floral scent. *Silene caroliniana* flowers are not entirely scentless as previously thought (Reynolds et al. 2009); however, the total VOC peak area produced by these flowers is much lower than that of other scented Caryophyllaceae species. For example, *Dianthus* species are over 40 times more fragrant than *S. caroliniana*, (J. Freeman, B.O. Johnson, A. Golonka, unpubl. data). Of the 25 volatile organic compounds detected from unvisited flowers of *S. caroliniana*, 13 are considered specific to the flower and indicate flowers have an odor that pollinators may be able to detect. There was also some indication that overall scent differed among the sampled populations, perhaps indicating different populations have variable scent profiles. The predominant VOCs detected were not similar to the compounds isolated from other *Silene* species (Dötterl and Jürgens 2005, Dötterl et al. 2005, Knudsen et al. 2006, Muhlemann et al. 2006, Waelti et al. 2008). Instead of lilac aldehydes or benzenoid compounds which are found in other species of *Silene*, over 62% of the scent profile of unvisited *S. caroliniana* flowers was attributed to four aliphatic compounds: ethyl acetate, 4-methyl-1-pentanol, 2-ethoxy-2-methyl-propane, and hexyl acetate. At least two of these, the aliphatic esters ethyl acetate and hexyl acetate are known to be associated with bee-pollinated flowers (Dobson 2006).

Of the 25 VOCs isolated from unvisited *S. caroliniana* flowers, 12 may be contributed by the microbes that are associated with the flowers and with the sticky hairs on this plant species. As implied by the nickname "catchfly," *Silene caroliniana* flowers are never entirely without microbes when collected from the field due to the sticky nature of the hairs present on stems and sepals of the flowers. It is difficult to collect

a sterile flower sample, even when using sterile forceps, and alcohol cannot be used when analyzing floral scent because it is volatile. Because of this aspect of the flower, unvisited flowers often contained small amounts of microbe oriented compounds which were excluded from the 13 floral scent compounds discussed above.

Contribution of nectar to overall scent. The nectar of *S. caroliniana* was predominantly unscented, with only 5 VOCs detected from unvisited nectar samples. However, two nectar-specific compounds were isolated: 1-methoxy-2-propanone and an unknown compound at retention time 13.00 min with a Kovats retention index of 1521 (Table 2). Both of these compounds were not detected in the scent profile of unvisited flowers or controls. Research on *Silene latifolia* and other plant species indicates that different parts of flowers may produce different scent compounds (Dötterl and Jürgens 2005, Dudareva and Pichersky 2006, Jetter 2006). This research indicates that *S. caroliniana* nectar contains few volatile organic compounds and may indicate that nectaries are not the source of the floral scent detected in unvisited flowers. It is unclear whether the petals, sepals, anthophores, gynoecium, or flower base of this plant produce different VOCs. Further research is needed to determine which floral structures produced the isolated VOCs and whether there are spatial fragrance patterns in this species as well.

Importance of yeast to *Silene caroliniana's* scent profile

Scent compounds emitted by flowers are known to attract nectar-feeding pollinators (Cunningham et al. 2004, Dötterl et al. 2006, Farré-Armengol et al. 2013, Galen and Kevan 1983, Junker and Bluthgen 2010, Knudsen and Tollsten 1993, Raguso 2008, Wright and Schiestl 2009). Previous studies suggest that yeast-like odors may also be attractive to insects (Goodrich and Raguso 2009, Goodrich et al. 2006, Guerenstein et al. 1995, Herrera et al. 2008), and that microbial organisms may mediate signaling in plant-pollinator interactions (Goodrich et al. 2006, Pozo et al. 2009, Raguso 2004). Several studies have also established that nectar is altered after a pollination event and that microbes are key components in altering nectar quality (Canto et al. 2007, 2008; de Vega et al. 2009; Goodrich et al. 2006; Herrera et al. 2008; Herrera and Pozo 2010). Alterations in nectar composition are also known to impact a plant's fitness (Golonka 2002, Vannette et al. 2013). This study indicates that the presence of microbes in or on flowers may alter the scent profile of a plant species. A comparison of the VOCs present in unvisited nectar samples (5 VOCs) to visited nectar samples (20 VOCs) indicates that the scent profile of *S. caroliniana* changes after being visited by pollinators. The ramifications of this alteration are not explored here, but these changes in scent could potentially alter pollinator visitation patterns among visited and unvisited flowers which could potentially alter plant fitness. Relatively high concentrations of yeast, ranging from 607 to 4350 CFU/μL, isolated and identified from visited nectar samples along with the difference in VOCs from visited and unvisited nectar samples indicate that microbes inhabiting the nectar of this plant contribute to the volatile compounds. These concentrations of yeast are similar to those found in *Silene latifolia* (Golonka and Vilgalys 2013) and other North American plant species (Belisle et al. 2012,

Golonka and Vilgalys 2013) but are lower than plant species studied on other continents (deVega et al. 2009, Herrera et al. 2009).

Of the 20 volatile organic compounds detected in the visited nectar samples, 13 were found exclusively in these samples and are attributed to the presence of microorganisms in nectar. Four of these VOCs are potentially associated with *M. reukaufii* and *M. koreensis*, as indicated by comparison of the visited nectar samples with samples from isolated yeast species grown in pseudonectar. The greater number of VOCs found in the nectar of visited flowers indicates that the compounds associated with visited nectar were produced by several different types of microorganisms, some of which were not included in this study (e.g., bacteria). In this study we focused on the yeast species isolated from nectar (Table 1), particularly species of *Metschnikowia*. The compounds produced by these microorganisms and found in visited nectar are associated with metabolic processes and include fermentation by-products such as ethanol, 2-methyl-1-propanol, acetone, 3-methyl-1-butanol, and by-products of other metabolic processes (Table 2, italicized compounds).

Characterization of yeast-specific scent chemistry

Ethanol and other aliphatic alcohols were produced by *Metschnikowia* species found in visited nectar of *Silene caroliniana*. Six VOCs were common between the 2 species of *Metschnikowia* grown in pseudonectar with over twice as many compounds produced by *M. koreensis* (15 VOCs) versus *M. reukaufii* (7 VOCs). Volatile organic compounds from these species grown in pseudonectar included a high relative abundance of ethanol with other secondary fermentation by-product alcohols, such as 2-methyl-1-propanol, 3-methyl-1-butanol, 2-methyl-1-butanol, 2-methyl-2-propanol, and 2-methyl-2-butanol in varying relative abundances (Tables 2 and 4). *Metschnikowia reukaufii* and *M. koreensis* appear to produce significantly different metabolic by-products (Table 5); however, ethanol was the major component of VOCs in the headspace for both of these species. For both species, there was a negative correlation between the relative abundance of ethanol in the headspace and that of the other common secondary metabolic alcohols for cultures aged over 24 h, 48 h, and 72 h (Table 5). As cultures aged, the abundance of ethanol decreased whereas the abundance of secondary metabolic alcohols increased. There was also some indication that *M. koreensis* grew faster in the pseudonectar than *M. reukaufii* Both of these species appear to be well adapted to an osmotically difficult habitat as indicated by the positive growth rates of these species in pseudonectar (Table 5) and the consistent presence of these species in *S. caroliniana* and several other plant species (A. Golonka, unpubl. data; Golonka and Vilgalys 2013; Herrera et al. 2009; Pozo et al. 2011, 2012).

Conclusion

Silene caroliniana flowers do have a scent, but it appears to not be as rich in composition or strength as other *Silene* species. Of the 25 VOCs detected in the flowers of this plant, 12 were contributed by microbial organisms. Nectar was predominantly unscented and usually did not contribute to the overall scent of

the flower. We found only 2 unique nectar-associated compounds. This study established that microbial organisms alter the scent profile of *Silene caroliniana* after pollinator visitation, as seen by the increase in VOCs isolated from visited floral-nectar. Although *Metschnikowia* species contributed to the overall scent profile of this plant, they were not the only microbes that produced VOCs that may alter post-pollination scent or future pollinator visitation. Our study of the 2 most common yeast species in *S. caroliniana* flowers, *M. koreensis* and *M. reukaufii*, indicated that these 2 microorganisms contributed significantly to the overall scent of the flowers. These 2 yeast species produced 6 of the same VOCs; however, they differed in the remaining compounds produced, and *M. koreensis* produced twice as many VOCs as *M. reukaufii*. For both yeast species, ethanol was the major VOC with various aliphatic alcohols as secondary by-products of yeast metabolism. Future research on this plant species may help determine which floral parts contribute the majority of the scent compounds and which microorganisms found in the nectar and on the sticky hairs of this plant contribute the other VOCs found in the scent profile.

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