agronomy

Article

Accumulation and Distribution of Fertilizer Nitrogen and Nodule-Fixed Nitrogen in Soybeans with Dual Root Systems

Rui Zhang [1], Cong Wang [1], Wenzhi Teng [1], Jing Wang [1], Xiaochen Lyu [1], Shoukun Dong [1], Shuang Kang [2], Zhenping Gong [1,*] and Chunmei Ma [1,*]

1 College of Agriculture, Northeast Agricultural University, Harbin 150030, China; zhang134rui@163.com (R.Z.); w15774517432@163.com (C.W.); twz1024@163.com (W.T); crystal900313@163.com (J.W.); xiaochenlyu@163.com (X.L.); shoukundong@163.com (S.D.)
2 Heilongjiang Academy of Land Reclamation Sciences, Harbin 150038, China; ks92511@163.com
* Correspondence: gzpyx2004@163.com (Z.G.); chunmm518@163.com (C.M.)

Received: 28 February 2020; Accepted: 10 March 2020; Published: 14 March 2020

Abstract: The soybean (*Glycine max L. Merr.*) is a crop with a high demand for nitrogen (N). The root nodules that form in soybeans can fix atmospheric N effectively, yet the goal of achieving high yields cannot be met by relying solely on nodule-fixed N. Nonetheless, the application of N fertilizer may inhibit nodule formation and biological N fixation (BNF), but the underpinning mechanisms are still unclear. In this study, we grafted the roots of non-nodulated soybeans onto nodulated soybeans to generate plants with dual root system. The experiment included three treatments conducted under sand culture conditions with NO_3^- and NH_4^+ as N sources. Treatment I: The non-nodulated roots on one side received 50 mg·L^{-1} $^{15}NO_3^-$ or $^{15}NH_4{}^+$, and the nodulated roots on the other side were not treated. Treatment II: The non-nodulated roots received 50 mg·L^{-1} $^{15}NO_3^-$ or $^{15}NH_4^+$, and the nodulated roots received 50 mg·L^{-1} $^{14}NO_3^-$ or $^{14}NH_4^+$. Treatment III: Both non-nodulated and nodulated roots received 50 mg·L^{-1} $^{15}NO_3^-$ or $^{15}NH_4^+$. The results showed the following: (1) Up to 81.5%–87.1% of the N absorbed by the soybean roots and fixed by the root nodules was allocated to shoot growth, leaving 12.9%–18.5% for root and nodule growth. Soybeans preferentially used fertilizer N in the presence of a NO_3^- or NH_4^+ supply. After the absorbed fertilizer N and nodule-fixed N was transported to the shoots, a portion of it was redistributed to the roots and nodules. The N required for root growth was primarily derived from the NO_3^- or NH_4^+ assimilated by the roots and the N fixed by the nodules, with a small portion translocated from the shoots. The N required for nodule growth was primarily contributed by nodule-fixed N with a small portion translocated from the shoots, whereas the NO_3^- or NH_4^+ that was assimilated by the roots was not directly supplied to the nodules. (2) Based on observations of the shoots and one side of the roots and nodules in the dual root system as an N translocation system, we proposed a method for calculating the N translocation from soybean shoots to roots and nodules during the R_1–R_5 stages based on the difference in the ^{15}N abundance. Our calculations showed that when adding N at a concentration of 50 mg·L^{-1}, the N translocated from the shoots during the R_1–R_5 stages accounts for 29.6%–52.3% of the N accumulation in nodulated roots (Root$_n$) and 9.4%–16.6% of the N accumulation in Nodule$_n$ of soybeans. Through the study of this experiment, the absorption, distribution and redistribution characteristics of fertilizer N and root nodule N fixation in soybean can be clarified, providing a theoretical reference for analyzing the mechanisms of the interaction between fertilizer N and nodule-fixed N.

Keywords: soybean dual root system; N accumulation; distribution; remobilization; NO_3^- ; NH_4^+

1. Introduction

Leguminous crops and rhizobia form nodules in soybean roots through complex interactions to efficiently fix atmospheric N for the N nutrient supply. However, achieving the goal of high yields in leguminous crops is not possible by relying solely on nodule-fixed N. The application of N fertilizer can considerably increase the yields of leguminous crops [1–4]. In peanut growing fields, the application of urea-N is a better way to increase the supply of N from root nodules and improves the N use efficiency [5]. Nonetheless, N application may inhibit nodule formation and N fixation [6–15]. Many researchers have reported that the application of NO_3-N reduces the weight of the root nodules because high levels of NO_3-N lead to a sharp decrease in the proportion of photosynthetic products transported to the root nodules and a corresponding increase to the stem and root [16–19]. In addition, Minchin et al. [18] and Carroll et al. [20] indicated that NO_3^- inhibits the nitrogenase activity in root nodules by increasing their O_2 diffusion barrier. Moreover, Munns [21] and Wahab et al. [22] considered that NO_3^- affects the number of root nodules by inhibiting root hair formation and rhizobial infections in leguminous crops. Gan et al. [23] found that applying a higher concentration of N fertilizer markedly reduced the nodule number and nodule-fixed N in soybeans, whereas a lower concentration of N fertilizer did the opposite.

Many experiments have used the split-root system [24]. Xia et al. [25] conducted a study using dual root system, in which a high concentration of N was added to one side of the roots and no N was added to the other side. They found that the number of root nodules decreased in the side receiving a high concentration of N, whereas the number increased in the side without added N. This finding shows that high N concentrations have a local contact effect in inhibiting the formation and growth of root nodules. Fujikake et al. [26] reported that following the addition of NO_3^- to soybeans, the diameter growth of the root nodules completely stopped; however, after NO_3^- withdrawal from the nutrient solution, the growth of the root nodules rapidly recovered to the original normal rate. This observation suggests that the NO_3^--induced inhibition of root nodule growth is a reversible process. Using a split-root system, Kosslak et al. [27] inoculated rhizobia into one side of soybean roots, and 10 days later, they inoculated the other side of the roots. They showed that earlier rhizobia inoculation on one side of the roots inhibited nodulation on the other side. Using peas, van Brussel et al. [28] came to a similar conclusion in that the presence of nodules on one side of the roots inhibits nodule formation on the other side, which shows autoregulation.

The N utilization rate of plants determined by the ^{15}N isotope tracer method can truly reflect the status of fertilizer utilization by plants. Oghoghorie and Pate [29] divided the pea roots into the upper and lower parts and separated the roots into different treatments, with ^{15}N-labeled N_2 being added to the upper roots in one treatment and ^{15}N-labeled NO_3^- being added to the upper roots in the other treatment. Following treatment, ^{15}N was detected not only in the shoots but also in the lower roots and nodules. Silva et al. [30] applied ^{15}N urea to soybean leaves and stems, and found that in 71 days after marking, the content of ^{15}N aboveground decreased with time, whereas it increased in the roots. Moreover, Oghoghorie and Pate [29] added ^{15}N-labeled NO_3^- onto the 3rd, 7th, and 12th leaves and detected ^{15}N in both the aboveground and belowground parts of the peas. Akria et al. [9] used the soybean double root system to supply $^{15}NO_3^-$ at different concentrations on one side and no N on the other side. This study found that ^{15}N markers were also detected in the roots and root nodules on the supplied side and increased with the increase in N concentration. Reynnolds et al. [31] used the soybean root-dividing system, where $^{13}NH_4^+$ was applied on one side and no N was supplied on the other side, and detected an abundance of ^{13}N amino acids in the N-supplied side of the root, and ^{13}N markers were detected on both the nodes and the supplied side, indicating that N in the leaf stem was also transported to the root and root nodules.

Previous studies have shown that the N absorbed externally by legumes is transferred to other organs in the plant, but most studies do not show the ratio of N accumulation and distribution in the plant. In this study, ^{15}N-labeled NO_3^- and NH_4^+ were added to the root system planted under sand culture conditions. The N accumulation and ^{15}N abundance in soybean plants at the R_1 and R_5 stages were measured and analyzed to understand how N is absorbed, distributed, and remobilized in soybeans. The results provide reference data to understand the characteristics of N translocation and unravel the systematic regulation of nodule formation in soybeans.

2. Materials and Methods

This study was conducted at an experimental area on the campus of Northeast Agricultural University in 2018 and 2019. The experimental area (N: 45°74′ and E: 126°73′) is located in the Xiangfang District of Harbin, Heilongjiang Province, China. The annual precipitation is 500–550 mm, and the ≥10 °C accumulated temperature is 2700 °C.

2.1. Experimental Design and Treatments

2.1.1. Preparation of Plant Materials with a Dual Root System

The soybean plants with dual root system was prepared based on the method in Xia et al. [25]. The treatments were conducted using plastic pots with a diameter of 0.3 m and a height of 0.3 m. Each pot was divided into two equal, independent spaces by vertically inserting a custom-made polycarbonate plate that was fitted for the inner shape of the pot and sealed with glue in the middle of the pot. The top of the partition plate was 2 cm below the rim of the pot. For each partitioned space, a drainage hole with a 1 cm diameter was drilled in the bottom of each pot. The hole was capped with a piece of gauze to prevent clogging by river sand. Each pot was filled with 20 kg of washed sand for cultivating the soybean plants with dual root.

Seeds of nodulated soybeans (*Glycine max* L. *cv*. Kenfeng 16) and non-nodulated soybeans (*Glycine max* L. *cv*. WDD01795, L8-4858, provided by the Crop Research Institute, Chinese Academy of Agricultural Sciences) were drilled into a fine sand medium at a depth of 2 cm and incubated in a growth chamber at 30 °C for 3 days. When the distance between the growing point of the cotyledon and the tip of the root was 7 to 10 cm, the roots of the soybean seedlings were rinsed with water and then used for grafting. Two seedlings of nodulated and non-nodulated soybeans were chosen and an incision of 0.5–1.0 cm (without cutting off) was made with a sterilized blade, which extended upward or downward slightly above the middle point of the hypocotyl. The non-nodulated seedling was cut from the cotyledon toward the root (Figure 1A), whereas the nodulated seedling was cut from the root toward the cotyledon (Figure 1B). The two seedlings were cross-inserted into their cuts (Figure 1C) and clipped with a grafting clip. The root system of the two seedlings were planted separately into the fine sand medium on both sides of the partition plate in the pot, with the grafting site exactly on the top of the partition plate. The grafted seedlings were allowed to grow inside a weather-tight enclosure for a week. The grafting clip was then removed and the upper part of each non-nodulated seedling was cut from the grafting site, leaving its combined site and lower parts. This procedure generated seedlings with dual root system (nodulated and non-nodulated) and the shoots of a nodulated cultivar. The plants were grown under farmland conditions and treated experimentally. (Figure 1D) shows the roots of a soybean plant with a dual root system at the time of sampling, with non-nodulated roots ($Root_{non}$) on the left and nodulated roots ($Root_n$) on the right side.

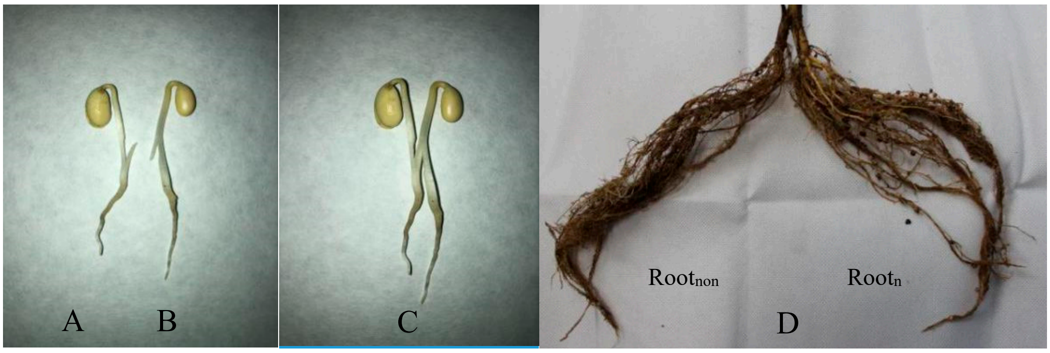

Figure 1. Soybean plant with dual root system. (**A**) Non-nodulated seedling, (**B**) nodulated seedling, (**C**) two seedlings cross-inserted into their cuts and clipped with a grafting clip, and (**D**) roots of a soybean plant with a dual root system at the time of sampling, with non-nodulated roots (Root$_{non}$) on the left and nodulated roots (Root$_n$) on the right side.

2.1.2. Experimental Treatments

Three treatments were conducted with NO_3^- and NH_4^+ as N sources (50 mg·L^{-1} each). In Treatment I, a nutrient solution containing ^{15}N-labeled NO_3^- or NH_4^+ was added to Root$_{non}$, whereas an N-free nutrient solution was added to Root$_n$. In Treatment II, a nutrient solution containing ^{15}N-labeled NO_3^- or NH_4^+ was added to Root$_{non}$, whereas a nutrient solution containing unlabeled NO_3^- or NH_4^+ was added to Root$_n$. In Treatment III, a nutrient solution containing ^{15}N-labeled NO_3^- or NH_4^+ was added to both Root$_{non}$ and Root$_n$. Three treatments are detailed in Table 1. The N free nutrient solution contained the following: 136 mg·L^{-1} KH_2PO_4, 240 mg·L^{-1} $MgSO_4$, 220 mg·L^{-1} $CaCl_2$, 4.9 mg·L^{-1} $MnCl_2.4H_2O$, 2.86 mg·L^{-1} H_3BO_3, 0.22 mg·L^{-1} $ZnSO_4.7H_2O$, 0.08 mg·L^{-1} $CuSO_4.5H_2O$, 0.03 mg·L^{-1} $Na_2MoO_4{\cdot}H_2O$, 5.57 mg·L^{-1} $FeSO_4{\cdot}7H_2O$, and 7.45 mg·L^{-1} Na_2EDTA. The NO_3^--containing nutrient solution was prepared by adding 360.7 mg·L^{-1} KNO_3 to the N-free nutrient solution, and the NH_4^+-containing nutrient solution was formulated by adding 235.7 mg·L^{-1}$(NH_4)_2SO_4$ to the N-free nutrient solution. The ^{15}N abundance of both ^{15}N-labeled NO_3^- and NH_4^+ was 3.63%.

Table 1. Experimental treatments.

	NO_3^-		**NH_4^+**	
	Root$_{non}$	**Root$_n$**	**Root$_{non}$**	**Root$_n$**
I	$^{15}NO_3^-$	N free	$^{15}NH_4^+$	N free
II	$^{15}NO_3^-$	$^{14}NO_3^-$	$^{15}NH_4^+$	$^{14}NH_4^+$
III	$^{15}NO_3^-$	$^{15}NO_3^-$	$^{15}NH_4^+$	$^{15}NH_4^+$

Before the full expansion of the opposite true leaves, the soybean seedlings were irrigated once a day with 250 mL of distilled water on each side of the root system [25]. Following the full expansion of the opposite true leaves, the seedlings were irrigated once a day with 250 mL of the corresponding nutrient solution on each side of the root system until the R_1 stage. From the R_1 stage on, the seedlings were irrigated twice a day, once in the morning and once in the evening, with 250 mL of the corresponding nutrient solution for each side of the root system until the end of the experiment. When the opposite true leaves were fully expanded, all the roots were inoculated with rhizobia as follows: Soybean root nodules harvested from the field during the previous year and stored in a freezer were washed, ground, and then added to the nutrient solutions at 5 g·L^{-1}. This method was used to inoculate soybean with soil rhizobia continuously for 5 days. Previous experiments showed that this method was feasible for soybean inoculation, and there would be spontaneous nodulation of rhizobia in the soil community [25,32]. The main N-fixing rhizobia in soil belonged to *Bradyrhizobium*, *Bradyrhizobium japonicum*, and *B. liaoningense* were the dominant bacteria [33].

2.2. Sampling and Parameter Analysis

Samples were taken at the R_1 and R_5 stages [34]. The plants were separated into different parts, deactivated at 105 °C for 30 min, and then dried at 85 °C. Dry samples were used to analyze the ^{15}N abundance, dry weight, and N content of each part.

Plant N content analysis: The plant N content was determined using a B324 automatic Kjeldahl analyzer after digestion with concentrated H_2SO_4 (K_2SO_4 and $CuSO_4$ as catalysts).

^{15}N abundance analysis: After plant N content analysis by the Kjeldahl method, the titrated samples were concentrated and allowed to react with lithium hypobromite to produce N_2 under freezing-vacuum conditions. The ^{15}N abundance was determined using a mass spectrometer (Thermo-Fisher Delta V Advantage IRMS) equipped with a dual-inlet system.

2.3. Data Calculations

The percent of ^{15}N-labeled N derived from fertilizer ($^{15}Ndff\%$) in plants was calculated as:

$$^{15}\mathrm{Ndff}\% = \frac{\mathrm{f_{treatment}} - \mathrm{f_{nature}}}{\mathrm{f_{fertilizer}} - \mathrm{f_{nature}}} \times 100\% \tag{1}$$

where f_{nature} is the natural ^{15}N abundance, $f_{fertilizer}$ is the ^{15}N abundance of the fertilizer, and $f_{treatment}$ is the ^{15}N abundance of the treatment.

The percent of N derived from atmosphere (Ndfa%) in plants was calculated as:

$$\mathrm{Ndfa}\% = 1\text{-}^{15}\mathrm{Ndff}\% \tag{2}$$

The percent of ^{14}Nitrogen derived from fertilizer ($^{14}Ndff\%$) plus the percent of N derived from atmosphere (Ndfa%) was calculated as:

$$^{14}\mathrm{Ndff}\% + \mathrm{Ndfa}\% = 1\text{-}^{15}\mathrm{Ndff}\% \tag{3}$$

Based on the ^{15}N abundance of each organ, the ratio of N sources from ^{15}N, ^{14}N, and N-fixing root nodules in each organ can be calculated; if these values are then multiplied by the N accumulation, the N accumulation from ^{15}N, ^{14}N, and root nodules in each organ can be obtained.

2.4. Statistical Analyses

Descriptive statistics, one-way ANOVA, and correlation tests were performed on the data by IBM SPSS Software version 17.0. The results were mean ± standard deviation (SD) of three replicates. Duncan test was used for comparison between treatments ($\alpha = 0.05$). All data were tested for normality and homogeneity of variance.

3. Results

3.1. Ratio of Fertilizer N and Root Nodule N Fixation in Soybean Plants

3.1.1. ^{15}N Abundance (%) Difference in Soybean Plants with Dual Root

Table 2 shows the ^{15}N abundance in the various parts of soybean plants with dual root system for Treatments I, II, and III. The ^{15}N abundance in the vegetative organs of soybean plants differed significantly among the three treatments, indicating that the different treatments resulted in considerable differences in the ^{15}N abundance in various organs.

Table 2. ^{15}N abundance (%) in plant organs of soybean with dual root

Stages	Organs	NO_3^-			NH_4^+		
		I	II	III	I	II	III
R_1	$Root_{non}$	2.73 ± 0.11ab	2.58 ± 0.06b	2.91 ± 0.04a	2.79 ± 0.05b	2.59 ± 0.06c	2.99 ± 0.02a
	$Root_n$	0.96 ± 0.01b	0.77 ± 0.04c	2.45 ± 0.02a	1.01 ± 0.01b	0.71 ± 0.02c	2.70 ± 0.03a
	$Nodule_n$	0.63 ± 0.02b	0.58 ± 0.03b	0.88 ± 0.01a	0.62 ± 0.01b	0.56 ± 0.01c	0.96 ± 0.01a
	Shoot	1.45 ± 0.04b	1.34 ± 0.06b	2.16 ± 0.03a	1.51 ± 0.02b	1.34 ± 0.01c	2.38 ± 0.02a
R_5	$Root_{non}$	2.46 ± 0.09b	2.27 ± 0.08b	2.74 ± 0.01a	2.55 ± 0.17a	2.47 ± 0.11a	2.76 ± 0.09a
	$Root_n$	0.73 ± 0.02b	0.76 ± 0.03b	1.87 ± 0.08a	0.80 ± 0.04b	0.70 ± 0.03b	1.98 ± 0.05a
	$Nodule_n$	0.51 ± 0.00b	0.52 ± 0.02b	0.76 ± 0.00a	0.52 ± 0.01b	0.51 ± 0.01b	0.75 ± 0.01a
	Shoot	0.94 ± 0.01b	0.94 ± 0.03b	1.44 ± 0.03a	0.94 ± 0.02b	1.01 ± 0.02b	1.61 ± 0.04a

In the dual root system, $Root_{non}$ represents non-nodulated roots, $Root_n$ represents nodulated roots, and $Nodule_n$ represents nodules on the same side of nodulated roots. The values are the means ± standard error (n = 3). Different lowercase letters indicate significant differences between treatments at the 5% level. Treatments I, II, and III are compared horizontally.

In Treatment I, under NO_3^- and NH_4^+ N sources, the ^{15}N abundance in $Root_{non}$ at the R_1 and R_5 stages was lower than that of the N fertilizer (3.63%). However, the ^{15}N abundance in $Root_n$ and $Nodule_n$ at the R_1 and R_5 stages remained higher than the natural ^{15}N abundance (0.365%). The ^{15}N abundance in the soybean Shoot at the R_1 stage was higher than the natural ^{15}N abundance (0.365%) and lower than the ^{15}N abundance of fertilizer N (3.63%). In Treatments II and III, under NO_3^- and NH_4^+ N sources, the ^{15}N abundance at the R_1 and R_5 stages in $Root_{non}$ was lower than that of fertilizer N (3.63%) and higher than the natural ^{15}N abundance (0.365%). There were no significant differences in the ^{15}N abundance between the NO_3^- and NH_4^+ N sources for Treatments I, II, and III. However, during three treatments, the ^{15}N abundance in all the soybean organs under different treatments at R_5 was lower than it was at the R_1 stage.

3.1.2. Ratio of N Absorbed from Different Sources in Dual Root Soybeans

Table 3 shows that for Treatment I under NO_3^- and NH_4^+ sources at the R_1 and R_5 stages, most of the N in $Root_{non}$ came from the fertilizer N that was self-absorbed by the roots. However, these results illustrate that at the R_1 and R_5 stages, most of the N in $Root_n$ and $Nodule_n$ came from the nodule-fixed N in this root system. At the R_1 and R_5 stages, nodule-fixed N contributed a larger proportion to the supply for the Shoot.

Table 3. Ratio of N absorbed from different sources in dual root soybeans (%).

	Stages	Organs	NO_3^-		NH_4^+	
			^{15}Ndff%	Ndfa%	^{15}Ndff%	Ndfa%
I	R_1	$Root_{non}$	72.4 ± 3.46a	27.6 ± 3.46b	74.2 ± 1.58a	25.8 ± 1.58b
		$Root_n$	18.3 ± 0.34b	81.7 ± 0.34a	19.7 ± 0.34b	80.3 ± 0.34a
		$Nodule_n$	8.0 ± 0.51b	92.0 ± 0.51a	7.9 ± 0.33b	92.1 ± 0.33a
		Shoot	33.1 ± 0.27b	66.9 ± 0.27a	34.9 ± 0.60b	65.1 ± 0.6a
	R_5	$Root_{non}$	64.1 ± 2.74a	35.9 ± 2.74b	67.0 ± 5.21a	33.0 ± 5.21b
		$Root_n$	11.2 ± 0.54b	88.8 ± 0.54a	13.3 ± 1.15b	86.7 ± 1.15a
		$Nodule_n$	4.3 ± 0.10b	95.7 ± 0.10a	4.8 ± 0.18b	95.2 ± 0.18a
		Shoot	17.6 ± 0.27b	82.4 ± 0.26a	17.6 ± 0.28b	82.4 ± 0.28a
			^{15}Ndff%	^{14}Ndff%+Ndfa%	^{15}Ndff%	^{14}Ndff%+Ndfa%
II	R_1	$Root_{non}$	68.0 ± 1.92a	32.0 ± 1.92b	68.1 ± 1.81a	31.9 ± 1.81b
		$Root_n$	12.4 ± 1.24b	87.6 ± 1.24a	10.7 ± 0.63b	89.3 ± 0.63a
		$Nodule_n$	6.6 ± 0.80b	93.4 ± 0.80a	6.0 ± 0.30b	94.0 ± 0.30a
		Shoot	30.0 ± 0.49b	70.0 ± 0.49a	30.0 ± 0.35b	70.0 ± 0.35a
	R_5	$Root_{non}$	58.5 ± 2.58a	41.5 ± 2.58b	64.6 ± 3.42a	35.4 ± 3.42b
		$Root_n$	12.2 ± 0.88b	87.8 ± 0.88a	10.2 ± 0.95b	89.8 ± 0.95a
		$Nodule_n$	4.7 ± 0.42b	95.3 ± 0.42a	4.6 ± 0.28b	95.4 ± 0.28a
		Shoot	17.6 ± 0.66b	82.4 ± 0.66a	19.8 ± 0.37b	80.2 ± 0.37a
			^{15}Ndff%	Ndfa%	^{15}Ndff%	Ndfa%
III	R_1	$Root_{non}$	78.0 ± 1.15a	22.0 ± 1.15b	80.5 ± 0.68a	19.5 ± 0.68b
		$Root_n$	63.7 ± 0.66a	36.3 ± 0.66b	71.4 ± 0.96a	28.6 ± 0.96b
		$Nodule_n$	15.8 ± 0.25b	84.2 ± 0.25a	18.3 ± 0.30b	81.7 ± 0.30a
		Shoot	55.0 ± 1.23a	45.0 ± 1.23b	61.6 ± 1.48a	38.4 ± 1.48b
	R_5	$Root_{non}$	72.7 ± 0.18a	27.3 ± 0.18b	73.4 ± 2.71a	26.6 ± 2.71b
		$Root_n$	46.0 ± 2.52a	54.0 ± 2.52a	49.6 ± 1.41a	50.4 ± 1.41a
		$Nodule_n$	11.9 ± 0.04b	88.1 ± 0.04a	11.8 ± 0.31b	88.2 ± 0.31a
		Shoot	32.9 ± 0.88b	67.1 ± 0.88a	38.1 ± 0.78b	61.9 ± 0.78a

In the dual root system, $Root_{non}$ represents non-nodulated roots, $Root_n$ represents nodulated roots, and $Nodule_n$ represents nodules on the same side as the nodulated roots. ^{15}Ndff% represents the proportion of ^{15}N-labeled fertilizer N, Ndfa% represents the proportion of nodule-fixed N, and ^{14}Ndff%+Ndfa% represents the proportion of nodule-fixed N plus unlabeled fertilizer N. The values are the means ± standard error (n = 3). Different lowercase letters indicate significant differences between treatments at the 5% level. The two N sources are compared horizontally.

In Treatment II under NO_3^- and NH_4^+ N sources, the results indicate that when N was added to both sides of the roots, the N in $Root_{non}$ primarily came from fertilizer N that was self-absorbed by the roots with small proportions from absorbed fertilizer N and nodule-fixed $Root_n$ N (translocated from the Shoot). A comparison with Treatment I revealed that there was little difference in the nutrient proportions of different N sources in $Root_{non}$ as contributed by the two roots of the dual root system with an addition to both sides versus one side. Furthermore, the proportions of different N sources in various parts of the soybean plants in Treatments I and II were compared. Similarly, the nutrient proportions contributed by the two roots of the dual root system did not change markedly in the $Root_n$, $Nodule_n$, or Shoot. These results indicate that the soybean plants contributed similarly to the plant N with or without N addition, showing the integrity of fertilizer N absorption and nodule N fixation.

No significant differences were detected in the proportions of various N sources between the NO_3^- and NH_4^+ N sources among Treatments I, II, and III, indicating that the nutritional effect of NO_3^- vs. NH_4^+ addition was not markedly different in soybean plants. However, compared with the R_1 stage, the R_5 stage was associated with a higher proportion of nodule-fixed N in various organs under different conditions for Treatments I, II, and III, indicating a larger contribution of nodule-fixed N to soybean plants at the R_5 stage than at the R_1 stage.

In both Treatments II and III, the same N concentration was added to both sides of the root system, and the only difference was related to the ^{15}N abundance. To distinguish among the three N sources in Treatment II, we calculated the proportion of absorbed ^{14}N-labeled fertilizer N in various parts of the soybean plants by summing up the proportion of absorbed ^{14}N-labeled fertilizer N and the proportion of N from nodule fixation in each part of the soybeans from Treatment II and subtracting the proportion of N from nodule fixation in the same parts of the soybean plants from Treatment III (Table 4).

Table 4. Proportions of N from different sources in various organs of soybean plants in Treatment II (%).

Stages	Organs	NO_3^-			NH_4^+		
		^{15}Ndff%	^{14}Ndff%	Ndfa%	^{15}Ndff%	^{14}Ndff%	Ndfa%
R_1	$Root_{non}$	68.0 ± 1.92a	10.0 ± 2.83c	22.0 ± 1.15b	68.1 ± 1.81a	12.4 ± 1.84c	19.5 ± 0.68b
	$Root_n$	12.4 ± 1.24c	51.3 ± 0.81a	36.3 ± 0.66b	10.7 ± 0.63c	60.7 ± 0.54a	28.6 ± 0.96b
	$Nodule_n$	6.6 ± 0.80c	9.2 ± 1.00b	84.2 ± 0.25a	6.0 ± 0.30c	12.3 ± 0.60b	81.7 ± 0.30a
	Shoot	30.0 ± 0.49b	25.0 ± 1.68c	45.0 ± 1.23a	30.0 ± 0.35b	31.6 ± 1.18b	38.4 ± 1.48a
R_5	$Root_{non}$	58.5 ± 2.58a	14.2 ± 2.63c	27.3 ± 0.18b	64.6 ± 3.42a	8.8 ± 0.72c	26.6 ± 2.71b
	$Root_n$	12.2 ± 0.88c	33.8 ± 2.63b	54.0 ± 2.52a	10.2 ± 0.95c	39.4 ± 0.52b	50.4 ± 1.41a
	$Nodule_n$	4.7 ± 0.42c	7.2 ± 0.46b	88.1 ± 0.04a	4.6 ± 0.28c	7.2 ± 0.03b	88.2 ± 0.31a
	Shoot	17.6 ± 0.66b	15.3 ± 1.20b	67.1 ± 0.88a	19.8 ± 0.37b	18.3 ± 0.57b	61.9 ± 0.78a

In the dual root system, $Root_{non}$ represents non-nodulated roots, $Root_n$ represents nodulated roots, and $Nodule_n$ represents nodules on the same side as the nodulated roots. ^{15}Ndff% represents the proportion of ^{15}N-labeled fertilizer N, ^{14}Ndff% represents the proportion of unlabeled fertilizer N, and Ndfa% represents the proportion of nodule-fixed N. The values are the means ± standard error (n = 3). Different lowercase letters indicate significant differences between treatments at the 5% level. Three N sources are compared horizontally.

In Treatment II, there were three N sources for soybean plants, i.e., absorbed fertilizer N from $Root_{non}$, absorbed fertilizer N from $Root_n$, and nodule-fixed N from $Root_n$. Under the NO_3^- and NH_4^+ N sources in the R_1 stage, $Root_{non}$ preferentially absorbed the self-assimilated N. At the R_1 stage, 51.3% and 60.7%, respectively, were contributed by absorbed $Root_n$ fertilizer N, and 36.3% and 28.6%, respectively, were contributed by nodule-fixed $Root_n$ N. At the R_5 stage, the corresponding proportions were 33.8% and 39.4%, 54.0% and 50.4% for absorbed fertilizer N from $Root_{non}$, absorbed fertilizer N from $Root_n$, and nodule-fixed N from $Root_n$, respectively. These results show that when N was added to both sides of the root system, $Root_n$ also preferentially absorbed the N that was assimilated by this root system; the proportion of nodule-fixed N in $Root_n$ increased with the increasing N fixation capacity of the nodules. For the N in $Nodule_n$ at the R_1 and R_5 stages, nodule-fixed N was preferentially absorbed by $Nodule_n$. Moreover, the proportion of N in the root nodules contributed by the absorbed fertilizer N of the two roots of the dual root system was significantly different; that is, the N from $Root_{non}$ was less than that from $Root_n$. These results show that when N was added to both sides of the root system, the Shoot primarily absorbed fertilizer N at the R_1 stage, whereas nodule-fixed N was primarily absorbed during the R_5 stage. The supplies of fertilizer N from the two roots of the dual root system to the Shoot were almost identical, suggesting the same supply of absorbed fertilizer N to Shoot by each side in the dual root system.

A comparison of Treatments I and II revealed no major change in the proportions of N supplied to the Shoot by each root of the dual root system, irrespective of whether N was added to one or both sides. However, after the addition of N to both sides of the root system, the proportion of nodule-fixed N decreased, whereas the proportions of absorbed NO_3^- and NH_4^+ supplied to the Shoot by $Root_{non}$ and $Root_n$ were similar.

3.2. N Accumulation and Source in Dual Root Soybeans

3.2.1. N Accumulation in Dual Root Soybeans

Table 5 shows the N accumulation of organs in the dual root system of single-nodule soybeans. In Treatments I, II, and III under the NO_3^- N source for soybean plants, the N accumulation of $Root_{non}$

at the R_1 stage was significantly higher in Treatment III than in Treatments I and II. During the R_5 stage, there was no significant difference among Treatments I, II, or III. At the R_1 stage, the N accumulation of $Root_n$ was significantly lower in Treatment I than in Treatments II and III. During the R_5 stage, there was no significant difference among Treatments I, II, and III. The N accumulation of $Nodule_n$ at the R_1 and R_5 stages was significantly higher in Treatment I than in Treatment II or III. In Treatment I, the N accumulation in the Shoot at R_1 was significantly lower than in Treatment II or III, and that at R_5 it was significantly lower than in Treatment II. The total N accumulation of Treatment I at the R_1 stage was significantly lower than in Treatment II or III, and the difference at the R_5 stage was not significant. In Treatments I, II, and III under the NH_4^+ N source for soybean plants, the N accumulation of $Root_{non}$ in Treatment I at the R_1 and R_5 stages was significantly lower than in Treatments II and III. At the R_1 stage, the N accumulation of $Root_n$ in Treatment I was significantly lower than in Treatments II and III, whereas the difference was not significant at the R_5 stage. The N accumulation of $Nodule_n$ in Treatment I at the R_1 and R_5 stages was significantly higher than in Treatments II and III. At the R_1 stage, the N accumulation in the Shoot of Treatment I was significantly lower than in Treatments II and III. The R_1 and R_5 stages showed the opposite pattern. In Treatment I, the N accumulation of the Shoot in the R_1 stage was significantly lower than that in Treatments II and III, and the difference at the R_5 stage was not significant.

Table 5. N accumulation in each organ of soybean in the dual root system (mg/plant).

Stages	Organs	NO_3^-			NH_4^+		
		I	II	III	I	II	III
R_1	**$Root_{non}$**	18.08 ± 1.19c	23.38 ± 1.40b	25.20 ± 0.77a	20.66 ± 1.76b	25.05 ± 1.55a	23.15 ± 2.39ab
	$Root_n$	22.54 ± 1.15b	31.19 ± 2.71a	34.53 ± 1.30a	26.52 ± 1.00b	39.69 ± 1.23a	38.29 ± 1.22a
	$Nodule_n$	35.79 ± 2.34a	23.28 ± 2.63b	24.70 ± 2.20b	33.51 ± 0.83a	19.66 ± 2.18b	20.94 ± 1.87b
	Shoot	355.50 ± 5.98b	418.10 ± 14.75a	432.48 ± 7.85a	312.18 ± 16.06b	408.87 ± 12.33a	401.98 ± 6.56a
	Total	431.91 ± 10.66b	495.95 ± 21.49a	516.91 ± 12.12a	392.87 ± 19.65b	493.27 ± 17.29a	484.36 ± 12.04a
R_5	**$Root_{non}$**	46.89 ± 4.92a	41.80 ± 4.86a	37.05 ± 4.91a	37.28 ± 0.49b	51.63 ± 7.80a	47.72 ± 4.97ab
	$Root_n$	69.22 ± 3.15a	76.83 ± 7.06a	82.07 ± 7.89a	68.46 ± 6.79a	83.41 ± 5.54a	78.15 ± 10.63a
	$Nodule_n$	109.14 ± 9.68a	76.94 ± 8.53b	88.15 ± 6.63ab	102.49 ± 5.37a	67.09 ± 6.84b	72.07 ± 7.57b
	Shoot	1012.07 ± 28.58b	1105.38 ± 48.17a	1045.11 ± 32.50ab	1169.88 ± 32.55a	1023.60 ± 32.29b	1041.89 ± 23.84b
	Total	1237.32 ± 46.33a	1300.95 ± 68.62a	1252.38 ± 51.93a	1378.11 ± 45.20a	1225.73 ± 52.47a	1240.1 ± 47.01a

The values are the means ± standard error (n = 3). Different lowercase letters indicate significant differences between treatments at the 5% level. Treatments I, II, and III are compared horizontally.

A comparison of Treatments I, II, and III showed that the N accumulation of $Root_{non}$ and $Root_n$ at the R_1 stage in Treatment I was lower than in Treatments II and III, whereas the N accumulation at the R_5 stage was not significantly different among the treatments. This finding indicates that local N application in the early stage of soybean growth affects the accumulation of N in the root, but local N application in the late stage of soybean growth has a weak effect on the accumulation of N in the root, possibly because the ratio of N fixation in the root nodules was increasing during the late stage of soybean growth. N accumulation of $Nodule_n$ in Treatment I was significantly higher than in Treatments II and III. This result indicates that the local application of N can inhibit the growth of root nodules on the N-treated side and promotes the growth of root nodules on the N-treated side, thus improving the N-fixing ability of root nodules and leading to an increase in N accumulation. There was no significant difference in N accumulation in each part of the treatment with a NO_3^- vs. NH_4^+ N source, indicating that under an N concentration of 50 $mg{\cdot}L^{-1}$, there was no difference in the nutritional effect of NO_3^- vs. NH_4^+ on each part.

3.2.2. Accumulation of Fertilizer N and N-Fixing Root Nodules in Dual Root Soybeans

As seen from Table 6, when N was applied on one side, the majority of $Root_{non}$ N came from the fertilizer N absorbed by $Root_{non}$, whereas a small part came from the root nodules of $Root_{n.}$ When N was not applied to $Root_n$, most of the N came from root nodule N fixation, and a small part came from the fertilizer N absorbed by $Root_{non}$. Under unilateral N application to soybean roots, most of the N in

$Nodule_n$ came from the N-fixing root nodules of $Root_n$, and a small part came from fertilizer N absorbed from $Root_{non}$, which also indicates that not all N needed for root nodule growth came from internal N fixation and some N needed to be absorbed from the roots. In comparing the two N sources in the R_1 and R_5 stages, N fixation from the root nodules was significantly higher than N from fertilizer N absorbed by $Root_{non}$. The total N accumulation from N-fixing root nodules was much higher than that from fertilizer.

Table 6. The accumulation of N fixation from fertilizer and nodule-fixed N in each parts of the dual root soybean (mg/plant).

	Stages	Organs	NO_3^-		NH_4^+	
			^{15}N	Nodule	^{15}N	Nodule
I	R_1	$Root_{non}$	13.10 ± 0.63a	4.99 ± 0.62b	15.32 ± 0.33a	5.34 ± 0.33b
		$Root_n$	4.13 ± 0.08b	18.41 ± 0.08a	5.23 ± 0.09b	21.29 ± 0.09a
		$Nodule_n$	2.87 ± 0.18b	32.92 ± 0.18a	2.64 ± 0.11b	30.88 ± 0.11a
		Shoot	116.7 ± 3.45b	238.8 ± 3.45a	109.99 ± 1.25b	202.18 ± 1.25a
		Total	136.8 ± 4.33b	295.12 ± 4.33a	133.27 ± 1.78b	259.69 ± 1.78a
	R_5	$Root_{non}$	30.04 ± 1.28a	16.85 ± 1.28b	24.97 ± 1.94a	12.30 ± 1.94b
		$Root_n$	7.74 ± 0.37b	61.49 ± 0.37a	9.11 ± 0.78b	59.34 ± 0.79a
		$Nodule_n$	4.68 ± 0.11b	104.46 ± 0.11a	4.87 ± 0.18b	97.63 ± 0.18a
		Shoot	178.12 ± 3.78b	833.95 ± 3.78a	222.20 ± 4.69b	947.67 ± 4.69a
		Total	220.58 ± 5.54b	1016.75 ± 5.54a	261.15 ± 7.59b	1116.94 ± 7.59a

	Stages	Organs	^{15}N	^{14}N	Nodule	^{15}N	^{14}N	Nodule
II	R_1	$Root_{non}$	15.90 ± 0.43a	2.34 ± 0.63c	5.14 ± 0.26b	17.06 ± 0.45a	3.11 ± 0.46c	4.88 ± 0.17b
		$Root_n$	3.87 ± 0.39c	16.00 ± 0.25a	11.32 ± 0.21b	4.25 ± 0.25c	24.09 ± 0.21a	11.35 ± 0.38b
		$Nodule_n$	1.54 ± 0.19c	2.14 ± 0.23b	19.60 ± 0.06a	1.18 ± 0.06c	2.42 ± 0.12b	16.06 ± 0.06a
		Shoot	125.43 ± 6.71b	104.53 ± 5.86c	188.14 ± 3.18a	122.66 ± 2.01c	129.20 ± 2.89b	157.01 ± 3.00a
		Total	146.74 ± 7.72b	125.01 ± 6.97c	224.20 ± 3.71a	145.15 ± 2.77c	158.82 ± 3.68b	189.30 ± 3.61a
	R_5	$Root_{non}$	24.45 ± 1.08a	5.94 ± 1.10c	11.41 ± 0.07b	33.35 ± 1.77a	4.54 ± 0.37c	13.73 ± 1.40b
		$Root_n$	9.37 ± 0.67c	25.97 ± 2.02b	41.49 ± 1.94a	8.51 ± 0.79c	32.86 ± 0.43b	42.04 ± 1.17a
		$Nodule_n$	3.62 ± 0.32c	5.54 ± 0.35b	67.78 ± 0.03a	3.09 ± 0.19c	4.83 ± 0.02b	59.17 ± 0.21a
		Shoot	194.55 ± 8.10b	169.12 ± 10.64c	741.71 ± 8.15a	202.67 ± 5.96b	187.32 ± 5.90c	633.61 ± 9.24a
		Total	231.99 ± 10.17b	206.57 ± 14.11c	862.39 ± 10.19a	247.62 ± 8.71b	229.55 ± 6.72b	748.55 ± 12.02a

In the dual root system, $Root_{non}$ represents non-nodulated roots, $Root_n$ represents nodulated roots, and $Nodule_n$ represents nodules on the same side as the nodulated roots. ^{15}N represents the N accumulation of ^{15}N-labeled fertilizer N, ^{14}N represents the N accumulation of unlabeled fertilizer N, and Nodule represents the N accumulation of nodule-fixed N. The values are the means ± standard error (n = 3). Different lowercase letters indicate significant differences between treatments at the 5% level. The three N sources are compared horizontally.

Under the condition of bilateral N application, most of the N in $Root_{non}$ came from the fertilizer N absorbed by $Root_{non}$, and a small part came from the N absorbed and fixed by $Root_n$. However, most of the N in $Root_n$ came from the N absorbed and fixed by $Root_n$, whereas a small part came from the fertilizer N absorbed by $Root_{non}$ and transferred to $Root_n$. Most of the N in $Nodule_n$ came from N absorbed and fixed by $Root_n$, and a small part came from fertilizer N absorbed by $Root_{non}$ and transferred to $Root_n$ nodules. At the same time, it can be observed that not all the N required for root nodule growth comes from self-fixing N, and it is also necessary to absorb N via the roots. In comparing the three N sources in the Shoot at the R_1 and R_5 stages, there was no significant difference between N accumulation in $Root_{non}$ and $Root_n$, but N accumulation was significantly lower than in the N-fixing root nodules of $Root_n$. This suggests that the supply of absorbed fertilizer N to Shoot was the same from the two parts of the dual root systems. As a whole, the total accumulation of N absorbed and fixed by $Root_n$ was much higher than the fertilizer N absorbed by $Root_{non}$, mainly because $Root_{non}$ had no root nodules for N fixation.

3.2.3. N Translocation from Shoot to Roots and Nodules of Soybeans

The fertilizer N absorbed by the two roots of the dual root system and the N fixed by the root nodules were transported to the Shoot, and after assimilation, they were transported to the roots and nodules in certain forms. Due to the different ^{15}N abundance in various parts of the soybean plants in the treatments (Table 2), we regarded the $Root_n$ and the Shoot as one system, in which the Shoot served as a source of ^{15}N for the $Root_n$. From the R_1 to the R_5 stage, the amount of ^{15}N increasing in $Root_n$ can be obtained from the ^{15}N accumulation of the R_5 stage minus the ^{15}N accumulation of the R_1 stage, and this should be equal to the amount of ^{15}N translocated from the Shoot plus the amount of naturally occurring ^{15}N that was self-absorbed by the roots of $Root_n$ (including the supply by nodules) from the R_1 to the R_5 stage. If we let the amount of N translocated from the Shoot to $Root_n$ be x, then the ^{15}N abundance of the N translocated downwards from the Shoot can be calculated using the mean ^{15}N abundance in the Shoot at the R_1 and R_5 stages, where $x \times (f_{shootR1}+f_{shootR5})/2$ represents the amount of ^{15}N translocated from the Shoot to $Root_n$ during R_1–R_5, N_{R5}–N_{R1} represents the N accumulation in $Root_n$ during R_1–R_5, N_{R1}-N_{R5}-x represents the N accumulation from $Root_n$ absorbed during R_1–R_5 (including the N supplied by nodules), $(N_{R5}–N_{R1}–x) \times f_{nature}$ represents the amount of ^{15}N in $Root_n$ that is self-absorbed by the roots and supplied by the nodules during R_1–R_5, $N_{R5} \times f_{R5}$ represents the total ^{15}N in $Root_n$ at the R_5 stage, and $N_{R1} \times f_{R1}$ represents the total ^{15}N in $Root_n$ at the R_1 stage. This method can also be used to calculate the amount of N translocated from the Shoot to $Nodule_n$. For Treatments I and II, the calculation is as follows:

$$x \times (f_{shootR1} + f_{shootR5})/2 + (N_{R5}\text{-}N_{R1}\text{-}x) \times f_{nature} = N_{R5} \times f_{R5}\text{-}N_{R1} \times f_{R1}$$

$$x = \frac{N_{R5} \times f_{R5} - N_{R1} \times f_{R1} - (N_{R5} - N_{R1}) \times f_{nature}}{(f_{shootR1} + f_{shootR5})/2 - f_{nature}} \tag{4}$$

where x is the amount of N translocated from the Shoot to $Root_n$ or $Nodule_n$, N_{R1} is the N accumulation in $Root_n$ or $Nodule_n$ at the R_1 stage, N_{R5} is the N accumulation in $Root_n$ or $Nodule_n$ at the R_5 stage, f_{nature} is the natural ^{15}N abundance, f_{R1} is the ^{15}N abundance in $Root_n$ or $Nodule_n$ at the R_1 stage, f_{R5} is the ^{15}N abundance in $Root_n$ or $Nodule_n$ at the R_5 stage, $f_{shootR1}$ is the ^{15}N abundance in the Shoot at the R_1 stage, and $f_{shootR5}$ is the ^{15}N abundance in the Shoot at the R_5 stage.

Similarly, when calculating the amount of N translocated from the Shoot to $Root_{non}$, we regarded the $Root_{non}$ and the Shoot as one system. Because ^{15}N-labeled fertilizer N was added to $Root_{non}$, the calculation is as follows:

$$x \times (f_{shootR1} + f_{shootR5})/2 + (N_{R5}\text{-}N_{R1}\text{-}x) \times f_{fertilizer} = N_{R5} \times f_{R5}\text{-}N_{R1} \times f_{R1}$$

$$x = \frac{(N_{R5} - N_{R1}) \times f_{fertilizer} - N_{R5} \times f_{R5} + N_{R1} \times f_{R1}}{f_{fertilizer} - (f_{shootR1} + f_{shootR5})/2} \tag{5}$$

where x is the amount of N translocated from the Shoot to $Root_{non}$, N_{R1} is the N accumulation in $Root_{non}$ at the R_1 stage, N_{R5} is the N accumulation in $Root_{non}$ at the R_5 stage, $f_{fertilizer}$ is the ^{15}N abundance in the fertilizer, f_{R1} is the ^{15}N abundance in $Root_{non}$ at the R_1 stage, f_{R5} is the ^{15}N abundance in $Root_{non}$ at the R_5 stage, $f_{shootR1}$ is the ^{15}N abundance in the Shoot at the R_1 stage, and $f_{shootR5}$ is the ^{15}N abundance in the Shoot at the R_5 stage.

The amount of N translocated from the Shoot to $Root_{non}$, $Root_n$, and $Nodule_n$ can be calculated using Equations (4) and (5), as shown in Table 7.

Table 7. N translocation from the Shoot to roots and nodules of soybean plants from the R_1–R_5 stage.

	Organs	NO_3^-			NH_4^+		
		NIA (mg/plant)	NTFS (mg/plant)	NTFS/NIA (%)	NIA (mg/plant)	NTFS (mg/plant)	NTFS/NIA (%)
I	$Root_{non}$	28.8	16.1	55.9	16.6	9.5	57.0
	$Root_n$	46.8	13.9	29.6	41.9	14.6	34.9
	$Nodule_n$	73.4	6.9	9.4	68.9	8.4	12.2
	Total	148.9	36.9	24.8	127.4	32.5	25.5
II	$Root_{non}$	18.4	13.0	70.5	26.6	13.8	51.8
	$Root_n$	45.7	22.9	50.0	43.6	17.6	40.3
	$Nodule_n$	53.7	8.9	16.6	47.5	7.3	15.3
	Total	117.7	44.8	38.1	117.6	38.7	32.9

In the dual root system, $Root_n$ represents nodulated roots, $Root_{non}$ represents non-nodulated roots, and $Nodule_n$ represents nodules on the same side as the nodulated roots. ANT (mg) represents the accumulation of N translocated from the Shoot to roots and nodules during R_1–R_5, AIN (mg) represents the accumulation of increased N in various parts during R_1–R_5, and ANT/AIN (%) represents the proportion of total N from Shoot (NTFS) under AIN in various plant parts.

Table 7 shows that for Treatment I, in the NO_3^- and NH_4^+ N sources between the R_1 and R_5 stages, the accumulation of increased N in $Root_{non}$ was 28.8 and 16.6 mg, respectively, and the accumulation of N translocated from the Shoot to $Root_{non}$ was 16.1 and 9.5 mg, respectively. The N translocated from the Shoot to $Root_{non}$ accounted for 55.9% and 57.0%, respectively, of the increased accumulation of N in $Root_{non}$. In $Root_n$, the increased accumulation of N was 46.8 and 41.9 mg, respectively, and the accumulation of N translocated from the Shoot was 13.9 and 14.6 mg, accounting for 29.6% and 34.9%, respectively, of the increased accumulation of N in $Root_n$. In $Nodule_n$, the increased accumulation of N was 73.4 and 68.9 mg, respectively, and the accumulation of N translocated from the Shoot was 6.9 and 8.3 mg, contributing to 9.4% and 12.2%, respectively, of the increased accumulation of N in $Nodule_n$. In Treatment II with NO_3^- and NH_4^+ N sources between the R_1 and R_5 stages, the increased accumulation of N in $Root_{non}$ was 18.4 and 26.6 mg, respectively, and the accumulation of N translocated from the Shoot to $Root_{non}$ was 13.0 and 13.8 mg, making up 70.5% and 51.8%, respectively, of the increased accumulation of N in $Root_{non}$. In $Root_n$, the increased accumulation of N was 45.7 and 43.6 mg, respectively, and the accumulation of N translocated from the Shoot was 22.9 and 17.6 mg, accounting for 50.0% and 40.3%, respectively, of the increased accumulation of N in $Root_n$. In $Nodule_n$, the increased accumulation of N was 53.7 and 47.5 mg, respectively, and the accumulation of N translocated from the Shoot was 8.9 and 7.3 mg, contributing to only 16.6% and 15.3%, respectively, of the increased accumulation of N in $Nodule_n$.

4. Discussion

4.1. Sources of N in the Roots and Nodules of Soybeans

After adding $^{15}NO_3^-$ to soybean (*Glycine max L. Merr.*) seedlings, Crafts-Brandner and Harper [35] detected ^{15}N in the reduced N from xylem sap and found that the ^{15}N abundance tended to increase with time, leading to a conclusion that soybean roots can reduce $^{15}NO_3^-$. Sprent and Thomas [36] indicated that *Phaseolus vulgaris* and *Glycine max* directly absorb and transport fertilizer NO_3^- into the shoots for assimilation, whereas *Pisum sativum* and *Vicia faba* transport NO_3^- into the shoots after assimilation in the roots. Following the application of different concentrations of NO_3^- to six leguminous crops, Andrews [37] found that the assimilation of absorbed NO_3^- occurred primarily in the roots of *Cajanus cajan*, *Lupinus albus*, *Trifolium repens*, and *Pisum sativum*, whereas *Glycine max*, and *Phaseolus vulgaris* primarily assimilated the absorbed NO_3^- in the shoots. Kiyomiya et al. [38] treated the roots of rice with $^{13}NH_4^+$ and then observed the ^{13}N at the bottoms of the shoots within 2 min. However, the rapid upward transport of ^{13}N was inhibited after the addition of glutamine synthetase inhibitor, indicating that most of the NH_4^+ was assimilated in the roots. In the present study, under $^{15}NO_3^-$ and

$^{15}NH_4^+$ treatments, the ^{15}N abundance in the roots on the N-receiving side was always higher than in the shoots, indicating that part of the $^{15}NO_3^-$ and $^{15}NH_4^+$ absorbed by the roots was used for root growth after assimilation in the roots (otherwise, if all the absorbed fertilizer N was transported to the shoots for assimilation, the ^{15}N abundance would have been the same in both the shoots and roots). Tanaka et al. [9] and Reynolds et al. [31] established split-root system in soybeans and added $^{15}NO_3^-$ and $^{13}NH_4^+$ to one side of the roots, and they then detected the ^{15}N and ^{13}N in the roots and nodules on the N-free side. In the present study, we added ^{15}N-labeled fertilizer N to non-nodulated roots on one side of the dual root system, and yet a ^{15}N concentration higher than the natural abundance was detected in both the nodulated roots and root nodules on the other side. This finding indicates that the N absorbed by the roots on one side was translocated to the roots and nodules on the other side via the Shoot.

Sato et al. [39] added $^{13}NO_3^-$ to the culture nutrient solution of nodulated soybeans and then recorded data at 1-min intervals for 1 h. They found that ^{13}N-labeled NO_3^- first appeared in soybean petioles and then in the leaves, with little detected in the nodules. This observation suggests that the NO_3^- absorbed by the roots was not translocated into the nodules within a short period of time. In Treatments I and II, we added ^{15}N-labeled fertilizer N to non-nodulated roots in the dual root system, and yet a higher ^{15}N was detected in the nodules on the other side than would be naturally available (0.365%). By combining these findings with the results of Sato et al. [39], we believe that the absorbed fertilizer N present in the nodules was translocated from the Shoot, rather than directly absorbed and supplied by the roots. In Treatment III, we added the same concentration of ^{15}N-labeled fertilizer N to both sides of the dual root system. The ^{15}N abundance in $Root_{non}$ was higher than that in $Root_n$ at both the R_1 and R_5 stages, reflecting that nodule-fixed N was directly supplied to $Root_n$ and nodule growth.

Wery et al. [40] conducted an experiment using alfalfa with and without NH_4^+ addition, and no significant difference was found in N accumulation between the two treatments. However, with an N supply, the rate of N fixation in the nodules decreased, whereas the absorbed N increased. This phenomenon indicates that alfalfa preferentially selected combined N in the presence of both combined N and N_2. In Treatment I, 50 mg·L^{-1} N was supplied to $Root_{non}$, and in Treatment II, there was a bilateral supply of 50 mg·L^{-1} N to $Root_{non}$ and $Root_n$. It was found that the ratio of $Root_n$ N supplied to aboveground N supply at the R_1 and R_5 stages was similar, but the proportion of nodule-fixed N supplied to aboveground N supply in root nodules was smaller; this further indicates that the fertilizer N was preferentially selected by soybeans in the presence of a fertilizer N supply.

Many researchers believe that N application can considerably increase soybean yields [1–4], yet there are divergent opinions regarding the differences in the effects of NO_3^- and NH_4^+ on plants. In a study using the soybean split-root system, Chaillou et al. [41] found that the dry weight of the roots on one side that received NO_3^- was higher than on the other side that received NH_4^+. However, Gan et al. [23] treated soybeans with NO_3^- and NH_4^+ and found that NH_4^+ application alone resulted in a higher biomass accumulation, nodule dry weight, total N accumulation, and N fixation in this crop. Saravitz et al. [42] found that when NH_4^+ and NO_3^- were applied to the dual roots of soybean, the cumulative absorption of NH_4^+ was about half that of NO_3^-. Abdellaoui et al. [43] believed that both NH_4^+ and NO_3^- were easily absorbed by the root system in wheat seedlings, but only NO_3^- could accumulate in plants. The phloem in the root of the castor oil plant only absorbs NO_3^-, not ammonium salt, and NO_3^- is rapidly transported in xylem [44]. Although plants can effectively use NH_4^+, it is generally considered that NO_3^- is the main absorption form of plant N, mainly because NO_3^- is more soluble [45]. Savvas et al. [46] found that NH_4^+ could be nitrated in both soil and a hydroponic nutrient solution and that NO_3^- could be formed by nitration even when NH_4^+ was supplied. Kumar [47] suggested that NH_4^+ was more easily converted into NO_3^- in soils with good ventilation. In this experiment, no significant differences were observed in the ^{15}N abundance or N accumulation in various parts of the soybean plants after NO_3^- and NH_4^+ treatments, indicating that there were no major differences in the effects of NO_3^- and NH_4^+ on N nutrition in soybeans under the experimental conditions (50 mg·L^{-1} N addition). This may be because NH_4^+ was nitrated to NO_3^-.

4.2. N Distribution in Shoot, Roots, and Nodules of Soybean Plants

In this study, a significantly higher N accumulation was observed in the Shoot than in the roots and nodules of soybeans (Table 6), suggesting that the N absorbed by the roots and fixed by the nodules was primarily transported to the aboveground part for Shoot growth, with only a small fraction supplied to the roots and nodules growth. After adding $^{15}NO_3^-$ or $^{15}NH_4^+$ to the leaf surface of sunflower, Ito, O. et al. [48] determined the ^{15}N abundance in N-treated leaves and their upper and lower internodes. ^{15}N was detected in both the upper and lower internodes, with a lower value for the upper than for the lower internode, revealing that the N added to the leaf surface can be transported not only upwards but also downwards and that the downward transport exceeds the upward transport. Tanaka et al. [9], Reynolds et al. [31], and our present study have all demonstrated that the N absorbed by roots on one side was transferred to the roots and nodules on the other side through the shoots, suggesting that the N assimilated by shoots can be translocated and redistributed to the roots and nodules.

In this study, using the Shoot and one side of the roots and nodules in the dual root system as an N translocation system, we established a method for calculating the translocation of N from the Shoot to the roots and nodules during the R_1–R_5 stages based on the difference in ^{15}N abundance. The calculation showed that when N was added at a concentration of 50 mg·L^{-1}, the N translocated from the Shoot to $Root_n$ accounted for 29.6%–52.3% of the N accumulation in $Root_n$, whereas the N translocated from the Shoot to $Nodule_n$ made up 9.4%–16.6% of the N accumulation in $Nodule_n$ during the R_1–R_5 stage. In the dual root system, the N absorbed and fixed by $Root_n$ and $Nodule_n$ was absorbed and utilized by the aboveground part whereas the fertilizer N was absorbed by $Root_{non}$. After the assimilation by the aboveground part, the N can be transported to the root and root nodules in a certain form.

5. Conclusions

1. Up to 81.5%–87.1% of the N absorbed by the soybean roots and fixed by the root nodules was supplied for shoot growth, leaving 12.9%–18.5% for root and nodule growth. Soybeans preferentially used fertilizer N in the presence of the NO_3^- or NH_4^+ supply. After the absorbed fertilizer N and nodule-fixed N was transported to the shoot, a portion of it was redistributed to the roots and nodules. The nitrogen required for root growth was primarily derived from the NO_3^- or NH_4^+ assimilated by the roots and the N fixed by the nodules, with a small portion translocated from the shoot. The N required for nodule growth was primarily contributed by nodule-fixed N with a small portion translocated from the shoot, whereas the NO_3^- or NH_4^+ assimilated by the roots was not directly supplied to the nodules.

2. Using the shoot and one side of the roots and nodules in the dual root system as an N translocation system, we established a method for calculating the translocation of N from the shoots to the roots and nodules during the R_1–R_5 stages based on the difference in ^{15}N abundance. The calculation showed that when N was added at a concentration of 50 mg·L^{-1}, the N translocated from the Shoot to $Root_n$ accounted for 29.6%–52.3% of the N accumulation in $Root_n$, whereas the N translocated from the Shoot to $Nodule_n$ made up 9.4%–16.6% of the N accumulation in $Nodule_n$ during the R_1–R_5 stage.

3. This experiment systematically studied the transport characteristics of soybean N and the interaction mechanism of fertilizer N and root nodule N fixation, providing a theoretical basis and guidance for the rational application of N fertilizer.

Author Contributions: Conceptualization, Z.G. and X.L.; Data curation, W.T.; Funding acquisition, C.M. and Z.G.; Investigation, S.D.; Methodology, J.W. and S.K.; Resources, C.M.; Software, C.W.; Writing – original draft, R.Z.; Writing – review & editing, R.Z. and J.W. All authors have read and agreed to the published version of the manuscript.

Funding: We are grateful for the support from the National Key Research and Development Programme, Physiology and Regulation of High Quality Soybean Production (Grant Number: 2018YFD1000905) and by Research and Demonstration on the Key Technology of High Yield and High Efficiency of Soybean (Grant Number: GA19B101).

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Taylor, R.S.; Weaver, D.B.; Wood, C.; van Santen, E. Nitrogen Application Increases Yield and Early Dry Matter Accumulation in Late-Planted Soybean. *Crop Sci.* **2005**, *45*. [CrossRef]
2. Mahon, J.D.; Child, J.J. Growth response of inoculated peas (Pisum sativum) to combined nitrogen. *Can. J. Bot.* **1979**, *57*, 1687–1693. [CrossRef]
3. Ranđelović, V.; Prodanović, S.; Tomić, Z.; Bijelić, Z. Genotypic response of two soybean varieties with reduced content of KTI to application of different nitrogen level. *Biotechnol. Anim. Husb.* **2010**. [CrossRef]
4. Đukić, V.; Đorđević, V.; Popović, V.; Balešević-Tubić, S.; Petrović, K.; Jakšić, S.; Dozet, G. Effect of nitrogen and Nitragin application on soybean yield and protein content. *Ratar. Povrt.* **2010**, *47*, 187–192.
5. Wang, C.B.; Zheng, Y.M.; Shen, P.; Zheng, Y.P.; Wu, Z.F.; Sun, X.W.; Yu, T.Y.; Feng, H. Dterming N supplied sources and N use efficiency for peanut under applications of four forms of N fertilizers labeled by isotope ^{15}N. *J. Integerative Agric.* **2016**, *15*, 432–439. [CrossRef]
6. Conejero, G.; Tirado, J.L.; Robin, P. Effect of nodulation on the nitrate assimilation in vegetative soybean plants. *Plant Soil* **1986**, *91*, 385–389. [CrossRef]
7. Saito, A.; Tanabata, S.; Tanabata, T. Effect of Nitrate on Nodule and Root Growth of Soybean(Glycine max (L.)Merr.). *Int. J. Mol. Sci.* **2014**, *15*, 4464–4480. [CrossRef]
8. Hinson, K. Nodulation Responses from Nitrogen Applied to Soybean Half-Root Systems. *Agron. J.* **1975**, *67*, 799–804. [CrossRef]
9. Tanaka, A.; Fujlta, K.; Terasawa, H. Growth and Dinitrogen Fixation, of Soybean Root System Affected by Partial Exposure to Nitrate. *Soil Sci. Plant Nutr.* **1985**, *31*, 637–645. [CrossRef]
10. Carroll, B.J.; Gresshoff, P.M. Nitrate Inhibition of Nodulation and Nitrogen Fixation in White Clover. *Z. Pflanzenphysiol.* **1981**, *110*, 77–88. [CrossRef]
11. Daimon, H.; Yoshioka, M. Responses of Root Nodule Formation and Nitrogen Fixation Activity to Nitrate in a Split-Root System in Peanut (Arachis hypogaea L). *J. Agron. Crop Sci.* **2001**, *187*, 89–95. [CrossRef]
12. Streeter, J.G. Effect of nitrate on acetylene reduction activity and carbohydrate composition of Phaseolus vulgaris nodules. *Physiol. Plant.* **1986**, *68*, 294–300. [CrossRef]
13. Arnone, J.A., III; Kohls, S.J.; Baker, D.D. Nitrate Effects on Nodulation and Nitrogenase Activity of Actinorhizal Casuarin a Studied in Split-Root Systems. *Soil Biol. Biochem.* **1994**, *26*, 599–606. [CrossRef]
14. Imsande, J. Inhibition of Nodule Development in Soybean by Nitrate or Reduced Nitrogen. *J. Exp. Bot.* **1986**, *37*, 348–355. [CrossRef]
15. Dazzo, F.B.; Brill, W.J. Regulation by Fixed Nitrogen of Host-Symbiont Recognition in the Rhizobium-Clover Symbiosis. *Plant Physiol.* **1978**, *62*, 18. [CrossRef]
16. Small, J.G.C.; Leonard, O.A. Translocation of ^{14}C labeled photosynthate in nodulated legumes as influenced by nitrate nitrogen. *Am. J. Bot.* **1969**, *56*, 187–194. [CrossRef]
17. Khan, A.A.; Khan, A.A. Effects of nitrate nitrogen on growth, nodulation and distribution of ^{14}C-labelled photosynthates in cowpea. *Plant Soil* **1981**, *63*, 141–147. [CrossRef]
18. Minchin, F.R.; Minguez, M.I.; Sheehy, J.E.; Witty, J.F.; Skøt, L. Relationships between nitrate and oxygen supply in symbiotic nitrogen fixation by white clover. *J. Exp. Bot.* **1986**, *37*, 1103–1113. [CrossRef]
19. Wong, P.P. Nitrate and carbohydrate effects on nodulation and nitrogen fixation (acetylene reduction) activity of lentil (Lens esculenta Moench). *Plant Physiol.* **1980**, *66*, 78–81. [CrossRef]
20. Carroll, B.J.; Hansen, A.P.; McNeil, D.L.; Gresshoff, P.M. Effect of oxygen supply on nitrogenase activity of nitrate-and dark-stressed soybean(Glycine max (L.) Merr.) plants. *Funct. Plant Biol.* **1987**, *14*, 679–687. [CrossRef]
21. Munns, D.N. Nodulation of Medicago sativa in solution culture:III. Effects of nitrate on root hairs and infection. *Plant Soil* **1968**, *29*, 33–47. [CrossRef]
22. Wahab, A.M.A.; Zahran, H.H.; Abd-Alla, M.H. Root-hair infection and nodulation of four grain legumes as affected by the form and the application time of nitrogen fertilizer. *Folia Microbiol.* **1996**, *41*, 303–308. [CrossRef]

23. Gan, Y.; Stulen, I.; van Keulen, H.; Kuiper, P.J. Low concentrations of nitrate and ammonium stimulate nodulation and N_2, fixation while inhibiting specific nodulation (nodule DW g^{-1}, root dry weight) and specific N_2, fixation (N_2, fixed g^{-1}, root dry weight) in soybean. *Plant Soil* **2004**, *258*, 281–292. [CrossRef]
24. Harper, C.J.E. Effect of Localized Nitrate Application on Isoflavonoid Concentration and Nodulation in Split-Root Systems of Wild-Type and Nodulation-Mutant Soybean Plants. *Plant Physiol.* **1991**, *95*, 1106–1112. [CrossRef]
25. Xia, X.; Ma, C.; Dong, S.; Xu, Y.; Gong, Z. Effects of nitrogen concentrations on nodulation and nitrogenase activity in dual root systems of soybean plants. *Soil Sci. Plant Nutr.* **2017**, *63*, 470–482. [CrossRef]
26. Fujikake, H.; Yamazaki, A.; Ohtake, N. Quick and reversible inhibition of soybean root nodule growth by nitrate involves a decrease in sucrose supply to nodules. *J. Exp. Bot.* **2003**, *54*, 1379–1388. [CrossRef]
27. Kosslak, R.M.; Bohlool, B.B. Suppression of Nodule Development of One Side of a Split-Root System of Soybeans Caused by Prior Inoculation of the Other Side. *Plant Physiol.* **1984**, *75*, 125–130. [CrossRef]
28. van Brussel, A.A.; Tak, T.; Boot, K.J.; Kijne, J.W. Autoregulation of Root Nodule Formation:Signals of Both Symbiotic Partners Studied in a Split-Root System of Vicia sativa subsp.nigra. *Mol. Plant Microbe Interact.* **2002**, *15*, 41–349. [CrossRef]
29. Oghoghorie, C.G.; Pate, J.S. Exploration of the nitrogen transport system of a nodulated legume using ^{15}N. *Planta* **1972**, *104*, 35–49. [CrossRef]
30. Silva, C.F.; Paredes, D.S.; Guimarães, A.P.; Araújo, E.S.; Urquiaga, S.; Alves, B.J.R.; Boddey, R.M. Comparação entre duas técnica de marcação com ^{15}N para avaliação do N residual subterrâneo da soja. In Proceedings of the Poster Presented at the Congress Fertbio, Guarapari, Brazil, 13–17 September 2010.
31. Reynolds, P.H.S.; Boland, M.J. Induction of ammonium assiniilation:leguminous roots compared with nodules using a split root system. *Physiol. Plant.* **1990**, *79*, 359–367. [CrossRef]
32. Lyu, X.; Xia, X.; Wang, C.; Ma, C.; Dong, S.; Gong, Z. Effects of changes in applied nitrogen concentrations on nodulation, nitrogen fixation and nitrogen accumulation during the soybean growth period. *Soil Sci. Plant Nutr.* **2019**, 1–11. [CrossRef]
33. Guan, D.W.; Li, L.; Jiang, X.; Ma, M.; Cao, F.; Zhou, B.; Li, J. Influence of long-term fertilization on the community structure and diversity of soybean rhizobia in black soil. *Biodivers. Sci.* **2015**, *23*, 68–78. [CrossRef]
34. Fehr, W.R.; Caviness, C.E.; Burmood, D.T.; Pennington, J.S. Stage development description for soybean Glycine max (L.)Memll. *Crop Sci.* **1977**, *11*, 929–931. [CrossRef]
35. Crafts-Brandner, S.J.; Harper, J.E. Nitrate Reduction by Roots of Soybean (Glycine max [L.] Merr.) Seedlings. *Plant Physiol.* **1982**, *69*, 1298–1303. [CrossRef]
36. Sprent, J.I.; Thomas, R.J. Nitrogen nutrition of seedling grain legumes: some taxonomic, morphological and physiological constraints. *Plant Cell Environ.* **1984**, *7*, 637–645. [CrossRef]
37. Andrews, M. Nitrate and reduced-N concentrations in the xylem sap of Stellaria media, Xanthium strumarium and six legume species. *Plant Cell Environ.* **1986**, *9*, 605–608. [CrossRef]
38. Kiyomiya, S.; Nakanishi, H.; Uchida, H.; Tsuji, A.; Nishiyama, S.; Futatsubashi, M.; Tsukada, H.; Ishioka, N.S.; Watanabe, S.; Ito, T.; et al. Real Time Visualization of ^{13}N-Translocation in Rice under Different Environmental Conditions Using Positron Emitting Tracer Imaging System. *Plant Physiol.* **2001**, *125*, 1743–1753. [CrossRef]
39. Osa, A.; Sekine, T.; Uchida, H.; Tsuji, A.; Matsuhashi, S.; Ito, T.; Kume, T. Analysis of Nitrate Absorption and Transport in Non-nodulated and Nodulated Soybean Plants with NO_3^- and NH_4^+. *Radioisotopes* **1999**, *48*, 450–458.
40. Wery, J.; Turc, O.; Salsac, L. Relationship between growth, nitrogen fixation and assimilation in a legume (Medicago sativa L). *Plant Soil* **1986**, *96*, 17–29. [CrossRef]
41. Chaillou, S.; Rideout, J.W.; Raper, C.D., Jr.; Morot-Gaudry, J.F. Responses of soybean to ammonium and nitrate supplied in combination to the whole root system or separately in a split-root system. *Physiol. Plant.* **1994**, *90*, 260–270. [CrossRef]
42. Saravitz, C.H.; Chaillou, S.; Musset, J.; Raper, C.D., Jr.; Morot-Gaudry, J.F. Influence of nitrate on uptake of ammonium by nitrogen-depleted soybean: is the effect located in roots or shoots. *J. Exp. Bot.* **1994**, *45*, 1575–1584. [CrossRef]
43. Abdellaoui, A.; Talouizte, A. Effect of Previous Nitrogen Starvatio on NO_3^- and NH_4^+ Uptake and Assimilation Associated with the Endogenous Soluble Carbohydrate Utilization in Moroccan Wheat Seedlings. *J. Plant Nutr.* **2007**, *24*, 1995–2007. [CrossRef]

44. Schobert, C.; Komor, E. Transport of Nitrate and Ammonium into the Phloem and the Xylem of Ricinus communis seedlings. *J. Plant Physiol.* **1992**, *140*, 306–309. [CrossRef]
45. Daniels, W.L. *The Nature and Properties of Soils*, 15th ed.; Soil Science Society of America: Madison, WI, USA, 2016; p. 1428. [CrossRef]
46. Gianquinto, G.; Muñoz, P.; Pardossi. Soil Fertility and Plant. *Good Agricultural Practices Principles for Greenhouse Vegetable Production in the Mediterranean Region*. 2013, p. 205. Available online: https://www.researchgate.net/publication/256334829_Good_Agricultural_Practices_for_Greenhouse_Vegetable_Crops_-Principles_for_Mediterranean_Climate_Areas_FAO_Ministry_of_Agriculture_Good_Agricultural_Practices_for_greenhouse_vegetable_crops (accessed on 13 March 2020).
47. Kumar, V.; Rawat, A.K.; Rao, D.L.N. Slow and fast-growing soybean rhizobial population, their symbiotic efficiency and soil nitrogen behavior under different cropping systems in Vertisols of Madhya Pradesh. *Legume Res. Int. J.* **2018**, *41*, 617–623. [CrossRef]
48. Ito, O.; Kumazawa, K. Nitrogen assimilation in sunflower leaves and upward and downward transport of nitrogen. *Soil Sci. Plant Nutr.* **1976**, *22*, 181–189. [CrossRef]

© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).