

Enhanced emissions of floral volatiles by *Diplotaxis eruroides* (L.) in response to folivory and florivory by *Pieris brassicae* (L.)

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1 **Abstract**

2 The main function of floral emissions of volatile organic compounds (VOCs) in
3 entomophilous plants is to attract pollinators. Floral blends, however, can also contain
4 volatile compounds with defensive functions. These defensive volatiles are specifically
5 emitted when plants are attacked by pathogens or herbivores. We characterized the
6 changes in the floral emissions of *Diplotaxis erucoides* induced by folivory and
7 florivory by *Pieris brassicae*. Plants were continually subjected to *folivory*, *florivory*
8 and *folivory+florivory* treatments for two days. We measured floral emissions with
9 proton transfer reaction/mass spectroscopy (PTR-MS) at different times during the
10 application of the treatments. The emissions of methanol, ethyl acetate and another
11 compound, likely 3-butenitrile, increased significantly in response to florivory.
12 Methanol and 3-butenitrile increased 2.4- and 26-fold, respectively, in response to the
13 *florivory* treatment. Methanol, 3-butenitrile and ethyl acetate increased 3-, 100- and 9-
14 fold, respectively, in response to the *folivory+florivory* treatment. Folivory alone had no
15 detectable effect on floral emissions. All VOC emissions began immediately after attack,
16 with no evidence of delayed induction in any of the treatments. Folivory and florivory
17 had a synergistic effect when applied together, which strengthened the defensive
18 response when the attack was extended to the entire plant.

19 **Keywords:** Methanol, glucosinolates, ethyl acetate, floral scent, VOCs, folivory-
20 florivory synergy.

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25 **1 Introduction**

26 Flowers are visited by many organisms that can have positive, neutral or negative
27 effects on plants (Irwin et al., 2004). Such visits can have important repercussions on
28 plant fitness (Soper Gorden, 2013). The main visitors to flowers can be classified as
29 pollinators, larcenists (nectar thieves) and florivores. Pollinators have positive effects on
30 flowers by acting as effective vectors of pollination (Dafni, 1992; Dafni et al., 2005),
31 but larcenists and florivores have detrimental effects on flowers (Field, 2001; Irwin et
32 al., 2001; Mothershead and Marquis, 2000). Larcenists affect plant fitness negatively by
33 exploiting and exhausting floral rewards, which are produced to attract pollinators,
34 without contributing to successful pollination (Irwin et al., 2010). Florivory can reduce
35 the attractiveness of flowers by altering the quality and quantity of diverse floral traits,
36 such as petal size or nectar production (Cardel and Koptur, 2010; McCall and Irwin,
37 2006; McCall, 2008). Florivory can also critically damage floral structures that are
38 important for fruit and seed development (Cardel and Koptur, 2010; McCall, 2008).
39 Visitors to flowers thus have multiple and diverse effects on plants (Farré-Armengol et
40 al., 2013; Kessler and Halitschke, 2009).

41 Plants have several strategies to attract pollinators to their flowers for pollination
42 and reproductive outcrossing (Chittka & Raine, 2006; Sheehan *et al.*, 2012; Schiestl &
43 Johnson, 2013). Plants have also evolved different mechanisms (toxins, deterrents and
44 physical barriers) and strategies (escape in time or space) to prevent visits from visitors
45 such as larcenists and herbivores that can have significant negative effects on fitness
46 (Irwin et al., 2004). Among these mechanisms, the emission of volatile organic
47 compounds (VOCs) such as terpenoids, benzenoids and fatty acid derivatives serves
48 plants to attract or deter various visitors to flowers (Kessler *et al.*, 2008, 2013; Junker &
49 Blüthgen, 2010; Farré-Armengol *et al.*, 2013). Benzenoids mostly function as

50 attractants in floral scents, while floral terpenoids can both attract and deter visitors
51 (Farré-Armengol et al., 2013).

52 Some VOCs are instantaneously released in high amounts from damaged plant
53 tissues(Matsui, 2006). Herbivore-induced plant volatiles (HIPVs) play a crucial role in
54 tritrophic interactions by being involved in a mechanism of indirect defense that attracts
55 predators and parasitoids of the herbivores (Dicke, 2009; Hopkins et al., 2009; Llusà
56 and Peñuelas, 2001; Whitman and Eller, 1990). HIPVs also mediate plant-to-plant
57 communication by inducing defensive responses against herbivores in neighboring
58 undamaged plants or in undamaged tissues of the same plant (Blande et al., 2010; Heil,
59 2014; Rodriguez-Saona and Frost, 2010; Seco et al., 2011).

60 The emission of HIPVs by flowers may indiscriminately deter both pollinators
61 and florivores and thus interfere with pollination (Dicke and Baldwin, 2010). In
62 addition to the direct damage caused to plant tissues and other derived negative impacts,
63 herbivory could thus have major detrimental effects on plant fitness when HIPVs are
64 emitted by attacked flowers but also when the systemic transduction of defensive
65 chemical responses is induced from damaged leaves or flowers to undamaged flowers
66 (Lucas-Barbosa et al., 2011). Few studies, however, have demonstrated the induction of
67 defensive VOCs in flowers in response to florivory (Muhlemann et al., 2014) or to the
68 interaction between folivory and florivory.

69 We characterized the floral VOC emissions of *Diplotaxis erucoides* subjected to
70 folivory and florivory by *Pieris brassicae* larvae. We hypothesized that folivory and
71 florivory could induce the emission of floral HIPVs and that florivory would
72 immediately induce the emission of VOCs. We thus compared the floral VOC
73 emissions from plants subjected to florivory and folivory. Most herbivores feed on both

74 flowers and leaves, so plants infested by herbivores are expected to experience folivory
75 and florivory at the same time (when in flower). We thus also subjected plants to a
76 combined treatment of both folivory and florivory to test for additive or synergistic
77 effects.

78

79 **2 Materials and methods**

80 **2.1 Experimental design of bioassays**

81 Twenty *D. erucoïdes* plants of 40-60 cm height were collected near Cerdanyola del
82 Vallès (Barcelona, Catalonia, NE Spain) and were transplanted in 3 dm³ pots with the
83 soil from the field, whose properties were consistent among all the plants. We tested
84 four different treatments: control, folivory, florivory and folivory+florivory. The floral
85 emissions of four plants, one plant per treatment, were periodically monitored during
86 two days. The process was repeated 5 times (with 5 different plants for each treatment)
87 during two weeks. VOCs were measured once in the morning (8:00-12:00) from each
88 plant in each treatment before larvae were applied and four times once the larvae started
89 to feed on the flowers and leaves. The first post-treatment measurement was conducted
90 immediately after applying *P. brassicae* larvae (all treatments except the control) and
91 verifying that they began to eat leaves and/or flowers. The second post-treatment
92 measurement was on the same day in the afternoon (14:00-17:00), and the third and
93 fourth post-treatment measurements were on the following morning (8:00-11:00) and
94 afternoon (12:00-15:00), respectively. The larvae were allowed to feed on the plants
95 continuously during the two days of measurement.

96 The *P. brassicae* larvae had been captured from the field at the 1st and 2nd instar
97 stages. They were fed on *D. erucoïdes* plants until the 3rd instar stage when they begin

98 to feed more and cause significant amounts of damage to their host plants and begin to
99 show a preference for plant tissues other than leaves, such as flowers, which present
100 more attractive nutritional properties (Smallegange et al., 2007). We applied larvae from
101 the 3rd to the 5th (last) instar to the *D. eruroides* plants to feed on the flowers and/or
102 leaves, depending on the treatment. The larvae were deprived of food for two hours
103 before application to ensure that they would begin to feed immediately. Five larvae
104 were applied to basal leaves in the folivory treatment, and two larvae were applied to an
105 inflorescence in the florivory treatment. Seven larvae, two on an inflorescence and five
106 on the basal leaves, were applied in the florivory+folivory treatment. We controlled the
107 location of the larvae by enclosing the inflorescences in gauze bags or by preventing
108 access to flowers.

109 We used a portable infrared gas analyzer (IRGA) system (LC-Pro+, ADC
110 BioScientific Ltd., Herts, England) with a conifer leaf chamber (175 cm³) to sample
111 floral VOC emissions at standard conditions of temperature (30 °C) and light
112 (PAR=1000 μmol m⁻² s⁻¹). An inflorescence containing 4-11 open flowers was enclosed
113 in the chamber without detaching the flowers from the plant. For samples in the
114 florivory and folivory+florivory treatments, we put the inflorescences with the larvae in
115 the chamber and recorded the times at which the larvae began to feed for detecting and
116 measuring floral VOCs instantaneously released by wounded floral tissues. We also
117 measured several blank samples containing only larvae to identify possible larval
118 emissions and to distinguish them from the floral emissions.

119

120 **2.2 Biogenic VOC (BVOC) exchange measurements**

121 Flower samples were clamped into the leaf chamber (175 cm³) of an LC-Pro+
122 Photosynthesis System (ADC BioScientific Ltd., Herts, England). Flow meters
123 monitored the air flowing through the LC-Pro+ chamber to determine and quantify
124 BVOC exchange, and the air exiting the chamber was analyzed by proton transfer
125 reaction-mass spectrometry (PTR-MS; Ionicon Analytik, Innsbruck, Austria). The leaf
126 chamber was connected to the PTR-MS system using a Teflon[®] tube (50 cm long and 2
127 mm internal diameter). The system was identical for all measurements in all treatments
128 and blanks. Floral emission rates were calculated for those masses that showed positive
129 emissions after subtracting the concentrations measured for the blanks from the
130 concentrations of the samples. The floral emission rates were calculated from the
131 difference between the concentrations of VOCs passing through the chamber clamped
132 to the flowers and the chamber without flowers, considering the flow rates and the dry
133 masses of open flowers. Finally, we selected only those VOC masses that showed
134 statistically significant responses to any of the treatments tested, thus discussing and
135 showing the floral emissions of these compounds but not describing the whole floral
136 scent profile of *D. erucoides* that includes those VOCs that are constitutively emitted
137 and did not change their emission rates in response to folivory and/or florivory.

138 PTR-MS is based on chemical ionization, specifically non-dissociative proton
139 transfer from H₃O⁺ ions to most of the common BVOCs and has been fully described
140 elsewhere (Peñuelas et al., 2005). The PTR-MS drift tube was operated at 2.1 mbar and
141 50 °C, with an E/N (electric field/molecule number density) of approximately 130 Td
142 (townsend) (1 Td = 10⁻¹⁷ V cm²). The primary ion signal (H₃O⁺) was maintained at
143 approximately 6 × 10⁶ counts per second. The instrument was calibrated with a mixed

144 aromatic standard gas (TO-14A, Restek, Bellefonte, USA) and a monoterpene standard
145 gas (Abello Linde SA, Barcelona, Spain).

146

147 **2.3 Statistical analyses**

148 We conducted analyses of variance (ANOVAs) with R software (R Development Core
149 Team, 2011) to test the differences between pre- and post-treatment measurements for
150 each compound and treatment. Relative increases in mean floral emission rates between
151 post- and pre-treatment measurements were calculated for each individual. We
152 conducted t-tests with STATISTICA 8 to analyze if relative increases in floral emission
153 rates were significantly higher than 1.

154

155 **3 Results**

156 The feeding by *P. brassicae* larvae on floral tissues produced immediate and radical
157 changes in floral emission rates (Figure 1). The rates of emission of masses 33
158 (methanol), 68 (likely 3-butenenitrile) and 89 (ethyl acetate) increased immediately in
159 the florivory and folivory+florivory treatments (Figure 1). The peaks of 3-butenenitrile
160 and ethyl acetate fluctuated highly on a short timescale. The emissions of methanol
161 were more constant and continuous after the initial increase compared to 3-butenenitrile
162 and ethyl acetate.

163 The floral emissions of the measured masses did not change significantly in the
164 folivory treatment relative to the control treatment throughout the monitored period
165 (Figure 2). The emission rates of methanol, 3-butenenitrile and ethyl acetate from the
166 flowers increased 2.4- ($P=0.055$), 26- ($P=0.099$) and 2.8-fold ($P=0.38$), respectively, in

167 the florivory treatment and 2.9- ($P=0.009$), 100- ($P=0.047$) and 9-fold ($P=0.025$),
168 respectively, in the folivory+florivory treatment relative to the control treatment (Figure
169 3).

170

171 **4 Discussion**

172 **4.1 Floral volatiles enhanced by folivory and florivory**

173 The emission rates of masses 33, 68 and 89 did not increase significantly in the folivory
174 treatment, increased only marginally significantly in the florivory treatment but
175 increased significantly in the folivory+florivory treatment (Figure 2). Only methanol
176 has been detected with PTR-MS at mass 33 (Warneke et al., 2011, 2003). The
177 protonated mass 68 detected by PTR-MS is very likely a glucosinolate derivative, such
178 as 3-butenitrile (molar mass 67). Glucosinolates are a group of chemicals typical in
179 plants of the family Brassicaceae and are usually released after tissue damage,
180 especially due to herbivorous attack (Tsao et al., 2002). Mass 89 is the primary PTR-
181 MS mass for ethyl acetate (Steeghs et al., 2004). The emission rates of mass 89 have
182 also been correlated with those of masses 61 and 71, which are secondary masses of
183 ethyl acetate (Steeghs et al., 2004).

184 Florivory caused an immediate increase in the emission rates of methanol, 3-
185 butenenitrile and ethyl acetate in both the florivory and folivory+florivory treatments
186 (Figure 1). All these compounds are released in high amounts immediately after damage
187 to plant tissues. Methanol is a ubiquitous and well-known VOC that is normally emitted
188 at high rates by undamaged plants but is also locally released in high amounts by
189 wounded tissues (Peñuelas et al., 2005). Methanol is produced from pectin
190 demethylation in the cell walls (Galbally and Kirstine, 2002; Seco et al., 2007), so

191 significant methanol emissions are expected from damaged plant tissues because pectin
192 demethylation occurs in the apoplast, and methanol is a common constituent of the
193 transpiratory stream in plants (Fall and Benson, 1996). Additionally, alkaline oral
194 secretions from lepidopteran larvae induce a change in pH at the wound site that can
195 strongly enhance methanol emissions (von Dahl et al., 2006). The compound emitted
196 most by flowers subjected to florivory, 3-butenitrile, is a glucosinolate derivative and
197 thus has insecticidal activity in plants attacked by herbivores (Tsao et al., 2002). Some
198 degradation products of glucosinolates, such as isothiocyanates, nitriles and
199 thiocyanates, also participate in the induction of stomatal closure after herbivorous
200 attack, suggesting that these degradation products regulate stomatal movements against
201 attacks by phytophagous insects (Hossain et al., 2013). Ethyl acetate is emitted by some
202 plant species in response to herbivorous and pathogenic attack from various plant
203 structures, such as leaves (Zhang et al., 2008), roots (Steeghs et al., 2004) and fruits
204 (Benelli et al., 2013).

205

206 **4.2 Dynamic response of floral emissions to florivory**

207 Floral emissions increased quickly in response to the attack on flowers by *P. brassicae*
208 larvae (Figure 1) but did not change significantly in the final 28 h of the treatments.
209 This immediate response indicated that the VOCs in the flowers were released from the
210 wounded tissues once the larvae had begun to feed. The floral emission rates of 3-
211 butenenitrile and ethyl acetate fluctuated highly on a short timescale (Figure 1), which
212 may indicate a very fast response of these compounds to the dynamic fluctuations in the
213 intensity of the damage caused by the feeding *P. brassicae* larvae. The emission rates of
214 methanol, however, were more constant after the initial increase in response to attack.

215 An increase in methanol emissions by wounded plant tissues can be mostly due to the
216 direct release from internal tissues after damage (Peñuelas et al., 2005).

217

218 **4.3 Herbivore-induced plant volatiles and systemic defensive responses**

219 Defensive compounds can deter both detrimental and beneficial visitors to flowers in a
220 similar way. The constitutive emission of repellent compounds to deter herbivores can
221 thus imply disadvantages to plant fitness by the interference of pollination, which can
222 sometimes exceed the benefits of avoiding enemies (Lucas-Barbosa et al., 2011).

223 Selective pressures may then reduce or eliminate such deterrent compounds from floral
224 emissions, due to the negative impact they have on plant fitness. From this viewpoint,
225 plants may benefit from presenting defenses that are activated only when necessary,
226 such as the HIPVs emitted after herbivorous attack. Induced defensive responses
227 provide benefits to plants compared to constitutive defenses, such as their activation
228 only when needed, representing a more optimal investment of resources for defense
229 (Pare and Tumlinson, 1999).

230 The induced emission of HIPVs during the flowering season, however, can
231 imply detrimental effects on plant pollination (Lucas-Barbosa et al., 2011). The
232 emission of HIPVs can be systemically induced from damaged to undamaged leaves
233 (Dong et al., 2011; Rodriguez-Saona et al., 2009) and to undamaged flowers (Kessler
234 and Halitschke, 2009; Theis et al., 2009). This systemic induction of deterrent emissions
235 from damaged to undamaged plant tissues can also interfere with the attraction of
236 pollinators, but some species can avoid the induction of HIPVs when they can interfere
237 with pollinator attraction. HIPV emissions from *Datura wrightii*, for example, are high
238 during the vegetative phase but decline after the beginning of flowering and fruit

239 production (Hare, 2010). This timing may avoid the counterproductive effect of HIPVs
240 on pollinator visits.

241 We found no evidence for a systemic induction of defensive floral VOC
242 emissions in response to folivory in *D. erucoides*. Folivory combined with florivory,
243 however, increased floral VOC emissions, perhaps by inducing a synergistic systemic
244 effect. *D. erucoides* plants grow quickly and flower early and for a substantial portion
245 of their lives. The long flowering period may have generated selection pressures to
246 suppress herbivory-induced systemic responses in this species to avoid interference with
247 pollinator attraction. Florivory caused only a local immediate increase in the emission
248 rates of some volatiles in flowers damaged by *P. brassicae* larvae. This local defensive
249 response may only deter herbivores temporarily at the site of damage so may not
250 interfere with the pollination of distant undamaged flowers that are still attractive and
251 viable. Similarly, *Nicotiana suaveolens* plants subjected to green-leaf herbivory emitted
252 HIPVs from leaves but not from flowers, suggesting that the response to herbivory was
253 systemic among leaves but was not transmitted to flowers (Effmert et al., 2008). In fact,
254 flowers can show no induction of enhanced floral emissions in response to folivory and
255 can even reduce their emissions due to tradeoffs between pollinator attraction and
256 indirect defenses induced in other plant tissues (Schiestl et al., 2014).

257

258 **4.4 Synergistic effect of the folivory+florivory treatment**

259 Folivory alone had no clear significant effects on the emissions rates of floral volatiles.
260 A synergistic effect on the emission rates of floral VOCs, however, was evident when
261 folivory was combined with florivory. The relative increases in the emission rates of
262 methanol, 3-butenenitrile and ethyl acetate between pre and post-treatment were 1.2-, 4-

263 and 3-fold higher, respectively, in the plants subjected to the combined treatment than in
264 the plants subjected only to florivory (Figure 3).

265 All these results strongly suggest a synergistic effect of folivory and florivory.
266 Such an effect may intensify the magnitude of the chemical defensive response when
267 both flowers and leaves are attacked, which usually indicates a wider degree of
268 infestation. Plants may benefit from increasing their defenses when herbivorous attack
269 is more severe and generalized compared to mild and local attacks. These results are the
270 first reported indication of a synergistic effect of folivory and florivory on floral
271 emissions.

272

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431 **Figure captions**

432 **Figure 1.** Dynamics of floral emission rates of masses 33 (methanol), 68 (likely 3-butenitrile)
433 and 89 (ethyl acetate) from one individual of each treatment on a short timescale before and
434 after herbivorous attack. The dashed line shows the time point when herbivores were applied on
435 the plants and treatments started.

436 **Figure 2.** Mean floral emission rates of masses 33 (methanol), 68 (likely 3-butenitrile) and 89
437 (ethyl acetate) before and after treatment application ($n=5$ plants). For the after treatment
438 floral emission rates we first calculated a mean value for each of the four post-treatment
439 measurements per each individual plant. Then, after observing that post-treatment floral
440 emissions were sustained and did not significantly change along successive
441 measurements, a mean value among the four post-treatment measurements was
442 calculated. Finally we calculated the mean and the standard error for floral emission
443 rates of each treatment with the means obtained for the five plant replicates. Error bars
444 indicate standard errors of the means. Asterisks indicate significant differences between
445 pre- and post-treatment measurements ($(^*) P<0.1$, $* P<0.05$).

446 **Figure 3.** Mean relative increase (relative to 1, dotted lines) in floral emission rates of masses
447 33 (methanol), 68 (likely 3-butenitrile) and 89 (ethyl acetate) after treatment ($n=5$ plants). The
448 whole post-treatment means calculated with the means for the four post-treatment
449 measurements were divided by the respective pre-treatment means to obtain a relative increase
450 in floral emission rates. Error bars indicate standard errors of the means. Asterisks indicate
451 statistically significant relative increases (t -test, $(^*) P<0.1$, $* P<0.05$, $** P<0.01$).

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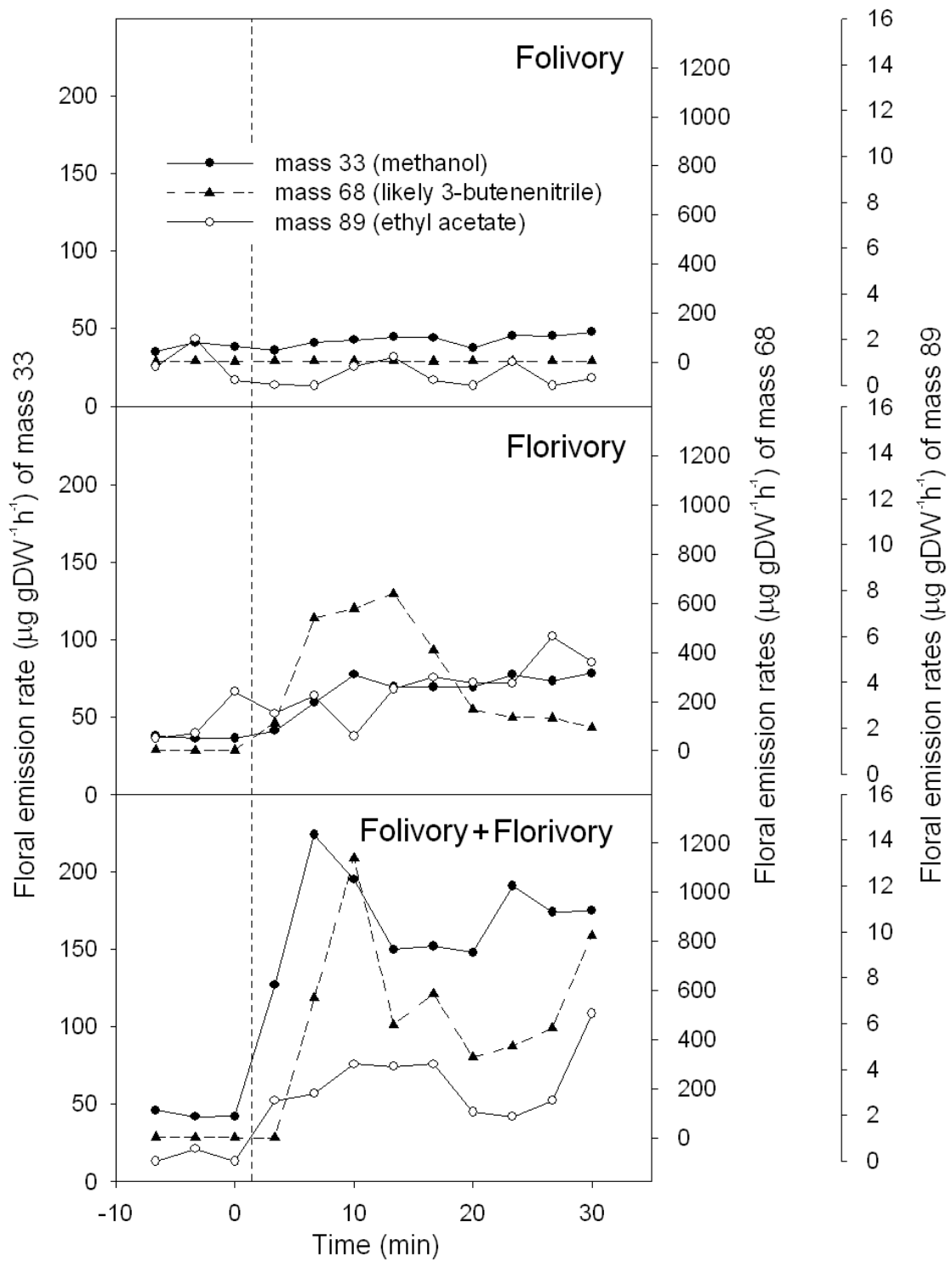
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457 **Figure 1**



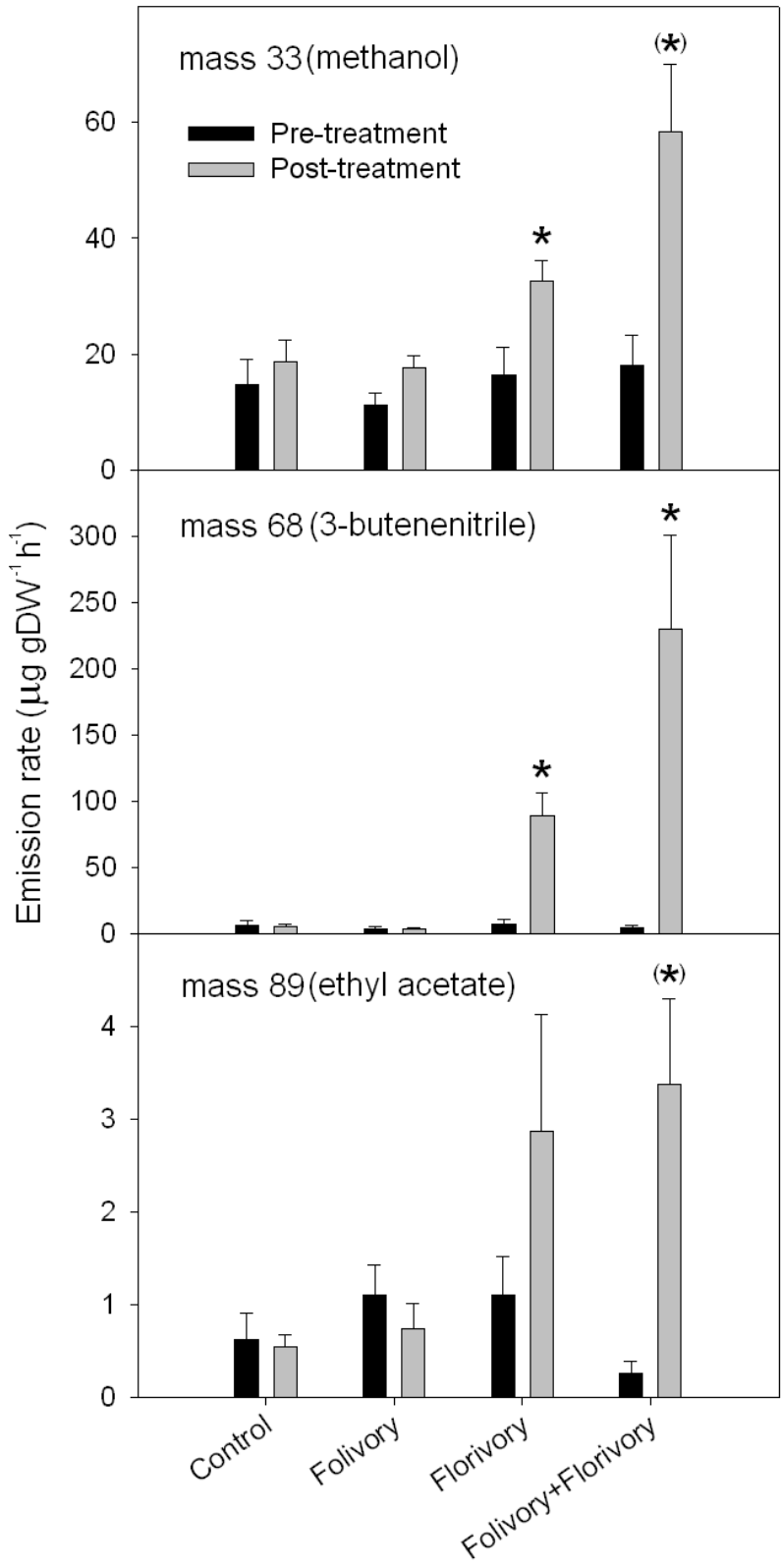
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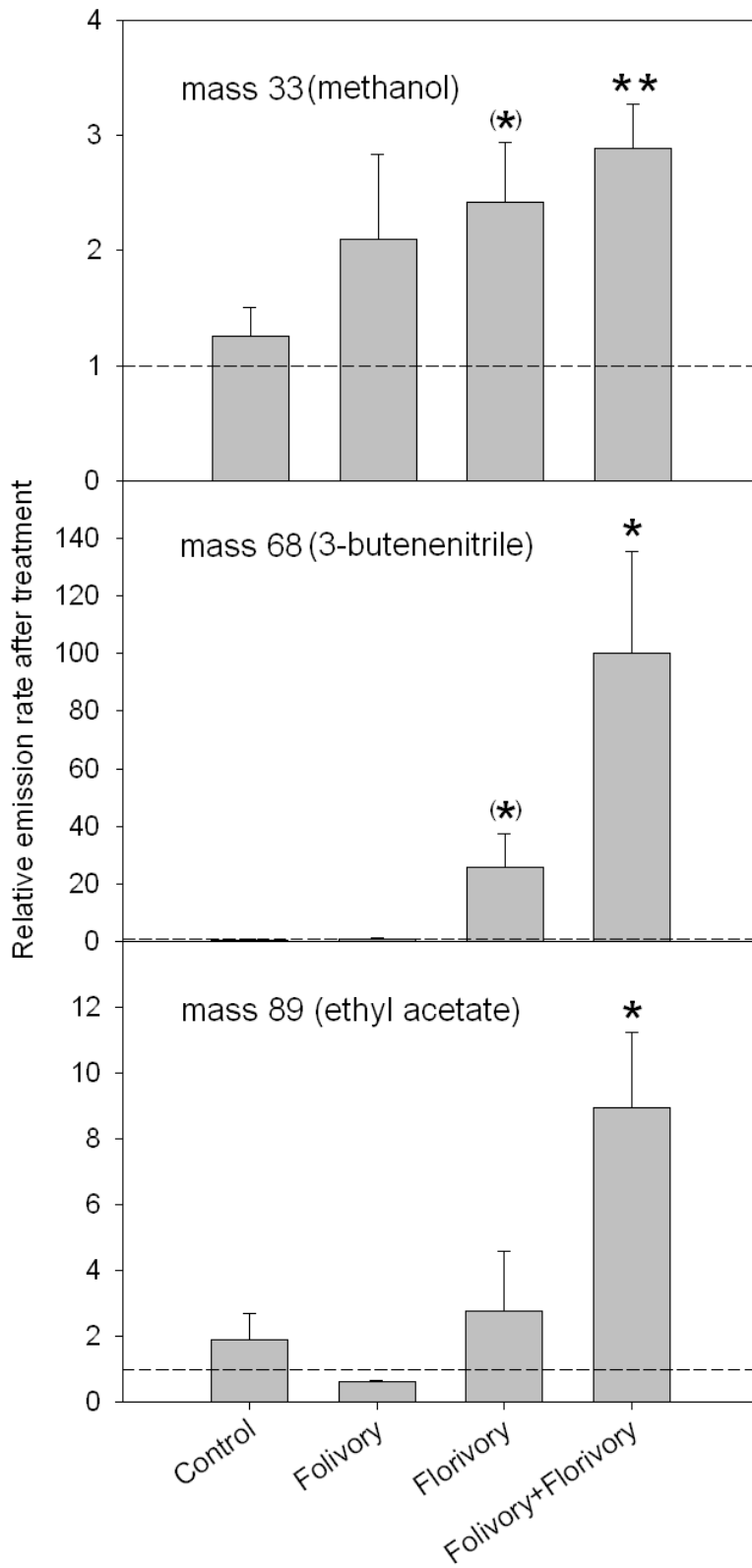
462 **Figure 2**



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465 **Figure 3**



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