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Quantification of Biologically Fixed Nitrogen by White Lupin (*Lupins albus* L.) and Its Subsequent Uptake by Winter Wheat Using the ^{15}N Isotope Dilution Method

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Abstract: A field experiment was carried out in 2016–2018 in a white lupin (*Lupinus albus* L.)-winter wheat (*Triticum aestivum* cv. 'Bogatka') crop rotation. The aim of this study was to determine the amount of nitrogen (N) that was biologically fixed by the white lupin crop in the first year of the rotation and to estimate how much of this N was then taken up from the lupin residues by winter wheat in the second and third years of the rotation. Biologically fixed N was determined by the isotope-dilution method ($ID^{15}N$) by applying 30 kg N ha^{-1} of ^{15}N-labeled fertilizer $(^{15}NH_4)_2SO_4$ (containing 20.1 at.% ^{15}N) to the white lupin and the reference plant spring wheat. The yields of white lupin seeds and crop residues were 3.92 t ha^{-1} and 4.30 t ha^{-1}, respectively. The total amount of N in the white lupin biomass was 243.2 kg ha^{-1}, which included 209.3 kg ha^{-1} in the seeds and 33.9 kg ha^{-1} in the residues. The ^{15}N-labeled residue of white lupin was cut and ploughed into soil. Our results indicate that 111.2 kg N ha^{-1} was fixed from the atmosphere by the lupin plants, with 93.7 kg ha^{-1} found in the seeds and 17.5 kg ha^{-1} in the residues. In the second and third years of the rotation when winter wheat was cultivated, the plots were divided into two groups of subplots (1) without N-fertilization (control) and (2) with an application of 100 kg N ha^{-1}. In the first year of winter wheat cultivation, 20.0% and 21.0% of N from the crop residues was taken up by the control and N-fertilization plots, respectively, while in the second year, uptake was lower at 7.12% and 9.27% in the control and N-fertilized plots, respectively.

Keywords: ^{15}N isotope; leguminous; biologic nitrogen fixation; crop rotation; post-crop

1. Introduction

An increasing global population will require more products from animal and plant production. The cultivation of leguminous plants could help in the delivery of plant protein, as well as enhancing biodiversity in the crop rotation [1]. These plants can be cultivated in both organic and sustainable production systems [2]. Leguminous plants, such as white lupin (*Lupinus albus* L.), yellow lupin (*Lupinus luteus* L.) and narrow-leafed lupin (*Lupinus angustifolius* L.) are native European plants and could provide an excellent source of plant protein [3,4]. Lupins grow in almost all European countries, although the total area under cultivation remains modest and yields are highly variable. A slight increase in cultivation area was recorded in the period 2000–2013 [5]. Leguminous plants are also very important in plant production because they have the capacity to biologically fix molecular nitrogen (N_2) from the atmosphere, thereby increasing the concentration in the soil of one the most important plant nutrients. As such, it is then possible to decrease the application rates of N in inorganic fertilizers and thereby reduce potential environmental pollution. In many cases, the introduction to the soil–plant system of additional amounts of N causes changes in plant-biomass yield, N-accumulation in the seeds and plant residues, the nutritional value of the seeds and the amount of biologically fixed N [6–8]. It should be emphasized that when studies presented results from biological N-fixation, it is mainly the aboveground parts of the leguminous plant that have been used to calculate the N balance. Indeed, Mayer et al. [9], Wichern et al. [10] and Fustec et al. [11] have reported that the belowground parts of legumes may account for up to 35–45% of the total N accumulated in the residual parts of the plants.

Decomposition of legume plant residues in the soil delivers available forms of N for plants and soil microorganisms, which leads to competition and acceleration of the biological sorption of N (immobilization process). The interaction process between organic residues and soil organic matter mineralization determines the amount of available N for the subsequent plant, usually a cereal, in the crop rotation. The amount of N fixed depends on the species of legume. Work by Unkovich et al. [12] that examined the quantities of N_2 fixed per unit area revealed that the principal crop legumes were (ranked in descending order) soybean, lupin, field pea, faba bean, common bean, lentil and chickpea. In European conditions, the most widely cultivated grain legumes, such as lupin, faba bean and field pea are able to accumulate (from biologic fixation) an average of 130–153 kg N ha^{-1} in their aboveground biomass [13]. In addition, a substantial quantity of N may accumulate in the belowground biomass of a legume, mainly in the nodules and roots, which can account for up to 30–60% of the content. Carranca et al. [14] reported that 7–11% of total N was associated with the roots and nodules and allocated 11–14 fixed N kg t^{-1} belowground dry matter, which represents half the amount contained in the aboveground plant.

The isotope-dilution method with ^{15}N can be very useful in determining the amount of N that is biologically fixed by leguminous plants and the uptake of this fixed N by subsequent plants from the crop rotation [15–18]. However, most studies that have applied this method have focused on the aboveground parts of the legumes. Therefore, the aim of this study was to determine the amounts of biologically fixed N taken up by white lupin (*Lupinus albus* L.) in the first year of a 3-year crop rotation and percentage utilization of the fixed N by winter wheat (*Triticum vulgare*) in the second and third years of the rotation.

2. Materials and Methods

2.1. Site Description

The field experiment was conducted in 2016–2018 at the research station in the Wielkopolska region, Złotniki, Poland (52°29′ N, 16°49′ E) on a soil developed from sandy loam having the following granulometric composition: granulometric fraction above 2 mm, 2.20%; sand fraction 2.0–0.05 mm, 73%; clay fraction 0.05–0.002 mm, 22%; silt fraction ≤0.002 mm, 5%. The pH of the soil was 6.1 (in 1 mol dm^{-3} KCl). The carbon (C) content of the organic compounds was 5.24 g kg^{-1}, which equated to 9.01 g kg^{-1} of the soil organic matter. Total N-content in the soil was 0.572 g kg^{-1}, plant available phosphorus (P) was 15.0 mg kg^{-1} soil, potassium (K) was 31.5 mg kg^{-1} soil and magnesium (Mg) was 75.0 mg kg^{-1} soil.

The weather conditions in Poland are suitable for the cultivation of most cereal and legume species, which require 500–600 mm annual precipitation. Weather conditions during the growing season in the years of study (2016–2018) are presented in Table 1.

Table 1. Mean monthly air temperature (°C) and rainfall (mm) sum in 2016–2018 and long-term average (recorded at the agrometeorological observatory in Złotniki, Poland).

Year /Month	Mean Monthly Air Temperature (°C)												$\bar{x}$
	J	F	M	A	M	J	J	A	S	O	N	D	
2016	−1.9	3.4	3.7	8.6	15.4	18.3	18.8	17.5	16.5	8.0	2.9	1.7	9.4
2017	−2.2	0.4	6.2	7.3	13.7	17.4	18.0	18.9	13.3	10.6	5.1	2.6	9.3
2018	1.8	−3.0	0.6	12.9	16.8	18.5	20.1	21.4	15.8	11.0	5.1	2.5	10.3
1951–2015	−1.2	−0.2	3.5	8.8	14.3	17.5	19.3	18.6	13.9	9.1	3.9	0.2	15.6
	Monthly Rainfall Sum (mm)												Σ
2016	31.6	36.8	49.0	37.4	43.0	83.6	149	40.6	5.6	105	47.8	42.6	672
2017	17.7	18.4	45.4	40.6	56.8	68.2	168	82.0	45.6	91.8	50.0	33.8	720
2018	44.6	5.0	22.6	36.2	17.4	25.4	70.5	11.6	44.2	24.8	11.4	46.2	360
1951–2015	31.5	27.7	31.7	31.0	50.5	59.4	77.2	55.4	45.2	34.1	35.6	38.9	518

2.2. Experiment Design and Agronomic Management

The field experiment was established in the following crop rotation: white lupin (*L. albus* L. cv. 'Butan'), winter wheat (*T. aestivum* cv. 'Bogatka') and winter wheat (*T. aestivum* cv. 'Bogatka'). Spring wheat (*T. aestivum* cv. 'Jarlanka') was cultivated simultaneously with white lupin as a reference crop. The use of the same crop (winter wheat) species in the second and third years of the rotation enabled a comparison of the effects of leguminous crop residues on the yield of the subsequent plant. During the growth and development stages of the studied plants, agricultural practices were carried out in accordance with established management principles. White lupin and spring wheat were sown on 29 March 2016; both crops were harvested 19 August 2016. After harvest, crop residues were cut off and ploughed in to 25-cm deep. Winter wheat was sown after white lupin on 29 September 2016, then harvested on 27 July 2017. Winter wheat was sown again, as the second subsequent plant, on 17 October 2017 and harvested on 24 July 2018. The crop rotation in 2016–2018 is shown in Table 2.

Table 2. Crop rotation in 2016–2018.

Crop Rotation (Harvest Year)		
2016	**2017**	**2018**
white lupin + spring winter (reference plant)	winter wheat	winter wheat

The field experiment was carried out in three plots of 9 m^2. Sowing and harvest dates depended on species and weather conditions. The recommended sowing rates used were: 100 seeds m^{-2} for lupin and 400 grains m^{-2} for the cereals. During the growing season, recommended pesticides were used for particular target species. In each year of the experiment, the plants in the study plots were harvested at the mature stage, and the following were then separated: for white lupin—seeds and crop residues (roots, stems, leaves and deseeded pods) and for spring and winter wheat—grains and crop residues (roots, stems, leaves and husks). After the emergence of white lupin in 2016 the 4 m^2 plots were established. Here, ^{15}N-labelled fertilizer, in the form of ammonium sulfate $(^{15}NH_4)_2SO_4$ solution (containing 20.1 at.% ^{15}N), was applied at a rate of 30 kg N ha^{-1}. This was also carried out on the spring wheat plots (as the reference plant) that were sown in the spring of 2016. A reference crop is a plant that does not biologically fix nitrogen.

The experiment was conducted according to following scheme (Figure 1a–c). Plot, where white lupin was sown in 2016 had size 3 m × 3 m = 9 m^2. In year 2016 during vegetation of white lupin on smaller area 2 m × 2 m = 4 m^2 the ^{15}N-labeled fertilizer was applied (leaving 0.5 m around whole plot as unfertilized area). White lupin was sown on 9 m^2 but harvested from area of 4 m^2 where the

^{15}N-labeled fertilizer was applied. In autumn 2016 winter wheat was sown on whole area of 9 m^2. In spring 2017 this plot was divided for two subplots with equal area (9 m^2 / 2 = 4.5 m^2). Then, on the first subplot, the dose 100 kg N ha^{-1} was applied, and the second part was without nitrogen fertilizer (control). Winter wheat was harvested in 2017 from area 2 m^2 from each subplot, where in 2016 the ^{15}N-labeled fertilizer was applied. In autumn 2017, the winter wheat was sown again on whole area of 9 m^2. In spring 2018 this plot was again divided for two subplots with equal area (4.5 m^2). On the first subplot the dose 100 kg N ha^{-1} was applied, and the second part was without nitrogen fertilizer (control). Winter wheat was harvested in 2018 from area 2 m^2 from each subplot, where in 2016 the ^{15}N-labeled fertilizer was applied.

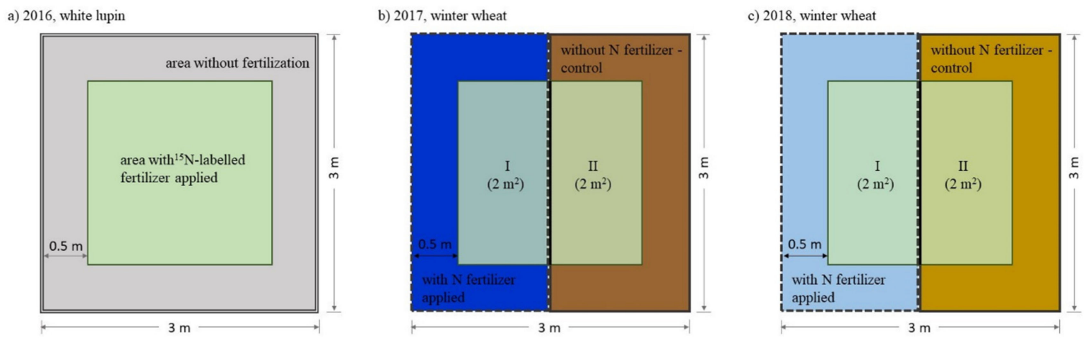

Figure 1. Scheme of plots experiment in each year. (**a**) 2016: in spring white lupin was sown on a plot of 9 m^2 (square with double line); on green area (2 m × 2 m = 4 m^2) ^{15}N-labeled fertilizer was applied during the white lupin vegetation season. In the experiment the ^{15}N-labeled fertilizer, $(^{15}NH_4)_2SO_4$, using a dosage of 30 kg N ha^{-1} was applied; gray area—area without fertilization. White lupin was next harvested only from the green area. In autumn 2016 whole area (9 m^2) was sown by winter wheat; (**b**) 2017: in spring, the original plot (9 m^2) was divided into two subplots with equal area (4.5 m^2). On the first subplot (blue area, dashed line), a dose of 100 kg N ha^{-1} of N fertilizer was applied; on the second subplot (brown area, solid line), N-fertilizer was not used (control). Winter wheat was then harvested from each subplot from a 2 m^2 area (marked as I and II), where in 2016 the ^{15}N-labeled fertilizer was applied. In autumn 2017, the whole area (9 m^2) was sown with winter wheat; (**c**) 2018: in spring, the original plot (9 m^2) was divided into two subplots with equal area (4.5 m^2)—the same as previous year. On the first subplot (light blue area, dashed line), a dose of 100 kg N ha^{-1} of N fertilizer was applied; on the second subplot (light brown area, solid line), N-fertilizer was not used (control). Winter wheat was then harvested from each subplot from a 2 m^2 area (marked as I and II), where in 2016 the ^{15}N-labeled fertilizer was applied.

From an agricultural perspective, an important factor in the crop rotation and fertilization system is the total amount of N that is contained in the crop residues, as well as its availability to subsequent plants. The application of the ^{15}N isotope in a study examining the process of biologic N-fixation enables a full determination of the amount of N that is fixed by the white lupin crop during the growing season and an evaluation of the amount of N remaining in the crop residues of these plants when they are introduced into the soil.

2.3. Chemical and Isotopic Analysis

After the harvesting of the crops, plant samples were collected and ground to a particle diameter size of <0.15 mm. Total N and organic C contents were determined using a PerkinElmer CHNS/O elemental analyzer (PerkinElmer Series RI 2400, Whaltham, MA, USA).

Measurements of $^{13}C/^{12}C$ and $^{15}N/^{14}N$ isotopic ratios in the plant samples were performed in the Stable Isotope Laboratory, Institute of Geological Sciences Polish Academy of Sciences with a Flash 1112HT elemental analyzer coupled to a Delta V Advantage IRMS (both: Thermo Scientific) in continuous flow mode. Samples were wrapped in tin capsules and combusted at 1020 °C. The obtained carbon dioxide (CO_2) and N_2 were purified on a water trap and separated on a gas chromatograph column. Subsequently, CO_2 and N_2 were transferred sequentially to an isotope ratio mass spectrometer where their isotopic composition was measured. The measurement results were normalized to three international standards USGS 40, USGS 41 and IAEA 600. The reproducibility of standards analysis was generally better than ±0.1‰ for $\delta^{13}C$ and ±0.3‰ for $\delta^{15}N$.

2.4. Calculations

The obtained results (yield and chemical analyses) provided the basis for the calculation of analyzed parameters, according the formulas provided by IAEA [15] using the following equations:

(1) %N derived from atmosphere

% Ndfa = (1 − at% ^{15}Nexcess in white lupin/at% ^{15}Nexcess in spring wheat) × 100

(2) Amount of N fixed by white lupin from atmosphere (kg ha^{-1})

N fixed from atmosphere (kg ha^{-1}) = (% Ndfa × TN)/100
where: TN—total amount of N in white lupin (kg ha^{-1})

(3) % N derived from fertilizer

% Ndff = (at% ^{15}Nexcess in plant/at% ^{15}Nexcess in fertilizer) × 100

(4) Amount of N derived from fertilizer (kg ha^{-1})

Ndff (kg ha^{-1}) = (% Ndff × TN)/100

(5) % N derived from soil

% Nfs = 100 − (% Ndfa +% Ndff)

(6) Coefficient of N-utilization (N-use efficiency) from fertilizer

NUE (%) = (Ndff kg ha^{-1}/D kg ha^{-1}) × 100
where: D—dose of nitrogen applied in nitrogen fertilizer

(7) % N in the winter wheat from white lupin residue

% Ndf_R = (at% ^{15}Nexcess in winter wheat/at% ^{15}Nexcess in white lupin residue) × 100

(8) % N derived from residue

% Ndf_R = (at% ^{15}Nexcess in winter wheat × TN in winter wheat)/(at% ^{15}Nexcess in white lupin residues × TN in white lupin residues)

(9) Amount of N derived from residue (kg ha^{-1}) = (% Ndf_R in wheat × TN in winter wheat/N in residues kg ha^{-1}) × 100

(10) Coefficient of nitrogen utilization by winter wheat from crop residue of white lupin (%) =

(Amount of N derived from residue kg ha^{-1}/Amount of N in white lupin residue kg ha^{-1}) × 100

(11) Total amount of ^{15}N in biomass of lupin and winter wheat (kg ha^{-1})

^{15}N (kg ha^{-1}) = (at% ^{15}Nexcess in plant × TN in plant)/100

(12) % of ^{15}N uptake = (^{15}N kg ha^{-1} in plant/^{15}N kg ha^{-1} in fertilizer) × 100.

2.5. Statistical Analysis

The experiment design for white lupin was established as randomized complete blocks. The results for winter wheat were statistically analyzed using one-way ANOVA. Significant differences were determined using Tukey's test at a significance level of $p < 0.05$. For significant differences, simple regression equations and correlation coefficients between selected traits were determined using the Statistica PL 12 software (Statsoft 2019, TIBCO Software, Palo Alto, CA, USA).

3. Results and Discussion

During the study period, mean air temperatures were similar in 2016 and 2017, but were 1 °C higher in 2018 (Table 1). Annual precipitation differed considerably between the years of the study: it was highest in 2017 and lowest in 2018. The weather conditions significantly influenced the yield of white lupin. A rainfall deficit, combined with high temperatures, has been shown to be particularly unfavorable during flowering and pod formation, resulting in the shedding of flowers and pod setting and consequently reducing the seed yield [19].

As a consequence of the thermal and rainfall conditions in the 2016 growing season (May–August), the yields of white lupin seeds and crop residues were high; 3.92 t ha^{-1} and 4.30 t ha^{-1}, respectively, accounting for 47.7% and 52.3% of total biomass, respectively (Table 3). The ratio of seeds to crop residues was 1:1.09. White lupin requires grain yield improvements to realize its potential as a high-protein grain crop [4]. As white lupin is a N_2 fixing legume, it can also play a role in enhancing soil fertility [20]. Total N-content in the crop residues was 0.79%, and the mean weighed value for N-content in total white lupin biomass was 2.95% (Table 3). On the basis of these values, the total N-content in the biomass of white lupin was estimated at 243.2 kg ha^{-1}, which included 209.3 kg ha^{-1} in the seeds, which was removed from the field (soil–plant system). The amount of N in the crop residues was 33.9 kg ha^{-1} and this was introduced into the soil because after harvest the crop residues were cut off and ploughed up to 25 cm deep as potential source of N for the winter wheat crop. Of course, the amount of N that will be available for the winter wheat crop will depend upon the turnover rates of N in the soil, which are driven by immobilization—mineralization processes.

Table 3. Parameters of white lupin and spring wheat (reference plant) harvested in 2016.

Specification	Seeds/ Grain	Crop Residues	Sum/Mean Weighted *
White lupin			
Yield (t ha^{-1})	3.92	4.30	8.22
Total nitrogen content (%)	5.34	0.79	2.95 *
Total nitrogen content in biomass (kg ha^{-1})	209.3	33.9	243.2
Atomic-enrichment percentage (at% ^{15}Nexcess)	1.071	0.972	1.058 *
Nitrogen fixed from atmosphere (kg ha^{-1})	93.7 (44.8%) **	17.5 (51.7%) **	111.2
Nitrogen uptake from $(^{15}NH_4)_2SO_4$ (kg ha^{-1})	11.3 (5.42%) **	1.7 (5.01%) **	13.0
Nitrogen uptake from the soil (kg ha^{-1})	104.2 (49.8%) **	15.2 (43.3%) **	119.4
Spring wheat (reference plant)			
Yield (t ha^{-1})	1.24	2.93	4.17
Total nitrogen content (%)	2.30	0.65	1.14 *
Atomic-enrichment percentage (at% ^{15}Nexcess)	1.940	2.014	1.956 *

* mean weighted; ** percentage value in brackets represent amount of total nitrogen in plants.

The use of the ^{15}N-isotope-dilution method enabled a full observation and determination of the amount and pathways of the process of biological fixation of molecular N from the atmosphere and soil air by leguminous plants, as a result of the mutual symbiosis between plants and *Bradyrhizobium* bacteria, as well as the amount of N taken up by the leguminous plants from the soil and the fertilizers containing the ^{15}N isotope [21]. In this study, the at% 15Nexcess was found to be 1.071 in the seeds and 0.972 in the crop residues. In the biomass of spring wheat (reference plant) the at% 15N excess was 1.940 in the grain and 2.014 in the crop residues. These values provide the basis to calculate the amount of N fixed from the atmosphere by white lupin. Mayer et al. [9] reported that white lupin derived 89%

of its N from the atmosphere. Hence, the low value of% Ndfa in our experiment is probably caused by the high content of N-NO_3 (17 mg kg^{-1} of soil) which inhibited the activity of the nitrogenase. High content of NO_3^- in soil inhibited activity of nitrogenase.

The results presented in Table 3 show that 44.8% of the total N-content in the white lupin seeds and 51.7% of the total N in the white lupin crop residues was fixed from the atmosphere, which equates to 93.7 and 17.5 kg N ha^{-1}, respectively and a total of 111.2 kg N ha^{-1}. This value is very important for N-utilization and uptake by plants cultivated in crop rotations, although this value can vary considerably in practice and is mainly dependent on the environmental conditions in which the legume plants are cultivated [5]. It should be emphasized that the amount of biologically fixed N determined above is calculated only for the aboveground part of the white lupin crop.

In our study, a secondary source of N for white lupin was the inorganic fertilizer, $(^{15}NH_4)_2SO_4$, enriched with the ^{15}N isotope. Pampana et al. [22] studied N-fixation in four legume species (lupin, chickpea, bean, pea) and, depending on the type of N-fertilization, found that species and the rate of N application were critical factors in determining symbiotic N_2—fixation responses to N-fertilization. In our study, the amount of inorganic N taken up from fertilizer by white lupin and accumulated in the seeds and residues was 11.3 kg ha^{-1} (5.42%) and 1.7 kg ha^{-1} (5.01%), respectively (Table 3). The sum of N utilized from $(^{15}NH_4)_2SO_4$ by white lupin was 13.0 kg ha^{-1} (Table 3). A significant amount of the N in the white lupin biomass was derived from the soil (as a third source of N); 104.2 kg ha^{-1} (49.8%) in the seeds and 15.2 kg ha^{-1} (43.3%) in the residues. This means that the greatest amount of N was taken up by white lupin from the soil.

The grain yield of spring wheat (reference plant) was 1.24 t ha^{-1} and the crop residues 2.93 t ha^{-1}. In biomass was found ^{15}N (at% ^{15}Nexcess) 1.940 in grain and 2.014 in crop residue. Moreover, there was noticed the total nitrogen content 2.30% and 0.65%, respectively.

An additional aim of this study was to determine the effects of white lupin residues on the subsequent crop in the rotation (i.e., winter wheat). In practice, winter wheat is often cultivated in a monoculture, which can decrease its yield [8,23]. According to Rahimizadeh et al. [24], the forecrop affects the N-use efficiency, N-uptake efficiency, N-utilization efficiency, N harvest index and the grain protein content of wheat.

The biomass of legume plants partly contains biologically fixed N, which is normally taken up by the subsequent crop in the rotation after turnover in soil. Therefore, legume crop residues can supply more mineral forms of N to the subsequent crop than the residues of cereals (due to their relatively higher content of N and lower C:N ratio compared to cereal residues). Decomposition of legume residues in the soil increases the solubilization of insoluble P compounds, soil microbial activity and the replenishment of soil organic matter. Kumar and Goh [25] observed that the significantly lower wheat grain yields obtained from non-leguminous rather than leguminous residues were related to N additions; residue-N added from ryegrass and wheat residues (64 and 72 kg N ha^{-1}) were lower than those provided by white clover and field pea residues (223 and 141 kg N ha^{-1}).

In 2017, the yield of winter wheat was 4.31 t ha^{-1} grain and 7.58 t ha^{-1} of crop residues in the control subplots and 6.31 t ha^{-1} grain and 8.42 t ha^{-1} of crop residues in the N-fertilization subplots (Table 4). Therefore, the N applied as inorganic fertilizer increased grain and crop residue yields by 2.0 t ha^{-1} and 0.8 t ha^{-1}, respectively. This means that 1 kg N ha^{-1} increased the yield of grain by 20 kg ha^{-1}. The application of N in the inorganic fertilizer also resulted in an increase in total N-content in the grain from 1.5% to 1.9% (a 26.5% increase), with no changes in the N-content in the crop residues. The additional application of N resulted in substantial differences in the total amount of N accumulated in the winter wheat biomass; 77.7 kg N ha^{-1} in the control subplots and 132.5 kg ha^{-1} in the N-fertilized subplots. The N-utilization coefficient of the inorganic fertilizer showed that the N mainly accumulated in the grain (54.1%), with only 0.7% in the crop residues, to give a total of 54.8% (Table 4). The utilization coefficient value is a very important factor in the application of N in inorganic fertilizers; a high value is indicative of the effectiveness of the applied N. This value can range from 30 to 80%, is very changeable and is influenced by the conditions of wheat cultivation, the application rates of N fertilizer and the type of crop residues.

Table 4. Parameters of winter wheat harvested in 2017.

Specification	Nitrogen Dose kg ha^{-1}	Grain	Crop Residues	Sum/Mean Weighted *
Yield (t ha^{-1})	0 100	4.31 ^{b} 6.31 ^{a}	7.58 ^{b} 8.42 ^{a}	11.89 ^{b} 14.73 ^{a}
Effect of fertilization 100 kg ha^{-1} N		+2.0	+ 0.8	+2.8
Total nitrogen content (%)	0 100	1.47 ^{b} 1.89 ^{a}	0.19 ^{a} 0.18 ^{a}	0.65 ^{b,*} 0.90 ^{a,*}
Nitrogen in winter wheat biomass (kg ha^{-1})	0 100	63.3 ^{b} 117.4 ^{a}	14.4 ^{a} 15.1 ^{a}	77.7 ^{b} 132.5 ^{a}
Coefficient of nitrogen utilization (%)		54.1	0.7	54.8
Atomic-enrichment percentage (at% ^{15}Nexcess)	0 100	0.256 ^{a} 0.149 ^{a}	0.232 ^{a} 0.193 ^{a}	2.240 ^{a,*} 0.171 ^{a,*}
Nitrogen content in winter wheat derived from white lupin residues (%)	0 100	26.3 ^{b} 15.3 ^{a}	23.8 ^{b} 19.8 ^{a}	25.0 ^{b,*} 17.6 ^{a,*}
Nitrogen in winter wheat derived from white lupin residues (kg ha^{-1})	0 100	16.6 ^{b} 18.0 ^{a}	3.4 ^{b} 3.0 ^{a}	20.0 ^{b} 21.0 ^{a}
Coefficient of nitrogen utilization by winter wheat from white lupin residues (%)	0 100	48.9 ^{b} 53.1 ^{a}	10.1 ^{b} 8.8 ^{a}	59.0 ^{b} 61.9 ^{a}

* mean weighted; ^{a,b} averages with different letters in the columns are significantly different ($p \leq 0.05$).

The use of the isotope-dilution method allows the percentage of N in the subsequent crop in the rotation (cultivated after legumes) to be calculated [15]. The N-content in the winter wheat crop (derived from the white lupin residues) was 25.0% and 17.6% in the control and N-fertilized subplots, respectively (equivalent to 20.0 and 21.0 kg N ha^{-1}) (Table 4). In the control subplots, the amount of N taken up from the soil by the winter wheat crop was estimated at 57.7 kg ha^{-1} (total amount of N (77.7 kg ha^{-1})—amount of N (20.0 kg ha^{-1}) from white lupin residues). In the N-fertilized subplots, the amount of N taken up from the soil by winter wheat was estimated at 111.5 kg ha^{-1} (total amount of N (132.5 kg ha^{-1})—amount of N (21.0 kg ha^{-1}) from white lupin residues and inorganic fertilizer). It should be noted that the N-utilization coefficient for winter wheat for the white lupin residues was 59.0% in the control and 61.9% in the N-fertilized subplots (Table 4). These results are similar to other studies, e.g., according to Rutkowska and Pikuła [26], the percentage of N derived from fertilizers was significantly greater in wheat (56%) compared to field pea (15–17%).

In 2018, the yield of winter wheat was 3.10 t ha^{-1} of grain and 7.46 t ha^{-1} of crop residues, with a total biomass yield of 10.56 t ha^{-1} in the control subplots (Table 5). In contrast, the yields in the N-fertilized subplots were 4.20, 8.20 and 12.40 t ha^{-1}, respectively (Table 5). Differences between treatments were significant. In 2018, yields in the control subplots were 28% lower for grain, 1.32% lower for crop residues and 10.9% lower for total yield compared to the yields in 2017 (Tables 4 and 5), and yields were lower by 33.3% (grain), 23.8% (crop residues) and 15.6% (total yield) in the N-fertilized subplots. The reason for the decrease in winter wheat yield is probably due to the amount of rainfall during the growing season (May–July) in both years: 689 and 149.5 mm, respectively. Many authors have reported substantial changes in the yield of winter wheat caused by differences in fertilization rates, climatic conditions and crop rotation [23,27–29]. Total N in the winter wheat harvested in 2018 amounted to 93.2 kg ha^{-1} in the control subplots and 146.6 kg in the N-fertilized subplots. In both treatments, more N accumulated in the grains than in the crop residues. Of importance to our investigation with ^{15}N, is the enrichment of the biomass, which in this case was much lower than in the previous year but did not differ between treatments (control and N-fertilized subplots). Based on the mean weighted value (Table 5), the amount of N taken up from the white lupin residues in the control subplots was 6.99% but was 6.22% in the N-fertilized subplots, which equates to 7.12 kg N ha^{-1} in the control subplots and 9.27 kg ha^{-1} in the N-fertilized subplots (Table 5). In the total biomass, 21.0 and 27.2% of N was derived from white lupin residues in the control and N-fertilized subplots, respectively (Table 5). In an experiment conducted by Mayer et al. [9], the N derived from the residues of white lupin in the subsequent winter wheat crop constituted 31% of total N.

Table 5. Parameters of winter wheat harvested in 2018.

Specification	Nitrogen Dose kg ha^{-1}	Grain	Crop Residues	Sum/Mean Weighted *
Yield (t ha^{-1})	0 100	3.10 ^{b} 4.20 ^{a}	7.46 ^{b} 8.20 ^{a}	10.56 ^{b} 12.40 ^{a}
Effect of fertilization 100 kg ha^{-1} N		+1.10	+0.74	+1.84
Total nitrogen content (%)	0 100	1.71 ^{b} 1.97 ^{a}	0.54 ^{b} 0.78 ^{a}	0.88 ^{b,*} 1.18 ^{a,*}
Nitrogen in winter wheat biomass (kg ha^{-1})	0 100	53.0 ^{b} 82.7 ^{a}	40.2 ^{b} 63.9 ^{a}	93.2 ^{b} 146.6 ^{a}
Coefficient of nitrogen utilization (%)		29.7	23.7	53.4
Atomic-enrichment percentage (at% ^{15}Nexcess)	0 100	0.080 ^{a} 0.069 ^{a}	0.065 ^{a} 0.052 ^{a}	0.068 ^{a,*} 0.065 ^{a,*}
Nitrogen in winter wheat derived from crop residues of white lupin (%)	0 100	8.23 ^{b} 7.09 ^{a}	6.88 ^{b} 5.34 ^{a}	6.99 ^{b,*} 6.22 ^{a,*}
Nitrogen in winter wheat derived from crop residues of white lupin (kg ha^{-1})	0 100	4.36 ^{b} 5.86 ^{a}	2.76 ^{b} 3.41 ^{a}	7.12 ^{b} 9.27 ^{a}
Coefficient of nitrogen utilization by winter wheat from crop residues of white lupin (%)	0 100	12.9 ^{b} 17.2 ^{a}	8.1 ^{b} 10.0 ^{a}	21.0 ^{b} 27.2 ^{a}
33.9 kg ha^{-1} of nitrogen introduced in crop residues of white lupin				

* mean weighted; $^{a, b}$ averages with different letters in the columns are significantly different ($p \leq 0.05$).

Molecular N, which is contained in the atmosphere, is introduced into the soil during biologic fixation. This form of N is unavailable to plants and its transformation into a form that can be taken up by successive crops is mediated by microorganisms in the process of mineralization whereby NH_4^+ ions are produced [30,31]. The total amount of N taken up by winter wheat (cultivated twice in the crop rotation) was 27.14 and 30.26 kg ha^{-1} in the control and N-fertilized subplots, respectively (Table 6). Taking into consideration the white lupin residues (33.9 kg ha^{-1}), the N-utilization coefficient value was 80% in the control subplots and 89.1% in the N-fertilized subplots. It should be noted that the application of additional N increased the utilization coefficient value of winter wheat by 9.1%, mainly in the grain (8.5%) and less so in the crop residues (0.6%).

In our study, $(^{15}NH_4)_2SO_4$ (containing 20.1 at% ^{15}N) was applied at a rate of 30 kg N ha^{-1}. At this dosage, 5.92 kg ha^{-1} ^{15}N isotope (expressed as at% ^{15}Nexcess) was applied in the first year of the experiment to the white lupin crop. The enrichment of ^{15}N was determined each year at the end of the growing season (as presented in Tables 3–5), which enabled calculation of the amount of ^{15}N in the plant biomass harvested in the first, second and third years of the rotation (Table 7).

Table 6. Nitrogen uptake (kg ha^{-1}) and nitrogen-utilization coefficient (%) derived from the white lupin residues taken up by winter wheat in the second and third years of the rotation (2017 and 2018).

Specification	Nitrogen Dose kg ha^{-1}	Grain	Crop Residues	Sum
Total nitrogen uptake by winter wheat in the second and third years of rotation (kg ha^{-1})	0 100	20.96 ^{b} 23.86 ^{a}	6.18 ^{b} 6.40 ^{a}	27.14 ^{b} 30.26 ^{a}
Coefficient of nitrogen utilization derived by winter wheat from white lupin residues (%)	0 100	61.8 ^{b} 70.3 ^{a}	18.2 ^{b} 18.8 ^{a}	80.0 ^{b} 89.1 ^{a}
Change in nitrogen utilization rate as a result of nitrogen fertilization		+8.5	+0.6	+9.1

$^{a, b}$ averages with different letters in the columns are significantly different ($p \leq 0.05$).

Table 7. Quantity (kg ha^{-1}) and percentage share of the ^{15}N isotope in the biomass of cultivated plants, in relation to the amount used in the form $(^{15}NH_4)_2SO_4$.

Specification	Nitrogen Dose kg ha^{-1}	Seeds/ GRAIN	Crop Residues	Sum	Percentage Share in Relation to the Initial Quantity
Amount of isotope ^{15}N in the biomass of white lupin harvested in 2016 (kg ha^{-1})		2.241	0.329	2.570	43.4
Amount of ^{15}N isotope in winter wheat harvested in 2017 (kg ha^{-1})	0 100	0.162 ^{b} 0.174 ^{a}	0.033 ^{b} 0.029 ^{a}	0.195 ^{b} 0.203 ^{a}	3.29 3.42
Amount of ^{15}N isotope in winter wheat harvested in 2018 (kg ha^{-1})	0 100	0.042 ^{b} 0.057 ^{a}	0.026 ^{b} 0.033 ^{a}	0.068 ^{b} 0.090 ^{a}	1.14 1.52
Sum for dose N: 0 kg ha^{-1}/100 kg ha^{-1}					47.83/48.34

In the first year of the experiment (2016), 5.92 kg ha^{-1} ^{15}N isotope was applied in the form $(^{15}NH_4)_2SO_4$; $^{a, b}$ averages with different letters in the columns are significantly different ($p \leq 0.05$).

The largest amount of ^{15}N isotope (2.570 kg ha^{-1}) accumulated in the biomass of white lupin; the plant harvested in the first year of the rotation. This amount corresponds to 43.4% of the total ^{15}N applied, with the majority (2.241 kg ha^{-1}) contained in the seeds and a very low amount in the crop residues. This confirmed the fact that legume plants mainly accumulate N in their seeds. Moreover, 3.29% of the ^{15}N isotope accumulated in the winter wheat biomass (harvested after white lupin in the rotation) in the control subplots and 3.42% in the biomass in the N-fertilized subplots (Table 7). The ^{15}N-content was much lower in the winter wheat biomass harvested in 2018; 0.07 and 0.09 kg ha^{-1} in the control and N-fertilized subplots, respectively, which equates to 1.14% and 1.52% of the total ^{15}N applied. In both treatments, a substantial decrease in ^{15}N was observed in the winter wheat biomass compared to the white lupin biomass, 12.9-fold lower in 2017 and 32.5-fold lower in 2018. The application of an additional N dose (100 kg ha^{-1}) in the first year of winter wheat cultivation increased the ^{15}N-content by 0.13% and by 0.38% in the second year. Probably this is due to a priming effect. It means that the addition of mineral nitrogen fertilizer accelerates the mineralization of organic nitrogen compounds in soil. Moreover, 2.83 kg ha^{-1} ^{15}N was found in the plant biomass in the control subplots and 2.86 kg ha^{-1} in the N-fertilized subplots, what equates to 47.8% and 48.3% of the total amount of ^{15}N applied in the first year of the rotation to the white lupin crop. This means that more than 50% of the applied ^{15}N isotope may remain in the organic and inorganic parts of the soil, be lost or be contained in the belowground parts of the plants [9,10]. The introduction of legume plants into a cereal crop rotation is common in agricultural practice and may significantly increase the yield of cereals cultivated after legume crops and may also strongly increase soil fertility levels. The positive effects of legumes on subsequent crops may extend to the second or even the third crop [7]. These effects are greater on low fertility soils [1] than on soils with an adequate N-supply. According to the results from a European experiment, the yield of cereals cultivated after legumes was 0.5–1.6 t ha^{-1} higher than if cereals were the pre-crop [32,33]. It should be noted that legumes have considerable potential to increase the content of C and N in the seeds. Deep rooting species (such as lupin) as well as low C:N ratios in crop residues stimulate the process of mineralization– immobilization of those elements in the soil.

4. Conclusions

The total amount of N taken up by the white lupin crop was 209.3 kg ha^{-1} in the seeds and 33.9 kg ha^{-1} in the crop residues. The amount of N biologically fixed by white lupin was 111.2 kg ha^{-1} (93.7 kg ha^{-1} in the seeds and 17.5 kg ha^{-1} in the crop residues). The winter wheat crop cultivated after white lupin (second year of the crop rotation) took up 20 kg N ha^{-1} (59.0%) in the control subplots and 21.0 kg N ha^{-1} (61.9%) in the N-fertilized subplots. In the third year of the rotation, winter wheat accumulated 7.12 kg N ha^{-1} (21.0%) in the control subplots and 9.27 kg N ha^{-1} (27.2%) in the N-fertilized subplots. Total uptake of N (derived from white lupin residues) by winter wheat in the second and third years of the rotation was 27.1 kg ha^{-1} (80% of introduced N) and 30.3 kg ha^{-1} (89.1%) in the control and N-fertilized subplots, respectively. The results obtained in this experiment are very important for sustainable agriculture, especially in the nitrogen balance sheet. Our findings clearly show how much nitrogen is supplied to the soil–plant system by biologic nitrogen fixing plants in a three-year crop rotation, and how much of this can be utilized by cereals in subsequent plantings. This knowledge will support a decrease in the amount of nitrogen applied as mineral fertilizer.

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