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Variation in volatile profiles of *Arabidopsis thaliana* Col-0 Heynh plants due to herbivorous infestation: piercing-sucking versus chewing insects

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***Abstract*:** *There has been a long-standing hypothesis that plant volatiles induced by herbivorous insects vary with different feeding habits. For Arabidopsis-insect interactions, it has been hypothesized that chewers cause the greater induction of plant defense mechanisms compared to phloem-sap feeders in terms of volatile organic compound (VOC) emission. In the present study, we obtained more knowledge on this by comparing the profile of VOCs emitted by Arabidopsis thaliana exposed to piercing-sucking insect (green peach aphid Myzus persicae) and chewing insect (diamondback moth Plutella xylostella). Untreated plants were used as controls. By using headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography–mass spectrometry (GC-MS), the results of Arabidopsis VOC analyses showed that P. xylostella larvae-infested plants emitted sulfide (dimethyl disulfide (DMDS)) and ester (2-Methylcyclopentyl acetate) instead of the 4-methylpentyl isothiocyanate as it is the case with M. persicae. While the proportions of ketones, and aldehydes were higher in plants infested by larvae than by aphids, the alcohol and terpene proportions were higher in aphid-infested plants. The study expand our understanding of plant volatile productions in response to different herbivore feeding behaviors. It can be expected that the variation in volatile profile of Arabidopsis plants in this study can be explained more deeply about the expression of gene/protein related to such chemical cues in further studies.*

Keywords: *Arabidopsis thaliana, Myzus persicae, Plutella xylostella, herbivorous infestation, volatile organic compounds*

1. Introduction

Being sessile forms, plants have evolved to live in environments where they are often exposed to multiple simultaneous factors. Such factors can be abiotic, biotic or a combination of both [1-6]. Therefore, plants must have specific mechanisms that allow them to defend against stressors [3, 6, 7]. Evidence shows that plants activate these protective mechanisms, resulting in the production and/or translocation of metabolites (e.g., nonvolatile compounds, volatile organic compounds (VOCs)), which may act directly and/or indirectly on stressors [2, 8, 9]. Concerning the influence of insect on plant VOC emission, it is well documented that plant volatiles induced by herbivorous insects vary with different feeding behaviors.

Based on the feeding guild on plants, the insect community is separated into two groups: (i) phloem-feeding insects, and (ii) chewing insects [10, 11]. It is well established that chewing insects (e.g., caterpillars and beetles) cause extensive physical damage to plants in comparison to phloem-feeding herbivores (e.g., aphids and leafhoppers) [8, 10-12]. Furthermore, data indicate that plants respond to phloem-feeding and chewing herbivores with differently altered chemical cues and proteomic levels [8, 12-16]. Amongst the insect species that have been described, green peach aphid (GPA, *Myzus persicae* Sulzer) and diamondback moth (DBM,

Plutella xylostella L.) are two highly invasive pests of many crops, especially *Brassica* plants. They have adapted to a wide range of insecticides, and their growth rate has rapidly increased [12, 17-19]. Moreover, some GPAs are the vector of viral diseases. Therefore, GPA and DBM are considered important insects to manage in agriculture [18, 19]. Existing studies illustrate that the plant defense against these herbivorous stresses are complicated mechanical responses, which are associated with the specific and coordinated regulation of metabolites, genes, and proteins [8, 12, 16]. Vogel, Kroymann [20] for instance found that the responses of *Boechera divaricarpa* (A. Nelson) to *P. xylostella* are determined by direct effects associated with the ethylene (ET) and JA pathways. Lu, Li [21] proved that *AtMYB44* gene take an important role in *Arabidopsis* response to *M. persicae* and *P. xylostella*. The *Arabidopsis* defense-related pathway involving aliphatic glucosinolate by-products was down-regulated by GPA, but up-regulated by DBM [10].

In general, changes to plant defense responses according to the type and damage degree of given herbivorous insects are well-documented; substantial knowledge on pest density and infestation time has also been provided [12, 22]. With aiming to justify changes in volatile profiles of plants due to type of herbivorous insect infestation, the present work was conducted to assess the alteration in volatile emission of *Arabidopsis thaliana* Col-0 Heynh under piercing-sucking, *M. persicae*, and chewing insects, *P. xylostella* over three time periods (0-24, 24-48, and 48-72 h). The results are expected to expand our knowledge of plant volatile productions in response to different herbivore feeding habits.

2. Materials and Methods

3. Plants and Insects

All of the experimental treatments were carried out with five-week-old *A. thaliana* (L.) Heynh (ecotype Columbia (Col-0), Lehle Company, Texas, USA). The plant seeds were sown in plastic pots (0.20 l) with potting soil, and cultivated in a growth chamber at 22 ± 0.6 °C, 16L: 8D (LED lighting: 43 µmol m^{-2} s^{-1} photosynthetically active radiation during the light period), and 64.5 ± 2.6 % relative humidity (RH). Plants were watered twice a week (tap water, 10–20 ml/pot) for 5 weeks, before starting the experimental procedures.

The piercing-sucking insects, green peach aphid *M. persicae* Sulzer, were reared on broad bean plants (*Vicia faba* L.), under controlled environmental conditions in a room at 20 ± 2 °C with a 16L: 8D photoperiod.

Diamondback moth, *P. xylostella* (L.), larvae (2^{nd}–3^{rd} instar) were used as chewing insects in this study. Butterflies were kept in insect cages in a chamber at 22 ± 2 °C, 16L:8D light regime and 50–70% RH. To maintain the *P. xylostella* population, pupae were collected, transferred to a gauze cage, and provided with 15% sugar solution as food source in a climate room. Eggs laid by the butterflies obtained from field were grown on cabbage plants (*Brassica oleracea* L.) until hatching into larvae.

3.1. Herbivorous Insect-Infested *Arabidopsis* Plants

Plants were infested by exposure to adult aphids and diamondback moth larvae (2^{nd}–3^{rd} instar) under controlled conditions. 70 adults and one *P. xylostella* larvae were released onto randomly selected *Arabidopsis*, and the VOC analyses were carried out with plants maintained at at 22 °C over three time periods (0–24, 24–48, and 48–72 h). Plants without insects were as controls. Each experiments were replicated three times.

3.2. Plant Volatile Extraction And Analysis

HS-SPME using the PDMS/DVB (65 µm) fiber (Supelo, Bellefonte, PA, USA) was made to collect VOCs from *Arabidopsis* plants under different experimental treatments during three time courses (0–24, 24–48, and 48–72 h). Collections started after an 18 h period treatment of each time course and lasted 6 h. The fibers were conditioned at 225 °C for 30 min to avoid contaminations prior to the onset of VOC collection.

At the end of the each stress treatment (24, 48 and 72 h), the fibers were desorbed in the splitless injector (220 °C) of a gas chromatograph (Trace GC Ultra) coupled to a quadrupole-type mass spectrometer Trace Finnigan (Thermo-Fisher Scientific; Waltham, MA, USA). The GC was equipped with an apolar column (30 m; 0.25 mm i.d.; 0.25 µm film thickness, Optima-5-MS, from Macherey-Nagel, Düren, Germany).

The SPME fibers were desorbed in the GC splitless injection port, held at 220 °C. Blank samples were regularly conducted to check possible carry over and peaks originating from the fibers. After each sampling, the fibers were immediately inserted into the GC injector for 5 min at 220 °C to prevent contaminations. After desorption, the oven temperature program was: from 40 to 220 °C (with 1-min final hold) at 4 °C min^{-1}, and then from 220 to 320 °C (10-min hold) at 100 °C min^{-1}. Helium was used as the carrier gas (constant flow rate of 1.5 ml min^{-1}).

The mass spectra were obtained with a mass selective detector operating in electron ionization mode at 70 eV with a multiplier voltage of 275 V and a scanned mass range from 39 to 400 amu at a rate of 1 scan s^{-1}. The transfer line and ion source temperatures were maintained at 230 °C and 250 °C, respectively. Detected VOCs were identified based on their retention data and by careful examination of their mass spectra in comparison with the Wiley and NIST MS 2.0 mass spectral databases. When available, the comparison between the retention time of the detected VOC peaks with those of the commercial compounds (provided by Sigma-Aldrich, Germany) analysed under the same experimental conditions was carried out. Further identification of VOCs was also conducted by comparing their calculated Retention index with the literature.

3.3. Statistical Analyses

The relative abundance of individual VOCs was expressed as the ratio between their peak area and the total area of all detected VOCs from *Arabidopsis* plants exposed to unifested and infested by insects. In a second step, the proportions of the VOC classes (terpenes, sulfides, ketones, alcohols, aldehydes, ITCs, and ester) were calculated.

Principal component analysis (PCA) was performed on a dataset containing the relative abundance of either the individual compounds or the VOC classes for each experimental treatment, and the PCs were calculated using a correlation matrix. Three-way ANOVA, and subsequent *post hoc* Tukey's tests, were used to compare the mean relative abundances of the individual VOCs, as well as the relative proportions of the VOC chemical families emitted by plants subjected to different treatments after two time intervals. For each subset, the $\log(x + 1)$ transformation of data was carried out when necessary, to meet assumptions of normality and homogeneity of variances. These tests were performed with Minitab® 16.2.2 software (State College, Pennsylvania, USA).

4. Results and Discussion

The results described herein were mainly obtained by using the PDMS/DVB (65 µm) fiber in the HS in order to extract the VOCs emitted from *Arabidopsis* plants in different stress treatments over three time periods (0-24, 24-48, and 48-72 h).

Twenty-two compounds divided into seven VOC groups were collected in the headspace of *A. thaliana* subjected to uninfested and infested by aphids *M. persicae* and *P. xylostella* larvae in laboratory conditions. When aphids and larvae were present a total of 14 components were detected, whereas there were only 10 compounds were observed in uninfested *Arabidopsis* plants.

Principal component analysis (PCA) resulted in a spatial visualization of volatile profiles from *Arabidopsis* infested with different insect behaviors (piercing-sucking and chewing) at 22 °C over 0-24, 24-48 and 48-72 h. In particular, the results showed that the first two components explain 77.1% of the observed variation, i.e. PC1 57.2% and PC2 19.9%, forming three main groups: (1) uninfested plants, (2) *M. persicae*-infested plants, and (3) *P. xylostella*-infested plants (Fig. 1).

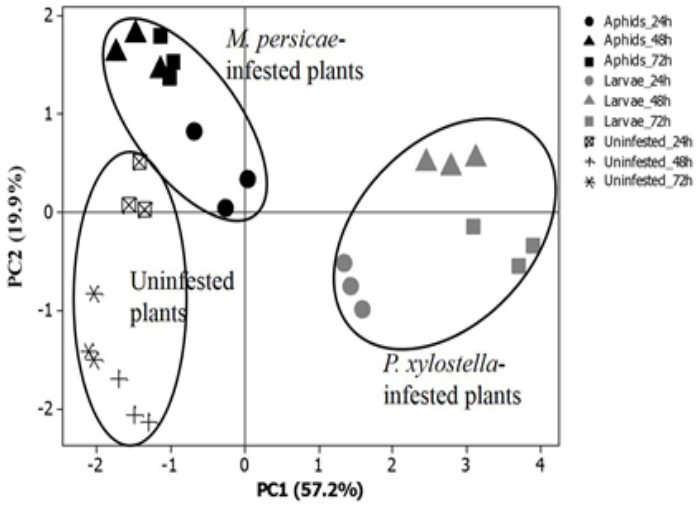

Fig. 1: Principal component analyses (PCAs) and hierarchical clustering analysis of VOC profiles emitted by *A. thaliana* exposed to uninfested or infested by either aphid *M. persicae* or *P. xylostella* larval over three time periods (0–24, 24-48, and 48-72 h). PCAs show the first (PC1) and second (PC2) principal components.

A three-way full crossed ANOVA model was carried out for both VOC classes to identify differences between *Arabidopsis* volatile profiles. The proportion of alcohols ($p < 0.001$) and terpenes ($p < 0.001$) significantly decreased when *Arabidopsis* plants were subjected to larval infestation compared to aphid-infested and uninfested plants over three time periods (Fig. 2). In contrast, ketone ($p < 0.001$) and aldehyde ($p < 0.001$) proportions exhibited a significant increase in plants exposed to larval infestation (Fig. 2). Interestingly, ester (2-Methylcyclopentyl acetate) and sulfide (dimethyl disulfide) were only detected in *P. xylostella*-infested *Arabidopsis* plants, whereas isothiocyanate (ITC, 4-methylpentyl ITC) were observed in *M. persicae*-infested plants.

These findings confirm that the emission of *Arabidopsis* volatiles is closely associated with herbivore feeding mode. Evidence indicates that the *M. persicae*-infested *Arabidopsis* plants exhibited an increase in the content of aliphatic GS metabolites, whereas the *P. rapae*-infested plants established a rising in indolyl GS derivative content [23]. Bidart-Bouzat and Kliebenstein [10] proved that in the interactions between *A. thaliana* and insects, lepidopteran species upregulated different genes in JA-related pathways, leading to an increase in sulfate metabolism and aliphatic metabolites content, whereas these genes were all down-regulated by phloem-sap feeders. In addition, given that *P. xylostella* larvae do not seem to suffer from the GS aliphatic level, they can detoxify GSs by desulfating them through the activity of enzymatic sulfase and the expression of genes within their gut [8]. These evidences can explain why the emission of GS derivative volatiles (e.g., ITC, sulfide) occurred in our current study.

Overall, our findings indicate that *M. persicae* adults and *P. xylostella* larval feeding induce different volatile production in *Arabidopsis* plants under the same environmental conditions, and the variations occurred not only in GS hydrolysis volatiles but also ketones, aldehydes, esters, alcohols, and terpenes. It can be expected that the variation in volatile profile of *Arabidopsis* plants in this study can be explained more deeply about the expression of gene/protein related to such chemical cues in further studies. These studies can expand our understanding of plant volatile productions in response to different herbivore feeding behaviors.

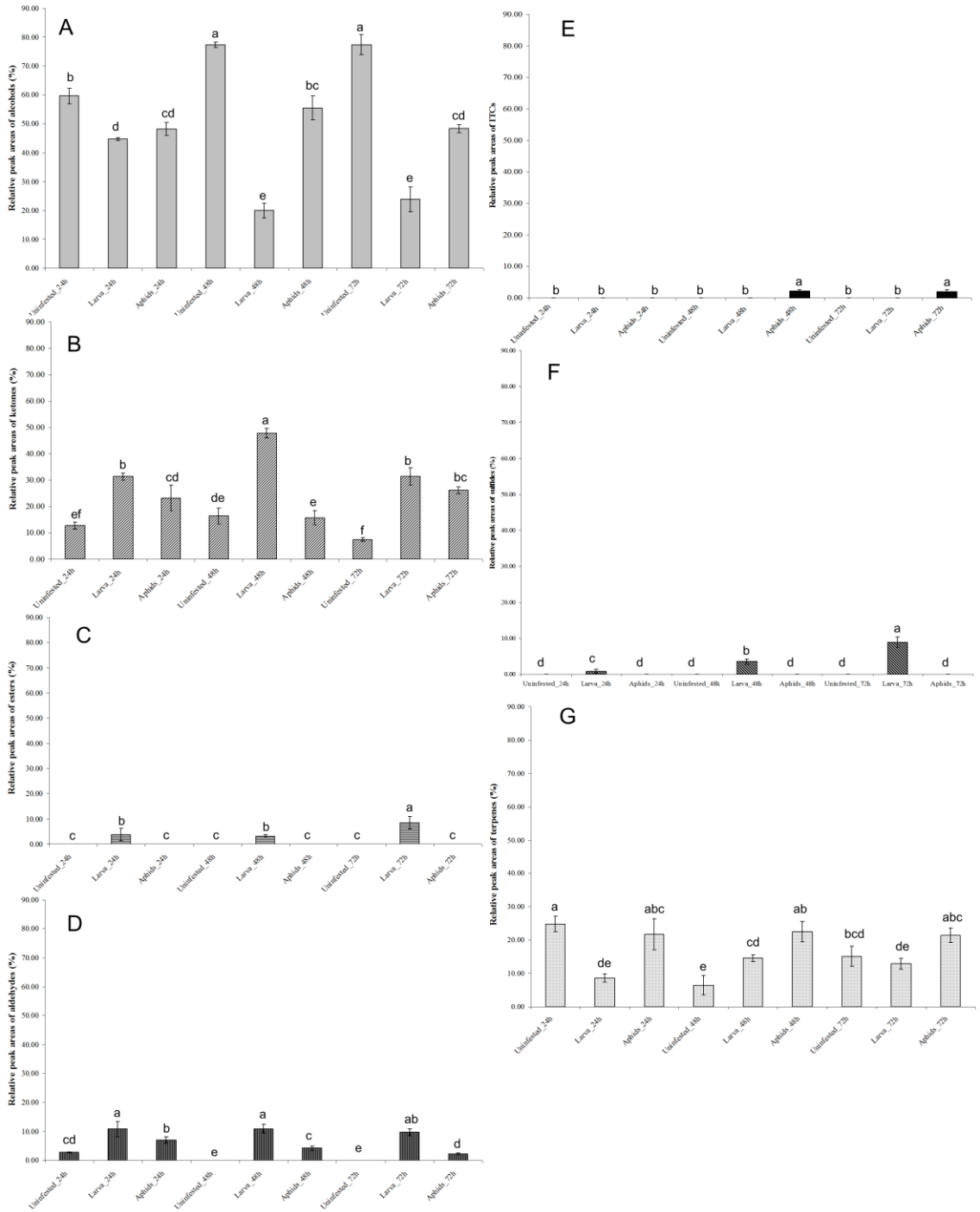

Fig. 2: VOC groups (A, alcohols; B, ketones; C, esters; D, aldehydes; E, isothiocyanates (ITCs); and F, terpenes) from *Arabidopsis* plants uninfested or infested by *M. persicae* (70 aphids per plant) or by *P. xylostella* larva (1 larva per plant) over three time periods. Means (n = 3; ± SD) followed by the different letter are significantly different ($p > 0.05$, two-way ANOVAs, *post hoc* Tukey's HSD test).

5. Conclusions

The comparison of adults *M. persicae* and *P. xylostella* larvae in *Arabidopsis* plants indicates that the profile of plant volatiles is closely associated with the type of attacking insects. Under the same experimental conditions, larval feeding on *Arabidopsis* plants led to sulfide and ester emission, whereas ITC production was induced by aphid sucking on plants. The variation was also found in the proportion of some other chemical cues (i.e., ketones, aldehydes, and terpenes).

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