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Mathilde Baude, Julie Leloup, Séverine Suchail, Béatrice Allard, Danielle Benest, et al.. Litter inputs and plant interactions affect nectar sugar content. *Journal of Ecology*, 2011, 99 (3), pp.828-837. 10.1111/j.1365-2745.2011.01793.x . hal-02047775

HAL Id: hal-02047775

<https://hal-univ-avignon.archives-ouvertes.fr/hal-02047775>

Submitted on 25 Feb 2019

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Litter inputs and plant interactions affect nectar sugar content

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Summary

1. Declines in availability of plant resources to pollinators are a major cause of pollinator loss. The management of plant communities to enhance floral resources is often proposed as a way to sustain pollinator populations. Nectar, the main energetic resource for pollinators, plays a central role in behaviour and composition of pollinator communities. Abiotic and biotic factors are known to influence nectar traits at both the species and community levels, but the impact of plant community composition itself has never been investigated.

2. Below-ground interactions in plant communities can induce changes in plant development through (i) plant-derived litter amendment of the soil and (ii) competition for soil resources between plants. We tested how plant below-ground interactions affect above-ground nectar traits involved in plant attractiveness to pollinators.

3. A short-term pot experiment was carried out with three temperate grassland species *Mimulus guttatus*, *Lamium amplexicaule*, and *Medicago sativa*, showing distinct litter stoichiometry and competitive abilities for soil resources. Litter amendment (none, mono and tri-specific litter) and plant interaction treatments (monocultures, two- and three-species mixtures) were crossed in a factorial design.

4. Litter amendment to the soil led to an increase in total nectar sugar content in *L. amplexicaule* plants but not in the two other species. We also found that the presence of *M. guttatus*, a competitive species, reduced the total nectar sugar content in *L. amplexicaule* through a concomitant decrease in nectar volume per flower and in floral display size, but not in other species. Species-specific responses of nectar traits to variation in soil nitrogen availability were thus observed, suggesting consequences for plant species and community attractiveness to pollinators. However, we did not find evidence that the legume *M. sativa* affected nectar traits of any neighbouring plants.

5. *Synthesis.* Our results demonstrate that litter inputs and competition between plants for soil resources can alter nectar traits linked to plant attractiveness to pollinators. This supports the idea that below-ground plant–plant interactions for soil resources can influence above-ground plant–plant interactions for pollination services. This offers promising perspectives in studying the role of below-ground–above-ground interactions on higher trophic levels.

Key-words: above-ground–below-ground interactions, attractiveness, competition, diversity, floral display, nectar, plant–plant interactions, plant–pollinator interactions, plant–soil interactions

Introduction

A global pollination crisis has recently been recognized (Allen-Wardell *et al.* 1998; Steffan-Dewenter, Potts & Packer 2005).

Pollinators and services they provide are under increasing pressure from multiple perturbations of anthropogenic origin (Kearns, Inouye & Waser 1998; Kremen & Ricketts 2000). A decline in floral resources used by pollinators is considered to be one of the major causes involved in pollinator loss (Biesmeijer *et al.* 2006; Carvell *et al.* 2006; Potts *et al.* 2010).

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Nectar, which is mainly composed of sugars, provides energy required by active adult bees and other pollinating insects, whereas protein rich pollen is collected for larval growth (Proctor, Yeo & Lack 1996). Pollen and nectar resources produced by flowers are therefore fundamental to sustaining pollinator populations. Conservation measures often promote the establishment of pollen and nectar-rich floral communities to preserve pollinator populations and enhance pollination services (Pywell *et al.* 2006; Carvell *et al.* 2007).

Nectar plays a key role in plant–pollinator systems as it influences pollinator behaviour (Klinkhamer & de Jong 1990; Cresswell 1999; Klinkhamer, de Jong & Linnebank 2001; Kudo & Harder 2005) and pollinator community structure (Potts *et al.* 2003, 2004). Nectar is essentially an aqueous solution of sugars but also contains amino acids, proteins, lipids and a variety of secondary compounds (Dafni 1992). Among the glucidic fraction, three sugars are dominant: glucose, fructose (monosaccharides) and saccharose (a disaccharide of glucose and fructose). Nectar traits are highly variable among species (Wykes 1952; Percival 1961; Baker & Baker 1979) and recent studies have also reported high intra-specific variability that occurs at several scales, from plant populations to nectaries (Lanza *et al.* 1995; Pierre *et al.* 1999; Herrera, Perez & Alonso 2006). Nectar traits respond to biotic factors such as nectar sampling or herbivory (e.g. Castellanos, Wilson and Thomson 2002; Adler *et al.* 2006) as well as abiotic factors such as light, water, fertilization, temperature and CO₂ concentration (e.g. Osborne *et al.* 1997; Rusterholz & Erhardt 1998; Gardener & Gillman 2001; Burkle & Irwin 2009a). Indeed, soil nutrient availability to plants is known to influence the production of flowers (Muñoz *et al.* 2005; Burkle & Irwin 2010), pollen (Lau & Stephenson 1993) and nectar (Campbell & Halama 1993; Gardener & Gillman 2001; Burkle & Irwin 2009a) and can lead to changes in subsequent pollinator visitation (Muñoz *et al.* 2005; Burkle & Irwin 2010).

Studies on nectar traits have mostly been conducted at the species level and only few recent papers have considered the whole community (Burkle & Irwin 2009b, 2010). In particular, the effects of plant community composition on individual nectar traits have never been explicitly studied, despite the fact that these individual traits contribute to community attractiveness to pollinators. In terrestrial communities, plant individuals interact with each other above-ground for light and below-ground for water and nutrients. Because below-ground interactions between individuals affect plant nutrition, they might also affect floral and nectar traits. It can therefore be hypothesized that within plant communities, competitive species for soil resources should have a negative impact, whereas facilitative ones should have a positive impact on floral and nectar traits of neighbouring plants. Nutrient fluxes in soil result from nutrient uptake by plants and organic matter inputs from plants to the soil (Wardle *et al.* 2004). Some nutrients such as nitrogen are commonly derived from the decomposition of the litter of species present in the plant community. In nitrogen-limited ecosystems, positive effects of litter inputs to the soil on nectar traits of plants can be expected and the magnitude of

this effect is likely to depend upon litter C:N stoichiometry (Trinsoutrot *et al.* 2000).

Here, we present a study aimed at investigating how below-ground–above-ground interactions affect floral and nectar traits in plant communities. More precisely, we tested whether litter amendments and plant interactions within the community alter the traits involved in plant attractiveness to pollinators. A synthetic diagram of the main tested hypotheses is presented in Fig. 1. A short-term pot experiment was carried out using a complete factorial design in which three temperate grassland plants were grown in communities of one, two or three species with different types of litter amendment derived from the same plants (no litter, addition of litter from a single species or addition of a mix of litter of the three studied species). The plant species (*Mimulus guttatus* Fisch. ex DC., *Lamium amplexicaule* L. and *Medicago sativa* L.) were chosen because they can occur simultaneously in temperate grasslands, show distinct competitive abilities and have distinct litter stoichiometry.

Materials and methods

EXPERIMENTAL TREATMENTS

A pot experiment was set up based on different plant associations with different litter amendments in a complete factorial design. The ‘plant interaction’ treatment was induced by the manipulation of the assemblage of the three plant species in monocultures, two- and three-species mixtures (seven combinations in total). The ‘litter’ treatment consisted of five combinations of above-ground biomass of the

Fig. 1. Synthetic diagram of our experiment aimed at investigating how below-ground–above-ground interactions mediated through litter input (‘Litter’) and plant interaction (‘Plant’) affect plant traits involved in attractiveness to pollinators (‘Attractiveness’). Large boxes represent soil resources, plant and pollinator communities. Black arrows indicate positive effects and grey arrows indicate negative ones. Solid arrows indicate tested effects in our experiment and dashed arrows represent extrapolated effect. A putative hierarchy of plants in their below-ground competitive abilities (‘Facilitative’, ‘Neutral’ and ‘Competitive’ species) composed plant communities. It was hypothesized that the availability of soil resources positively impacted plant traits linked to attractiveness to pollinators. Litter inputs and plant interaction with facilitative species should positively affect plant attractiveness to pollinators whereas plant interaction with competitive species should negatively affect plant attractiveness.

same plant species: no litter, single species litters and three-species litter. The full factorial design thus comprised 35 combinations: seven plant associations $\times$ five litter inputs, in four replicates. This experimental design enabled us to test experimentally the effects of 'plant interaction' and 'litter' as well as the interaction between these factors on below-ground and above-ground parameters. Details on the choice of plant species, experimental set-up, measurements and statistical analyses are given below.

PLANT SPECIES

Three European grassland plant species were used: *M. guttatus* (MG, Scrophulariaceae), *L. amplexicaule* (LA, Lamiaceae) and *M. sativa* (MS, Fabaceae). These species were chosen among a pool of grassland plant species that are bee-pollinated and that can co-occur in natural situations since they share similar ecological requirements (Hill *et al.* 1999). The species also had to have flowers with easily accessible nectar to be suitable for the experiment.

Mimulus guttatus is a semi-perennial herb that occurs in semi-dry to wet grasslands. This species, native to North America, is invasive in some European countries. It exhibits strong competitive attributes including a short germination period, rapid growth and effective long- and short-distance dispersal mechanisms (Truscott *et al.* 2006). It presents a mixed mating system with insect pollination and up to four modes of self-pollination. The main pollinators of *M. guttatus* are *Apis mellifera*, *Bombus* sp. and solitary bees (Robertson *et al.* 1999).

Lamium amplexicaule is an annual herb, native to Europe, that frequently occurs in grassland communities. Both cleistogamous (closed, autogamy) and chasmogamous (open, possible xenogamy) flowers are found on plants of *L. amplexicaule* (Lord 1978). *Bombus* sp. is the most important pollinator of this species (Proctor, Yeo & Lack 1996).

Medicago sativa is a common perennial legume that frequently occurs in European grassland communities. Partial self-incompatibility is observed in *M. sativa* (Brink & Cooper 1938). Pollinators of *M. sativa* are *Bombus* spp. and solitary bees, especially Megachilidae (Brookes *et al.* 1994). Due to their ability to fix N₂, legumes are known to facilitate growth of non-N-fixing neighbouring plant species (Temperton *et al.* 2007). Therefore, the three species in our experiment belong to a putative competitive hierarchy for below-ground interactions, from the competitive *M. guttatus* to the facilitative species *M. sativa*.

LITTERS

Prior to the beginning of the experiment, litter was obtained by growing individuals of the three study species in optimal conditions. The above-ground biomass of each species was harvested, oven-dried (65 °C, 48 h), and ground to 0.08 mm (Retsch ZM 100, Fisher Bio-block Scientific, Illkirch, France). The resulting litter of *M. guttatus*, *L. amplexicaule* and *M. sativa* had C:N ratios of 13.95, 18.17 and 23.98 and C:P ratios of 9.01, 12.74 and 27.49, respectively. The three-litter mixture was obtained by mixing an equal ratio of each specific litter.

EXPERIMENTAL SET-UP

During the experiment, plants were grown in plastic pots (14 cm diameter $\times$ 10.6 cm height; 1540 cm³) on a loamy sand soil. The soil was taken from depths of 5–20 cm in a grassland site near Paris (CEREPEP Foljuif, Saint-Pierre-les-Nemours, Seine-et-Marne, France). The soil had a C:N ratio of 10.40 and a C:P ratio of

26.03. The soil was sieved (< 1 cm) to remove rocks and large plant material.

The bottom of each pot was covered with a 118- μ m mesh and 10 g of vermiculite to prevent roots from growing out. Pots were then half-filled with a mixture of 600 g soil and 18 g vermiculite. Then 5.8 g of litter were incorporated into a second mixture of 500 g soil and 15 g vermiculite and added to the pots. The amount of litter that was added to the pots corresponded to an addition of 2.46 g of carbon, and c. +30% C. This amount is approximately equivalent to the annual carbon deposit in natural grassland ecosystems (0.16 kg C m⁻², Schlesinger 1997). Seeds of *M. guttatus* (MNHN, France), *L. amplexicaule* (Herbiseed, UK) and *M. sativa* (Herbiseed, UK) were germinated in Petri dishes, transplanted into experimental pots, and grown for 74 days. A substitutive design was adopted with a constant total number of six plants per pot (6, 3:3, 2:2:2 plants of each species in monocultures, two- and three-species mixtures, respectively). In mixtures, different species were arranged in alternation. Artificial light conditions differed during the growth and flowering periods (growth period: Metal Halide 250 W Philips; flowering period: Sodium lighting 250 W Sylvania), with a 16-h photoperiod. Pots were watered from below and their spatial position in the growth chamber was randomized throughout the experiment.

TRAIT MEASUREMENTS

In order to investigate the role of litter and plant interactions in determining above-ground traits linked to plant attractiveness to pollinators through modifications in below-ground functioning, we measured soil, plant and nectar variables.

Soil traits

Nitrogen and water are the most important common soil resources for plant growth. Total nitrogen content of biomass in plant communities was measured in order to determine the nitrogen supplied to the plants during the experiment (see methods below). When comparing similar communities, this measure can be seen as a proxy for the nitrogen supply capacity of the soil (van Eekeren *et al.* 2010). We also measured the final soil humidity in pots with a TethaProbe ML2 (Delta-T Devices, Cambridge, UK) to estimate water requirements of plant communities. Soil traits thus encompassed final humidity (%) and nitrogen supplied to communities (mg pot⁻¹).

Plant traits

At the end of the experiment, above-ground material of all plants was harvested, oven-dried (65 °C; 48 h) and weighed. Shoots from all individual plants of the same species from each pot were pooled to have enough dry matter for quantification. Shoot carbon and nitrogen contents were measured using a CHN Elemental Analyser (NA 1500 Series 2, Fisons, Manchester, UK). Throughout the experiment, the number of flowers of each individual plant was recorded weekly and the flower display size (i.e. largest number of opened flowers counted at once on individuals) was determined. Plant traits thus encompassed final above-ground biomass (g plant⁻¹), final shoot C:N ratio (g g⁻¹) and floral display size (number of flowers plant⁻¹) as indicators of quality of plant nutrition.

Nectar traits

Nectar was sampled from the beginning of the flowering period (after 35, 42 and 49 days for *L. amplexicaule*, *M. guttatus* and

M. sativa, respectively) until the end of the experiment. Because flower age influences nectar composition (Petanidou, VanLaere & Smets 1996), nectar was only collected in flowers that had been open for one day: in total 604 flowers of *M. guttatus*, 551 of *L. amplexicaule* and 236 of *M. sativa*. The volume of nectar was determined by measuring the length of liquid in the microcapillary used for nectar sampling (1 µL Drummond Microcaps®, Drummond Scientific Co., Broomall, Pa., USA). Nectar sugar composition (e.g. glucose, fructose and saccharose contents) was analysed by High Performance Anion Exchange Chromatography Pulsed Amperometric Detection (HPAEC-PAD, ICS-3000 Dionex). Due to the quantification threshold, samples were pooled by plant species for each pot. Consequently, chemical analyses were done on 66, 87 and 23 distinct samples of nectar from *M. guttatus*, *L. amplexicaule* and *M. sativa*, respectively. Separation was achieved on a CarboPac PA-1 anion-exchange column (4 × 250 mm, Dionex) thermostated at 30 °C. Elution was performed with a 48:52 (vol:vol) mixture of ultrapure water (Milli-Q, Millipore Corporation, Billerica, MA., USA) and a solution of NaOH (250 mM) with NaOAc (4 mM) at constant flow (0.7 mL min⁻¹). Nectar traits thus encompassed nectar volume (µL flower⁻¹), nectar sugar concentration (nmol µL⁻¹) and nectar di-/monosaccharide ratio (g g⁻¹) to depict nectar quantity, quality and composition. In order to compute an integrative index of plant attractiveness to pollinators, total sugar content in nectar potentially available to pollinators at plant level was estimated as the product of the mean quantity of sugar per flower by the mean floral display size per plant (µg of sugars plant⁻¹).

STATISTICAL ANALYSES

The three sets of variables: soil traits (humidity and nitrogen supplied), plant traits (final biomass, shoot C:N ratio and floral display size) and nectar traits (nectar volume, sugar concentration and di-/monosaccharide ratio) were analysed by multivariate analyses of variance (MANOVA Pillai tests) after log-transformation. Protected ANOVAS were subsequently used for each response variable only when multivariate significance was granted. Type III analyses of variance were done after backward simplification. Tukey *post hoc* tests were used to determine the significant differences among treatments. Total sugar content in nectar was analysed by linear model fitting using square-root-transformed data. Because plant interactions within plant communities are, *per se*, linked to focal plant species, statistical analyses of plant and nectar traits were performed for each focal plant species, with 'plant interaction', 'litter' and their interaction as fixed factors. All analyses were carried out using R 2.10.1 (R development core team 2010).

Results

SOIL TRAITS

The MANOVA performed on the set of soil properties (humidity and nitrogen supplied) revealed a highly significant effect of litter amendments (d.f. = 8, $F = 5.6$, $P < 0.001$) and plant interactions (d.f. = 12, $F = 7.6$, $P < 0.001$). However, no interaction between litter and plant interaction treatments was observed.

Pots that received litter were significantly drier than pots without litter (d.f. = 4, $F = 1.0$, $P < 0.01$), with the exception of litter of *M. guttatus* (Tukey test: $P = 0.16$). Regardless of the identity of litter, the addition of litter to the soils improved the soil nitrogen supply capacity since plant communities incorporated more nitrogen when grown on soil with litter (d.f. = 4, $F = 0.7$, $P < 0.001$). The observed significant effect of plant interactions on soil humidity (d.f. = 6, $F = 1.19$, $P < 0.001$) was only due to the *M. sativa* monocultures, the soils of which were drier than those of all other plant communities (least significant Tukey tests: $P = 0.04$), with the exception of *M. sativa* and *M. guttatus* mixtures ($P = 0.63$). The effect of plant interactions on nitrogen supplied to plants (d.f. = 6, $F = 1.84$, $P < 0.001$) was due, on the one hand, to monocultures of *L. amplexicaule* that contained less nitrogen than other communities (least significant: $P = 0.02$), and on the other hand, to monocultures of *M. sativa* (least significant: $P < 0.001$) and mixtures of *M. sativa* and *M. guttatus* (least significant: $P = 0.06$) that contained more nitrogen than other communities.

PLANT TRAITS

Values of plant and nectar traits of each plant species used in the experiment are summarized in Table 1. The MANOVAS performed over the set of plant traits (biomass, shoot C:N ratio and floral display size) revealed significant effects of litter inputs and plant interactions, but no interaction between these treatments was detected (Table 2).

Litter treatments significantly impacted final above-ground biomass and shoot C:N ratio in *L. amplexicaule* and floral display size in *M. guttatus* but did not influence any plant traits in *M. sativa* (Table 3; see Fig. S1 in Supporting Information).

Table 1. Plant and nectar traits for the three plant species *M. guttatus*, *L. amplexicaule* and *M. sativa* used in the experiment

	<i>Mimulus guttatus</i>		<i>Lamium amplexicaule</i>		<i>Medicago sativa</i>	
	Mean ± SE	<i>n</i>	Mean ± SE	<i>n</i>	Mean ± SE	<i>n</i>
Above-ground biomass (g plant ⁻¹)	0.95 ± 0.04	(77)	0.30 ± 0.02	(76)	0.36 ± 0.03	(72)
Shoot C:N ratio (g g ⁻¹)	37.9 ± 0.7	(77)	31.6 ± 0.9	(76)	20.6 ± 0.8	(72)
Floral display size (number of flowers plant ⁻¹)	2.6 ± 0.2	(77)	7.1 ± 0.5	(76)	0.3 ± 0.05	(72)
Nectar volume (µL flower ⁻¹)	0.12 ± 0.01	(30)	0.35 ± 0.02	(63)	0.04 ± 0.01	(21)
Nectar sugar concentration (nmol µL ⁻¹)	101.7 ± 8.1	(30)	115.6 ± 5.5	(63)	287.7 ± 25.3	(21)
Nectar di-/monosaccharide ratio (g g ⁻¹)	110.9 ± 35.3	(30)	2.4 ± 0.1	(63)	0.9 ± 0.1	(21)
Nectar sugar content (µg plant ⁻¹)	9.1 ± 1.0	(62)	68.3 ± 5.7	(65)	2.4 ± 0.7	(22)

Table 2. Summary of the effects of litter input and plant interaction on plant traits and nectar traits in *M. guttatus*, *L. amplexicaule* and *M. sativa*. Multivariate tests (MANOVA Pillai tests)

Source of variation	<i>Mimulus guttatus</i>					<i>Lamium amplexicaule</i>					<i>Medicago sativa</i>				
	Num d.f.*	Den d.f.*	Pillai test	F	P	Num d.f.*	Den d.f.*	Pillai test	F	P	Num d.f.*	Den d.f.*	Pillai test	F	P
Plant traits															
Litter input	12	207	0.46	3.11	0.001	12	204	0.48	3.23	0.001	12	192	0.30	1.76	0.057
Plant association	9	207	0.61	5.86	<.0001	9	204	0.81	8.42	<.0001	9	192	0.27	2.10	0.031
Nectar traits															
Litter input	12	66	0.39	0.81	0.639	12	165	0.19	0.93	0.521	12	39	0.62	0.84	0.605
Plant association	9	66	0.66	2.06	0.045	9	165	0.41	2.94	0.003	9	39	0.65	1.20	0.321

Data were log-transformed before multivariate analyses; see text for details of analyses.

*Num d.f. and Den d.f. correspond to numerator and denominator degrees of freedom, respectively.

Table 3. Summary of the effects of litter input and plant interaction on plant traits (above-ground biomass, shoot C:N ratio, floral display size) and nectar traits (nectar volume, nectar sugar concentration, nectar sugar ratio) in *M. guttatus* and *L. amplexicaule* and *M. sativa*. Univariate tests (Protected ANOVAS)

	<i>Mimulus guttatus</i>				<i>Lamium amplexicaule</i>				<i>Medicago sativa</i>			
	d.f.*	MS*	F	P	d.f.*	MS*	F	P	d.f.*	MS*	F	P
Litter input effect												
Above-ground biomass	4	0.009	2.26	0.071	4	0.009	5.68	0.001	†	†	†	†
C:N ratio	4	0.007	1.70	0.159	4	0.020	2.99	0.025	†	†	†	†
Floral display size	4	0.126	3.28	0.016	4	0.082	0.19	0.189	†	†	†	†
Plant interaction effect												
Above-ground biomass	3	0.023	5.99	0.001	3	0.016	10.31	<.0001	3	0.021	4.35	0.007
C:N ratio	3	0.042	10.68	<.0001	3	0.118	17.91	<.0001	3	0.046	3.38	0.023
Floral display size	3	0.006	0.15	0.926	3	0.298	5.73	0.001	3	0.039	2.74	0.050
Nectar volume	3	0.001	1.83	0.171	3	0.007	3.46	0.022	†	†	†	†
Nectar sugar concentration	3	0.008	0.22	0.882	3	0.032	1.72	0.172	†	†	†	†
Nectar sugar ratio	3	1.54	2.39	0.096	3	0.020	2.53	0.067	†	†	†	†

Data were log-transformed before univariate analyses; see text for details of analyses.

*d.f. corresponds to degrees of freedom and MS to square means.

†indicated non-allowed protected ANOVAS.

More precisely, the addition of *M. sativa* litter increased the biomass of *L. amplexicaule* plants by 68% relative to no litter, *M. guttatus* litter or tri-species mixture litter treatments (least significant: $P = 0.03$). The addition of litter, especially that of *L. amplexicaule*, also led to an 18% decrease of the shoot C:N ratio of *L. amplexicaule* plants ($P = 0.01$). The effect of litter inputs on the floral display size of *M. guttatus* was due to litter of *L. amplexicaule* that induce an increase of 91% compared to no litter or to *M. sativa* litter treatments (least significant: $P = 0.05$). More generally, the addition of litter improved biomass and C:N ratio plant traits of *L. amplexicaule* (d.f. = 3, $F = 5.45$, $P < 0.01$) and *M. guttatus* (d.f. = 3, $F = 5.44$, $P < 0.01$) but not those of *M. sativa* (d.f. = 3, $F = 2.24$, $P = 0.09$; see Fig. S1).

Regardless of the focal plant species, the plant interaction treatment had a significant impact on plant traits (Table 2). In the cases of *M. guttatus* and *L. amplexicaule*, the effects of plant interaction on plant traits were seen mainly when

M. guttatus was present in the communities (Table 3; see Fig. S2). When grown with *M. guttatus*, the above-ground biomass of *L. amplexicaule* plants was reduced by 42% and its shoot C:N ratio increased by 44% compared to those in monoculture or in two-species mixtures with *M. sativa* (least significant: $P = 0.02$). In three-species mixture, plants of *L. amplexicaule* also showed a reduced biomass and a higher shoot C:N ratio than those in two-species mixture with *M. sativa* (least significant: $P = 0.04$). Then, floral display size of *L. amplexicaule* was significantly reduced when grown in three-species mixture relative to plants in monoculture and in two-species mixtures with *M. sativa* (least significant: $P = 0.01$). *Mimulus guttatus* also had its biomass reduced by 30% and C:N increased by 20% when grown in monoculture compared to all other treatments (least significant: $P = 0.02$). In the case of *M. sativa*, analyses indicated significantly higher biomass and lower C:N ratio in monocultures relative to three-species mixtures (Table 3; least significant: $P = 0.05$; see Fig. S2).

NECTAR TRAITS

None of the nectar traits (nectar volume per flower, sugar concentration and di-/monosaccharide ratio) of the three plant species was significantly affected by litter regardless of its identity (see Fig. S3), and no interaction between litter and plant interaction treatments was observed (Table 2). However, MANOVAS indicated that the nectar traits of *L. amplexicaule* and *M. guttatus*, but not those of *M. sativa*, were sensitive to plant interactions (Table 2).

In *L. amplexicaule*, plant interactions strongly affected the volume of nectar per flower but did not significantly influence the sugar concentration or the di-/monosaccharide ratio in the nectar (Table 3; see Fig. S4). *Lamium amplexicaule* plants grown in two-species mixtures with *M. guttatus* showed a tendency to reduce volume of nectar per flower compared with mono ($P = 0.06$) and three-species mixtures ($P = 0.02$). In *M. guttatus*, univariate ANOVAS did not reveal significant effects of plant interactions on independent nectar traits (Table 3).

TOTAL SUGAR CONTENT OF NECTAR AVAILABLE TO POLLINATORS AT PLANT LEVEL

Once again, no significant interaction between plant interaction and litter treatments was detected on sugar content in nectar at the plant level in any species, but simple significant effects were found in *L. amplexicaule*.

Regardless of the focal plant species, no effect of litter treatment on the total sugar content of nectar was found. Nevertheless, analyses performed by grouping litter treatments according to 'with' or 'without' litter showed that the addition of litter significantly increased total sugar content in nectar of *L. amplexicaule* (d.f. = 1, $F = 4.53$, $P = 0.04$) but not in *M. guttatus* (d.f. = 1, $F = 1.26$, $P = 0.27$) or in *M. sativa* (d.f. = 1, $F = 3.98$, $P = 0.07$). On average, litter amendments led to an increase of 65% of sugar content in nectar at plant level in *L. amplexicaule*, compared to treatments without litter (Fig. 2).

Plant interaction within communities also significantly affected the total sugar content of nectar in plants of *L. amplexicaule* (d.f. = 3, $F = 5.09$, $P < 0.01$) but not in *M. guttatus* and *M. sativa*. When grown with *M. guttatus*, plants of *L. amplexicaule* provided a 56% lower nectar sugar content to pollinators than those in monoculture or in mixture with *M. sativa* (Fig. 2; least significant: $P = 0.02$).

Discussion

We investigated how modifications in below-ground functioning in plant communities influenced above-ground floral traits involved in plant attractiveness to pollinators. Our results indicate that total sugar content in nectar of *L. amplexicaule* was increased by litter amendment and strongly reduced by competitive interaction with *M. guttatus*, which is in accordance

Fig. 2. Mean total sugar content in nectar available to pollinators at the plant level ($\mu\text{g plant}^{-1}$) in *M. guttatus*, *L. amplexicaule* and *M. sativa* according to plant association and litter addition. Open bars represent treatments without litter amendment; shaded bars represent treatments with litter amendment (all litter identity confound). Plant interaction is coded with the first two letters referring to the focal plant species and the last two letters to the associated plant species. Numbers in brackets under bars indicate the number of replicates (pots). Error bars are + 1 SE.

with our predictions in Fig. 1. However, nectar traits of *L. amplexicaule* were not affected by facilitative interaction with *M. sativa*. On the other hand, nectar traits of *M. guttatus* and *M. sativa* were less sensitive to experimental treatments, showing that the validity of the predictions in Fig. 1 depends upon the focal plant species considered. In the following, we will discuss sequentially the effects of litter and plant interactions on soil, plant and nectar traits. We will then discuss the implications for pollination.

LITTER INPUTS

Litter quantity, identity and diversity are known to affect below-ground functioning (Bardgett & Shine 1999; Bowman *et al.* 2004; Meier & Bowman 2008) as well as above-ground primary productivity (Nilsson, Wardle & Dahlberg 1999; Bowman *et al.* 2004; Dehlin, Nilsson & Wardle 2006; Quedstedt & Eriksson 2006; Nilsson, Wardle & DeLuca 2008). The role of below-ground on above-ground biota including pollinators has already been investigated (Poveda *et al.* 2003, 2005; Wolfe, Husband & Klironomos 2005), but less is known about how litter affects floral and nectar traits.

Regardless of origin, litter amendment increased the nitrogen supply capacity of the soil, as expected (Fig. 1). This resulted in more plant growth and a greater water requirement of plant communities, hence the observed low soil humidity in pots with litter. However, litter effects on plant traits were very dependent on the focal species considered. The increase in above-ground biomass and the decrease in shoot C:N ratio with litter amendment in *L. amplexicaule*, and similarly, but less intensively, in *M. guttatus*, suggest that litter amendment lowered nutrient limitations. This was probably due to the release of nutrients from the litter amendment (Quedstedt, Callaghan & Press 2003) or to the stimulation of soil organic matter mineralization (Bardgett & Shine 1999). Positive (Quedstedt & Eriksson 2006; Nilsson, Wardle & DeLuca 2008) and negative effects of litter inputs (Bowman *et al.* 2004; Dehlin, Nilsson and Wardle 2006) on vegetation have been reported previously. In these latter cases, litter with high C:N ratio could have caused the soil micro-organisms to immobilize nutrients necessary for plant growth (Bowman *et al.* 2004). Litter amendments, on the other hand, did not affect plant traits of *M. sativa*. The fact that nodules were observed on the root system of *M. sativa* (data not shown) suggests that this species was able to symbiotically fix atmospheric nitrogen, making it less dependent on soil nitrogen availability.

The effects of litter identity varied depending upon the plant trait and the focal plant species considered, supporting the great variability of litter inputs on vegetation (Quedstedt & Eriksson 2006). Whilst litter of *L. amplexicaule* and *M. sativa* occasionally affected plant traits, that of *M. guttatus* never had any effect despite the lower C:N and C:P ratios. The magnitude of litter amendment effects on plant traits did not correlate with the initial litter stoichiometry, which is in accordance with the results of Bardgett & Shine (1999) that show that the

decomposition rate of litters from grassland was not necessarily related to litter nutrient content. There might have been a lower release of nutrients from *M. guttatus* litter due to the carbon quality of this litter (Hättenschwiler and Jorgensen 2010). However, we cannot exclude the possibility that such variations in responses are the result of a lack of statistical power in the experiment.

Mineral soil fertilization is known to stimulate the production of flowers (Campbell & Halama 1993; Juenger & Bergelson 1997; Muñoz *et al.* 2005; Burkle & Irwin 2009a, 2010), but the presence of litter did not have the same effect, with the exception of a positive effect of litter of *L. amplexicaule* on the floral display size of *M. guttatus*. A poor synchronization between the release of nutrients from the litter amendment and the flowering might explain the lack of strong effects, especially in *L. amplexicaule*. Finally, none of the nectar traits (nectar volume per flower, sugar concentration and di-/monosaccharide ratio) of the three species studied was affected by the presence of litter, regardless of litter identity. Nevertheless, the addition of litter was found to enhance the total nectar sugar content in *L. amplexicaule* at the plant level due to the integration of several modifications in nectar and plant properties. Plants of *L. amplexicaule* grown with litter supplied nearly 56% more nectar sugars than plants without litter (Fig. 2). This suggests that soil litter amendment can enhance individual plant attractiveness to pollinators at the plant scale as expected (Fig. 1), but only in some species. Differential responses to nitrogen addition are known to depend on life-history. Annual species are more sensitive to variations in nitrogen availability, whereas the response of perennials is often delayed due to their ability to store resources and their flexibility in resource allocation to biomass and reproduction (Burkle & Irwin 2009a; 2010).

PLANT INTERACTIONS

By taking up nutrients and providing carbon to soil, plants affect below-ground processes (Casper & Jackson 1997), which in turn can affect above-ground plant communities (Wardle *et al.* 2004). These effects can be due to particular species (e.g. legumes; Temperton *et al.* 2007) or particular assemblages of species (Milcu *et al.* 2006).

We found that *M. sativa*, whether alone or in mixture with *M. guttatus* had a positive influence on total nitrogen acquisition. This was due to the nitrogen fixing capacity of *M. sativa*. *Medicago sativa* plants in monocultures presented the highest biomass and lowest shoot C:N ratio, which explained the greater water consumption in these communities. In contrast to our initial predictions (Fig. 1), the effect of *M. sativa* on the neighbouring plants in the communities was weak: we found no statistical evidence that the presence of *M. sativa* in two-species mixtures improved plant traits of other species. Although it has been shown that legumes can improve the growth of neighbouring plants (Temperton *et al.* 2007), this is particularly true for grasses, but less so for herbs. Consequently, none of the nectar traits of plant species were improved by the presence of *M. sativa* within communities. Despite the fact that

vegetative traits of *M. sativa* were improved in monoculture, we did not find significant effects on floral display size, probably because the flower production was very low.

Plant traits of *L. amplexicaule* and *M. guttatus* were negatively affected by competitive interaction with *M. guttatus* in communities. When grown in association with *M. guttatus*, *L. amplexicaule* and *M. guttatus* plants presented a reduced biomass and a higher shoot C:N ratio. This explicitly revealed the effects of inter- and intra-specific competition with *M. guttatus*, possibly through an increased exploitation of soil nitrogen. Indeed, rapid growth of *M. guttatus* constituted a strong competitive attribute of this species (Truscott *et al.* 2006) for soil nitrogen acquisition. As vegetative traits of *L. amplexicaule* and *M. guttatus* positively responded to nutrient addition released by litter, they also negatively responded to nutrient depletion by the presence of *M. guttatus*. This translated to a reduction in floral display size only in *L. amplexicaule* but not in *M. guttatus*.

Most importantly, the competitive attributes of *M. guttatus* led to a reduced sugar content in the nectar of *L. amplexicaule* (Fig. 2). This resulted from a combined decrease in mean volume of nectar per flower and in floral display size. To our knowledge, this is the first experimental demonstration that competition among plants for soil resources can have an impact on plant traits linked to attractiveness to pollinators, as predicted in Fig. 1. On the other hand, nectar traits of *M. guttatus* and *M. sativa* were not affected by plant interactions within communities, contrary to what was expected (Fig. 1). The literature seems to suggest that the responses of nectar traits to soil mineral fertilization are idiosyncratic: positive (Campbell & Halama 1993; Petanidou, Goethals & Smets 1999; Burkle & Irwin 2009a), neutral (Petanidou, Goethals & Smets 1999; Erhardt, Rusterholz & Stocklin 2005; Burkle & Irwin 2009a) or negative effects (Petanidou, Goethals & Smets 1999) have been observed. The species *M. guttatus* and *M. sativa* might be less sensitive to competition for resources for the reasons explained above (Burkle & Irwin 2009a; 2010). The effects may also have been too weak to be detected because of the small nectar volumes per flower (especially in *M. sativa*, see Table 1).

Finally, no significant interactions between plant association and litter inputs were detected, suggesting that the presence of litter did not affect the magnitude of plant–plant interactions. It differed from observations made by Ladd & Facelli (2008) who demonstrated that litter could modify plant interactions. Litter effects on seedling emergence, which were not considered in our experiment, were found as the main mechanism in modification of plant interaction by litter.

IMPLICATIONS FOR POLLINATION

According to foraging economics, nectar availability and floral displays are the most important traits influencing attractiveness of plants to pollinators (Kudo & Harder 2005). Flower visitors' foraging benefits are directly linked to nectar availability and foraging costs depend on the flight distance among flowers, which is likely to be reduced with larger floral displays.

Plants with larger floral displays (Ohashi & Yahara 2001; Kudo & Harder 2005) or with higher nectar production per flower (Klinkhamer & de Jong 1990; Klinkhamer, de Jong & Linnebank 2001; Kudo & Harder 2005) attract more pollinators.

In our pot experiment, we showed that amendment of a quantity of litter of the same order of magnitude as that found in natural grasslands (Schlesinger 1997) can enhance the total sugar content of nectar in *L. amplexicaule* but not in *M. guttatus* and *M. sativa*. In natural situations, litter amendment to the soil from species that composed the community should thus enhance attractiveness of plant species that are the most dependent on below-ground resources. Due to species-specific sensitivity of plants to litter inputs, we thus hypothesize that litter management practices would alter plant–pollinator interaction networks at the community level (Burkle & Irwin 2009b).

Furthermore, we found that *M. guttatus*, the most competitive species in our experiment, strongly altered nectar sugar content in *L. amplexicaule*. Since *M. guttatus* and *L. amplexicaule* share common pollinators, the introduction of *M. guttatus* in European natural grasslands might decrease *L. amplexicaule* attractiveness to pollinators in favour of *M. guttatus* (Fig. 1). Pollination of *L. amplexicaule* might therefore suffer from competitive interactions with *M. guttatus* (Waser 1978; Brown, Mitchell & Graham 2002; Bell, Karron & Mitchell 2005), which could have negative consequences for *L. amplexicaule* fitness since the quality of its reproduction depends upon pollinators (Lord 1978). This highlights alternative mechanisms by which invasive plant species could alter natural community dynamics.

Overall, our experiment underlines the importance of taking into account interactions between plants for soil resources in studying plant–plant interactions for pollinator access (Waser 1978; Chittka & Schurkens 2001; Brown, Mitchell & Graham 2002; Moeller 2004; Bell, Karron & Mitchell 2005; Torang, Ehrlen & Agren 2006). Since non-attractive plants such as wind-pollinated species are also involved in plant interactions for soil resources, such species might indirectly alter plant–pollinator networks, by affecting insect-pollinated floral and nectar traits involved in plant attractiveness to pollinators.

Acknowledgements

We are grateful to Elodie Alapetite, Adélaïde Roguet and Marion Demarcy for their valuable help with experiment setting up and data collection. We also thank Colin Fontaine, Gérard Lacroix, Florence Maunoury-Danger and Elisa Thébaud for productive discussion on the experiment. Earlier drafts of the manuscript benefited from insightful comments from Richard Michalet, Luc Gigord and Theodora Petanidou. We also thank the Associate Editor and two anonymous referees for constructive and useful comments.

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Received 19 May 2010; accepted 4 January 2011

Handling Editor: Sedonia Sipes

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Effects of litter on plant traits of *M. guttatus*, *L. amplexicaule* and *M. sativa*.

Figure S2. Effects of plant interaction on plant traits of *M. guttatus*, *L. amplexicaule* and *M. sativa*.

Figure S3. Effects of litter on nectar traits of *M. guttatus*, *L. amplexicaule* and *M. sativa*.

Figure S4. Effects of plant interaction on nectar traits of *M. guttatus*, *L. amplexicaule* and *M. sativa*.

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