Above- and belowground mutualisms in diverse cropping systems: The effect of strip-cropping and insect pollination on faba bean (*Vicia faba*) nodulation and yield

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Abstract

Strip-cropping with legumes is a promising strategy in the transition towards sustainable agriculture. These systems have the ability to produce high yields while delivering ecosystem services, including pollination and biological nitrogen fixation. Whereas strip-cropping and pollination are separately known to benefit yield, the effects of pollination on legume nodulation remains unclear, as well as the interactive effects on yield in strip-cropping systems. Therefore, we conducted a field experiment in a faba bean-pumpkin strip-crop combination where pollinator visitation was manipulated by bagging individual faba bean plants. The effects of strip-cropping (strip edge and strip centre) and insect pollination on faba bean nodulation and yield parameters were investigated and interactions between nodulation and pollination were explored.

The strip edges showed higher nodulation (nodule mass and active nodules) and higher yield parameters (bean biomass, total beans, total pods, beans per pod) compared to the monoculture. Reduced pollination decreased root biomass and nodule biomass, and negatively impacted yield parameters (bean biomass, total beans and total pods). Pollination interacted with the proportion of active nodules in shaping faba bean yield (total beans and beans per pod), whereby reduced-pollinated plants benefitted more from higher proportions of active nodules.

These findings show the benefits of strip-cropping and pollination on faba bean nodulation and yield. Effects of strip-cropping were most pronounced in the edges, likely driven by resource use complementarity. We reveal an effect of pollination on belowground roots and nodules. Reduced pollination possibly altered resource allocation away from nodules, resulting in less available nitrogen to invest in yield. This provides a novel mechanism via which pollination indirectly affects yield. Improved understanding how pollination and nodulation shape yield in strip-cropping systems is important in the transition towards resource-efficient, sustainable agriculture. We strengthen the case for strip-cropping with legumes and pollinator management to maximize benefits on nodulation and yield.

Keywords: strip-cropping, pollination, nodulation, yield, faba bean, mutualism, resource allocation, ecosystem services

Introduction

Industrial agriculture, characterized by large-scale homogeneously cultivated fields, is considered a major driver of environmental pollution, soil degradation and biodiversity loss (Campbell et al., 2017). These systems heavily rely on agrochemical inputs to maintain high yields (Emmerson et al., 2016). Alternative farming systems are needed to maintain food production while minimizing negative impacts on the environment (Campbell et al., 2017). Crop diversification, in which the diversity of cultivated crops is increased, has been proposed a promising strategy in the transition towards sustainable agriculture (Juventia et al., 2022). Crop diversification showed to benefit soil fertility, nutrient cycling and biodiversity while maintaining crop yield (Beillouin et al., 2021; Tamburini et al., 2020).

One means of crop diversification is strip-cropping, a type of intercropping in which alternating crops are planted in parallel strips. Strip-cropping systems promotes efficient use of resources when crop traits (e.g. root structure or leaf area) are complementary, meaning that traits occupy different niches (Duchene et al., 2017; Glaze-Corcoran et al., 2020). Complementarity results in more complete use of above- and belowground resources, which often promotes crop productivity in strip-cropping systems (Duchene et al., 2017; Wang et al., 2023). This effect is often pronounced at the strip edges, where interspecific interactions are most likely to occur (Wang et al., 2020).

Legumes play a key role in resource-efficient strip-cropping system due to their ability to biologically fix nitrogen (Duchene et al., 2017). Faba bean (*Vicia Faba*) is a legume with high protein- and micronutrient-containing seeds (Karkanis et al., 2018), and interest in including faba bean in European strip-cropping systems is growing (Mínguez & Rubiales, 2020). Faba bean, together with other legumes, acquire the ability to biologically fix nitrogen (N) from the atmosphere through a symbiosis with *Rhizobia* (Lepetit & Brouquisse, 2023). These bacteria form nodules in roots of leguminous plants, where atmospheric nitrogen (dinitrogen, N_2) is converted to a biologically available form (ammonium, NH_4^+ and nitrate, NO_3^-) (Lepetit & Brouquisse, 2023). Biological nitrogen fixation (BNF) is an energy costly process and is mainly driven by nitrogen demand (Duchene et al., 2017). Excessive soil N inhibits nodulation, whereas soil N deficits stimulate nodulation (Duchene et al., 2017). Nitrogen competition drives faba bean nodulation in legume-cereal intercropping systems through interspecific interactions in close proximity to a neighbouring crop (Li et al., 2016; Li et al., 2009; Liu et al., 2019). However, whether nodulation in strip-cropping is impacted further away from the edges of the strips remains unclear. Also, whether faba bean nodulation is similarly impacted in intercropping with a non-cereal remain to be investigated.

Apart from a belowground mutualism with *Rhizobia*, faba bean yield benefits from an aboveground mutualism via insect pollination (Bishop & Nakagawa, 2021). Insect pollinators aid transfer of pollen, resulting in more successful ovule fertilization and thereby enhance seed set (Brünjes & Link, 2021). Faba bean is partially dependent on insect pollinators - it can produce seeds via both self- and cross-pollination; the latter requires insects to transfer pollen between plants (Suso et al., 1996). Faba bean pollination is mainly performed by bumblebees and honeybees in Northern Europe (Karkanis et al., 2018; Nayak et al., 2015). Studies report variable results on the extent to which insect pollination benefits faba bean yield (Lundin & Raderschall, 2021; Nayak et al., 2015), but a recent meta-analysis reports an estimated yield reduction of 21-43% without insect pollination (Bishop & Nakagawa, 2021).

Absence of pollinators has been reported a clear biotic stressor to faba bean (Riggi et al., 2022). Plants can respond to biotic stressors by distributing resources between growth and reproduction (Fairhurst et al., 2022). In oilseed rape, absence of pollinators shifted resource allocation towards growth and flowering, away from seed production (Adamidis et al., 2019; Fairhurst et al., 2022). However, whether pollinators convey similar shifts in resource allocation in faba bean remains unknown. Despite the importance of pollination for faba bean yield, the impact on resource allocation towards nodulation has seldom been studied.

Although strip-cropping and pollination are known to separately benefit yield, it remains unclear whether and how pollination affects faba bean nodulation, and how these above- and belowground mutualisms interactively shape yield in strip-cropping systems. To tackle this knowledge gap, we carried out a field experiment in a long-term strip-cropping experimental farm where faba bean was planted with pumpkin. Pollinator visitation was manipulated by bagging individual faba bean plants. The effects of strip-cropping (strip edge and strip centre) and pollinator visitation on faba bean nodulation- and yield parameters was assessed and interactions were explored. Nodulation is expected to be enhanced in the strip-cropping fields compared to the monoculture due to increased competition for nitrogen (Duchene et al., 2017), and most pronounced at the edge of the strip where resource complementarity is most pronounced (Wang et al., 2023). Stress caused by absence of pollinators is expected to cause a shift in resource allocation from nodulation towards aboveground growth. This early-season investment in growth in non-pollinated plants is expected to leave less resources available to invest in yield, making poorly pollinated plants more dependent on nitrogen derived from nodules compared to their pollinated counterparts.

Materials and Methods

Site description

To assess the effect of the field position and reduced-pollination on faba bean nodulation and yield, a field experiment was conducted at Droevendaal, the organic experimental farm of Wageningen University & Research (51°59'27.4"N; 5°39'36.0"E). At Droevendaal, a long-term strip-cropping experiment started in 2018 in which multiple crop combinations are investigated (Cropmix, 2023). The current experiment focused on the combination of faba bean and pumpkin. Faba bean-pumpkin strips were grown in four replicated blocks with a reference monoculture for each block (Figure 1A). Strips in block 1-3 are 55m x 3m with a monoculture field of 9m x 9m. Strips in block 4 are 72m x 3m with a monoculture field of 72m x 72m. Soil characteristics in the faba bean monoculture, strip centre and strip edge can be found in Table 1. Faba bean variety "Tiffany" was sown on May 2nd. No fertilizer was applied in the faba bean strips. Pumpkin strips were fertilized with cattle slurry on May 16 (nutrient content: see Table S1).

Table 1: Soil characteristics in the faba bean monoculture, strip centre and strip edge. Data was obtained on February 22nd and values show the average of different blocks. Soil characteristics between field positions did not significantly differ.

Field position	*Nitrogen (%)*	*Carbon (%)*	*pH*	*OM (%)*
Monoculture	0.083 ± 0.007	1.657 ± 0.119	6.72 ± 0.07	4.076 ± 0.174
Strip centre	0.067 ± 0.014	1.425 ± 0.111	6.62 ± 0.04	3.867 ± 0.130
Strip edge	0.070 ± 0.012	1.500 ± 0.098	6.58 ± 0.05	3.935 ± 0.071

Experimental setup

To investigate the effects of crop diversity and pollination on faba bean nodulation and yield parameters (Table 2), faba bean plants were sampled in the strip centre (SC), strip edge (SE) and monoculture (M) in pollinated (P+) and reduced-pollinated (P-) treatments. Sampling occurred using plant quartets (Figure 1B), consisting of two plant pairs with similar height at the onset of crop bloom. One plant pair was used to assess nodulation parameters, the other plant pair was used to assess yield parameters. In each plant pair, one plant was bagged with a tulle bag (15cm x 20cm, mesh size <1mm) to exclude insect pollinators (Figure 1B).

Per block, five plant quartets were located in each field position (SC, SE, M), resulting in a total of 60 plant quartets. Within each block, the same strip was used to reduce spatial variation, except for block 1 and 3, where management factors made it impossible to assess nodulation and yield in the same strip. Strips used in the experiment are indicated with an arrow in Figure 1A. Strips consisted of six rows of faba bean. Within strips, plant quartets were located ten meters apart in the strip edge (south-side, 6th row) and strip centre (3rd or 4th row) (Figure S1). The outer seven (block 1-3) or sixteen meters (block 4) of each strip were regarded a 'buffer zone' and were not used for sampling. Within monocultures (block 1-3), quartets were spaced approximately six meters apart throughout the field with a buffer zone of two meters. In the monoculture of block 4, quartets followed a similar spacing as in the strip-cropping field of block 4 (Figure S1). Coordinates of plant quartet locations were recorded and used for spatial analysis.

Table 2: Parameters related to nodulation, yield and plant response, measured during the study.

Assessment moment (growing stage)	*Category*	*Parameters*
Mid-season (BBCH65-75)	Nodulation	Nodule mass-ratio (g nodule DM /g root DM) Nodule number-ratio (nodule number/g root DM) Proportion active nodules
	Plant response	Leaf N content (g/kg) Root DM (g) Shoot DM (g)
Full maturity (BBCH90)	Yield	Bean DM per plant (g) Total beans per plant Total pods per plant Average amount of beans/pod Bean protein content (g/100 g)
	Plant response	Root DM (g) Shoot DM (g)

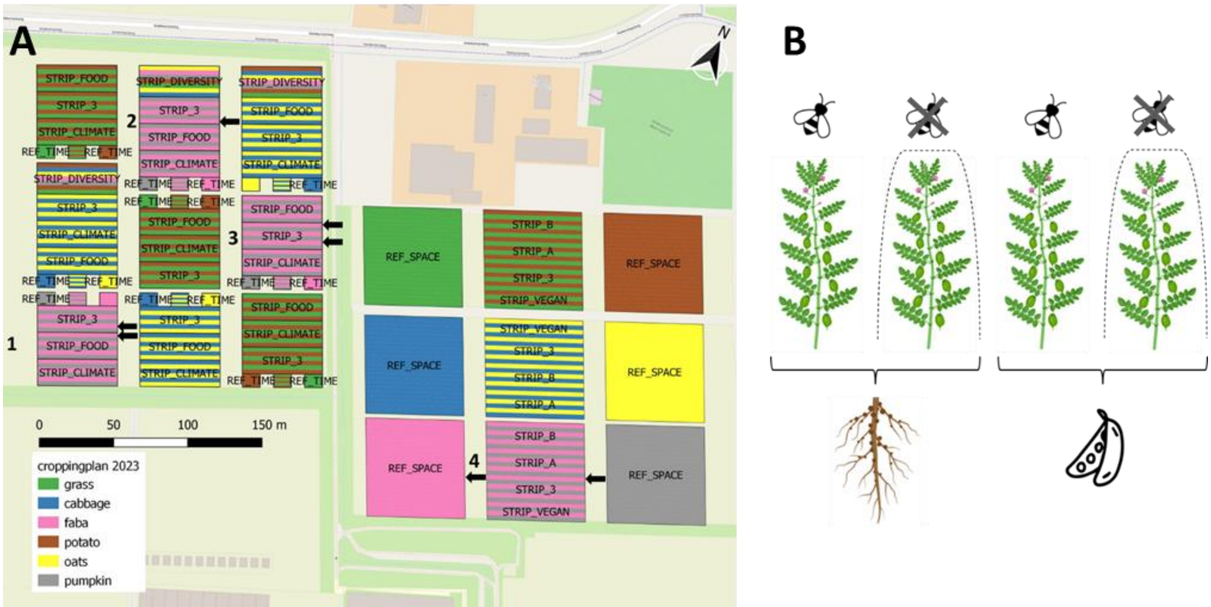

***Figure 1**: **A)** Field setup of Droevendaal experimental farm. Block 1-4 (faba bean - pumpkin combination) are used for the experiment. Arrows indicate the strips used for the experiment. Similar faba bean management occurred in all strips. REF_TIME fields serve as a monoculture reference for block 1-3; REF_SPACE serves as a monoculture reference for block 4. **B)** Setup of plant quartets. One pair in each quartet is used to assess nodulation parameters; the other plant pair is used to assess yield parameters. One plant in each pair is bagged to exclude insect pollinators.*

Bagging of individual plants was used as a method to assess the effect of pollination on the plant-level while reflecting true conditions in the field. Bags were adjusted twice per week to only cover open flowers and were removed after crop bloom (growth stage BBCH69) to minimise the effect on plant growth and pod development. We expect limited effects of that the negative effects of bagging on yield, as a recent meta-analysis on the effects of pollination on faba bean yield report no negative effects of bagging (Bishop & Nakagawa, 2021). Ideally, bagging would have occurred before the onset of crop bloom to completely prevent insect pollination. However, due to early flowering caused by early season drought, plants were bagged during the onset of crop bloom (June 21st, growth stage BBCH60). Bagged plants could have been pollinated during the first days of crop bloom and are therefore referred to as 'reduced-pollinated' instead of 'non-pollinated'.

Pollinator visitation rate

To assess whether pollinator visitation rates differed between field positions, pollinators were surveyed during faba bean flowering (growth stage BBCH60-69). Pollinator visitations were recorded by walking along transects situated in the strip-crop and monoculture of each block. Strip-crop transects were 30m long and located in the middle of the 3m wide strip. Monoculture transects were 2x 6m long (block 1-3) or 30m long (block 4). Transects were walked for 1 minute observation time per meter. The number of pollinator flower visits was counted and the pollinators were identified as bumblebee, honeybee or 'other'. Additionally, to quantify flower resources available to pollinators, the number of open faba bean flowers (crop bloom), open weed flowers (weed bloom) and number of weed plants (weed density) were counted in four 0.25 m^2 quadrats per transect. Pollinator surveys were performed four times during crop bloom between June 23rd and July 10th. Surveys were conducted during favourable conditions for pollinator activity (no rain, temperature of at least 15°C, skies partly sunny, wind speed <4 on Beaufort scale) (Westphal et al., 2008).

Nodulation parameters

Nodulation parameters (Table 2) were assessed during the onset of pod filling (growth stage BBCH65-75, between the 3rd and 7th of July) as this period is regarded the most suitable stage for nodulation assessment (Seeger et al., 2022). Plants were carefully excavated circa 30 cm deep using a spade, leaving the root system as intact as possible. Roots were cut off and washed using a sieve. Nodules were detached from the roots and counted. To determine the proportion of active nodules, 20 randomly chosen nodules per plant were dissected to assess the pigmentation status. Active nodules are red pigmented by the presence of leghaemoglobin; changes of nodule colour indicates inactivity (Puppo et al., 2005). Pigmentation was categorized as pink/red (active nodule) or white/green/brown (inactive nodule) (Seeger et al., 2022). The proportion of active nodules was estimated by dividing the amount of red/pink nodules by 20 (total subsample size). Nodules, roots and aboveground biomass were oven-dried (72 hours, 70°C) and dry matter (DM) was weighed. Nodule mass and nodule number were divided by the root DM to correct for variability in plant size. This enables to determine whether plants truly nodulate more, or whether differences are simply a result of changes in the roots (Friel & Friesen, 2019). Resulting parameters are the nodule mass-ratio (g nodule DM / g root DM) and the nodule number-ratio (nodule number / g root DM). Leaf nitrogen (N) (g/kg) and phosphorus (P) (g/kg) content were spectrophotometrically determined in dried, grinded leaves following H_2SO_4-Se digestion, according to the protocol of Houba et al (1988).

Yield parameters

Yield parameters (Table 2) were assessed at full plant maturity (BBCH90, August 21st). Plants were carefully excavated and roots were cut off and washed. For each plant, the amount of pods, beans per pod and total beans (yield quantity parameters) were recorded. The average amount of beans per pod was calculated by dividing the total beans by the total pods. Beans, pods, aboveground biomass and roots were oven-dried for each plant (72 hours, 70°C) and weighed. Bean N (g/kg) and P (g/kg) content were spectrophotometrically determined in dried, grinded beans following H_2SO_4-Se digestion according to the protocol of Houba et al (1988). The standard nitrogen-to-protein conversion factor of 6.25 was used to obtain the protein content (Mariotti et al., 2008).

Statistical analysis

All statistical analyses were performed using (generalized) linear mixed-effect models (package 'glmmTMB', Brooks et al., 2023) in R version 2023.09.1+494 (R core team, 2019). Model assumptions were validated using residual diagnostics and count data models were checked for overdispersion (package 'DHARMa', Hartig, 2022). The amount of variance attributable to different factors was analysed using a type-II ANOVA (package 'car', Fox et al, 2023). Estimated marginal means (EMMs) were obtained using the 'emmeans' package (Lenth et al., 2023). Model outcomes were visualised using packages 'ggplot2' (Wickham et al., 2023) and 'sjPlot' (Lüdecke, 2023). R-squared values were calculated using the package 'MuMin' (Bartón, 2023) and represent the proportion of variance explained by the fixed effects (marginal R-squared) and both fixed and random effects (conditional R-squared) of the model.

First, we tested whether the total amount of pollinator flower visits differed between the strip-crop and monoculture using a generalized linear mixed-effect model with a Poisson distribution. The round of pollinator survey (n=4) nested within block (n=4) was added as a random effect (1|block/round). Explanatory variables included cropping system (strip-crop and monoculture), crop bloom (number of open faba bean flowers), weed bloom (number of open weed flowers) and weed density (number of weed plants). Based on the Akaike Information Criterion (AIC), the best-fitting model only included crop bloom as a fixed effect. Cropping system was kept in the model because this was our main interest. The final model structure can be found in Table S2.

Then, the effects of field position, pollination treatment and their interaction on (a) nodulation parameters (nodule mass-ratio, nodule number-ratio, proportion of active nodules), (b) yield parameters (bean DM per plant, number of beans per pod, pods per plant, beans per plant and bean protein content) and (c) plant response parameters (root DM, shoot DM, leaf N and P content) were analysed. All response variables followed a Gaussian distribution except for total pods and total beans per plant, where a Poisson distribution was used. Explanatory variables included field position (SC, SE and M), pollination treatment (P+ and P-) and their two-way interaction. The interaction term between field position and pollination treatment was never significant and was therefore excluded from all models. Random effects included quartet (n=60) nested within block (n=4) (1|block/quartet). Model structures are presented in Table S2. Spatial autocorrelation was assessed by calculating the Moran's I statistic and its associated *p*-value using coordinates of the plant quartets. For all models, residuals did not show spatial autocorrelation, therefore, no spatial correlation structure was added to the models.

Finally, in order to explore interactions between pollination and nodulation on yield, data of plant pairs (Figure 1B) were merged. This was needed because destructive sampling to assess nodulation made it impossible to assess nodulation and yield parameters on the same plant. Each plant pair was treated as one observation so that pairs contained data for nodulation and yield parameters in pollinated (n=60) and reduced-pollinated (n=37) treatments. Separate correlation matrices for P+ and P- plant pairs were constructed (package 'corrplot', Wei et al., 2017) in which the Pearson correlation coefficients between yield parameters (bean DM, beans per pod, total beans, total pods) and nodulation- and plant parameters (nodule mass-ratio, nodule root ratio, active nodules, leaf N content) were explored (Figure S2). In case the Pearson correlation coefficient differed >0.20 between the P+ and P- treatment, a (generalized) linear mixed-effect model was constructed to analyze whether the interaction between nodulation and pollination was significant. In these models, fixed effects included the field position, pollination treatment, a nodulation parameter and the two-way interaction term of the latter. Quartet nested within block was added as a random effect (1|block/quartet). Model structures can be found in Table S2.

Results

Pollinator flower visits

In a total of four pollinator surveys, 144 pollinator flower visits were observed (Table S3). The amount of pollinator flower visits did not significantly differ between the cropping systems (p = 0.995) and was positively affected by the amount of open faba bean flowers (p <0.001).

Nodulation

The effect of the field position and insect pollination on faba bean nodulation parameters was assessed in 99 plant roots in the strip edge, strip centre and monoculture in pollinated (n=60) and reduced-pollinated (n=39) treatments. No interactions between the fixed effects were found, therefore, field position and pollination treatment were analysed as main effects (Table 3).

The nodule mass-ratio (g nodule DM/g root DM) was 78% higher in the strip edge compared to the strip centre (estimate ± SE = 0.07 ± 0.02, p<0.001) and 37% higher compared to the monoculture (0.05 ± 0.01, p=0.007) (Figure 1A). Similarly, the proportion of active nodules was significantly higher in the strip edge compared to the strip centre (0.16 ± 0.05, p = 0.009) and monoculture (0.17 ± 0.05, p=0.003) (Figure 1C). In contrast, the nodule number-ratio (nodule number/g root DM) was highest in the monoculture and significantly reduced in strip centre with 23% (-20.31 ± 7.43, p=0.02) (Figure 1B). Leaf N and P content (g/kg) and root DM (g) were not affected by field position (Table 3).

Reduced insect pollination resulted in a reduction of the nodule mass-ratio (-0.02 ± 0.01, p = 0.038) and root DM (-0.21 ± 0.08, p = 0.010) of respectively 16% and 13% (Figure 1D,F). On the contrary, leaf N and P content increased with respectively 7% (2.46 ± 0.61, p <0.001) and 15% (0.47 ± 0.10, p <0.001) in the reduced-pollination treatment (Figure 1E, S3). Shoot DM decreased in reduced-pollinated conditions (-1.64 g ± 0.98, p <0.001) (Figure S3). The pollination treatment did not affect the nodule number-ratio nor the proportion of active nodules (Table 3, Figure S3).

*Table 3: Anova results for the nodules and plant response variables collected in June. Chi-square values (X2) and associated p-values are shown. R-squared values represent the percentage of variance explained by solely the fixed effects (R2m) and both the fixed and random effects (R2c). Significance is shown as *p<0.05, **p<0.01, ***p<0.001. Plots for significant results and raw data are shown in Figure 2 and Figure S3.*

Explanatory variable (levels)	**Field position** (SC, SE, M)		**Pollination treatment** (P+, P-)		**R^2m (%)**	**R^2c (%)**
Response variables	*X2*	*p-value*	*X2*	*p-value*		
Nodule mass-ratio (g/g root DM) *Sample size = 99*	24.41	**<0.001*****	4.43	**0.035***	24.2	47.2
Proportion active nodules *Sample size = 99*	13.48	**0.001****	0.20	0.657	12.9	38.4
Nodule number-ratio (number/g root DM) *Sample size = 99*	7.49	**0.023***	1.12	0.290	7.6	12.1
Leaf N content (g/kg) *Sample size = 99*	0.19	0.911	16.41	**<0.001*****	11.3	44.6
Leaf P content (g/kg) *Sample size = 99*	1.306	0.521	22.46	**<0.001*****	14.3	51.7
Root DM (g) *Sample size = 99*	2.70	0.259	6.84	**0.009****	8.0	54.4
Shoot DM (g) *Sample size = 99*	5.761	0.056	14.08	**<0.001*****	16.0	62.7

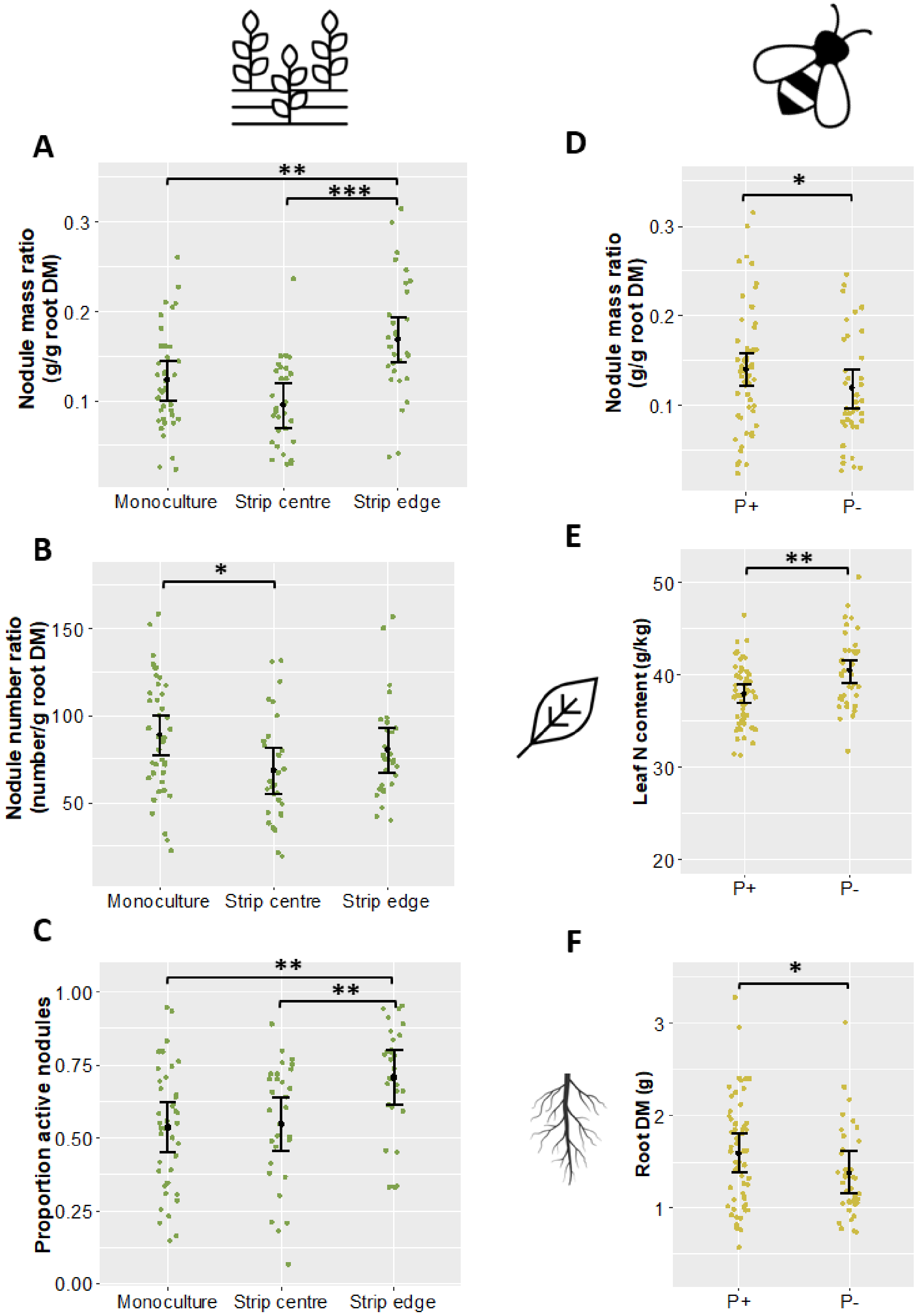

*Figure 2: Model predictions for faba bean nodulation parameters in relation to the field position (A, B, C; averaged over pollination treatment) and pollination treatment (D, E, F; averaged over field position). Whiskers represent 95% confidence intervals and a point estimate. Significance is shown as *p<0.05, **p<0.01, ***p<0.001. Note that y-axis for the leaf N content does not start at 0.*

Yield parameters

The effect of field position and reduced-pollination on faba bean yield quantity (bean DM, beans per pod, total pods, total beans) and quality (bean protein content) was assessed in 138 plants in the strip edge, strip centre and monoculture in pollinated (n=60) and reduced-pollinated (n=78) treatments.. No interactions between the main effects were found, therefore, field position and pollination treatments were analysed as main effects (Table 4).

Bean DM per plant was 64% higher (4.19 g ± 1.00, $p<0.001$) in the strip edge compared to the monoculture and marginally significantly increased compared to the strip centre (2.28 g ± 0.98, $p=0.054$) (Figure 2A). Total beans and total pods per plant were respectively 53% (0.43 ± 0.12 (log scale), $p<0.001$) and 31% (0.27 ± 0.09 (log scale), $p<0.001$) higher in the strip edge compared to the monoculture (Figure 3B, C). Similarly, the amount of beans per pod was 18% (0.44 ± 0.17, $p=0.03$) higher in the strip edge compared to the monoculture (Figure S4). Bean protein content was highest in the monoculture and showed a 6% reduction in the strip centre (-1.74 g ± 0.71, $p=0.04$) (Figure 2D). The shoot DM was not affected by the field position (Table 3).

Reduced insect pollination resulted in a 25% reduction of both the bean DM (-2.48 g ± 0.45, $p<0.001$) and total pods (-0.29 ± 0.07 (log scale), $p<0.001$) and a 26% reduction in total beans (-0.29 ± 0.04 (log scale), $p<0.001$) (Figure 2E,F,G). The amount of beans per pod, bean protein content and shoot DM were not affected by the pollination treatment (Table 3, Figure Sx).

Table 4: Anova results for the yield and plant parameters collected in August. Chi-square values (X2) and associated p-values are shown. R-squared values represent the variance explained by solely the fixed effects (R2m) and the fixed and random effects (R2c). Plots for significant results and raw data are shown in Figure 2 and Figure S4.

Explanatory variable (levels)	**Field position** (SC, SE, M)		**Pollination treatment** (P+, P-)		**R^2m (%)**	**R^2c (%)**
Response variables	*X2*	*p-value*	*X2*	*p-value*		
Bean DM (g) *Sample size = 138*	15.64	**<0.001 *****	21.67	**<0.001 *****	19.6	60.1
Beans per pod *Sample size = 137*	7.32	**0.026****	0.60	0.439	6.0	12.4
Total beans per plant *Sample size = 138*	14.07	**<0.001 *****	50.93	**<0.001 *****	20.0	75.6
Total pods per plant *Sample size = 138*	8.53	**0.014****	18.40	**<0.001 *****	14.4	24.5
Bean protein content (g/100g) *Sample size = 88*	7.38	**0.025****	0.18	0.671	8.4	9.8
Shoot DM (g) *Sample size = 138*	2.66	0.265	2.34	0.126	3.9	27.7

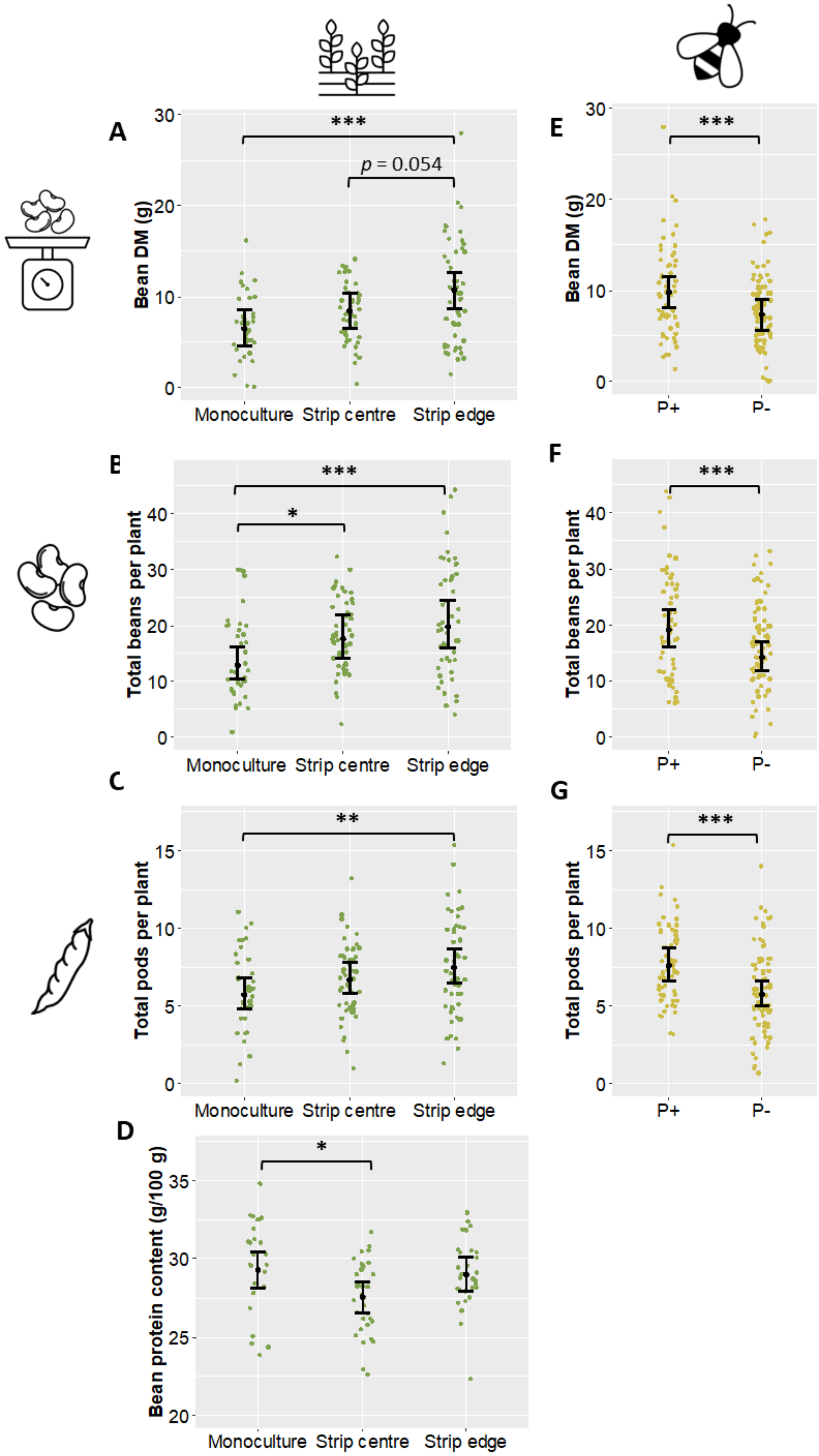

*Figure 3: Model predictions for faba bean yield parameters in relation to the field position (A, B, C, D, averaged over pollination treatment) and pollination treatment (E, F, G, averaged over field position). Whiskers represent 95% confidence intervals and a point estimate. Note that x-axis of D does not start at 0. Significance is shown as *P<0.05, **P<0.01, ***P<0.001.*

Interactive effects of pollination and nodulation on yield

Pollination and the proportion of active nodules interactively affected total beans per plant (Poisson distribution, *p*=0.001) and beans per pod (Gaussian distribution, *p*= 0.001) (Table 5). In absence of pollination, there was a positive relationship between active nodules and beans per pod (estimated slope ± SE = 2.36 ± 0.64) and total beans (0.89 ± 0.29), which was not found in the presence of insect pollinators (estimated slopes -0.03 ± 0.48 (beans per pod) and -0.12 ± 0.22 (total beans)) (Figure 4A,B).

Table 5: Anova results for the interaction between nodulation and pollination on faba bean yield. Chi-square values (X2) and associated p-values are shown. R-squared values represent the variance explained by solely the fixed effects (R2m) and the fixed and random effects (R2c).

Response variables	**Beans per pod** $R^2m = 0.170$ $R^2c = 0.290$		**Total beans** $R^2m = 0.332$ $R^2c = 0.792$	
Fixed effects	*X2*	*p-value*	*X2*	*p-value*
Field position (SC, SE, M)	2.24	0.326	9.74	**0.008****
Pollination treatment (P+, P-)	9.82	**0.002****	22.47	**<0.001*****
Proportion active nodules	0.01	0.941	0.30	0.581
*Pollination treatment * Proportion active nodules*	10.14	**0.001****	10.17	**0.001****

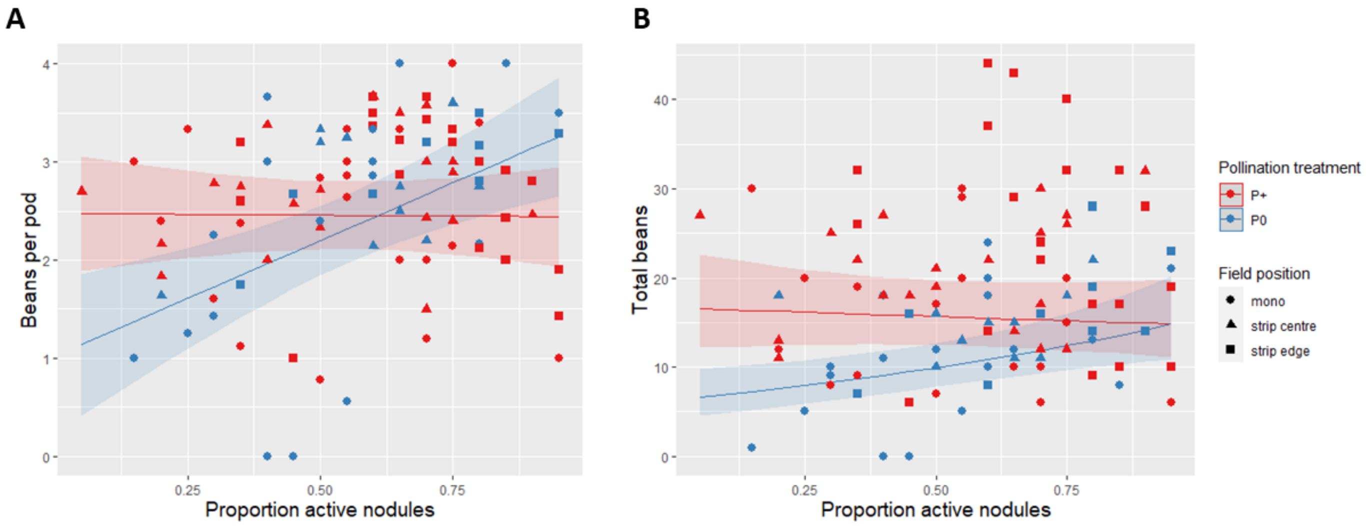

Figure 4: Model predictions for faba bean yield components in relation to the proportion of active nodules and pollination (P+ red line, P- blue line): (A) beans per pod, (B) total beans per plant. Different shapes indicate different field positions (monoculture, strip edge, strip centre). Bands represent 95% confidence intervals.

Discussion

The effects of strip-cropping (strip edge and strip centre) and reduced insect pollination on faba bean nodulation and yield parameters were investigated in a long-term strip-cropping field experiment where faba bean was planted with pumpkin. In line with our hypothesis, nodulation and yield parameters were highest in the strip edge and negatively affected by reduced insect pollination. No interactions between field position and pollination were found. The proportion of active nodules and pollination interactively affected total beans and beans per pod, where non-pollinated plants benefitted more from higher proportions of active nodules in shaping total beans and beans per pod.

Effects of the field position on nodulation and yield

In our research, the nodule mass-ratio and proportion of active nodules were significantly higher in the strip edge compared to the strip centre and monoculture. Increased absolute nodule biomass in faba bean-cereal intercropping systems has often been reported (Bargaz et al., 2021; Li et al., 2016; Li et al., 2009; Liu et al., 2017). Cereals are strong competitors for nitrogen (Yu et al., 2016), reducing the amount of available soil N which drives nodulation in legume-cereal intercropping systems (Duchene et al., 2017). Enhanced nodulation through competition for soil N likely drove enhanced nodulation in our results as well, despite additional fertilization of pumpkin strips. Naderi et al (2017) reports highest pumpkin yield with 250 kg N/ha – the amount applied in our study (60 kg N/ha) was likely insufficient to fully eliminate nitrogen competition. Soil sample data from June (not obtained by us; data not shown) showed reduced bioavailable N in the faba bean strip edge compared to the strip centre and monoculture, further suggesting that pumpkin caused sufficient nitrogen competition to limit soil N, thereby enhancing faba bean nodulation in the strip edge. This competitive effect likely remained limited to the strip edge, because nodulation was not enhanced in the centre of the strip. Our findings suggest that the benefits of strip-cropping on nodulation extend beyond legume-cereal intercropping systems, but not beyond the edges of the strip. A future experiment could determine nodulation in all six rows of faba bean to investigate the maximum distance from the edge to enhance.

Surprisingly, while the nodule mass-ratio was highest in the strip edge, the nodule number-ratio was highest in the monoculture. Faba bean in the monoculture invested more in nodule numbers and less in nodule mass – raising the question whether nitrogen fixation relies most on nodule number, or on nodule mass. Recently, Iqbal et al (2022) found that water stress resulted in large variability in nodule numbers, whereas nodule mass and nitrogen fixation remained conserved. When we use the proportion of active nodules as an indicator for nitrogen fixation, our data shows a higher correlation with nodule mass compared to nodule number (Table S4). These results could suggest that nodule mass is a better predictor of nitrogen fixation compared to nodule number. However, future studies, in which the nodule number and nodule mass are correlated to the amount of nitrogen fixation, are needed to determine which is a better predictor of nitrogen fixation.

In our study, we corrected the nodule mass and number for the root DM to account for variability in plant size. Nodule DM and total nodules were significantly and positively correlated with the root DM (Table S4), confirming the need for a correction in order to disentangle whether resources were allocated to the roots or nodules. We propose future nodulation research to include a nodule-root ratio in the analyses, as this is a more standardized measure which allows for comparison between treatments and cultivars (Friel & Friesen, 2019).

Faba bean yield parameters were assessed at full maturity, six week after assessing nodulation. All yield quantity parameters (bean DM, total beans, total pods, beans per pod) were higher in the strip edge compared to the monoculture. No significant differences between the strip centre and the monoculture were detected (apart from total beans), indicating that beneficial effects on faba bean yield remained limited to the strip edge. Increased faba bean yield in an intercrop with cereals compared to monocrops has often been shown (Li et al., 2016; Li et al., 2009; Mei et al., 2021) and is often pronounced in the border rows due to direct interactions between species (Wang et al., 2020). In our study, faba bean in the strip edges likely benefitted from above- and belowground resource complementarity. Belowground, enhanced nodulation likely benefitted the amount of available N to invest in faba bean yield (Allito et al., 2021). Aboveground, increased light interception could occur because faba bean was sown two weeks prior to pumpkin, resulting in reduced shading effects (Wang et al., 2017). The effects of light interception was likely pronounced because sampling occurred in the south-side of the strips. Alternatively, plants could have been sampled on both the north- and south-side of the strip to reduce pronounced effects of light interception in the edges. Our results show benefits of the strip edges on individual plants; field data should confirm whether our findings translate to the field level.

Positive effects of the strip edge on both nodulation and yield parameters would opt for narrow strips to optimally make use of resource complementarity and interspecific interactions in strip-cropping systems (Wang et al., 2020). The benefits of strip-cropping drop when strips are wider than one meter (van Oort et al., 2020). However, agricultural intensification has shifted focus to large and homogenously cultivated areas, making it challenging to manage narrow strips with conventional equipment (van Oort et al., 2020). Research into the optimal strip width is ongoing (Hu et al., 2020; Raza et al., 2023; Wang et al., 2020), and should focus on the trade-off between efficient resource complementarity and enabling management with conventional machinery.

Interestingly, the bean protein content was highest in the monoculture (29.3 g/100g) and did not significantly differ from the strip edge (29.0 g/100g). Despite highest nodule mass in the strip edge, nitrogen investment into beans did not increase. Possibly, nitrogen fixed by faba bean in the strips partly transferred to pumpkin – nitrogen transfer from legumes to neighbouring non-legume species has often been detected, indicated by higher amounts of nitrogen derived from the atmosphere (Ndfa) in non-leguminous crops (Peoples et al., 2015; Reilly et al., 2022; Stoltz & Nadeau, 2014). Future research into the Ndfa content of pumpkin in the strip edge, strip centre and monoculture should point out whether nitrogen transfer occurred, and to what distances in the strip. Although we found no differences between the strip edge and monoculture, bean protein content the strip centre was slightly, but significantly lower compared to the monoculture (6% decrease). Previously, studies found no effects of intercropping on faba bean protein content (Lepse et al., 2017; Marcos-Pérez et al., 2023). However, our finding suggests that strip-cropping could affect bean protein content. It would be interesting to further investigate whether differences in nodulation or nitrogen transfer could be the cause of this change, in order to determine if cropping systems can impact protein content of leguminous crops. This research could be of interest given the current upward trend of using plant-based proteins for human consumption (Langyan et al., 2022).

Effects of pollination on nodulation and yield

Interestingly, we found an effect of reduced pollination on belowground traits - the nodule mass-ratio and root DM decreased with respectively 16% and 13%. Although the effect of pollination on nodule mass (16% decrease) is small compared to the effect of strip-cropping on nodule mass (78% increase), we provide the first indications that pollination can alter nodulation. Pollinator absence previously showed to alter resource allocation within plants, enabling plants to adapt to changes in their environment (Dewitt & Scheiner, 2004; Fairhurst et al., 2022). In oilseed rape, non-pollinated plants allocated resources towards growth and abundant flowering (Adamidis et al., 2019; Fairhurst et al., 2022). In faba bean, another aboveground stressor (aphid herbivory) previously showed to reduce root biomass, possibly due to allocation of resources into plant recovery (Raderschall, Vico, et al., 2021). Likewise, we could hypothesize that stress caused by poor pollination altered resource allocation away from nodules and towards growth. However, the shoot biomass was reduced in absence of pollination (Figure S3), which is not in line with this hypothesis. On the other hand, we observed that several bagged plants continued flowering for a longer period compared to non-bagged plants (personal observation; no data obtained) and we found increased leaf N and P content in reduced-pollinated conditions, which could still indicate altered resource allocation into aboveground plant parts. The effects of pollination on nodulation remained limited to the nodule mass-ratio, no effects on nodule number nor active nodules were found. Clearly, underlying mechanisms of the effect of insect pollination on nodulation should be further elucidated. Our findings open up an interesting avenue for research in which resource allocation towards growth, flowering and nodulation in response to pollination could further be explored.

Pollination also altered aboveground faba bean yields. In reduced-pollinated conditions, we found a reduction in all yield quantity parameters (bean DM, total beans and total pods per plant), except for beans per pod. By transferring pollen, pollinators benefit successful ovule fertilization (Brünjes & Link, 2021), which explains the higher amount of beans in presence of pollination. Our findings are line with a previous meta-analysis by Bishop & Nakagawa (2021), reporting an average 32.9% faba bean yield loss in absence of pollination, with beans per pod being the least responsive parameter towards pollinator absence. Within the same cultivar ('Tiffany'), variable yield responses towards pollinator absence were reported, including reduced beans per pod (Beyer et al., 2022; Raderschall, Vico, et al., 2021; Riggi et al., 2022) and bean mass per plant (Raderschall, Vico, et al., 2021; Riggi et al., 2022), but also no effect of pollinators on yield was reported (Lundin & Raderschall, 2021). Variation in pollination benefits can be explained by multiple factors, including differences in soil type (St-Martin & Bommarco, 2016), heat stress (Bishop et al., 2016) or weather conditions (Bishop & Nakagawa, 2021). In our specific growing context, characterized by early-season heat followed by prolonged rainfall, we found a clear benefit of pollination on faba bean yield despite late bagging of plants. Our results show the effect of insect pollination on the plant-level, reflecting true conditions in the field. These results strengthen the case for pollinator management, which will become more urgent in the future given the rapid decline in pollinating insects (Dicks et al., 2021).

Apart from directly impacting yield by benefitting ovule fertilization, our results indicate a novel mechanism in which pollination indirectly affects yield via nodulation. We found an interaction between pollination and the proportion of active nodules on total beans and beans per pod, where reduced-pollinated plants showed higher proportional benefits of active nodules in shaping faba bean yield. As we observed that reduced-pollinated plants had a lower nodule mass-ratio, it is possible that these plants shifted resource allocation away from nodules towards flowering. This could have resulted in less available nitrogen to invest in yield, causing a higher dependency on nitrogen from nodules in shaping yield. The interaction only occurred between pollination and active nodules – no interactions with nodule mass nor nodule number on yield parameters were found. Possibly, higher proportion of active nodules directly benefits available nitrogen for the plant, which is not necessarily the case for higher nodule mass or numbers as these can partly be inactive, cancelling out any interactive effects on yield. Previously, pollination showed to interact with heat stress (Bishop et al., 2016), herbivory (Raderschall, Vico, et al., 2021) and presence of semi-natural habitats (Raderschall, Bommarco, et al., 2021) in shaping faba bean yield. As far as we know, we are the first to provide indications of an interactive effect between nodulation and pollination. However, results should be interpreted with caution because nodulation and yield parameters were not assessed on the same plant – destructive sampling was needed to assess nodulation. Future research could experiment with using a minirhizotron as a non-destructive method to assess nodulation (Gray et al., 2013) to make inferences about nodulation and yield on the same plant. Our results open up an interesting avenue to further explore the interplay between nodulation and pollination in shaping faba bean yield.

Conclusion

In summary, our field study showed beneficial effects of strip-cropping and insect pollination on faba bean nodulation and yield. The benefits of strip-cropping were most pronounced in the strip edge, where nitrogen competition with pumpkin likely drove nodulation, and enhanced resource complementarity benefitted yield. Reduced pollination decreased biomass of belowground roots and nodules, and negatively impacted yield. Reduced resource investment nodules possibly resulted in less available nitrogen to invest in yield. We have provided the first indications of an interactive effect between pollination and nodulation, where non-pollinated plants benefitted more from higher proportions of active nodules in shaping faba bean yield. These findings provide a novel mechanism via which pollination can indirectly affect yield via altering resource allocation belowground. Future studies into resource allocation (partitioned in above- and belowground growth, flowering and yield) are required to fully elucidate the effects of pollination on faba bean nodulation and, ultimately, yield. Our results strengthen the case for strip-cropping with legumes and pollinator management to enhance nodulation and yield. Inclusion of legumes in strip-cropping systems provides additional benefits apart from enhancing nitrogen availability, as legumes showed to enhance water retention, nutrient cycling and soil fertility (Stagnari et al., 2017). We opt for narrow strips to maximize the benefits of resource complementarity in strip-cropping systems. By investigating a faba bean-pumpkin crop combination, we showed that the benefits of strip-cropping on yield and nodulation extend beyond the commonly investigated legume-cereal intercropping systems. Improved understanding of the (interactive) effects of pollination and nodulation in shaping resource allocation and yield in diverse cropping systems with legumes is important for building resource-efficient, sustainable cropping systems.

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Appendix

Table S 1: Nutrient content of cattle slurry used to fertilize pumpkin strips.

Component	Content
Nitrogen (N)	60.4 kg/ha
Phosphorus (P)	47.9 kg/ha
Potassium (K)	195 kg/ha

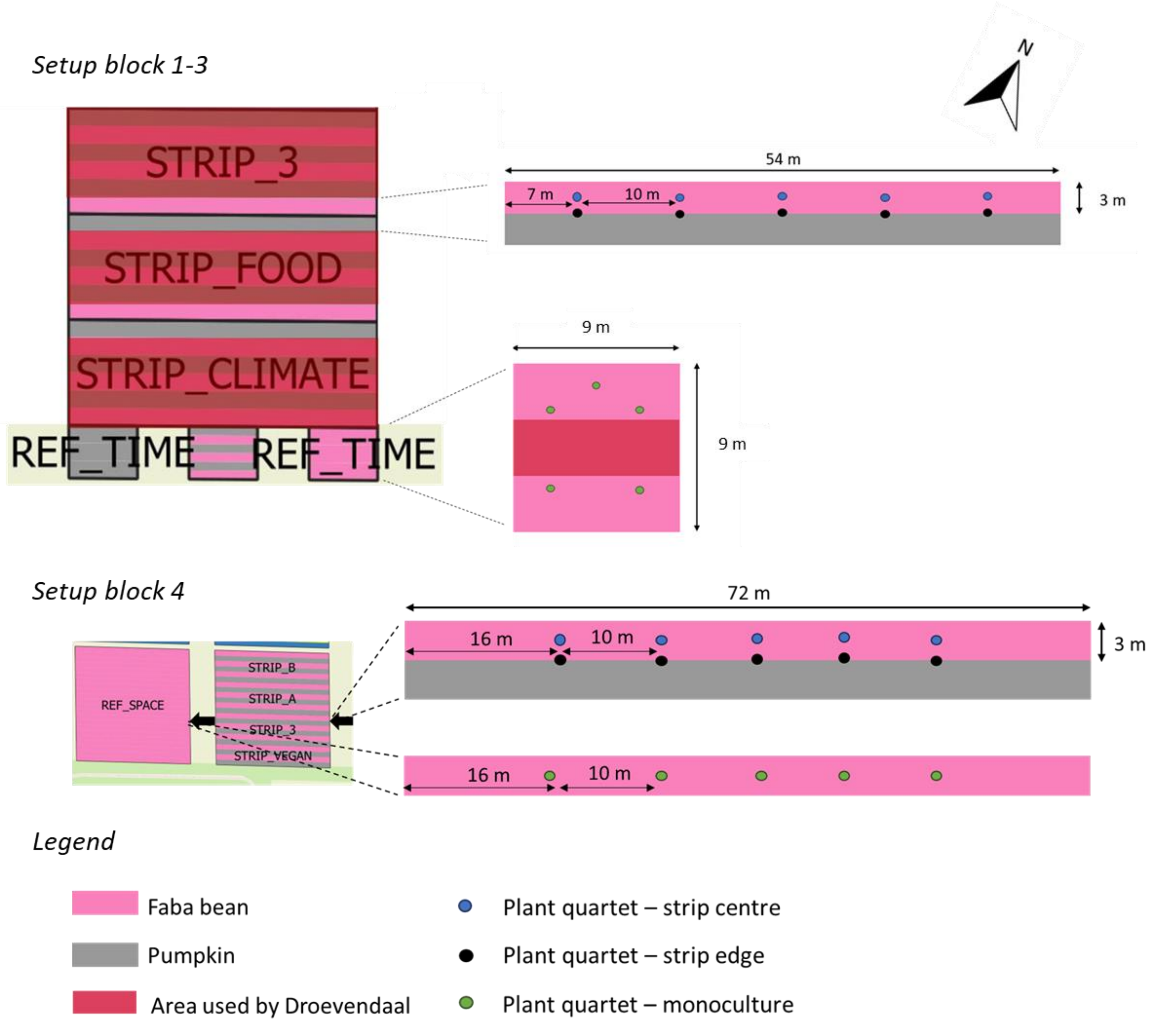

Figure S 1: Spacing of plant quartets in block 1-3 (upper) and 4 (lower) of Droevendaal experimental farm.

Table S 2: Model structures

Response variable	***Distribution***	***Fixed factor***	***Random structure***
Insect visitation rate	Poisson	Crop bloom Strip treatment	1|block/round
Nodulation & growth parameters			
Nodule mass-ratio	Gaussian	Field position treatment	1|block/quartet
Nodule number-ratio	Gaussian	Pollination treatment	
Proportion of active nodules	Gaussian		
Leaf N content	Gaussian		
Leaf P content	Gaussian		
Root DM	Gaussian		
Yield parameters			
Bean DM	Gaussian	Field position treatment	1|block/quartet
Average beans / pod	Gaussian	Pollination treatment	
Total beans per plant	Poisson		
Total pods per plant	Poisson		
Bean protein content	Gaussian		
Interaction nodulation - pollination			
Total beans per plant	Gaussian	Field position treatment	1|block/quartet
Beans per pod	Poisson	Pollination treatment	
		Proportion active nodules	
		Pollination*active nodules	

Table S 3: amount of observed pollinating events in a total of four pollinator surveys, separated by type of insect pollinator.

	Honeybee	**Bumble bee**	**Hummingbird hawk-moth**	**Total visit**
Strip-crop	16	49	1	66
Monocrop	25	47	6	78
Total	41	96	7	144

Table S 4: Pearson correlation coefficients and associated p-values.

Variables	**Pearson's correlation**	***p* - value**
Nodule DM – root DM	0.455	<0.001 ***
Nodule number – root DM	0.473	<0.001 ***
Nodule DM - proportion active nodules	0.567	<0.001 ***
Nodule number - proportion active nodules	0.392	<0.001 ***

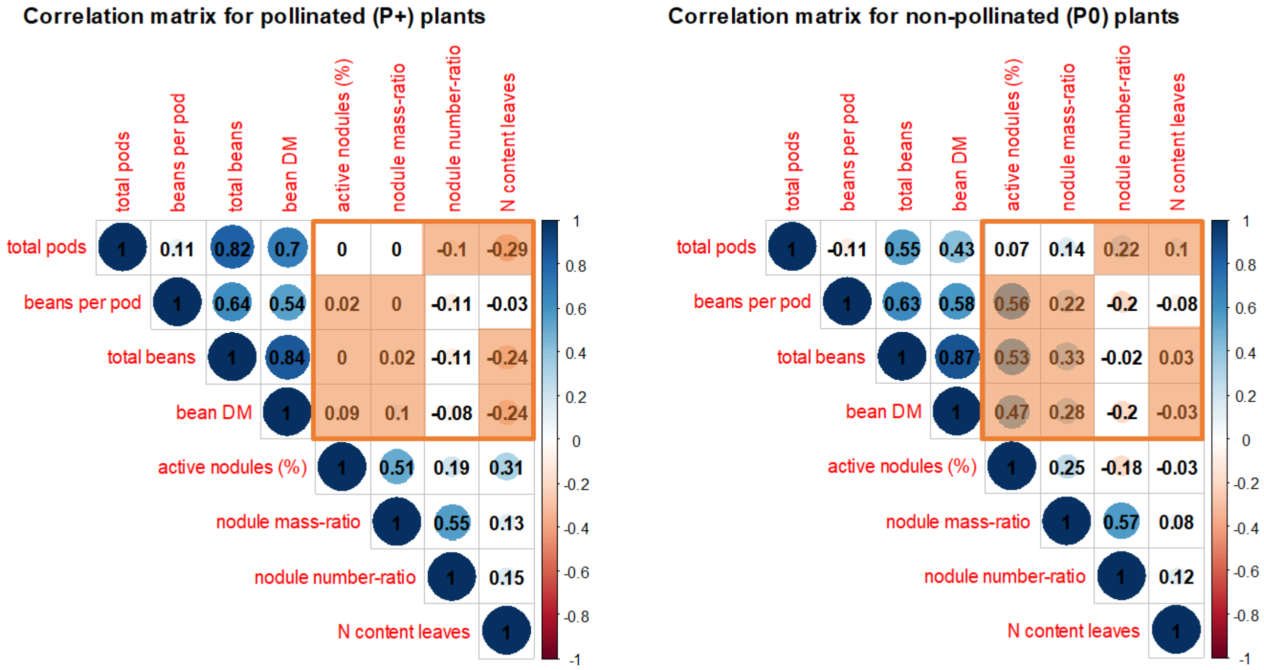

Figure S 2: Correlation matrixes for nodulation- and yield parameters per pollination treatment

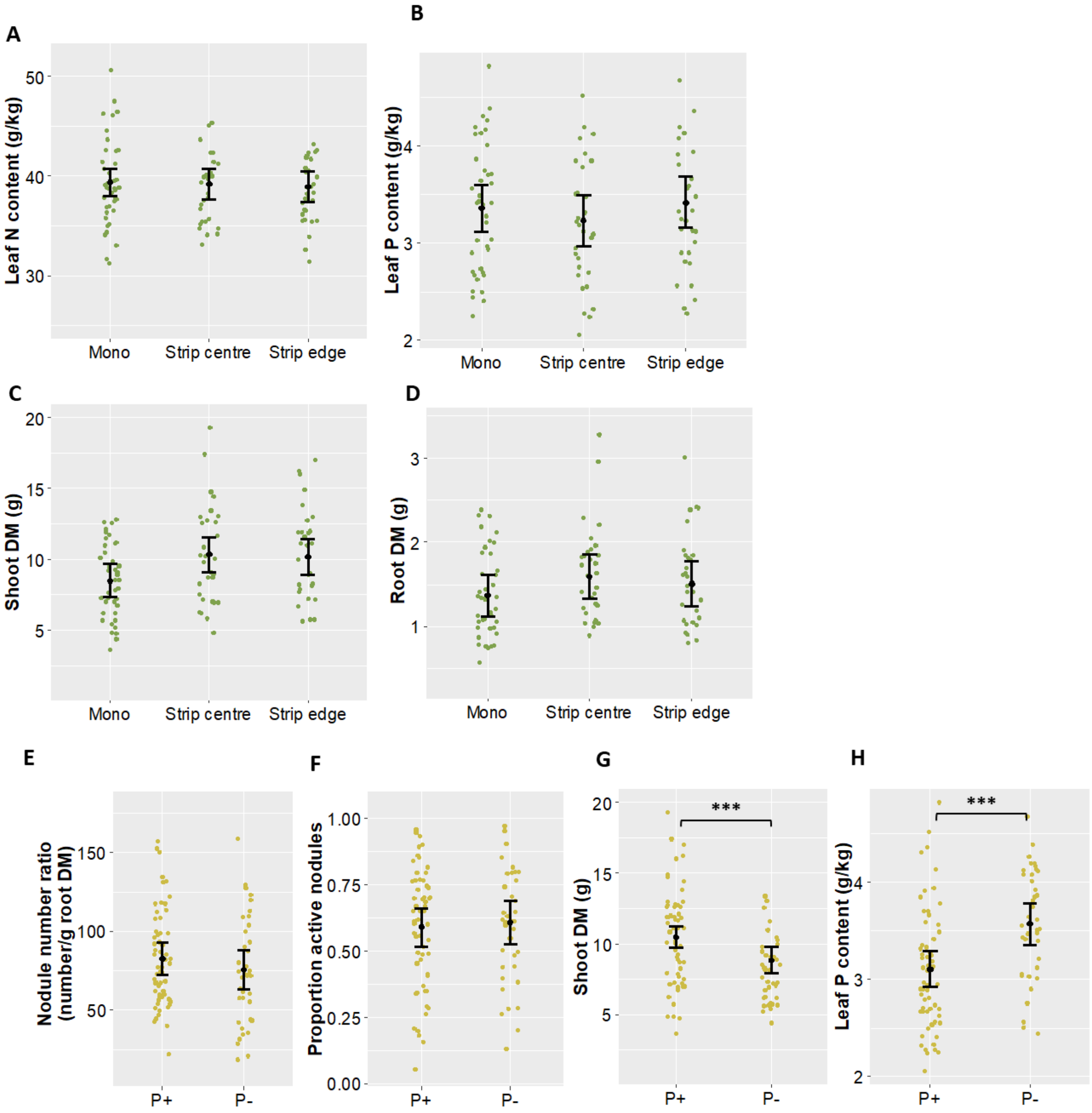

Figure S 3: Model predictions for faba bean nodulation and plant response parameters mid-season in relation to the field position (A, B, C, D, averaged over pollination treatment) and pollination treatment (E, F, G, H, *averaged over field position). Whiskers represent 95% confidence intervals and a point estimate. Note that x-axis does not always start at 0. Significance is shown as *P<0.05, **P<0.01, ***P<0.001.*

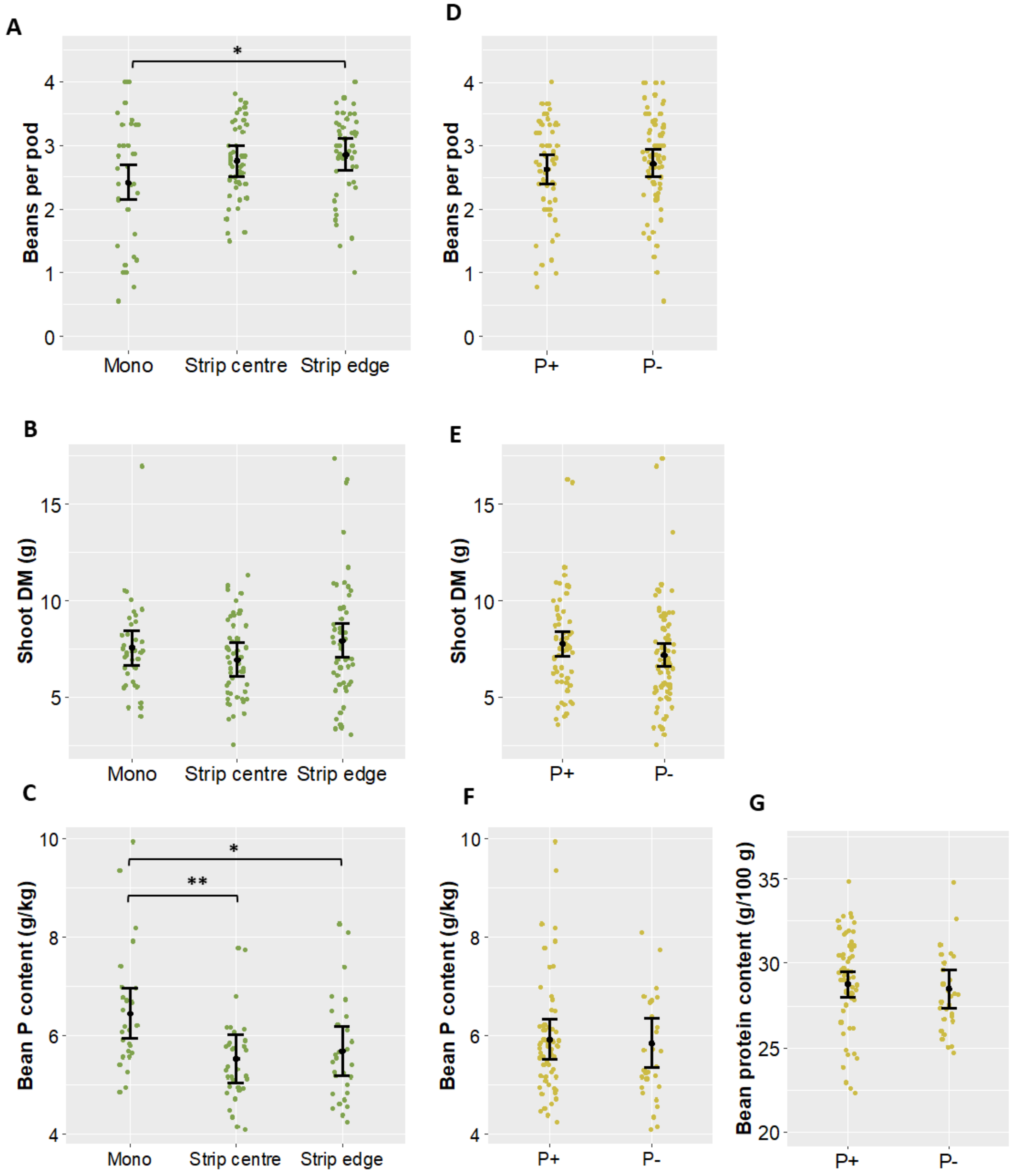

*Figure S 4: Model predictions for faba bean yield and plant response parameters at full maturity in relation to the field position (A, B, C, averaged over pollination treatment) and pollination treatment (D, E, F, G, averaged over field position). Whiskers represent 95% confidence intervals and a point estimate. Note that x-axis does not always start at 0. Significance is shown as *P<0.05, **P<0.01, ***P<0.001.*