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Short communication

Profiling of volatile organic compounds released from individual intact juvenile and mature citrus leaves

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ABSTRACT

Plants release volatiles to communicate with each other and to attract or repel insects. The methods used to collect volatiles are varied. Here, we describe a simple solvent-less, solid phase microextraction-based method to collect the volatiles released from intact citrus leaves. We were able to collect up to 39 volatiles from both juvenile and mature leaves. Our results indicated that juvenile leaves produced both monoterpenes and sesquiterpenes, and while mature leaves continued to produce a variety of monoterpenes, their release of sesquiterpenes decreased dramatically. The finding that juvenile leaves emitted higher levels of sesquiterpenes while mature leaves released mostly monoterpenes suggests that younger leaves of plants may be involved in a more complex chemical communication system.

1. Introduction

The release of volatile organic compounds (VOCs) is the primary means of cellular and plant-to-plant communication (Ueda et al., 2012). Plant VOCs are released to attract insect pollinators such as bees and moths, and can affect the behavioral interactions between herbivorous insects and their natural enemies, an affect termed "allelobiosis" (Glinwood et al., 2011). This is a different chemical response than that induced by herbivory, mechanical wounding, or pathogen invasion (Mayer et al., 2008; Kigathi et al., 2009; Hijaz et al., 2013).

Citrus VOCs consist primarily of monoterpene hydrocarbons ($C_{10}H_{16}$) and sesquiterpene hydrocarbons ($C_{15}H_{24}$), with molecular weights of 136 and 204, respectively. Previously, our group described changes in citrus leaf volatiles due to herbivory by the phloem feeding Asian citrus psyllid, the insect vector of citrus greening disease or Huanglongbing (Hijaz et al., 2013). Greening disease is currently the most significant disease in citrus worldwide. In our earlier study, leaves were ground in liquid nitrogen and the stored leaf VOCs were extracted with hexane (Hijaz et al., 2013). Many other researchers in plant volatile studies have also preferred to extract large quantities of aroma compounds, including leaf and flower oils, from leaving using a solvent or hydrodistillation to test for biological activity (either attraction or repulsion) against agricultural pests (Silva et al., 2016) and anti-microbial or anti-fungal activity for pharmaceutical use (Kim et al., 1995). Solvent-extracted plant volatiles are useful for determining the composition of stored VOCs. However, using a solvent dilutes the extracted compounds, which then need to be concentrated using a distillation apparatus or nitrogen streams, both of which can lead to losses of low molecular weight volatiles. "Whole plant" volatile experiments are often conducted by enclosing plants within an airtight bag or container, and circulating the air through an organic vapor trap, which is then thermally desorbed or eluted with solvent. Additionally, "whole plant" systems do not discriminate between volatiles released from leaves of different maturities.

In contrast, solid phase microextraction (SPME) can detect a wide array of volatile compounds without solvent interference or dilution. In citrus, SPME has gained wide acceptance for monitoring juice and peel oil quality, but is less often utilized for leaf volatile characterization. When it has been used, the leaf samples were detached (Flamini et al., 2007) and the solvent extracted (Cevallos-Cevallos et al., 2011) or frozen and ground prior to volatile extraction (Azam et al., 2013; Zhu et al., 2013). We found that, while Beck et al. (2008) used a similar approach with enclosed leaves of *Centaurea* spp. to study the oviposition of a weevil pest, they only detected a few VOCs from undamaged control leaves. To the best of our knowledge, there are no other studies examining the profiles of released VOCs from intact juvenile and mature citrus leaves. Here, we describe a SPME method of collecting leaf volatiles (collected *in vivo*) emitted from juvenile and mature leaves of the sweet orange (*Citrus sinensis* (L.) Osbeck, var. 'Midsweet') that minimizes

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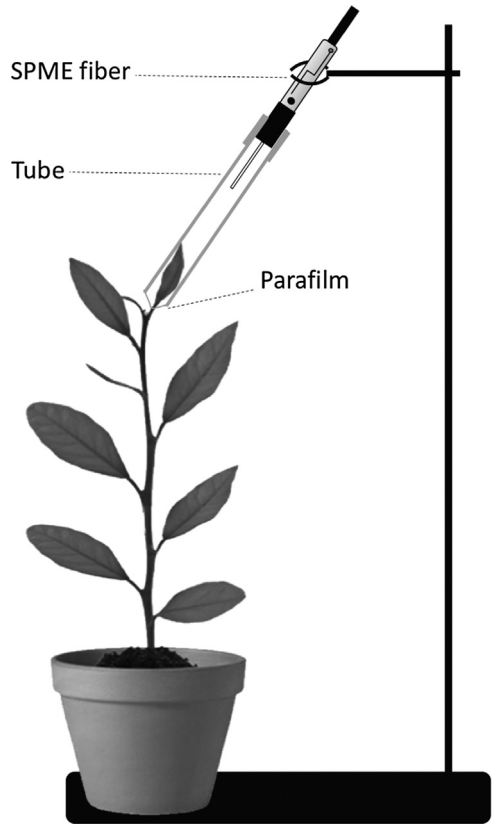

Fig. 1. Schematic representation of the device used to collect released volatile organic compounds from the headspace of isolated living leaves of 'Midsweet' orange trees using a solid phase microextraction (SPME) fiber.

the production of green leaf volatiles (GLVs), which are an indication of wounding, and better reflects the underlying metabolism of citrus leaves during maturation. Therefore, we hypothesized that the profiles of *in vivo* released volatiles from intact juvenile and mature leaves would differ and might reveal important indicators of physiological status.

2. Material and methods

2.1. Plant material

In this study, we used a variety of mid-season maturing sweet orange, *Citrus sinensis* 'Midsweet' scion on Kuharsky rootstock, sold by Southern Citrus Nurseries Inc., Dundee, Florida. Plants were maintained in our plant growth chamber for one month before use. The plants were maintained at 27 °C with 70% relative humidity (RH), with water and full spectrum artificial light (16 h light:8 h dark photoperiod) provided as needed. In this study, three plants were used, with two juvenile and two mature leaves were sampled per plant.

2.2. In vivo volatile collection system

A simple, portable volatile collection device was designed to collect the naturally released volatiles from orange leaves of different ages using two joined 10 mL pipette tips (one inserted partially inside the other, with the tapered ends trimmed off) (Fig. 1). This small volume allowed the isolation of either single mature leaves or small new shoots of citrus to be sampled while still attached to the trees (*in vivo*). Thus, dilution by either solvent or air, which can occur with traditional forced air circulation systems, and contamination by the GLVs associated with plant wounding and herbivory, was avoided. The tapered end of the pipette tip was trimmed precisely to fit tightly to the SPME fiber holder and was then wrapped with Parafilm® (Menasha, WI) to minimize the loss of any volatiles. The union between the two pipette tips (Finntip, #9402151, ThermoFisher Scientific, Waltham, MA) was also wrapped in Parafilm. Either a single mature leaf or a juvenile leaf bundle was inserted into the open end of the tube. The tube was secured on a ring stand held in a finger clamp, and the stem end of the plant shoot or leaf was sealed into the tube with Parafilm.

2.3. SPME fiber selection

The SPME fiber selected for this study was a 1 cm triple-coated 50/30 μm Carboxen/Divinylbenzene/Polydimethylsiloxane (CAR/DVB/PDMS; #57328-U, Supelco) using a manual fiber holder. Fibers were conditioned for 1 h at 250 °C, according to the manufacturer's instructions, and desorbed for 5 min prior to each collection to insure the fiber was clean.

2.4. Volatile sampling

After 5 min of equilibration time, the SPME fiber was exposed to the leaf volatiles for 2 h, and then carefully retracted. Citrus leaves were considered mature if they were dark green, fully expanded and hardened. Juvenile leaves were soft, light green and not fully expanded. After sampling, the sampled leaves were removed from the trees and weighed. Chromatogram peak areas were normalized to a leaf weight of 1 g. Blank samples from empty devices were run to determine the presence of background signals. Traces of limonene and linalool were detected from device-only samples (blanks), but were considered negligible when compared to the amounts collected from leaf samples. Collection devices were cleaned with ethanol and water between samples.

2.5. GC–MS conditions and analysis

After the 2 h collection period, the SPME fiber was inserted into the GC inlet for 5 min for thermal desorption and analysis. The GC–MS injector temperature was 220 °C, and it was equipped with a 2 mm splitless liner (Restek, State College, PA). Compounds were separated on a Perkin Elmer Elite 5-MS column, 30 m × 0.25 mm, 0.25 μm film thickness, using ultra-pure helium gas at a rate of 1.0 ml min^{-1}. The GC conditions were as described by Hijaz et al. (2013). The Wiley 9th ed., NIST 2011, and Wiley Flavor and Fragrance mass spectral libraries were used for volatile identification in addition to comparing the sample spectra to those of authentic reference standards when available.

2.6. Statistical analysis

Normalized peak areas were used to generate significant difference *P*-values using the paired *t*-test function in Excel 2010 (Microsoft, Redmond, WA) using a two-tailed test assuming unequal variance.

3. Results and discussion

Overall, we detected 39 VOCs released from 'Midsweet' leaves using the leaf isolating device with SPME. The use of the mixed-coating fiber allowed for the collection of both semi-volatile and volatile organic compounds simultaneously, unlike single- or dual-coated fibers, which are more selective. Preliminary trials with a 100 μm PDMS fiber yielded fewer peaks and an excess of D-limonene when compared to the CAR/DBV/PDMS fiber.