JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

Cite This: J. Agric. Food Chem. 2019, 67, 7530−7537

pubs.acs.org/JAFC

Rapid In Situ Analysis of Plant Emission for Disease Diagnosis Using a Portable Gas Chromatography Device

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Supporting Information

ABSTRACT: We developed and applied a fully automated portable gas chromatography (GC) device for rapid and in situ analysis of plant volatile organic compounds (VOCs) to examine plant health status. A total of 42 emission samples were collected over a period of 5 days from 10 milkweed (*Asclepias syriaca*) plants, half of which were infested by aphids. Thirty-five VOC peaks were separated and detected in 8 min. An algorithm based on machine learning, principal component analysis, and linear discriminant analysis was developed to evaluate the GC results. We found that our device and algorithm are able to distinguish between the undamaged control and the aphid-infested milkweeds with an overall accuracy of 90−100% within 48−72 h of the attack. Such rapid in situ detection of insect attack attests to the great potential of VOC monitoring in plant health management.

KEYWORDS: *portable gas chromatography, volatile organic compounds, plant emission, plant disease diagnosis, common milkweed (Asclepias syriaca)*

1. INTRODUCTION

Early detection of pest infection and plant diseases can minimize economic losses in agricultural industries.[1] Many plants respond to internal and external stimuli (such as drought and insect attacks) by releasing volatile organic compounds (VOCs) from their tissues.[2−5] Monitoring plant VOCs can therefore provide important information about past and current levels of abiotic and biotic stress.[6−10] Numerous studies of plant VOCs in the past 2 decades have shown that plants change their VOC blends in response to damage and herbivore attacks[11−14] and that those VOCs are significantly different from those of healthy plants.[9,15−19] In short, VOCs can potentially be used as a noninvasive means to monitor plant health.

Traditional laboratory-based techniques to diagnose diseases can be time-consuming and expensive. For example, enzyme-linked immunosorbent assays (ELISA) of pathogen proteins and polymerase chain reaction (PCR) amplification of pathogen gene sequences have been employed extensively to identify plant diseases and to inform an appropriate treatment strategy.[20−26] However, such diagnostic techniques typically demand substantial skill in molecular biology and access to well-equipped laboratories. Therefore, there is a strong need for a fast, sensitive, and selective method for rapid detection of plant diseases. In contrast to the laboratory-based technologies described above, VOC detection may provide a rapid, reliable, nondestructive, and cost-effective alternative for plant disease diagnosis that can also be used routinely as a real-time monitoring tool in greenhouses and the field. The VOC-based detection methods currently in use for monitoring the disease states of plant are reviewed by Sankaran et al. and Bicchi and Maffei,[24,27] among which gas chromatography (GC) in tandem with mass spectrometry (MS) is the conventional and commonly used technique for quantitative as well as qualitative analyses of VOCs released from plants.[28−34] However, GC-MS methods involve several complicated and time-consuming stages to prepare, collect, and analyze samples. In addition, benchtop GC-MS instruments are expensive, bulky, and power-intensive, and require considerable training to use. Furthermore, because of the long turnaround time between sample collection and analysis, benchtop GC-MS cannot be used to continuously monitor the dynamic changes in plant VOCs. There are a few commercially available portable GC-MS such as Torion from PerkinElmer and Hapsite from Inficon. They are fast and reliable and can provide chemical identification. However, those instruments are expensive, power-intensive (2−3 h of battery operation), and heavy (15−20 kg). In addition, they usually use manual sample injection based on the SPME (solid-phase microextraction) technique (a headspace sampling module adds an additional 10 kg). Therefore, they are not suitable for plant emission monitoring. Recently, an alternative technology called electronic nose (E-nose) was developed for rapidly assessing VOCs. It consists of an array of gas sensors that are sensitive to a range of organic compounds and has been used to assess food quality, identify human disease, and detect microorganisms in food products.[7,9,11,16,24,27,35] However, E-nose is highly susceptible to background or interference VOCs and has poor chemical selectivity, device-to-device repeatability, and stability.[24,27]

Here, we report testing a fully automated portable GC device that can rapidly separate complex mixtures of plant VOCs with subppb sensitivity.[36−38] The device weighs only about 4.5 kg, including a preconcentrator, separation columns, heater, valves,

Received: April 21, 2019
Revised: June 8, 2019
Accepted: June 11, 2019
Published: June 11, 2019

DOI: 10.1021/acs.jafc.9b02500
J. Agric. Food Chem. 2019, 67, 7530−7537

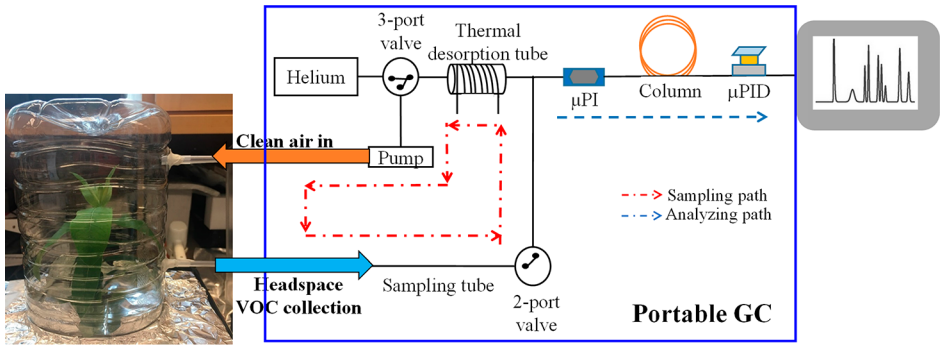

Figure 1. Illustration of the collection and analysis system for studying plant VOC emission. A push−pull headspace collection chamber was used to collect plant VOCs. The top tube was connected to the output of the pump that provided clean air to the chamber. Aluminum foil-covered blades closed the base of the chamber around the plant stem, allowing trapping of VOCs from the upper part of the plant. The bottom tube was connected to the portable GC device via a sampling tube and moisture filter. A picture of the entire setup is given in Figure S1, Supporting Information. μPI, micropreconcentrator; μPID, microphotoionization detector.

detector, pump, helium cartridge, and a user interface. Sample collection and analysis can be conducted autonomously with predetermined parameters (such as vapor collection duration and interval). It has low power consumption and can be operated with batteries (the operation time is estimated to be >10 h). The aim of the current work was to adapt the above portable GC device for rapid (every 40 min), in situ, dynamic monitoring of the VOCs produced by plants (milkweeds) under insect (aphids) attack. Through machine learning, principal component analysis (PCA), and linear discriminant analysis (LDA), we demonstrate that our portable GC device can differentiate between healthy and infested plants with an overall accuracy of 90−100%. Importantly, we could distinguish between the VOCs of infested and aphid-free milkweeds within 48−72 h of attack, comparable to the fastest effects reported previously,[39−41] and 3−4 days earlier than VOC changes detected in some other studies.[42,43] Such rapid in situ detection of insect attack attests to the great potential of VOC monitoring in plant health management and the advantages of using a portable vapor analyzer.

2. MATERIALS AND METHODS

2.1. Milkweed Plants. We chose to study the VOC emissions of common milkweed, *Asclepias syriaca*. Milkweed VOC blends have been shown previously to change in response to damage by caterpillars[44,45] and aphids.[46] Common milkweed is a widespread native plant in eastern North America, with a range that extends north to Canada and south to Louisiana.[45] The milkweeds used in our experiments were grown from seed collected from the University of Michigan Biological Station, near Pellston, Michigan, USA. Seeds were stratified for 6 weeks at 4 °C and planted in potting soil (Metromix 360, Sun Gro Horticulture Canada CM Ltd., Vancouver, BC, Canada) in conical depots (D40H, Stuewe and Sons Inc., Corvallis, OR, USA). Plants were grown in an environmental chamber under 16:8 light:dark at 24 ± 1 °C. After emergence, plants were fertilized weekly with 30 mL of 15−15−15 N−P−K fertilizer (JR Peters Inc., Allentown, PA) and watered every second day. Plants were used in experiments when they were 12 weeks old with 8−10 fully developed leaves.

2.2. Herbivore Insects. We used oleander aphids (*Aphis nerii*) to induce changes in milkweed VOC emissions. *A. nerii* reproduces parthenogenetically, and all aphids were derived from a single individual female collected from milkweed near the University of Michigan Biological Station and reared in the laboratory on *A. syriaca*. On the basis of previous work,[46−48] we used 20 reproductive aphids to induce changes in VOCs on experimental plants (details below).

2.3. Experimental Setup. To analyze the VOCs from aphid-infested or control plants, one whole plant was enclosed in a plastic transparent chamber (20 cm (L) × 20 cm (W) × 30 cm (H)), as shown in Figure 1. A push−pull headspace collection assembly was used for collecting VOCs from above-ground tissues of the plant. The collection assembly had an inlet (top) and an outlet (bottom). The air inlet port was connected to a pump via 30 cm long polytetrafluoroethylene (PTFE) tubing. To ensure a purified air flow into the chamber, the air was passed through a thermal desorption tube containing Carbopack X and B. For VOC collection, the bottom output port was connected to the thermal desorption tube via a 30 cm long PTFE tubing and a moisture filter (Nafion drying tube from BIOPAC Systems, Inc., Part no.: RX-AFT20-NAFION). The Nafion drying tube is essentially Teflon with sulfonic acid groups interspersed within it. The sulfonic acid has a very high affinity for water, thus retaining water in the Nafion tube. Because the Nafion tube uses sulfur ions to selectively remove water molecules, it is not gas permeable. Therefore, there is no introduction of contaminants or loss of target compounds (except ammonia and amines, which we do not detect in this work). As such, the Nafion tube can be used successfully to dry virtually any gas.[49] During operation, purified air entered the chamber at a controlled rate of 70 mL/min. Meanwhile, the VOCs were collected and loaded onto the thermal desorption tube at the same flow rate (70 mL/min). Since both the input and the output port were located symmetrically near the top and bottom of the chamber, a continuous flow of pure air and VOC sample could be maintained during sampling. The soil was isolated from the upper leaves and stem using two removable plates coated with aluminum foil. The blades were fitted together, leaving only a small opening for the stem. Glass wool was used to cover the soil in the pot. The above arrangements allowed for the collection of VOCs from the upper portion of the milkweed plants while isolating the lower section of the plant. During VOC collection, aphids were allowed to remain on plants, as this represents the most ecologically relevant plant conditions under which rapid detection of infestation would be of value.

2.4. Portable GC Operation. The detailed description and specification of our portable GC device can be found in refs 36−38. Briefly, as illustrated in Figure 1 and Figure S1, the portable GC consisted of an in-house made thermal desorption tube (5 cm long copper tube; inside diameter (i.d.): 1 mm) loaded with both Carbopack X and B granules (10 mg each, 60−80 mesh), a micropreconcentrator (μPI) loaded with Carbopack X and B (60−80 mesh), a 10 m long nonpolar DB-1 ms column (250 μm × 0.25 μm, Agilent J&W Scientific), and a microphotoionization detector (μPID). During 15 min of VOC sampling, the VOCs were trapped by the thermal desorption tube. Then the tube was heated to 300 °C in 1 min and maintained at 300 °C for 4 min to transfer the trapped VOCs to the μPI. Finally, the μPI was heated to 250 °C in 0.3 s and maintained at 250 °C for 5 s to inject the VOC pulse into the separation column. The column temperature started from and stayed at 25 °C for 2 min and was then heated to 80 °C with a ramping rate of 10 °C/min. Then the temperature was raised to 120 °C with a ramping rate of

DOI: 10.1021/acs.jafc.9b02500
J. Agric. Food Chem. 2019, 67, 7530−7537

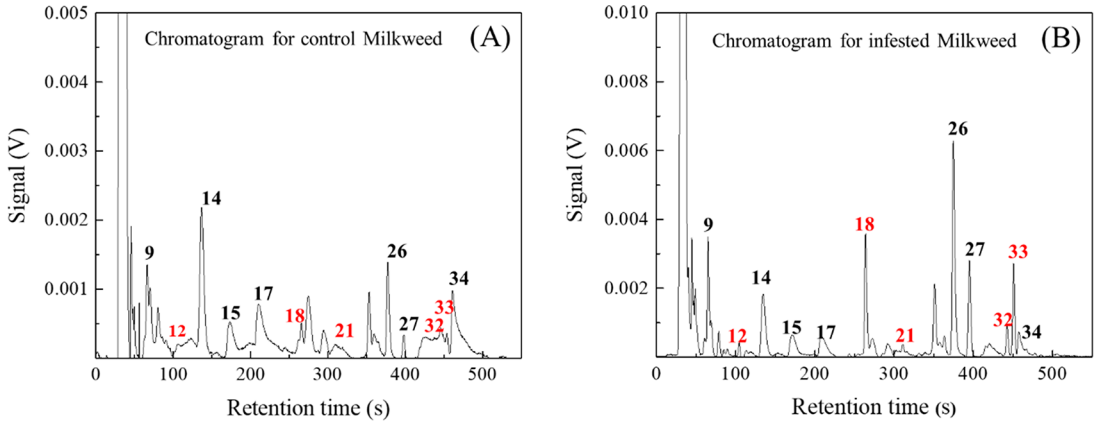

Figure 2. Representative GC chromatogram for a control milkweed (A) and an aphid-infested milkweed (B). A total of 35 peaks were separated out in about 8 min. Note that not all 35 peaks appear in a GC chromatogram of a particular milkweed. Also note that some of the 35 peaks may contain coeluted VOCs. The peaks with numbers are identified by their retention times using a reference library constructed with authentic standards (see VOC list in Table 1). For the purpose of illustration, five peaks (12, 18, 21, 32, and 33) are highlighted in the chromatograms to show the difference between the control and infested milkweed.

40 °C/min and held at 120 °C for 1 min. Overall, the total assay time was 40 min, including 15 min of sample collection time, 5 min of desorption/transfer time, 10 min of separation time, and 10 min of cleaning time. The flow rate of the carrier gas (helium) was 2 mL/min. The entire system was controlled by a homemade LabView program for automation. The material detail, device assembly, and operation of the GC system are elaborated in detail in the Supporting Information, section S1.

2.5. Experimental Protocols. A total of 10 milkweed plants were selected, five of which were aphid-free controls and five of which were infested with *A. nerii*. The 10 milkweeds were divided into three temporal groups (below) and each group was sampled repeatedly over 5 days, with groups of milkweeds sampled during 3 consecutive weeks.

The first group was tested in the first week. It consisted of one healthy control milkweed and one infested with 20 aphids. Aphids were transferred onto experimental plants on day 0, 24 h prior to the first VOC analysis. Experimental and control plants were then sampled daily over 5 consecutive days (day 1 to day 5). Each day, the VOC sampling and analysis were conducted during two time periods that is, 10 a.m. to 12 p.m. and 3 p.m. to 5 p.m., which fall within the 10 a.m. to 5 p.m. time window during which VOC emissions are typically high.[41,50] The control and infested plants were analyzed alternately between these two time periods during the 5-day monitoring: that is, on the first day, the control plant was tested during the first period and the infested plant during the second period, and on the following day, the infested plant was tested during the first window. Within each window, the VOCs from each individual plant were collected and analyzed twice to validate repeatability. Although either one of the two measurements proved acceptable for data analysis, we always used the data from the second run to maintain consistency among trials. On each occasion, volatiles were sampled for 15 min at a sampling rate of 70 mL/min and analyzed using the portable GC device. Chambers and bottom plates were rinsed with hexane between samples, to ensure that there were no residual VOCs. The above processes continued each day for 5 consecutive days and a total of 20 GC chromatograms were obtained including the duplicate measurements, from which 10 GC chromatograms were used for each individual plant for data analysis (i.e., we used the chromatograms obtained from the second run for each set of duplicate measurements). Once the first group of experiments was over, all aphids were removed from the milkweed plants using a paintbrush, and the leaves were dried and weighed to provide a biomass estimate for each plant.

The second and third groups were tested as above during the second and third week, respectively. Each of those groups consisted of two aphid-free control milkweeds and two aphid-infested milkweeds.

Table 1. List of VOCs Detected from Control and Aphid-Infested Milkweeds during VOC Analysis Using a Portable GC[a]

peak no.	compound (VOCs)
1	unknown
2	unknown
3	unknown
4	unknown
5	unknown
6	unknown
7	unknown
8	unknown
9*	benzyl alcohol*
10	unknown
11	unknown
12*	6-methyl-5-hepten-2-one*
13	unknown
14*	methyl ester benzoic acid*
15*	cis-ocimene*
16	unknown
17*	3-carene*
18*	(*E*)-4,8-dimethyl-1,3,7-nonatriene*
19	unknown
20	unknown
21*	3-hexenyl-acetate*
22	unknown
23	unknown
24	unknown
25	unknown
26*	hexyl acetate*
27*	phenyl methyl ester acetic acid*
28	unknown
29	unknown
30	unknown
31	unknown
32*	methyl salicylate*
33*	linalool*
34*	methyl 2-methoxybenzoate*
35	unknown

[a]"*" symbols denote the VOCs identified by their retention times using a reference library constructed with authentic standards.

DOI: 10.1021/acs.jafc.9b02500
J. Agric. Food Chem. 2019, 67, 7530−7537

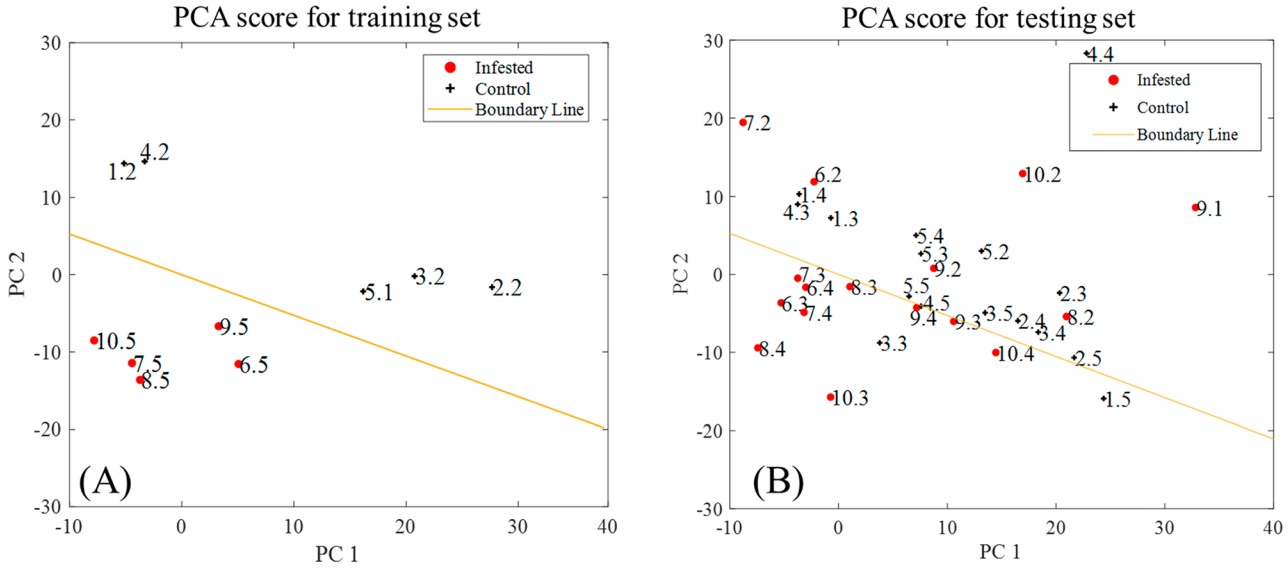

Figure 3. PCA plot for (A) all training set (10 data points) and (B) all testing set (32 data points). The region below and above the boundary line represents respectively the aphid-infested and control plants using the VOC analysis method. The plant ID and the test day are labeled as "plant ID.test day". For example, "1.2" refers to plant no. 1, day 2 test result and "7.5" refers to plant no. 7, day 5 test results. The corresponding statistics are given in Table 2.

Because the VOCs of infested and control plants were indistinguishable on day 1 (based on group 1 samples; see below for detailed description), we collected VOCs from day 2 to day 5 in groups 2 and 3. Altogether, for data analysis we used a total of 42 GC chromatograms obtained from 10 plants, that is, 10 for the first week, 16 for the second week, and 16 for the third week.

3. RESULTS AND DISCUSSION

3.1. Chromatograms for Aphid-Infested and Aphid-Free Milkweed Plants. Representative GC chromatograms for an aphid-infested milkweed plant and a control milkweed plant are shown in Figure 2. A total of 35 peaks were separated out in about 8 min, as shown in Figure S5. Note that not all 35 peaks appear in a GC chromatogram of a particular milkweed and some of the peaks may contain coeluted VOCs. Figure 2 illustrates that VOCs of aphid-infested milkweed plant are different from those of controls, allowing us to differentiate the two groups using a noninvasive VOC measurement technique. Since no mass spectrometry was used in our portable GC measurement, we tentatively identified some of the plant-related VOCs in Figure 2 by their retention times, using a reference library constructed with authentic VOC standards. The standard VOCs used to generate the library were selected on the basis of the VOCs identified previously by the Hunter group using GC-MS to be emitted by milkweed plants under attack by insects.[44−46] The list of identified VOCs is included in Table 1. We found that VOCs such as (*E*)-4,8-dimethyl-1,3,7-nonatriene (peak 18 in Figure 2), 3-hexenyl-acetate (peak 21 in Figure 2), hexyl acetate (peak 26 in Figure 2), methyl salicylate (peak 32 in Figure 2), and linalool (peak 33 in Figure 2) were emitted in significantly higher amounts from aphid-infested plants than from control plants.

3.2. Plant VOCs Data Classification Using PCA and LDA. Principal component analysis (PCA) and linear discriminant analysis (LDA) are commonly used techniques for data classification.[51] Here, we applied PCA-LDA to the 42 chromatograms obtained with the portable GC to distinguish between control and aphid-infested milkweeds by their VOC emission. As described previously, we detected a total of 35 peaks in the GC chromatograms. However, we need to determine which peaks are useful indices of herbivore damage. Some may be from indoor air or reflect constitutive levels of milkweed VOC production unrelated to herbivore activity. It is therefore critical to determine which subset of the 35 peaks discriminates between infested and aphid-free milkweed plants. To select the optimal subset of peaks, 10 data sets (5 control and 5 infested) were used as the training set. For the 5 infested milkweeds, we used the chromatograms obtained 5 days after adding aphids (i.e., chromatograms on day 5) to ensure that the changes in VOCs are maximal among all days and that the distinction between the control and the infested plant is maximal. The remaining 32 data sets (16 control and 16 infested) were used as the testing set. PCA-LDA was developed to first select the optimal subset of peaks using the training set. The details of the algorithm are given in the Supporting Information, section S2. With this algorithm, we selected an 8-peak subset (peak no. (9, 14, 21, 32, 18, 26, 33, 34)) in the chromatogram as the final optimal peak subset, which yields the best classification accuracy (100%) and the maximum boundary distance on the PCA plot for the training data set (see Figure 3A and the corresponding statistics in Table 2).

Table 2. Statistics of Training and Testing Sets

	training set			testing set		
	infested	control	row total	infested	control	row total
positive	5	0	5	10	2	12
negative	0	5	5	6	14	20
column total	5	5	10	16	16	32
specificity	1−0/5 = 100%			1−2/16 = 87.5%		
sensitivity	1−0/5 = 100%			1−6/16 = 62.5%		
positive predictive value	5/5 = 100%			10/12 = 83.3%		
negative predictive value	5/5 = 100%			14/20 = 70%		
total accuracy	10/10 = 100%			24/32 = 75%		

Then with the PCA coefficients acquired from the training set, the PCA scores for the testing set (a total of 32 chromatograms)

DOI: 10.1021/acs.jafc.9b02500
J. Agric. Food Chem. 2019, 67, 7530−7537

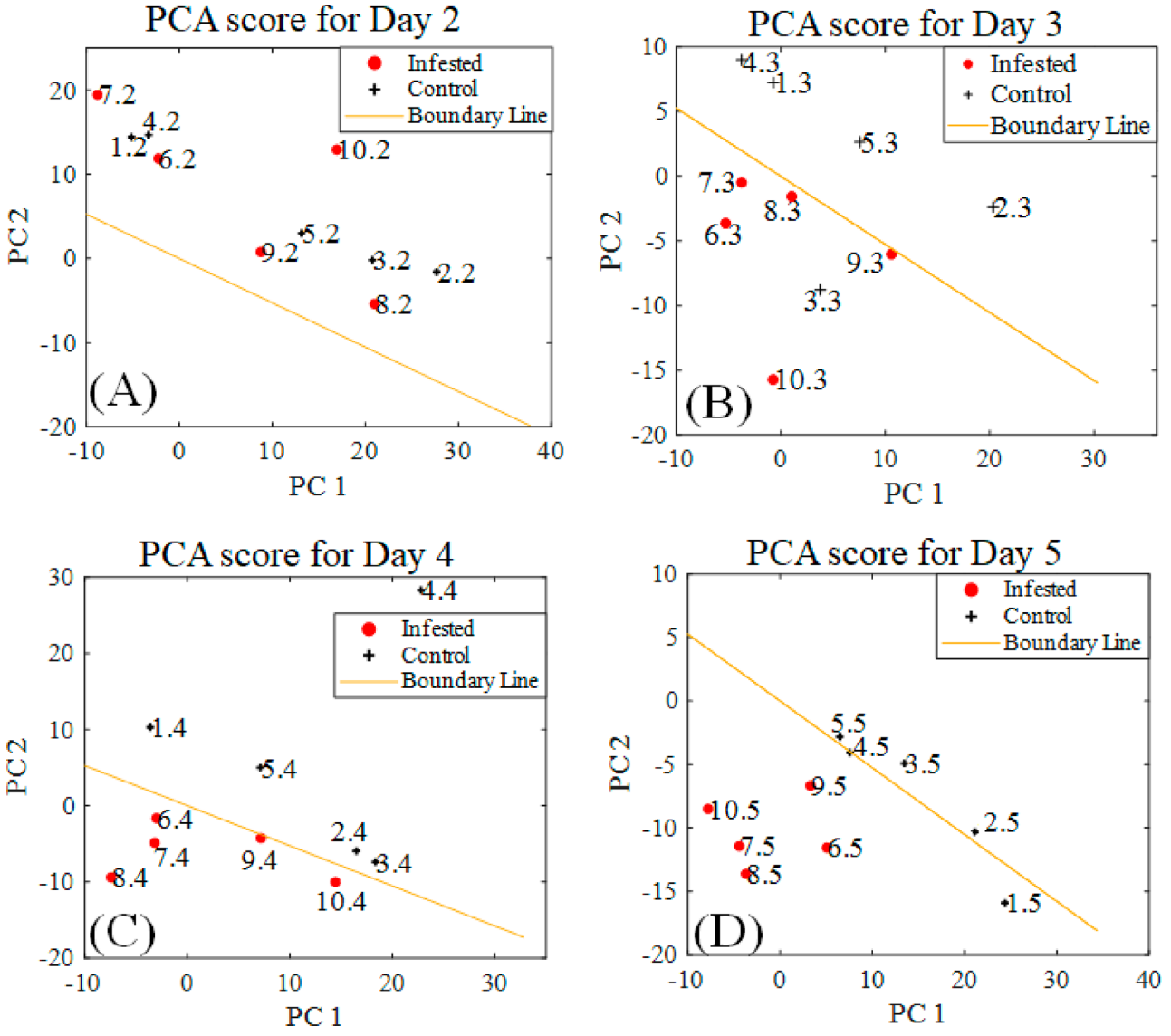

Figure 4. PCA plot for all 10 plants (a total of 42 chromatograms) measured on different days. PCA score for all 10 plants on (A) day 2, (B) day 3, (C) day 4, and (D) day 5. Note that for all measurements on different days, the same set of 10 plants were used. The region below and above the boundary line represents respectively the aphid-infested and control plants using the VOC analysis method. It suggests that the change in VOCs resulting from aphid attack becomes clearly distinguishable from day 3 onward. All data points shown in the figure above belong to the testing set, except 10 data points (1.2, 2.2, 3.2, 4.2, 5.1, 6.5, 7.5, 8.5, 9.5, and 10.5) that belong to the training set (see Figure 3A). Note that the boundary line is generated by the algorithm itself during the data analysis. It appears different because of the different *x*- and *y*-axis scales used in the subfigures.

can be calculated (see Figure 3B and the corresponding statistics in Table 2). As compared to the training set, the accuracy for the testing set is significantly lower. This is because it takes some time for plants to respond to aphid damage[46−48] and early changes in VOC emission may be too subtle to be detected by our GC device. We can therefore explore whether the quality of discrimination improves over time since aphid infestation began. To accomplish this, we plotted the PCA scores for the entire 42 chromatograms (containing both training and testing data sets) separately for each testing day, as shown in Figure 4. Changes in VOC emissions due to aphid attack are not very prominent until day 3. From then onward, VOC emissions from aphid-infested milkweeds are distinguishable from the controls (Figure 4), which is confirmed by significantly improved accuracy (90−100%) (Table 3). Note that "3.3" and "1.5" in Figure 4 are on the wrong side of the boundary line, which could reflect some remaining lack of accuracy in the system.

One of the advantages of using a portable GC is the ability to monitor in situ the VOC trajectories of individual plants (Figure 5). This allows us to calculate and illustrate the dynamic changes in plant phenotype after infection or damage, to facilitate early remediation and timely damage/treatment assessment. For example, using the portable GC, we found that the milkweeds started to respond definitively 48−72 h after aphid attack, which is in agreement with some other work[39−41] and 3−4 days earlier than reported in some other previous studies,[42,43] attesting to the advantage of rapid in situ VOC detection in plant health monitoring. In addition, using a portable GC provides a rapid assay time (a total of 40 min) and sampling time (15 min) compared to conventional GC-MS, as reported in other research work,[35,41,43,45] where sampling time itself ranges from 1 to 96 h.

We note that our present portable GC is only able to separate VOCs with a maximal molecular weight of approximately

DOI: 10.1021/acs.jafc.9b02500
J. Agric. Food Chem. 2019, 67, 7530−7537

Table 3. Statistics of All 42 Chromatograms, Separated by Collection Day, Containing Both Training and Testing Data Sets[a]

	(A) day 2			(B) day 3		
	infested	control	row total	infested	control	row total
positive	0	0	0	5	1	6
negative	5	5	10	0	4	4
column total	5	5	10	5	5	10
specificity	1−0/5 = 100%			1−1/5 = 80%		
sensitivity	1−5/5 = 0%			1−0/5 = 100%		
positive predictive value	0/0 = 0%			5/6 = 83.3%		
negative predictive value	5/10 = 50%			4/4 = 100%		
total accuracy	5/10 = 50%			9/10 = 90%		
	(C) day 4			(D) day 5		
	infested	control	row total	infested	control	row total
positive	5	0	5	5	1	6
negative	0	5	5	0	4	4
column total	5	5	10	5	5	10
specificity	1−0/5 = 100%			1−1/5 = 80%		
sensitivity	1−0/5 = 100%			1−0/5 = 100%		
positive predictive value	5/5 = 100%			5/6 = 83.3%		
negative predictive value	5/5 = 100%			4/4 = 100%		
total accuracy	10/10 = 100%			9/10 = 90%		

[a]All aphid-infested and control (total 10 no.) plants for (a) day 2, (b) day 3, (c) day 4, and (d) day 5.

160 g/mol. Probable reasons include (a) selection of Carbopack X and B, (b) maximum working temperature of μPI (250 °C), (c) short column length, and (d) low maximum column operating temperature (120 °C). While these constraints did not prevent us from discriminating between infested and aphid-free plants, it would be possible to increase the separation and detection capability of the present portable GC to include additional VOCs. Specifically, further improvement in our portable would allow for the detection of sesquiterpenoids (molecular weight: ~204 g/mol) group, which plays a critical role in plant VOCs.[19,40,41] In addition, the present one-dimensional portable GC could be upgraded to a two-dimensional portable GC for better separation capability, as some of the peaks might be coeluted in the present chromatograms.[37] Future research will include investigation of the effects of variations in plant species and of different herbivores on VOC emissions and rigorous studies of dynamic changes in plant VOC emissions within and beyond the duration of herbivore attack. Finally, we will use the portable GC system on plants growing under field conditions to assess our ability to differentiate between infested and uninfested plants under typical management conditions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.9b02500.

Portable GC description and operation; GC chromatogram construction; description of algorithm (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors acknowledge the University of Michigan Biological Station at Pellston, Michigan. They are also thankful for

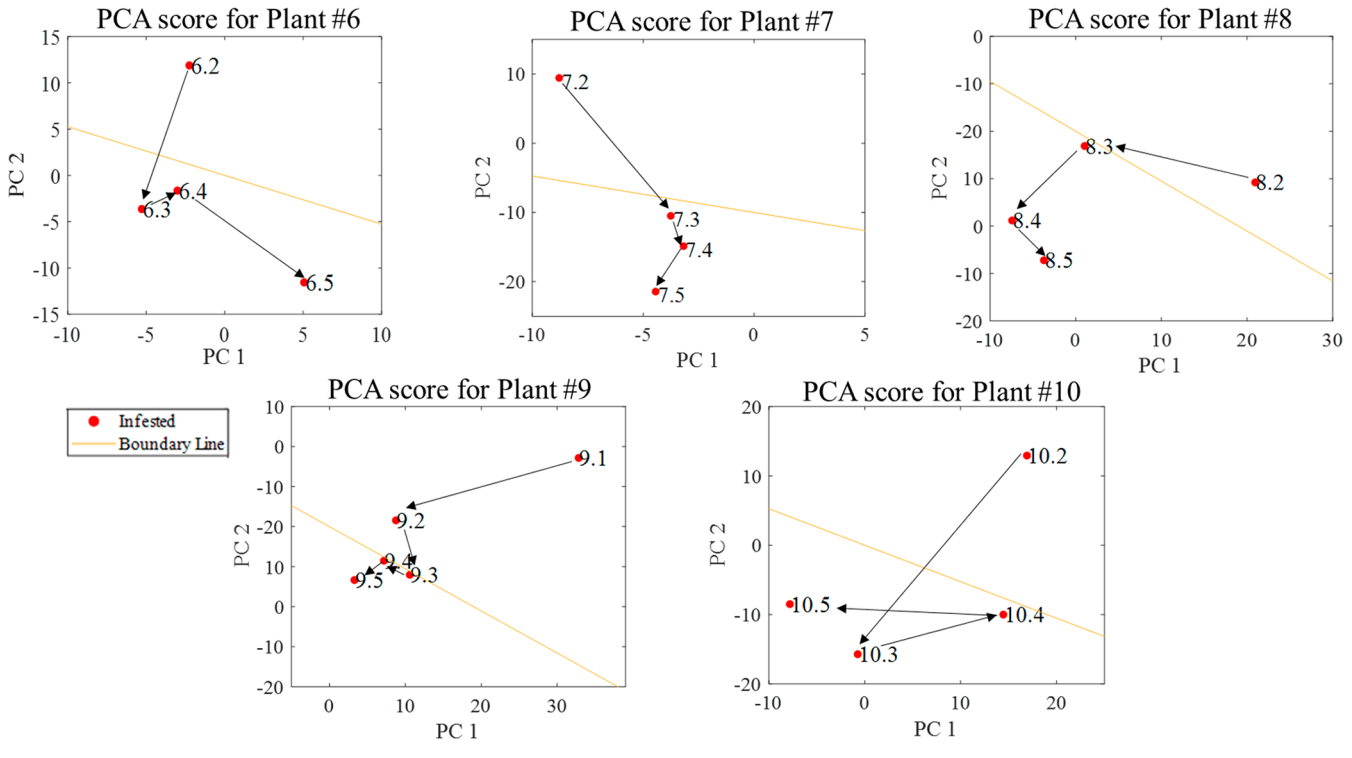

Figure 5. PCA plots showing daily VOC trajectories of individual aphid-infested milkweeds from day 1 or 2 to day 5.

DOI: 10.1021/acs.jafc.9b02500
J. Agric. Food Chem. 2019, 67, 7530−7537

the support from Flux HPC Cluster provided by the University of Michigan Office of Research and Advanced Research Computing – Technology Services (ARC-TS).

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