

Optimum temperature for floral terpene emissions tracks the mean temperature of the flowering season

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1 **Abstract**

2 Emissions of volatiles from leaves exhibit temperature dependence with maximums, but
3 optimum temperatures for the release of floral volatiles or the mechanism of
4 optimization of these emissions have not been determined. We hypothesized that
5 flowers have an optimum temperature for the emission of volatiles and, because the
6 period of flowering varies highly among species, that this optimum is adapted to the
7 temperatures prevailing during flowering. To test these hypotheses, we characterized the
8 temperature responses of floral terpene emissions of diverse widespread Mediterranean
9 plant species flowering in different seasons by using dynamic headspace sampling and
10 analysis with gas chromatography mass spectrometry. The floral emissions of terpenes
11 across species exhibited maximums at the temperatures corresponding to the season of
12 flowering, with the lowest optimal temperatures observed in winter-flowering and the
13 highest in summer-flowering species. These trends were valid for emissions of both
14 total terpenes and the various terpene compounds. The results show that the optimum
15 temperature of floral volatile emissions scales with temperature at flowering and
16 suggest that this scaling is the outcome of physiological adaptations of the biosynthetic
17 and/or emission mechanisms of flowers.

18 **Keywords:** flower scent, interspecific variation, phenology, seasonal variability.

19

20 **Introduction**

21 Floral emissions of volatile organic compounds (VOCs) constitute important olfactory
22 signals for pollinators to locate and identify flowers and thus mediate pollination in
23 entomophilous angiosperms (Dudareva et al. 2006). Floral emissions, however, are
24 susceptible to diverse biotic and abiotic factors that can lead to significant changes in

25 emission rates and composition, thereby interfering with or affecting chemical
26 communication between plants and pollinators (Farré-Armengol et al. 2013; Farré-
27 Armengol et al. 2014). Several environmental factors can affect the emission of VOCs
28 from various plant tissues; the effects of temperature and light on foliar terpene
29 emissions are the best studied (Peñuelas and Llusia 2001; Niinemets et al. 2004; Grote
30 et al. 2013). The responses of terpene emissions from leaves to temperature are well
31 characterized (Niinemets et al. 2010) and are known to be determined by temperature
32 dependencies of the physicochemical properties of terpenes, such as volatility, solubility
33 and diffusivity, and by the effects of temperature on foliar physiology, such as terpene
34 biosynthesis or stomatal resistance (Reichstein et al. 2002; Niinemets et al. 2004;
35 Harley 2013). The responses of terpene emissions from flowers to temperature are less
36 known. However, we argue here that the need of maximization of the intensity of floral
37 olfactive signals to enhance the ability of pollinators to locate flowers has likely exerted
38 a selective pressure on floral physiology to tune the maximum floral emissions to the
39 temperature ranges to which the flowers of each species are typically exposed.

40 Species from cooler environments have lower optimum temperatures for
41 photosynthesis than do species living in warmer environments, which reveals a positive
42 correlation between species-specific optimum temperature for photosynthesis and the
43 range of ambient temperatures in which the species live (Berry and Björkman 1980;
44 Niinemets et al. 1999; Medlyn et al. 2002). The optimum foliar temperature for
45 photosynthesis also varies within species, depending on the range of temperatures under
46 which individuals grow, indicating an additional physiological process of acclimation
47 (Cleveland et al. 1992; Kattge and Knorr 2007). In species that do not store terpenes,
48 the rates of terpene emission have temperature response curves similar to those of
49 photosynthesis (Copolovici and Niinemets 2005; Llusia et al. 2006; Niinemets et al.

50 2010). In fact, terpene biosynthesis and physiological processes related to the emission
51 of terpenes are affected by temperature in a way similar to that of photosynthetic rates.
52 Moreover, the biosynthetic pathways responsible for the production of terpenes are
53 dependent on the rates of carbon assimilation, and the acclimation of temperature
54 responses of the rates of terpene emission has also been proposed (Staudt et al. 2003;
55 Niinemets 2004). We hypothesized that plant species may thus be expected to
56 experience adaptive trends to fine-tune the temperature responses of floral emissions to
57 match the thermal environment the flowers typically encounter throughout the period of
58 flowering. In this study, we aimed to test this hypothesis in Mediterranean species
59 flowering at different times of the year.

60 Most Mediterranean angiosperms flower in spring. Some species, however,
61 flower in summer, autumn or even winter. Flowers are thus exposed to different
62 temperature ranges and can potentially evolve different temperature sensitivities of their
63 floral emissions. The flowers of winter-flowering species are exposed to low
64 temperatures and therefore are expected to adapt their optimal floral emissions to low
65 temperature ranges. In contrast, summer-flowering species may adapt their floral
66 emissions to high temperatures. Such different responses can result from differences in
67 the composition of volatiles emitted by the species and from physiological
68 modifications in the production and release of volatiles.

69 We tested the hypothesis that optimum temperatures maximizing floral terpene
70 emissions depend on the temperatures prevailing during the flowering period. The
71 hypothesis was tested with seven Mediterranean species flowering at different times of
72 the year for which we had previously studied the responses of floral BVOCs emission
73 rates to warming (Farré-Armengol et al. 2014). We also sampled terpene emissions at
74 two different times during the flowering period in the Mediterranean perennial herb

75 *Dittrichia viscosa* to explore whether the optimum temperatures for floral emissions can
76 also vary within species having prolonged flowering periods extending over widely
77 differing temperatures.

78

79 **Methods**

80 ***Study site and species sampled***

81 The study was conducted at various field locations within the province of Barcelona
82 (Catalonia, Spain). Six common Mediterranean species of anemophilous plants in
83 Garraf national park (UTM: 31T, 409km, 4570km; *Dorycnium pentaphyllum* Scop.,
84 *Erica multiflora* L., *Globularia alypum* L.) and Cerdanyola del Vallès (UTM: 31T,
85 426km, 4595km; *Spartium junceum* L., *Sonchus tenerrimus* L., *Dittrichia viscosa* (L.)
86 Greuter), and one anemophilous plant in Collserola national park (UTM: 31T, 427km,
87 4592km; *Quercus ilex* L.) were included in the analysis. Floral emissions from *D.*
88 *viscosa* were collected in late summer and again in early autumn. In each of the two
89 series of measurements conducted on *D. viscosa* we sampled individuals from two
90 different populations from very close locations (2-3 km) in Cerdanyola del Vallès. The
91 species sampled include a wide range of flowering periods with different mean
92 temperatures (Table S1, Suppl. Mat.). For a same location, we measured floral
93 emissions for species flowering during different seasons.

94

95 ***Temperature-response curves***

96 Samples of emissions were collected using a dynamic headspace technique. A portable
97 infrared gas analyzer (IRGA) system (LC-Pro+, ADC BioScientific Ltd., Great Amwell)

98 was employed to measure gas exchange and to provide a constant light intensity of 1000
99 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and the required temperatures. The temperature responses of floral
100 emissions were measured in the field over a range of temperatures of 15-40 °C at
101 intervals of 5 °C. The IRGA system used reached a maximum temperature of 40°C. The
102 maximum temperature reached in the winter measurements, however, was only 30 °C
103 because the IRGA system was unable to heat the ambient air to higher temperatures.

104 One or several attached flowers were enclosed in the chamber of the IRGA (*G.*
105 *alypum*: 1 capitula, *E. multiflora*: 8-12 flowers, *Q. ilex*: 1 male inflorescence, *D.*
106 *pentaphyllum*: 10-15 flowers, *S. junceum*: 4-5 flowers, *S. tenerrimus*: 1 capitula, *D.*
107 *viscosa*: 5-9 capitula). We used two different chambers depending on the size of the
108 flowers of each species. A 12 cm³ chamber was used at a flow rate of 450-500 ml min⁻¹
109 for *G. alypum*, *E. multiflora*, *Q. ilex*, *D. pentaphyllum* and *S. tenerrimus*, and a 175 cm³
110 chamber was used at a flow rate of 250-300 ml min⁻¹ for *S. junceum* and *D. viscosa*. We
111 collected the samples of terpene emissions after setting the required quantum flux
112 density and temperature and after an acclimation period of approximately 10 min or the
113 time needed to reach a steady-state exchange of CO₂ and H₂O. The enclosed flowers
114 were sequentially submitted to different temperatures, and their emissions were sampled
115 for additional 10 min. The air exiting the chamber of the IRGA, at a mean flux of air of
116 approximately 200-250 ml min⁻¹, was directed through a Teflon tube to a stainless
117 steel tube (89 mm in length and 6.4 mm external diameter) filled with the adsorbents
118 Tenax (114.6 mg, 50% vol.) and Carbotrap (236.8 mg, 50% vol.), separated by sorbent-
119 retaining springs (Markes International Inc. Wilmington, USA) fixed using gauze-
120 retaining springs (Markes International Inc. Wilmington, USA) and closed with air-tight
121 caps (Markes International Inc. Wilmington, USA), which collected the terpenes
122 emitted by the flower(s) over a period of 10-15 min. The same process was repeated

123 with empty chambers of the IRGA that served as blanks of the system. At least two
124 blank samples were collected for each curve, one at the beginning of the emission
125 samplings and another at the end. We collected 3-5 replicate samples of emissions per
126 species (*G. alypum*: 5, *E. multiflora*: 4, *Q. ilex*: 4, *D. pentaphyllum*: 5, *S. junceum*: 5, *S.*
127 *tenerrimus*: 4, *D. viscosa* late summer: 3, *D. viscosa* early autumn: 3). Each replicate was
128 collected from a different plant. At the end of each sampling sequence we collected the
129 flower samples from which emissions were collected and we dried and weighed the
130 flowers for emission rate calculations. Sampled tubes were stored in a freezer at -25°C
131 until we conducted the analyses by GC-MS.

132

133 ***Terpene analyses***

134 The terpene samples in the adsorbent tubes were thermally desorbed using an injector
135 (Unity, Series 2, Markes International Inc. Wilmington, USA) and released with an
136 automatic sample processor (TD Autosampler, Series 2 Ultra, Markes International Inc.
137 Wilmington, USA) to be analyzed by an Agilent gas chromatography mass spectrometry
138 (GC-MS) system (Agilent Technologies, GC: 7890A, MS: 5975C inert MSD with
139 Triple-Axis Detector, Palo Alto, CA, USA). The desorbed sample was retained in a
140 cryo-trap at -25°C. The split was 2:1. The sample was desorbed again at 320°C for 15
141 and 10 min and injected into the column with a transfer line at 250°C. Samples were
142 injected into a 30 m x 0.25 mm x 0.25 µm capillary column (HP-5MS, Agilent
143 Technologies). Helium flow was 1 ml min⁻¹, and total run time was 26 min. After
144 injection, the sample was maintained at 35 °C for 1 min, the temperature was then
145 increased at 15 °C min⁻¹ to 150 °C and maintained for 5 min, then increased at 50 °C

146 min⁻¹ to 250 °C and maintained for 5 min and then increased at 30 °C min⁻¹ to 280 °C
147 and maintained for 5 min.

148

149 The terpenes were identified by comparing the retention times with standards
150 from Fluka (Buchs, Switzerland) that had been injected into clean adsorbent tubes, and
151 the fractionation mass spectra were compared with standard spectra and spectra in the
152 Nist05a and wiley7n mass spectral libraries. Terpene concentrations were determined
153 from the calibration curves. Calibration curves for the common terpenes α -pinene, β -
154 pinene, limonene, γ -terpinene, linalool and α -humulene were determined daily. The
155 terpene calibration curves ($n=4$ different terpene concentrations from $0.33 \cdot 10^{-4}$ to 0.33
156 mL L⁻¹) were always highly significant ($R^2 > 0.99$ for the relationship between the signal
157 and the amount of compound injected).

158

159 *Statistical analysis*

160 We used the *loess* function of the *stats* package from R (R Development Core Team
161 2011) to characterize the shape of the curve of the temperature responses of floral
162 terpene emissions and to determine the optimum temperature for floral terpene
163 emissions. Optimum temperature for floral terpene emissions was considered to be the
164 temperature at which flowers emit the maximum terpene emission rates. The *loess*
165 function fits local polynomial functions to the data in different ranges of the
166 independent variable (Cleveland et al. 1992). We used SigmaPlot 11.0 to visualize the
167 data and to determine the relationship between optimum temperature for floral terpene
168 emissions (the temperature at which floral terpene emissions of a particular species

169 reached highest emission rates) and mean temperature of the month of the flowering
170 peak by linear regression models.

171

172 *Optimum temperature for floral emissions*

173 The mean ambient temperature for the month of the flowering peak for each species in
174 the region from which the species was sampled was calculated as the average for the
175 period 1971-2000 (Servei Meteorològic de Catalunya 2010). The optimum temperatures
176 for floral emissions of each species were obtained from the maxima of the fitted
177 temperature-response curves. Optimum temperatures for each terpene present in the
178 floral emissions from each species were estimated as the temperatures at the highest
179 emission of that compound.

180

181 **Results**

182 *G. alypum* and *E. multiflora* flowers emitted detectable amounts of α -pinene, camphene,
183 3-carene and D-limonene (Table S2, Suppl. Mat.). *Q. ilex* male flowers emitted α -
184 pinene, β -pinene, camphene, 3-carene and D-limonene. *D. pentaphyllum* flowers
185 emitted 3-carene, (E)- β -ocimene and (Z)- β -ocimene. *S. junceum* flowers emitted α -
186 pinene and α -farnesene. *S. tenerrimus* flowers emitted α -pinene and 3-carene. *D.*
187 *viscosa* flowers of late summer emitted α -pinene, β -pinene, α -phellandrene, β -
188 phellandrene, camphene, 3-carene, D-limonene, eucalyptol, γ -terpinene, α -terpinolene
189 and α -thujene. *D. viscosa* flowers of early autumn emitted α -pinene, β -pinene, α -
190 phellandrene, camphene, 3-carene and D-limonene (Table S2, Suppl. Mat.).

191 The rates of terpene emission initially increased with temperature in all species
192 and generally reached a maximum (Fig. 1). The temperature-response curves of floral
193 terpene emissions showed species-specific differences. The rates of floral emission of
194 winter-, autumn- and spring-flowering species began to decline at different temperatures,
195 usually between 30 and 40 °C, and the emissions from summer-flowering species did
196 not decline within the range of temperatures included in our measurements. The winter-
197 flowering species *G. alypum* and *E. multiflora* exhibited maximum floral terpene
198 emissions at 25 °C and 30 °C, respectively. Floral emissions from *Q. ilex* reached a
199 maximum at approximately 30 °C. In the spring-flowering *D. pentaphyllum*, the rates of
200 floral terpene emission increased with increasing temperature up to 35 °C, and a
201 moderate reduction was observed at 40 °C. The rates of terpene emission in the flowers
202 of *S. junceum*, *D. viscosa* and *S. tenerrimus* sampled in late spring and summer
203 increased with increasing temperature, even up to 40 °C, whereas the summer flowers
204 of *D. viscosa* and *S. tenerrimus* experienced a maximum increase only from 35 to 40 °C.
205 In early autumn, the maximum emission from *D. viscosa* flowers was at 25-30 °C (Fig.
206 1).

207 The optimum temperature for floral emissions of all terpenes for each species
208 were positively and linearly correlated with the mean temperature of the month of the
209 flowering peak (Pearson's $r=0.91$, $P=0.002$, Fig. 2). Across the species sampled, the
210 optimum temperatures for floral emissions of each terpene compound were also
211 positively and linearly correlated with the mean temperature of the month of the
212 flowering peak (α -pinene, $r=0.85$, $P=0.02$; camphene, $r=0.91$, $P=0.03$; $r=0.96$, β -pinene,
213 $P=0.17$; 3-carene, $r=0.88$, $P=0.008$; D-limonene, $r=0.99$, $P<0.001$; Fig. 3).

214

215 **Discussion**

216 Our data demonstrate that the well-known temperature-dependent increase of terpene
217 emissions previously reported for leaves also occurs in flowers (Fig. 1). The
218 temperature responses of floral volatile emission generally exhibited an optimum,
219 suggesting that these emissions reflect de novo synthesis of terpenes (Niinemets et al.
220 2010; Li and Sharkey 2013; Monson 2013). The temperature dependence function for
221 de-novo synthesized isoprenoids considers an Arrhenius type response which describes
222 a curve with an optimum (Niinemets et al. 2010). This optimum represents a threshold
223 temperature from which physiological processes involved in isoprenoid biosynthesis are
224 limited or completely inhibited. On the other hand, the emission rates for species that
225 store monoterpenes in specialized plant tissues are suggested to be controlled only by
226 physical evaporation and diffusion, two processes that do not decline but present a
227 sustained increase with temperature.

228 As we hypothesized, species flowering in different seasons had optimum
229 temperatures for floral emissions that paralleled the mean temperature of the month of
230 the flowering peak (Fig. 2). The positive correlation between the temperature optimum
231 for floral emission and ambient temperature generally resembled the correlation
232 between optimum temperature for photosynthesis and ambient temperature (Berry and
233 Björkman 1980; Niinemets et al. 1999; Kattge and Knorr 2007). Species flowering in
234 cold seasons had maximum emissions at lower temperatures than did species flowering
235 in warm seasons. Our results thus supported the hypothesis that the temperature
236 responses of floral terpene emissions were adapted to the temperature ranges to which
237 the flowers were exposed during flowering. Even though we were not able to determine
238 the precise optimum temperature for floral emissions in summer species, we clearly
239 demonstrated that it was above 40°C. If we could obtain the real optimum for these

240 species, the difference between optimums for species flowering in cold and warm
241 seasons would increase, strengthening the significance of our conclusions. The faster
242 increases in floral terpene emission rates with temperature in early-flowering
243 entomophilous species show that these species are more sensitive to temperature
244 increases than species flowering in spring or summer, which is in accordance with the
245 observed higher responsiveness of early-flowering plants to climate warming by
246 advancing more their flowering phenology (Dunne et al. 2003; Cleland et al. 2007).
247 Also, different flowering seasons combine changes in temperatures with changes in the
248 length of the day (hours of daylight), which may also play a role on floral terpene
249 emissions (Colquhoun et al. 2013).

250 Our results also showed that the emission rates of each terpene compound also
251 tended to have an emission optimum, and that this optimum was positively correlated
252 with the mean temperature of the month of the flowering peak of that species (Fig. 3).
253 This response of the individual terpene compounds indicated that the differences in the
254 optimum temperature for total terpene emissions among species was not due to the
255 differences in the compounds that constitute the scents of flowers, but reflected
256 physiological adaptation of underlying biochemical processes. Terpene production in
257 summer-flowering species has thus been adapted such that floral terpene emissions are
258 maximized at high temperatures and are strongly curbed at low temperatures. In contrast,
259 terpene production in winter-flowering species has been adapted to maximize floral
260 emissions at low temperatures. This pattern is clearly supported in the insect-pollinated
261 species explored in this study. We only studied one wind-pollinated species, *Q. ilex*.
262 *Quercus ilex* also fits into this pattern, indicating that adaptation of optimum
263 temperature for floral terpene emissions to ambient temperature of the flowering season
264 might not be exclusively linked to biotic pollination.

265 We observed different temperature responses of floral terpene emissions in *D.*
266 *viscosa* in late summer and early autumn. *Dittrichia viscosa* plants can flower
267 abundantly over a long period of 4-5 weeks, which allowed us to conduct a second
268 series of measurements some weeks after the first measurements. The two series of
269 measurements were thus conducted during the same flowering event, but at different
270 moments (Table 1, 17-25 September and 23-30 October). Analogous intraspecific
271 seasonal differences in the responses of terpene emissions to environmental conditions
272 have been observed for leaves (Llusia et al. 2006; Helmig et al. 2013). These results
273 suggest that temperature dependencies of floral emissions can vary even within
274 individuals of the same species, at least in those species that can flower under different
275 temperature conditions, and indicate some degree of phenotypic, epigenetic or
276 genotypic plasticity in the physiology of the flowers of these species, which clearly
277 constitutes an important adaptive modification to optimize flower emissions at diverse
278 temperature ranges.

279 Such plasticity in the physiology of flowers controlling terpene floral emissions
280 could be adaptations of the terpene biosynthetic and/or release mechanisms of floral
281 volatiles. The biosynthetic pathways involved in the production of some terpene
282 volatiles are well described (Dewick 2002; Dubey et al. 2003; Kuzuyama and Seto
283 2003), and the mechanisms that regulate terpene biosynthetic rates have been
284 extensively investigated (Dudareva and Pichersky 2000; Fischbach et al. 2002;
285 Dudareva et al. 2004; van Schie et al. 2006). The key controls operating in terpene
286 production are the transcription, production and activity of enzymes and the
287 concentrations of the substrates of these enzymes (Dudareva and Pichersky 2000;
288 Fischbach et al. 2002; Dudareva et al. 2004; van Schie et al. 2006). On the other hand,
289 some mechanisms that mediate and control terpene release (e.g. stomatal closure,

290 compound volatility and mechanisms of transport of terpenes across the cell) can
291 regulate the rates of diffusion from internal terpene pools to the exterior and can thereby
292 also limit the rates of terpene release by direct regulation of the resistance to terpene
293 diffusion from the sites of synthesis to the external gas phase (Dudareva et al. 2004).
294 The convergent modifications in temperature adaptation of floral terpene release
295 demonstrate a very high temperature-driven plasticity of plant physiological traits and
296 clearly emphasize the need to consider genotypic, epigenetic and phenotypic plasticity
297 in estimating and modeling floral emissions.

298 Our data demonstrate important variation in the temperature dependencies of
299 floral terpene emissions. In particular, the lower optimum temperatures for emission
300 maximum observed in species flowering in colder seasons and the higher optimum
301 temperatures observed in species flowering in warmer seasons indicate species-specific
302 temperature responses. This relationship suggests an adaptive mechanism that tunes
303 floral emissions to the temperatures to which the species are exposed during their
304 flowering season. Furthermore, our results also show this adaptive trend among
305 individuals of the same species, for example in *D. viscosa*, a species that has a long
306 flowering period and that was sampled in late summer and early autumn. This observed
307 seasonal change in the physiology of floral scent emission within a species indicates
308 intraspecific plasticity and can constitute an additional major source of variability in
309 floral emissions in the field. New measurements are warranted at different points in time
310 in species with long flowering periods or with separate flowering periods throughout the
311 year to gain a more detailed insight into the intraspecific plasticity of the physiology of
312 flowers under different temperatures.

313

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437 **Figure captions**

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439 **Figure 1.** Rates of total terpene emission per dry weight of floral tissue ($\mu\text{g g DW}^{-1} \text{h}^{-1}$)
440 throughout the temperature gradient from 15 to 40 °C. The quantum flux density was
441 maintained at $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ during the measurements. The data were fitted by local
442 polynomial functions (discontinuous lines indicate the 95% confidence intervals). Error
443 bars indicate SE ($n=3-6$ plants).

444

445 **Figure 2.** Relationships between the optimum temperature for floral emissions of
446 terpenes and the mean temperature for the month of the flowering peak of the species.
447 Colors indicate the flowering season of the species (blue, winter; green, autumn; yellow,
448 spring; red, summer).

449

450 **Figure 3.** Correlations between the optimum temperature for floral emissions of each
451 terpene compound and the mean temperature for the month of the flowering peak of the
452 species. Colors indicate the flowering season of the species (blue, winter; green, autumn;
453 yellow, spring; red, summer).

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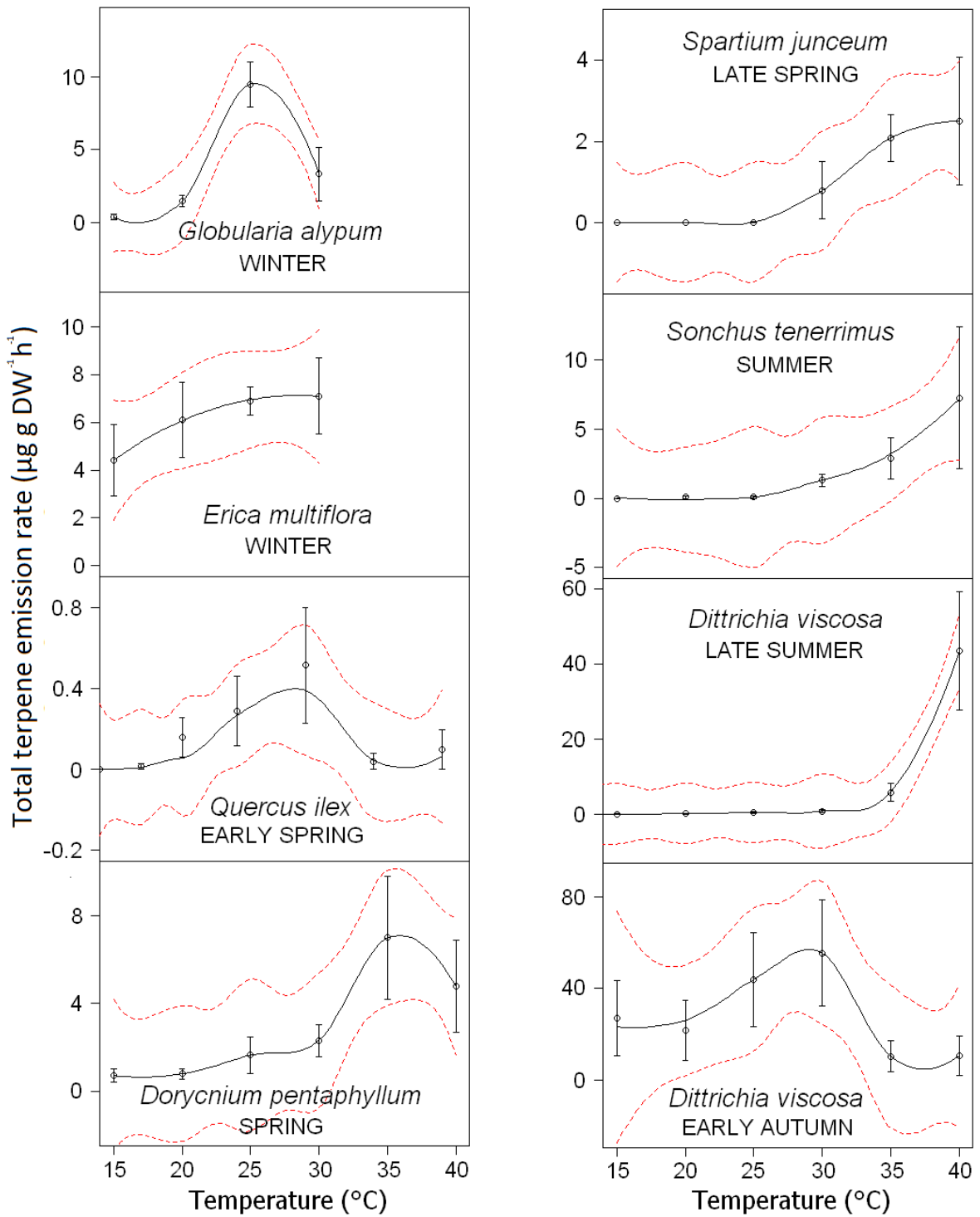
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459 Figure 1

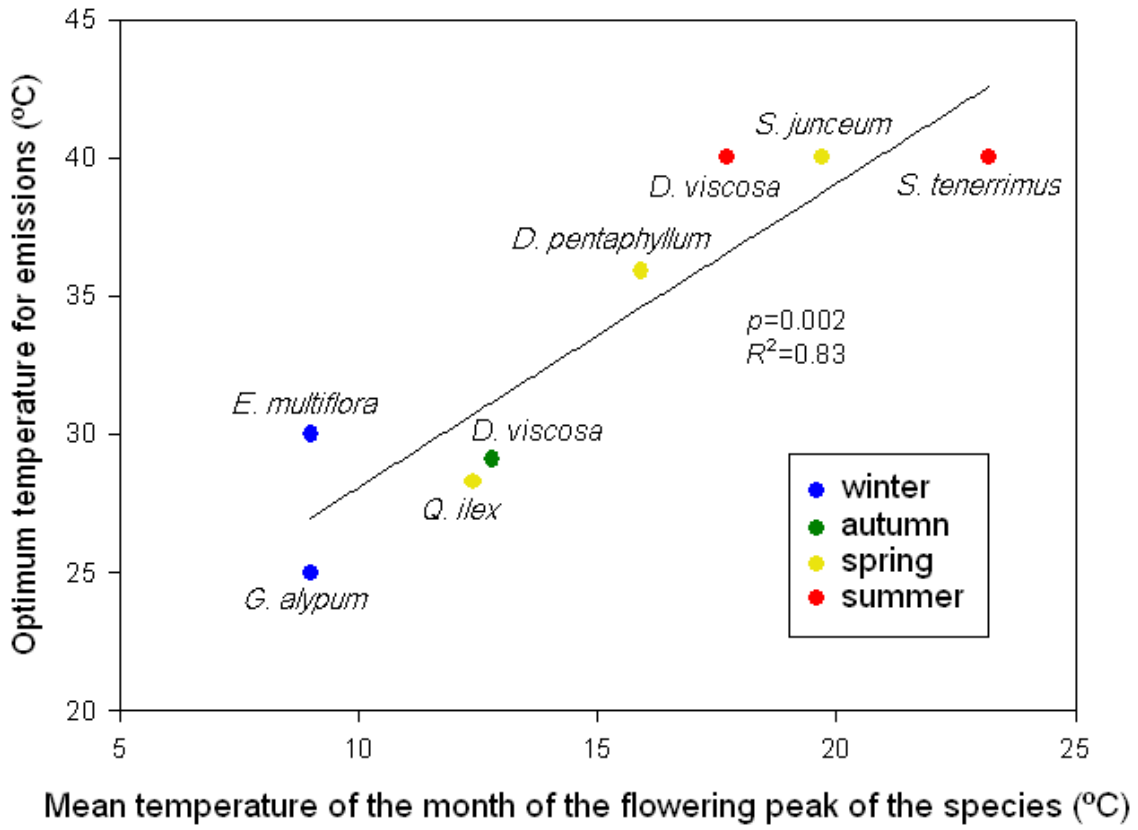


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463 Figure 2



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