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Quantitative Determination of Nitrogen Fixed by Soybean and Its Uptake by Winter Wheat as Aftercrops Within Sustainable Agricultural Systems

Karolina Ratajczak [1,*], Marcin Becher [2], Stanisław Kalembasa [2], Agnieszka Faligowska [1], Dorota Kalembasa [2], Barbara Symanowicz [2], Katarzyna Panasiewicz [1], Grażyna Szymańska [1] and Hanna Sulewska [1]

[1] Department of Agronomy, Faculty of Agriculture, Horticulture and Biotechnology, Poznan University of Life Sciences, Dojazd 11 Street, 60-632 Poznan, Poland; agnieszka.faligowska@up.poznan.pl (A.F.); katarzyna.panasiewicz@up.poznan.pl (K.P.); grazyna.szymanska@up.poznan.pl (G.S.); hanna.sulewska@up.poznan.pl (H.S.)

[2] Faculty of Agricultural Sciences, University of Siedlce, Prusa 14/8 Street, 08-110 Siedlce, Poland; marcin.becher@uws.edu.pl (M.B.); europrof171@gmail.com (S.K.); dorka@ap.siedlce.pl (D.K.); barbara.symanowicz@uws.edu.pl (B.S.)

* Correspondence: karolina.ratajczak@up.poznan.pl

Abstract: The future of agricultural production involves sustainable production systems with a balance between nutrients in soil–plant systems. These production systems are based on limiting the use of mineral fertilizers while introducing natural sources that increase soil fertility. The best example of such a system is plant rotation, including legumes as a forecrop for cereal plants. For this reason, the goal of the present study was to determine the possibility of obtaining nitrogen from the air using ^{15}N isotopes and to determine the quantity of nitrogen biologically fixed and taken up by winter wheat cultivated as a succeeding plant. In field experiments, we investigated the cycle of nitrogen fixed by legume plants in rotation under sustainable conditions, as follows: soybean–winter wheat–winter wheat. After soybean seedling emergence, a mineral fertilizer $(^{15}NH_4)_2SO_4$ containing 20.1 at% ^{15}N (a dose of 30 kg·ha^{-1}) was applied, with summer wheat as a reference plant. The yield of soybean reached 2.48 t·ha^{-1} for seeds and 8.73 t·ha^{-1} for crop residue (CR), providing a total yield of 11.21 t·ha^{-1}. The total biomass of soybean contained 149.1 kg·ha^{-1} of total nitrogen, with 108.1 kg·ha^{-1} in the seeds and 41.0 kg·ha^{-1} in the residue, of which 34.0 kg·ha^{-1} in the seeds and 11.4 kg·ha^{-1} in the residue was biologically fixed. CR was ploughed into the soil. Plots with winter wheat cultivated after soybean (2017) were divided into two sub-plots for the application of 0 and 100 kg·ha^{-1} of mineral N. The scheme was repeated in 2018. Overall, winter wheat cultivated for two subsequent years took up 8.12 kg·ha^{-1} of the total nitrogen from the CR from the control sub-plot and 15.51 kg·ha^{-1} from the fertilized sub-plot, of which 2.61 and 2.98 kg·ha^{-1} was biologically fixed by soybean plants, respectively. The dose of fertilizer contained 5.920 kg·ha^{-1} of ^{15}N, of which 3.024 kg·ha^{-1} was accumulated in soybean. In wheat cultivated as the first subsequent crop, the accumulation of ^{15}N was as follows: 0 kg N (control)—0.088 kg·ha^{-1}; 100 kg N—0.158 kg·ha^{-1}. Meanwhile, in winter wheat cultivated as the second aftercrop, 0.052 and 0.163 kg·ha^{-1} of ^{15}N was accumulated, respectively. This study demonstrates that biological nitrogen fixation in soybeans is an underappreciated solution for enhancing crop productivity within sustainable agricultural systems. It holds significant implications for planning rational fertilizer management, reducing the application of chemical fertilizers, and improving nitrogen use efficiency within crop rotation systems.

Keywords: ^{15}N isotope; legume; biological N reduction; yield; sustainable production; ID^{15}N; soybean–winter wheat–winter wheat

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1. Introduction

Nitrogen is one of the most important elements in relation to the production of food; the preservation of water, soil, and air quality; the mitigation of climate change; and the conservation of biodiversity in the environment. It is a constituent of nucleotides and proteins, which are essential for life [1]. Most nonlegume plants require 20–50 g of nitrogen, which must be taken up by their roots, to synthesize a 1 kg dry mass of biomass. The natural supply of nitrogen provided by different soil types is strongly limited in its ability to deliver a suitable quantity of nitrogen to guarantee high yields of plant biomass. Therefore, in order to obtain a proper balance of this element in plant–soil systems, additional doses of nitrogen are necessary. The main means by which nitrogen is introduced into plant–soil systems is biological nitrogen fixation (BNF), which is a common activity in sustainable agriculture (SA) and fertilization practices, mainly involving mineral nitrogen. Legume plants in symbiosis with *Rhizobium* possess the ability, developed over more than 60 million years [2], to reduce nitrogen from N_2 ($N \equiv N$) into forms which can be utilized by plants and synthesized into proteins. During recent years, great progress has been made in the field of research on the intensification of BNF [3]. Global nitrogen fixation contributes 413 Tg of reactive nitrogen compounds (Nr) to terrestrial and marine ecosystems each year, compared to anthropogenic activity, which contributes 210 Tg $N \cdot y^{-1}$. Nitrogen-fixing agricultural crops substantially contribute to introducing Nr into the soil and plant environment [4], and the total amount of nitrogen (AN) biologically fixed by legume plants can reach up to 70 Tg $N \cdot y^{-1}$ [5]. The second largest source of Nr in plant production is chemical reduction through the Haber–Borsch process, where N_2 and H_2 are combined at high temperatures and pressures with a catalyst [6]. In the first decade of the 21st century, 120 Tg of N was produced as NH_3 per year, of which 80% was used in agriculture as a fertilizer. In recent years, intensive research has been carried out to reduce and limit the amount of greenhouse gasses (CO_2) in the air, since the increase in their content in the Earth's atmosphere is a serious ecological problem. Plants use CO_2 in the process of photosynthesis to create biomass, which is called 'biosequestration' [7]; this is an interesting and important process that is receiving more and more attention in the cultivation of agricultural plants [8,9]. Legumes are the best example of carbon biosequestration, while providing nitrogen to plants as a result of the biological nitrogen reduction (BNR) process [8]. The process of BNR requires a large amount of energy from photosynthesis, reaching up to 2.5–4.0 g of C per 1 g of biologically reduced nitrogen (BRN). Despite these beneficial features of legume plants, the amount of BRN is too low to fully cover their nitrogen demand in plant production, even when using various methods to increase the efficiency of this process [10]. Increasing the area of legume crops and research on the biological activity of nitrogen reduction by these plants do not provide a sufficient (AN) to produce an adequate amount of food for the increasing human population [11]; therefore, it is necessary to use mineral nitrogen fertilizers. It should be pointed out that the production of nitrogen fertilizers is very expensive, and their use increases the carbon footprint of agriculture. In accordance with the GHGenius model [12], the emission levels attributable to the stages of mineral fertilizer production and distribution based on the average amounts of fertilizers used and emission factors were calculated as 2.792 kg $CO_2 \cdot kg^{-1}$ N, 0.738 kg $CO_2 \cdot kg^{-1}$ P_2O_5, and 0.382 kg $CO_2 \cdot kg^{-1}$ K_2O, respectively. The production and use of mineral fertilizers account for 13% of the total CO_2 emissions from agriculture [13].

Among the legumes, soybean is one of the most important plants cultivated worldwide as a source of plant protein and oil [14], and it also has a positive effect on soil fertility [15]. Currently, the main producers of soybean are the United States, Brazil, and Argentina, which accounted for approximately 82% of global soybean production in 2021 [14]. During recent years, the seed yield of soybean has increased due to plant breeding efforts (new varieties) [16], BRN, and an increase in the portioning efficiency of nitrogen (PEN), but still the increase in seed biomass and PEN mainly in seeds require a huge AN [17,18]. The total nitrogen (CTN) in soybean biomass taken up from the air by means of the BNR process (N_2 fixation) can range from 0 up to 98% [19], and also it can be taken up from the soil as available forms during the mineralization of organic nitrogen compounds.

Nitrogen biologically reduced by soybean is found mainly in the seeds, and a much smaller part of the total pool is contained in aboveground and underground post-harvest residues [20]. Nitrogen is released from post-harvest residues introduced into the soil in available forms to subsequent plants as a result of their decomposition and mineralization. Post-harvest residues of legume plants release more nitrogen in mineral forms due to their higher nitrogen content and narrower C/N ratio in comparison to cereal post-harvest residues [21,22]. Moreover, post-harvest residues can supplement the C_{org} and N stock in soil after mineralization, making soybean plants very valuable in sustainable plant production due to reducing the use of synthetic N fertilizers in plant fertilization [22]. In addition to the seed yield obtained, the nitrogen balance is often used to assess the benefits of soybean cultivation within sustainable agricultural systems [23]. Soybean accumulates large AN in its seeds due to its plant physiology, often more than the AN contained in the biomass as a result of the BNR process, which makes the balance in the cultivation of this plant negative [24]. Legumes' ability to biologically reduce molecular nitrogen contained in atmospheric air with the participation of *Rhizobium* and *Bradyrhizobium* bacteria reduces the AN doses used in mineral fertilizers [25]. Nitrogen from the air is contained in the seeds and post-harvest residues of legumes and remains in the soil, which increases the content of this element and improves soil productivity. Grain legumes were estimated to fix a global total of 35.5 Tg N in the year 2018, with soybean (25.0 Tg) dominating global grain legume N_2 fixation [26]. Therefore, it is assumed that, after water, nitrogen is the main factor determining plant yield [25]. Introducing legumes into crop rotation is very beneficial from an environmental protection point of view; it reduces the use of chemicals and introduces additional AN. The cultivation of legumes has thus become very beneficial in developing a sustainable plant production system [27].

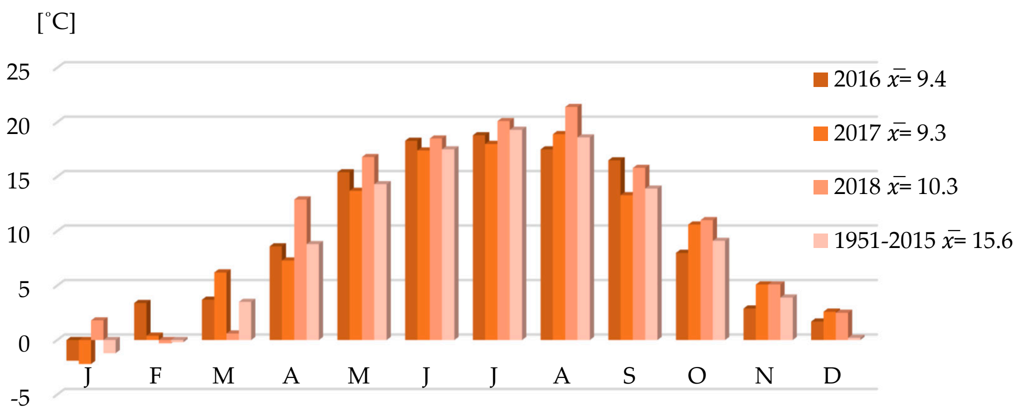
The role of BNR, especially performed by large-seeded legumes, has been well documented in the literature [28]. Various methods have been used to assess the AN reduced by legumes in natural conditions [29]. It is estimated that using the isotopic dilution ($ID^{15}N$) method with the application of mineral nitrogen fertilizer containing the isotope ^{15}N in research on the BNR process by legumes plants provides the most accurate results [30]. It was assumed that the use of ^{15}N has a significant effect and advantages in agricultural research connected with the management of nitrogen with the most important elements in the sustainable production of food and feed. It enables us to observe the rate of N_2 biologically reduced by soybean expressed as a percentage or $kg \cdot ha^{-1}$ in whole legume plants, AN in plant residue, and its uptake by subsequent plants cultivated after legume crops.

The aim of this study was to determine the quantity of nitrogen fixed by soybean in the first year of a 3-year crop rotation and its uptake by winter wheat (WW) as aftercrops in the second and third years of the rotation with this cereal. It was assumed that the method of monitoring the cycle of nitrogen ($ID^{15}N$) fixed by soybean would determine the AN derived from the atmosphere through the soybean in crop rotation with cereal, thus indicating a solution for sustainable agriculture practice. Moreover, based on current European policy [31] and the challenges related to reducing external inputs and emissions, especially with the rising costs of fertilizers, the results highlight the benefits of introducing legumes into crop rotations, demonstrating that this practice is justified as a recommendation that, although known, is still underestimated, but nevertheless future-proof.

2. Materials and Methods

2.1. Field Experiment Description

In Złotniki Research Station (52°29′ N, 16°49′ E) in the region of Wielkopolska (Poland), belonging to the University of Life Sciences in Poznan, we conducted field experiments with soybean and winter wheat (WW) in the years 2016–2018. The soil comprised sandy loam with the following granulometric composition: fractions above 2.0 mm in diameter—2.10%; sand, 2.0–0.05 mm—73%; clay, 0.05–0.002—22.0%; and silt, $\leq$ 0.002 mm—6.0%. Also, the following chemical properties were determined: pH (1M KCl)—6.1; total carbon in organic compounds—5.84 $g \cdot kg^{-1}$; total nitrogen—0.592 $g \cdot kg^{-1}$ of soil. The pH was measured with a laboratory pH device (Elmetron CPC-400, Elmetron, Zabrze, Poland) by means of the potentiometric method; soil samples were flooded with a 1 M KCl solution, at a ratio of 1/2.5 (m/v). However, the nutritional analysis revealed that the experimental site has levels of available P, K, and Mg of 170, 340, 72 mg·kg^{-1} of soil, respectively. Meteorological conditions were recorded during the experiment (Figure 1). Measurements were performed automatically every 24 h, and the data were downloaded from the Pessl Instruments server at www.fieldclimate.com (accessed on 10 December 2018) to calculate average daily temperatures and precipitation.



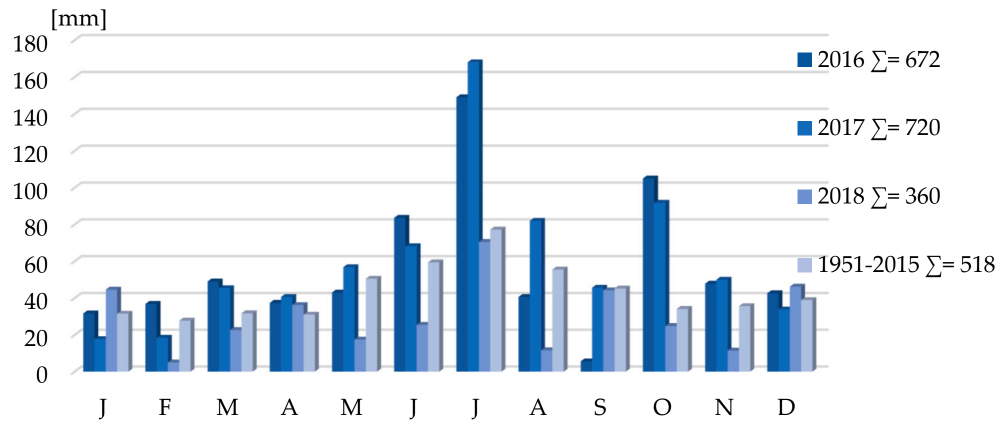


Figure 1. Weather conditions during the study period, January–December (J–D) (2016–2018).

2.2. Experimental Design

The field experiment was established using randomized complete blocks with three replications. In the first year of crop rotation (2016), soybean 'Aldana' and spring wheat 'Jarlanka' (as reference crop) were sown (29 April 2016) on plots with a 9 m^2 area. After the emergence of soybean and spring wheat, plots with areas of 4 m^2 (2 × 2 m) were marked out within the 9 m^2 plots, where the ^{15}N fertilizer in the form of ammonium sulphate $(^{15}NH_4)_2SO_4$ in solution (containing 20.1 at% ^{15}N) was applied at a dose of 30 kg·ha^{-1} (5.920 kg·ha^{-1} of ^{15}N isotope) in the first year of the experiment to the soybean plants. Soybean and spring wheat, after being harvested (19 August 2016), were divided into seeds and CR, which was ploughed into the soil. In the autumn of 2016 (29 September 2016), 'Bogatka' WW was sown. In the spring of 2017, each 9 m^2 plot was divided into 2 sub-plots with an area of 4.5 m^2. No nitrogen fertilizer was applied to the first of these sub-plots (control), while the second of these sub-plots received nitrogen at a dose of 100 kgN·ha^{-1} in the form of ammonium nitrate, divided over two instances: 60% at the emergence stage and 40% at the early tilling stage (fertilized sub-plots). After WW was harvested (27 July 2017), CR was ploughed and WW was resown (17 October 2017). The cultivation of the same crop (WW) in the second and third years of the crop rotation enabled a comparison of the effect of soybean residues on the yield of WW as a subsequent crop. The application of two crop rotations allowed us to compare significant differences in plant cultivation: (1) 100% cereal plant and (2) 33% leguminous plants and 66% cereal crop. During the growing season, no irrigation system was used. The biological fixation of atmospheric N was determined on the basis of visible plant root nodules. After each growing season, the atomic enrichment of at% $^{15}N_{exc}$ was determined in the biomass of harvested plants. These values were multiplied by the biomass of tested plants to obtain the amount of ^{15}N expressed in kg·ha^{-1}. The application of ^{15}N in the ID^{15}N method in agricultural research is based on the assumption that the assimilation of N by plants from the soil, fertilizers, and atmosphere differs between fixing and non-fixing plants. The application of the ID^{15}N method enabled the determination of the amount and pathways of biological fixation of N_2 from the air by legume plants as a result of symbiosis between plants and *Bradyrhizobium* bacteria, as well as nitrogen taken up from the soil and fertilizers.

2.3. Chemical Analysis

Plant material samples were collected during harvesting at the phase of technological ripeness and analyzed in a chemical laboratory. Samples for tests (1000g) were taken from the soil and plant material (ground down to a particle diameter of < 0.15 mm), air-dried, and then dried at 105 °C in order to determine the dry weight. The content of total carbon and nitrogen in soil and plant samples was determined by means of dry combustion using the ® 2400 Series II elemental analyzer with thermal conductivity detection (TCD) (Whaltham, MA, USA) and acetanilide (C—71.09%; N—10.34%) as a reference calibration standard. The $^{15}N/^{14}N$ and $^{13}C/^{12}C$ isotope ratios in analyzed samples were established using the elemental analyzer Flash EA 1112HT (Thermo Scientific, Whaltham, MA, USA) coupled with the mass spectrometer Delta V Advantage (Thermo Scientific, Whaltham, MA, USA) in a continuous-flow system with helium.

2.4. Calculation of the Results and Statistical Analysis

The obtained results (yield and chemical analysis) provided a base for the calculation of analyzed parameters according to the formulas presented by Kalembasa et al. [32]. The experiment with soybean was established using randomized complete blocks, but the results for WW showing the effect of nitrogen fertilizer were statistically analyzed using one-way ANOVA followed by Tukey's HSD post hoc test to assess significant differences between the dose of N at a significance level of $p < 0.05$.

3. Results and Discussion

Temperature and precipitation are important factors influencing the vegetative and generative periods of plant productivity from the perspective of plant biomass production. During the study period, significant differences among the different years were observed. The mean air temperature and sum of precipitation for the period of vegetation (from April to July) were as follows in each year: 15.3, 14.1, and 17.1 °C and 313.0, 333.6, 149.5 mm, respectively. A negative correlation between air temperature and the sum of precipitation in the vegetative period ($r = -0.95$, $y = 19.3 - 0.014x$) was clearly observed. Under the climatic conditions of 2016, the total yield of soybean biomass reached 11.21 t·ha^{-1}, including 2.48 t·ha^{-1} as seeds and 8.73 t·ha^{-1} as CR, indicating that 22.1% of the total yield was seeds and 77.9% was CR (Table 1). The yield of seeds was slightly higher than that reported by Panasiewicz et al. [33] in the same soil and climatic conditions. Similar yields were reported by Kotecki and Lewandowska [34] and Prusiński et al. [35]. Similarly, Wang et al. [25] pointed out that wet years have a better seed yield and economic benefits than dry years, and thus precipitation is the decisive factor in determining crop yield [36–38].

Table 1. Detailed foliar and seed analysis of the soybean cv. 'Aldana' harvested in the year 2016.

Specification	Seeds	Crop Residues	Sum/Mean Weighted
Yield ($t \cdot ha^{-1}$)	2.48	8.73	11.21
CN (%) [1]	4.36	0.47	1.33
ANb ($kg \cdot ha^{-1}$) [2]	108.1	41.0	149.1
Atomic enrich% (at% $^{15}N_{exc}$) [3]	2.135	1.750	2.029
ANa (%) and ($kg \cdot ha^{-1}$) [4]	31.5 (34.0)	27.8 (11.4)	45.4
$AN_{(15NH4)2SO4}$ in % and ($kg \cdot ha^{-1}$) [5]	10.8 (11.7)	8.86 (3.63)	15.3
ANs in % and ($kg \cdot ha^{-1}$) [6]	57.7 (62.4)	63.3 (25.9)	88.4
CCorg (%) [7]	49.5	39.7	
C/N [8]	11.3	84.5	
AOC ($kg \cdot ha^{-1}$) [9]		3456	
$\delta\ ^{13}C/^{12}C$ (‰)	−28.6	−26.8	

[1] CN—content of nitrogen; [2] ANb—amount of nitrogen in biomass; [3] Atomic enrich%—atomic enrichment percentages, at% $^{15}N_{exc}$—at% of ^{15}N excess; [4] ANa—amount of nitrogen from the air; [5] $AN_{(15NH4)2SO4}$—amount of nitrogen taken up from $(^{15}NH_4)_2SO_4$; [6] ANs—amount of nitrogen taken up from the soil; [7] CCorg—content of carbon in organic compounds; [8] C/N—values of C/N=1; [9] AOC—amount of organic carbon introduced into the soil with CR.

The CTN in seeds of soybean was 4.36%, and by multiplying this value by the seed yield, we obtained the total AN harvested in seeds (108.1 $kg \cdot ha^{-1}$) (Table 1). A value equal to 676 kg of plant protein was obtained in harvested seeds. This value is similar to results reported by other authors [34,35].

The AN in seeds was acquired from the soil and the plant environment. The AN in the CR of soybean was estimated to be 0.47%, what was 0.32 percentage point lower than in white lupin residue [32]. Multiplying the value of total nitrogen in CR by its yield, we found that the total nitrogen in the yield of CR amounted to 41.0 $kg \cdot ha^{-1}$, which is half that in seeds. This AN was ploughed into the soil, providing a potential source of nitrogen for succeeding plants. It should be pointed out that this amount was accumulated in the aboveground part of soybean plants. According to the results of other authors, 50% more nitrogen is accumulated in the underground residue of soybean plants [39–41]. This nitrogen is, of course, mainly formed in organic compounds and may increase soil fertility when in available forms (NH_4^+ or NO_3^-) for succeeding plants after the processes of nitrogen and carbon transformation in the soil as a result of mineralization–immobilization turnover [42,43].

Isotopic chemical analysis revealed that ^{15}N enrichment in the biomass of soybean with this isotope amounted to 2.135 at% $^{15}N_{exc}$ in seeds and 1.750 at% $^{15}N_{exc}$ in the CR, but in the biomass of spring wheat cultivated as a reference plant, it was 1.940 and 2.014 at% $^{15}N_{exc}$ in the grain and CR, respectively. These values were used for the calculation of the AN biologically reduced from the atmosphere by soybean. The data presented in Table 1 show that the AN, expressed as percentages and $kg \cdot ha^{-1}$, was as follows: seeds, 31.5% (34.0 $kg \cdot ha^{-1}$); CR, 27.8% (11.4 $kg \cdot ha^{-1}$). The total AN accumulated in the aboveground biomass of soybean reached 59.3% and 45.4 $kg \cdot ha^{-1}$, which means that 30.4% of the total nitrogen was reduced from the air. Similar results were obtained in 2017 in the same soil conditions for soybean [28]. The AN derived from the air by legume plants mainly depends on the amount of mineral forms of nitrogen in the soil [44]. Therefore, this explains why, in soils to which high doses of mineral fertilizers are applied, the yield of legume plants is lower in comparison to that in soils without N fertilizers' application [45]. Mineral nitrogen ions made nodulation difficult in the root zones with the inhibition of nitrogenase activity [46]. In these circumstances, plants use less energy to take up nitrogen from the soil than that biologically reduced from the atmosphere [47]. The AN taken up by soybean in this experiment from the fertilizer $(^{15}NH_4)_2SO_4$ at a dose of 30 $kg \cdot ha^{-1}$ reached 10.8 $kg \cdot ha^{-1}$ (11.7%) for seeds and 8.86 $kg \cdot ha^{-1}$ (3.63%) for the CR. These results indicate that 3.63 $kg \cdot ha^{-1}$ of nitrogen derived from soybean and accumulated in the CR was returned to the soil by ploughing the CR. The highest AN in the biomass of soybean that was taken up from the soil amounted to 88.4 $kg \cdot ha^{-1}$, which accounted for 59.2% of the total nitrogen in the aboveground soybean biomass. This nitrogen was accumulated in the seeds at a rate of 57.7% (62.4 kg·ha^{-1}) and in the CR at a rate of 63.3% (25.9 kg·ha^{-1}). The reason for this distribution is probably connected to the low nitrogenase activity and high concentration of mineral N (mainly N-NO_3) in the soil. In the cultivation of legume plants, in addition to the BNR, the amount of carbon accumulated in plants during the photosynthetic process is very important and is referred to as the 'biosequestration' of CO_2 [48]. The accumulation of organic compounds of carbon in soybean reached 49.5% (1228 kg·ha^{-1} of carbon) and 39.7% (3466 kg·ha^{-1} of carbon) in the seeds and CR, respectively. As well as the amount of carbon in the seeds and CR, the ratio of C:N is an important indicator, which amounted to 11.3 and 84.5 kg·ha^{-1}, respectively. Taking into consideration the plant rotation and the balance of carbon and plant nutrients, this value can be used to determine the immobilization–mineralization process in the soil and, as a consequence, the amount of available nutrients for plants in the soil [43]. The content of δ^{13}C in soybean biomass reached −28.6‰ in the seeds and −26.8‰ in the CR, which are normal values for *Dicotyledoneae* plants [49,50].

The literature has demonstrated a significant effect of European crop rotations including legumes plants on the yields of succeeding plants and environmental quality [51–53]. The yields of grains and CR of WW cultivated after soybean as the first succeeding plant also confirmed this trend (Table 2). In the sub-plot without additional nitrogen fertilization (control sub-plot), the yield of grain reached 3.60 t·ha^{-1} and that of CR reached 5.23 t·ha^{-1}, giving a total yield of the WW biomass of 8.83 t·ha^{-1}. The yield of WW in the sub-plots fertilized with 100 kgN·ha^{-1} amounted to 5.20 and 7.25 t·ha^{-1} for grain and CR, respectively, and provided a total yield of 12.45 t·ha^{-1}, which was significantly higher than in the control sub-plots ($F_{1,4\ \mathrm{grain}} = 116.87$, $p < 0.001$; $F_{1,4\ \mathrm{crop\ residue}} = 281.56$, $p < 0.001$). In our experiment, the effect of nitrogen fertilization was shown as an increase in yield by 1.60 and 2.02 t·ha^{-1} for grain and crop residue, respectively. This means that 1 kg of nitrogen increased grain and CR yield by 16 and 20.2 kg, respectively. In addition to the positive effect of nitrogen on yield, a significant increase in the CTN in the biomass of WW was also observed (Table 2). A significant increase in the total nitrogen in grains from 1.35% (unfertilized sub-plot) to 1.84% (fertilized sub-plot) ($F_{1,4} = 68.81$, $p < 0.001$), with no changes in this element's content in CR, was also observed ($F_{1,4} = 4.17$, $p = 0.11$). Changes in the yield and content of nitrogen in the biomass of WW resulted from the total AN in the biomass, which affects the value of the nitrogen utilization coefficient (NUC%). This value reached 47.0% for grains and 4.2% for CR for plants from the sub-plot fertilized with 100 kgN·ha^{-1}. Nitrogen use efficiency depends on a few factors, including plant characteristics, soil conditions, temperature, moisture, and the crop rotation, and ranges from 30% up to 80% [54,55]. The uptake, distribution, and accumulation of ^{15}N influence its concentration in WW biomass.

The content of the nitrogen isotope ^{15}N, expressed as at% $^{15}N_{exc}$, in the biomass of wheat harvested from the sub-plot without nitrogen fertilization (control) was 0.134 at% $^{15}N_{exc}$ in grains and 0.164 at% $^{15}N_{exc}$ in CR, but in the biomass of wheat harvested from the sub-plot with additional fertilization (100 kg·ha^{-1}), it was 0.125 at% $^{15}N_{exc}$ in grains and 0.208 at% $^{15}N_{exc}$ in CR ($F_{1,4\ \mathrm{grain}} = 0.89$, $p = 0.39$; $F_{1,4\ \mathrm{crop\ residue}} = 0.75$, $p = 0.30$). The increase in the content of ^{15}N in biomass harvested from the fertilized sub-plot was a result of the higher uptake of total nitrogen than in the control sub-plot. Taking into consideration the ^{15}N content in the biomass of WW and the residue of soybean ploughed into the soil, it was possible to calculate the percentages and AN derived from the soybean residue to the WW. In this case, the percentage of nitrogen in the WW harvested in 2017 from the residue of soybean in the sub-plot without additional nitrogen fertilization amounted to 7.65% and 9.37% in grains and CR, respectively, but from the fertilized sub-plot, it was 7.14% and 11.88%, respectively. These percentage values indicate that, from the total nitrogen ploughed with soybean residue (41.0 kg·ha^{-1}), the biomass of WW derived from it in the control sub-plot was 3.71 kg·ha^{-1} in grains and 1.36 kg·ha^{-1} in CR, and in the fertilized sub-plot, it was 6.82 in grains and 2.23 kg·ha^{-1} in CR. The coefficient of nitrogen utilization (CNU %) from soybean by the total biomass of WW amounted to 12.35% and

22.06% in the control sub-plot and fertilized sub-plot, respectively. However, these values are not high, but from an agricultural and fertilization (balance of available plant nutrients) point of view, they are very important. The results of CNU% for nitrogen from mineral fertilizer (ammonium nitrate) are more than two times higher in comparison to those for soybean residue. This is connected to the availability of nitrogen in applied materials, whereas NH_4NO_3 is much more available than organic compounds introduced into the soil with soybean residue [43]. In the present study, the rate of BRN by soybean accumulated in CR was 11.4 kg·ha^{-1}, and this was ploughed into the soil (Table 1), which constitutes a very important point. From this amount, in the control sub-plot, the WW derived 0.87 kg·ha^{-1} in the grains and 1.06 kg·ha^{-1} in the CR, with a total of 1.93 kg·ha^{-1}, while in the fertilized sub-plot, it derived 0.81 kg·ha^{-1} in the grains and 1.35 kg·ha^{-1} in the CR, for a total of 2.16 kg·ha^{-1}, respectively. These amounts are equal to 16.92% and 18.94% of the total BRN (11.4 kg·ha^{-1}).

The yield of WW biomass harvested in 2018 as the third crop in the rotation, as well as the second succeeding crop in the soybean–winter wheat rotation (Table 3), in the control sub-plot amounted to 4.30 t·ha^{-1} of grain and 7.37 t·ha^{-1} of CR, which in total amounted to 11.67 t·ha^{-1}. The application of 100 kg N·ha^{-1} significantly increased the yield of grain up to 5.20 t·ha^{-1} ($F_{1,4}$ = 234.94, $p < 0.001$), that of CR to 8.90 t·ha^{-1} ($F_{1,4}$ = 352.79, $p < 0.001$), and the total biomass to 14.10 t·ha^{-1}. The effect of an additional dose of nitrogen was lower than in 2017 and reached +0.90 for grains, +1.53 for CR, and +2.43 t·ha^{-1} for total biomass. The CTN in the biomass of WW harvested from the control sub-plot was 1.53% in grains, 0.40% in CR, and 0.81% for the total biomass, while for the fertilized sub-plot, it was 1.93% (grain), 0.81% (CR), and 1.22% (total biomass). Increases in the CTN caused by the dose of nitrogen were significantly higher ($F_{1,4\ grain}$ = 12.64, p = 0.02; $F_{1,4\ crop\ residue}$ = 129.13, $p < 0.001$) in comparison to the CTN in WW harvested in 2017. The CTN in the total biomass of WW harvested in 2018 was nearly two times higher in plants from the fertilized sub-plot than in those from the control. This means that the content of proteins was also higher. The accumulation of total nitrogen in CR harvested from the fertilized sub-plot was over two times higher than that from the control.

Table 2. Detailed foliar and seed analysis of winter wheat harvested in the year 2017.

Specification	Nitrogen Dose (kg·ha^{-1})	Grain	Crop Residues	Sum/Mean Weighted
Yield (t·ha^{-1})	0 100	3.60 b 5.20 a	5.23 b 7.25 a	8.83 b 12.45 a
Effect of 100 kg N·ha^{-1}		+1.60	+2.02	+3.62
Content of total nitrogen %	0 100	1.35 b 1.84 a	0.28 a 0.26 a	0.70 b 0.82 a
ANb (kg·ha^{-1}) [1]	0 100	48.6 b 95.6 a	14.6 b 18.8 a	63.2 b 114.4 a
CNU (%) [2]		47.0	4.2	51.2
(%) at % $^{15}N_{exc}$	0 100	0.134 0.125	0.164 0.208	0.141 0.142
N (%) (kg·ha^{-1}) [3]	0 100	7.65 7.14	9.37 11.88	8.05 8.10
N (kg·ha^{-1}) [4]	0 100	3.71 6.82	1.36 2.23	5.07 9.05

[1] ANb—amount of nitrogen in biomass; [2] CNU—coefficient of N utilization from fertilizer; [3] N (%)—nitrogen (%) in winter wheat taken up from soybean residue (kg·ha^{-1}); [4] N (kg·ha^{-1})—amount of N (kg·ha^{-1}) in winter wheat from soybean residue; a,b—averages with different letters in the same column are significantly different ($p \leq 0.05$).

Table 3. Detailed foliar and seed analysis of winter wheat harvested in the year 2018.

Specification	Nitrogen Dose ($kg \cdot ha^{-1}$)	Grain	Crop Residues	Sum/Mean Weighted
Yield ($t \cdot ha^{-1}$)	0	4.30 b	7.37 b	11.67 b
	100	5.20 a	8.90 a	14.10 a
Effect of 100 kg $N \cdot ha^{-1}$		+0.90	+1.53	+2.43
Content of total nitrogen %	0	1.53 b	0.40 b	0.81 b
	100	1.93 a	0.81 a	1.22 a
ANb ($kg \cdot ha^{-1}$) [1]	0	65.8	29.4	95.2 b
	100	100.3	72.0	172.3 a
CNU (%) [2]		34.5	42.6	77.1
(%) at % $^{15}N_{exc}$	0	0.062	0.044	0.056
	100	0.075	0.053	0.065
N (%) ($kg \cdot ha^{-1}$) [3]	0	3.54	2.51	6.05/3.20
	100	4.28	3.02	7.30/3.71
N ($kg \cdot ha^{-1}$) [4]	0	2.32 b	0.73 b	3.05 b
	100	4.29 a	2.17 a	6.46 a
CNU soya by wheat (%) [5]	0	5.65 b	1.78 b	7.43 b
	100	10.46 a	5.29 a	15.75 a
AN_BRN ($kg \cdot ha^{-1}$) [6]	0	0.40	0.28	0.68
	100	0.48	0.34	0.82
CNU_BRN (%) [7]	0	3.50	2.45	5.95
	100	4.21	2.98	7.19

[1] ANb—amount of nitrogen in biomass; [2] CNU—coefficient of N utilization from fertilizer; [3] N (%)—nitrogen (%) in winter wheat taken up from soybean residue ($kg \cdot ha^{-1}$); [4] N ($kg \cdot ha^{-1}$)—amount of N ($kg \cdot ha^{-1}$) in winter wheat from soybean residue; [5] CNU soya by wheat—coefficient of nitrogen utilization (CNU) from soybean residue by winter wheat (%); [6] AN_BRN—amount of N ($kg \cdot ha^{-1}$) in winter wheat from BRN by soybean; [7] CNU_BRN—coefficient of nitrogen utilization (CNU) as a percentage of BRN; a,b—averages with different letters in the same column are significantly different ($p \leq 0.05$).

The value of the N utilization coefficient applied in mineral fertilizer at a dose of 100 kg $N \cdot ha^{-1}$ in this year reached 34.5% for grains and 42.8% for CR, amounting to 77.1% in total. The concentration of ^{15}N in the biomass of WW harvested in 2018 was two times lower than in 2017, and as a mean weighed expressed in at% $^{15}N_{exc}$, it amounted to 0.056 at% $^{15}N_{exc}$ for the control sub-plot and 0.065 for the fertilized sub-plot, with much more in the grains than in the CR. Similar results were obtained for WW cultivated after faba bean cultivation [32]. The percentage of nitrogen in wheat ($kg \cdot ha^{-1}$) taken up and the nitrogen utilization coefficient displayed by wheat from the CR of soybean (Table 3) were affected by mineral fertilization. AN ($kg \cdot ha^{-1}$) and CNU% were significantly higher after the application of mineral fertilizer. All of these values were nearly two times higher for the biomass of WW harvested in 2018 than in 2017. A similar situation was noticed for the AN from biological reduction by soybean, applied as a pre-crop for winter wheat. The amount of BNR by soybean ploughed as CR amounted to 12 $kg \cdot ha^{-1}$. In the total biomass of WW cultivated in the second year as a succeeding crop, the AN from soybean was determined as 0.68 $kg \cdot ha^{-1}$ from the control sub-plot and 0.82 $kg \cdot ha^{-1}$ from the fertilized one, equal to 5.95% and 7.19%, respectively.

The amount of total nitrogen ($kg \cdot ha^{-1}$) in WW harvested in 2017 and 2018 and derived from soybean residue, its distribution in grains and residue, and the values of CNU% are presented in Figure 2. The additional application of mineral nitrogen significantly increased all analyzed parameters. For example, the total AN taken up increased from 8.12 to 15.51 $kg \cdot ha^{-1}$. This is equal to a CNU of 19.8% to 37.8%. These results are very important in agricultural plant production and help in planning proper fertilizer management with sustainable practices through decreasing the AN applied using fertilizers in the form of mineral N. The data shown in Figure 2 indicate that nitrogen taken up from soybean residue is mainly accumulated in the grains of WW. The additional application of mineral nitrogen caused an increase in AN from soybean CR up to +18.0%, which was accumulated mainly in the grains (12.4%) and CR (+5.6%).

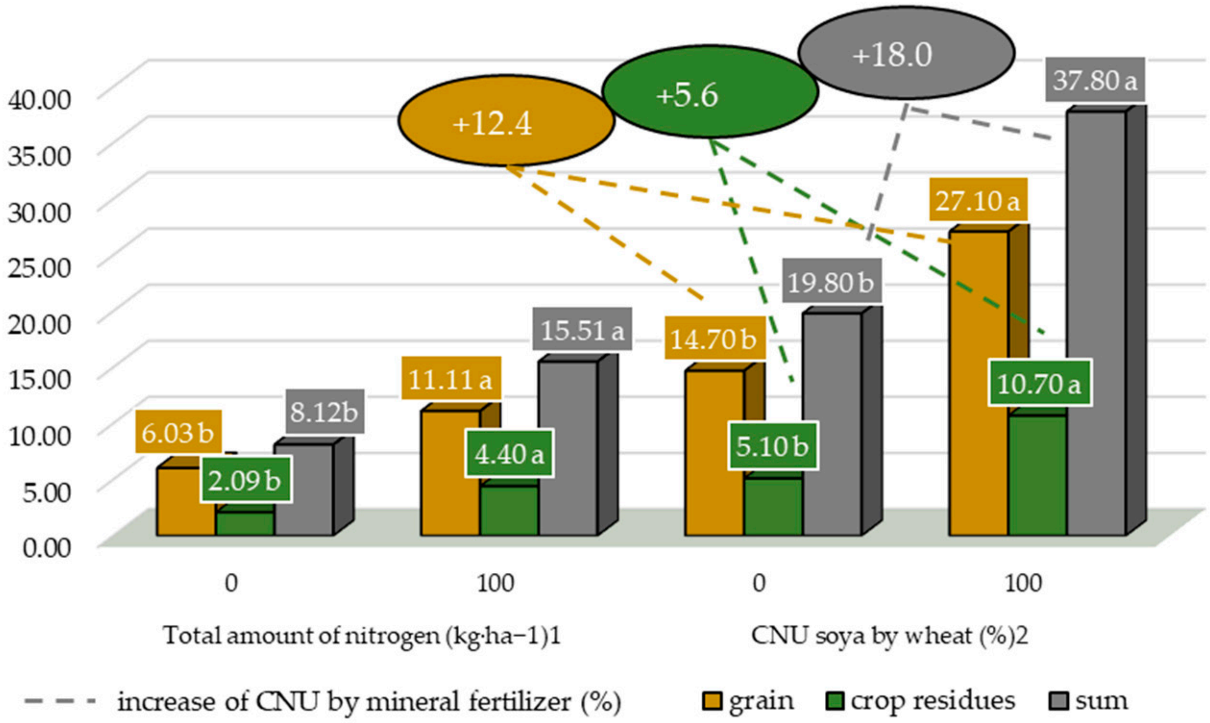


Figure 2. Amount of nitrogen (kg·ha^{-1}) and coefficient of nitrogen utilization (CNU) as a percentage of total nitrogen from soybean crop residue taken up by winter wheat in the second and third years of the rotation. [1] Total amount of nitrogen (kg·ha^{-1})—total amount of nitrogen in winter wheat harvested in 2017 and 2018; [2] CNU soybean by wheat—coefficient nitrogen utilization (CNU) (%) from soybean residue by winter wheat; a,b—averages with different letters in the same column are significantly different ($p \leq 0.05$).

In addition to the total nitrogen rate, knowledge on the BRN by soybean and the amount taken up by WW in the second and third years of cultivation is very important (Figure 3). The CR of soybean ploughed into the soil contained 11.74 kgN·ha^{-1} as effect of the biological reduction of N_2. Similarly, as with the total nitrogen, the additional application of mineral nitrogen fertilization caused an increase in the analyzed parameters. The sum of BRN taken up by WW ranged from 2.61 kg·ha^{-1} for the control sub-plot to 2.98 kg·ha^{-1} for the fertilized sub-plot, which showed a higher accumulation of nitrogen in CR than in grains. The value of CNU% was significantly higher for the fertilized sub-plot in comparison to the control one, and reached 26.13% and 22.87%, respectively. Therefore, additional fertilization increased the value of CNU% (+3.26%) (Figure 3).

Using the ID^{15}N method allowed us to not only determine the activity of the biological reduction process of N_2 but also the uptake, distribution and accumulation of ^{15}N in plants and the soil. The highest amount of ^{15}N isotope (3.024 kg·ha^{-1}) was accumulated in the biomass of soybean, including the seeds (2.307) and CR (0.717), which was equal to 51.08% of the initial amount of ^{15}N applied (Table 4). The amount of ^{15}N in WW biomass harvested in the second year of crop rotation was several times lower and significantly different under the influence of additionally applied mineral nitrogen at a dose of 100 kg·ha^{-1}. The amount of ^{15}N in the plants harvested from the control sub-plot reached 0.088 kg·ha^{-1}, including the seeds (0.065 kg·ha^{-1}) and CR (0.023 kg·ha^{-1}), equal to 1.48% of the initial total. For the fertilized sub-plot, these values were as follows: 0.158, 0.119, and 0.039 kg·ha^{-1} and 2.66%, respectively.

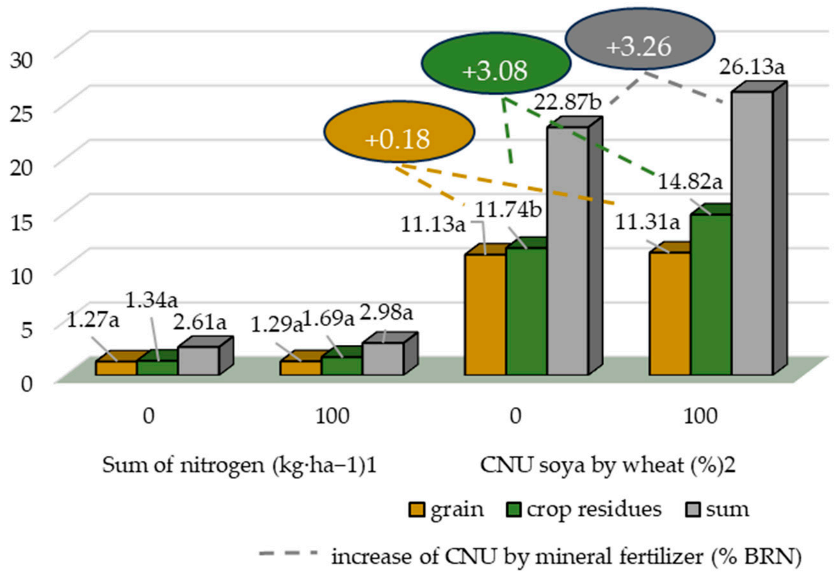


Figure 3. Amount of nitrogen (kg·ha^{-1}) and coefficient of nitrogen utilization (CNU) as a percentage of biologically reduced nitrogen (fixed) (BRN) from soybean crop residue taken up by winter wheat in the second and third years of rotation. [1] Sum of nitrogen (kg·ha^{-1})—sum of nitrogen in winter wheat harvested in 2017 and 2018; [2] CNU soya by wheat—coefficient nitrogen utilization (CNU) (%) of biologically reduced nitrogen (fixed) (BRN) from soybean residue by winter wheat harvested in 2017 and 2018; a,b—averages with different letters in the same column are significantly different ($p \leq 0.05$).

Table 4. Quantity (kg·ha^{-1}) and percentage share of ^{15}N isotopes in the biomass of cultivated plants in relation to the amount used in the form $(^{15}N)_2SO_4$.

Specification	Nitrogen Dose (kg·ha^{-1})	Seeds/Grain	Crop Residues	Sum	% Share [1]
Isotope ^{15}N_biomass (kg·ha^{-1}) [2]		2.307	0.717	3.024	51.08
Isotope ^{15}N_wheat^{2017} (kg·ha^{-1}) [3]	0 100	0.065 b 0.119 a	0.023 0.039	0.088 b 0.158 a	1.48 2.66
Isotope ^{15}N_wheat^{2018} (kg·ha^{-1}) [4]	0 100	0.040 b 0.075 a	0.012 b 0.038 a	0.052 b 0.163 a	0.87 2.75
Sum for the rotation (%) [5]	0 100				52.63 56.49

[1] % share—percentages share in relations to the initial amount; [2] isotope ^{15}N_biomass—the amount (kg·ha^{-1}) of isotope ^{15}N in the biomass of soybean harvested in 2016; [3] isotope ^{15}N_wheat^{2017}—the amount (kg·ha^{-1}) of ^{15}N isotope in winter wheat harvested in 2017; [4] isotope ^{15}N_wheat^{2018}—the amount (kg·ha^{-1}) of ^{15}N isotope in winter wheat harvested in 2018; [5] Sum for the rotation—sum for the rotation in % soybean–winter wheat–winter wheat; a,b—averages with different letters in the same column are significantly different ($p \leq 0.05$).

The analyzed values in the biomass of WW harvested in 2018 (third year of crop rotation) were similar to those for 2017 and also significantly affected by mineral fertilizer, with 0.052 kg·ha^{-1} for the control and 0.163 kg·ha^{-1} for the fertilized sub-plot, equal to 0.87 and 2.75% of the initial total, respectively. These results show that additional mineral fertilizer slightly increased the uptake of ^{15}N in the third year, 2018 (1.88%), in comparison to the value obtained in the second year, 2017 (1.18%), respectively. The mean value for the uptake of ^{15}N for the whole experiment including crop rotation in the control sub-plot reached 52.63% of the applied dose. The mean recovery of 56.49% showed that about 57% of the ^{15}N introduced into the soil in the first year was present in the tested plants in the root zone and in the mineral and organic compounds of nitrogen in the soil, while the rest was lost to the soil layer below 20 cm. This is higher than in Aulakh et al.'s [56] study, in which the recovery of applied fertilizer N (at a dose of 120 and 150 kg N·ha^{-1}) in the soil–plant system at the harvest of wheat showed that 36–47% was utilized by the crop after soybean cultivation. These authors found that the recovery of applied fertilizer N in different layers of the soil profile at the harvest of wheat revealed that the majority of the residual fertilizer N was present in the top soil layer.

4. Conclusions

These results clearly show that AN (kg·ha^{-1}) is reduced by soybean and that this nitrogen is recovered by WW as succeeding plants. The use of the ID^{15}N method in this study allowed for determining the amount of BRN by soybean in a crop rotation with cereals. The amount of BRN by soybean accumulated in the aboveground biomass reached 34.0 kg·ha^{-1} (seeds), 11.4 kg·ha^{-1} (CR), and 45.4 kg·ha^{-1} in total. The biomass of soybean cultivated in the crop rotation of soybean–winter wheat–winter wheat, harvested in the first year, contained 108.1 kg·ha^{-1} of the total nitrogen in the seeds and 41.0 kg·ha^{-1} in the CR. This study shows that nitrogen uptake by WW cultivated in the second and third years of the rotation derived 2.61 kg·ha^{-1} of N from the CR of soybean in the control sub-plot. An additional mineral nitrogen dose (100 kg N·ha^{-1}) increased the recovery of BRN up to 2.98 kg·ha^{-1}, representing a 3.26% increase. ^{15}N isotopes mainly accumulated in the soybean biomass, at a rate of over 51.08%, while this rate was drastically lower in WW harvested in the second and third years of crop rotation.

The mean value for total recovery throughout the crop rotation was above 50% of the ^{15}N applied in the first year of the experiment. The substitution of mineral nitrogen fertilizers is a necessity from the perspective of the current ecological and economical requirements. These results demonstrate the proper dose of nitrogen fertilizer in the following crop rotation: soybean–winter wheat–winter wheat. This demonstration of the possibility to calculate the nitrogen balance in a crop rotation involving legumes and cereal plants can be applicable in sustainable agricultural systems. The use of BFN by grain legumes as a mineral fertilizer substitution can affect GHG emission, and thus, the obtained results can constitute a base for future research on GHG emission estimations in agriculture.

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