Temperature regimes and aphid density interactions differentially influence VOC emissions in *Arabidopsis*

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Abstract

The effects of volatile emissions from plants exposed to individual abiotic and biotic stresses are well documented. However, the influence of multiple stresses on plant photosynthesis and defense responses, resulting in a variety of volatile profiles has received little attention. In this study, we investigated how temperature regimes in the presence and absence of the sucking insect *Myzus persicae* affected volatile organic compound emissions in *Arabidopsis* over three time periods (0-24 h, 24-48 h, and 48-72 h). Headspace solid-phase microextraction coupled with gas chromatography-mass spectrometry was used to evaluate *Arabidopsis* volatile organic compounds. The results showed that under laboratory conditions, eight volatile classes [alcohols (mainly 2-ethyl-hexan-1-ol), ketone (6-methyl hept-5-en-2-one), esters (mainly (*Z*)-3-hexenyl acetate), aldehydes (mainly phenylacetaldehyde), isothiocyanates (mainly 4-methylpentyl isothiocyanate), terpenes (mainly (*E*,*E*)-α-farnesene), nitrile (5-(methylthio) pentanenitrile), and sulfide (dimethyl trisulfide)] were observed on plants exposed to stress combinations, whereas emissions of six volatile classes were observed during temperature stress treatments alone (with the exception of nitriles and sulfides). Aphid density at high temperature combinations resulted in significantly higher isothiocyanate, ester, nitrile and sulfide proportions. The results of the present study provide an insight into the effects of temperature - aphid interactions on plant volatile emissions.

Key words: *Arabidopsis thaliana*. *Myzus persicae*. Temperature regimes. Stress combination. Volatile organic compounds (VOCs).

Introduction

Plants in diverse continental environments might experience the effects of a variety of biotic and abiotic constraints (Vickers et al. 2009). Evidence provides support for a long-standing hypothesis that emissions of volatile organic compounds (VOCs) from plants vary substantially depending on changes in environmental factors (Hatano et al. 2008; Holopainen and Gershenzon 2010; Loreto and Schnitzler 2010). In fact, a plant reaction to pathogen or insect attack often results in development of different defense mechanisms, both direct, e.g. phytoalexins, reactive oxygen species (ROS) and indirect, e.g. mediated by VOCs (Maffei 2010). Induced resistance involves defense strategies, and results in the production and/or translocation of secondary products within plants, which might act directly and/or indirectly on plant attackers (Van Poecke 2007; Unsicker et al. 2009; Winter and Rostás 2010).

Abiotic factors can affect primary metabolism by changing photochemical or biochemical reactions of the photochemical cycle, or secondary metabolism by affecting VOC emissions (Holopainen and Gershenzon 2010; Loreto and Schnitzler 2010; Krasensky and Jonak 2012). For example, increasing drought stress inhibited isoprene emissions and photosynthesis in black poplar (*Populus nigra* L.) (Fortunati et al. 2008; Loreto and Schnitzler 2010). Vickers et al. (2009) proposed the “single biochemical mechanism for multiple stressors” hypothesis to explain how plants respond to abiotic stress. Based on this hypothesis, volatile isoprenoids are emitted from plants to prevent cell damage caused by many environmental factors as follows: (1) direct isoprenoid reactions with oxidizing species; (2) indirect ROS signaling changes; and/or (3) membrane stabilization (Vickers et al. 2009). Confirming this hypothesis, temperature, light, fertilization rate, soil and air humidity, salt concentration, and high ozone levels were found to change the quantity of VOC emissions in plants (Gouinguené and Turlings 2002; Vuorinen et al. 2004; Pinto et al. 2010).

Feeding and oviposition by herbivorous insects are well-documented biotic factors involved in plant-biotic stress interactions (Dicke and Baldwin 2010; Holopainen and Gershenzon 2010; Kos et al. 2012). Herbivorous insect damage to vegetative organs has been reported to cause increased VOC emissions, particularly green leaf volatiles (GLVs) and terpenes (Mumm et al. 2003; Dudareva et al. 2006; Holopainen and Gershenzon 2010). Studies have demonstrated that plants respond to biotic stresses by emitting a specific blend of volatiles depending on the type of insect pests and experimental conditions (Mewis et al. 2006; De Vos et al. 2007; Snoeren et al. 2010; Huang et al. 2012). Plant-insect relationships are regulated by plant responsive mechanisms resulting from long-term co-evolutionary processes, and pest survival adaptations due to plant defensive mechanisms (Van Poecke 2007; De Vos and Jander 2009). The establishment of induced systemic resistance (ISR) or systemic acquired resistance (SAR)

involves reactions regulated by signaling pathways, jasmonate (JA) and ethylene (ET) for ISR, or salicylate (SA) for SAR, which are major signals in the release of volatile compounds against elicitors (Vallad and Goodman 2004). Some studies demonstrated that in aphid infestations, plants induced SAR as a pest avoidance mechanism via the SA signaling pathway (Vallad and Goodman 2004; Van Poecke 2007). In addition, aphid feeding investigations found changes in the induction of plant volatile emissions, resulting in reduced tissue damage at aphid feeding sites (Louis and Shah 2013; Verheggen et al. 2013). Under natural conditions, stresses rarely occur individually; plants may be subjected to a combination of different biotic and/or abiotic stress factors affecting plant VOC emissions (Holopainen and Gershenzon 2010; Winter et al. 2012). Much less is known regarding the emission of VOC blends during plant response to such multiple stress factors (Holopainen and Gershenzon 2010; Winter and Rostás 2010).

In the present study, we examined the combined stress effects of temperature and densities of the sucking aphid *Myzus persicae* (Sulzer) on *Arabidopsis thaliana* (L.) Heynh ecotype Columbia (Col-0), primarily with respect to VOC emissions. The objectives of this study were as follows: (i) investigate *Arabidopsis* VOC emissions induced by different *M. persicae* leaf densities; (ii) evaluate the effects of environmental temperature regimes on volatile emissions; and (iii) assess the effects of temperature stress and aphid density interactions on *Arabidopsis* VOC emissions over different time periods.

Materials and Methods

Plants and insects

Five-week old *Arabidopsis thaliana* (L.) Heynh (ecotype Columbia (Col-0), Lehle Company, Texas, USA) were used for all experiments. 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. Plants were watered twice a week (tap water, 10-20 ml/pot) for five weeks, before starting the experiments at different temperatures. The green peach aphid *Myzus persicae* (Sulzer) was reared on broad bean plants (*Vicia faba* L.), under controlled environmental conditions in a room at 20 ± 2 °C with a 16L: 8D photoperiod.

Experimental design and protocols

Arabidopsis plant treatments

Five-week old *Arabidopsis* plants, including root balls (9-10 leaves, 0.25-0.40 g/plant) were carefully removed from the plastic pots, individually wrapped in aluminum foil around the root balls to reduce the detection of soil volatiles. Three plants were carefully placed in the same reaction vessels (100 ml, Duran Group, Germany) and submitted to each stress treatment. Reaction vessels were carefully cleaned by methanol, milli-Q water and put in the oven at 180 °C during 24 h before the plants were put into them.

Temperature regimes

Temperature regimes were applied using a thermostat water bath (IKA®-Werke GmbH & Co. KG, Germany), and the reaction vessels containing three plants were immersed in water. The temperature treatments inside the collection reaction vessels were 17 °C, 22 °C, 27 °C, and 32 °C, and controlled using a HOBO Pro temp/RH/light/text channel data logger (Onset Computer Corp., Bourne, MA 02532, USA). Plants were kept 18 h at such temperatures before collecting VOCs. Light intensity was kept constant during all collections (600-800 Lux, HOBO Pro temp/RH/light/text channel data logger). Three replications were conducted for each treatment.

Myzus persicae infestation

Arabidopsis were either untreated, as a control, or infested with aphids (Online resource 1). Plants were infested by exposure to adult aphids under controlled conditions. In a preliminary trial (Exp. 1), 30, 70 and 100 adults were released onto randomly selected *Arabidopsis*, and the volatiles were collected from plants maintained at 22 °C during 48 h. The samplings were carried out from 42-48 h. In order to test temperature and aphid interaction (Exp. 2), 70 aphids were placed on plants at different temperatures (17 °C, 22 °C, 27 °C, and 32 °C) over three time periods (0-24 h, 24-48 h, and 48-72 h). Plants without aphids were subjected to the same temperature. Population growth rate of aphids was measured at the end of each test. Both experiments were replicated three times.

Plant volatile collection and analysis

To select the best SPME fibers suitable to extract VOCs from *Arabidopsis*, preliminary experiments were conducted on control plants with different stationary phases and various film thicknesses: carboxen-polydimethylsiloxane (CAR/PDMS, 75 μm), polydimethylsiloxane-divinylbenzene (PDMS/DVB, 65 μm) and divinylbenzene-carboxen-polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm) (Supelco, Bellefonte, PA, USA). The results (data not shown)

demonstrated that 65 µm PDMS/DVB fibers were most efficient for *A. thaliana* VOC sampling. Our results were consistent with Rohloff and Bones (2005) who profiled volatiles in *Arabidopsis*.

Volatile collection was conducted from *Arabidopsis* under stress treatments during three time courses (0-24 h, 24-48 h, and 48-72 h). Collections started after an 18 h period treatment of each time course and lasted 6 h. Prior to the onset of VOC collection, the fibers were conditioned at 225 °C for 30 min to avoid contaminations.

At the end of each treatment of the sample periods (24, 48 and 72 h), the fibers were desorbed in the injector of a gas chromatograph coupled to a mass spectrometer (SPME-GC/MS). GC/MS analyses were performed using a Trace GC Ultra (Thermo-Fisher Scientific; Waltham, MA, USA) gas chromatograph coupled to a quadrupole-type mass spectrometer Trace Finnigan (Thermo-Fisher Scientific; Waltham, MA, USA). The GC was equipped with an apolar column (Optima-5-MS, 30 m; 0.25 mm i.d.; 0.25 µm film thickness, Macherey-Nagel, Düren, Germany). The oven temperature program was from 40 °C to 220 °C (1-min hold) at 4 °C/min, then from 220 °C to 320 °C (10-min hold) at 100 °C/min. Carry over and peaks originating from the fibers were regularly assessed by running blank samples. After VOC collections, fibers were immediately thermally desorbed in the GC injector for 5 min at 220 °C to prevent contamination. The injections were performed in splitless mode using SPME liner (1 min). The carrier gas was helium at a constant flow rate of 1.5 ml/min. The mass spectra were obtained using a mass selective detector operating in electron impact mode at 70 eV, 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 to the mass spectrometer was maintained at 230 °C, and the ion source temperature was set at 250 °C. The volatile components were identified based on retention times, and by careful examination of their main spectra in comparison with the Wiley and NIST MS 2.0 computed spectral databases.

Statistical analyses

The relative abundances of each detected volatile were calculated as the ratio between their area and the total area of all detected VOCs from plants subjected to each treatment. In a second step, the proportions of the main chemical families [alcohols, terpenes, ketones, aldehydes, isothiocyanates (ITCs), esters, nitriles and sulfides] were calculated.

In order to reveal the patterns of variation and clustering among treatments, and identify the volatile chemicals responsible for variation in volatile profiles among *Arabidopsis* subjected to different treatments, a principal component analysis (PCA) followed by hierarchical clustering analysis on principal components (HCPC) was performed respectively using R 3.0.1 software (R-Development-Core-Team 2013) and FactoMineR 1.25 package (Husson et al. 2013). Multivariate analyses (PCA and HCPC) were preformed on a dataset containing the mean relative abundances of either the individual compounds or eight groups for each treatment. Principal components (PCs) were calculated using a correlation matrix. The optimal group number defined by hierarchical clustering on principal components was chosen

159 automatically by the statistical software, and was between 3 and 10 clusters. Equality of variances among clusters for
160 each chemical family was tested using a Bartlett test ($P < 0.05$). For each chemical family, the variation in emitted
161 proportions between clusters was analyzed by one-way ANOVA ($P < 0.05$) using Minitab® 16.2.2 software (State
162 College, Pennsylvania, USA). Data used for multivariate analyses were plotted with heatmap 2 functions in the gplots
163 2.11.3 package (Warnes et al. 2013) using R 3.0.1 software. Heatmap was constructed using standardized mean values.
164 In the final heatmap, stress factors were ordered according to a dendrogram calculated with the Ward algorithm (R-
165 Development-Core-Team 2013).

166 ANOVAs (one-way and three-way), and subsequent *post hoc* Tukey's test were also applied to analyze the
167 variation in volatile blends among treatments and compare mean relative abundance in VOC classes as well as the
168 specific compounds. For each subset, data were log (x + 1) transformed when necessary to meet assumptions of
169 normality and homogeneity of variances. These tests were conducted with Minitab® 16.2.2 software.
170
171 **Results**
172
173 Volatile emission changes in *Arabidopsis* following aphid density treatments
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175 PCA resulted in a spatial visualization of volatile profiles from *Arabidopsis* infested with different *M. persicae* densities
176 (0, 30, 70 and 100 adult aphids/plant) at 22 °C for 24-48 h. The first two principal components (PC1 and PC2)
177 explained 88.5% of the variance, i.e. PC1 69.9% and PC2 18.6% (Fig. 1A). Analysis of the score plots constructed with
178 PC1 and PC2 revealed the VOCs responsible for differences among experimental conditions. According to statistical
179 analyses, the correlations between variables on first two principal components (PCs) indicated that PC1 was positively
180 correlated with terpenes (0.98), ITCs (0.82), aldehydes (0.74), but negatively correlated with ketones (-0.64) and
181 alcohols (-0.96). PC2 was positively correlated with alcohols (0.24), ITCs (0.21) and terpenes (0.06), whereas
182 negatively correlated with aldehydes (-0.59) and ketones (-0.69).

183 A one-way ANOVA was conducted on VOC classes to identify significant differences between *Arabidopsis*
184 volatile blends (Fig. 1B). The proportion of terpene significantly increased, and was the highest with 100 adults per
185 plant ($F_{3,8} = 54.39$, $P < 0.001$). In contrast, total alcohol percentage significantly decreased according to aphid densities
186 in infested plants ($F_{3,8} = 25.73$, $P < 0.001$). Significant differences were observed for total aldehyde proportion between
187 control and infested plants, irrespective of aphid numbers ($F_{3,8} = 51.62$, $P < 0.001$). The percentage of 4-methylpentyl
188 isothiocyanate significantly increased with the number of aphids feeding on plant leaves ($F_{3,8} = 25.74$, $P < 0.001$). This
189 compound has not been detected in uninfested plants. However, based on the aphid densities no significant difference in
190 the emission of 6-methyl hept-5-en-2-one was observed ($F_{3,8} = 3.64$, $P = 0.064$) (Table 1).

With respect to the specific terpenes showing significant differences between treatments, the proportions of limonene ($F_{3,8}$ = 10.58, P = 0.004) and menthol ($F_{3,8}$ = 15.78, P = 0.001) generally increased in infested *Arabidopsis* plants. Moreover, α-terpineol ($F_{3,8}$ = 731.93, P < 0.001) and (*E*,*E*)-α-farnesene ($F_{3,8}$ = 745.26, P < 0.001) were only detected in highly infested *Arabidopsis* (100 aphids/plant) (Table 1).

With respect to the single alcohol compounds, decan-3-ol ($F_{3,8}$ = 158.69, P < 0.001) was only observed in highly infested plants, while the levels of two major molecules from this class: 4-methyl-1-penten-3-ol ($F_{3,8}$ = 64.86, P < 0.001) and 2-ethyl-hexan-1-ol ($F_{3,8}$ = 40.23, P < 0.001) generally showed a significant decrease in infested plants (Table 1).

Changes in *Arabidopsis* volatile profiles due to combined effects of temperature and aphid density

Twenty compounds divided into eight VOC classes were recorded in the headspace of *A. thaliana* exposed to different temperatures (from 17 °C to 32 °C). When aphids were present a total of 29 compounds were detected. Volatile analyses were performed for the three time periods (0-24 h, 24-48 h, and 48-72 h) (Fig. 2; Online resource 2). First, a PCA using the mean proportion of each individual compound was conducted. The first two principal components (PCs) showed 43.6% of the observed variation, i.e. PC1 29.9% and PC2 13.7% (Online resource 3). In addition, PCA and HCPC using the mean percentage of each VOC class were applied to reveal patterns of variation and clusters among treatments. The goal also was to identify the chemical families responsible for volatile profile variation among *Arabidopsis* under different stress treatments. PCA results showed that the first two components accounted for 65.0% of the total variance, with PC1 and PC2 explaining 47.6 and 17.4% of the total chemical composition variation, respectively (Fig. 2A). Correlations between variables with the first two principal components indicated that PC1 was positively correlated with the emission of esters (0.80), ITCs (0.78), and aldehydes (0.75), but negatively correlated with ketones (-0.43) and alcohols (-0.80). PC1 and PC2 were equally positively correlated with nitriles (PC1: 0.62; PC2: 0.62) and sulfides (PC1: 0.60; PC2: 0.64). Terpenes were positively correlated with PC1 (0.66), but negatively correlated with PC2 (-0.55).

HCPC on principal components using the eight VOC classes defined four clusters corresponding to the four groups already identified by PCAs (Fig. 2A-B). A careful examination of the data clearly indicated differences between cluster 1 and cluster 4 (Group 1 and 4 in PCA). Indeed, for the former, it corresponded to treatments at low temperatures without insects and for the later to the most stressful conditions (highest temperature with the occurrence of aphids). Cluster 2 (Group 2 in PCA) represented the effects of temperature increase (from 22 °C to 32 °C) on the VOCs emitted by uninfested plants. It is noteworthy that this cluster also grouped the chemical profiles observed when infestation occurred at low temperatures (17 °C and 22 °C). That brought out both effects (temperatures and occurrence

of *M. persicae*) on the VOC profiles. This tendency was the same and well depicted in cluster 3 (Group 3 in PCA) for higher temperatures (with infested plants at 27 °C during the three sampling durations, at 32 °C after 24 h, and with uninfested plants stressed at 32 °C).

To identify the differences between clusters of the HCPC, a one-way ANOVA was carried out for each VOC class, followed by a *post hoc* Tukey's test (Fig. 2C). The results showed that the emission of alcohols was highest for plants from Cluster 1 (without aphids at 17 °C and 22 °C) ($P < 0.001$) (Fig. 2C). In contrast, the ketone proportion significantly increased from plants exposed to stresses in Cluster 2 (with aphids at 17 °C and 22 °C; without aphids at 27 °C and 32 °C) ($P < 0.001$), whereas terpene emissions showed a significant increase in their proportions from plants subjected to treatments in Cluster 3 (with aphids at 27 °C) ($P < 0.01$). The proportions of esters ($P < 0.001$), ITCs ($P < 0.001$) and aldehydes ($P < 0.001$) significantly increased when *Arabidopsis* plants were exposed to stress treatments described in Cluster 3 and 4. Finally, plants infested by aphids and kept at the highest temperature (32 °C) emitted significantly higher proportions of nitriles ($P < 0.001$) and sulfides ($P < 0.001$) (Cluster 4) (Fig. 2C). These observations have been summarized in the heat-map (Fig. 2B) which clearly demonstrated the effects of treatments on volatile chemicals.

A three-way full crossed ANOVA model was carried out for both VOC classes and each individual compound to identify differences between *Arabidopsis* volatile profiles. Alcohol proportions exhibited a significant decrease in plants exposed to combined temperature and aphid density ($F_{6,42} = 17.10$, $P < 0.001$) (Online resource 2). Considering individual compounds, the percentage of 2-ethyl-hexan-1-ol significantly decreased according to the conditions of experimentations ($F_{6,42} = 13.19$, $P < 0.001$), whereas 4-methyl-1-penten-3-ol generally increased ($F_{6,42} = 74.65$, $P < 0.001$). (*Z*)-3-hexen-1-ol ($F_{6,42} = 7.18$, $P < 0.001$) and decan-3-ol ($F_{6,42} = 513.31$, $P < 0.001$) were only detected from infested samples at the three highest temperatures during the three sampling times (Online resource 2).

Most of the emission of esters ($F_{6,42} = 65.44$, $P < 0.001$), aldehydes ($F_{6,42} = 26.24$, $P < 0.001$) and 6-methyl-hept-5-en-2-one, the only detected ketone, ($F_{6,42} = 21.61$, $P < 0.001$) were affected by high temperature – aphid interactions on *Arabidopsis* plants. With respect to the individual ester compounds, 1-methylcyclopentyl acetate ($F_{6,42} = 39.27$, $P < 0.001$), methyl 2-ethylpentanoate ($F_{6,42} = 7.10$, $P < 0.001$) and (*Z*)-3-hexenyl acetate ($F_{6,42} = 47.60$, $P < 0.001$) generally showed a significant increase in infested plants at high temperatures (27 °C and 32 °C) (Online resource 2).

Considering aldehydes, the release of phenylacetaldehyde only appeared above 17 °C in uninfested and infested plants ($F_{6,42} = 41.70$, $P < 0.001$). While benzaldehyde ($F_{6,42} = 34.85$, $P < 0.001$) was only detected from *Arabidopsis* infested by aphids at all temperatures after 0-24 h, 24-48 h, and 48-72 h, (*Z*)-2-heptenal ($F_{6,4} = 60.72$, $P < 0.001$) was found in uninfested and infested plants at highest temperatures over the second and the third time periods (Online resource 2).

The combined effects of temperature and occurrence of aphids led to an increase of the terpene proportion when compared to temperature effects alone (17 °C, 22 °C, and 27 °C) during three sampling periods ($F_{6,42}$ = 25.53, P < 0.001) (Online resource 2). With respect to the specific compounds, (*E*,*E*)-α-farnesene ($F_{6,42}$ = 40.38, P < 0.001) was only found in the case of temperature and aphid interaction. Nevertheless, when compared to the same temperature, the release of menthol was generally lower in infested leaves than in uninfested ones over the three periods ($F_{6,42}$ = 19.78, P < 0.001). Increasing temperature led to the emission of α-terpineol ($F_{6,42}$ = 15.88, P < 0.001). The same observation has been made for 2,3,6-trimethyl-1,5-heptadiene ($F_{6,42}$ = 75.67, P < 0.001) in the presence or absence of aphids after 24-48 h and 48-72 h at 27 °C and 32 °C (Online resource 2).

With respect to the emission of glucosinolate derivative volatile compounds, most of them were only found in the aphid-infested *Arabidopsis* at different temperatures for all sampling times. With respect to ITC, 4-methylpentyl ITC mainly increased continuously with time and temperature in the case of aphid infestation, but decreased from plants exposed to 32 °C ($F_{6,42}$ = 37.85, P < 0.001). Among all different conditions, we only observed 5-(methylthio) pentanenitrile ($F_{6,42}$ = 173.05, P < 0.001) and dimethyl trisulfide ($F_{6,42}$ = 51.86, P < 0.001) when the temperature was set to 32 °C for infested plants after the two last sampling periods (Online resource 2).

Population of aphids

The population of aphids was established at the end of each stress treatment. The results of Fig. 3 showed that temperature influenced the fecundity (population growth rate) and mortality of aphids. At 22 °C and 27 °C, the population significantly increased, whereas it decreased at 17 °C and 32 °C resulted in decreased growth rate ($F_{(3,8)}$ = 54.31; P < 0.001). These observations are in line with Ma et al. (2004) who found that the growth, development, and reproduction of aphids (*Sitobion avenae* and *Rhopalosiphum padi*) were strongly reduced by heat stresses.

Discussion

Herbivorous insects, especially when combined with abiotic stresses (e.g. temperature, drought, salinity) can alter the production of plant secondary metabolites (Vos et al. 2001; Van Poecke 2007; Holopainen and Gershenzon 2010). Some recent evidence indicates that drought stress and herbivore interactions cause changes in the emission of plant VOCs (Tariq et al. 2013; Copolovici et al. 2014). Our results found that simultaneous stress treatments by temperatures and *M. persicae* infestation led to VOC emission changes in *A. thaliana* after the three investigated time periods (0-24 h, 24-48 h, and 48-72 h) (Fig. 2). In addition, it was also observed that VOC induction was dependent on aphid density on leaves (Fig. 1).

Changes in volatile emission from *Arabidopsis* upon aphid infestation have been studied previously (Mewis et al. 2005; Van Poecke 2007; De Vos and Jander 2009; Louis et al. 2012). So far little attention has been paid to the investigation of pest density effects on the release of volatile blends. Very recently, Cai et al. (2014) found that the density of the piercing-sucking leafhopper (*Empoasca vitis*) influenced the concentration of VOCs in tea plants. The results described herein demonstrate that aphid density has great effects on the composition of *Arabidopsis* volatiles, including alcohols, aldehydes, ketones, ITCs and terpenes (Fig. 1). Specifically, the relative abundance of 4-methylpentyl ITC and terpene (mainly (*E,E*)-α-farnesene) significantly increased with aphid density (Table 1). According to Blande et al. (2004) and Van Poecke (2007), ITCs and terpenes exert both direct defense effects (repellent, feeding deterrents, or toxicity) against herbivorous insects and indirect protection (natural enemies attractants and/or oviposition stimulants).

The combination of abiotic and biotic stresses led to either reduced or elevated VOC emission of plants in comparison to single stress treatments (Mewis et al. 2012; Winter et al. 2012). Here, we found variation in the induced volatiles emitted by *Arabidopsis* subjected to interaction between temperature regimes (17 °C, 22 °C, 27 °C and 32 °C) and *M. persicae* density over the three time periods (65.0% of the total variance, Fig. 2A).

Similarly to previous studies on plant – aphid interactions (Mewis et al. 2005; Van Poecke 2007; Ma and Ma 2012), we found that the release of ITCs, including 4-methylpentyl ITC, 1-isothiocyanato-3-methylbutane and heptyl ITC, significantly increased with the population growth rate of aphids on plants (Figs. 2B-3; Online resource 2). The proportion of ITCs greatly increased under plant infestation at 27 °C and decreased at 32 °C after 48-72 h treatment. These molecules are generated by myrosinase-mediated degradation of GSs occurring when aphid stylet is inserted into the phloem (favoring enzyme-substrate contact) (Kim et al. 2008; Louis and Shah 2013). In addition the accumulation of indole GS of *Arabidopsis* has been shown to increase in response to aphid *M. persicae* feeding, which require the presence of two functional proteins: nonexpressor of pathogenesis-related proteins1 (NPR1) and ethylene receptor ethylene response 1 (ETR1) (Mewis et al. 2005; Kim and Jander 2007).

In contrast to ITC emissions, the total sulfide and nitrile releases significantly increased from *Arabidopsis* exposed to aphid feeding at 32 °C over 24-48 h and 48-72 h treatments (Online resource 2). Van Poecke (2007) proposed that degradation of GSs in plants infested by insects can lead to the production of either ITC or sulfides and nitriles according to the activity of ethiospecifier modifier1 (ESM1) and ethiospecifier protein (ESP), respectively. Moreover, it was noted that the activity of enzymatic ESP could be affected by temperature (Lambrix et al. 2001; Matusheski et al. 2004). Lambrix et al. (2001) demonstrated that the production of nitrile was contrasted to ITC. Therefore, the release of sulfides, nitriles and ITCs may be related to the combination of aphids and temperature stresses in this study.

In our findings, the proportion of terpenes in the volatile profile of infested *A. thaliana* significantly increased in comparison to uninfested plants at the same temperatures alone (Fig. 2). In particular, limonene, 2,3,6-trimethyl-1,5-heptadiene, α-terpineol , menthol and (*E*,*E*)-α-farnesene showed significant changes (Online resource 2). Many studies found that aphid infestation led to altered terpene emissions in plants (Van Poecke 2007; Giorgi et al. 2012; Verheggen et al. 2013). In general, under abiotic and/or biotic stress commonly emitted terpenes come from the mevalonate (MVA) or 2-C-methyl-D-erythritol 4-phosphate (MEP) pathways (Van Poecke 2007; Loreto and Schnitzler 2010; Tholl and Lee 2011). Moreover, Loreto and Schnitzler (2010) reported that under rising temperatures, enzymatic reactions in MEP pathway induced the release of terpenes.

Green leaf volatiles (GLVs), including C6 and C9 aldehydes, alcohols and associated acetate esters, are produced by the lipoxygenase/hydroperioxide lyase (LOX/HPL) pathway from plants damaged during abiotic and/or biotic stresses (Arimura et al. 2009; Holopainen and Gershenzon 2010; Loreto and Schnitzler 2010; Matsui et al. 2012). In *A. thaliana*, it has been demonstrated that the genome contains six LOX genes (Liavonchanka and Feussner 2006; Bannenberg et al. 2009; Nalam et al. 2012). Nalam et al. (2012) investigated that the LOX5-encoded 9-LOX–derived oxylipin(s) functions as a susceptibility factor in *Arabidopsis – M. persicae* interaction. From the present study, some products like (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate arising from LOX/HPL pathway occurred when *Arabidopsis* plants were subjected to both stresses (temperature and sucking insects) (Online resource 2). In addition, we observed a significant decrease of 2-ethyl-hexan-1-ol according to an increase of temperature. This is in line with Sung et al. (2003); Loreto and Schnitzler (2010) and Copolovici et al. (2012) who suggested that plant alcohol production is dependent on stomatal behavior under temperature changes.

Evidence indicated that plants stressed by aphids and temperature led to the release of benzadehyde (Schade and Goldstein 2001; Staudt et al. 2010; Giorgi et al. 2012). Similarly to these observations, we found this molecule from infested *Arabidopsis* plants at different temperatures (Online resource 2). Staudt et al. (2010) proposed that benzaldehyde was produced by the enzymatic breakdown of prunasin, a cyanogenic glycoside often found in plant leaves.

The results presented herein demonstrated that the application of abiotic (temperature regimes) and biotic (feeding *M. persicae*) stresses simultaneously influenced volatile compound emissions from *Arabidopsis*. Interestingly, significant differences were observed in volatile profiles based on changes in aphid densities as well as temperatures over different time periods (Figs. 1-2). *Arabidopsis* microarray database and analysis toolbox sets with the Genevestigator tool (https://www.genevestigator.com/) (Zimmermann et al. 2004; Grennan 2006) indicated that abiotic stresses (like cold, heat and drought) relate to the expression of the AT-00120 gene. Moreover, some studies also proposed that green peach aphid infestation resulted in the expression of genes like *M. persicae*-induced lipase1 (MPL1) and senescence associated genes (SAG) (Louis et al. 2012; Louis and Shah 2013). We suggest further research

to focus on gene and protein expression related to VOC emissions which could provide new insights on the role and pattern of VOC production in relation to multiple stresses.

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References

Arimura GI, Matsui K, Takabayashi J (2009) Chemical and molecular ecology of herbivore-induced plant volatiles: Proximate factors and their ultimate functions. Plant and Cell Physiology 50:911-923

Bannenberg G, Martínez M, Hamberg M, Castresana C (2009) Diversity of the enzymatic activity in the lipoxygenase gene family of arabidopsis thaliana. Lipids 44:85-95

Blande JD, Pickett JA, Poppy GM (2004) Attack rate and success of the parasitoid Diaeretiella rapae on specialist and generalist feeding aphids. Journal of Chemical Ecology 30:1781-1795

Cai XM, Sun XL, Dong WX, Wang GC, Chen ZM (2014) Herbivore species, infestation time, and herbivore density affect induced volatiles in tea plants. Chemoecology 24:1-14

Copolovici L, Kännaste A, Pazouki L, Niinemets Ü (2012) Emissions of green leaf volatiles and terpenoids from Solanum lycopersicum are quantitatively related to the severity of cold and heat shock treatments. Journal of Plant Physiology 169:664-672

Copolovici L, Kännaste A, Remmel T, Niinemets T (2014) Volatile organic compound emissions from Alnus glutinosa under interacting drought and herbivory stresses. Environmental and Experimental Botany 100:55-63

De Vos M, Jae HK, Jander G (2007) Biochemistry and molecular biology of Arabidopsis-aphid interactions. BioEssays 29:871-883

De Vos M, Jander G (2009) Myzus persicae (green peach aphid) salivary components induce defence responses in Arabidopsis thaliana. Plant Cell Environ 32:1548-1560

Dicke M, Baldwin IT (2010) The evolutionary context for herbivore-induced plant volatiles: beyond the 'cry for help'. Trends in Plant Science 15:167-175

Dudareva N, Negre F, Nagegowda DA, Orlova I (2006) Plant Volatiles: Recent Advances and Future Perspectives. Critical Reviews in Plant Sciences 25:417-440

Fortunati A, Barta C, Brilli F, Centritto M, Zimmer I, Schnitzler JP, Loreto F (2008) Isoprene emission is not temperature-dependent during and after severe drought-stress: A physiological and biochemical analysis. Plant Journal 55:687-697

Giorgi A, Panseri S, Masachchige Chandrika Nanayakkara N, Chiesa L (2012) HS-SPME-GC/MS analysis of the volatile compounds of Achillea collina: Evaluation of the emissions fingerprint induced by Myzus persicae infestation. J Plant Biol 55:251-260 doi:10.1007/s12374-011-0356-0

Gouinguené SP, Turlings TCJ (2002) The effects of abiotic factors on induced volatile emissions in corn plants. Plant Physiology 129:1296-1307

Grennan AK (2006) Genevestigator. Facilitating web-based gene-expression analysis. Plant Physiology 141:1164-1166

Hatano E, Kunert G, Michaud JP, Weisser WW (2008) Chemical cues mediating aphid location by natural enemies. European Journal of Entomology 105:797-806

Holopainen JK, Gershenzon J (2010) Multiple stress factors and the emission of plant VOCs. Trends in Plant Science 15:176-184

Huang M, Sanchez-Moreiras AM, Abel C, Sohrabi R, Lee S, Gershenzon J, Tholl D (2012) The major volatile organic compound emitted from Arabidopsis thaliana flowers, the sesquiterpene (E)-beta-caryophyllene, is a defense against a bacterial pathogen. New Phytol 193:997-1008

Husson F, Josse J, Le S, Mazet J (2013) FactoMineR: Multivariate exploratory data analysis and data mining with R. R package version 1.25. <http://CRAN.R-project.org/package=FactoMineR>.

Kim JH, Jander G (2007) Myzus persicae (green peach aphid) feeding on Arabidopsis induces the formation of a deterrent indole glucosinolate. Plant J 49:1008-1019

Kim JH, Lee BW, Schroeder FC, Jander G (2008) Identification of indole glucosinolate breakdown products with antifeedant effects on Myzus persicae (green peach aphid). Plant J 54:1015-1026

Kos M, Houshyani B, Achhami BB, Wietsma R, Gols R, Weldegergis BT, Kabouw P, Bouwmeester HJ, Vet LEM, Dicke M, van Loon JJA (2012) Herbivore-Mediated Effects of Glucosinolates on Different Natural Enemies of a Specialist Aphid. Journal of Chemical Ecology 38:100-115

Krasensky J, Jonak C (2012) Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. Journal of Experimental Botany 63:1593-1608

Lambrix V, Reichelt M, Mitchell-Olds T, Kliebenstein DJ, Gershenzon J (2001) The arabidopsis epithiospecifier protein promotes the hydrolysis of glucosinolates to nitriles and influences Trichoplusia ni herbivory. Plant Cell 13:2793-2807

413 Liavonchanka A, Feussner I (2006) Lipoxygenases: Occurrence, functions and catalysis. Journal of Plant Physiology
414 163:348-357
415 Loreto F, Schnitzler JP (2010) Abiotic stresses and induced BVOCs. Trends in Plant Science 15:154-166
416 Louis J, Shah J (2013) Arabidopsis thaliana-Myzus persicae interaction: shaping the understanding of plant defense
417 against phloem-feeding aphids. Front Plant Sci 4 doi:10.3389/fpls.2013.00213
418 Louis J, Singh V, Shah J (2012) Arabidopsis thaliana-Aphid Interaction. Arabidopsis Book / The American Society of
419 Plant Biologists 10:e0159 doi:10.1199/tab.0159
420 Ma CS, Hau B, Poehling HM (2004) The effect of heat stress on the survival of the rose grain aphid, Metopolophium
421 dirhodum (Hemiptera: Aphididae). European Journal of Entomology 101:327-331
422 Ma G, Ma CS (2012) Effect of acclimation on heat-escape temperatures of two aphid species: Implications for
423 estimating behavioral response of insects to climate warming. Journal of Insect Physiology 58:303-309
424 Maffei ME (2010) Sites of synthesis, biochemistry and functional role of plant volatiles. South African Journal of
425 Botany 76:612-631
426 Matsui K, Sugimoto K, Mano J, Ozawa R, Takabayashi J (2012) Differential metabolisms of green leaf volatiles in
427 injured and intact parts of a wounded leaf meet distinct ecophysiological requirements. PLoS ONE 7:e36433
428 Matusheski NV, Juvik JA, Jeffery EH (2004) Heating decreases epithiospecifier protein activity and increases
429 sulforaphane formation in broccoli. Phytochemistry 65:1273-1281
430 Mewis I, Appel HM, Hom A, Raina R, Schultz JC (2005) Major signaling pathways modulate Arabidopsis
431 glucosinolate accumulation and response to both phloem-feeding and chewing insects. Plant Physiology
432 138:1149-1162
433 Mewis I, Khan MAM, Glawischnig E, Schreiner M, Ulrichs C (2012) Water Stress and Aphid Feeding Differentially
434 Influence Metabolite Composition in Arabidopsis thaliana (L.). PLoS ONE 7:e48661
435 Mewis I, Tokuhisa JG, Schultz JC, Appel HM, Ulrichs C, Gershenzon J (2006) Gene expression and glucosinolate
436 accumulation in Arabidopsis thaliana in response to generalist and specialist herbivores of different feeding
437 guilds and the role of defense signaling pathways. Phytochemistry 67:2450-2462
438 Mumm R, Schrank K, Wegener R, Schulz S, Hilker M (2003) Chemical analysis of volatiles emitted by Pinus sylvestris
439 after induction by insect oviposition. Journal of Chemical Ecology 29:1235-1252
440 Nalam VJ, Keeretaweep J, Sarowar S, Shah J (2012) Root-derived oxylipins promote green peach aphid performance
441 on arabidopsis foliage. Plant Cell 24:1643-1653
442 Pinto DM, Blande JD, Souza SR, Nerg AM, Holopainen JK (2010) Plant volatile organic compounds (VOCs) in ozone
443 (O3) polluted atmospheres: the ecological effects. J Chem Ecol 36:22-34

444 R-Development-Core-Team (2013) R: A language and environment for statistical computing. Vienna, Austria: R
445 Foundation for Statistical Computing <http://wwwR-projectorg/>
446 Rohloff J, Bones AM (2005) Volatile profiling of Arabidopsis thaliana - Putative olfactory compounds in plant
447 communication. Phytochemistry 66:1941-1955
448 Schade GW, Goldstein AH (2001) Fluxes of oxygenated volatile organic compounds from a ponderosa pine plantation.
449 Journal of Geophysical Research D: Atmospheres 106:3111-3123
450 Snoeren TAL, Kappers IF, Broekgaarden C, Mumm R, Dicke M, Bouwmeester HJ (2010) Natural variation in
451 herbivore-induced volatiles in Arabidopsis thaliana. Journal of Experimental Botany 61:3041-3056
452 Staudt M, Jackson B, El-Aouni H, Buatois B, Lacroze JP, Poëssel JL, Sauge MH (2010) Volatile organic compound
453 emissions induced by the aphid Myzus persicae differ among resistant and susceptible peach cultivars and a
454 wild relative. Tree Physiology 30:1320-1334
455 Sung DY, Kaplan F, Lee KJ, Guy CL (2003) Acquired tolerance to temperature extremes. Trends Plant Sci 8:179-187
456 Tariq M, Wright DJ, Bruce TJA, Staley JT (2013) Drought and Root Herbivory Interact to Alter the Response of
457 Above-Ground Parasitoids to Aphid Infested Plants and Associated Plant Volatile Signals. PLoS ONE
458 8:e69013
459 Tholl D, Lee S (2011) Terpene Specialized Metabolism in Arabidopsis thaliana. Arabidopsis Book 9:e0143
460 doi:10.1199/tab.0143
461 Unsicker SB, Kunert G, Gershenzon J (2009) Protective perfumes: the role of vegetative volatiles in plant defense
462 against herbivores. Current Opinion in Plant Biology 12:479-485
463 Vallad GE, Goodman RM (2004) Systemic acquired resistance and induced systemic resistance in conventional
464 agriculture. Crop Science 44:1920-1934
465 Van Poecke RM (2007) Arabidopsis-insect interactions. Arabidopsis Book / The American Society of Plant Biologists
466 5:e0107 doi:10.1199/tab.0107
467 Verheggen FJ, Haubruge E, De Moraes CM, Mescher MC (2013) Aphid responses to volatile cues from turnip plants
468 (Brassica rapa) infested with phloem-feeding and chewing herbivores. Arthropod-Plant Interactions 7:567-577
469 Vickers CE, Gershenzon J, Lerdau MT, Loreto F (2009) A unified mechanism of action for volatile isoprenoids in plant
470 abiotic stress. Nature Chemical Biology 5:283-291
471 Vos M, Berrocal SM, Karamaouna F, Hemerik L, Vet LEM (2001) Plant-mediated indirect effects and the persistence
472 of parasitoid - Herbivore communities. Ecology Letters 4:38-45
473 Vuorinen T, Nerg AM, Holopainen JK (2004) Ozone exposure triggers the emission of herbivore-induced plant
474 volatiles, but does not disturb tritrophic signalling. Environmental Pollution 131:305-311

Warnes GR, Bolker B, Bonebakker L, Gentleman R, Liaw WHA, Lumley T, Maechler M, Magnusson A, Moeller S, Schwartz M, Venables B (2013) Gplots: Various R programming tools for plotting data. R package version 2.11.3. <http://CRAN.R-project.org/package=gplots>.

Winter TR, Borkowski L, Zeier J, Rostás M (2012) Heavy metal stress can prime for herbivore-induced plant volatile emission. Plant, Cell and Environment 35:1287-1298

Winter TR, Rostás M (2010) Nitrogen deficiency affects bottom-up cascade without disrupting indirect plant defense. Journal of Chemical Ecology 36:642-651

Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W (2004) GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. Plant Physiology 136:2621-2632

Table caption

Table 1 Major compounds released from *A. thaliana* infested by different densities of *M. persicae* over 24-48 at 22 °C. Data show mean percentage ± SD of values of three replicates. Means followed by the same letter are not significantly different ($P > 0.05$, one-way ANOVA, *post hoc* Tukey's HSD test).

Figure captions

Fig. 1 (A) Principal Components Analysis (PCA) for PC1 and PC2 shows the chemical profile based on *A. thaliana* emissions resulting from the effects of different aphid densities on plant leaves. ⊙ uninfested plants; ▲ infested by 30 aphids/plant; ⊡ infested by 70 aphids/plant, and ◆ infested by 100 aphids/plant. (B) VOC classes from *Arabidopsis* uninfested and infested plants resulting from different aphid densities within 24-48 h under 22 °C temperature conditions. Uninfested = control plants; Infested_30, Infested_70, and Infested_100 correspond to 30, 70, and 100 adult aphids feeding on individual plants. Means (n = 3; ± SD) followed by the same letter are not significantly different ($P > 0.05$, one-way ANOVA, *post hoc* Tukey's HSD test).

Fig. 2 (A) Principal Component Analysis (PCA) for PC1 and PC2 shows the chemical profile based on *A. thaliana* emissions resulting from the effects of temperature regimes and aphid feeding on plant leaves over three time periods. Treatments were coded as follow: temperature (17 °C, 22 °C, 27 °C and 32 °C), Infested and Uninfested (Inf and Uninf), and Time periods (24, 48 and 72 correspond to 0-24 h, 24-48 h and 48-72 h, respectively). (B) Hierarchical Cluster Analysis on Principal Components (HCPC) for both stress treatments and defined groups of similar chemical

profiles based on PC1 and PC2. Heatmap colors represent the fold change, based on the provided color key scale; red for higher levels, green for lower levels. Uninf and Inf: uninfested and infested plants. (C) Relative abundance of VOC classes from total *Arabidopsis* expression profiles due to temperature stress (17 °C, 22 °C, 27 °C, and 32 °C) exposure in the absence and presence of *M. persicae* grouped based on HCPC. Means followed by the same letter are not significantly different ($P > 0.05$, one-way ANOVA, *post hoc* Tukey's HSD test).

Fig. 3 Population growth rate of *M. persicae* at 17 °C, 22 °C, 27 °C and 32 °C over the last period of time treatments (48-72 h). Means followed by the same letter are not significantly different ($P > 0.05$, one-way ANOVA, *post hoc* Tukey's HSD test).

Supplementary data

Online Resource 1 *Arabidopsis* leaves (a) uninfested, and (b) infested by 70 *M. persicae* under 22 °C conditions for 48-72 h.

Online Resource 2 Mean percentage (n = 3, ± SD) of different compounds from *Arabidopsis* volatile emissions resulting from temperature stress (17 °C, 22 °C, 27 °C, and 32 °C) in the absence and presence of *M. persicae* over different time periods (0-24 h, 24-48 h and 48-72 h). Means followed by the same letter are not significantly different ($P > 0.05$, three-way ANOVA, *post hoc* Tukey's HSD test).

Online Resource 3 Principal Component Analysis (PCA) of the individual volatiles based on *A. thaliana* emissions resulting from the effects of temperature regimes and aphid feeding on plant leaves. The PCA shows the first and second components (PC1 and PC2).