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

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Abstract

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

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

1 Introduction

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

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

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

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

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

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

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

2 Materials and methods

2.1 Experimental design of bioassays

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

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

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

We used a portable infrared gas analyzer (IRGA) system (LC-Pro+, ADC BioScientific Ltd., Herts, England) with a conifer leaf chamber (175 cm^3) to sample floral VOC emissions at standard conditions of temperature (30 ºC) and light (PAR=1000 μmol m^{-2} s^{-1}). An inflorescence containing 4-11 open flowers was enclosed in the chamber without detaching the flowers from the plant. For samples in the florivory and folivory+florivory treatments, we put the inflorescences with the larvae in the chamber and recorded the times at which the larvae began to feed for detecting and measuring floral VOCs instantaneously released by wounded floral tissues. We also measured several blank samples containing only larvae to identify possible larval emissions and to distinguish them from the floral emissions.

2.2 Biogenic VOC (BVOC) exchange measurements

Flower samples were clamped into the leaf chamber (175 cm^3) of an LC-Pro+ Photosynthesis System (ADC BioScientific Ltd., Herts, England). Flow meters monitored the air flowing through the LC-Pro+ chamber to determine and quantify BVOC exchange, and the air exiting the chamber was analyzed by proton transfer reaction-mass spectrometry (PTR-MS; Ionicon Analytik, Innsbruck, Austria). The leaf chamber was connected to the PTR-MS system using a Teflon® tube (50 cm long and 2 mm internal diameter). The system was identical for all measurements in all treatments and blanks. Floral emission rates were calculated for those masses that showed positive emissions after substracting the concentrations measured for the blanks from the concentrations of the samples. The floral emission rates were calculated from the difference between the concentrations of VOCs passing through the chamber clamped to the flowers and the chamber without flowers, considering the flow rates and the dry masses of open flowers. Finally, we selected only those VOC masses that showed statistically significant responses to any of the treatments tested, thus discussing and showing the floral emissions of these compounds but not describing the whole floral scent profile of *D. erucoides* that includes those VOCs that are constitutively emitted and did not change their emission rates in response to folivory and/or florivory.

PTR-MS is based on chemical ionization, specifically non-dissociative proton transfer from H_3O^+ ions to most of the common BVOCs and has been fully described elsewhere (Peñuelas et al., 2005). The PTR-MS drift tube was operated at 2.1 mbar and 50 ºC, with an E/N (electric field/molecule number density) of approximately 130 Td (townsend) (1 Td = 10^{-17} V cm^2). The primary ion signal (H_3O^+) was maintained at approximately 6×10^6 counts per second. The instrument was calibrated with a mixed

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

2.3 Statistical analyses

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

3 Results

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

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

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

4 Discussion

4.1 Floral volatiles enhanced by folivory and florivory

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

Florivory caused an immediate increase in the emission rates of methanol, 3-butenenitrile and ethyl acetate in both the florivory and folivory+florivory treatments (Figure 1). All these compounds are released in high amounts immediately after damage to plant tissues. Methanol is a ubiquitous and well-known VOC that is normally emitted at high rates by undamaged plants but is also locally released in high amounts by wounded tissues (Peñuelas et al., 2005). Methanol is produced from pectin 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-butenenitrile, 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.

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

4.3 Herbivore-induced plant volatiles and systemic defensive responses

Defensive compounds can deter both detrimental and beneficial visitors to flowers in a similar way. The constitutive emission of repellent compounds to deter herbivores can thus imply disadvantages to plant fitness by the interference of pollination, which can sometimes exceed the benefits of avoiding enemies (Lucas-Barbosa et al., 2011). Selective pressures may then reduce or eliminate such deterrent compounds from floral emissions, due to the negative impact they have on plant fitness. From this viewpoint, plants may benefit from presenting defenses that are activated only when necessary, such as the HIPVs emitted after herbivorous attack. Induced defensive responses provide benefits to plants compared to constitutive defenses, such as their activation only when needed, representing a more optimal investment of resources for defense (Pare and Tumlinson, 1999).

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

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

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

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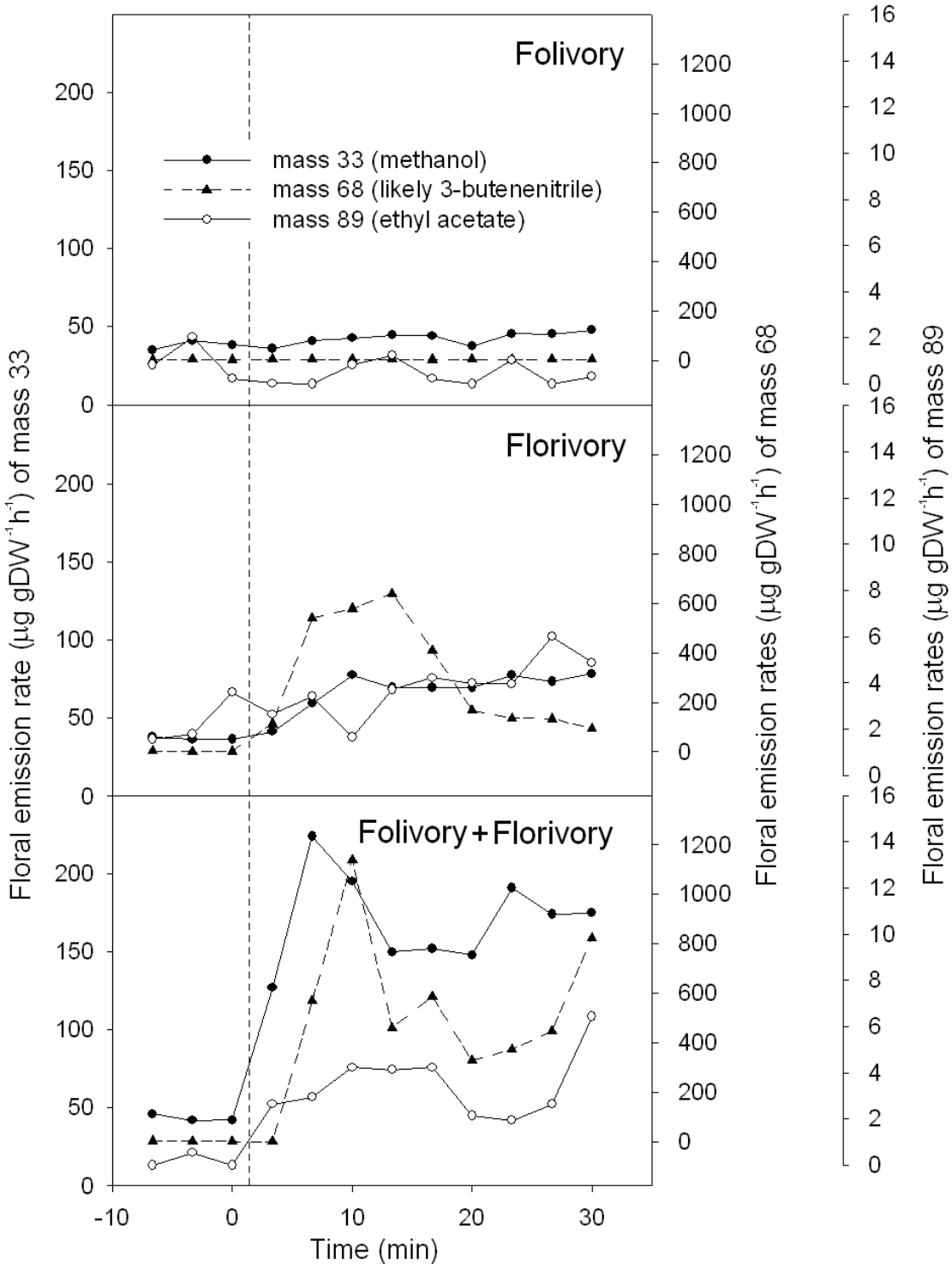
Figure captions

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

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

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

457 **Figure 1**



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462 **Figure 2**

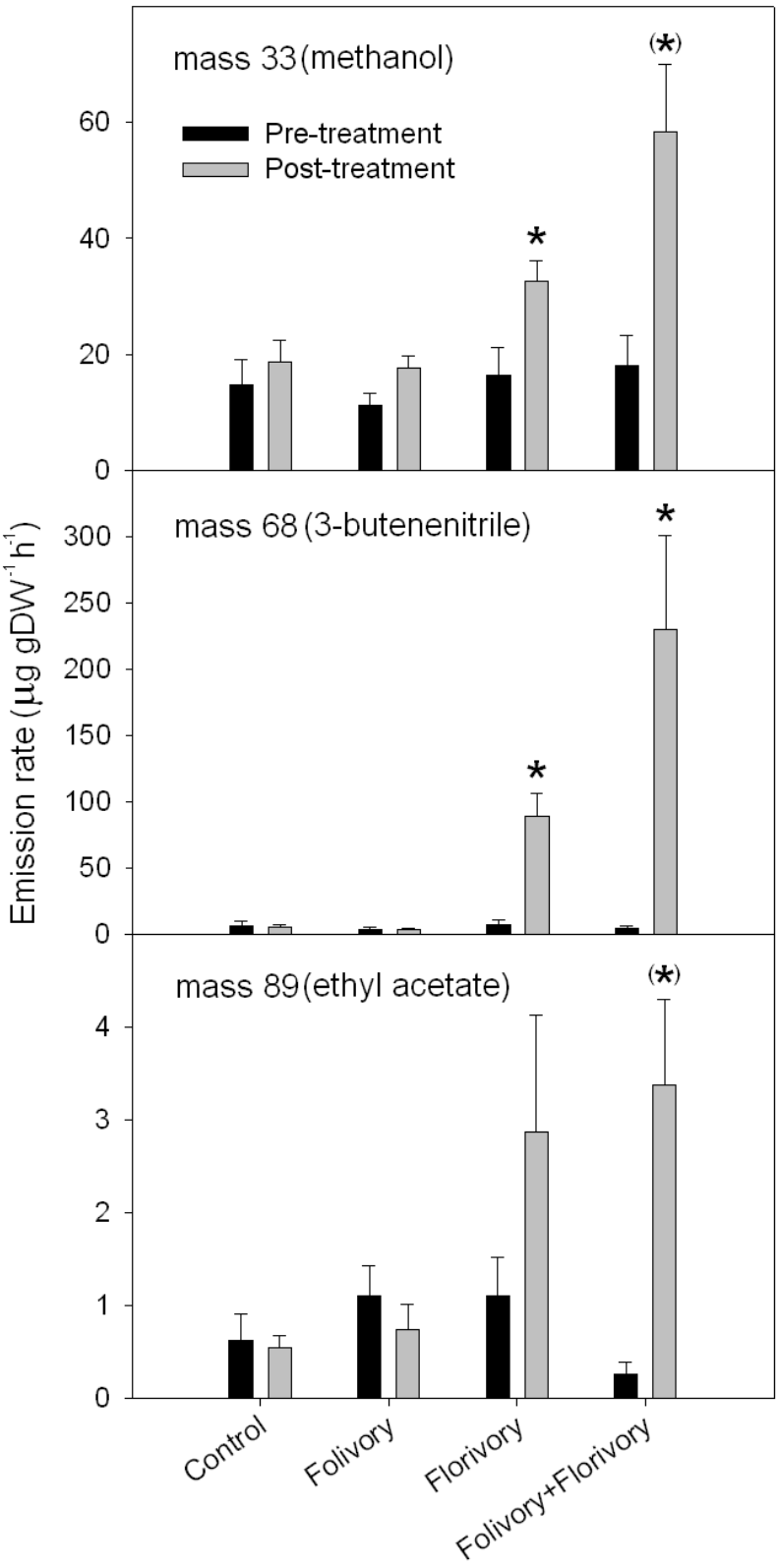

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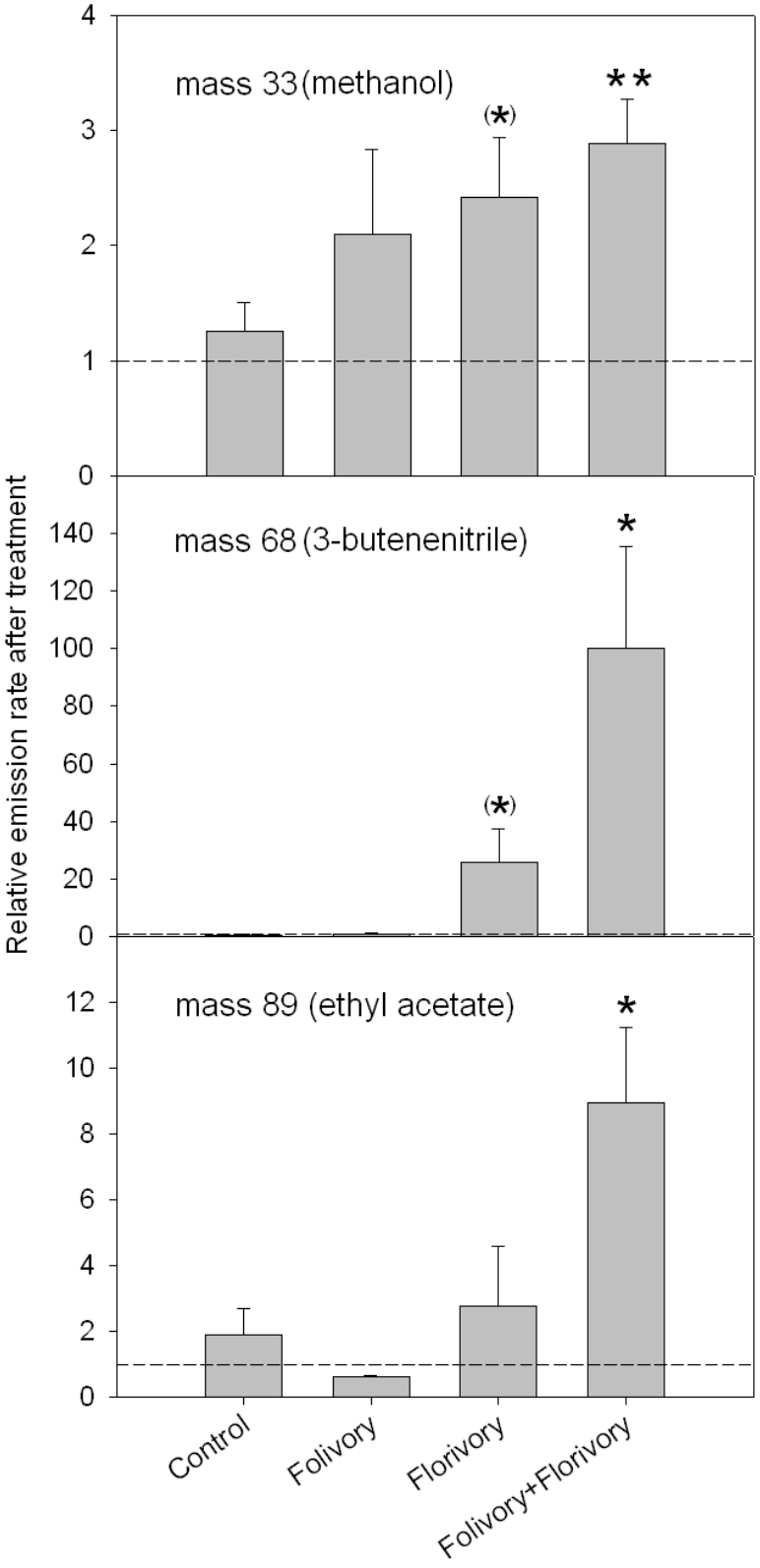
Figure 3


mass 33 (methanol)
(*)
**
mass 68 (3-butenenitrile)
(*)
*
mass 89 (ethyl acetate)
*
Relative emission rate after treatment
Control
Folivory
Florivory
Folivory+Florivory