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

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16

17 **Abstract**

18   The effects of volatile emissions from plants exposed to individual abiotic and biotic stresses are well

19 documented. However, the influence of multiple stresses on plant photosynthesis and defense responses, resulting in a

20 variety of volatile profiles has received little attention. In this study, we investigated how temperature regimes in the

21 presence and absence of the sucking insect *Myzus persicae* affected volatile organic compound emissions in

22 *Arabidopsis* over three time periods (0-24 h, 24-48 h, and 48-72 h). Headspace solid-phase microextraction coupled

23 with gas chromatography-mass spectrometry was used to evaluate *Arabidopsis* volatile organic compounds. The results

24 showed that under laboratory conditions, eight volatile classes [alcohols (mainly 2-ethyl-hexan-1-ol), ketone (6-methyl

25 hept-5-en-2-one), esters (mainly (*Z*)-3-hexenyl acetate), aldehydes (mainly phenylacetaldehyde), isothiocyanates

26 (mainly 4-methylpentyl isothiocyanate), terpenes (mainly (*E,E*)-α-farnesene), nitrile (5-(methylthio) pentanenitrile), and

27 sulfide (dimethyl trisulfide)] were observed on plants exposed to stress combinations, whereas emissions of six volatile

28 classes were observed during temperature stress treatments alone (with the exception of nitriles and sulfides). Aphid

29 density at high temperature combinations resulted in significantly higher isothiocyanate, ester, nitrile and sulfide

30 proportions. The results of the present study provide an insight into the effects of temperature - aphid interactions on

31 plant volatile emissions.

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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 $64.5 \pm 2.6\%$ 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

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96   Five-week old *Arabidopsis* plants, including root balls (9-10 leaves, 0.25-0.40 g/plant) were carefully removed

97   from the plastic pots, individually wrapped in aluminum foil around the root balls to reduce the detection of soil

98   volatiles. Three plants were carefully placed in the same reaction vessels (100 ml, Duran Group, Germany) and

99   submitted to each stress treatment. Reaction vessels were carefully cleaned by methanol, milli-Q water and put in the

100 oven at 180 °C during 24 h before the plants were put into them.

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102 Temperature regimes

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104 Temperature regimes were applied using a thermostat water bath (IKA®-Werke GmbH & Co. KG, Germany),

105 and the reaction vessels containing three plants were immersed in water. The temperature treatments inside the

106 collection reaction vessels were 17 °C, 22 °C, 27 °C, and 32 °C, and controlled using a HOBO Pro temp/RH/light/text

107 channel data logger (Onset Computer Corp., Bourne, MA 02532, USA). Plants were kept 18 h at such temperatures

108 before collecting VOCs. Light intensity was kept constant during all collections (600-800 Lux, HOBO Pro

109 temp/RH/light/text channel data logger). Three replications were conducted for each treatment.

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111 *Myzus persicae* infestation

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113 *Arabidopsis* were either untreated, as a control, or infested with aphids (Online resource 1). Plants were

114 infested by exposure to adult aphids under controlled conditions. In a preliminary trial (Exp. 1), 30, 70 and 100 adults

115 were released onto randomly selected *Arabidopsis*, and the volatiles were collected from plants maintained at 22 °C

116 during 48 h. The samplings were carried out from 42-48 h. In order to test temperature and aphid interaction (Exp. 2),

117 70 aphids were placed on plants at different temperatures (17 °C, 22 °C, 27 °C, and 32 °C) over three time periods (0-24

118 h, 24-48 h, and 48-72 h). Plants without aphids were subjected to the same temperature. Population growth rate of

119 aphids was measured at the end of each test. Both experiments were replicated three times.

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121 Plant volatile collection and analysis

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123 To select the best SPME fibers suitable to extract VOCs from *Arabidopsis*, preliminary experiments were conducted on

124 control plants with different stationary phases and various film thicknesses: carboxen-polydimethylsiloxane

125 (CAR/PDMS, 75 μm), polydimethylsiloxane-divinylbenzene (PDMS/DVB, 65 μm) and divinylbenzene-carboxen-

126 polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm) (Supelco, Bellefonte, PA, USA). The results (data not shown)

127   demonstrated that 65 μm PDMS/DVB fibers were most efficient for *A. thaliana* VOC sampling. Our results were

128   consistent with Rohloff and Bones (2005) who profiled volatiles in *Arabidopsis*.

129   Volatile collection was conducted from *Arabidopsis* under stress treatments during three time courses (0-24 h,

130   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

131   onset of VOC collection, the fibers were conditioned at 225 °C for 30 min to avoid contaminations.

132   At the end of each treatment of the sample periods (24, 48 and 72 h), the fibers were desorbed in the injector of

133   a gas chromatograph coupled to a mass spectrometer (SPME-GC/MS). GC/MS analyses were performed using a Trace

134   GC Ultra (Thermo-Fisher Scientific; Waltham, MA, USA) gas chromatograph coupled to a quadrupole-type mass

135   spectrometer Trace Finnigan (Thermo-Fisher Scientific; Waltham, MA, USA). The GC was equipped with an apolar

136   column (Optima-5-MS, 30 m; 0.25 mm i.d.; 0.25 μm film thickness, Macherey-Nagel, Düren, Germany). The oven

137   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

138   100 °C/min. Carry over and peaks originating from the fibers were regularly assessed by running blank samples. After

139   VOC collections, fibers were immediately thermally desorbed in the GC injector for 5 min at 220 °C to prevent

140   contamination. The injections were performed in splitless mode using SPME liner (1 min). The carrier gas was helium

141   at a constant flow rate of 1.5 ml/min. The mass spectra were obtained using a mass selective detector operating in

142   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

143   1 scan s^{-1}. The transfer line to the mass spectrometer was maintained at 230 °C, and the ion source temperature was set

144   at 250 °C. The volatile components were identified based on retention times, and by careful examination of their main

145   spectra in comparison with the Wiley and NIST MS 2.0 computed spectral databases.

146

147   Statistical analyses

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149   The relative abundances of each detected volatile were calculated as the ratio between their area and the total area of all

150   detected VOCs from plants subjected to each treatment. In a second step, the proportions of the main chemical families

151   [alcohols, terpenes, ketones, aldehydes, isothiocyanates (ITCs), esters, nitriles and sulfides] were calculated.

152   In order to reveal the patterns of variation and clustering among treatments, and identify the volatile chemicals

153   responsible for variation in volatile profiles among *Arabidopsis* subjected to different treatments, a principal component

154   analysis (PCA) followed by hierarchical clustering analysis on principal components (HCPC) was performed

155   respectively using R 3.0.1 software (R-Development-Core-Team 2013) and FactoMineR 1.25 package (Husson et al.

156   2013). Multivariate analyses (PCA and HCPC) were preformed on a dataset containing the mean relative abundances of

157   either the individual compounds or eight groups for each treatment. Principal components (PCs) were calculated using a

158   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**

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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).

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191   With respect to the specific terpenes showing significant differences between treatments, the proportions of

192   limonene ($F_{3,8}$ = 10.58, P = 0.004) and menthol ($F_{3,8}$ = 15.78, P = 0.001) generally increased in infested *Arabidopsis*

193   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

194   detected in highly infested *Arabidopsis* (100 aphids/plant) (Table 1).

195   With respect to the single alcohol compounds, decan-3-ol ($F_{3,8}$ = 158.69, P < 0.001) was only observed in

196   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

197   < 0.001) and 2-ethyl-hexan-1-ol ($F_{3,8}$ = 40.23, P < 0.001) generally showed a significant decrease in infested plants

198   (Table 1).

199

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

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202   Twenty compounds divided into eight VOC classes were recorded in the headspace of *A. thaliana* exposed to different

203   temperatures (from 17 °C to 32 °C). When aphids were present a total of 29 compounds were detected. Volatile

204   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

205   PCA using the mean proportion of each individual compound was conducted. The first two principal components (PCs)

206   showed 43.6% of the observed variation, i.e. PC1 29.9% and PC2 13.7% (Online resource 3). In addition, PCA and

207   HCPC using the mean percentage of each VOC class were applied to reveal patterns of variation and clusters among

208   treatments. The goal also was to identify the chemical families responsible for volatile profile variation among

209   *Arabidopsis* under different stress treatments. PCA results showed that the first two components accounted for 65.0% of

210   the total variance, with PC1 and PC2 explaining 47.6 and 17.4% of the total chemical composition variation,

211   respectively (Fig. 2A). Correlations between variables with the first two principal components indicated that PC1 was

212   positively correlated with the emission of esters (0.80), ITCs (0.78), and aldehydes (0.75), but negatively correlated

213   with ketones (-0.43) and alcohols (-0.80). PC1 and PC2 were equally positively correlated with nitriles (PC1: 0.62;

214   PC2: 0.62) and sulfides (PC1: 0.60; PC2: 0.64). Terpenes were positively correlated with PC1 (0.66), but negatively

215   correlated with PC2 (-0.55).

216   HCPC on principal components using the eight VOC classes defined four clusters corresponding to the four

217   groups already identified by PCAs (Fig. 2A-B). A careful examination of the data clearly indicated differences between

218   cluster 1 and cluster 4 (Group 1 and 4 in PCA). Indeed, for the former, it corresponded to treatments at low

219   temperatures without insects and for the later to the most stressful conditions (highest temperature with the occurrence

220   of aphids). Cluster 2 (Group 2 in PCA) represented the effects of temperature increase (from 22 °C to 32 °C) on the

221   VOCs emitted by uninfested plants. It is noteworthy that this cluster also grouped the chemical profiles observed when

222   infestation occurred at low temperatures (17 °C and 22 °C). That brought out both effects (temperatures and occurrence

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223 of *M. persicae*) on the VOC profiles. This tendency was the same and well depicted in cluster 3 (Group 3 in PCA) for

224 higher temperatures (with infested plants at 27 °C during the three sampling durations, at 32 °C after 24 h, and with

225 uninfested plants stressed at 32 °C).

226   To identify the differences between clusters of the HCPC, a one-way ANOVA was carried out for each VOC

227 class, followed by a *post hoc* Tukey's test (Fig. 2C). The results showed that the emission of alcohols was highest for

228 plants from Cluster 1 (without aphids at 17 °C and 22 °C) ($P < 0.001$) (Fig. 2C). In contrast, the ketone proportion

229 significantly increased from plants exposed to stresses in Cluster 2 (with aphids at 17 °C and 22 °C; without aphids at

230 27 °C and 32 °C) ($P < 0.001$), whereas terpene emissions showed a significant increase in their proportions from plants

231 subjected to treatments in Cluster 3 (with aphids at 27 °C) ($P < 0.01$). The proportions of esters ($P < 0.001$), ITCs ($P <$

232 0.001) and aldehydes ($P < 0.001$) significantly increased when *Arabidopsis* plants were exposed to stress treatments

233 described in Cluster 3 and 4. Finally, plants infested by aphids and kept at the highest temperature (32 °C) emitted

234 significantly higher proportions of nitriles ($P < 0.001$) and sulfides ($P < 0.001$) (Cluster 4) (Fig. 2C). These observations

235 have been summarized in the heat-map (Fig. 2B) which clearly demonstrated the effects of treatments on volatile

236 chemicals.

237   A three-way full crossed ANOVA model was carried out for both VOC classes and each individual compound

238 to identify differences between *Arabidopsis* volatile profiles. Alcohol proportions exhibited a significant decrease in

239 plants exposed to combined temperature and aphid density ($F_{6,42} = 17.10$, $P < 0.001$) (Online resource 2). Considering

240 individual compounds, the percentage of 2-ethyl-hexan-1-ol significantly decreased according to the conditions of

241 experimentations ($F_{6,42} = 13.19$, $P < 0.001$), whereas 4-methyl-1-penten-3-ol generally increased ($F_{6,42} = 74.65$, $P <$

242 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

243 infested samples at the three highest temperatures during the three sampling times (Online resource 2).

244   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-

245 hept-5-en-2-one, the only detected ketone, ($F_{6,42} = 21.61$, $P < 0.001$) were affected by high temperature – aphid

246 interactions on *Arabidopsis* plants. With respect to the individual ester compounds, 1-methylcyclopentyl acetate ($F_{6,42} =$

247 39.27, $P < 0.001$), methyl 2-ethylpentanoate ($F_{6,42} = 7.10$, $P < 0.001$) and (*Z*)-3-hexenyl acetate ($F_{6,42} = 47.60$, $P <$

248 0.001) generally showed a significant increase in infested plants at high temperatures (27 °C and 32 °C) (Online

249 resource 2).

250   Considering aldehydes, the release of phenylacetaldehyde only appeared above 17 °C in uninfested and

251 infested plants ($F_{6,42} = 41.70$, $P < 0.001$). While benzaldehyde ($F_{6,42} = 34.85$, $P < 0.001$) was only detected from

252 *Arabidopsis* infested by aphids at all temperatures after 0-24 h, 24-48 h, and 48-72 h, (*Z*)-2-heptenal ($F_{6,4} = 60.72$, $P <$

253 0.001) was found in uninfested and infested plants at highest temperatures over the second and the third time periods

254 (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.

318   In our findings, the proportion of terpenes in the volatile profile of infested *A. thaliana* significantly increased

319   in comparison to uninfested plants at the same temperatures alone (Fig. 2). In particular, limonene, 2,3,6-trimethyl-1,5-

320   heptadiene, α-terpineol , menthol and (*E,E*)-α-farnesene showed significant changes (Online resource 2). Many studies

321   found that aphid infestation led to altered terpene emissions in plants (Van Poecke 2007; Giorgi et al. 2012; Verheggen

322   et al. 2013). In general, under abiotic and/or biotic stress commonly emitted terpenes come from the mevalonate (MVA)

323   or 2-C-methyl-D-erythritol 4-phosphate (MEP) pathways (Van Poecke 2007; Loreto and Schnitzler 2010; Tholl and Lee

324   2011). Moreover, Loreto and Schnitzler (2010) reported that under rising temperatures, enzymatic reactions in MEP

325   pathway induced the release of terpenes.

326   Green leaf volatiles (GLVs), including C6 and C9 aldehydes, alcohols and associated acetate esters, are

327   produced by the lipoxygenase/hydroperioxide lyase (LOX/HPL) pathway from plants damaged during abiotic and/or

328   biotic stresses (Arimura et al. 2009; Holopainen and Gershenzon 2010; Loreto and Schnitzler 2010; Matsui et al. 2012).

329   In *A. thaliana*, it has been demonstrated that the genome contains six LOX genes (Liavonchanka and Feussner 2006;

330   Bannenberg et al. 2009; Nalam et al. 2012). Nalam et al. (2012) investigated that the LOX5-encoded 9-LOX–derived

331   oxylipin(s) functions as a susceptibility factor in *Arabidopsis – M. persicae* interaction. From the present study, some

332   products like (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate arising from LOX/HPL pathway occurred when *Arabidopsis*

333   plants were subjected to both stresses (temperature and sucking insects) (Online resource 2). In addition, we observed a

334   significant decrease of 2-ethyl-hexan-1-ol according to an increase of temperature. This is in line with Sung et al.

335   (2003); Loreto and Schnitzler (2010) and Copolovici et al. (2012) who suggested that plant alcohol production is

336   dependent on stomatal behavior under temperature changes.

337   Evidence indicated that plants stressed by aphids and temperature led to the release of benzadehyde (Schade

338   and Goldstein 2001; Staudt et al. 2010; Giorgi et al. 2012). Similarly to these observations, we found this molecule from

339   infested *Arabidopsis* plants at different temperatures (Online resource 2). Staudt et al. (2010) proposed that

340   benzaldehyde was produced by the enzymatic breakdown of prunasin, a cyanogenic glycoside often found in plant

341   leaves.

342   The results presented herein demonstrated that the application of abiotic (temperature regimes) and biotic

343   (feeding *M. persicae*) stresses simultaneously influenced volatile compound emissions from *Arabidopsis*. Interestingly,

344   significant differences were observed in volatile profiles based on changes in aphid densities as well as temperatures

345   over different time periods (Figs. 1-2). *Arabidopsis* microarray database and analysis toolbox sets with the

346   Genevestigator tool (https://www.genevestigator.com/) (Zimmermann et al. 2004; Grennan 2006) indicated that abiotic

347   stresses (like cold, heat and drought) relate to the expression of the AT-00120 gene. Moreover, some studies also

348   proposed that green peach aphid infestation resulted in the expression of genes like *M. persicae*-induced lipase1

349   (MPL1) and senescence associated genes (SAG) (Louis et al. 2012; Louis and Shah 2013). We suggest further research

350 to focus on gene and protein expression related to VOC emissions which could provide new insights on the role and

351 pattern of VOC production in relation to multiple stresses.

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353 **Acknowledgements**

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361

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485

486 **Table caption**

487

488 **Table 1** Major compounds released from *A. thaliana* infested by different densities of *M. persicae* over 24-48 at 22 °C.

489 Data show mean percentage ± SD of values of three replicates. Means followed by the same letter are not significantly

490 different ($P > 0.05$, one-way ANOVA, *post hoc* Tukey's HSD test).

491

492 **Figure captions**

493

494 **Fig. 1** (A) Principal Components Analysis (PCA) for PC1 and PC2 shows the chemical profile based on *A. thaliana*

495 emissions resulting from the effects of different aphid densities on plant leaves. ⊙ uninfested plants; ▲ infested by 30

496 aphids/plant; ⊡ infested by 70 aphids/plant, and ◆ infested by 100 aphids/plant. (B) VOC classes from *Arabidopsis*

497 uninfested and infested plants resulting from different aphid densities within 24-48 h under 22 °C temperature

498 conditions. Uninfested = control plants; Infested_30, Infested_70, and Infested_100 correspond to 30, 70, and 100 adult

499 aphids feeding on individual plants. Means (n = 3; ± SD) followed by the same letter are not significantly different ($P >$

500 0.05, one-way ANOVA, *post hoc* Tukey's HSD test).

501 **Fig. 2** (A) Principal Component Analysis (PCA) for PC1 and PC2 shows the chemical profile based on *A. thaliana*

502 emissions resulting from the effects of temperature regimes and aphid feeding on plant leaves over three time periods.

503 Treatments were coded as follow: temperature (17 °C, 22 °C, 27 °C and 32 °C), Infested and Uninfested (Inf and

504 Uninf), and Time periods (24, 48 and 72 correspond to 0-24 h, 24-48 h and 48-72 h, respectively). (B) Hierarchical

505 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).