RESEARCH ARTICLE

Changes in belowground interactions between wheat and white lupin along nitrogen and phosphorus gradients

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Abstract

Background and aims The positive effects of cereal-legume intercropping can occur through complementarity and facilitation for the acquisition of soil nitrogen (N) and phosphorus (P). However, the magnitude of the benefits depends on the availability of such resources. To improve our understanding and design best-performing systems, we evaluated the performance and underlying mechanisms of a cereal-legume mixture on two crossed gradients of N and P.

Methods In a pot experiment, wheat (*Triticum aestivum* L.) and white lupin (*Lupinus albus* L.) were grown separately or together on four levels of N combined with four levels of P. We compared biomass, N content, P content, shoot-to-root biomass ratios and white lupin production of cluster roots and nodules of sole crops with those of intercrops. We used the relative interaction intensity index to determine how the nature of the interaction between the two species changed along the two resource gradients.

Results Regardless of N and P supply, combined biomass and N content of intercropped wheat and white lupin were 29% and 23% greater than those of sole crops, respectively. Intercropping increased P content by 34%, on average, particularly at the lowest P supply. While wheat benefited from intercropping in all treatments, white lupin's performances decreased with increasing N and P supply, resulting in a shift from mutualism to competition along the two gradients and compensation mechanisms between the two species.

Conclusion Nutrient availability negatively influenced the nature and mechanisms of wheat-lupin interaction. However, we only observed mutualistic interactions under low resource supply, which led to the lowest production, while competition was associated with the most productive situations.

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Introduction

In recent decades, agricultural production has increased dramatically to support the strong global

human population growth. This mostly resulted from a significant increase in crop productivity, which has been achieved mainly through an intensive use of chemical inputs such as fertilizers and pesticides or irrigation water, and through the selection of high-yielding crop varieties. This has led to the dominance of poorly diversified agricultural systems (short rotations or monocultures), heavily relying on chemical inputs with various negative consequences on the environment and biodiversity (Steffen et al. 2015; Tilman et al. 2001). It is therefore increasingly recognized that agriculture must evolve towards more sustainable alternatives based on ecological knowledge, such as proposed in the framework of agroecology (Altieri 1989; Duru et al. 2015; Foley et al. 2011; Malézieux 2012).

Among the agroecological solutions proposed for an ecological intensification of agroecosystems, the use of diversity in multiple cropping systems has received considerable attention (Barot et al. 2017; Gaba et al. 2015). This is particularly the case for intercropping, a practice involving two or more species grown together and coexisting for a significant time of their growth cycle (Li et al. 2014; Martin-Guay et al. 2018; Tilman 2020). The benefits of intercropping can arise through different ecological mechanisms such as complementarity between plants and facilitation (Hinsinger et al. 2011). Complementarity occurs when plants differ in their use of a resource, either spatially, temporally or by exploiting different chemical forms (Kahmen et al. 2006; Loreau and Hector 2001). This process has been well documented in intercropping systems, particularly concerning nitrogen (N) acquisition in cereal-legume intercropping systems, in which the cereal exploits soil mineral N while the legume fixes atmospheric N symbiotically, thus lowering the negative effect of interspecific competition for N (Bedoussac et al. 2015; Jensen et al. 2020; Li et al. 2009). Facilitation refers to the benefit of one species in the presence of another without any harm; it has also been observed for phosphorus (P) acquisition in cereal-legume intercropping systems (Callaway 1995; Hinsinger et al. 2011; Li et al. 2020, 2014; Tang et al. 2016). Indeed, many legume species have the ability to increase P availability through the acidification of the rhizosphere and the release of carboxylates and phosphatases, which in turn, benefits the cereal (Betencourt et al. 2012; Cu et al. 2005; Li et al. 2016, 2003).

Knowledge of these ecological mechanisms offers interesting potential for the design of intercropping systems. However, they are known to depend considerably on the availability of resources shared by intercropped species. For example, various studies show that cereal-legume intercrops are particularly efficient in low-N-input systems, as reviewed by Bedoussac et al. (2015). Similarly, the benefits of P acquisition through facilitation are greater in conditions where available P is low (e.g., Betencourt et al. 2012). This general observation and the ecological concept of the stress gradient hypothesis lead to the prediction that positive interactions will be more frequent under harsh conditions, and conversely negative interactions more likely under favorable environments (Brooker et al. 2008; Callaway 2007; Yu et al. 2021). Conversely, intercropping can lead to large yield increases, even in highly fertilized systems (Li et al. 2020). In order to intensify the ecological processes of facilitation and complementarity for the acquisition of limiting resources in intercropping systems, a better understanding of how these ecological mechanisms and plant-plant interactions occur in different scenarios of N and P input appears fundamental. Cereal-legume intercropping systems are particularly relevant to consider in this respect, as many studies highlighted its benefits for both crops, especially concerning their N and P acquisition (Brooker et al. 2015; Bedoussac et al. 2015; Jensen et al. 2020; Tilman 2020; Justes et al. 2021; Li et al. 2021).

In the present study, a cereal (wheat: *Triticum aestivum* L.) and a legume (white lupin: *Lupinus albus* L.) were grown either separately or together along two crossed gradients of N and P supply in a pot experiment. Benefits from intercropping wheat with white lupin have already been demonstrated in previous studies (Cu et al. 2005; Gardner and Boundy 1983; Mariotti et al. 2009). For instance, Cu et al. (2005) showed an increase of wheat shoot growth and shoot P content of 33 and 45%, respectively, without significantly affecting lupin growth. This result may be due to the ability of white lupin to produce cluster roots that release large quantities of protons and carboxylates such as malate and citrate, especially under low-P conditions, which in turn increase the availability of P for plants (Gardner et al. 1981; Gardner and Boundy 1983; Hinsinger 2001; Lambers 2022; Lambers et al. 2006). The intermingling of cereal roots with those of white lupin would thus be expected to

increase P availability through facilitation, especially in low-P conditions.

The objective of this study was to address the following questions: (i) Does the growth of wheat and white lupin increase in an intercropping system and does the change in performance vary along N and P gradients? (ii) How do the cereal and the legume affect each other along the N and P gradients? (iii) What are the nature (i.e. positive, neutral or negative) and the underlying mechanisms of their interactions along the N and P gradients? (iv) Is there a relationship between the nature of cereal-legume interactions and the mixture performances? The performances of sole crops and intercrops (i.e. biomass, N uptake and P uptake) have been compared. Ecological indices of plant mixture and relative performances have been calculated, while belowground traits of lupin (biomass of nodules and cluster roots) have been measured.

Materials and Methods

Experimental design

A pot experiment was conducted under glasshouse conditions using a complete factorial design with four levels of N, four levels of P, and three cropping treatments (C): one with the two species intercropped, and two with each crop grown separately. The levels of N and P supplied were based on a preliminary experiment to ensure contrasting responses of plants between different treatments. Plants were grown in a randomized block design with five replicates, so that each combination of factors was represented in each of the five blocks, leading to a total number of 240 pots. Plant density was kept constant through a substitutive design, and chosen to be close to agricultural practices. When grown as sole crops, four individuals were planted in each pot for wheat, while two individuals were planted for white lupin. When intercropped, half the number of individuals of each species were grown.

Plant and soil material

Lupinus albus L. cv. Feodora and *Triticum aestivum* L. cv. Lennox were selected for their phenological synchrony. For the sake of simplicity, these species are hereafter referred to simply as lupin and wheat, respectively. Topsoil (0–10 cm depth) of a Luvisol (FAO 2014) with a silty-clay texture was collected from the highest (+P) and lowest (-P) P supply treatments of a long-term P-fertilizer field trial at INRAE (Auzeville, France, 43°31'N, 1°30'E) (Colomb et al. 2007). The composition and properties of these two soils were almost identical apart from the levels of total and available (Olsen-extractable) P (Betencourt et al. 2012). The -P treatment had received no P for 40 years, while +P was over-fertilized at a rate equivalent to three- to four-fold the actual P removal by crops. The main properties of the -P soil were: $pH_{H20} = 7.6$, organic $C = 7.4$ mg kg^{-1} dry soil (heat-loss at 1000 °C), total $P = 287$ mg kg^{-1} dry soil (HCl extractable), extractable $P = 6.7$ mg kg^{-1} dry soil (Olsen method), NO_3^{-}-N $= 19$ mg kg^{-1} dry soil and NH_4^{+}-N $= 0.5$ mg kg^{-1} dry soil (KCl extractable). The main properties of the +P soil were: $pH_{H20} = 7.8$, organic $C = 8.9$ mg kg^{-1} dry soil, total $P = 721$ mg kg^{-1} dry soil, extractable $P = 33.5$ mg kg^{-1} dry soil, NO_3^{-}-N $= 20$ mg kg^{-1} dry soil and NH_4^{+}-N $= 0.6$ mg kg^{-1} dry soil. These two soils were used either alone or in mixtures of -P and +P to obtain four different levels of P. In this way it was not necessary to add phosphate salts, which would have resulted in different dynamics, less realistic compared to aged P. The P1 treatment was made of 2/3 of -P and 1/3 of +P, the P2 treatment was made of 1/3 of -P and 2/3 of +P, while P0 and P3 treatment comprised pure -P and pure +P, respectively. The mixtures of soils and the resulting levels of Olsen-P (mg P kg^{-1}) dry soil were as follows: P0 (-P) 6.7; P1 (1:2 -P:+P) 13.5; P2 (2,1 -P:+P) 22.5; and P3 (+P) 33.5.

Plant growth conditions

Pots (20 cm diameter, 15 cm height) were lined with polyethylene bags to prevent nutrient leaching. The pots were filled with 2 kg of air-dried soil mixed with 60 g of perlite to avoid soil compaction due to watering. Soil moisture content was adjusted to 75% of water holding capacity. A ^{15}N-enriched urea solution (5% atom in excess) was added at the beginning of the experiment to determine the proportion of N derived from symbiotic fixation in lupin. The ^{15}N-enriched urea solution was diluted at four rates resulting in four N levels: 5, 20, 40 and 100 mg N kg^{-1} dry soil

for N0, N1, N2 and N3 treatments, respectively. No other nutrients were added, because previous studies had shown that only N and P were growth limiting. In each sole crop treatment, 12 seeds of wheat or six seeds of lupin were sown, and plants were thinned to four per pot for wheat and two for lupin a week after emergence. In the intercrop treatment, six seeds of wheat were sown with three seeds of lupin, and plantlets were thinned a week after emergence to two and one plants per pot, respectively. Lupin seeds were inoculated with rhizobia (*Rhizobium lupini* LL13 strain, INRAE, Dijon, France) directly after sowing to ensure good nodulation. Plants were grown in February – April 2015 in a glasshouse with a night temperature of 19 °C and a day temperature of 24 °C. Light intensity was kept at about 400 W m^{-2} during 16 h day^{-1}. Pots were weighed daily and watered when necessary to maintain soil water content near 75% of water holding capacity. Plants were harvested 51 days after sowing, at the start of flowering stage, when first pods of lupin had reached 1 cm and wheat entered the anthesis stage. At the end of the experiment, plants did not show any nutrient deficiency symptom except for N and P in some treatments along the gradients.

Plant measurements

At harvest, shoots from each pot were cut and roots were gently removed from the soil and washed. In the intercrop treatment, plant parts from each species were collected separately. For lupin, cluster roots and nodules were collected separately from the rest of the root system. Plant material was then oven-dried at 65 °C for three days, weighed and ground (MM 400, Retsch, Düsseldorf, Germany). Biomass values are expressed per plant in each pot (dividing the whole biomass by the number of plants per pot). N-fixation in lupin was calculated with the ^{15}N isotope dilution method (Unkovich et al. 2008), using the *%Ndfa* equation (i.e. proportion of N derived from the atmosphere):

$$\%Ndfa = \left(\frac{\delta^{15}N_{Ref} - \delta^{15}N_{Lupin}}{\delta^{15}N_{Ref}} \right) \times 100. \qquad (1)$$

$\delta^{15}N_{Ref}$ was obtained with wheat in the sole crop treatment, and for each *%Ndfa* calculation, we used the $\delta^{15}N_{Ref}$ value obtained in the same plant parts, for

identical N and P treatments, and in the same replication block as for $\delta^{15}N_{Lupin}$. %Ndfa was calculated for shoots (*%Ndfa$_{Shoots}$*) and roots (*%Ndfa$_{Roots}$*), and the total *%Ndfa* was then obtained with the following equation:

$$\%Ndfa_{Total} = \frac{\%Ndfa_{Shoots} \times B_{Lupin\_Shoots} + \%Ndfa_{Roots} \times B_{Lupin\_Roots}}{B_{Lupin\_Total}}, \qquad (2)$$

where B_{Lupin_Shoots}, B_{Lupin_Roots} and B_{Lupin_Total} are the mean individual biomass of shoots, roots and the mean individual total biomass of lupin, respectively. $\delta^{15}N$ and N content were determined with a Stable Isotope Ratio Mass Spectrometer coupled with an Elemental Analyzer (EA/GA-IRMS, Carlo Erba, Elementar, Finnigan, Isoprime, Nancy, France). Plant P content was determined using the vanado-molybdate method, as described in Sauvadet et al. (2021).

Interaction indices

To compare the growth of wheat and lupin grown separately with their growth in mixture (intercrop), we used two interaction indices: the Relative Yield of Mixture (hereafter called *RYM* (Wilson 1988)) and the Relative Interaction Intensity (hereafter called *RII* (Armas et al. 2004)). The first quantifies the overall effect of the interaction on both species – at the scale of the mixture. It was calculated as follows:

$$RYM = \frac{X_{Lupin\_Intercrop} + 2 \times X_{Wheat\_Intercrop}}{X_{Lupin\_Sole\_Crop} + 2 \times X_{Wheat\_Sole\_Crop}}, \qquad (3)$$

where $X_{Lupin\_Intercrop}$ and $X_{Wheat\_Intercrop}$ are, respectively, the mean individual values of any variable measured in lupin and wheat in the intercrop treatment, and $X_{Lupin\_Sole\_Crop}$, $X_{Wheat\_Sole\_Crop}$ are the mean individual values of the same variable of lupin and wheat in the sole crop, respectively. *RYM* calculations were made for each N and P level combination and with variables measured in the same replication block. In this study, we considered three variables: total biomass (*RYM$_{Biomass}$*), total N content (*RYM$_N$*) and total P content (*RYM$_P$*). The factor of 2 (see Eq. 3) takes account of the greater number of wheat individuals relative to lupin in the intercrops. When $0 < RYM < 1$, there was an overall negative effect of intercropping on the studied plant variable, whereas when $1 < RYM$, the overall effect was positive. When

RYM was not significantly different from *1*, intercropping had no effect on the studied variable.

To measure the effect of intercropping at the species level, a second index was calculated for lupin and wheat, respectively:

$$RII_{Lupin} = \frac{B_{Lupin\_Intercrop} - B_{Lupin\_Sole\_Crop}}{B_{Lupin\_Intercrop} + B_{Lupin\_\_Sole\_Crop}} \text{ and}$$

$$RII_{Wheat} = \frac{B_{Wheat\_Intercrop} - B_{Wheat\_Sole\_Crop}}{B_{Wheat\_Intercrop} + B_{Wheat\_Sole\_Crop}}. \tag{4}$$

RII_{Lupin} and RII_{Wheat} were calculated for each combination of N and P level, using biomass from the same replication block. A negative value of *RII* indicates that intercropping had an overall negative effect on species biomass, and a positive value indicates a positive influence. *RII* thus provides information on the effects of the interaction at the species level. This allows some determination of the nature of the wheat-lupin interaction.

Statistics

For each variable, means and standard deviations were calculated from replicates of each treatment ($n = 5$ replicates) and three-way ANOVA was performed to determine the effects of "N supply", "P supply" and "Cropping" treatments. For plant indices (*RYM* and *RII*), comparisons of means among treatments were performed using two-way ANOVA (using "N supply" and "P supply" as factors), and Student's t-test was carried out to determine the sign of the interaction (positive, negative or neutral) for *RII*. Following ANOVAs, Tukey HSD post hoc tests and Eta-squared (Eta^2) calculations were carried out. Eta-squared is a measure of effect size for analysis of variance models and can be interpreted as the proportion of a variance in a variable explained by an effect. It is calculated as the ratio between the sum of squares of the considered effect on the studied variable, and the total sum of squares in the ANOVA model. Residuals were verified for normality and homogeneity of variance using Wilk-Shapiro and Levene's tests, respectively. All tests were computed with R 4.4.1 (R Development Core Team 2021) and statistical significance was set at $P < 0.05$.

Analysis of net biodiversity, selection and complementarity effects

Net biodiversity, selection and complementarity effects were estimated for plant biomass, N content and P content, and are presented and discussed in a separate section (see Supplementary Information). These results were obtained with the Loreau and Hector's (2001) analysis method of diversity effects which allows unravelling the net effect of biodiversity by additive partitioning of complementarity effect and selection effect.

Results

Plant biomass

Total wheat biomass was significantly influenced by each of the three two-way interactions between the three factors, namely "N supply", "P supply" and "Cropping" treatments (Table 1, Fig. 1). There was a significant effect of the interaction between "N" and "P supply" (N×P) on wheat total biomass ($P < 0.001$, $Eta^2 = 0.07$). For a given supply of N, wheat biomass increased with P supply, especially beyond N1 (see Table S1 for Tukey's range post hoc test results). Moreover, with the exception of plants grown at P0, total biomass increased markedly with increasing N supply.

These results highlight a threshold effect between N1 and N2 levels, beyond which wheat biomass had a stronger response to P addition. There was another threshold between P0 and P1 levels beyond which biomass was substantially greater and responded strongly to N addition. Furthermore, the positive effects of N and P addition on wheat biomass were more pronounced in intercropping than in sole cropping treatments ($P < 0.001$ for both N×C and P×C interactions). As indicated by the analysis of the main factors, wheat total biomass increased with N and P addition, and when intercropped with lupin, explaining 22%, 37% and 22% of the variation observed in wheat total biomass for "N supply", "P supply" and "Cropping" treatments, respectively (see Eta^2 column in Table 1). Consequently, the lowest biomass was achieved at the lowest N and P supply (N0P0) in sole

Table 1 Three-way ANOVA and Eta-squared results for total dry biomass, nitrogen (N) content and phosphorus (P) content in plants with "N supply", "P supply" and "Cropping" treatments as factors. Significant results are in bold

		Total dry biomass				Nitrogen content				Phosphorus content			
		Wheat		Lupin		Cluster roots		Nodules		Wheat		Lupin	
		P	Eta²	P	Eta²	P	Eta²	P	Eta²	P	Eta²	P	Eta²
Main effects	N supply (N)	**<0.001**	0.22	0.10	0.03	**<0.001**	0.35	**0.008**	0.07	**<0.001**	0.20	**<0.001**	0.08
	P supply (P)	**<0.001**	0.37	0.44	0.01	**<0.001**	0.14	0.65	<0.01	**<0.001**	0.43	**<0.001**	0.25
	Cropping Treatment (C)	**<0.001**	0.22	0.20	<0.01	**<0.001**	0.26	0.98	<0.01	**<0.001**	0.20	0.47	<0.01
Interactions	N×P	**<0.001**	0.07	0.52	0.04	**<0.001**	0.07	0.61	0.04	**<0.001**	0.07	**0.014**	0.07
	N×C	**<0.001**	0.01	**0.02**	0.05	**<0.001**	0.02	0.17	0.03	**<0.001**	0.01	**0.009**	0.04
	P×C	**<0.001**	0.04	**<0.001**	0.18	**<0.001**	0.05	**0.005**	0.08	**<0.001**	0.04	**<0.001**	0.11
	N×P×C	0.31	<0.01	0.72	0.03	0.59	<0.01	0.93	0.02	0.29	<0.01	0.42	0.03

cropping while the greatest biomass was reached at the highest N and P supply (N3P3) in intercropping.

For lupin, "P supply" interacted strongly with the "Cropping" treatment ($P < 0.001$, Eta² = 0.18; Table 1, Fig. 1). When plants were grown as sole crops, there was a significant increase of total biomass between P0 and P3 (Table S1). Conversely, we observed a decrease of total biomass between P0 and other P levels when lupin was intercropped with wheat. In addition, there was a small but significant effect of N supply on the total biomass of lupin in the intercropping treatment ($P < 0.05$, Eta² = 0.05). This was only found between lupins grown at N1, which led to the greatest total biomass, and lupins grown at N3, which led to the lowest total biomass.

Nitrogen content

The total N content of wheat followed a similar trend to that of biomass. There was a significant effect of each of the two-way interactions between the three factors ($P < 0.001$ for N×P, N×C and P×C; Table 1, Fig. 2). However, the main factors influenced N content differently, explaining 35%, 14% and 26% of the variation observed for "N supply", "P supply" and "Cropping" treatments, respectively.

The total N content of lupin was significantly influenced by N supply ($P < 0.01$, Eta² = 0.07) and by the interaction between P supply and cropping treatment ($P < 0.01$, Eta² = 0.08; Table 1 and Fig. 2). The N content was significantly greater in lupin grown at the lowest N level (N0) compared with the highest (N3) level (Table S1). Moreover, at P0, greater N contents were observed in lupin grown with wheat in comparison with lupin grown as sole crop (Table S1, Fig. 2).

Phosphorus content

The total P content of wheat followed very similar trends to biomass. There was a significant effect of each of the two-way interactions between the three factors ($P < 0.001$ for N×P, N×C and P×C; Table 1, Fig. 3). Moreover, the main factors influenced P content almost identically to what we observed for biomass ($P < 0.001$ for "N supply", "P supply" and "Cropping" treatments), with a slightly greater effect of P supply (Eta² = 0.40; Table 1).

There was also a significant effect for lupin of each of the three two-way interactions between the three
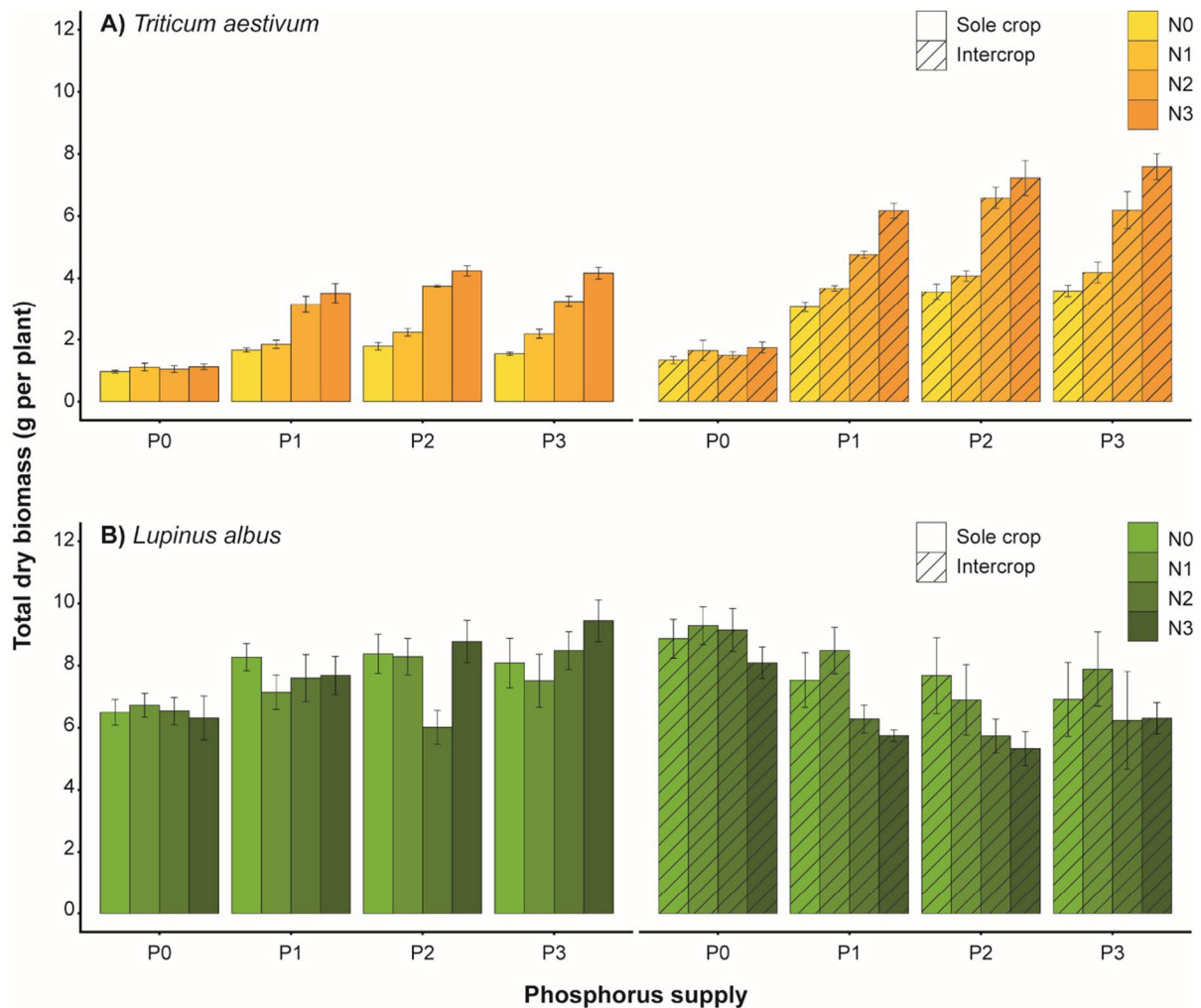
Fig. 1 Total biomass of wheat (A) and lupin (B) at the end of the experiment. Vertical bars represent standard error. N0, N1, N2, N3 stand for the four levels of N, and P0, P1, P2, P3 stand for the four levels of P

factors ($P < 0.05$ for N×P, $P < 0.01$ for N×C and $P < 0.001$ for P×C; Table 1, Fig. 3). The P content did not respond significantly to N supply in the sole crop treatment. However, it was negatively affected by N addition when lupin was intercropped with wheat (Table S1). In contrast, P supply had a strong positive effect in lupin grown alone, although it did not greatly influence lupin P content when intercropped with wheat. While no clear pattern emerged from the Tukey's range post hoc test results of the N×P interaction, the analysis of the main factors shows a strong positive effect of P addition ($P < 0.001$, Eta^2=0.25; Table 1), and a negative effect of N addition ($P < 0.001$, Eta^2=0.08) on lupin P content.

Relative Yield of Mixture

Intercropping significantly increased the total biomass of plants compared with sole cropping. At the scale of the complete experimental design, the value of $RYM_{Biomass}$ was 1.29 ± 0.02. Student's t-test showed that this value was significantly greater than 1 ($t_{79} = 13.134$, $P < 0.001$), indicating a positive effect of intercropping on total biomass. This indicates that intercropping led, on average, to an increase of $\approx 29\%$ of the total biomass produced by two wheat individuals and one lupin individual.

Similarly, plant N content ($RYM_N = 1.23 \pm 0.04$) and P content ($RYM_P = 1.34 \pm 0.03$) increased in
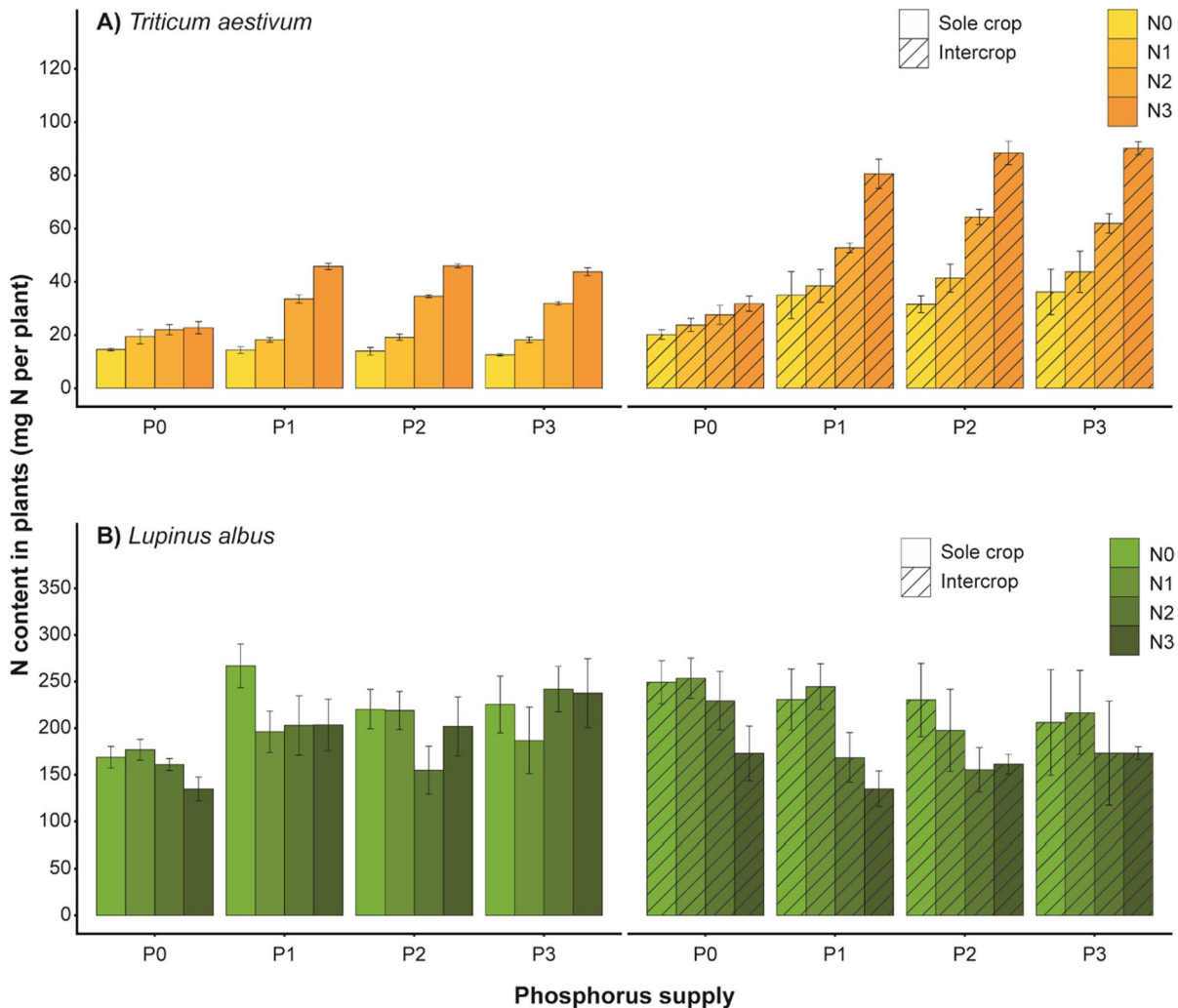
Fig. 2 Nitrogen (N) content in wheat (A) and lupin (B) at the end of the experiment. Vertical bars represent standard error. N0, N1, N2, N3 stand for the four levels of N supply, and P0, P1, P2, P3 stand for the four levels of P supply

intercropping compared with sole cropping, with values significantly greater than 1 ($t_{79} = 5.734$, $P < 0.001$ and $t_{79} = 11.647$, $P < 0.001$ for RYM_N and RYM_P, respectively).

While the increase in biomass and N content were not significantly affected by N and P supply, we found a significant impact of P supply on RYM_P ($P < 0.001$, $Eta^2 = 0.27$; Table 2). Tukey's range post hoc showed a higher value of RYM_P at P0 compared with other P levels (Table S2), indicating that the positive effect of intercropping on P content was significantly stronger at P0 than at the other P levels.

Relative Interaction Intensity

At the species level, intercropping did not affect the total biomass of wheat and lupin in the same way. RII_{Lupin} was not significantly different from 0, as indicated by Student's t-test ($RII_{Lupin} = -0.04 \pm 0.02$, $t_{79} = -1.65$, $P = 0.104$; Fig. 4), meaning that, at the scale of the complete experimental design, lupin total biomass did not significantly change when intercropped with wheat.

In contrast, wheat total biomass generally increased when mixed with lupin. The RII_W index was positive and significantly different from 0
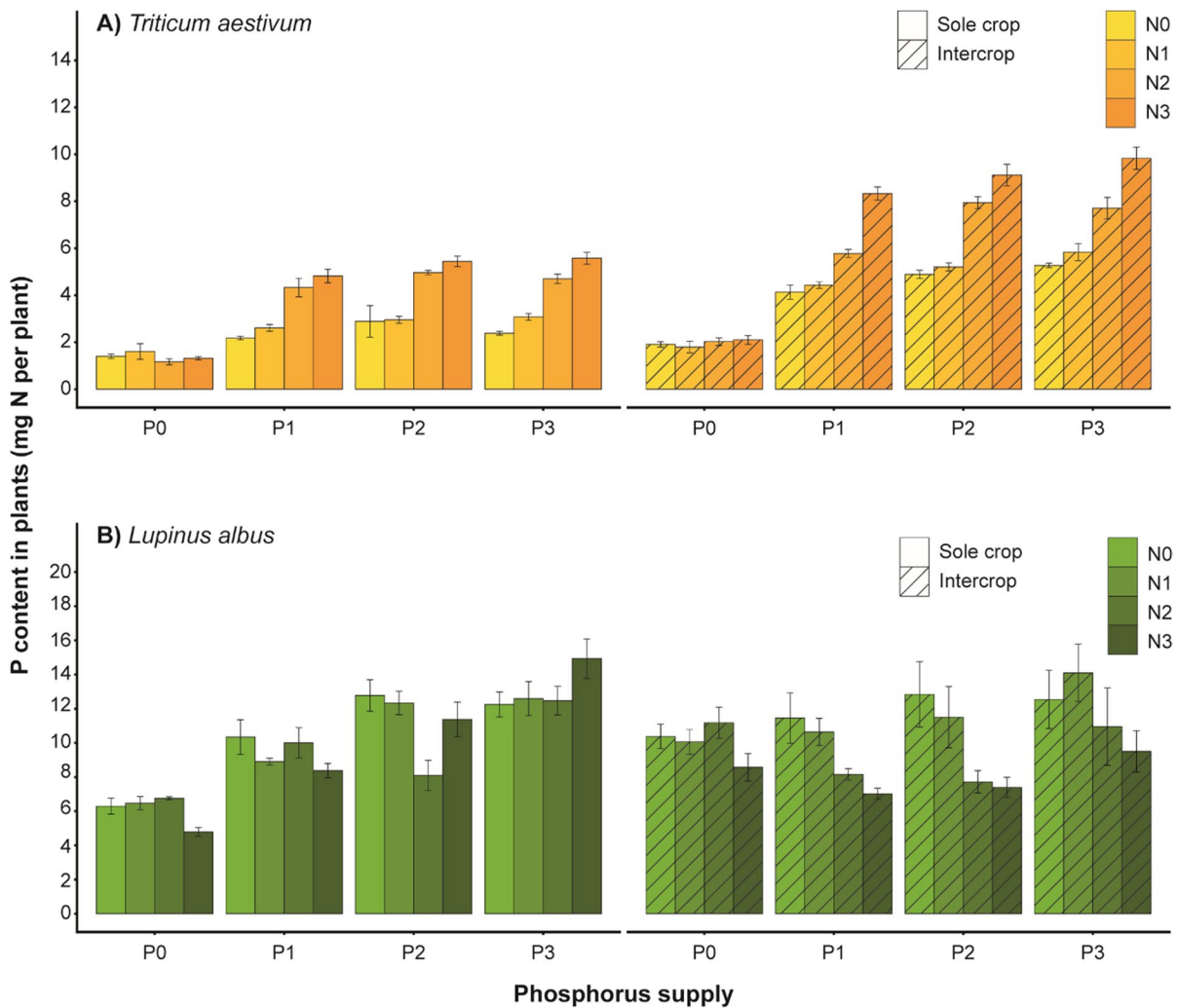
Fig. 3 Phosphorus (P) content in wheat (A) and lupin (B) at the end of the experiment. Vertical bars represent standard error. N0, N1, N2, N3 stand for the four levels of N, and P0, P1, P2, P3 stand for the four levels of P

Table 2 Two-way ANOVA and Eta-squared results for Relative yield of mixture (*RYM*) and Relative interaction intensity (*RII*) indices, with "N supply" and "P supply" as factors. Significant results are in bold

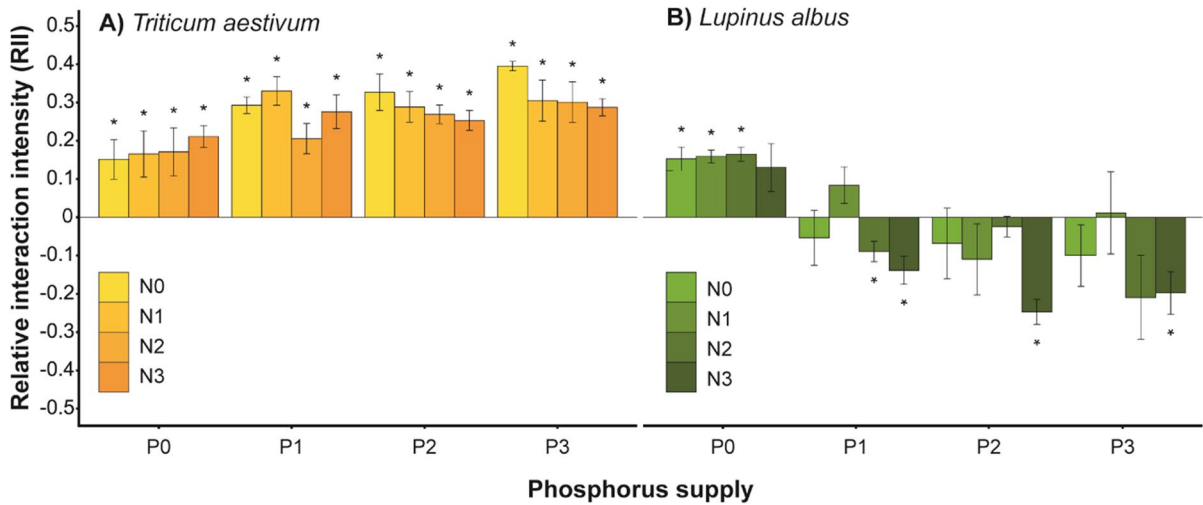|		Relative yield of mixture(*RYM*)						Relative interaction intensity (*RII*)			
		Biomass		Nitrogen		Phosphorus		Wheat		Lupin	
		P	Eta^2	*P*	Eta^2	*P*	Eta^2	*P*	Eta^2	*P*	Eta^2
Main effects	*N supply (N)*	0.43	0.03	0.31	0.05	0.68	0.01	0.32	0.04	0.021	0.08
	P supply (P)	0.07	0.09	0.08	0.09	**<0.001**	0.33	**<0.001**	0.27	**<0.001**	0.37
Interactions	*N×P*	0.23	0.14	0.46	0.11	0.05	0.15	0.57	0.08	0.38	0.08

Fig. 4 *RII* values of wheat (A) and lupin (B). Vertical bars represent standard error. N0, N1, N2, N3 stand for the four levels of N, and P0, P1, P2, P3 stand for the four levels of P. Stars (*) denote *RII* values significantly different from 0

($RII_{Wheat} = 0.26 \pm 0.01$, $t_{79} = 21.74$, $P < 0.001$; Fig. 4), indicating a general beneficial effect of intercropping on wheat biomass. This positive effect was not affected by N supply, but there was a strong effect of P treatment on RII_{Wheat} ($P < 0.001$, Eta$^2 = 0.27$; Table 2). More precisely, Tukey's range post hoc test showed a lower value for RII_W obtained at P0 compared with the other P levels (Table S2). This result shows that the benefit to wheat of intercropping with lupin was less at P0 (+45% increase of biomass per plant) compared with the three other P levels (+72%, +77% and +92% increase of biomass for P1, P2 and P3 levels, respectively).

For lupin, the effect of intercropping on lupin total biomass depended on N and P treatments, although RII_{Lupin} was not significantly different from 0 at the scale of the complete experimental design. First, RII_{Lupin} was strongly affected by P supply ($P < 0.001$, Eta$^2 = 0.37$, see Table 2), and Tukey's range post hoc test highlighted a higher value of the RII_{Lupin} obtained at P0 than at the three other P levels (Table S2). Furthermore, while RII_{Lupin} was significantly positive at P0 ($t_{19} = 8.788$, $P < 0.001$), it was negative but not significantly different from 0 at P1 ($t_{19} = -1.993$, $P = 0.06$), and negative and significantly different from 0 at P2 ($t_{19} = -3.054$, $P < 0.01$) and P3 ($t_{19} = -2.671$, $P < 0.05$). Intercropping thus yielded a positive effect on lupin total biomass only at P0. Second, RII_{Lupin} was also significantly

affected by N supply ($P < 0.05$, Eta$^2 = 0.08$; Table 2), and Tukey's range post hoc test showed a significant difference between the RII_{Lupin} obtained at N1 and the RII_{Lupin} obtained at N3 (Table S2). No consistent trend of the effect of nutrient supply on lupin intercropped with wheat was apparent. However, Student's t-tests showed that N3 was the only level leading to a RII_L value significantly different from 0 ($t_{19} = -2.820$, $P < 0.05$), leading to a 21% decrease in lupin total biomass in the intercropping treatment.

Student's t-tests revealed a positive effect on wheat for every combination of N and P levels (Fig. 4). Intercropping significantly increased lupin biomass when grown at a low P supply ($RII_{Lupin} > 0$ in N0P0, N1P0 and N2P0 treatments). However, intercropping also significantly decreased lupin biomass at high N availability ($RII_{Lupin} < 0$ in N2P1, N3P1, N3P2 and N3P3 treatments). For the other N and P combinations, intercropping did not significantly change lupin biomass.

Changes in wheat-lupin interactions along N and P gradients

Figure 5 shows the lupin and wheat total biomass obtained for each N and P combination in intercropping (two individuals of wheat and one individual of lupin). A color code illustrates the three types of lupin–wheat interactions, namely: mutualism
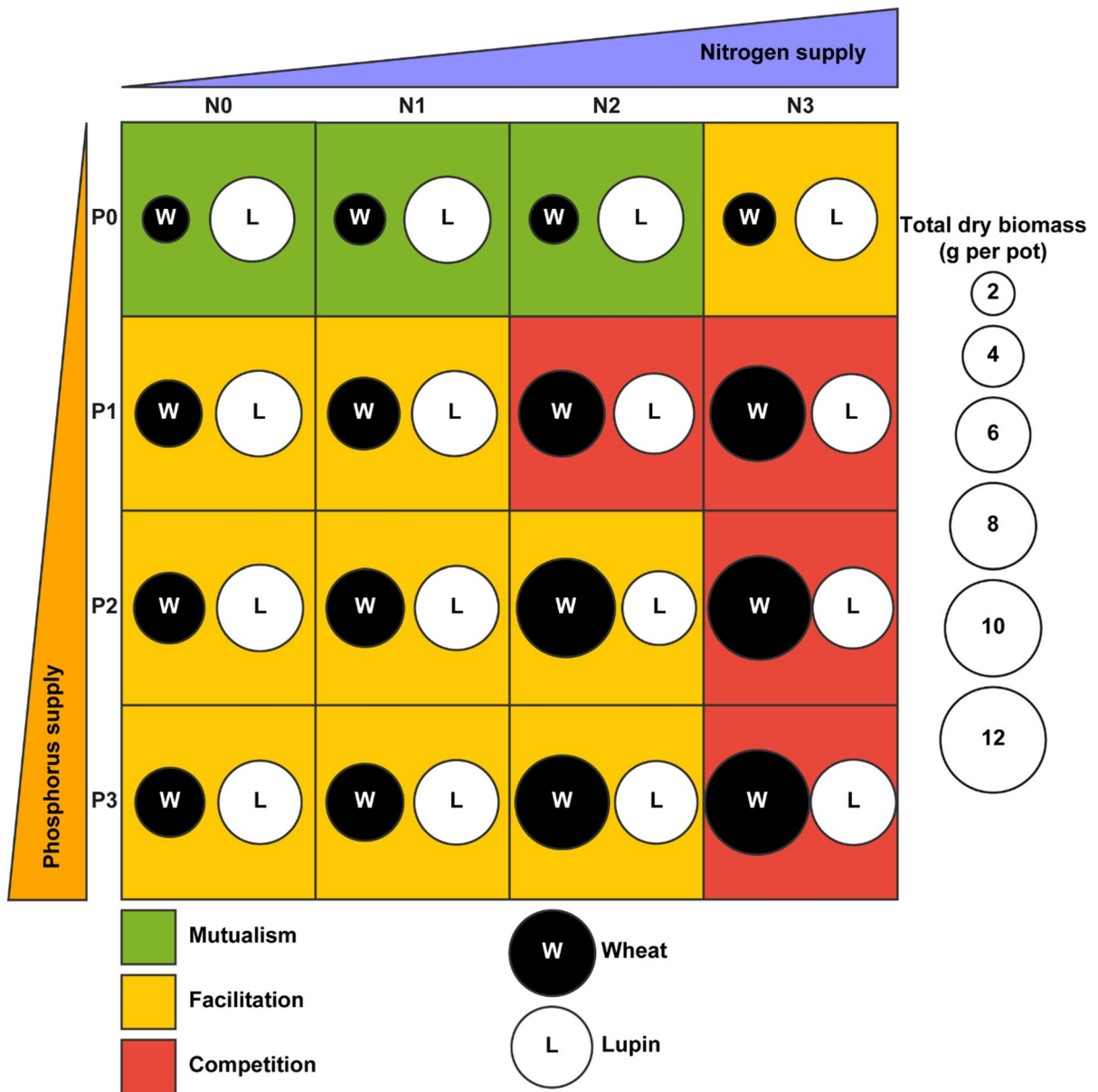
Fig. 5 Circle colors designate the plant species (black for wheat and white for lupin), and circle surface areas are proportional to the total biomass of each species in the intercropping treatment (in g of total dry biomass per pot). The background color corresponds with the nature of the interaction between wheat and lupin as defined by their *RII* (green for mutualism, yellow for facilitation of wheat by lupin, and red for competition)

($RII_{Wheat} > 0$ and $RII_{Lupin} > 0$), facilitation of wheat by lupin ($RII_{Wheat} > 0$ and $RII_{Lupin} = 0$), and antagonistic competition ($RII_{Wheat} > 0$ and $RII_{Lupin} < 0$).

The position along the nutrient gradients altered wheat-lupin interactions, from mutualism in the upper left corner of Fig. 5 – which corresponds to the most stressful conditions of our design – to antagonism at the bottom right corner of Fig. 5 – which corresponds to the least stressful conditions. More precisely, mutualistic interactions were observed in low-P treatments at all but the highest N supply (i.e. for N0P0, N1P0 and N2P0) while antagonistic interactions occurred in the N2P1 treatment and at high N treatments at all but the lowest P supply (i.e. for N3P1, N3P2 and N3P3).

All other N and P combinations led to facilitation of wheat by lupin.

When considering the total biomass of wheat and lupin in regard to the nature of wheat/lupin interactions, it is clear that positive interactions (i.e. mutualism) are associated with the lowest values of wheat biomass, and negative interactions (i.e. competition) are associated with high wheat biomass. As stated above, lupin biomass was much less affected by N and P supply than wheat biomass.

Shoot to root ratios

The wheat shoot to root ratio was significantly influenced by "N supply", "P supply" and "Cropping" treatments ($P < 0.01$, Eta$^2 = 0.05$, $P < 0.001$, Eta$^2 = 0.40$, $P < 0.001$, Eta$^2 = 0.09$, respectively; Table 3).

When grown as a sole crop, wheat exhibited a higher shoot to root ratio than when intercropped with lupin (Table S3, Fig. S1). In addition, although the shoot to root ratio increased with N addition, it decreased with increasing P supply. Taken together, the highest wheat shoot to root ratio was obtained in sole cropping at high N and low P supply, while low wheat shoot to root ratios were exhibited in intercropping at low N and high P supply. Cropping treatment was the only factor that significantly affected the lupin shoot to root ratio (for lupin root biomass included all belowground parts, namely non-cluster roots, cluster roots and nodules) ($P < 0.001$, Eta$^2 = 0.13$). Contrary to wheat, the shoot to root ratio of lupin was greater in intercropping.

Biomass of cluster roots and nodules

In lupin, the percentage of cluster roots relative to the biomass of belowground parts, namely roots, cluster roots and nodules, was significantly affected by P supply ($P < 0.001$, Eta$^2 = 0.14$, see Table 3). Tukey's range post hoc test showed a higher percentage of cluster roots in lupin grown at P0 than at other P levels (Table S3, Fig. 6). There was also a small effect of cropping treatment on the percentage of cluster roots ($P < 0.05$, Eta$^2 = 0.02$; Table 3). Tukey's range post hoc test showed a higher percentage of cluster roots in lupin grown in sole cropping than in intercropping (Table S3, Fig. 6).

The percentage of nodules relative to the biomass of belowground parts was significantly impacted by the interaction between "N supply" and "Cropping" ($P < 0.05$, Eta$^2 = 0.03$; Table 3). Tukey's range post hoc test indicated that the decrease of the percentage of nodules with the addition of N was sharper in sole cropping than in intercropping treatments (Table S3). Moreover, P addition positively affected the percentage of nodules ($P < 0.001$, Eta$^2 = 0.10$; Tables 3 and S3).

N-fixation

Percentages of N derived from the atmosphere in lupin were significantly impacted by each of the

Table 3 Three-way ANOVA and Eta-squared results for the shoot to root ratio of wheat and lupin, fraction of belowground biomass as cluster roots and nodules in lupin, and percentages of nitrogen (N) derived from atmosphere in lupin, with "N supply", "P supply" and "Cropping" treatments as factors

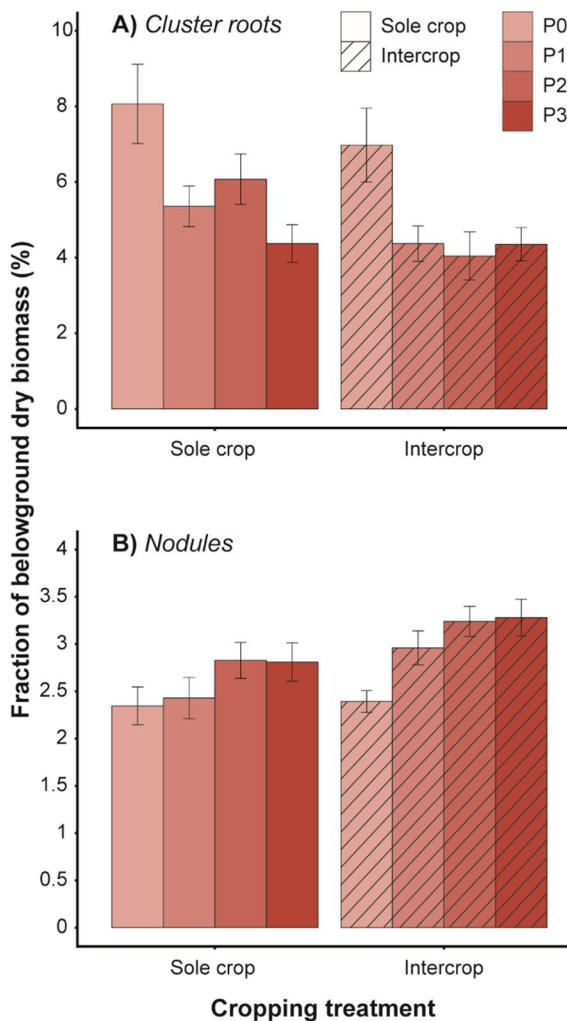|		Shoot to root biomass ratio				Fraction of belowground dry biomass (%)				Nitrogen derived from atmosphere (%)	
		Wheat		Lupin		Cluster roots		Nodules			
		P	Eta^2	P	Eta^2	P	Eta^2	P	Eta^2	P	Eta^2
Main effects	*N supply (N)*	**0.002**	0.05	0.76	<0.01	0.20	0.02	**<0.001**	0.28	**<0.001**	0.37
	P supply (P)	**<0.001**	0.40	0.13	0.03	**<0.001**	0.14	**<0.001**	0.10	**<0.001**	0.05
	Cropping Treatment (C)	**<0.001**	0.09	**<0.001**	0.13	**0.035**	0.02	**<0.001**	0.04	**<0.001**	0.23
Interactions	*N×P*	0.53	0.03	0.70	0.04	0.10	0.08	0.08	0.06	**<0.001**	0.06
	N×C	0.87	<0.01	0.34	0.02	0.12	0.03	**0.028**	0.03	**<0.001**	0.04
	P×C	0.71	<0.01	0.46	0.02	0.53	0.01	0.37	0.01	**0.024**	0.02
	N×P×C	0.62	0.02	0.71	0.04	0.71	0.03	0.42	0.03	0.07	0.03

Fig. 6 Fraction of belowground biomass as cluster roots (A) and nodules (B) in lupin. Vertical bars represent standard error. P0, P1, P2, P3 stand for the four levels of P supply

three two-way interactions between the three factors, namely "N supply", "P supply" and "Cropping" (Table 3, Fig. 7). While the percentage of nitrogen fixation decreased with the addition of N, this effect was stronger in lupins grown alone than when intercropped with wheat ($P < 0.001$, Eta$^2 = 0.04$ for N×C interaction; Table S3). In addition, the decrease in the percentage of nitrogen fixation with the addition of N was even more noticeable at low P supply ($P < 0.001$, Eta$^2 = 0.06$ for N×P interaction). Finally, while the percentage of nitrogen fixation was not significantly influenced by the addition of P in sole cropped lupins, it increased significantly in intercropped lupins

($P < 0.05$, Eta$^2 = 0.02$ for P×C interaction). Overall, the percentage of nitrogen fixation decreased with N addition, but increased with P addition and in the intercropping treatment (Table S3), explaining 37%, 5% and 23% of the variation in nitrogen fixation for "N supply", "P supply" and "Cropping" treatments, respectively, as shown by the analysis of main factors (Table 3).

Discussion

Benefits of intercropping

Intercropping of wheat with lupin always led to a substantial increase in total biomass, N content and P content compared to sole cropping. In addition, the increase in total biomass and N content was stable across the different combinations of N and P treatments, $RYM_{Biomass}$ and RYM_N not responding to N and P supply. This result is in line with the recent meta-analysis by Li et al. (2020), showing that intercropping can lead to large benefits under both low- and high-input conditions. The average value of $RYM_{Biomass} = 1.29$, corresponding to a global gain of 29% in biomass, is in line with other studies comparing cereals and legumes grown separately versus intercropped, and confirms the general observation that intercropping markedly enhances plant growth (Bedoussac et al. 2015; Li et al. 2020; Tilman 2020). Similarly, the 23% increase in N content matches results from previous studies showing a higher total N content in cereal-legume intercropping than in sole cropping for equivalent plant densities and land surface areas (Bedoussac and Justes 2010; Hauggaard-Nielsen et al. 2009; Nyfeler et al. 2011). Regarding RYM_P, the 34% global increase in P content observed in intercropping is consistent with previous studies (Dissanayaka et al. 2015; Li et al. 2001). For example, Li et al. (2018) found an average increase in P acquisition of 28.4% and 27.6% in intercropped faba bean/maize and chickpea/maize, respectively, compared with their monocultures. However, the P-application rate did not influence this increase in that study, whereas in the present study there was a strong effect of P supply on RYM_P; P0 led to a 39% increase in P content, while other P levels led to increases of about 25%. This result highlights a P threshold effect between P0 (soil Olsen P level: 6.7 mg P kg^{-1}) and
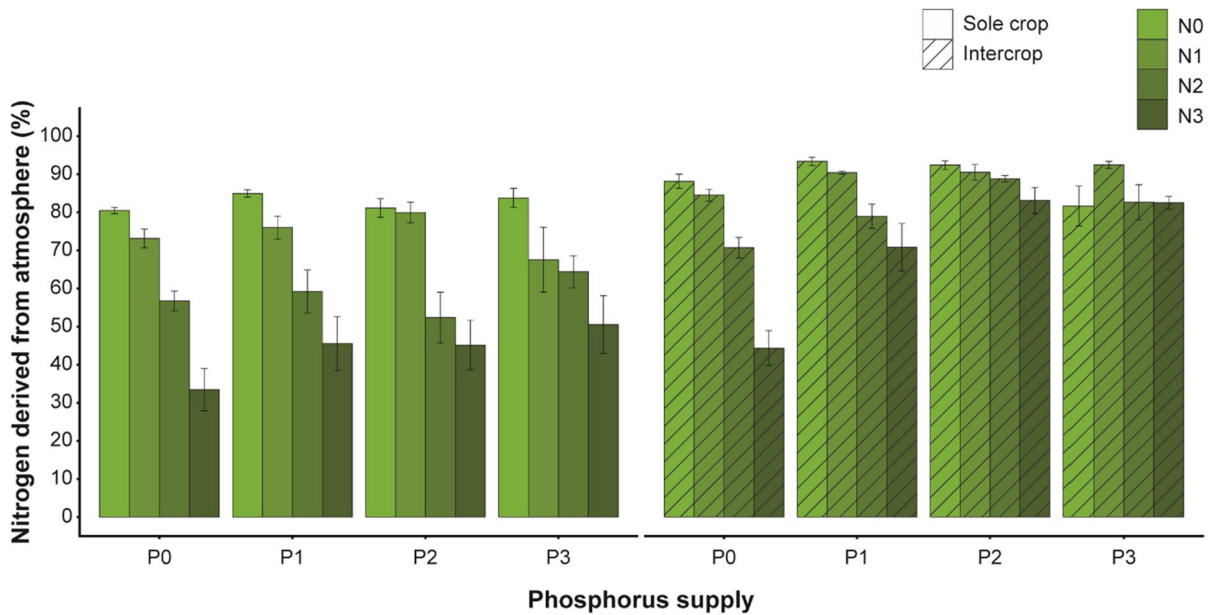
Fig. 7 Percentages of nitrogen (N) derived from the atmosphere in lupin. Vertical bars represent standard errors. N0, N1, N2, N3 stand for the four levels of N, and P0, P1, P2, P3 for the four levels of P

P1 (soil Olsen P level: 15.6 mg P kg^{-1}). This agrees with reports of critical soil P levels for wheat from 5 to 26 mg kg^{-1}, depending on soil type and climate (Khan et al. 2018). Below this threshold, wheat was very strongly affected by P deficiency, inhibiting its response to the addition of N.

Changes in wheat-lupin interactions

Although an overall positive effect of intercropping on total biomass was observed, this does not imply that the biomasses of wheat and lupin obtained in the intercrop treatment were greater for both species compared with the sole crop treatment. Cumulative biomass of both species was greater in the intercrop treatment, taking into account their relative individual densities. While *RYM* gives a good overall representation of the global effect of intercropping compared with sole cropping, it does not describe how each species is impacted, nor the magnitude of the change at the species level.

RII allows a better understanding at the species level. *RII*$_{Lupin}$ gives a measure of the effect that intercropping had on lupin biomass; it was significantly positive only below the P threshold. Above the P threshold, lupin was either not impacted (P1)

or negatively impacted by wheat (P2 and P3). Consequently, the higher the P supply, the more negative the effect of intercropping on lupin total biomass. In contrast, *RII*$_{Wheat}$ – though always positive – was significantly greater above the P threshold than below. Concerning the influence of N supply on wheat-lupin interaction, *RII*$_{Wheat}$ was always positive and did not significantly vary with N supply. For lupin, for all P supply levels combined, there was a negative effect of intercropping on lupin biomass at the highest N level (N3) only, while other levels led to zero *RII*$_{Lupin}$. Taken together, a low P supply led to mutualistic interactions, while a high N supply led to antagonistic interactions. Although intercropping led to an increase in total biomass in all scenarios, we observed a change in the type of interaction along the N and P gradients, with mutualism under low-input conditions, facilitation of wheat by lupin under intermediate conditions, and antagonistic relationships under high-input conditions (Fig. 5). This pattern is in agreement with the stress gradient hypothesis (Brooker et al. 2008; Callaway 2007). This phenomenon has been widely described for various types of abiotic stress gradients such as aridity, elevation, or temperature mostly in natural ecosystems (He et al. 2013). In contrast, it has been seldom studied

for N and P gradients in agroecosystems, and former studies have addressed N and P gradients separately: Tang et al. (2016) for instance did not validate the stress gradient hypothesis in a field experiment along the same P gradient as that tested in the present work. However, both theoretical and field studies have addressed the responses of plant communities to a switch from N to P limitation (Koffel et al. 2018; Laliberté et al. 2012).

Underlying ecophysiological mechanisms of wheat-lupin interactions

While the relative increases in total biomass and N content were stable when lupin and wheat were intercropped, the changes in wheat-lupin interactions indicate that there was a compensation in biomass, N content, and P content variation between the two species along the N and P gradients. Indeed, responses in biomass, N content and P content tended to be opposite for wheat and lupin when intercropped (Figs. 1, 2 and 3). This compensation mechanism has been identified in several intercropping studies (Jensen et al. 2020; Rao and Willey 1980) and is met "when the failure of one species is compensated by the other(s) because they differ in their sensitivity to abiotic stress", as described in a recent review by Justes et al. (2021).

In the present study, the lupin shoot to root ratio was not influenced by N and P supply. Instead, lupin adjusted the production of nodules and cluster roots in response to the various N and P combinations in our experimental setup. In contrast, wheat shoot to root ratio was strongly influenced by N and P treatments, decreasing with increasing N supply. This result was expected, as it is well known that most plants, including wheat, decrease their allocation to roots with increasing N availability (Dreccer et al. 2000; Duncan et al. 2018; Reynolds and D'Antonio 1996). However, the observation of the highest shoot to root ratio at the lowest P supply was unexpected. This underlines the strong limiting effect of the availability of P below the P threshold, in particular with regard to the production of root biomass. Indeed, we found that root biomass was particularly low at this level of P (data not shown). In addition, we observed a decreasing effect of intercropping on wheat shoot to root ratio, whereas it increased in intercropped lupin. These observations may reflect a competitive advantage of wheat over lupin in their ability to

exploit the soil volume, particularly in a pot experiment where the soil volume is limited. Indeed, while belowground biomass of intercropped lupin tended to slightly decrease with increasing N supply (non-significantly), wheat more than doubled its root biomass when grown with the legume (data not shown). This result is supported by many studies describing the advantage in belowground competition that non-legumes have over legumes in moderately N-limited conditions (Bessler et al. 2012; Brooker et al. 2015; Jensen 1996; Jensen et al. 2020; White et al. 2013), including in wheat-white lupin intercropping systems (Mariotti et al. 2009). This is reflected here for lupin as the decrease in soil N and P availability is promoting an increase in the production of nodules and symbiotic fixation, ultimately leading to a niche complementarity mechanism through the simultaneous exploitation of soil N by wheat and atmospheric N by lupin (Bedoussac et al. 2015; Justes et al. 2021). It is also interesting to note that N fixation and production of nodules were never totally suppressed, even at the highest supply of N, though N availability did not affect lupin total biomass in the sole crop treatment. This observation indicates that N availability was always limiting in our experiment, even at the highest N supply, and that lupin was able to overcome N limitation through N fixation.

Lupin was able to mitigate P limitation through the production of cluster roots, as their biomass, relative to total belowground biomass, was greater below the P threshold than above. Although there was a small negative effect of P limitation on lupin total biomass, it was far less pronounced for lupin than for wheat. Moreover, the higher percentage of cluster roots observed at P0 than at other P levels suggests that cluster-root production has been promoted below this threshold, in agreement with previous studies showing an increase in cluster-root formation in low-P conditions (Keerthisinghe et al. 1998; Shen et al. 2003). In particular, this may explain the mutual benefits of intercropping in wheat and lupin below the P threshold. Even though P availability was too low to allow wheat to compete with lupin, the cereal benefited from the legume without harming it (RII_{Wheat} and $RII_{Lupin} > 0$), and improved its P uptake as well as N uptake and total biomass. While numerous studies have shown facilitation from intercropping legumes in P-deficient soil, through their ability to acidify rhizosphere and release carboxylates and phosphatases (e.g.

Cu et al. 2005; Hinsinger et al. 2011; Li et al. 2016, 2003), our results also show a facilitative effect from wheat at low P availability, leading to a mutualistic interaction between the cereal and the legume.

Positive interactions or competition with positive consequences?

If, as we hypothesized, the increase in N fixation when lupin was intercropped with wheat was due to a more efficient exploitation of soil N by wheat, thus stimulating the positive "niche complementarity" effect, we may have to change the way we consider positive interactions. Lupin's ability to fix N and solubilize sparingly-available P is controlled by nodules and cluster roots, respectively, whose production and activity both depend on N and P availability. When lupin was grown as a sole crop, N and P availability were determined by the levels we chose. However, when lupin was grown in mixture with wheat, N and P availability were determined not only by the supply we chose, but also by the impact of wheat on N and P availability. In turn, wheat modified the perception lupin had of its own chemical environment, leading to more pronounced responses to N and P deficiency. This is in line with the view of Bedoussac et al. (2015), who define interactions in multi-species mixtures as "the effect of one species on the environment and the response of the other(s) species to this change".

In the present experiment, when grown together, lupin was always beneficial to wheat since interspecific competition was less harmful than intraspecific competition ($RII_{Wheat} > 0$). For lupin, growth with wheat was beneficial below the P threshold, because wheat growth was so limited by P availability that it could not compete with lupin ($RII_{Lupin} > 0$). Above the P threshold, and for a relatively low N availability, competition with wheat did not negatively affect lupin total biomass, because the legume compensated the drawbacks from being intercropped with wheat ($RII_{Lupin} = 0$). However, at high N supply, and above the P threshold, growth conditions allowed wheat to outcompete lupin, leading to a decrease in biomass of the lupin in comparison with sole cropping ($RII_{Lupin} < 0$). A simplistic explanation would be that lupin "helped" wheat to overcome N and P limitation through N fixation and increased P availability; however, it seems likely that the ability of lupin to "escape" the competition from wheat is most important. Therefore, the term "positive interaction" – which refers to niche complementarity for N and facilitation for P in our experiment – is misleading.

Reconciling ecological intensification and production

Wheat-lupin intercropping is a promising way to increase biomass production, particularly if wheat is the main crop. Whatever the supply of N and P, wheat produced more biomass in intercropping than in sole cropping treatments. However, when comparing the least- with the most-productive treatments in intercropping, wheat total biomass increased more than four-fold (from 1.05 to 5.60 g dry matter plant^{-1}). More precisely, biomass of wheat within mutualistic situations averaged 1.21 g dry matter plant^{-1}, with means of 2.90 and 4.91 g dry matter plant^{-1} obtained in the cases of facilitation and competition, respectively. Consequently, though interactions tended to become positive as nutrient stress increased, wheat biomass was severely impacted when N and P were too severely limiting. Overall, our results confirm the claim from Tilman (2020), who developed the idea that "intercropping can bring the benefits of intensification within a reasonable footprint". This idea is partly based on the meta-analysis by Li et al. (2020) which demonstrates that intercropping can produce large yield increase in highly-fertilized systems. One challenge could be to find a balance between the use of chemical inputs and a satisfactory productivity.

Conclusion

Nitrogen and P availability strongly influenced the nature of wheat-lupin interactions, changing from mutualism in low-input conditions to competition in high-input conditions. Despite this, our results show that intercropping always led to a significant increase of wheat biomass – even compensating the decrease of lupin biomass in competition scenarios – and to a better exploitation of N and P as a result of the ability of lupin to fix atmospheric N and increase P availability depending on N and P availability. Therefore, the intensification of ecological interactions through intercropping of legumes and cereals – even in highly fertilized situations – offers potential for sustainable crop production. There are

obvious limitations to the present experiment in pots. For example, the root systems of both crops might not have interacted in the same way in the field as they did in our pots. Moreover, we only considered the vegetative stage of growth of wheat and lupin, while grain yield and N content are the main targets for farmers. Future studies carried out with a similar, combined N and P gradient design, in field conditions, with various crops and over the whole growth cycle would be a step forward in the design of ecologically intensive intercropping systems.

Authors' contributions Simon Boudsocq, Philippe Hinsinger and Hans Lambers contributed to the study conception and design. Material preparation, data collection and analysis were performed by Simon Boudsocq and Camille Cros. The first draft of the manuscript was written by Simon Boudsocq and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflicts of interest/Competing interests The authors have no relevant financial or non-financial interests to disclose.

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