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Understanding Plant Volatiles for Environmental Awareness

Chemical Composition in Response to Natural Light Cycles and Wounding

Simone S. Whitecloud, Holly H. VerMeulen, Franz J. Lichtner, Nadia A. Podpora, Timothy Cooke, Christopher Williams, Michael Musty, Irene E. MacAllister, and Jason R. Dorvee

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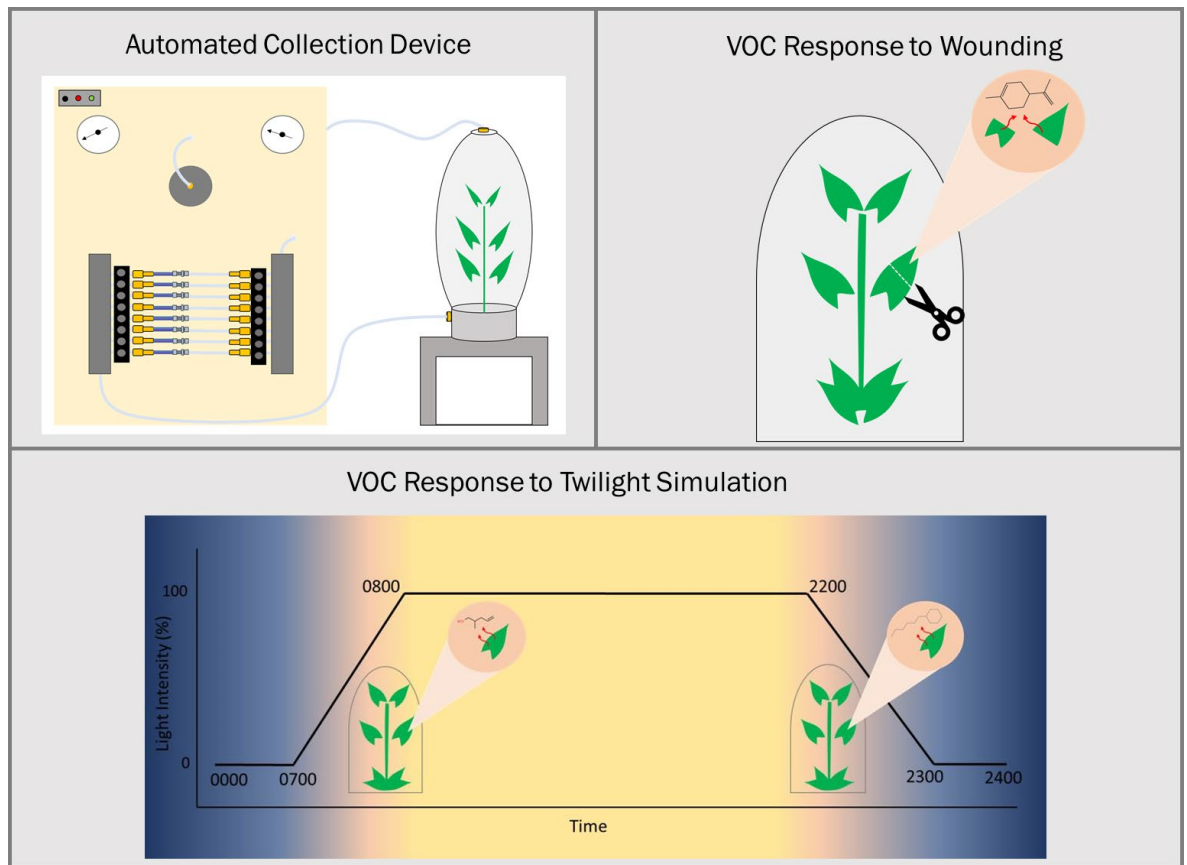
Cover image: Schematic of project accomplishments to date: construction of custom plant volatile collection apparatus, chemical analysis of plant response to wounding, and simulated sunrise and sunset. (Image by Nadia A. Podpora.)

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Abstract

Plants emit a bouquet of volatile organic compounds (VOCs) in response to both biotic and abiotic stresses and, simultaneously, eavesdrop on emitted signals to activate direct and indirect defenses. By gaining even a slight insight into the semantics of interplant communications, a unique awareness of the operational environment may be obtainable (e.g., knowledge of a disturbance within). In this effort, we used five species of plants, *Arabidopsis thaliana*, *Panicum virgatum*, *Festuca rubra*, *Tradescantia zebrina*, and *Achillea millefolium*, to produce and query VOCs emitted in response to mechanical wounding and light cycles. These plants provide a basis for further investigation in this communication system as they span model organisms, common house plants, and Arctic plants. The VOC composition was complex; our parameter filtering often enabled us to reduce the noise to fewer than 50 compounds emitted over minutes to hours in a day. We were able to detect and measure the plant response through two analytical methods. This report documents the methods used, the data collected, and the analyses performed on the VOCs to determine if they can be used to increase environmental awareness of the battlespace.

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Preface

1 Introduction

1.1 Background

Largely rooted in place, plants have evolved a highly specialized means of accomplishing interplant communications. Whether it be to identify the presence of an herbivore, alert neighbors to the presence of a pathogen, or defend a territory, plants produce and emit a suite of signaling chemicals called *plant secondary metabolites*. Volatile organic compounds (VOCs), a group of secondary metabolites, are emitted from both vegetative and floral plant parts, typically in response to both abiotic and biotic stressors. VOCs include a wide variety of biologically pertinent components, such as amino acid–derived metabolites, benzenoids, fatty acid derivatives, phenylpropanoids, and terpenes, that constitute many of the familiar plant scents, cut grass or mint, for example. VOC emissions may be constitutive (Kesselmeier and Staudt 1999) or induced and often contain a bouquet of compounds rather than a single volatile. Constitutive VOCs protect against abiotic factors such as high temperature or light, and it is the particular blend of chemicals that insects use to find their host plants for feeding and oviposition (Webster et al. 2009). Emissions induced by mechanical damage, herbivory, or pathogen infestation serve a variety of purposes, including within-plant signaling, plant-to-plant signaling, attraction of natural enemies of herbivores (Holopainen 2004), or pathogen antagonism through antifungal or antimicrobial properties (e.g., Guynot et al. 2003; Razzaghi-Abyaneh and Rai 2013; Dorman and Deans 2000). Induced plant emissions can be thought of as a "scream" by the wounded section to unwounded parts of the same plant or to adjacent plants. The plant response to these "screams" is either an increase in defense mechanisms, such as the production of toxic secondary metabolites, or a priming of the system, which prepares the plant to form these toxins should an attack take place (Bleeker et al. 2009).

"Screams" contain critical information; the literature has shown that different wound types, such as tearing versus puncturing, create unique signatures (e.g., Beck et al. 2008). Because of the potential for VOC use in agricultural pest and disease management programs, the majority of VOC research has focused on herbivore and pathogen effects on VOC emissions: Engelberth et al. (2013) reported the activation of defense responses by green leaf volatiles (GLV, a subset of VOCs, consisting of C6 aldehydes,

alcohols, and acetates) against insect herbivory in maize, Shiojiri et al. (2006) identified antifungal (Beck et al. 2008) activity of GLV against a *Botrytis* sp., and Kessler et al. (2006) reported that the bouquet of volatiles from clipped sagebrush successfully primed a tobacco trypsin proteinase inhibitor response. However, plants also endure repeated forms of mechanical wounding due to a variety of environmental factors such as wind, hail, and heavy snow loads. Research has identified that volatile emissions can be higher in scraped versus punctured leaves (Piesik et al. 2010). To date, however, there has been little research comparing wound type, severity of tissue damage, and plant age on VOC chemical composition and concentrations.

Given that human movement in a field is known to cause volatile emissions (Barney et al. 2009) and that emission concentrations are correlated with wound extent (Fall et al. 1999; Brilli et al. 2011), it is likely that plants may be used as biosensors for battlespace awareness. For example, Piesik et al. (2011) found that cereal grasses inflicted with mechanical damage released VOCs that differed, albeit slightly, from VOCs released due to herbivory and fungal infection. The difference was attributed to a variation in the ratios of the specific VOCs emitted. The intensity of VOCs emitted reflect the size of the wound (Brilli et al. 2011), where larger wounds result in greater VOC-emission concentrations (i.e., louder screams).

Once a wound is inflicted, a suite of reactions take place either instantaneously or up to hours later. The rate at which a particular VOC is emitted is linked to its biosynthesis and location within plant tissue. The first compounds released are C_6 aldehydes, which are normally synthesized at trace levels in plant tissue, primarily leaves, and are formed and released within seconds of damage (e.g., Matsui et al. 2006; Brilli et al. 2011; Fall et al. 1999). Aldehydes are then subsequently converted to C_6 alcohols and then to acetates (Brilli et al. 2011) within minutes of the damage occurrence. The C_6 compounds and their derivatives constitute the GLVs, which are ubiquitously released upon damage to leaf tissue and are known to play a key role in intra- and interplant communications (Ameye et al. 2017).

In addition to the GLVs, acetaldehyde, pentenone, isoprene, and methanol are also released within the first minute of a wound (Brilli et al. 2011). Terpenoids, which are a predominant group of plant-emitted volatiles (Das et al. 2013), along with aromatic compounds are the product of slower biosynthetic pathways. As a result, these VOCs are released later, typically more

than an hour after damage, and continue to be released up to 72 hr later, at which point VOC collection was stopped (Giacomuzzi et al. 2016). This early research suggests potential for the use of plant VOCs in ascertaining not only the type of wound inflicted but also the time since wounding.

Multiple studies have detected diurnal patterns in VOC emissions. For example, linalool and linalool-oxide emissions following a mechanical wound were observed to be highest in the morning and decreased in the afternoon, with overall photophase emissions exceeding scotophase emissions (Piesik et al. 2006a, 2006b). Hatanaka (1993) found that one enzyme required for GLV biosynthesis, lipoxygenase, nearly stopped GLV production in dark conditions in vitro, which is supported by prior work showing reduced constitutive GLV emissions in vivo at night (Webster et al. 2010). Few studies, however, have considered the effect of circadian rhythm on VOC emissions during wounding (see Loughrin et al. 1990; Beck et al. 2014). Although prior studies have detected a pulse of transient VOC emissions when plants were subjected to darkness (e.g., Brilli et al. 2011; Chamberlain et al. 2006; Giacomuzzi et al. 2017), the results represent an immediate switch from light to dark as opposed to a gradual change and therefore may reflect a stress response and not a diurnal pattern.

Additionally, plant developmental stage affects VOC emissions. Shiojiri et al. (2011) noted that intra-plant-species volatile communication was stronger between younger plants than between older plants. Also, Bergstrom et al. (1994) observed clear qualitative and quantitative differences between volatiles emitted by young and old cabbage (*Brassica oleracea*), milkweed (*Asclepias syriaca* and *A. curassavica*), bitter orange (*Citrus aurantium*), and lime (*C. aurantifolia*) leaves.

Our effort documents some of the constitutive and induced VOC emissions necessary to determine if plant volatiles may serve as discrete messages of battlespace disturbance. We measured constitutive emissions under a natural light cycle that includes twilight rather than rapid light/dark transitions. In addition, the study incorporates wound types, cutting or crushing, to mimic those expected from large mammals or vehicular damage. Understanding constitutive baseline emissions and wound-induced responses with regard to circadian rhythm lays the foundation for developing an operational tool for disturbance detection and timing calculation.

1.2   Objectives

In this effort from 2019–2021, we use two model organisms, *Arabidopsis thaliana* and *Panicum virgatum*; an easily cloned houseplant, *Tradescantia zebrina*; and two cold-region-relevant Arctic species, *Festuca rubra* and *Achillea millefolium,* to produce and query VOCs emitted in response to differing mechanical wounds and light intensities. The purpose of collecting and analyzing these VOCs is to create a baseline understanding of plant response to fluctuating environmental conditions and abiotic stressors such as vegetative damage. The original research objectives were to, under controlled conditions, (1) quantify baseline VOC-emission profiles as a function of plant age and associated circadian rhythm; (2) identify the effect that wound type (bending vs. tearing vs. compression vs. crushing) and severity (30%, 60%, and 100%) have on VOC chemical composition and concentration; (3) determine the persistence of specific VOC functional groups in an environmental gradient; and (4) develop a deterministic model to forecast plant VOC emissions in response to wounding.

1.3   Approach

We developed a systematic and technical approach to accomplish these objectives. For this data to be reproducible, our team followed detailed standard operating procedures and remained highly detail oriented throughout the experimentation, analysis, and final write up. Specific tasks were assigned to the individual experts in each field.

This report discusses the methods used to collect and analyze plant VOCs in a controlled environment. Because of issues with equipment, we reduced the project scope to address both plant wound response and VOC emission response in all of the species except *A. thaliana*. Yet, still the complexity and scope of this task to better understand the phenomena of plant signaling required a diverse collection of experts. Overall, this project brought together chemists, biologists, engineers, and modelers from the US Army Corps of Engineers' Engineer Research and Development Center to coordinate and accomplish the research. Ideally, this work will create a foundation of volatile emission data for future use in Army intelligence.

2   Methods and Materials

Because of challenges with equipment and technique refinement, our methods evolved over time. Our first studies were conducted with the adsorbent Porapak Q, which required elution; and the liquid eluents were analyzed on the gas chromatography flame ionization detector (GC-FID). Our later studies used thermal desorption (TD) tubes and a thermal desorption gas chromatography mass spectrometer (TD-GC-MS).

2.1   Plant material

2.1.1   Plant species

We selected five plant species for our study. *Arabidopsis thaliana*, a dicot, is an ideal test specimen as Aharoni et al. (2003) has described the metabolic pathways for certain VOCs it produces; and the *A. thaliana* genome has been sequenced, allowing for future validation of VOC defense-induced responses. We selected *Panicum virgatum*, a monocot, to assess responses within the broader plant kingdom, as previous studies have identified differential emission profiles between monocots and eudicots subjected to the same stress (Ameye et al. 2017). Additionally, previous studies have already documented constitutive emissions for both species (Eller et al. 2011; Rohloff et al. 2005), providing a list of chemicals needed as standards for accurate GC-MS analysis.

A. thaliana were young (preflowering) and were 24 to 29 days old. *P. virgatum* plants were 10 weeks old and preflowering. To understand how much variability may occur between plants, we selected an easily cloned houseplant, *Tradescantia zebrina*, a monocot that has never flowered in our care over the last 2 years. To compensate for the slow cultivation process of *P. virgatum*, we selected two Arctic species, *Festuca rubra* (a monocot) and *Achillea millefolium* (a dicot), both of which were queried preflowering.

2.1.2   Plant propagation

2.1.2.1 Arabidopsis thaliana

We used a hydroponic system (Araponics, University of Belgium, Leige, Belgium) to prevent soil intrusion into the automated collection device. Methods for hydroponic cultivation were adapted from Toquin et al. (2003). *A. thaliana* seeds were sown and germinated on 65% agar solution

(0.65 g* of micropropagation agar type I mixed with 100 mL grade 2 lab water). For initial setup and germination, we prepared 2000x diluted FloraSeries stock nutrient solution using grade 2 lab water and poured it into the Araponics holding container. The FloraSeries nutrient solution comprises three stock solutions: FloraGro (3-1-6), FloraMicro (5-0-1), and FloraBloom (0-5-4). Together, the diluted stocks can be mixed for optimal nutrition for each developmental phase of plant growth. After germination setup was complete, the Araponic holding container was covered with a clear plastic lid for two weeks while the plants germinated and the first true leaves emerged. During this time, the nutrient solution did not need to be replaced. Nutrient solutions were mixed and replaced according to the stage of plant growth and as needed. After they were two weeks old, *A. thaliana* plants were transferred with the seed holder from the Araponic holding container to 125 mL round, narrow-mouth, amber glass bottles for VOC collection. This cultivation method was conducted in the newly purchased Percival PGC-105 (Percival Scientific, Inc., Perry, Iowa) growth chamber. This growth chamber improved our cultivation precision with the capability to control relative humidity and to gradually increase and decrease light strength, capabilities previously not available. The Percival PGC-105 growth chamber operates on a multistep program. SciWhite LED lights follow a 18/6 hr light cycle. The multistep program produces 1 hr of gradual light increase and 1 hr of light decrease to simulate twilight (Figure 1). The light intensity remained at 290 μmol/m²/s for 16 hr of the day. The relative humidity was 60%, and the temperature was 23°C. Conditions were identical in the walk-in growth chamber where VOC collection occurred although there was no relative humidity control. We did, however, monitor relative humidity in the plant chamber during VOC collection.

2.1.2.2 Tradescantia zebrina

Plant propagation of *Tradescantia zebrina* began with cuttings from the "mother" plant in Dr. Whitecloud's home. Six cuttings, all with five leaves, were taken. Plant cuttings were transferred to 40 mL scintillation vials and filled with distilled water. All plants were kept in the Conviron E15 plant growth chamber at 20°C and full light intensity for 18 hr and at 15°C in

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complete darkness for 6 hr. VOC sampling did not occur until plants exhibited new foliar and root growth.

2.1.2.3 Festuca rubra and Achillea millefolium

Festuca rubra and *Achillea millefolium* seeds were purchased from Pickseed Inc., Halsey, Oregon. Plants were germinated and cultivated in potting soil. Potting soil was sieved through a number 8 sieve, sterilized at 120°C for 30 min, and then moistened with grade 2 lab water. Two-inch pots were gently packed with soil, and seeds were sown just below the soil surface. Plants were then covered with a vented plastic lid until emergence of the first true leaves. All plants were kept in the Conviron E15 plant growth chamber at 20°C and full light intensity for 18 hr and at 15°C in complete darkness for 6 hr. *F. rubra* and *A. millefolium* were fertilized weekly with 24-8-6 Miracle-Gro.

2.1.2.4 Panicum virgatum

Seeds purchased from Hancock Seed, Dade City, Florida, were germinated and cultivated in potting soil and followed the same cultivation protocol as the species above. After the purchase of the Percival Scientific PGC-105 growth chamber, *P. virgatum* was grown with the same multistep program as *A. thaliana*. The program includes a 18/6 hr light cycle with 1 hr of gradual light increase and 1 hr of light decrease to simulate twilight (Figure 1). The light intensity remained at 290 μmol/m²/s for 16 hr of the day. The relative humidity was 60%, and the temperature was 23°C.

Figure 1. *Panicum virgatum* and *Arabidopsis thaliana* plants were grown on an 18/6 hr light cycle that included 1 hr of transition from dark to light (ramp-up/sunrise) and 1 hr from light to dark (ramp-down/sunset). VOC collection times for *A. thaliana* captured sunrise and sunset; *circles* indicate sampling collection starting for sunrise, and the *triangles* are the sunset collection start times.
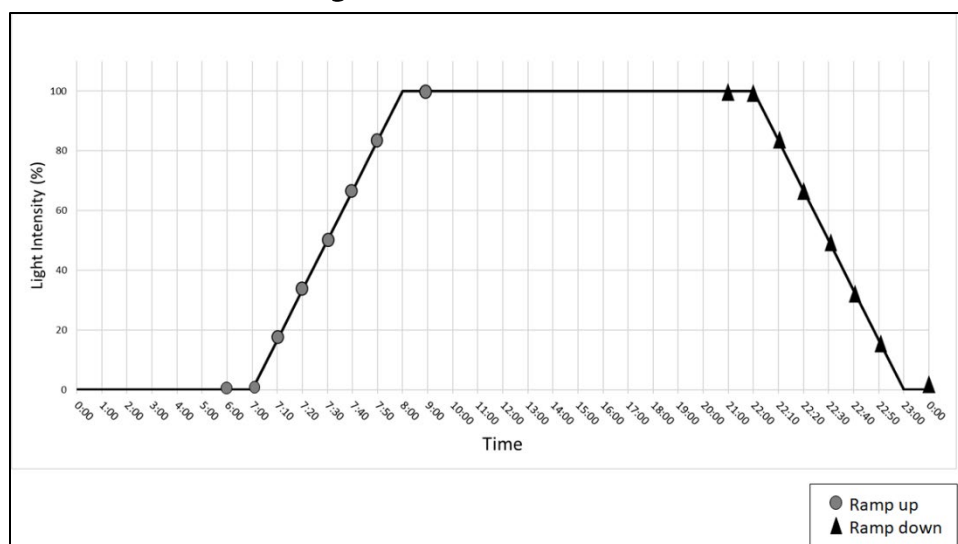
2.2 Volatile collection

2.2.1 Collection apparatus construction

We modified the design from Heath and Manukian (1994) for a programmable, automated volatile collection apparatus based on a push/pull/purge design described in their paper. Our design used a polyethylene terephthalate (PET) bag to seal the plant chamber rather than glass. The modification provides flexibility to wound the plant within the chamber without breaking the seal on the system. The system utilizes opposing air flow (~1200 mL/min) and vacuum pressure (~1000 mL/min), as well as a purge (~200 mL/min) through which the positive pressure created in the plant chamber flows to prevent soil volatiles from entering the plant chamber (Figure 2). Both the push and pull airflows are adjustable to maximize volatile collection. A guillotine base separates the soil and plant roots from the chamber to ensure only volatiles from the stem and leaves of the plant are collected (Figure 3). Additionally, plants are wrapped in charcoal filter cloth where the guillotine base closes around the plant to prevent soil volatiles from intrusion into the plant chamber. The system is sustained at approximately 60 kPa of pressure. To remove impurities, house air is passed through a charcoal filter prior to entering the system. Volatiles are carried from the collection chamber through inert polytetrafluoroethylene (PTFE) tubing to the volatile collection tubes

(VCTs) with a steady stream of air. Vacuum pressure connected by a separate valve on the other end of the tube helps to pull those volatiles through the VCT so that they are collected onto the adsorbent (Figure 4).

Figure 2. VOC automated collection apparatus with the plant chamber on the *left* and electronic ports for programmable VOC collection on the *right*. Tubes running between the two components are for the pushed and pulled air that flow through the plant chamber to enable VOC collection.
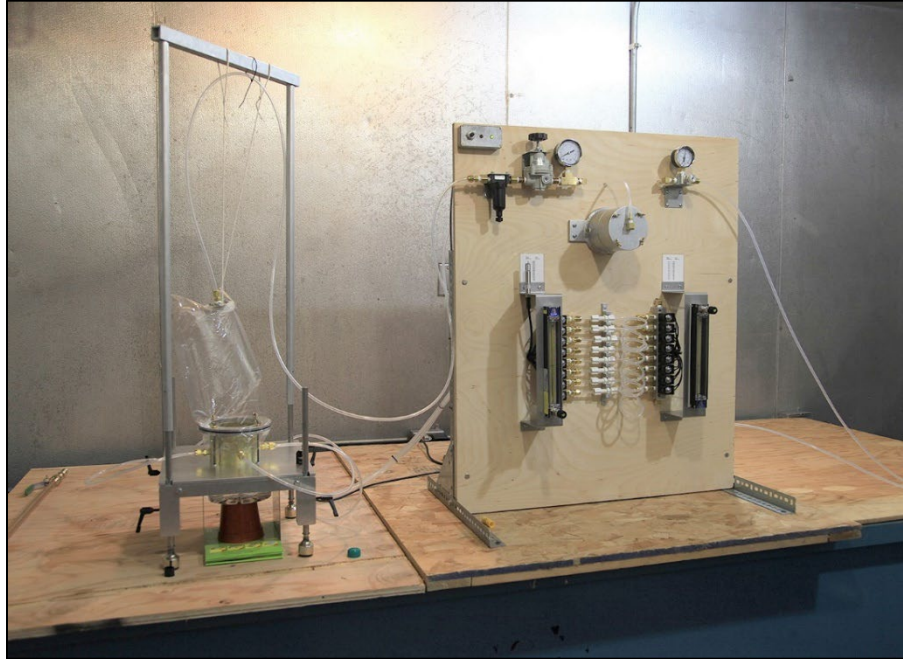
Figure 3. Mature (flowering) *A. thaliana* plant inserted in the guillotine base of the plant chamber, which isolates the vegetative components to the chamber, separating soil volatiles from plant volatiles. Also note the light sensor in the foreground.

Figure 4. Close-up photograph of eight borosilicate and Porapak Q volatile collection tubes fitted into the automated ports that are preprogrammed for collection duration and time.
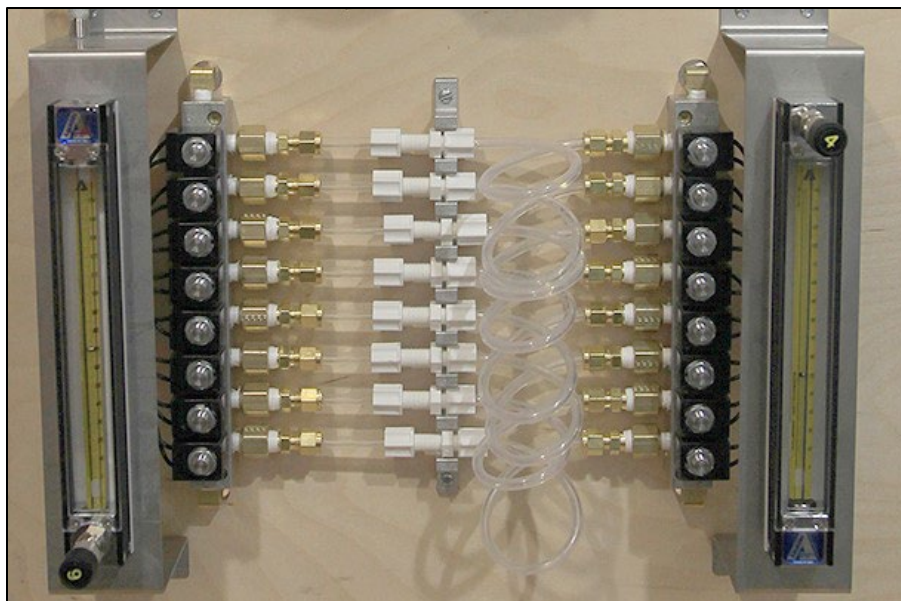
Above the guillotine base sits a brass ring fitted with the vacuum tubing for pulling air over the VCTs. The ring is fitted with a collar to which a PET oven bag (Wrapok Brand, Xiamen Huli Feng Yi Industry Co., Ltd., Xiamen, China) is fastened with a large O ring to create a closed system. The guillotine base is equipped with a pyranometer (CS 300, Campbell Scientific, Logan, UT) to log light levels. The apparatus is equipped with a HMP60 temperature and relative humidity probe (Campbell Scientific, Logan, UT) to log environmental data within the growth chamber.

2.2.2 Adsorption methods

2.2.2.1 Porapak Q, elution, and sorbent cleaning

Given that plant response to wounding is the release of C_6 compounds, we selected Porapak Type Q (ethylvinylbenzene-divinylbenzene) as an adsorbent due to its affinity for small (C_5–C_{12}) compounds (Tholl et al. 2006). We purchased 1/4 in. (6.4 mm; outside diameter) borosilicate tubes prepacked with 20 mg Porapak Q from Sigma Scientific LLC (Micanopy, Florida). The adsorbent was held in place with a stainless steel 316 screen on the tapered end and on the other end borosilicate glass wool followed by a PTFE Teflon retaining plug with a 3 mm hole.

For elution of the compounds from the adsorbent, we adapted methods from Edgewood Chemical Biological Center IOP-0910 for serial elution to use hexane, a nonpolar solvent, and methanol, a polar solvent. The methods in IOP-0910 were based on the work of Rosso et al. (1996) and an internal report, AM-036, *Qualitative Identification of Chemical Warfare Related Compounds in Sample Extracts Prepared Using Modified OPCS Sample Preparation Methods and Analyzed by a Hewlett-Packard (HP) Gas Chromatography/ Mass Selective Detector (GS/MSD)*. For each tube to be eluted, we added hexane (approximately 5–10 mL) to two 40 mL scintillation vials and filled two vials with methanol in the same manner. We placed the tube into the first hexane vial, packed-side down, added more hexane if needed to ensure that the solvent covered the Porapak Q, and capped the vial. The vial was shaken for 1 min and allowed to settle for 10 min. We moved the tube to the second hexane vial, repeated the shaking and settling process, and combined the two vials into one. We repeated the shake, settle, and combine process with the methanol vials.

To concentrate the eluent, 6 mL of each solvent were pipetted into separate 9 mL Pyrex vials. We ran the hexane samples for 10–11 min in the Thermo Scientific Savant SPD131DDA SpeedVac until less than 1 mL of solvent remained. The sample was then transferred to a GC vial, and hexane was added until the sample reached a total volume of 1 mL. The process was repeated for the methanol samples, but the run time in the SpeedVac was approximately an hour with intermittent checks to make sure the solvent did not completely evaporate.

To determine the best method for cleaning tubes following elution, we sequentially rinsed the tubes with 2 mL of hexane, dichloromethane or ethyl acetate, and finally with methanol, letting gravity pull the solvents through the traps. Each tube held approximately 1 mL of solvent. The tubes were then dried with a steady stream of nitrogen for about 2 min. Dry tubes were then eluted as described above, and the eluent was analyzed on the GC-MS to determine which cleaning method was most effective. From these trials, we determined that ethyl acetate is better at removing compounds than dichloromethane. Clean tubes were then stored in a glass jar under nitrogen.

2.2.2.2 Thermal desorption tubes and tube conditioning

We purchased coated steel Universal Inert TD tubes from Markes International Inc. Each tube contains a proprietary combination of three sorbents

selective for C_4 through C_{32} compounds. The TD tubes are coated stainless steel tubes packed with a matrix of adsorbent-coated small beads held within the tube by small round metal screens. The maximum gas flow rate through the tubes is 200 mL/min. We used the Markes International TC-20 to condition the tubes before use. The conditioning process requires a temperature of 325°C for 30 min with continuous nitrogen flow at a rate of 50 mL/min. The tubes were cooled gradually with nitrogen flow until they reach an ambient temperature. Dif-Lok caps were placed on the tubes after conditioning and used throughout the study.

2.2.3 Volatile collection methods

VOC collection required preprogrammed methods that enabled control over sample time, duration, volume, and time between sampling periods. All plants sampled were preflowering. Individual *T. zebrina, F. rubra, P. virgatum,* and *A. millefolium* plants were installed into the guillotine base of the chamber a minimum of 24 hr before collection to minimize the collection of volatiles released while handling the plant. A 24 hr normalization period occurred before VOC collection took place. An individual *A. thaliana* plant was placed in the chamber while in the hydroponic vial containing the nutrient solution used during propagation.

Once volatiles had been collected, Porapak Q tubes were capped with red silicone caps (Sigma Scientific LLC, Micanopy, Florida) or wrapped in Teflon tape and stored at −20°C for no longer than 2 weeks before elution. TD tubes were capped, stored at room temperature, and analyzed within 72 hr of collection.

2.2.3.1 Plant wound response (GC-FID)

T. zebrina (5 plants) and *F. rubra* (3 plants) samples for unwounded plants were collected 5 min, 10 min, and 1 hr after the plant was placed in the chamber. The PET bag was removed, all the leaves were crushed with sterile forceps, and the plant was quickly enclosed back into the PET bag. Samples for the wounded plants were collected 5 min, 10 min, and 1 hr after the bag was replaced. Respectively, volumes of 5 L, 10 L, and 60 L of air flowed over the collection tubes to determine the minimum volume of air required to detect a plant signal with the GC-FID.

Because we detected plant signals at low volumes, we adjusted our collection times to have higher resolution of the plant response to wounding

over time (Table 1). *A. millefolium* (4 plants) and *P. virgatum* (5 plants) collection times ranged from 1 min (1 L of air) to 5 min (5 L of air).

Table 1. Collection timetable for *A. millefolium* and *P. virgatum*.

Tube #	Plant Status	Sampling Duration (min)	Volume Collected (L)	Time Collected Since Start of Experiment (min)	Approximate Time since Wounding (min)	Delay since Previous Collection (min)
1	Unwounded	1	1	0–1	n/a	0
2	Unwounded	3	3	1–4	n/a	0
3	Unwounded	5	5	4–9	n/a	0
4	Unwounded	5	5	19–24	n/a	10
5	Unwounded	5	5	61–65	n/a	36
6	Wounded	1	1	70–71	5–6	5
7	Wounded	3	3	71–74	6–9	0
8	Wounded	5	5	74–79	9–14	0
9	Wounded	5	5	89–94	24–29	10
10	Wounded	5	5	130–135	65–70	36

2.2.3.2 P. virgatum wound response (TD-GC-MS)

The *P. virgatum* experimental sampling design included 16 sampling time points. The first sample was collected 65 min before wounding occurred, and the second collection was 5 min before wounding. The young *P. virgatum* was wounded at 1005 in the morning after the previously explained light cycle occurred. The third VOC collection started 5 min after wounding, beginning at 1010. Subsequent sampling occurred every 15 min starting at 1030 and continuing until 1100. We then collected VOCs every 60 min until 2100. The VOC were passed through the tube at a flow rate of 0.24 L/min for 4–5 min on average, for a total volume of 1 L.

2.2.3.3 A. thaliana sunset and sunrise response

Figure 1 shows the collection sequence for the twilight experiments conducted on *A. thaliana;* sampling instances are marked with circles and triangles. Briefly, the first sampling instance occurred an hour before the lights began to increase or decrease in intensity. The second sample occurred when the lights started to increase or decrease, then samples were collected every 10 min until 60 min had elapsed. The last collection occurred an hour after the lights either turned off or reached full intensity. The flow rate was 0.24 L/min for 1 L total volume.

2.3 Volatile analysis

2.3.1 GC-FID method

Because of a malfunction of the GC-MS, we used the GC-FID to analyze samples for *T. zebrina, F. rubra, A. millefolium, and P. virgatum.* The GC-FID detects compounds in the parts per million range and provides retention times for peaks but does not provide peak identification. The Agilent 6890 network GC system with a 5973 mass selective detector was used with the FID. The GC temperature program was 36 min of total run time, 40°C for 2 min, ramp to 280°C at 10°C/min, and a hold at 280°C for 10 min. The carrier gas was ultrahigh purity helium. The column in the GC was an Agilent Technologies HP-1 (25 m × 0.2 mm × 0.33 μm). The injection volume was 1 μL with a front inlet pressure of 9.14 psi for splitless injection. Each sample was injected three times to provide a measure of reproducibility.

2.3.2 TD-GC-MS method

An Agilent 8890A/5977B GC-MS was coupled with a Markes International Thermal Desorption Unity XR instrument to perform GC-MS analysis on the volatiles collected onto the Markes TD tubes.

The GC run time was set for 36 mins. with an initial oven temperature of 40°C and a 2-min hold time. The oven temperature after the run was also set for 40°C. The programmed temperature ramp rate was 10°C/min up to 280°C with a 10-min hold time. Equilibration occurred for 3 min with a temperature maximum set at 350°C. The front inlet was in splitless mode at 250°C with a pressure of 9.1471 psi. The total flow rate was 19.208 mL/min with the septum purge at 3 mL/min. The column was an Agilent 19091S-433UI with a description reading HP-5MS UI calibrated at 29.8 m × 250 μm × 0.25 μm. The Unity XR Ultra TD method followed general default settings, including a flow rate of 10 mL/min at a temperature of 200°C. The predesorption prepurge time was 1 min at a flow rate of 50 mL/min. The desorb time was 5 min with a trap flow of 50 mL/min at 320°C.

Calibration curves of common plant volatiles were generated and utilized for identification and subsequent quantification of emissions. An internal standard of 0.4 μL nonyl acetate dissolved in hexane at a concentration of 0.25 ng/μL was added to each sample. The results were also compared with multiple National Institute of Standards and Technology (NIST) mass spectral libraries.

Nonyl acetate was used to create a standard curve both for benchmarking each run and for creating a relative internal standard. Five concentrations of nonyl acetate were manually injected into the TD tubes through the calibration solution loading rig in a replicated fashion. The starting concentration was 75 ng with 25 ng incremental increases up to 175 ng. Figure 5 shows the absolute peak height with the standard deviation as error bars ($n = 3$). This standard curve will be used for a relative response ratio quantification in the data presented in this technical report.

Figure 5. Absolute peak height of response to GC-MS detection of internal standard (ISTD) nonyl acetate injected onto TD tube before being processed on the TD instrument. The concentrations varied from 75 ng to 175 ng. The results presented are the mean and standard deviation.
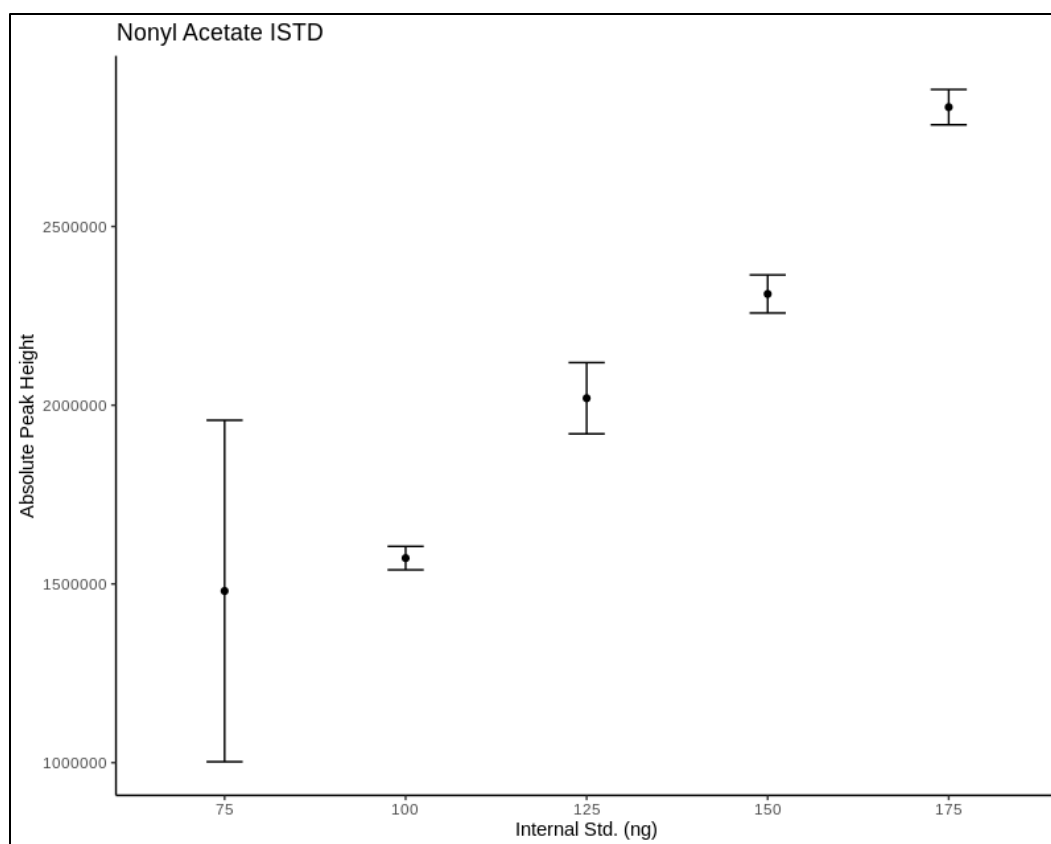
2.4 Statistical analysis

2.4.1 GC-FID data

Because the GC-FID did not resolve compound information from peaks or areas, peaks were identified by retention time and compared for presence or absence in each sample. We analyzed the results using two methods.

2.4.1.1 Manual compilation and alignment

For data for the first *T. zebrina* time point (5 min after wounding), we combined the three replicate injections for each of the three plants to determine if our methods could identify emission variations between our two treatments, wounded and unwounded plants. Although data for five plants were collected for this experiment, the intense nature of manual alignment prevented us from compiling the data from the remaining two plants and the methanol elutions. We compiled the raw .txt file output from the GC-FID into Excel for each individual plant (n = 3) and then aligned peaks from the three replicate injections manually. Alignment was necessary because the retention time for peaks varied slightly between replicated injections. Peaks within 0.010 s were considered to be the same compound. Within each treatment, all time points were counted using the COUNTA function where the presence of a peak (retention time > 0) was counted as 1, and the absence (no retention time reported by the GC-FID) was counted as 0. We then used an IF/THEN statement to assign peaks to one of three categories: occurring in only wounded samples, occurring in only unwounded samples, or occurring in both wounded and unwounded samples. Tabulation results were plotted in a bar graph.

2.4.1.2 Compilation and alignment with GCalignR

We used the R software package `GCalignR` to align and compare the entire peak profile of interest for all sampling efforts. `GCalignR` provides functions with user-defined parameters to align peak lists based on retention times. Initially, parsing .txt file outputs from the GC-FID, we formatted the data into a readable input for `GCalignR`. Code written by Dr. Michael Musty was modified by Dr. Franz Lichtner through correspondence with the package developer Meinolf Ottensmann to accept batch file inputs for `GCalignR` and order files to handle redundancy of names. Peak profiles were aligned with `GCalignR`, and peaks that occurred only in a single sample were automatically removed. Peak retention times that were retained in parsed data sets were only between 3 and 35 min and had a maximum linear shift of 0.05. The maximum–minimum distance of a peak to the mean across samples used was 0.05–0.03. Samples were further processed in groups based on chemical eluent (methanol or hexane) and plant species (*T. zebrina, F. rubra, A. millefolium, P. virgatum*). In total, we compiled over 600 sample GC-FID peak profiles from individual .txt files for processing.

After processing all samples for each group through `GCalignR`, we performed a log transformation on each individual sample's peak and associated retention time and area. There were 90 separate GC-FID peak profiles on average for each plant species for each of the two eluents, methanol and hexane. We then used these data to create either a Bray-Curtis or Jaccard dissimilarity matrix with the R statistical package `vegan` (Oksanen et al. 2020). With the dissimilarity matrix, we were then able to create a non-metric multidimensional scale plot of the samples of interest to show whether the individual samples were grouping together.

Through the `vegan` package, we were also able to statistically test the significance of within-group variation with the `adonis` function and between-group variation with the `betadisper` function (Table 2).

2.4.2 TD-GC-MS data

We performed the statistical analysis and visualization in R version 3.6.1, "Action of the Toes," and base R for the analysis of variance (ANOVA) and beta dispersion tests. We used `ggplot2` for the data visualization. Code is available upon request.

3   Results and Discussion

3.1   Plant wound response (GC-FID)

Our initial approach to understanding the VOC response to wounding involved the use of a GC-FID instrument. With this method, we were able to record chromatograph peak retention time and area for a given sample rather than compound identification. We would need to repeat these tests on the GC-MS for peak identification.

3.1.1   Manual compilation and alignment

Preliminary results from manually compiled data indicated our methods were successful in detecting differences in volatile emissions between wounded and unwounded *T. zebrina* plants (Figure 6). However, it is likely that a portion of the peaks are artifacts from the collection methods, such as siloxanes from column bleed and the manufacturing by-products of Porapak Q. If it is confirmed that these compounds are artifacts from the sampling process, we would expect the artifacts to be present in all samples. Removing those compounds from the analysis should reduce the number of peaks in the "Both" category. Of note is the high variability between individual plants, given that samples are genetically identical. Plants 1 and 4 had more compounds occurring in unwounded plants than in either the wounded or both categories. Plant 5 had no compounds that occurred only in the unwounded category. These results are striking, given that the majority of compounds occurred in the unwounded category for the other two plants. More replications are needed to determine if Plant 5 is an outlier or if such a range of differences is to be expected. Despite efforts to maintain consistent environmental conditions in the growth chamber, it is also possible that there was variation in temperature, light, or relative humidity during collection. Unfortunately, we have not paired the environmental data collected from the data loggers with the results presented here to confirm if the variation in emissions is the result of environmental factors. It is also possible that the plants were experiencing different levels of stress due to differences in environmental factors, which could in turn result in variations in emissions.

Figure 6. Total number of peaks detected in hexane by the GC-FID in three wounded and unwounded *T. zebrina* clones 5 min after wounding. For each plant, the bar on the left represents the total number of peaks unique to the wounded plant. The bar on the right represents the total number of peaks in both wounded and unwounded plants. Plants 1 and 4 contain a third bar showing the number of peaks present in unwounded plants.
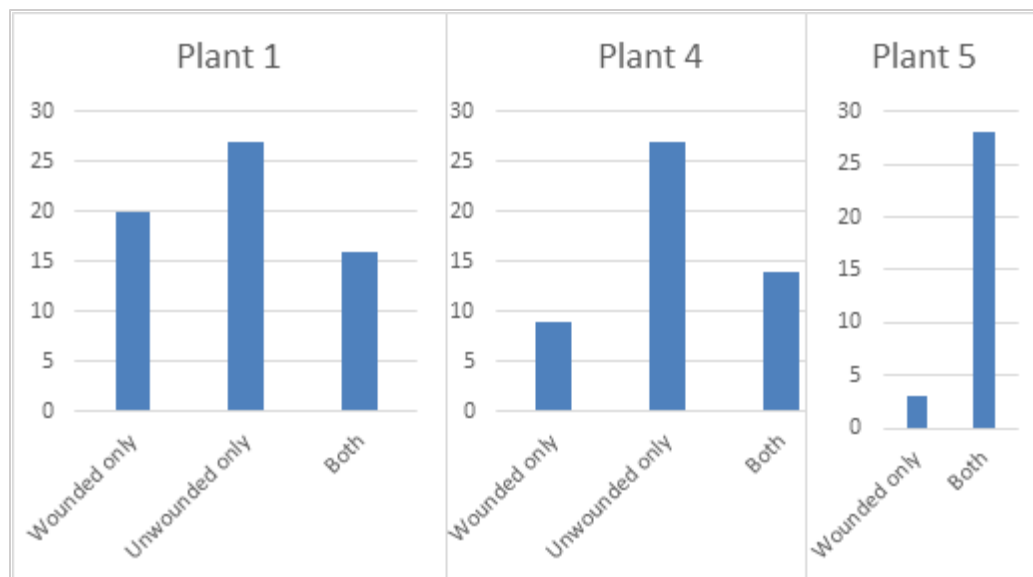
3.1.2 Compilation and alignment with GCalignR

Aligning and comparing peak profiles through the R software package `GCalignR` (Ottensmann et al. 2018) allowed for nonmetric multidimensional scaling techniques, based on Bray-Curtis dissimilarity matrices for each species, to plot the sample data in a visual space. Though we conducted the experiments with both methanol and hexane as the eluents, the results presented for *T. zebrina* and *F. rubra* are hexane alone, which elutes nonpolar molecules (Figure 7), and the results for *A. millefolium* and *P. virgatum* are for methanol, which elutes polar molecules (Figure 8). Table 2 contains the results from within- and between-treatment ANOVA analyses for all four species and both solvents. Both solvents proved similarly inconclusive.

The nonmetric multidimensional scale visualizations confirm what is evident in the ANOVA table above: with the methods used here, there is no pronounced difference between wounded and unwounded plants. One possible confounding factor for all four species is the high flow rates used for collection, which would push the smaller compounds, particularly the C_6 GLVs known to be a wound response, out of the collection tubes. This seems likely given the results of the breakthrough studies conducted (Appendix), and we ran subsequent experiments with the lowest possible flow

rate with our collection apparatus, 240 mL/min. Additionally, *T. zebrina* and *F. rubra* samples were collected at high volumes, which may also have forced compounds out of the tubes.

Figure 7. Nonmetric multidimensional scaling plot of a Bray-Curtis dissimilarity matrix for wounded and unwounded *T. zebrina* (*A*) and *F. rubra* (*B*). The analysis compares peak areas from three time/volume treatments (5 L sampled starting 5 min, 10 L sampled starting 10 min, and 60 L starting 60 min after wounding) and GC-FID injection replicates. Peaks with single occurrences were dropped. Note that the legends differ for each figure in the interest of visualizing the data in different ways.
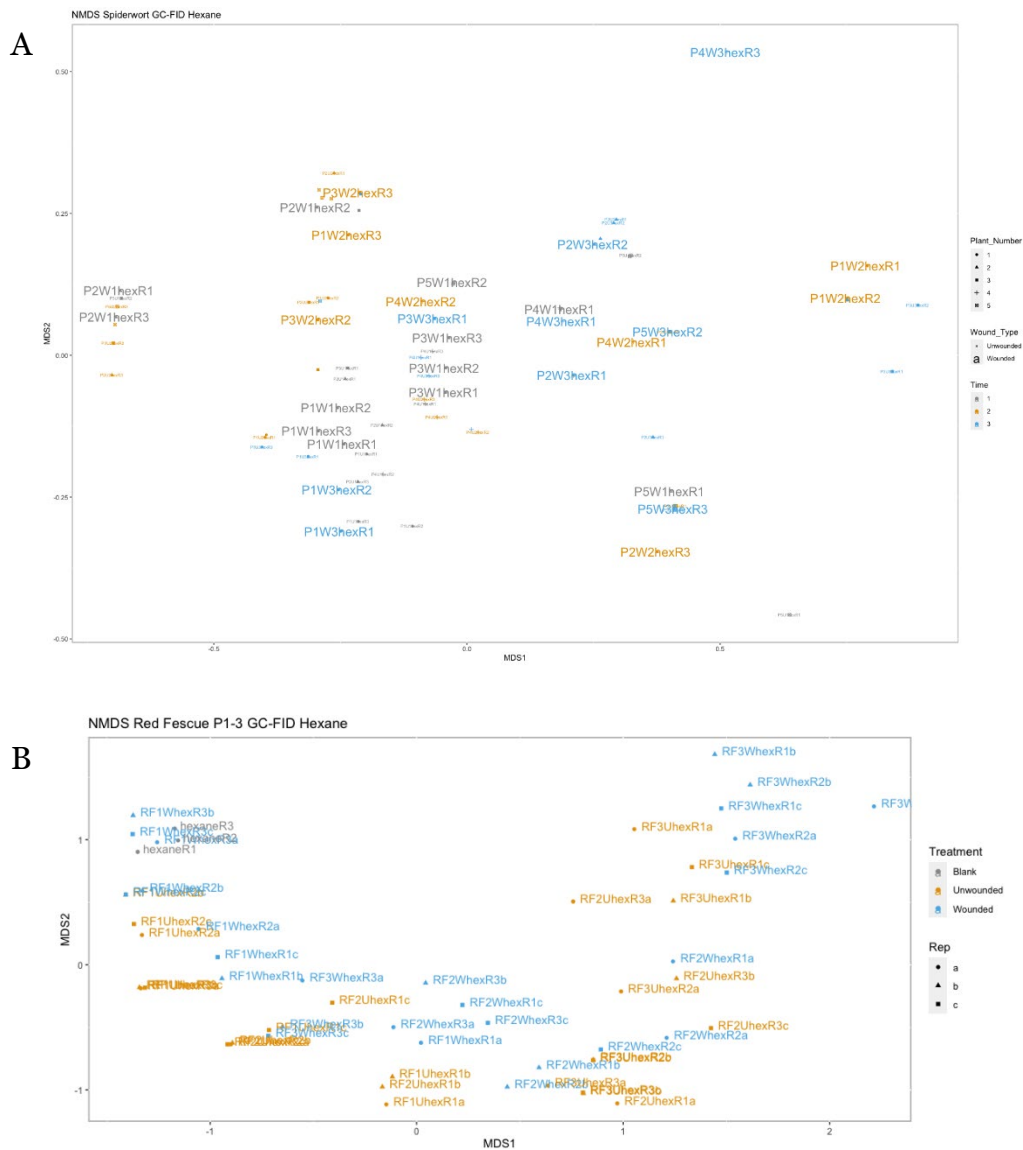
Figure 8. Nonmetric multidimensional scaling plot of a Bray-Curtis dissimilarity matrix for wounded and unwounded *A. millefolium* (*A*) and *P. virgatum* (*B*) from peaks detected with the methanol elution. The analysis compares log-transformed peak areas from five time/volume blocks with replication (see Table 1 for sample duration times and volumes) measured on the GC-FID. Peaks with single occurrences were dropped.
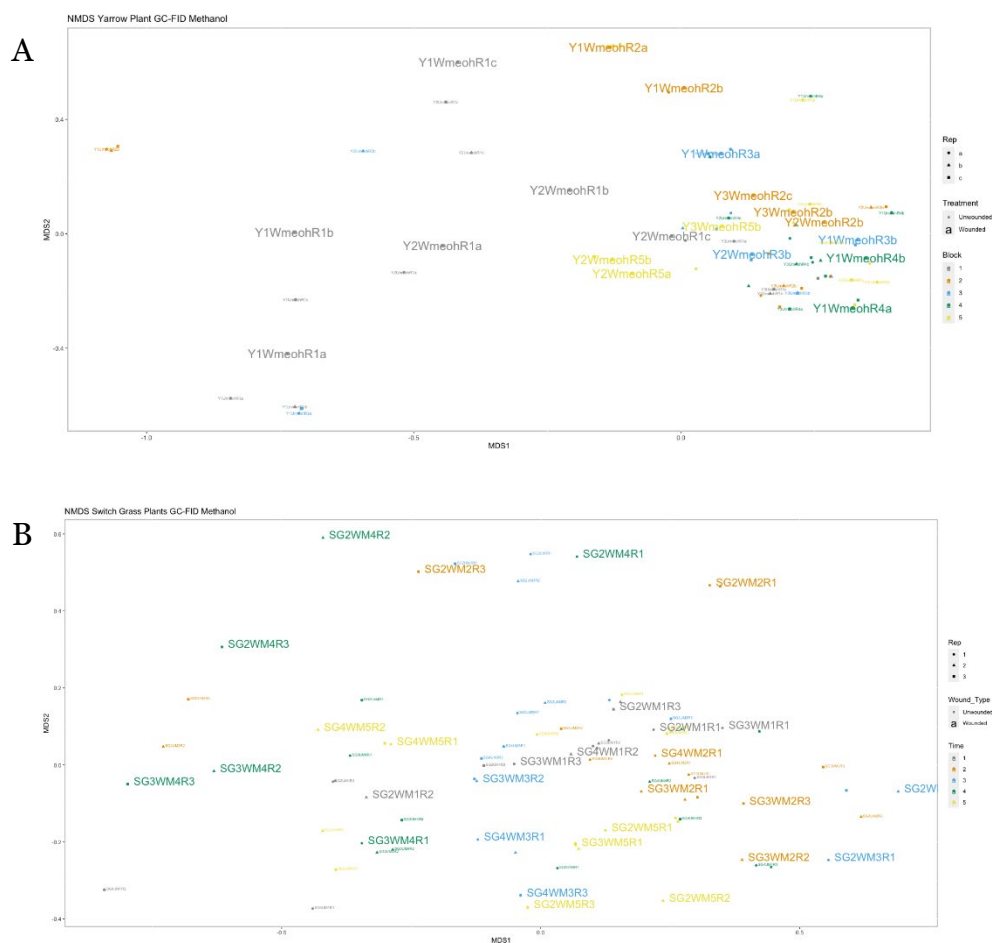
One interesting finding is that the manual compilation of data from three *T. zebrina* clones, which tabulated the presence and absence of compounds but did not include peak area, does indicate a difference between wounded and unwounded emissions. This implies that detecting wound response is possible with the mere presence or absence of compounds but is hindered by the inclusion of compound concentrations. Identification of compounds without concentrations would greatly simplify the sensors needed for using plants as biosensors.

Table 2. ANOVA *p*-values (*α* < 0.05) testing for differences between individual plants (within each treatment) and between wounded and unwounded plants. The *Peaks* columns include total number of peaks initially included in the GC-FID profile, the number of blanks of pure hexane or methanol included in the run for validation, and the singular peaks detected in an individual sample. The *Retained* column includes the total number of peaks included in the final downstream analyses after blanks and singular peaks were removed.

Plant	Sample	Within Treatment ANOVA	Between Treatment ANOVA	Peaks			
				Total	Blanks	Singular	Retained
T. zebrina (*n* = 5)	Hexane	0.08	0.3268	334	37	112	185
	Methanol	NA	NA	265	43	135	87
A. millefolium (*n* = 3)	Hexane	0.004	0.08531	501	25	187	289
	Methanol	0.113	0.0001993	116	50	15	51
F. rubra (*n* = 3)	Hexane	0.155	0.6671	553	25	319	209
	Methanol	0.083	0.843	496	50	265	181
P. virgatum (*n* = 5)	Hexane	0.251	0.1997	452	128	175	149
	Methanol	0.132	0.5445	138	56	16	66

3.2 *P. virgatum* wounding response (TD-GC-MS)

Panicum virgatum wounding experiments resulted in 41 compounds with a standard deviation greater than zero across each of the 16 time points for all three replicates (Figure 9). Of these 41 compounds, 5 are classified as siloxanes and can be attributed to the GC column. Interestingly, there is no D-Limonene or several other unique VOCs detected until the last sampling event at 2100 hr, 12 hr after sampling started. We are uncertain what could cause this trend but speculate it could be either due to stress related to being in the plant chamber for such a long duration or due to the nearness to simulated sunset, which would begin in 1 hr. We hesitate suggesting that this second explanation implies plant awareness of conditions to come.

Of the 41 compounds observed after filtering, we compared those that were observed before wounding with those observed after wounding. Though there was variability in the response of compounds formed after wounding over time, the amount of each VOC detected after wounding requires further investigation. Further replication of what has been completed thus far and multiple control experiments following the same sampling regime without wounding will be conducted at a later date to show which compounds are produced and to what degree they are produced. The 12 compounds, 1-thynyl-2-methyl-3-methylene-1-cyclobutene; 1-phenyl-4-[1]benzopyrano[4,3-c]pyrazolone; 3.alpha.-methylcholest-5-en-3-.beta.ol nitrite; 7-hydroxy-dibenzo[c,h]chromen-6-one; benzene, 1,1'-

sulfonylbis[4-chloro-; butane, 2,3-dimethyl-; D-Limonene; hexane; hexane, 1,6-dicyclohexyl; isopropyl (E)-3-decenoate; N-(4-(1-hydroxycyclobutyl)but-3-ynyl)benzenesulfonamide; and pentane, 2,3,3,4-tetramethyl-, were not present in the unwounded *P. virgatum* VOC matrix (Figure 10). Additionally, Figure 10 also shows greater variance in the response ratio for compounds released after wounding.

Figure 9. Heat map of the *P. virgatum* wounding experiment. The response ratio (RR) was filtered by including compounds with a standard deviation greater than zero to remove compounds seen at only a single time point across replicates. The RR was then log transformed to create a gradient for the heat map. Samples were ordered by time, starting with 0900 and wounding occurring at 1005.

Figure 10. *P. virgatum* wounding response as measured by VOC factored by treatment, unwounded and wounded. The unwounded response was measured at two time points (0900 and 1000). The compounds released after wounding are measured for the remaining 14 time points.
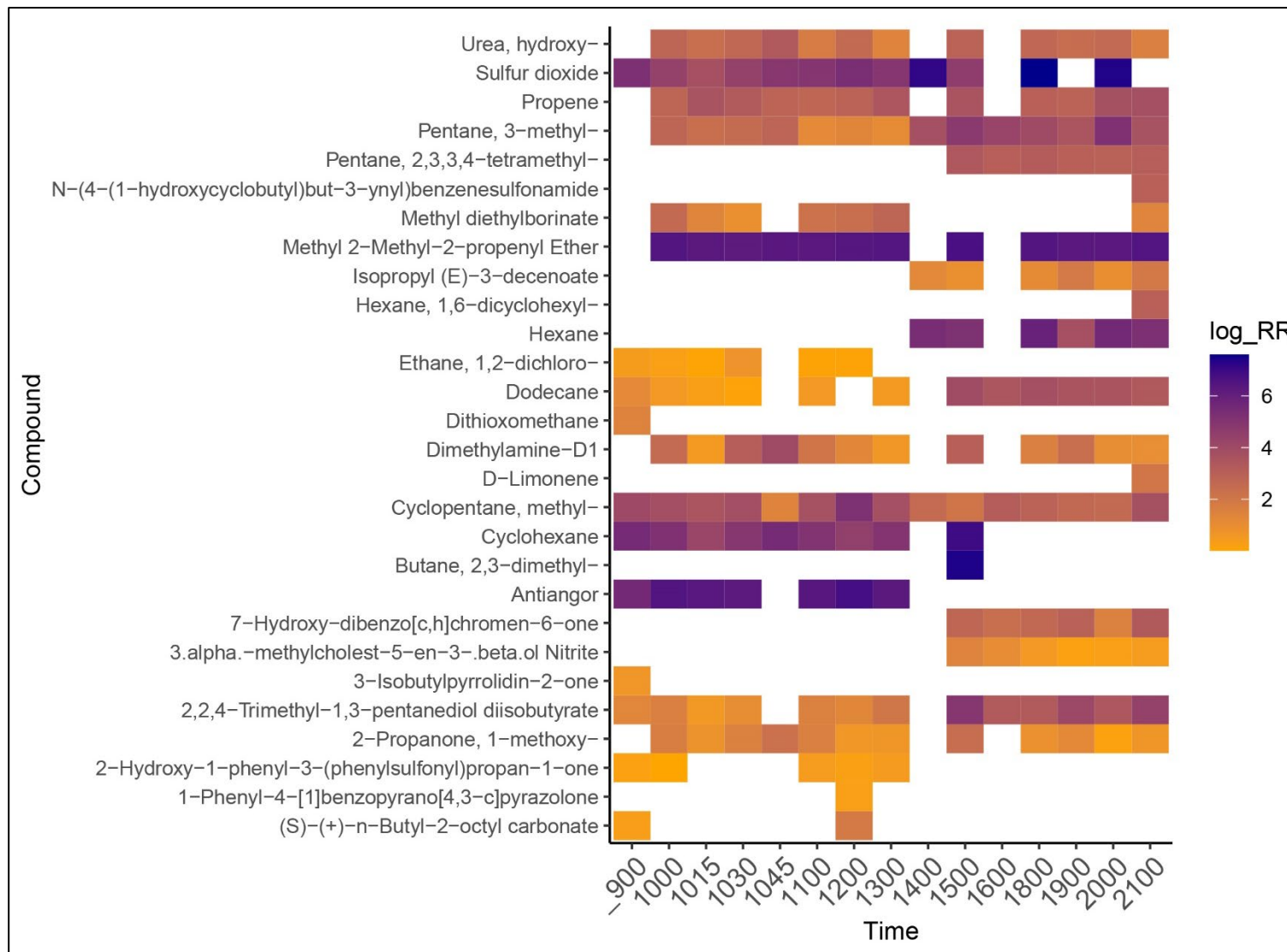
3.3   *A. thaliana* sunset and sunrise response

To capture the VOCs emitted during a simulated sunset and sunrise, we followed the collection schedule described in Figure 1. For three replicates, we collected VOCs at eight time points. With each collection effort analyzed on the TD-GC-MS, we included a blank control where no plant volatiles were collected. To reduce the variability in measuring the response across samples, we performed a batch analysis with MassHunter Quant-My-Way version 10.1 from Agilent Technologies (Santa Clara, California) of all samples from a single experimental event.

For sunset, we detected 178 compounds during the analysis across all eight time points. Due to high sample variability detected across replicates within a specific time point, we filtered out compounds with a standard deviation of zero. We next removed compounds that included "siloxanes" because these compounds are known by-products of the chemical analysis. Figure 11 presents the log-transformed response ratio of the remaining compounds over time as a heat map. GLV compounds detected in some but not all samples are 2-ethyl-1-hexanol; D-limonene; beta-pinene; and benzaldehyde.

From these data, we can observe both changes in compound detection over time and compound response ratio. The heat map indicates both plant perception of changing light and a dynamic plant response. The response may potentially provide a signal of how much photosynthate was produced during photophase or how much light is left until darkness occurs. Notably, the greatest change in both detection and response ratios occurs at 2220, 20 min after dimming begins and at approximately 65% light intensity. Several compounds are absent although occurring at all time points both before and after 2220. Additionally, several compounds present at all time points increase in response ratio at 2220, such as Bicyclo[3.1.1]heptane; 6,6-dimethyl-2-methylene-,(1S); and Benzene (2-ethylbutyl). Further work is needed to identify if the pronounced change at this time point, rather than when the lights first dim, is the result of a lag due to plant metabolic rate or results from the sampling protocol used, such as the flow rate. It could also be that an approximately 18% decrease in intensity, as found at 10 min after sunset, is not a great enough change to trigger a large metabolic response. Instead, a greater decrease, such as the 35% changed at 20 min after sunset, is required for plants to amplify their signal.

Figure 11. VOCs collected from young *A. thaliana* during a simulated sunset. The *log_RR* is the log-transformed response ratio.
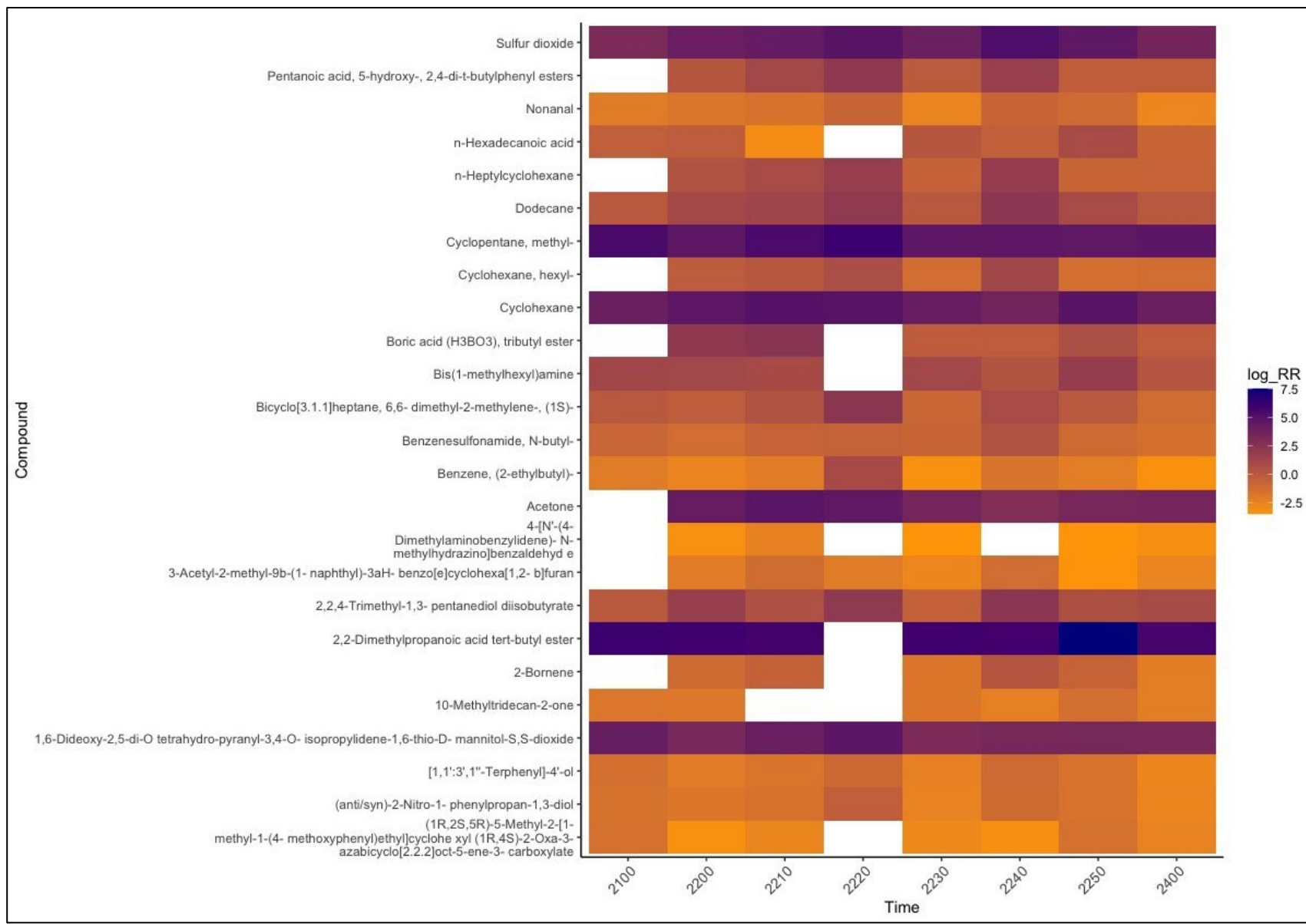
Simulated sunrise VOC analysis of *A. thaliana* differed from that of the sunset experiment. Variation in the 40 compounds presented in Figure 12 shows a time-dependent response in VOCs as *A. thaliana* receives increased light exposure. Compounds that also occurred under simulated sunset include D-limonene; cyclohexane; pentanoic acid; 5-hydroxy-, 2,4-di-t-butylphenyl esters; dodecane; and sulfur dioxide. Two compounds showed a pronounced increase in RR after only 10 min of increasing light: 7-hydroxy-dibenzo(c,h)chromen-6-one at 0710 and dimethylamine-D1. The response could signal the increase in light or be in response to the plant experiencing low-light conditions. Compounds unique to simulated sunrise included pyrimidinium; 5-carboxy-4-(1,1-dimethylethyl)-1,6-dimethyl-2-(1-methylethyl)-; hydroxide; inner salt; oxacyclotetradecan-2-one; methyl 2-methyl-2-propenyl ether; and 2-methyl-4-penten-1-ol.

Figure 12. VOC collected from young *A. thaliana* during a simulated sunrise.
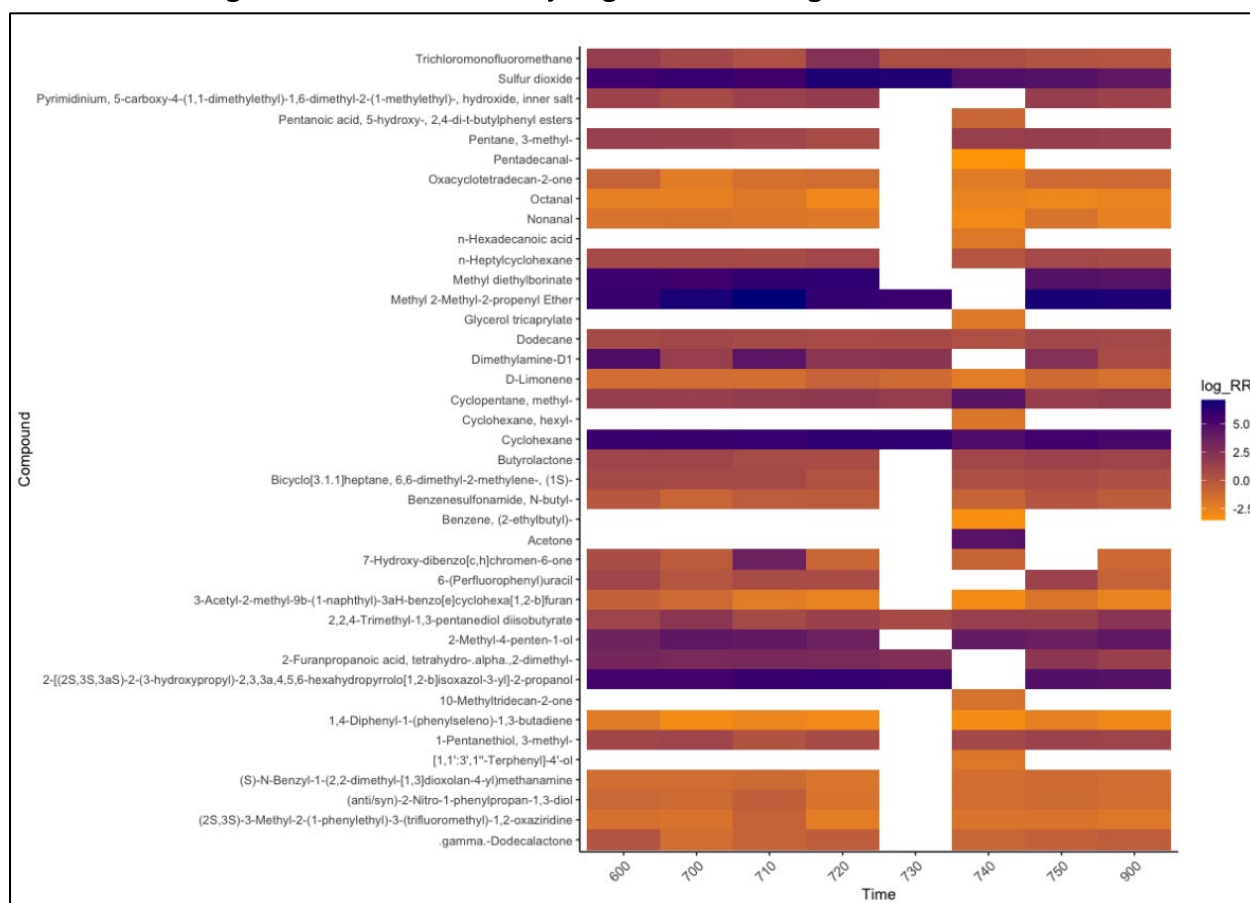
The detection of acetone in the sunrise and sunset experiments requires further investigation of the mass spectra being used to match to this compound. It is most likely an impurity from the system or a mistake.

Additional sampling needs to occur to make better statistical comparisons, especially at time points underrepresented in this study, such as during the ramping of lights, which simulates the rising sun. Compound name assignments by the MassHunter Quant-My-Way software need to be validated by comparison to the mass spectra detected.

4 Conclusions and Recommendations

The understanding of plant language is still in its nascency. The work performed here optimized techniques for collecting and analyzing plant volatile emissions. As a result, we were able to detect differences in emissions between wounded and unwounded plants, as well as unwounded plant response to changes in light. We preferred the TD-GC-MS paired with TD tubes to the GC-FID paired with Porapak Q tubes because it reduces sample processing time and chemical waste. Additionally, the peak identification capabilities of the TD-GC-MS enable recognition of collection artifacts such as column bleed and by-products of adsorbents. One additional step needed in method development is to determine the minimum sample collection time and volume required for TD tubes. Shorter collection times would enable higher resolution for the detection of compound and concentration changes over time.

The work here also highlights remaining knowledge gaps. Initial GC-FID results indicate that variation is greater between individuals than between the treatment of wounded and unwounded plants. However, given that peaks were unidentified by the GC-FID, our analysis included artifacts. Once artifacts of the collection method can be removed, as will be done with the TD-GC-MS, individual variation should be further explored. Of particular interest would be an investigation of plant species that reproduce both vegetatively (forming clones) and sexually, such as the strawberry, which would enable comparisons between both genetically identical individuals and between genetically distinct individuals from the same species. Other work (e.g., Beck et al. 2008) has detected differences in compounds and concentrations between different wound types. Here we report on wound responses to cutting and crushing, but the differences in methodology do not allow for robust comparison. Of interest for Army surveillance, reconnaissance, and mobility would be examining volatiles released from damage due to different vehicle types.

Additionally, more work is necessary to understand a plant's natural rhythm of emissions in response to the diel cycle of light. The work presented here examines one species' response to only sunrise and sunset without considering the possible changes throughout the day as photosynthate increases. However, the spike in the number of compounds detected from *P. virgatum* during the last hour of daylight implies plant signals

should be observed more holistically over the course of the day to fully understand response to changes in light and wounding.

Results reported here indicate that plants can function as biosensors for persistent intelligence, surveillance, and reconnaissance. We have developed a successful plant volatiles program at ERDC with capabilities for detecting and quantifying plant signals in a growth chamber. We are currently developing statistical and machine learning techniques for deciphering the meanings of these messages. The next logical step is to take these new tools outdoors. A wild world of plant communication awaits us beyond these laboratory experiments.

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Appendix: Plant Volatile Collection and Analysis with Gas Chromatography Mass Spectroscopy Flame Ionization Detector in Response to Wounding

This appendix describes the methods developed for identifying and quantifying the chemical compounds released by plants as baseline emissions and in response to wounding. The methods described here were ultimately rejected due to the purchase of the new thermal desorption gas chromatography mass spectrometer (TD-GC-MS) and universal TD tubes.

A.1 Objective

In the initial pilot study, we sought to differentiate between the compounds emitted from wounded and unwounded plants, between two different plant species, and between plants of different age classes within the same species.
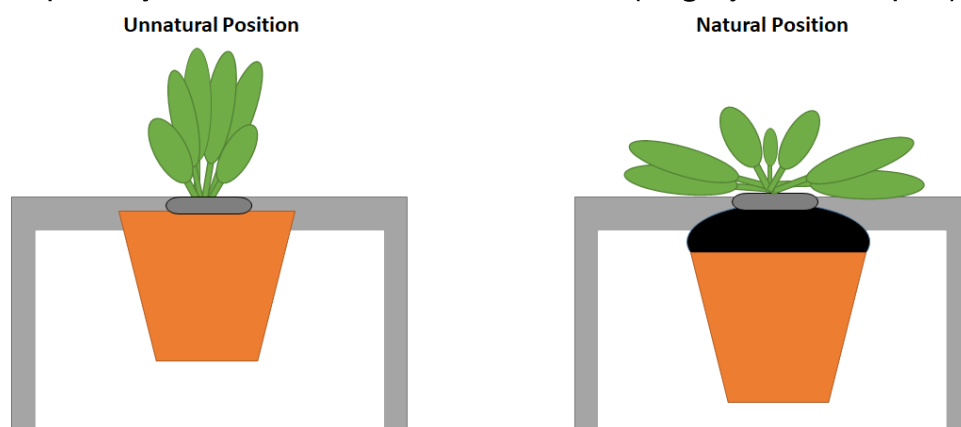
A.2 Methods

A.2.1 Plant material

Before we established a population of *Arabidopsis thaliana,* we used *Chlorophyllum comosum* (spider plant) spiderettes taken from the office of Dr. Whitecloud. Spiderettes were clipped from the mother plant and placed in a 100 mL beaker in deionized water before placement in the plant chamber.

Subsequently, we modified the method in Rivero et al. (2014) to cultivate wild type *Arabidopsis thaliana* seeds (*A. thaliana* ecotype Columbia [L.] Heynh. [Col-0]) in soil. Potting soil was sieved through a number 8 sieve and sterilized at 120°C for 30 min and mixed with grade 2 lab water until moist. Moistened soil was gently packed into 2-inch pots until it was slightly mounded above the container lip to form a dome. The dome shape ensured that *A. thaliana* grew above the container lip, allowing the plant to sit in a natural position in the guillotine base of the plant chamber. However, the installation of a plant into the guillotine base presented two issues. First, loose soil got onto the guillotine base, potentially contaminating volatile organic compound (VOC) samples with soil VOCs. Second, placing the charcoal filter around the base of the plant to prevent soil

volatiles from moving up through the purge trap disturbed the prostrate stature of *A. thaliana*. To minimize these impacts, we modified our cultivation as follows (Figure A-1). We placed a 7.6 cm × 7.6 cm square of landscaping fabric, cut with a small (~0.85 cm) hole in the center over the soil and secured it in place with a rubber band. The landscape fabric prevents soil from getting onto the guillotine base during plant installation. Next, we inserted a small 1/4 in. piece of Teflon tube in the soil and sowed the *A. thaliana* seed in the middle of the tube. The Teflon tube acts as a collar at the base of the plant and encourages vertical growth before horizontal growth. The greater upright posture of the plant minimized disturbance during installation. *A. thaliana* plants were grown in the Conviron E15 growth chamber programmed at 20°C and full light intensity for 18 hr and at 15°C in complete darkness for 6 hr prior to being moved to the volatile collection chamber.

Figure A-1. When wrapped in the carbon-filter collar and installed into the guillotine base, *A. thaliana* grown with traditional practices was forced into an unnatural posture with all leaves sticking upwards (*left*) rather than lying flat. A modified cultivation technique (*right*) that included mounded soil, shade cloth, and a Teflon collar to force the plant to grow upward before growing outward, created a natural, prostrate position for the plant when installed in the guillotine. The Teflon collar was replaced by the carbon-filter collar before installation. (Image by Nadia A. Podpora.)

A.2.2 VOC collection

We used Porapak Q and the elution methods described in "Methods and Materials" but with only hexane as a solvent, and eluents were concentrated under nitrogen rather than with the SpeedVac. Approximately 24 hr before collection began, an individual plant was placed in the VOC collection chamber with a new PET oven bag. Airflow over the collection tube was 1 L/min, and the sample was collected onto a single Porapak Q tube for 3 hr. We then injured the plant by tearing the leaves without removing

the polyethylene terephthalate (PET) bag and collected the wounded sample for 3 hr. Tubes were stored as described in section 2.2.3.

A.2.3 Gas Chromatography–Mass Spectrometry

We used an Agilent 6890 GC system coupled with a 5973 mass selective detector fit to a 7683 series injector and autosampler. The temperature program was 40°C for 2 min, ramp to 280°C at 10°C/min, and hold at 280°C for 10 min for a total run time of 36 min. The carrier gas was ultra-high-purity helium. We used an Agilent Technologies HP-1 column (25 m × 0.2 mm × 0.33 µm film thickness) and a splitless injection of 1 µl with the heater set to 250°C and a front inlet pressure of 9.14 psi (63 kPa). The solvent delay was set to 4 min.

A.3   Results

We were able to detect differences between species, plant ages, and wounded versus unwounded plant emissions (Figures A-2 to A-4). We visually identified differences in peak presence/absence and height from the chromatograms. Because we had no chemist on the team at the time, we were unable to confirm the compound names assigned by the ChemStation software and do not present them here. With the addition of Tim Cooke, a chemist, to the team, we were able to identify artifacts in our samples in the 4–10 min retention time range (Figure A-5). The peaks were identified and confirmed from the literature as artifacts from Porapak Q (Betti et al. 1985; Sturaro et al. 1992, 1994).

Figure A-2. GC-MS chromatogram for the spider plant *(Chlorophyllum comosum)* and *Arabidopsis thaliana*. *White boxes* highlight differences in peak location, indicating the presence of different compounds.
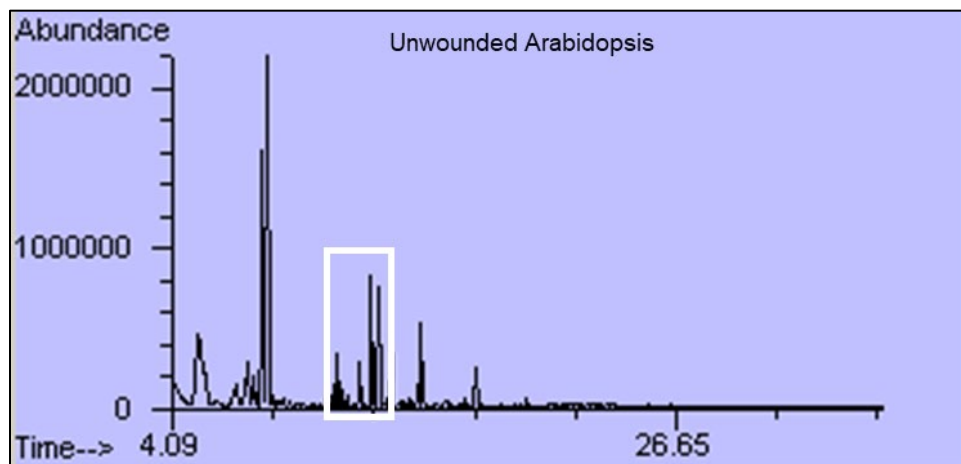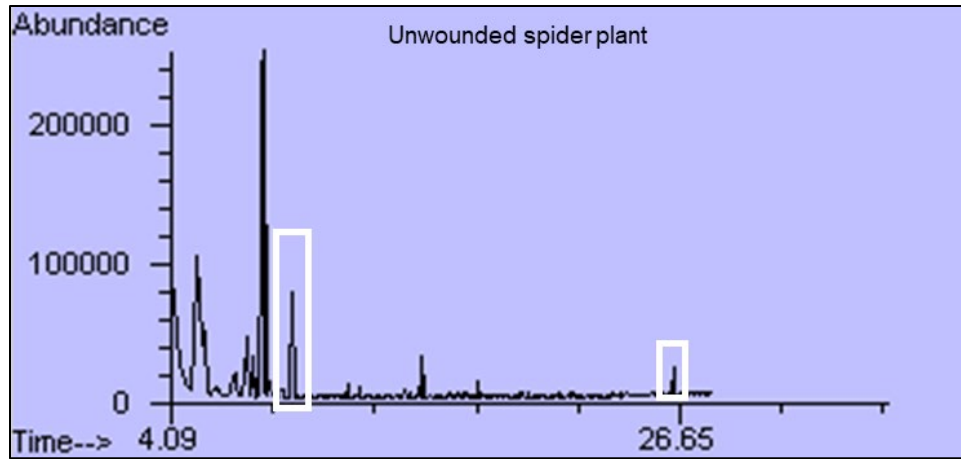
Figure A-3. GC-MS chromatogram of young (preflowering) and mature (flowering) *A. thaliana* plants. *White boxes* indicate differences in peak height and thus relative chemical concentration.
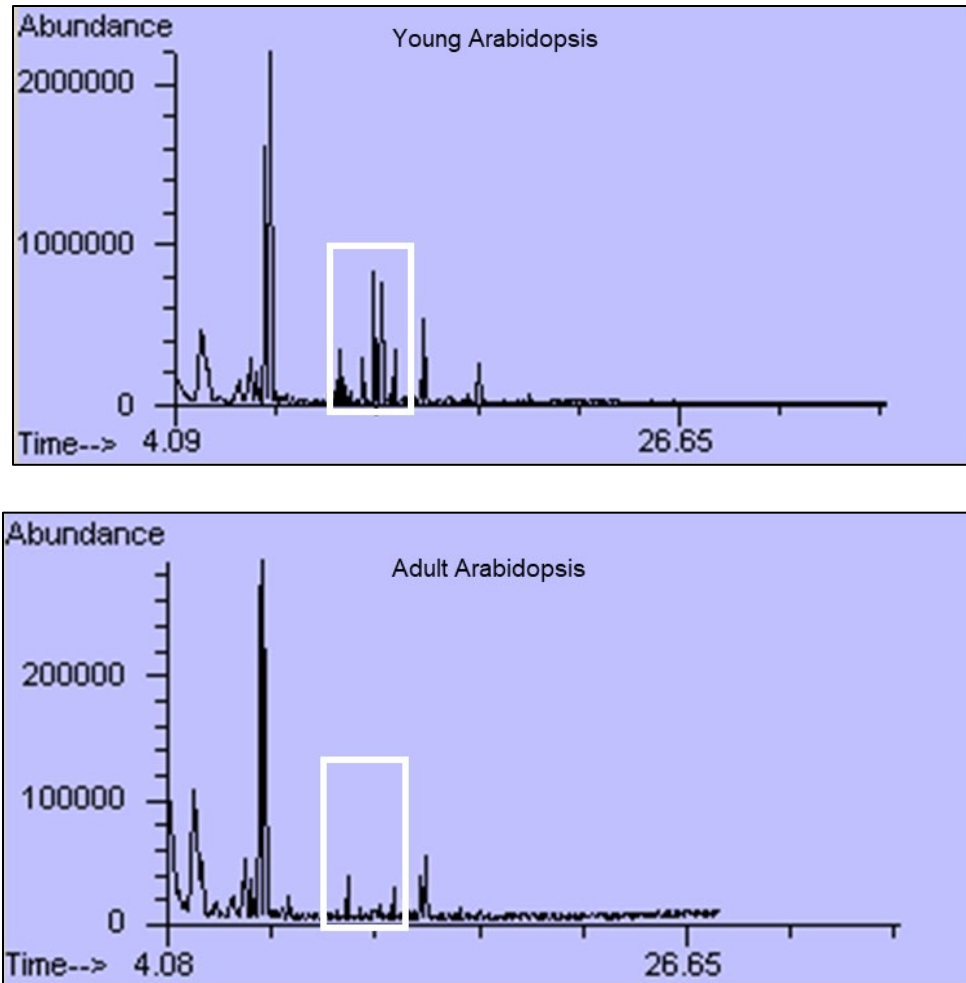
Figure A-4. GC-MS chromatogram of the same spider plant before and after wounding. *White boxes* highlight differences in peak height and thus relative concentrations of compounds.
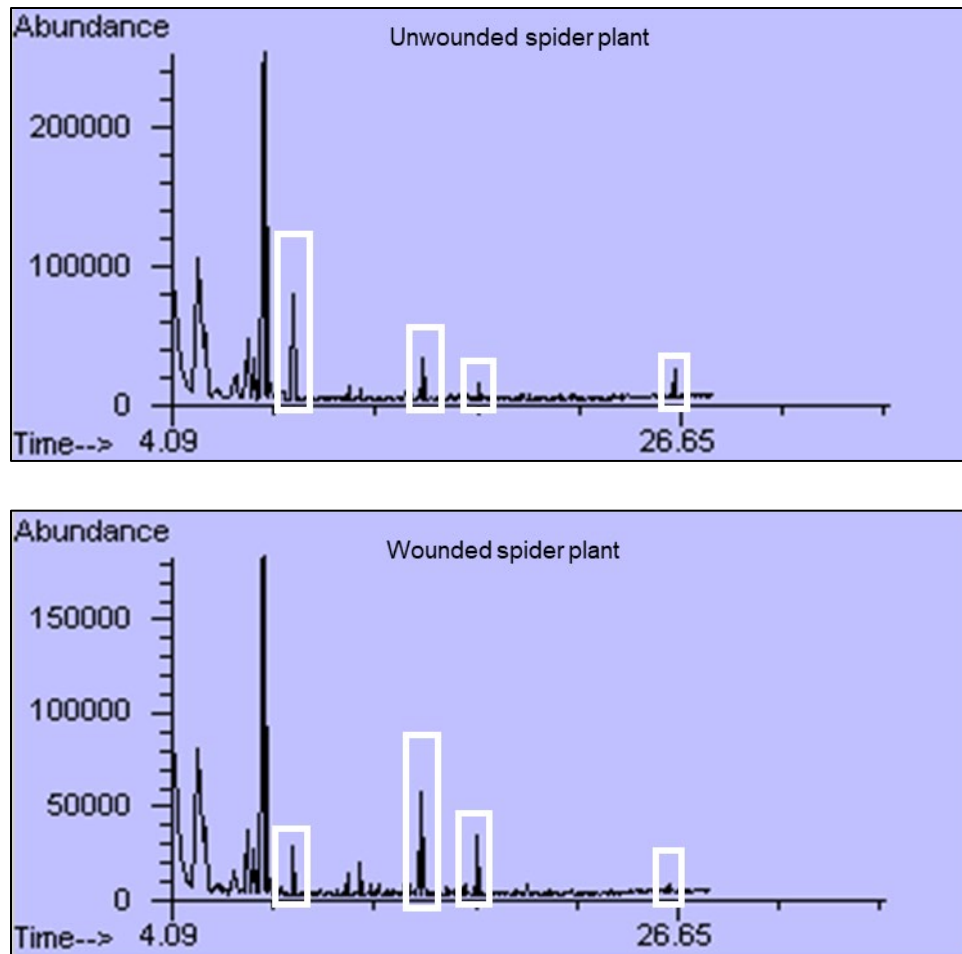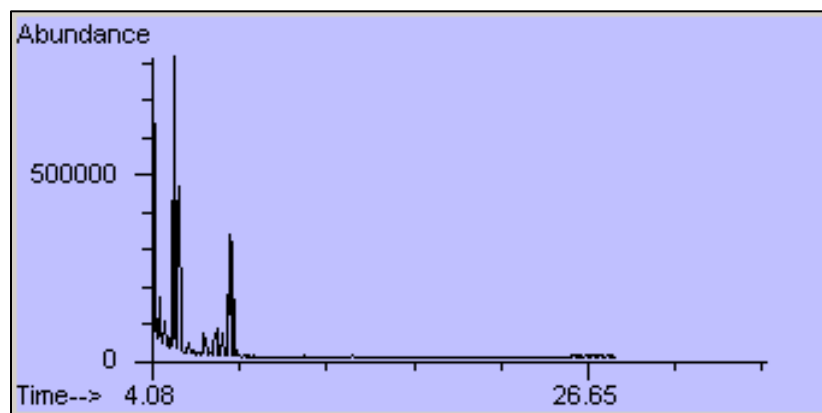

Figure A-5. Total ion chromatogram of a Porapak Q column that was flushed with 2 mL of hexane, dichloromethane, and methanol sequentially. The continued presence of peaks from 4 min to 10 min indicates that these contaminants are being released by the Porapak Q adsorbent.

A.4 Method refinements

After these initial results, we refined our methods. To ensure that we were collecting as many volatiles as possible, we added a second polar solvent, methanol, to create a serial elution process. Tubes were eluted in methanol as described above for hexane. We eliminated the concentration of eluents with nitrogen and switched to using the SpeedVac.

To increase green leaf volatiles peak intensity and to minimize the impact of adsorbent artifacts on the spectra, we conducted a set of experiments manipulating concentration methods. After 6 mL was removed for the previous concentration method, 24 mL of eluent remained a. The remaining 24 mL was concentrated with the SpeedVac to dryness. We placed 7 mL of solvent in each SpeedVac tube, ran until dry, and then repeated using one tube per sample (~10 min for hexane and ~45 min for methanol each run) until all remaining solvent was evaporated. Then, each tube was reconstituted with 1 mL each of hexane or methanol. We conducted a second trial by placing 7 mL of solvent in each SpeedVac tube, running until less than 1 mL was left, and then repeating using one tube per sample (~10 min for hexane and ~45 min for methanol each run) until nearly all remaining solvent was evaporated. Then, each tube was reconstituted with hexane or methanol so that each sample contained 1 mL total. Each reconstituted liquid was filtered using a 0.45 µm PTFE syringe filter to remove escaped adsorbent particles before analysis by GC-MS because particles could clog the instrument. Samples were analyzed using the same GC-MS method as with our standard analysis and a 4 min solvent delay.

Increasing the amount of eluent concentrated did not increase plant-derived-compound peak size substantially enough to mitigate the impacts of the adsorbent artifact peaks. We continued with the original protocol because it was more time efficient.

A.5 Breakthrough Study

We performed a breakthrough study to optimize flow rate and volume for the VOC collection apparatus and to ensure that all volatiles were being collected by the volatile collection tubes. Breakthrough can occur either because the volatiles move too fast to adsorb or they break free from the adsorbent and flow out of the tube. To test if breakthrough was occurring, two tubes were fitted in series, with the plant chamber air pulled into the first tube and directly into the second. Both tubes were then analyzed for

the presence of VOCs. If VOCs were present in the second tube, then breakthrough occurred. We manipulated flow rate and volume of air collected, as shown in Table A-1. Initial breakthrough studies used a liquid standard containing 2-phenylethanol and beta-pinene inside the chamber. All conditions tested using this standard showed breakthrough, but the concentration of volatiles inside the collection apparatus was significantly higher with a liquid standard compared to the VOCs generated by a plant. We repeated the study with a mature (flowering) *A. thaliana* plant to replicate experimental conditions. Minimizing breakthrough is important to accurately calculating the concentration of VOCs given off by the plant. Conditions for plant VOC collection were chosen from those only where no breakthrough occurred.

Table A-1. Results of breakthrough study conducted by connecting two collection tubes in series while manipulating flow rate and volume. Standards were a combination of two liquid standards, 2-phenylethanol and beta-pinene, in a vial. Plant material was mature *A. thaliana.*

Tube	Sample	Flow rate (L/min)	Volume (L)	Breakthrough?
Porapak Q	Standards	1.0	1	Yes
Porapak Q	Standards	1.0	3	Yes
Porapak Q	Standards	1.0	5	Yes
Porapak Q	Standards	1.0	10	Yes
Porapak Q	Plant	1.0	1	No
Porapak Q	Plant	1.0	3	No
Porapak Q	Plant	1.0	5	No
Porapak Q	Plant	1.0	10	Yes
TD Tube	Plant	0.3	1	No
TD Tube	Plant	0.3	3	No
TD Tube	Plant	0.3	5	No
TD Tube	Plant	0.3	10	No

No breakthrough occurred with the Markes TD tubes and a flow rate of 300 mL/min of air. These conditions were initially selected based on manufacturer's recommendations for flow rate (60–100 mL/min) and the limitations of our apparatus (300 mL/min is the lowest flow achievable in the current configuration). We are having the manufacturer recalibrate the flowmeter to 0–250 mL/min.

Acronyms and Abbreviations

ANOVA	Analysis of variance
CRREL	Cold Regions Research and Engineering Laboratory
ERDC	US Army Engineer Research and Development Center
FID	Flame ionization detection
GC	Gas chromatography
GLVs	Green leaf volatiles
ISTD	Internal standard
MS	Mass spectrometer
NIST	National Institute of Standards and Technology
PET	Polyethylene terephthalate
PTFE	Polytetrafluoroethylene
RR	Response ratio
TD	Thermal desorption
VCT	Volatile collection tube
VOC	Volatile organic compound

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14. ABSTRACT

Plants emit a bouquet of volatile organic compounds (VOCs) in response to both biotic and abiotic stresses and, simultaneously, eavesdrop on emitted signals to activate direct and indirect defenses. By gaining even a slight insight into the semantics of interplant communications, a unique awareness of the operational environment may be obtainable (e.g., knowledge of a disturbance within). In this effort, we used five species of plants, *Arabidopsis thaliana*, *Panicum virgatum*, *Festuca rubra*, *Tradescantia zebrina*, and *Achillea millefolium*, to produce and query VOCs emitted in response to mechanical wounding and light cycles. These plants provide a basis for further investigation in this communication system as they span model organisms, common house plants, and Arctic plants. The VOC composition was complex; our parameter filtering often enabled us to reduce the noise to fewer than 50 compounds emitted over minutes to hours in a day. We were able to detect and measure the plant response through two analytical methods. This report documents the methods used, the data collected, and the analyses performed on the VOCs to determine if they can be used to increase environmental awareness of the battlespace.

15. SUBJECT TERMS

Biosensor, Environmental awareness, Gases from plants--Detection, GC-FID, Plants--Effect of stress on, Plant volatiles, TD-GC-MS, Thermal desorption, PoraPak Q

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