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Does competition with wind-pollinated species alter *Echium plantagineum*'s attractiveness to a common pollinator *Bombus terrestris*?

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Abstract. 1. In insect-pollinated plants, pollinator attraction is influenced by flowers (e.g. number, size) and their associated rewards (e.g. pollen, nectar). These traits can depend on plant interactions. Indeed, below-ground competition between plants can lead to a decrease in flower or reward production in insect-pollinated species.

2. Wind-pollinated plants, in particular, which are almost never studied in plant–pollinator networks, can alter insect-pollinated plants' attractiveness through competition for nutrients. The response of pollinators to such changes has never been investigated.

3. A pot experiment was carried out in which an insect-pollinated species, *Echium plantagineum*, was grown in binary mixture with three wind-pollinated species selected to exert a panel of competitive interactions. Below-ground competition was controlled using dividers limiting interspecific root competition. Floral traits of *E. plantagineum* (*i.e.* flower production, floral display size, flower size and nectar production) were measured. For each species mixture, the visits (*i.e.* first visit, number of visits, 10-min sequences) of *Bombus terrestris* individuals released in a flight cage containing two pots were followed, one with and one without below-ground competition.

4. Below-ground competition significantly affected nectar's sucrose concentration but did not influence flower and nectar production. Likewise, pollinator visits were not influenced by below-ground competition. Competitor identity significantly influenced flower and reward production of *E. plantagineum*, with a decrease in the presence of the most competitive wind-pollinated species. A tendency for faster flower visitation events was also detected in the presence of the least competitive competitor. This study raises new questions regarding the influence of wind-pollinated plants on plant–pollinator interactions.

Key words. Attractiveness, competition, nectar, plant communities, pollination.

Introduction

Insect-pollinated plants' attractiveness to pollinators relies on the production of advertising as well as rewarding traits, such as flowers, nectar and pollen (Willmer, 2011). Flower and reward production are known to vary in time and space, at the plant (Herrera *et al.*, 2006) and population (Brink & de Wet, 1980) levels or between plant species (Galetto & Bernardello, 2004; Chalcoff *et al.*, 2006). Therefore, to optimise foraging and reward collecting efficiency, pollinators exhibit innate as well as learned preferences for some floral traits (e.g. for flower colour; Weiss, 1997). At the plant level, pollinators tend to visit more frequently plant species or individuals displaying a greater number of simultaneously open flowers (i.e. a larger floral display size; Mitchell *et al.*, 2004; Grindeland *et al.*, 2005; Miyake & Sakai, 2005; Makino *et al.*, 2007). Such a preference could be the result of better long-distance detection or limited flight cost between plants (Chittka & Thomson, 2001). At the flower level, features such as colour, shape or size can influence pollinators' choice. In particular, pollinators seem to prefer plants displaying larger flowers (Conner & Rush, 1996), as this may be correlated to reward production (Fenster *et al.*, 2006). The quantity and quality of nectar and pollen, key components of pollinators' diet, also influence pollinator visits on a plant (Dafni, 1992). Cnaani *et al.*, 2006 showed that bumblebees were more sensitive to variations in nectar concentration than to variations in volume and preferred more concentrated nectar, probably for a greater energy gain. Likewise, nectar composition might be of great importance as some studies showed that long-tongued bees and butterflies tend to prefer sucrose-rich nectar while short-tongued bees and flies prefer hexose-rich nectar (see references in Heil, 2011). Similarly, honeybees tend to prefer pollen with a greater amount of essential amino acids (Cook *et al.*, 2003).

Regarding plants, at the individual level, traits involved in attractiveness to pollinators can be modulated by availability of resources, especially below ground. Indeed, the addition of nutrients (e.g. nitrogen or phosphorus; Lau & Stephenson, 1993, 1994; Petanidou *et al.*, 1999; Muñoz *et al.*, 2005; Burkle & Irwin, 2009, 2010; Soper-Gorden & Adler, 2013) as well as litter (Baude *et al.*, 2011), compost (Cardoza *et al.*, 2012) or water (Petanidou *et al.*, 1999) can have a positive impact on flower production or pollen and nectar quantity (e.g. number of pollen grains and nectar volume) and quality (e.g. pollen grain size, pollen performance, nectar sugar content and nectar sugar ratios). Similarly, Burkle and Irwin (2009, 2010) and Muñoz *et al.* (2005), showed that the addition of nitrogen can increase flower width or length in *Ipomopsis aggregata* and *Potentilla pulcherrima*, and capitula size in *Chuquiraga oppositifolia*, respectively. Such modifications of soil resources levels can in turn positively affect pollinator visits (Burkle & Irwin, 2009). However, at the plant community level, resource availability can also be modulated (e.g. depleted) through interactions between plants such as competition for abiotic resources (Goldberg, 1990). Working with species mixtures of insect-pollinated plants, Baude *et al.* (2011) found that the presence of a strong plant competitor led to a decrease in the total amount of sugar allocated to nectar in the less competitive

one, probably through soil resource depletion. Likewise, the presence of a neighbouring wind-pollinated competitor reduced the flower production of an insect-pollinated one, *C. glomerata* (Partzsch & Bachmann, 2011), and Flacher *et al.* (2015) found that competition induced by wind-pollinated plants led to a decrease in floral display size and flower size, but also total flower production and total sugar allocated to nectar in two annual insect-pollinated species. In this last experiment, the stronger the competitor, the stronger the effect on attractiveness traits.

The neighbouring context of a plant, through 'indirect' interactions (i.e. competition for abiotic resources between plants), could thus play a key role in plant–pollinator networks. However, to our knowledge, while the effect of plant competition for abiotic resources on floral traits has been studied (Baude *et al.*, 2011; Partzsch & Bachmann, 2011; Flacher *et al.*, 2015), the indirect role of such competition on pollinator behaviour, through modifications of floral traits involved in plant attractiveness, has never been investigated. In addition, wind-pollinated species are almost never taken into account in plant–pollinator studies. However, they can affect floral traits in insect-pollinated plants through competitive interactions (Partzsch & Bachmann, 2011; Flacher *et al.*, 2015), and they may influence the attractiveness of an insect-pollinated focal plant through competition for abiotic resources while insect-pollinated competitors would influence the attractiveness of the focal plant through an additional process, competition for pollinators. In this context, to better understand pollinators' response to plant competition for abiotic resources, we set up a pot experiment in which a common pollinator, *Bombus terrestris*, could visit focal insect-pollinated plants (*Echium plantagineum*). These plants were grown in binary mixtures with wind-pollinated plants (*Chenopodium album*, *Agrostis capillaris* or *Holcus lanatus*) or in monocultures. These competitors were selected to expose insect-pollinated plants to varying degrees of below-ground competitive interactions (see Flacher *et al.*, 2015). We focused on below-ground competition as it is often cited as the most important part of competition in natural grassland communities (Kiaer *et al.*, 2013). Visits of *B. terrestris* as well as attractiveness traits of the focal species (flower and nectar production) were analysed. The objective of this study was to determine whether pollinator visits to an insect-pollinated plant could be influenced by the presence of wind-pollinated species. In particular, we studied: (i) if below-ground competition induced by wind-pollinated species can modify floral traits involved in the attractiveness of an insect-pollinated plant; (ii) if these variations of traits involved in attractiveness influence pollinator behaviour (see Karron *et al.*, 2004), especially at a fine scale (e.g. flower size, nectar production); and (iii) if the effect of below-ground competition on plant–pollinator interactions depends on plant identity (i.e., competition intensity, see Flacher *et al.*, 2015).

Material and methods

Plant species

Four plant species were selected, based on similar ecological preferences: one insect-pollinated species, *Echium*

plantagineum (EC, hereafter ‘focal species’) and three wind-pollinated species (hereafter ‘competitors’), *Agrostis capillaris* (AG), *Chenopodium album* (CH) and *Holcus lanatus* (HO). *Echium plantagineum* is an annual to biennial plant whose range spans from the Mediterranean basin to southeastern Europe (Fitter & Peat, 1994; FCBN – Fédération des Conservatoires Botaniques Nationaux, 2016). In natural conditions it blooms from June to August. Flowers are hermaphrodites and protandrous, generally blue to purple, but a rare white-flowered form exists. This species is entomophilous and visited mostly by bees (Dauber *et al.*, 2010), both for its pollen and its nectar (Corbet & Delfosse, 1984). In native areas, it is usually reported as a self-incompatible and cross-pollinated species (Dauber *et al.*, 2010; Petanidou *et al.*, 2012; the Ecoflora database, Fitter & Peat, 1994). The three wind-pollinated species were selected to exert varying degrees of competitive interactions on the focal insect-pollinated plant, from positive (*Chenopodium album*) to strongly negative (*Holcus lanatus*; see Flacher *et al.*, 2015 for details). All four plant species co-occur naturally in France (Muséum National d’Histoire Naturelle, 2017). More information on plant species descriptions and ecological preferences is given in Table S1. Plant seeds were supplied by (Graines-Baumaux, Mericourt, France) (*Echium plantagineum*, <http://www.graines-baumaux.fr/>), (Herbiseed, Twyford, England) and (B&T World Seeds, Aigues-Vives, France) (*Holcus lanatus*, *Agrostis capillaris* and *Chenopodium album*, <http://www.herbiseed.com/home.aspx>, <https://b-and-t-world-seeds.com/>).

Experimental setup

The experiment took place in a greenhouse at the CEREEP Ecotron Ile-de-France (St Pierre-lès-Nemours, France). In winter 2013, seeds of all plant species were sown in plastic germination trays (45 × 30 cm) in potting soil 3 cm deep (Floradur B fin, Puteaux SA, Villefranche sur Saône, France). All germination trays were kept at 20 °C, received 12 h day⁻¹ of light from neons (Solar ultra tropic T5 54 watt, JBL, Neuhofen, Germany) and were watered every 2–3 days. Seedlings were maintained in germination trays for 1–4 weeks depending on plant species. Seedlings of all plant species were then simultaneously transferred in multipots trays for 2–3 weeks. In spring 2013, seedlings of all plant species were simultaneously planted in plastic pots (14 cm diameter, 1.5 litres; Puteaux SA, France). For both multipots and pot transfers, seedlings were planted in soil taken from a nearby grassland site (sandy soil, pH 6). Soil was sieved (<4 mm) to remove rocks and plant material prior to the experiment. Each pot was divided into two compartments by plastic dividers that were glued inside the pots. Dividers were aimed at isolating roots from species in mixtures, controlling for interspecific below-ground competition. Such compartmentalization was aimed at discriminating below-ground from above-ground competition. In half of the pots, dividers were largely perforated to allow interactions between root systems of plants from the two compartments (with interspecific below-ground competition treatment, C+), whereas in the other half, dividers were not perforated, thus preventing interactions between root systems (without interspecific below-ground

competition treatment, C−). In pots containing species mixtures, one compartment was planted with three focal plants while the other was planted with three plants of a competitor species. Monocultures contained six plants of the focal species, three plants in each compartment. All in all, the experiment consisted of four plant treatments (three species mixtures and one monoculture) crossed with two competition treatments (with or without competition). Each combination of species mixture and competition was replicated 15 times, making a total of 120 pots (4 × 2 × 15 pots). Pots were randomly placed in the greenhouse and their position was changed each week. Air temperature in the greenhouse followed outdoor conditions but was maintained above 18 °C when outside temperatures were low. Photoperiod was initially set to 12 h day⁻¹ using natural light and sodium lamps when necessary (i.e. when solar irradiation was lower than 200 W m⁻² h⁻¹; HS2000 Hortilux Schréder, Monster, the Netherlands). Pots were watered daily for 5 min by sub-irrigation. No fertilisers were applied during the experiment. Among the 120 pots, 40 were used to measure floral traits (‘trait pots’; see the ‘Floral traits’ section) and 80 were used to observe pollinator visits (‘visited pots’; see the ‘Pollinator visits’ section). Indeed, pollinator visits as well as sampling deplete floral resources (e.g. nectar) or damage flowers (bumblebee marks, flower handling) so that using the same pots to follow both floral trait measurements and pollinator visits could have led to an estimation bias of floral traits production and/or pollinator visits.

Floral traits

Flower production was measured each day on all focal plants growing in the pot set dedicated to floral trait measurements. Buds ready to open were marked the day before each sampling. Flowers actually opened the day after were counted. The total number of opened flowers (new and old ones) was also counted to estimate the daily floral display size per plant. For each plant, three newly opened flowers were selected (at most) to measure flower size and sample nectar (see the section on ‘Nectar traits’). Flower size was measured from the bottom of the corolla to the tip of the upper petal using a digital caliper (Digit-Cal MK IV; Hexagon AB, Stockholm, Sweden).

Nectar traits

Nectar production was assessed every day on all focal plants of the same pot set. For a maximum of three newly opened flowers, nectar was sampled using microcapillary tubes that were calibrated in size and volume (0.5 and 1 µl; Minicaps end to end, Hirschmann Laborgeräte, Eberstadt, Germany). Nectar volume per flower was estimated by measuring the length of nectar in the microcapillary tube, with a digital caliper (Digit-Cal MK IV). As microcapillary tubes were calibrated in size, the ‘length’ of sampled nectar was measured and converted into nectar volume (µl). Nectar sugar concentration per flower was measured using hand-held refractometers for small volumes (Eclipse 45–81 and Eclipse 45–82; Bellingham + Stanley Ltd, Kent, U.K.) and converted to g l⁻¹ thanks to the conversion table of Kearns and Inouye (1993). When nectar volumes were too small (i.e. when

0.5 µl microcapillary tubes were not full) microcapillary tubes were completed up to 0.5 µl with MilliQ water (Merck millipore, Billerica, Massachusetts) before measurement. As a consequence, sampled nectar was diluted. The original concentration of sampled nectar was calculated using the following dilution equation: $C_1 V_1 = C_2 V_2 \leftrightarrow C_1 = C_2 V_2 / V_1$, where C_1 is the original nectar concentration (g l^{-1}), V_1 is the original nectar volume (sampled nectar, µl converted in litres), C_2 is the diluted nectar concentration (read on the refractometer in % brix and converted in g l^{-1}) and V_2 is the diluted nectar volume (0.5 µl converted in l). Refractometers were calibrated with a 30% sucrose solution at 20 °C and measurements were corrected according to temperature when necessary, following the manufacturer's instructions. Because nectar can contain sugars other than sucrose (Pacini & Nicolson, 2007), our concentration measurements thus correspond to sucrose equivalent ('sucrose' hereafter). The amount of sucrose allocated to nectar per flower was calculated by multiplying nectar volume per flower by nectar concentration per flower. To produce an integrative measure of plant attractiveness (Leiss & Klinkhamer, 2005; Baude *et al.*, 2011), the amount of sucrose allocated to nectar per flower was multiplied by the number of produced flowers per plant as a 'daily sucrose index'. This index was also summed all along the flowering period ('total sucrose index') to estimate the whole plant allocation to nectar. A similar index was calculated for nectar volume.

Bumblebee colonies

We studied visits to plants of a bumblebee species, *Bombus terrestris terrestris* (L.). This species is a common pollinator in France, providing a good pollination service, and is easy to breed. Colonies of *B. terrestris* are widely used in agriculture for crop pollination under greenhouses (Velthuis & van Doorn, 2006). Colonies were supplied by KOPPERT Biological Systems (Berkel en Rodenrijs, the Netherlands). They were stored in a temperature-controlled room (20 °C). Bumblebees were fed daily with a sucrose solution supplied by the company after the observations had ended (feeding for 2 h; Baude *et al.*, 2008). We also provided 4 g of pollen (the equivalent of a teaspoon) as a dietary supplement every 3–5 days. A total of nine colonies were used throughout the experiment.

Pollinator visits

From May to July 2013, we followed pollinator visits to plants from the 'visited pots' set. Observations took place in a flying arena, made of a nylon mesh cage (150 × 80 × 130 cm; mesh size, 0.93 × 1.6 mm; TIP 1700, Texinov, La Tour du Pin, France). The floor of the flying arena was given a green background to mimic outdoor conditions. We worked with naïve bumblebees in order to exclude acquired preferences from previous foraging experience which could have interfered with our treatments. Before observations, the colony was starved for at least 12 h. As the starvation period grew to hours, we took care to randomise starvation status among the treatments. For each observation, two pots of the same plant mixture, one of each competition treatment (with and without competition), were placed in the

flying arena. As much as possible, we chose pots with newly opened flowers to match the measurement of nectar done on the 'trait pots' set. As floral display size (total number of open flowers) can be involved in attractiveness, we chose pots having an identical, or similar, number of flowers to test the effect of modifications in fine attractiveness traits, i.e. rewards in particular (nectar). As a result, for each observation, pollinators were submitted to one plant treatment with a similar number of flowers per pot, but potentially different rewards induced by below-ground competition. As bumblebees can lay scented marks (Stout & Goulson, 2001) or damage flowers, which could influence the visit of a congener, pots were changed for every observation and a delay of 48 h was instituted before re-using a pot for observation. Before each observation, the colony was opened to allow an individual out to be transported to the flying arena a few metres away. An observation began as soon as the pollinator was released in the flying arena. Visits were recorded over 41 min using JWATCHER software (version 0.9; Blumstein *et al.*, 2000), the first minute being a habituation period. For each species mixture type, we recorded the time before the first visit, the identity of the first-visited pot (C+ or C−) and the number of visits per competition treatment. As we were also interested in the distribution of pollinator visits across time, we divided each observation round into four sequences of 10 min.

Plant biomass

At the end of the experiment, we carried out several biomass measurements to confirm the gradient of competitive interactions previously found (see Flacher *et al.*, 2015). For the 'trait pots' set, we harvested above-ground and below-ground biomass of all plant species (focal and competitors). For the 'visited pots' set, we also harvested above-ground biomass, as pollinators might be sensitive to above-ground biomass when foraging. Harvested plants were 18–22 weeks old, depending on plant species (as their germination date differed). For below-ground biomass we took special care to separate the roots of each plant. Harvested biomasses were oven-dried for 48 h at 60 °C before weighing.

Competitive interactions

In order to estimate the intensity of competitive interactions between each focal insect-pollinated plant and its wind-pollinated competitors, we calculated the log response ratio ($\ln \text{RR}$) as an index of competition (Weigelt & Jolliffe, 2003). This index is defined as:

$$\ln \text{RR} = \ln \left(\frac{P_{\text{mix}}}{P_{\text{control}}} \right)$$

where P_{mix} is the biomass of a focal plant when grown in species mixtures and P_{control} is the biomass of a focal plant in monoculture pots. In order to have a good assessment of $\ln \text{RR}$ values as well as a variance, $\ln \text{RR}$ values for each treatment were calculated as means of all possible combinations

of each focal plant in a mixture divided by each focal plant in a monoculture. Because three focal plants were present in mixtures, we considered monocultures as ‘mixtures’ of three focal plants with three ‘competitor’ plants of the same species. Values of $\ln RR$ are symmetrical around zero, with positive values indicating that focal plants grow better in mixture (i.e. foci are better competitors) and negative values indicating that focal plant growth is negatively affected by competitors (i.e. foci are lower competitors). $\ln RR$ values were calculated from above-ground, below-ground and total biomass but only $\ln RR$ calculated from total biomass was used to study the effect of competition on floral traits, as it is a better integrator of competition within both compartments.

Statistical analysis

All statistical analysis were performed using R 3.2.2 (R Core Team, 2015).

Plant traits. Biomass, flower size, nectar volume and nectar concentration were analysed by fitting linear mixed models (LMM, function lme in nlme package; Pinheiro *et al.*, 2015). ANOVAs were performed with competitor species and competition treatment set as fixed effects, while pots were set as random effects. The competition:competitor interaction was also included in models. The date was set as a random effect to take into account repeated measures on the same plants. Data were transformed when not normal (log, biomass and nectar volume; square, flower size; square root, amount of sugar allocated to nectar). As both linear and generalised linear mixed models (with Poisson error distribution) did not fit well to daily flower production, total flower production and floral display size (i.e. residuals not satisfying), we used non-parametric Kruskal–Wallis tests for these variables. For these tests, we tested the ‘competition effect’, on the one hand, and the ‘competitor identity effect’ on the other hand. The interaction between both was tested through differences between competition levels among each species mixtures. P -values were adjusted using the Holm method.

Pollinator visits. As only 32–46% of observation rounds lead to pollinator visits depending on mixture type ($N_{CH} = 12$, $N_{EC} = 11$, $N_{AG} = 13$, $N_{HO} = 10$, where N is the number of observation rounds including pollinator visits, for each species mixtures or monoculture) we performed non-parametric tests to study the effect of our two treatments (competition and competitor identity) on pollinator visits. For the choice of first visit, Pearson’s χ^2 analysis was performed for each species mixture. The null hypothesis was that the number of first visits would be equally distributed among pots submitted or not to competition. P -values for the test were calculated from Monte-Carlo simulations ($N = 2000$) as the number of visits for each category was not always superior or equal to 5. Time to first visit was analysed using survival analysis (Coxph function of the R package Survival; Therneau & Grambsch, 2000). Kruskal–Wallis tests were performed for the total number of visits and the number of

visits per 10-min sequence. We tested the ‘competition effect’ on the one hand, and the ‘competitor identity effect’ on the other. The interaction between both was tested through differences in the number of visits between competition levels among each species mixture. P -values were adjusted using the Holm method.

Results

Plant traits

Competitive interaction and biomass production. We did not detect significant effects of the competition treatment on above-ground, below-ground and total biomass in ‘trait pots’ and ‘visited pots’ but there was a significant effect of the competitor identity as well as a significant interaction between competitors (competition:competitor interaction; Table 1 and Figure S1). Above-ground and total biomasses of *E. plantagineum* were significantly higher in the mixture with *C. album* (even compared with monocultures) than in the mixtures with *H. lanatus* (Table 1, Figure S1). The significant competition:competitor interaction between competitors indicates that the impact of competition was different within mixture types. In the presence of below-ground competition (C+), *E. plantagineum* produced more biomass (above- and below-ground) in mixtures with *C. album*, compared with pots without competition (C−), while the opposite pattern was found in the presence of *H. lanatus* (Figure S1). There were no significant differences in biomass between mixtures in the absence of competition. Comparison of $\ln RR$ values calculated on above-ground biomasses did not reveal an effect of the competition treatment, but there was a significant effect of the competitor identity and a competition:competitor interaction. Whatever the competition treatment, *C. album* was the weakest competitor (even compared with monocultures) while *H. lanatus* acted as the strongest competitor and *A. capillaris* tended to be an intermediate competitor with *E. plantagineum* (Table 2). This confirmed the following panel of growing competition intensity: $C. album_{mixtures} < \text{monocultures} < A. capillaris_{mixtures} < H. lanatus_{mixtures}$. For $\ln RR$ values based on below-ground and total biomasses, an effect of the competition treatment was found in addition to the effect of the competitor identity and the competition:competitor interaction (Table 1). While the same panel of competitive interaction was found in presence of below-ground competition for both below-ground and total biomasses (Table 2), *C. album* acted as the strongest competitor in the absence of below-ground competition.

Flower traits. Daily flower production, total flower production, floral display size and flower size were not affected by the competition treatment (Table 3, Figures S2–S5, all $P > 0.05$). However, these traits were significantly affected by the competitor identity (Table 3) with a decrease along the competition gradient. Indeed, daily flower production, total flower production, floral display size and flower size were significantly higher when *E. plantagineum* was in the presence of *C. album* than in mixtures with *H. lanatus* ($\chi^2_3 = 77.09$,

Table 1. ANOVA table for biomass measurements and log response ratio (ln RR) values calculated on *Echium plantagineum* plants in ‘trait pots’ (pots only used for floral trait measurements) and ‘visited pots’ (pots submitted to pollinator visits).

	Fixed effect	F-value	P-value
Trait pots			
Above-ground biomass	Competition	$F_{1,32} = 0.09$	0.77 ns
	Competitor	$F_{3,32} = 14.74$	<0.01
	Interaction	$F_{3,32} = 5.71$	<0.01
Below-ground biomass	Competition	$F_{1,32} = 0.07$	0.79 ns
	Competitor	$F_{3,32} = 5.21$	<0.01
	Interaction	$F_{3,32} = 7.05$	<0.01
Total biomass	Competition	$F_{1,32} = 0.19$	0.67 ns
	Competitor	$F_{3,32} = 14.80$	<0.01
	Interaction	$F_{3,32} = 8.78$	<0.01
Above-ground ln RR	Competition	$F_{1,32} = 0.25$	0.62 ns
	Competitor	$F_{3,32} = 63.21$	<0.01
	Interaction	$F_{3,32} = 24.48$	<0.01
Below-ground ln RR	Competition	$F_{1,32} = 10.29$	<0.01
	Competitor	$F_{3,32} = 5.89$	<0.01
	Interaction	$F_{3,32} = 8.04$	<0.01
Total ln RR	Competition	$F_{1,32} = 8.89$	<0.01
	Competitor	$F_{3,32} = 36.57$	<0.01
	Interaction	$F_{3,32} = 21.75$	<0.01
Visited pots			
Above-ground biomass	Competition	$F_{1,72} = 3.25$	0.08 ns
	Competitor	$F_{3,72} = 12.77$	<0.01
	Interaction	$F_{3,72} = 4.96$	<0.01
Above-ground ln RR	Competition	$F_{1,72} = 1.44$	0.23 ns
	Competitor	$F_{3,72} = 22.56$	<0.01
	Interaction	$F_{3,72} = 8.89$	<0.01

$F_{a,b}$, F with a and b degrees of freedom for numerator and denominator, respectively; ns, not significant).

Table 2. Mean log response ratio (ln RR) values (\pm SE) per treatment for *Echium plantagineum* in monocultures (‘mono’) and mixtures with the three wind-pollinated competitors.

	Competition treatment	Mono	Mixture <i>C. album</i>	Mixture <i>A. capillaris</i>	Mixture <i>H. lanatus</i>
Traits pots					
Above-ground biomass	C+	0 (± 0.003)	0.453 (± 0.002)	-0.150 (± 0.002)	-0.710 (± 0.002)
Below-ground biomass	C+	0 (± 0.004)	0.036 (± 0.003)	-0.459 (± 0.003)	-1.292 (± 0.004)
Total biomass	C+	0 (± 0.002)	0.289 (± 0.002)	-0.273 (± 0.001)	-0.899 (± 0.002)
Above-ground biomass	C-	0 (± 0.0008)	0.043 (± 0.001)	-0.161 (± 0.001)	-0.215 (± 0.002)
Below-ground biomass	C-	0 (± 0.003)	-0.159 (± 0.002)	-0.063 (± 0.003)	0.0212 (± 0.003)
Total biomass	C-	0 (± 0.001)	-0.192 (± 0.001)	-0.145 (± 0.001)	-0.151 (± 0.001)
Visited pots					
Above-ground biomass	C+	0 (± 0.0008)	0.387 (± 0.0008)	0.009 (± 0.0004)	-0.442 (± 0.0004)
Above-ground biomass	C-	0 (± 0.0004)	0.203 (± 0.0004)	-0.115 (± 0.0003)	-0.176 (± 0.0004)

$P < 0.01$, $\chi^2_3 = 28.43$, $P < 0.01$ and $\chi^2_3 = 226.11$, $P < 0.01$ and $F_{3,116} = 3.48$, $P = 0.02$ respectively; Table 3, Figures S2–S5). Moreover floral display size of *E. plantagineum* was significantly higher in the presence of below-ground interactions (C+) with *C. album* than in plants without below-ground competition

(C–), while the opposite pattern was found for mixtures with *H. lanatus* (Kruskal–Wallis, $\chi^2 = 20.58$, d.f. = 1, $P < 0.01$ and $\chi^2 = 41.98$, d.f. = 1, $P < 0.01$, respectively). These significant contrasted effects of below-ground competition within mixtures suggest an interaction between competition and competitor

Table 3. Summary of statistical analysis on attractiveness traits in *Echium plantagineum*.

Attractiveness trait	Sample size	Fixed effect	Statistic value	P-value
Traits pots				
Daily floral display size	<i>N</i> = 8160	Competition	K-W χ_1^2 = 2.35	0.13 ns
		Competitor	K-W χ_3^2 = 226.11	<0.01
		Interaction	—	—
Daily flower production	<i>N</i> = 8160	Competition	K-W χ_1^2 = 0.71	0.40 ns
		Competitor	K-W χ_3^2 = 77.09	<0.01
		Interaction	—	—
Total flower production	<i>N</i> = 120	Competition	K-W χ_1^2 = 0.23	0.63 ns
		Competitor	K-W χ_3^2 = 28.43	<0.01
		Interaction	—	—
Flower size	<i>N</i> = 1915	Competition	$F_{1,32}$ = 0.83	0.37 ns
		Competitor	$F_{3,32}$ = 4.59	<0.01
		Interaction	$F_{3,32}$ = 2.39	0.09 ns
Daily concentration	<i>N</i> = 1912	Competition	$F_{1,32}$ = 6.36	0.02
		Competitor	$F_{3,32}$ = 1.64	0.20 ns
		Interaction	$F_{3,32}$ = 2.03	0.13 ns
Daily volume index	<i>N</i> = 1630	Competition	$F_{1,32}$ = 1.94	0.17 ns
		Competitor	$F_{3,32}$ = 3.38	0.03
		Interaction	$F_{3,32}$ = 0.75	0.53 ns
Daily sucrose index	<i>N</i> = 1621	Competition	$F_{1,32}$ = 1.41	0.24 ns
		Competitor	$F_{3,32}$ = 5.82	<0.01
		Interaction	$F_{3,32}$ = 3.06	0.04
Total volume index	<i>N</i> = 120	Competition	$F_{1,32}$ = 3.71	0.06 ns
		Competitor	$F_{3,32}$ = 8.17	<0.01
		Interaction	$F_{3,32}$ = 2.89	0.05 ns
Total sucrose index	<i>N</i> = 120	Competition	$F_{1,32}$ = 1.18	0.29 ns
		Competitor	$F_{3,32}$ = 9.90	<0.01
		Interaction	$F_{3,32}$ = 2.98	0.05 ns

K-W, Kruskal–Wallis test; χ_a^2 , χ^2 statistic with *a* degrees of freedom. $F_{a,b}$, *F* with *a* and *b* degrees of freedom for numerator and denominator, respectively; ns, not significant.

identity. Likewise, this interaction was significant for total flower production ($\chi^2 = 19.59$, d.f. = 3, *P* < 0.01).

Nectar traits. The competition treatment had no influence on any of the nectar traits except for the daily sucrose concentration ($F_{3,32} = 6.36$, *P* = 0.02; see Table 3). Competitor identity had an influence on total indices only (Table 3, Figures S6–S7). Both total volume and sucrose indices tended to decrease along the competitive gradient ($F_{3,32} = 8.17$, *P* < 0.01 and $F_{3,32} = 9.90$, *P* < 0.01 respectively, Figures S6–S7). In particular, these two variables increased in mixtures with *C. album* (even compared with monocultures) and decreased in mixtures with *H. lanatus*. The competitor:competition interaction was significant for daily sucrose index only ($F_{3,32} = 3.06$, *P* = 0.04, see Table 3). In particular, below-ground competition with *C. album* had a positive effect while below-ground with stronger competitors such as *H. lanatus* had a negative influence.

Pollinator visits

First visit. Within each species mixture, the competition treatment did not influence the choice of the first visit of a bumblebee to a pot (χ^2 , all *P* > 0.05). Between species mixtures, first visits tended to be made more rapidly on

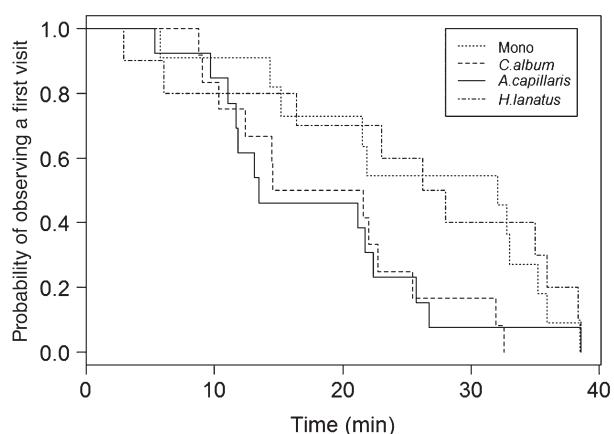


Fig. 1. Probability of observing a first visit to *Echium plantagineum* versus time, according to species mixtures types. Mono, monocultures of *E. plantagineum*; *Chenopodium album*, mixtures with *C. album*; *Agrostis capillaris*, mixtures with *A. capillaris*; *Holcus lanatus*, mixtures with *H. lanatus*.

E. plantagineum mixed with *C. album* and *A. capillaris* compared with monocultures and mixtures with *H. lanatus*, but not significantly so (likelihood ratio test = 6.83, d.f. = 3, *P* = 0.08; Fig. 1).

Total number of visits. There was no overall impact of the competitor identity on the total number of visits received by *E. plantagineum* (whatever the competition, Kruskal–Wallis, $\chi^2_3 = 3.61$, $P = 0.30$). This variable was not influenced by the competition treatment either (whatever the competitor, Kruskal–Wallis, $\chi^2_1 = 0.09$, $P = 0.76$). Moreover, the apparently contrasting effects of competition within competitor treatment on the number of visits were not significant (Kruskal–Wallis, for all competitors levels, $0.07 < \chi^2_1 < 3.52$, all $P > 0.05$). Likewise the effect of competitor within competition treatment was not significant (Kruskal–Wallis, for all competition levels, $2.06 < \chi^2_3 < 4.31$, all $P > 0.05$).

Visit sequences. The number of visits received by a plant of *E. plantagineum* was affected by time (Kruskal–Wallis, $\chi^2 = 71.66$, d.f. = 3, $P < 0.01$) as bumblebees made a greater number of visits to the focal plants between 30 and 40 min after the start of the observation than in the first 10 min. However, competition treatment and competitor identity had no influence on the distribution of such visits across time (all $P > 0.05$).

Discussion

With this experiment, our aims were to investigate if competition for abiotic resources, especially below-ground competition induced by wind-pollinated species, could influence bumblebees' behaviour on an insect-pollinated plant through modifications of floral traits. As wind-pollinated plants do not interact with pollinators, we could single out the effect of competition for abiotic resources on attractiveness traits. Moreover, these plant species are almost never taken into account in plant–pollinator studies, even though they can modulate the floral traits involved in the attractiveness of insect-pollinated plants (Partzsch & Bachmann, 2011; Flacher *et al.*, 2015). Our results demonstrated that competitor species' identity can influence floral traits involved in attractiveness while the effect of below-ground competitive status depended on competitor identity. The effect on pollinator behaviour, however, was tenuous.

Competitive interactions

As expected, biomass production of the focal species depended on its neighbouring context. Globally, there was an effect of competitor treatment, with *E. plantagineum* producing greater biomass in mixtures with *C. album* (even greater than in monoculture in the case of above-ground biomass) than in mixtures with *H. lanatus*. The associated ln RR values suggested a panel of interactions from 'facilitative', or at least slightly competitive (with *C. album*), to strongly competitive (with *H. lanatus*). These results are in accordance with Flacher *et al.* (2015) who found a similar pattern, although they did not control below-ground competition. In this study the significant competition:competitor interaction for all biomass and ln RR variables indicates a different effect of below-ground competition among species mixtures. Indeed, in mixtures with *C. album*, the presence of below-ground interactions had a positive

effect on above-ground, below-ground and total biomasses and ln RR values (compared with the same mixtures without competition) while below-ground competition with *H. lanatus* had a negative effect. Moreover, *E. plantagineum* produced a greater amount of above-ground biomass in the presence of *C. album* than in monocultures. This 'facilitative' effect of *C. album* might be due to the relatively small amounts of above-ground and below-ground biomass it produced: when the dividers were open (C+) the focal species probably had more space than in monocultures or in the absence of competition. This could have led to a greater access to soil resources, enabling more allocation to biomass production for *E. plantagineum*. The opposite pattern was observed in the presence of *H. lanatus* when dividers were open, probably because this wind-pollinated species can produce great amounts of biomass, especially below ground (from 10 to 20 times greater compared with *C. album*; see also Flacher *et al.*, 2015). Even though biomass allocations and competitive abilities are not always associated, it has been reported that larger plants can be greater competitors through resource depletion or space occupancy (Raynaud & Leadley, 2004; Gurevitch *et al.*, 2006). Therefore, the presence *H. lanatus* could have limited the access of *E. plantagineum* to soil resources, leading to a decrease in biomass production for the latter. Besides, even if we took care to limit overlap of above-ground plant parts, we cannot deny that *E. plantagineum* could also have experienced above-ground competition. Pairwise comparisons revealed that there were no significant differences of biomass production or ln RR values in the absence of below-ground competition, suggesting that, if there was above-ground competition, it was of the same intensity whatever the competitor's identity. The only exception concerns ln RR values calculated on above-ground biomass of C– treatments, which differed between the two extremes of the panel: consequently, *H. lanatus* probably induced above-ground competition on *E. plantagineum* (e.g. through shading) while *C. album* did not. It would be interesting in future experiments to study above-ground competition in order to discriminate the influence of both sources of competition.

Finally, the observed negative effect of *C. album* on below-ground and total biomass in the absence of competition was not expected. *Chenopodium album* can have some allelopathic effects on other plants (Batish *et al.*, 2006), especially through shoot residues (Qasem & Hill, 1989). We can hypothesise that in the absence of below-ground competition *C. album* could have limited growth of *E. plantagineum* through allelopathic effects of leaf residues on soil, for instance. However, in the presence of below-ground competition with *C. album*, this effect might have been counterbalanced by a greater access to soil space and resources.

Floral traits involved in attractiveness to pollinators

Competitor identity had an influence on floral and nectar traits. As previously stated, floral traits involved in attractiveness to pollinators can be sensitive to modifications of abiotic resource availability. (Petanidou *et al.*, 1999; Muñoz *et al.*, 2005; Burkle & Irwin, 2009, 2010; Soper-Gorden & Adler, 2013).

These resources can play a key role in metabolic pathways (Gurevitch *et al.*, 2006), especially those involved in the production of flowers and their associated rewards. Several studies have already found that allocation to reproductive structures, among which are traits involved in plant attractiveness to pollinators, can be negatively affected by competition (Weiner, 1988; Nötzold *et al.*, 1997; Baude *et al.*, 2011; Partzsch & Bachmann, 2011; Flacher *et al.*, 2015). In particular, Flacher *et al.* (2015), while they did not control for below-ground competition, found that the stronger the wind-pollinated competitor, the stronger the decrease of floral and nectar traits. In our study, as we controlled below-ground competition, we can hypothesise that *E. plantagineum* had greater access to resources in mixtures with *C. album* (and especially when in C+ pots), leading to an increase in allocation to both floral and nectar traits. By contrast, mixtures with *H. lanatus* may have led to a reduction in resource availability to *E. plantagineum*, inducing a decrease in both sets of traits.

Concerning the effect of competition, because of contrasting effects of below-ground competition between species mixtures according to biomass measurements of *E. plantagineum*, it was expected that there would be no overall impact of the competition treatment on floral traits (the only exception being the daily sucrose concentration; see earlier). However, we hypothesised that the influence of competition would rise through an interactive effect with the competitor identity. Within the C+ treatment, all flower and nectar traits followed the pattern of biomass. They tended to be higher or lower (compared to monocultures) when *E. plantagineum* was in mixture with *C. album* and *H. lanatus* respectively, however not always significantly. More importantly considering the above mentioned interaction, within each mixture (i.e. for each competitor identity), most attractiveness traits did not differ according to the competition treatment (i.e. between C+ and C– pots). The absence of such interaction was not expected. This suggests that, within each species mixture, *E. plantagineum* allocated the same amount of energy to the production of attractiveness traits, whatever its below-ground competitive status with neighbours. One explanation would be that there was a trade-off in energy allocation to vegetative and reproductive structures (Obeso, 2002): because *E. plantagineum* generally completes its life cycle within a year, it may favour energy allocation to reproductive parts (such as flowers and associated rewards) to ensure its reproductive success, leading to a trade-off with vegetative structures (such as biomass). Therefore energy allocation to vegetative parts might be more affected by competition than allocation to reproductive parts. Floral display size and total flower production did suffer from competition, however. As rewards ensure the repetition of visits to a plant (Dafni, 1992), one strategy for *E. plantagineum* could be to favour smaller floral display size but with constant reward production. Hence the significant effect of competitor identity on overall plant nectar production (daily and total nectar indices) was mostly due to modifications of flower production. Flacher *et al.* (2015) found the same pattern for two annual species, including *E. plantagineum*, with no impact of competition on daily nectar production but a negative influence at the plant level and on the whole flowering period. These results suggest a strong importance of flower production for plant

attractiveness. For nectar concentration and daily sucrose index, the effect of competition only (or through an interaction) could be the result of limited access to water in presence of below-ground competition. Indeed, nectar sucrose concentration was higher in plants submitted to below-ground competition (C+ pots) than in plants isolated from below-ground competition (C– pots). However, the daily amount of sucrose allocated to nectar was the same, whatever the below-ground competition context (i.e. C+ or C–). Therefore, an increase in nectar sucrose concentration in plants in C+ pots might be due to a limited access to water, concentrating nectar solution and revealing an interaction for daily sucrose index. Finally, *E. plantagineum* is visited by bees (e.g. honeybees) for its nectar, but also for its pollen (and sometimes for its pollen only; Corbet & Delfosse, 1984). According to Somerville & Nicol (2006) *E. plantagineum* produces a pollen of excellent quality, with a crude protein content above 30%. Some studies showed that pollinators have preferences for pollen with a greater amount of essential amino acids (Cook *et al.*, 2003) or visit more flowers with a higher protein content (Hanley *et al.*, 2008). Therefore, pollen (at least, its quality) may be an important attractiveness trait for *E. plantagineum*. As pollen quantity and quality can be sensitive to availability of soil resources (Lau & Stephenson, 1993, 1994), pollen production should be investigated in future work trying to assess the effect of below-ground competition on plant attractiveness. At a larger scale, floral odour or colour might be worth studying as they can both influence long-distance plant attractiveness and might be sensitive to resource availability (de Pascual-Teresa & Sanchez-Ballesta, 2008).

Pollinator visits

We found no effect of the below-ground competitive status on the first visit choice. The number of open flowers (or floral display size) is one of the cues pollinators rely on to assess plant attractiveness (Geslin *et al.*, 2014). As we used pots with a similar number of flowers in both competition statuses (C+ and C–) for every observation, this result could have been expected. However, we hypothesised that finer floral traits might be used by pollinators as cues. For instance, flower size or flower colour can be involved in pollinators' choice (Conner & Rush, 1996; Chittka & Thomson, 2001; Elle & Carney, 2003). However, there were no significant differences in flower size of *E. plantagineum* according to below-ground competitive status. Even though flower colour or flower scent investigations might be needed to conclude properly, we can assume that the absence of clear visual cues, such as differences in floral display size, did not allow bumblebees to discriminate among plants with different below-ground competitive status. Differences in flower production and floral display size among species mixtures suggest that future experiments should consider a two-pronged approach (i.e. global plant attractiveness at large scale and fine attractiveness at a smaller scale) to evaluate the effect of below-ground competition between plants on pollinator visits. The time to first visit tended to differ according to competitors' identity, though not significantly. Again we expected strong differences among species mixtures as competitors are part of a panel of contrasted interactions. For instance,

as *E. plantagineum* produced bigger flowers in mixtures with the small *C. album* (whatever the competition treatment), we assumed that bumblebees would make their choice more rapidly as flowers would be easier to discriminate. However, these differences might have been too small (only 2 mm) to act as cues for pollinators.

For the total number of visits, the lack of a competition effect was not surprising, according to our results on nectar traits. Indeed, the repetition of visits to a plant relies on reward quantity and quality. In our study there was neither an overall nor an interactive effect of competition on most nectar traits (except for daily sucrose concentration). As there were no strong differences between C+ and C– treatments, pollinators visited plants of *E. plantagineum* equally within each species mixtures. The lack of effect of the competitor identity was more unexpected as there were contrasting differences of floral and nectar traits between plant treatments. This underlines the fact that significant differences in floral traits do not necessarily lead to differences in pollinator visits, at least with our experimental design. Bumblebees are able to discriminate rewarding plants, and plants that offer more nectar received more visits (Leiss & Klinkhamer, 2005). However, it seems that bumblebees respond more rapidly to a change in the reward's sugar concentration than to a change in its volume (Cnaani *et al.*, 2006), and are more able to discriminate between rewarding and non-rewarding plants when plants have dissimilar flower colours (Internicola *et al.*, 2007). In our experiment, the observed modifications of floral and nectar traits might not have been strong enough to modulate the foraging behaviour of bumblebee between plant treatments, especially with flowers of the same colour. Finally, the distribution of visits across time (i.e. 10-min sequences) did not provide more information regarding the influence of both competition treatment and competitor identity.

Overall, these conclusions are made on a relatively small number of observations. Indeed, only 32–46% of observations led to at least one visit. Some studies indicate that *E. plantagineum* is visited by *B. terrestris* in natural conditions (Torretta *et al.*, 2006) and we took care to work with naïve bumblebees so that no acquired preference could interfere with their visits to *E. plantagineum*. Therefore, we were expecting numerous visits to our focal plant. Several hypotheses were tested to try to understand why we had such difficulty in observing visits: (i) the flying arena was taken outdoors to exclude the possibility of UV filtering through the glass of the greenhouse (UV being a part of the light spectrum used by hymenopterans; Chittka *et al.*, 1994); (ii) colonies were put in the flying arena for habituation to avoid stress; and (iii) starvation duration was modulated. Possible explanations regarding why we encountered such difficulties are that, in spite of all the care we took with the colonies, either they were not in good conditions to visit the focal plant or they were excessively stressed by handling prior to the experiments. An alternative hypothesis is that the density of flowers in the flying arena was too low, even though a previous behavioural study regarding bumblebees used an equivalent density (e.g. Kawaguchi *et al.*, 2007). Flowers might not have been easily discriminated from the green background we set up to mimic outdoor conditions. Finally, reward production can be influenced by flower age. Additional

measurements on 2- and 3-day-old flowers revealed that nectar volume and nectar concentration tended to be lower than in new flowers, especially in presence of below-ground interactions. In consequence, as we could not offer solely newly opened flowers to bumblebees, the contrast in floral and nectar traits among species mixtures might have been diluted by flower age, leading to the lack of difference in the number of visits.

Taken together, we found that the presence of wind-pollinated plants can influence floral traits through their identity. The effect of below-ground competition is, however, varied and dependent on competitor identity. Besides, the effect of below-ground competition might be trait-specific. The effect on attractiveness to pollinators was tenuous probably because we controlled floral display size, which is a major attractiveness trait. In this study we focused on only one pollinator species. In nature, most insect-pollinated species interact with a diverse community of pollinators that may have different preferences and thus different responses to floral and nectar traits modifications. Future research should therefore focus on field experiments to study the effect of competition induced by wind-pollinated plants on wild pollinator visits through modification of insect-pollinated attractiveness traits.

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Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference:
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Table S1. Plant species description (family names are given according to APG III classification).

Figure S1. Mean biomass (g) of *E. plantagineum* (\pm SE) per plant according to below-ground competition and competitor identity.

Figure S2. Mean floral display size of *E. plantagineum* per plant according to below-ground competition and competitor identity.

Figure S3. Mean daily flower production of *E. plantagineum* (\pm SE) per plant according to below-ground competition and competitor identity.

Figure S4. Mean total flower production of *E. plantagineum* (\pm SE) per plant according to below-ground competition and competitor identity.

Figure S5. Mean flower size (mm) of *E. plantagineum* (\pm SE) according to below-ground competition and competitor identity.

Figure S6. Mean total volume index (μl) per plant of *E. plantagineum* (\pm SE) according to below-ground competition and competitor identity.

Figure S7. Mean total sucrose index (g) per plant of *E. plantagineum* (\pm SE) according to below-ground competition and competitor identity.

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