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Pollination increases white and narrow-leaved lupin protein yields but not all crop visitors contribute to pollination

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ABSTRACT

Global biodiversity is declining under pressure of agricultural intensification and land-use change. Two-thirds of the agricultural lands is directly or indirectly devoted to the production of animal products. Replacing animal-based proteins by plant-based proteins can be an important step to a more sustainable agricultural system. Lupins (*Lupinus* sp.) are promising crop species due to a high protein content of up to 40 %, but crop yields are unstable in both quantity and quality. This might be due to a lack of effective pollinators, but the contribution of insect pollination to lupin crop yield is unknown. Here we studied for five varieties of two common lupin crop species (*L. albus* and *L. angustifolius*) which pollinators visit lupin flowers, whether this depends on nectar production, and what the contribution of insect pollination is to crop yield. We used a semi-experimental setup and placed bagged and open-pollinated plants in pots along an expected gradient of insect visitors and determined several yield parameters. We recorded 1355 pollinator visits of only eight bee species. None of the varieties tested produced nectar. Compared to bagged plants, protein yield increase of open-pollinated plants ranged from 3 to 11% depending on variety. Yield of open-pollinated plants was only consistently related to visitation of the large-bodied buff-tailed bumblebee (*B. terrestris* group; 59 % of all pollinators) with impact on seed set related yield parameters (number of seeds and pods) being generally larger than on seed filling related yield parameters (g/plant). Within the observed range, higher visitation rate of buff-tailed bumblebees increased protein yield of open-pollinated plants with 10–40 %. Visitation rates of the smaller common carder bee (*B. pascuorum*; 33 % of all pollinators), or all pollinators combined, were not significantly related to protein crop yield. This could indicate that only relatively large species are effective lupin pollinators. Lupins are generally considered self-pollinating, and therefore growers do not actively manage for insect pollination. Our results show that insect pollination, and in particular buff-tailed bumblebees, can contribute substantially to the crop yield, which suggests that management aimed at enhancing effective pollinator species can help to make lupin crop cultivation more profitable. Amongst others, such management should make sure that ample nectar is available in the surroundings of lupin crops, as lupin does not produce nectar.

1. Introduction

Global biodiversity is declining rapidly, mainly because of widespread habitat loss to agricultural land. Forty percent of the terrestrial world is devoted to agriculture (largest land-use in the world), and of this surface, 75 % is for the production of animal products, or producing animal feed (Foley et al., 2011). While animal products are rich in protein, they have a substantially larger carbon and ecological footprint than plant-based proteins (Willett et al., 2019). Replacing animal-based proteins by high-quality plant-based proteins would considerably reduce the land cover required to feed the growing world population, and would release some of the pressure on global biodiversity.

Lupins (*Lupinus* spec.) are promising protein-rich grain legumes for European agriculture (Lopez-Bellido and Fuente, 1986), with protein contents of up to 40 % (Linnemann and Dijkstra, 2002; Lucas et al., 2015). Cultivated lupins are generally biodiversity-friendly crops, as they are attractive to many insect pollinators and contribute to a healthy soil (Nemecek et al., 2008). However, some perennial lupin species (e.g. *L. polyphyllus*, *L. nootkatensis* and *L. arboreus*) are invasive species in for example Scandinavia, Iceland, and New Zealand, and at least one cultivated annual species (*L. luteus*) is invasive in South Africa (Yelenik et al., 2007), which can have net negative effects on biodiversity

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(Ramula and Sorvari, 2017). Several species of lupins native to Europe have been cultivated for about 4000 years, and two of them are still regularly cultivated as a seed crop for human consumption and animal feed (Lopez-Bellido and Fuente, 1986; Lucas et al., 2015): narrow-leaved *(L. angustifolius*) and white lupin (*L. albus*). Leguminous crops like lupins were traditionally being used to increase the soil fertility due to their symbiosis with nitrogen-fixing bacteria, and to provide valuable protein-rich crop yields. However, with the growth in fertilizer use after World War II, leguminous crops were increasingly lost in the crop rotation. Recently, leguminous crops are slowly gaining ground again, and lupins are in particular interesting, as they can also mobilize phosphate, and the flowers are frequently visited by insects (Lopez-Bellido and Fuente, 1986; Lucas et al., 2015). Currently, the area cultivated with lupins is small, largely because crop yields are still relatively low and unpredictable in both quality and quantity (Lucas et al., 2015) which limits its appeal to farmers and the food industry.

One of the reasons for the unreliable yields could be the lack of effective pollinators visiting lupin flowers. Like most leguminous crops, lupins are self-compatible, but cross-pollination by insects is likely to increase yields (Klein et al., 2007). Flowers are large and robust and pollinators need to be heavy to be able to push down the keel to effectively pollinate lupins (Williams, 1987). Mostly large and relatively hairy bees, such as bumblebees (*Bombus* sp.), megachilids (*Mechachile*, *Osmia* sp.), long-horn bees (*Eucera* sp.) and flower bees (*Anthophora* sp.) have been documented to visit (wild) lupins and to collect pollen (Kleijn and Raemakers, 2008; Peeters et al., 2012). A large number of these bee species have been declining in Northwestern Europe and are now rare or even extinct (Kleijn and Raemakers, 2008; Peeters et al., 2012; Rollin et al., 2020). A shortage of these lupin pollinators may have led to the relatively low lupin crop yields. However, little is known about the benefits of insect pollination to crop yields of lupin species (Langridge and Goodman, 1977; Williams, 1987), but this information is required to effectively manage for increasing lupin crop yields.

Surprisingly, it is unclear whether, in addition to pollen, (wild) lupins produce nectar that insect visitors require for their energy intake (Williams, 1987). Some studies have estimated the honey production of honeybees stocked in a narrow-leaved (Langridge and Goodman, 1977) and a white lupin crop field (Langridge and Goodman, 1985), and they observed honeybees foraging for nectar. From this, they concluded that lupins must produce nectar. However, other sources indicate that none of the lupins produce nectar (Williams, 1987; Heiling et al., 2019), but may produce sugar-rich phloem exudation near the flowers (Atkins, 1999). Lupins flower in June, the period in the season where nectar availability in agricultural landscapes is most limited (Timberlake et al., 2019). Knowing whether cultivated lupins produce nectar or not is important because nectar availability affects the attractiveness of the crop to pollinators and may indirectly act as a factor limiting the size of the local pool of pollinators available for pollination of this crop.

In this study the primary objective was to assess the contribution of insect pollination to crop yield. As a secondary objective, we wanted to know which pollinator species visit lupin and whether they could collect nectar. We studied the benefits of insect pollination on crop yield of five commercial varieties of both narrow-leaved and white lupin in a semi-controlled field experiment. We raised lupin plants in pots in a controlled setting, and just before flowering, we transported the pots to 15 different locations with varying background populations of wild pollinators. Half of the plants were bagged to exclude pollinators (i.e. only self-pollination), while the other half were exposed to the background pollinator populations. After flowering, the plants were transported back to the same location for seed ripening and harvest. We then addressed the following questions: 1) How does insect pollination contribute to lupin crop yield? 2) Does increasing pollinator visitation rate increase lupin crop yield, and does this depend on pollinator identity? And 3) Do narrow-leaved and white lupin produce nectar? We pay particular attention to the insect contribution to lupin protein yield, as this crop yield parameter determines crop revenue for lupin growers.

2. Methods

2.1. Study system

In this study we used the two commonly cropped species narrow-leaved lupin (*Lupinus angustifolius*) and white lupin (*L. albus*). Crop variety is one of the main management choices of farmers, and therefore it is important to test multiple varieties per species, as varieties can differ in their insect dependency. We tested for differences between insect dependency of five commonly used commercial varieties per lupin species (Fijen et al., 2018). For narrow-leaved lupin we used Primadonna (whitish flowers), Boruta (white flowers), Regent (blue flowers), Haags Blaue (blue flowers) and Iris (pinkish flowers), and for white lupin we used Boros (cream white flowers), Butan (cream white flowers), Dieta (cream white flowers), Feodora (cream-white flowers,) and Sulimo (off-white flowers).

The experiment took place in the surroundings of Wageningen, Gelderland, the Netherlands. The plants were sown and raised at the University's greenhouse complex (N51.995, E5.655) in a semi-open polytunnel. Just before flowering the plants were relocated to 15 University farm locations in the near surroundings, with the farthest location being eight kilometers away from the polytunnel (supplementary material S1). Locations were located at least 600 m from each other, which is about the mean maximum foraging distance of the pollinators foraging on lupins (Knight et al., 2005). To test for the effect of different levels of insect pollination we selected locations with a gradient in landscape complexity (% semi-natural habitat cover, including urban habitat) using Google Earth aerial pictures (final range: 12–68 % cover in 600 m radius). As shown by van Gils et al. (2016) this would result in a wide range of pollinator visitation rates in our study region.

2.2. Experimental setup

We used 10 L, black, plastic pots (26 cm top diameter) in our experiment to control for effects of management (e.g. fertilizer application, weed control, timing of sowing) and location (e.g. soil type and condition), and to be able to transport plants from a controlled environment to flowering locations with different pollinator backgrounds.

We filled the pots with former agricultural sandy soil that was poor in nitrogen (NO_3^-: 11.0 mg/kg dry soil, NH_4^+: 0 mg/kg dry soil) and phosphate (PO_4^-: 0.46 mg/kg dry soil) but rich in potassium (K^+: 18.98 mg/kg dry soil), and relatively high in organic matter content (4.65 %). We pre-germinated seeds on 30 March 2019 to be able to discard non-germinating seeds. We used plastic trays with filtration paper to keep the seeds moist, and we added inoculum (HiStick® Lupin inoculant, BASF) to ensure colonization of the nitrogen-fixating bacteria. On 2 April 2019, we sowed six and three seeds per pot to reach a plant density of 30 and 15 plants/m^2, respectively, for narrow-leaved and white lupin. After one week, we replaced dead seedlings with healthy seedlings that were also sown on 2nd of April. For some varieties (Haags Blaue, Iris and Primadonna), we had to re-sow seeds on the 9th of April because the seedling vigor was low. To prevent the plants from hanging over the pots, we placed bamboo sticks in the center of the pots and added a 30 cm wide plant support ring.

Because lupins can bind nitrogen through the symbiosis with *Bradyrhizobium*-bacteria (Lucas et al., 2015), and free up phosphate bound to soil particles by means of cluster roots (Lambers et al., 2012), we did not add any fertilizer. The pots were manually weeded several times. Plants were watered as required in the polytunnel, which meant every few days in the early season, but daily during hot days. On the flowering locations, the plants were watered every other day, and we placed a plate below the pots to ensure that there was some storage of water. Because up until flowering all plants had been growing under standardized conditions in a polytunnel, we assumed that the sets of nine or eighteen plants of each variety, provided a similar number of flowers (i.e. were equally attractive to pollinators) across flowering locations.

Just before flowering of the first variety (27 May 2019), we relocated the pots to the flowering locations. At this point, we bagged half of the plants with a transparent, white, fine-mesh bag made of bridal gown to exclude insect-pollinators from plants of our non-insect pollinated treatment. After all plants had finished flowering, the bags were removed and the pots were relocated back to the polytunnel on 24th of June.

We used three pots per lupin variety per flowering location per treatment. This resulted in a total of 3 pots * 2 pollination treatments * 10 varieties * 15 locations = 900 pots, with location being our unit of replication ($n = 15$). The pots of the same flowering location were put in blocks in the polytunnel (i.e. each flowering location is one block). Within each block, the three pots per treatment and variety (referred to as ‘set of plants’) were clustered, but different varieties and treatments were randomly located.

2.3. Pollinator visitation rate

From the onset of flowering we observed the lupin plants to estimate pollinator visitation rate. Each set of non-bagged plants was observed for 20 min per observation event, and we counted each pollinator that landed on the flowers (Fijen and Kleijn, 2017). We could usually follow an individual pollinator, and we counted that pollinator only as a new visit if it had visited another set of lupin plants or when it had left the patch of lupin plants. Pollinator species were identified on the wing, or if not possible caught and stored for later identification. We lumped the species of the *Bombus terrestris*-complex, as these species are not identifiable without DNA analysis (Williams et al., 2012). Locations were visited both in the morning and the afternoon to get a representative estimate of pollinator visitation rate. Observations only took place under favorable weather for pollinators (i.e. > 18 degrees Celsius, <5 Beaufort wind speed and not too cloudy). Sets of lupin plants that had none or few flowers open (maximum three) were not observed. A set of plants was observed on average 7.3 ± 0.3 SE times (range: 2–12 times).

2.4. Crop yield

Because some plants were lost due to disease or were too heavily infested with aphids, we counted the number of remaining plants with pods for each set of pots before harvest. The different varieties had different seed ripening durations, and not all pods were ripe at the same time. We therefore harvested the seed pods in different batches. We counted and harvested the seed pods as soon as the skin of the pods were dry, and combined all pods from the same set of lupin plants. We dried the pods completely in an artificial dryer at 28 degrees Celsius for 48 h. After drying, the pods were threshed and cleaned manually, total seed weight was determined, and the number of seeds was counted using a seed counter (Contador, Pfeuffer GmbH). To get representative samples of seed protein content - narrow-leaved lupin produced more, and smaller seeds than white lupin - we first calculated the average number of seeds produced for both crop species. We then ground 10 % of the average number of seeds produced (38 and 23 full seeds, respectively) per set of narrow-leaved and white lupin plants. The ground sample was destructed with H_2SO_4-salicylic acid-H_2O_2 and selenium (Walinga et al., 1989). Nitrogen content was measured using a segmented-flow system, and we multiplied these values by a conversion factor for nitrogen to crude protein content (%) per sample (Rhee, 2001). As this conversion factor is not available for lupin, we used the conversion factor of soy (5.71, c.f. (Rhee, 2001)), which has similar bean characteristics.

2.5. Nectar measurements

To measure nectar production, we used an extra set of three pots per variety. When the plants were flowering, we bagged them for 24 h using the bridal gown bags to potentially let nectar build up in the flowers (Cecala et al., 2020). We then used 1 μL micro-capillaries to suck up any liquids from the flowers, and counted how many flowers it takes to fill up the micro-capillaries (c.f. Fijen et al., 2020). We repeated this procedure on three occasions on different days.

2.6. Analysis

To test whether some varieties were visited more frequently than others, we calculated average visitation rate per variety per site. We calculated visitation rate for all pollinators (pollinators/20 min), and separately for the two most dominant pollinators (*Bombus terrestris* and *B. pascuorum*; together 92 % of all visits). We used a mixed effects model (function *lme* in R-package ‘nlme’; Pinheiro et al., 2015) with visitation rate as our response, and variety as our explanatory variable, using data of only the open-pollinated plants. We square root-transformed visitation rate to improve normality of residuals. To correct for the nested design of the experiment, we used lupin species, nested within location as our random structure. We controlled for the heterogeneity of variances due to lupin species by including this in the varIdent structure (weights = varIdent(form = ∼1|lupin species)).

We did not find nectar in any of the varieties on any of the three occasions, and we therefore did not statistically analyze this.

To test the effects of bagging treatment and visitation rate on crop yield, we used the total number of pods, number of harvested seeds, total harvested seed weight (g), protein content (% of dry weight) and total harvested protein weight (total seed weight (g) multiplied by protein content (%); g) as our crop yield parameters. We did not count the flowers, and could therefore not calculate seed or pod setting rates. To account for lost plants, we standardized all crop yield parameters back to yield per plant (e.g. g/plant). Effects of pollinator exclusion and varying pollinator visitation rates on crop yield parameters were examined in the same model (analyses using separate models for bagged and unbagged plants yielded qualitatively similar results). We calculated average visitation rate per site for all pollinators together, and for the two dominant pollinators separately. This makes visitation rate a variable without variation within site which guarantees that its effect is measured across sites, which is otherwise not guaranteed in mixed effects models. We also assigned this average visitation rate to the bagged plants, to avoid convergence issues in the model. The difference between the bagged plants and the non-bagged plants is then captured in the model by the factor treatment (bagged/open). We tested the effects of variety, treatment (bagged/open) and visitation rate, and their interactions on all crop yield measures separately, and we did this for all pollinators combined, and for the two dominant pollinators separately. For these analyses, we also included variety in our random structure (random = ∼1|location/lupin species/variety) to make sure that effects were measured across locations, even if the fixed effect variety was to be excluded in the final model. We corrected for the heterogeneity of variances as above. We constructed a full model, and used backward model simplification based on likelihood ratio tests (Burnham and Anderson, 2002). Pairwise comparisons between total protein crop yields, the main crop yield parameter, of different varieties were performed using a Tukey test in the multcomp R-package (Hothorn et al., 2008). Lupin species could not be included as a fixed effect, as lupin variety was completely nested within lupin species (i.e. full correlation). All models showed a normal distribution of residuals and no heterogeneity of variances.

Some pots of Primadonna of two different sites and treatments were mixed up during transport, and these whole sets ($n = 2$) were excluded from analyses. One set of plants had missing information on the number of pods, and was excluded from that analysis. All statistical analyses were performed in R version 3.6.3 (R Core Team, 2020).

3. Results

In a total of 371 h, we observed 1355 pollinator visits of only eight bee species. The two most common visitors were *Bombus terrestris* (59 %

of all visits) and *B. pascuorum* (33 %). Other occasional visitors were *Bombus lapidarius* (n = 40), *Megachile ericetorum* (n = 32), *Apis mellifera* (n = 22), *Bombus hortorum* (n = 3), *Andrena wilkella* (n = 3), and *Bombus pratorum* (n = 1). Eleven visitors moved on before they could be identified.

Overall pollinator visitation rate was similar between varieties (χ^2 (9) = 13.82, P = 0.13), as was visitation rate of *Bombus terrestris* (χ^2 (9) = 11.67, P = 0.23). The main effect of variety was significant for *Bombus pascuorum* visitation rate (χ^2 (9) = 18.19, P = 0.03), but marginal means did not differ significantly between varieties (pair-wise comparisons p > 0.08), suggesting that differences between visitation rates of different varieties were not very large.

None of the tested lupin varieties produced nectar on any of the three occasions measured. To double check, we dissected some flowers which confirmed that we did not miss nectar in hard to reach places.

Effects of bagging on number of pods, number of seeds and total harvested seed weight (g/plant) depended on the variety (i.e. significant interaction treatment*variety; Table 1; Fig. 1A). However, protein content (%) and total harvested protein weight (g/plant) was consistently higher (range: 3.1–11.3 %) in the open-pollinated treatment for all varieties (Table 1; Fig. 1B). Whether plants received few or many pollinator visits did not influence crop yield parameters when all pollinators considered together. In contrast, visitation rate of *Bombus terrestris* was significantly positively related to all crop yield parameters except protein content (Fig. 2; Table 1). Visitation rate effects were larger for seed set related crop yield parameters (number of seeds and pods) than for seed filling related crop yield parameters (g/plant yield; Fig. 2). All combined this resulted, within the observed range, in protein yield increasing 0.14 g per plant with each increase of one *Bombus terrestris* visit per 20 min (Fig. 3). This ultimately translated to a 20 % increase in the lowest yielding variety (Primadonna), and a 5% increase in the highest yielding variety (Sulimo) with one additional *Bombus terrestris* visit per 20 min. Visitation rate of *Bombus pascuorum* was not significantly related to yield parameters (Table 1), except for protein content (%), where the effect of visitation rate of *Bombus pascuorum* increased or decreased the protein content of the seeds (%), depending on the lupin variety. All narrow-leaved varieties had similar protein yields (g/plant; pairwise comparisons p > 0.12), but this was significantly lower than all the white lupin varieties (pairwise comparisons p < 0.001). Boros (white lupin) had intermediate yields and was significantly different from all other varieties (pairwise comparisons P < 0.001), but the other white lupin varieties showed similar protein crop yields (g/plant; pairwise comparisons PP > 0.77). Mean and SD values for the number of seeds per plant, 1000-seed weight (g), seeds per pod, and pods per plant are presented in Table S1.

4. Discussion

We found that insect pollination had differential effects on intermediate crop yield parameters, but consistently positive effects on the final protein crop yield. Lupin crop yields increased with increasing visitation rate of the relatively large buff-tailed bumblebee (*B. terrestris*-group) but not with the smaller common carder bee (*B. pascuorum*) or all pollinators combined (total of eight species). Protein yields differed between the two crop species and within crop species between varieties, but all responded similar to the bagging treatment and increased visitation rate of buff-tailed bumblebees. The benefits of increased buff-tailed bumblebee visitation were stronger for the more direct crop yield parameters such as number of seeds, and least strong, yet substantial, for total protein crop yield (g/plant). None of the varieties tested produced nectar, which suggests that lupin growers need to make sure there is ample nectar in the surroundings if they want to sustain a

Table 1

Treatment and visitation rate effects on lupin yield parameters. Tested separately for all pollinators combined, only *Bombus terrestris*, or only *Bombus pascuorum*. Significance values were obtained using type III anova tests (Chi-square) of the final model.

			All pollinators		***Bombus terrestris***		***Bombus pascuorum***	
	Response variable	df	Chi-square	p-value	Chi-square	p-value	Chi-square	p-value
Pods	Variety	9	242.92	<0.001	243.15	<0.001	242.92	<0.001
	Treatment	1	1.17	n.s.	1.167	n.s.	1.17	n.s.
	Visitation rate	1		n.s	10.81	0.001		n.s
	Variety*Treatment	9	30.67	<0.001	30.62	<0.001	30.67	<0.001
	Variety*Visitation rate	9		n.s		n.s.		n.s
Number of seeds								
	Variety	9	196.97	<0.001	196.8	<0.001	196.97	<0.001
	Treatment	1	0.56	n.s.	0.56	n.s.	0.56	n.s.
	Visitation rate	1		n.s.	8.33	0.004		n.s.
	Variety*Treatment	9	51.84	<0.001	51.87	<0.001	51.84	<0.001
	Variety*Visitation rate	9		n.s.		n.s.		n.s.
Total yield (g/plant)								
	Variety	9	864.67	<0.001	850.72	<0.001	864.67	<0.001
	Treatment	1	1.422	n.s.	1.43	0.038	1.422	n.s.
	Visitation rate	1		n.s.	11.83	<0.001		n.s.
	Variety*Treatment	9	22.75	0.007	22.9	0.006	22.75	0.007
	Variety*Visitation rate	9		n.s.		n.s.		n.s.
Protein content (%)								
	Variety	9	141.41	<0.001	141.41	<0.001	150.21	<0.001
	Treatment	1	4.68	0.031	4.68	0.031	5.3	0.021
	Visitation rate	1		n.s.		n.s.	2.87	0.09
	Variety*Treatment	9		n.s.		n.s.	39.58	<0.001
	Variety*Visitation rate	9		n.s.		n.s.		n.s.
Total protein yield (g/plant)								
	Variety	9	1481.47	<0.001	1470.37	<0.001	1481.47	<0.001
	Treatment	1	10.13	0.001	10.08	0.001	10.13	0.001
	Visitation rate	1		n.s.	10.78	0.001		n.s.
	Variety*Treatment	9		n.s.		n.s.		n.s.
	Variety*Visitation rate	9		n.s.		n.s.		n.s.

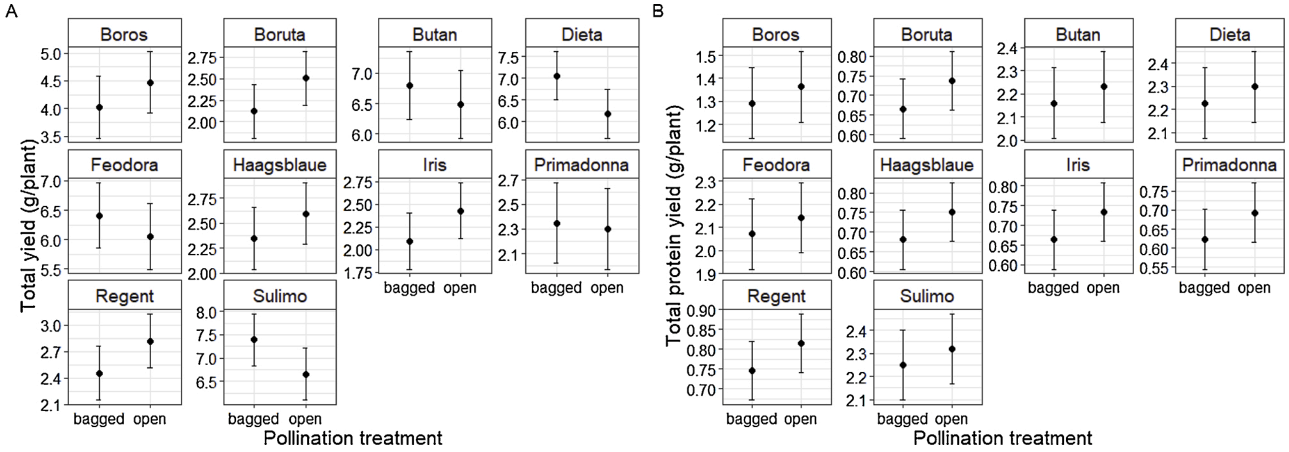

Fig. 1. The effect of bagging on (A) total yield (g/plant) and (B) total protein yield (g/plant) for all varieties. Patterns for number of pods and seeds were qualitatively similar to total yield (g/plant; panel A), and for protein content (%) similar to protein yield (g/plant; panel B). Points are estimated means, and error bars represent the 95 % confidence interval. Note that the y-axes are different for each graph.

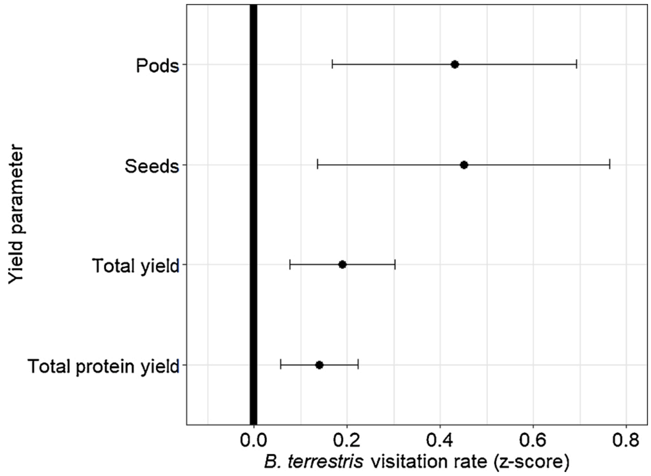

Fig. 2. The effect of *Bombus terrestris* visitation rate on the number of pods, number of seeds, total yield (gram), and total protein yield (gram) per plant. *Bombus terrestris* visitation rate was scaled and centered to compare slopes between analyses. Points are effect sizes, and error bars indicate 95 % confidence interval.

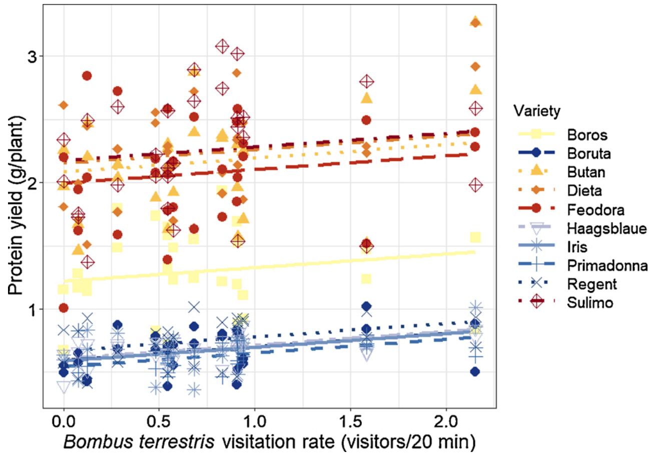

Fig. 3. The effect of *Bombus terrestris* visitation rate (visitors/20 min) on protein crop yield (g/plant). Varieties (indicated in different symbols, lines and color) have significantly different protein crop yields per plant. Visitation rate represents the average per site. Narrow-leaved lupin varieties have a blue-hue color, while white lupin varieties have a reddish-hue color. Results are back-transformed partial residuals, while controlled for treatment effects.

healthy crop pollinator community.

Total protein yields (g/plant) were 3–11 % higher with insect pollination depending on the lupin species and variety (Fig. 1b). These modest estimates are likely to be conservative, as the higher total yields (g/plant) for the four high-yielding varieties under the bagged treatment (Fig. 1a) indicate that the bags that cover the plants had a positive effect on growth conditions, for example by reducing pest pressure, evaporation and drought stress. Additionally, these estimates are based on yield differences between bagged plants and plants visited by all pollinators combined, where visitation rate of all pollinators was not significantly related to crop yield. Potentially more important, increasing visitation rate of buff-tailed bumblebees resulted in as much as 10–40 % increase in protein yield (Fig. 3). This is substantial given that lupin is often considered to be self-pollinated (Williams, 1987). While protein yield responses to increased insect pollination were similar in all tested varieties, white lupin varieties had twice as high protein yields per plant as narrow-leaved varieties. Protein content (%) of the seeds was higher in plants without bags, but did not increase with increasing visitation rate of buff-tailed bumblebees (Table 1). As the number of seeds increased with buff-tailed bumblebee visitation, this indicates that insect pollination was a stronger limiting factor for lupin seed production than the amount of nutrients that could be allocated to the seeds (Lopez-Bellido and Fuente, 1986; Fijen et al., 2020). Increasing the effective pollinator population could therefore result in higher lupin protein crop yields.

Only increasing visitation rate of buff-tailed bumblebees increased lupin yield, and coincidentally, they were the commonest and the largest lupin visitors, which might indicate that large pollinators are more efficient lupin pollinators. Lupin flowers need to be tripped to make the pollen available for cross-pollination (Williams, 1987; Williams et al., 1990). Common carder bees were able to trip flowers, as they collected substantial amounts of lupin pollen on the plants (~ 44 % lupin pollen in collected pollen clumps, ~ 56 % for *B. terrestris*; data not shown). However, their visitation rate did not increase crop yield, despite them being the second most abundant visitor. One possible explanation could be that they can only partially trip the flowers because of their relatively smaller size (Kendall et al., 2019), allowing them to collect the pollen, but do not deposit pollen on the stigma. Alternatively, inter-flower and inter-plant movement rates, and flower handling behavior can potentially explain why common carder bee did not contribute to crop yield, and buff-tailed bumblebees did (Russo et al., 2017). Such pollinator species-specific pollination effects are not uncommon for fabaceous crops (Garratt et al., 2014; Marzinzig et al., 2018). The absence of benefits of common carder bee visits may also have masked the contribution of buff-tailed bumblebee visitation rate in analyses with all pollinators combined. Whether pollinator body size is a good predictor of effective pollination in lupins needs to be studied in more detail, so that farmers can decide to manage for increasing average pollinator body size (Oliveira et al., 2016; Grab et al., 2019).

As may be expected, the few other lupin pollinator visitors were common crop pollinator species (Kleijn et al., 2015), except for two occasional visitors (*Andrena wilkella* & *Megachile ericetorum*), and we found none of the rare pollinator species that were historically found on lupin (Kleijn and Raemakers, 2008; Peeters et al., 2012). The large garden bumblebee (*Bombus ruderatus*) is extinct in the Netherlands, but made substantial use of lupins before it went extinct in the 1970s (Kleijn and Raemakers, 2008). Plantings of red clover have spectacularly increased large garden bumblebee numbers in the UK (Falk, 2011), and similarly, growing lupins on a larger scale may help bring this, and other, species back to the Netherlands. Leguminous crop cover has declined rapidly in the Netherlands, and bees that preferentially forage on Fabaceae have shown the steepest declines (Scheper et al., 2014). Furthermore, as lupins flower in June, they fill in the so-called ‘June gap’ of flowers in this period in time (Balfour et al., 2018), so they can contribute to a steady supply of flowers throughout the season. An increase in lupin crop cover might therefore also aid in the conservation and return of endangered pollinators.

One of the reasons of the rather limited set of lupin pollinators could be because none of our tested lupin varieties produced nectar. For example, honeybees are ubiquitous in our study area, yet comprised only 1.2 % of all pollinator visits, likely because honeybees are frequently nectar gatherers (Langridge and Goodman, 1985). Two studies in Australia found that honeybee hives produced a surplus of honey when placed in lupin fields, and observed honeybees searching for nectar (Langridge and Goodman, 1977, 1985). We too saw bumblebees frequently searching for nectar by sticking the tongue into the flower (TPMF & EM pers. obs). On a single occasion a fresh queen red-tailed bumblebee (*Bombus lapidarius*) flew in, searched for nectar, and flew off immediately. This suggests that flowers of lupin contain a nectar guide on the flowers to lure pollinators, but offer no reward (sometimes called false nectar guides (Lunau et al., 2020)). The most likely explanation of the studies finding a honey surplus, is that the honeybees were collecting nectar from other plants in the surroundings, or alternatively, were foraging on the sugar-rich phloem of lupins (Atkins, 1999). Because lupin does not produce nectar, lupin growers need to make sure that ample nectar is available in the direct surroundings of the crop fields, to ensure survival of the crop pollinators.

The relative contribution of buff-tailed bumblebee visitation rate to different crop yield parameters became smaller when more plant processes were involved (Fig. 2). Seed set related yield measures like number of seeds or pods largely depend on the number of ovules fertilized, whereas seed filling related yield measures (e.g. g/plant or kg/ha) are increasingly dependent on the seed filling process and protein assimilation (Lopez-Bellido and Fuente, 1986). We found the strongest effect of buff-tailed bumblebee visitation rate on seed set related yield parameters (i.e. ovule fertilization), but the contribution of insect pollination to seed filling related yield parameters was lower, yet substantial. Agricultural management should therefore focus on both raising high-quality crop plants, as well as increasing insect pollination to obtain the highest protein yields (Fijen et al., 2018, 2020). Measuring only the number of seeds, or seed yield (g/plant) would have overestimated the contribution to insect pollination, and this stipulates the importance of measuring pollinator effects on crop parameters that matter to farmers (Kleijn et al., 2019; Bishop et al., 2020).

By growing plants in pots, we isolated the contribution of insect pollination to crop yield of different lupin varieties and species from all other agricultural management practices. If we extrapolate our results to a field situation, our plants would yield approximately 1000 kg ha^{-1} of protein in white lupin (based on a field density of 45 plants m^{-2} (Prins, 2015)) and 700 kg ha^{-1} for narrow-leaved lupin (100 plants m^{-2} (Prins, 2015)), which is well within the average range of lupin yields (Lopez-Bellido and Fuente, 1986). This suggests that the contribution of pollinators to lupin yield found in this study is representative for field-grown lupins, but this should be validated in full-field trials.

In this study we found that insect pollination increases crop yield of lupins, and that increasing visitation rate of buff-tailed bumblebees can further increase crop yields. Incorporating management for increased crop pollination, and particularly by increasing buff-tailed bumblebee abundance, into the farm management will therefore likely be beneficial for biodiversity and farmer. As we found here, only a subset of all crop pollinators are effective (Rader et al., 2009; Fijen et al., 2018), and only a subset of the local pollinator community are crop pollinators (Kleijn et al., 2015; Senapathi et al., 2015; Fijen et al., 2019). Agricultural management aimed at the relatively large and effective lupin pollinators will, therefore, probably also be beneficial to non-contributing pollinator species (Sutter et al., 2017), also if particular attention is given to nectar plants (Timberlake et al., 2019). Whether managing for more pollinators to increase lupin crop yields is beneficial to farmers will also depend on the opportunity costs (Kleijn et al., 2019). If lupin crop yields can be successfully increased with increasing crop pollination, this might aid in enlarging the acreage of lupin cultivation, and in turn a shift from animal-based proteins to locally produced plant-proteins for human consumption. Because of the many beneficial characteristics that lupins have an increase of lupin cultivation can contribute to a more sustainable agricultural system where biodiversity conservation is placed centrally in daily agricultural management.

Author contributions

TF & DK conceived and designed the experimental setup. TF & EM collected data. TF performed analyses and wrote the initial manuscript. All co-authors contributed significantly to improving the manuscript. All authors gave final approval for publication.

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Data availability

Data supporting the results are available online through Mendeley Data (Fijen, 2021).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.agee.2021.107386.

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