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Root Contact between Maize and Alfalfa Facilitates Nitrogen Transfer and Uptake Using Techniques of Foliar ^{15}N -Labeling

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Abstract: Belowground nitrogen (N) transfer from legumes to non-legumes provides an important N source for crop yield and N utilization. However, whether root contact facilitates N transfer and the extent to which N transfer contributes to crop productivity and N utilization have not been clarified. In our study, two-year rain shelter experiments were conducted to quantify the effect of root contact on N transfer in a maize/alfalfa intercropping system. N transfer occurred mainly one direction from alfalfa to maize during the growth period. Following the N0 treatment, the amount of N transfer from alfalfa to maize was 204.56 mg pot⁻¹ with no root barrier and 165.13 mg pot⁻¹ with a nylon net barrier, accounting for 4.72% and 4.48% of the total N accumulated in maize, respectively. Following the N1 treatment, the amount of N transfer from alfalfa to maize was 197.70 mg pot⁻¹ with no root barrier and 139.04 mg pot⁻¹ with a nylon net barrier, accounting for 3.64% and 2.36% of the total N accumulated in the maize, respectively. Furthermore, the amount of N transfer without no root barrier was 1.24–1.42 times higher than that with a nylon net barrier regardless of the level of N addition. Our results highlight the importance and the relevance of root contact for the enhancement of N transfer in a maize/alfalfa intercropping system.

Keywords: intercropping; ^{15}N foliar labeling; root contact; N transfer; N utilization

1. Introduction

The north-eastern area of China is characterized as a semi-arid and temperate climatic region. The farming and pastoral area in this region represents an important grain commodity and animal husbandry base and covers $3.83 \times 10^5 \text{ km}^2$. This area plays a key role in the development of Chinese agriculture and animal husbandry. However, a long period of intensive agricultural production has led to serious problems, including soil degradation, chemical pollution, and the loss of biodiversity in the agricultural soil [1,2]. Furthermore, low vegetation cover and wind erosion have deteriorated the ecosystem in this region. Therefore, it is crucial to adjust the planting structure and reduce the amount of chemical fertilizer used in this region.

Compared to typical Chinese agriculture practices, intercropping can increase nutrient utilization efficiency and achieve optimal economic output as well as income [3–6]. These benefits occur

because N transfer between legumes and cereals can be an important source of nitrogen for cereal crops [7,8]. N transfer has been studied thoroughly in many cereal–legume intercropping systems, e.g., bean/maize [9], pea/barley [10], faba/wheat [11], peanut/rice [7], manures/lettuce [12], pea/maize [13], soybean/maize [14], and bean/garlic [15]. Literature data show that rates of N transfer range from 0 to 80% from legumes to cereal in mixed stands, depending on the legume species and the cultivar [16–18]. In the north-eastern area of China, maize (*Zea mays* L.) is a major food crop grown in farming and pastoral areas. Alfalfa (*Medicago sativa* L.) is a commonly grown perennial forage legume in this area [19] because it is high yielding, rich in nutrients, easy to digest, and palatable. Recent studies suggested that maize/alfalfa intercropping is a successful crop management strategy that could significantly enhance yield and economic benefits [5,20]. However, it is still unclear whether N transfer exists in an alfalfa/maize intercropping system.

Previous studies found that root contact plays an important role in the transfer of N. For example, one study revealed that N transfer from faba bean to the associated wheat without barrier was significantly higher than that with the nylon net barrier [11]. Meng et al. also found that N transfer was significantly increased under the no barrier treatment compared with the solid barrier in the soybean/maize intercropping system [21]. In addition, the amount of N transferred is also closely related to the supply of soil N fertilizer. For example, in peanut/rice intercropping systems, the amount of N transferred was greater, and its effects were more pronounced under low N conditions than under high N conditions [7]. Furthermore, the rate of N transfer from peanut to rice was decreased significantly with an increase in the N supply levels [7,22]. However, there have been no attempts to explore the effects of root contact on nitrogen transfer between alfalfa and maize at two different N levels.

It has been reported that belowground N transfer from legumes provides an important N source for coexisting cereals [23]. For example, the N transferred from faba bean to wheat corresponded to 15% of the total N uptake by wheat in a faba bean/wheat intercropping system [11]. Paddy rice/peanut intercropping significantly improved dry matter and N uptake in rice because 2–3.5% of the N fixed by the peanut was transferred to the rice [7]. One study revealed that root contact improved N uptake by 26.3% [24]. Zhang et al. found that soybean/maize intercropping could improve N transfer and uptake in the karst region of Southwest China [14]. However, it is unclear whether the high nitrogen transfer is associated with the total N uptake under maize/alfalfa root contact conditions. Therefore, two-year rain shelter experiments were carried out in a maize/alfalfa root separation experiment with the following objectives: (1) to clarify the effect of root contact on N transfer from alfalfa to maize at two N levels and (2) to determine the relationship between N transfer and total N uptake.

2. Materials and Methods

2.1. Site Description

Two pot experiments were conducted in the new rain shelter of the College of Resources and Environment of Jilin Agricultural University from 2015 to 2016 (125°24′50.38″ E and 43°48′28.59″ N, 248.5 masl). The climate in this area belongs to the north temperate continental monsoon category with four distinct seasons and moderate semi-arid characteristics. The annual frost-free period is 120–160 days, and the growing degree days (≥ 10 °C) are characterized by a temperature of 2200–3000 °C. The final frost and the first frost occur in April and September, respectively. The mean annual air temperature and precipitation in 2015 and 2016 were 5.2 °C and 671 mm, respectively. We used evaporative cooling and shade cloth to control the temperature on sunny days.

The experimental soil was collected from an area near the rain shelter (125°24′44.69″ E and 43°48′36.49″ N, an altitude of 231.7 m). The physico-chemical properties of the soil (0–20 cm) were as follows: bulk density, 1.33 ± 0.05 g cm⁻³; organic matter content, 21.85 g kg⁻¹; total N content, 1.48 g kg⁻¹; available N, 80.36 mg kg⁻¹; available P, 14.82 mg kg⁻¹; available K, 115.42 mg kg⁻¹; initial soil pH, 6.46 (soil: water = 1:2.5); soil cation exchange capacity (CEC), 23 cmol kg⁻¹; and clay

content, 20.5%. The soil organic C contents were determined by the Walkley–Black method [25]. The soil total N contents were determined by the micro-Kjeldahl method [26]. The available N was determined by the alkali diffusion method. The available phosphorus was determined by sodium bicarbonate-molybdenum antimony colorimetry. The available potassium was determined by flame photometry method [25]. The soil CEC was determined by the ethylene diamine tetraacetic acid (EDTA)-ammonium salt rapid method [27]. The soil clay content was determined by the pipette method [28]. The type of tested soil was black soil, equivalent to typical Phaeozem in the World Reference Base (WRB) system [29].

2.2. Experimental Design

A completely randomized block design was used in this experiment with two N-level treatments (N0 and N1), three methods of separation of maize and alfalfa roots, and four replicates. The N0 treatment (control) involved no N fertilizer, and the N1 treatment ($10.2 \text{ g N pot}^{-1}$) involved N addition with urea fertilizer (N content, 46%). The following three methods were used to separate the roots of maize and alfalfa: (1) plastic sheet separation (PSS) in which a plastic sheet (0.5 mm) was used to separate the roots and prevent the transfer of solutions and hyphae; (2) nylon net separation (NNS) in which a $30 \text{ }\mu\text{m}$ nylon net was used to prevent root contact but permit the transfer of solutions and hyphae; and (3) no separation (NS) in which contact between the maize and the alfalfa roots was permitted (Figure 1). To prevent root contact, each pot was cut in half, and a plastic sheet, a nylon net, or no barrier was placed between the halves. Then, the pot was reconstructed to implement the PSS, the NNS, and the NS treatments and was sealed to prevent water leakage (Figure 1). The spacing of the pots in the rain shelter was 1.2 m. The N transfer in the maize/alfalfa intercropping system was evaluated in the following two experiments.

Figure 1. Schematic diagram of the maize/alfalfa intercropping pot experiment. NS represents no root separation between the maize and alfalfa, NNS and PSS represent maize and alfalfa root separation with a nylon net barrier and a plastic sheet barrier, respectively.

2.2.1. Experiment I. Isotope (^{15}N) Labeling of Alfalfa Leaves (Alfalfa as the ^{15}N Donor)

The maize and the alfalfa plants were sown together in pots (length: 45 cm, width: 30 cm, and height: 50 cm) with 51 kg of soil that was passed through a 2 mm sieve. The application of NPK fertilizer to maize and alfalfa was conducted according to local fertilization practices [5]. Alfalfa was treated at 53 kg N ha^{-1} , 135 kg P ha^{-1} , and 90 kg K ha^{-1} , while 225 kg N ha^{-1} , 120 kg P ha^{-1} , and 60 kg K ha^{-1} were applied to maize. The NPK fertilizer contained the following components: urea (46% N content), superphosphate (46% P_2O_5 content), and potassium oxide (60% K_2O content). In addition, we calculated the fertilization amount for the pot experiment based on the fertilization amount above, and $6.12 \text{ g P}_2\text{O}_5 \text{ pot}^{-1}$ and $4.08 \text{ g K}_2\text{O pot}^{-1}$ were applied to all pots, and $10.2 \text{ g N pot}^{-1}$ was applied to half of the pots and mixed thoroughly at the time of sowing of the N1 treatments (29 May 2015 and 28 May 2016). Maize and alfalfa were planted in the pots at a ratio of 1:20 for the three separation methods (1 June 2015 and 23 May 2016). Maize was planted at a depth of 3 cm with 2–3 seeds per

pot and thinned to one plant at the second leaf stage. The plants were watered with deionized water to maintain the soil moisture at 60–70% of the field water holding capacity throughout the growth stage, and the soil moisture in each pot was monitored by micro-tensiometers (Nanjing Institute of Soil Science, Chinese Academy of Sciences). The maize and the alfalfa seeds were sown manually to ensure that all plants were placed at the same depth. Weeds were regularly controlled with a small shovel, and maize and alfalfa pests and diseases of alfalfa or maize were controlled while attempting to minimize the effects of pesticide application on the nontarget crop.

The alfalfa leaves were labeled at the vegetative stage of the second harvest (15 August 2015 and 18 August 2016). The maize leaves were labeled simultaneously with the alfalfa leaves. The stem-associated maize was surrounded by a plastic sheet (30 by 50 cm) before labeling to prevent ^{15}N contamination of the associated maize or soil from the runoff of ^{15}N -labeled solutions during foliar labeling. In addition, the surface of the pot soil was covered by two layers of plastic film and two layers of filter paper layered above the plastic film. One polyvinyl chloride cylinder (25 by 50 cm) open at both ends was used to enclose the alfalfa canopy. A 1.5% (W/W) solution of ^{15}N -labeled urea (5.14% ^{15}N enrichment) was spread on the surface of the alfalfa leaves as described by Shen and Chu (2004). Then, the alfalfa leaves were immediately covered with white plastic bags. Once the leaf surfaces were dry, the leaves were washed three times with deionized water. In total, 10 mL of the ^{15}N -labeled urea solution was sprayed, and each labeling was replicated five times. The ^{15}N labeling processes were carefully controlled to ensure that neither the soil nor the associated maize leaves were contaminated by ^{15}N . The same amount of urea solution was used as a control to determine the natural ^{15}N abundance under the test conditions. To ensure the nodulation and the nitrogen fixation of alfalfa, a suspension of *Rhizobium* sp. strain ACCC177512 (supplied by the College of Resources and Environment, Jilin Agriculture University, Jilin) was applied to the alfalfa at 20 mL pot⁻¹ (density was 3.0×10^7 mL⁻¹ CFU) at 7 d after sowing.

2.2.2. Experiment II. Isotope (^{15}N) Labeling of Maize Leaves (Maize as the ^{15}N Donor)

This experiment was conducted only in 2016 in the same manner as in experiment I. In this experiment, ^{15}N was applied to the maize leaves in the same way that it was applied to the alfalfa leaves in experiment I such that maize was the donor plant, and alfalfa was the receiver plant.

2.3. Sampling and Measurements

On 1 October 2015 and 2016, maize and alfalfa plants under the different N and root-separation treatments were harvested by cutting near the soil surface. The roots were excavated and washed with running tap water. The seeds, the shoots, and the fresh roots were heated at 105 °C for 0.5 h to stop all enzymatic activity, and the plants were dried at 70 °C to constant weight. The dry weights of both the maize and the alfalfa plants were measured. The total N contents in the plants were measured by a K-05 automatic N analyzer (Shanghai Sheng Sheng Automation Instrument Co., Ltd., Shanghai, China). The ^{15}N abundance was measured by an isotope mass spectrometer (DELTA V Advantage, Thermo-Fisher Ltd., Waltham MA., USA).

2.4. Data Calculations

The soil N use efficiency of each crop (NU_N) was calculated using the following equation [30]:

$$\text{NU}_\text{N} = (\text{U} - \text{U}_0) / \text{F} \times 100\%, \quad (1)$$

where U is the total amount of N uptake by the aboveground part of the crop after N application, U₀ is the total N uptake in the upper part of the crop without N application, and F is the amount of applied N fertilizer.

The natural ^{15}N abundance (atom %) in the maize and the alfalfa under the control treatments was used as the reference value. The excess ^{15}N (atom %) was calculated by the following equation [22]:

$$^{15}\text{N atom \% excess} = ^{15}\text{N atom \% excess in labeled crop} - ^{15}\text{N atom \% excess in unlabeled crop.} \quad (2)$$

The ^{15}N contents in the maize and the alfalfa plants were calculated based on the following equation:

$$^{15}\text{N content} = (^{15}\text{N atom \% excess}_{\text{plant}} \times \text{total N}_{\text{plant}}) / ^{15}\text{N atom \% excess}_{\text{labeled N}}. \quad (3)$$

The N transfer rate (% NT) represents the amount of total N transferred from alfalfa (^{15}N donor plant) to maize (^{15}N receiver plant). The percentage of total N transferred from donor to receiver (% NT) was estimated by the following equation [31]:

$$\% \text{NT} = ^{15}\text{N content}_{\text{receiver}} \times 100 / (^{15}\text{N content}_{\text{receiver}} + ^{15}\text{N content}_{\text{donor}}). \quad (4)$$

The amount of N (mg pot^{-1}) transferred from alfalfa was calculated as follows:

$$\text{N}_{\text{transfer}} = \% \text{N}_{\text{transfer}} \times \text{total N}_{\text{donor}} / (100 - \% \text{N}_{\text{transfer}}). \quad (5)$$

The percentage of N in the receiver plant derived from the transfer (% NDFT) was calculated according to the following equation [22]:

$$\% \text{NDFT} = (\text{N}_{\text{transfer}} \times 100) / \text{total N}_{\text{receiver}}. \quad (6)$$

2.5. Statistical Analysis

The experimental data were analyzed using Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA, USA), and the figures were plotted using GraphPad Prism 6.02 (GraphPad Software Inc., La Jolla, CA). The total dry biomass, the N content, and the ^{15}N abundance in the maize and the alfalfa were statistically analyzed using the PROC MIXED procedure in SAS (SAS Institute, Inc., Cary, North Carolina). In the mixed model, the N level and the root separation method were treated as fixed factors, and the year and all interactions were considered random effects. The LSMEANS statement was used for the mean estimation of the fixed effects, and significant differences were determined with Tukey's HSD test at $\alpha = 0.05$. The final model used in SAS was as follows:

$$Y_{ijkl} = \mu + \tau_i + \beta_j + \gamma_k + \tau\beta_{ij} + \tau\gamma_{ik} + \beta\gamma_{jk} + \tau\beta\gamma_{ijk} + \delta_l + \epsilon_{ijk} \quad (7)$$

where μ is the overall mean; τ_i is the effect of the N level; β_j is the effect of the root separation method; γ_k is the effect of the year; $\tau\beta_{ij}$ is the effect of the N level $\times$ root separation method; $\tau\gamma_{ik}$ is the effect of the N level $\times$ year; $\beta\gamma_{jk}$ is the effect of the root separation method $\times$ year; $\tau\beta\gamma_{ijk}$ is the effect of the N level $\times$ root separation method $\times$ year; δ_l is the block effect; and ϵ_{ijk} is the error term. A linear regression analysis was conducted, and the Pearson correlation coefficients were calculated using GraphPad Prism 6.02. The multivariate ordination method was used to analyze the relationships among N transfer, dry biomass, and N uptake in maize and alfalfa.

3. Results

3.1. Dry Biomass

The total dry biomass of both the maize and the alfalfa was significantly affected by N level, root separation method, and year, as well as their interactions (Table 1). The total dry biomass of maize was the highest when the roots were not separated, and shoot and root dry biomasses of alfalfa were the highest when the roots were separated, regardless of when the N treatment was applied (Figure 2).

On average, maize shoot and root biomasses in the N1 treatment exceeded those in the N0 treatment by 16.07% and 28.58% with no root barrier, 15.16% and 16.93% with the nylon net barrier, and 67.61% and 37.07% with the plastic sheet barrier, respectively. Under the N0 treatment, the maize shoot and root biomasses with no root barrier were 82.69% and 65.82% higher than those with the plastic sheet barrier and 63.48% and 54.47% higher than those with the nylon net barrier, respectively. Under the N1 treatment, the maize shoot and root biomasses with no root barrier were 26.51% and 55.52% higher than those with the plastic sheet barrier and 12.32% and 31.74% higher than those with the nylon net barrier, respectively. In the N1 treatment, the alfalfa shoot and root biomasses exceeded those of the N0 treatment by 10.77% and 16.72% with no root barrier, 7.05% and 11.16% with the nylon net barrier, and 3.20% and 7.68% with the plastic sheet barrier, respectively. Under the N0 treatment, the alfalfa shoot and root biomasses with the plastic sheet barrier exceeded those of the treatment with no root barrier by 77.63% and 72.55% and exceeded those with the nylon net barrier by 36.41% and 40.36%. Under the N1 treatment, the alfalfa shoot and root biomasses with the plastic sheet barrier exceeded those of the treatment with no root barrier by 65.47% and 59.24% and exceeded those with the nylon net barrier by 31.81% and 33.72%, respectively.

Figure 2. Effects of root separation and N addition on maize shoot and root (A,B), alfalfa shoot and root (C,D) and total dry biomass (E,F) in 2015 and 2016. Bars are means $\pm$ SD. Different lowercase letters indicate significant differences in maize and alfalfa dry biomass among different root separation treatments at the same nitrogen level at the $p < 0.05$ probability level. N0 and N1 represent no N fertilizer addition and N fertilizer addition (10.2 mg N pot⁻¹). NS represents no root separation between the maize and the alfalfa, NNS and PSS represent maize and alfalfa root separation with a nylon net barrier and a plastic sheet barrier, respectively.

Table 1. Results of the repeated measures ANOVA for the dry biomass of maize and alfalfa, with the nitrogen level (N) and the root separation method (RS) as the independent variables and year (Y) as the repeated measure. ×

Factor	Df	Maize dry Biomass (g pot ^{−1})		Alfalfa Dry Biomass (g pot ^{−1})	
		Shoot	Root	Shoot	Root
Y	1	6.43 *	0.77ns	116.38 **	234.64 **
N	1	556.16 **	421.09 **	36.40 **	42.69 **
RS	2	470.64 **	545.17 **	905.97 **	310.23 **
Y × N	1	0.00ns	4.71 *	2.36ns	15.54 **
Y × RS	2	2.51ns	6.21 **	5.71 **	16.82 **
N × RS	2	62.28 **	11.66 **	1.14ns	0.19ns
Y × N × RS	4	2.15ns	10.50 **	1.24ns	0.86ns

Analysis of variance (ANOVA) *p* values and symbols are defined as: * *p* < 0.05; ** *p* < 0.01; NS: *p* > 0.05.

3.2. N Uptake

The total N uptake of both the maize and the alfalfa was significantly affected by N level, root separation method, and planting year, as well as their interactions (Table 2). The total N uptake of maize was the highest when the roots were not separated, and the N uptake in the alfalfa plants' shoots and roots was the highest when the roots were separated, regardless of the N level applied (Figure 3). On average, in the N1 treatment, the N uptake in the maize shoots and roots exceeded that in the N0 treatment by 25.71% with no root barrier, by 82.53%, and 66.84% with the nylon net barrier, and by 139.41% and 134.38% with the plastic sheet barrier, respectively. Under the N0 treatment, the N uptake in the maize shoots and roots with no root barrier exceeded that with the plastic sheet barrier by 112.90% and 215.93% and exceeded that with the nylon net barrier by 92.55% and 101.32%. Under the N1 treatment, the N uptake in the maize shoots and roots with no root barrier exceeded that with the plastic sheet barrier by 72.78% and 65.49% and exceeded that with the nylon net barrier by 47.22% and 39.95%, respectively. The N uptake in the alfalfa shoots and roots exceeded that of the N0 treatment by 20.58%, 25.28% with no root barrier, 9.35% and 21.49% with the nylon net barrier, and 17.78% and 18.14% with the plastic sheet barrier under the N1 treatment. Under the N0 treatment, the N uptake in the alfalfa shoots and roots with the plastic sheet barrier exceeded that with no root barrier by 61.77% and 66.51% and exceeded that with the nylon net barrier by 38.31% and 37.12%. Under the N1 treatment, the N uptake in the alfalfa shoots and roots with the plastic sheet barrier exceeded that with no root barrier by 59.01% and 56.37% and exceeded that with the nylon net barrier by 26.21% and 32.41%, respectively.

Table 2. Results of repeated measures ANOVA for the nitrogen uptake of maize and alfalfa, with nitrogen level (N) and root separation method (RS) as the independent variables and year (Y) as the repeated measure.

Factors	Df	Maize nitrogen Uptake (g pot ^{−1})		Alfalfa Nitrogen Uptake (g pot ^{−1})	
		Shoot	Root	Shoot	Root
Y	1	33.63 **	2.09ns	450.14 **	101.10 **
N	1	4444.12 **	544.04 **	153.68 **	67.15 **
RS	2	1044.18 **	286.31 **	613.31 **	408.66 **
Y × N	1	10.86 **	43.58 **	0.48ns	54.72 **
Y × RS	2	0.27ns	3.81 *	17.24 **	2.63ns
N × RS	2	36.38 **	7.42 **	6.46 **	11.81 **
Y × N × RS	4	0.89ns	14.56 **	0.50ns	25.63 **

Analysis of variance (ANOVA) *p* values and symbols are defined as: * *p* < 0.05; ** *p* < 0.01; NS: *p* > 0.05. The other symbols are the same as for Table 1.

Figure 3. Effects of root separation and N addition on maize (A,B), alfalfa (C,D), and total N uptake (E,F) in 2015 and 2016. Bars are means $\pm$ SD. Different lowercase letters indicate significant differences in maize and alfalfa N uptake among different root separation treatments at the same nitrogen level at the $p < 0.05$ probability level. Other symbols are the same as in Figure 2.

3.3. ^{15}N Abundance

When alfalfa as ^{15}N -donor plant in 2015 and 2016, the ^{15}N abundance in the shoots of maize only was significantly affected by the root separation method. The ^{15}N abundance in the roots of maize was significantly affected by N levels, root separation method, and their interactions (except that the interactions among the year, the N levels, and the root separation method were not significant). The ^{15}N abundance in the shoots of alfalfa was significantly affected by the year, the interactions between year and N levels, and the interactions between year and root separation method; in the roots, it was significantly affected by year, N levels, and root separation method, as well as their respective interactions (Table 3). On average, under the N0 treatment, the ^{15}N abundances in maize and alfalfa shoots and roots were 0.387%, 0.392%, 0.773%, and 0.516% with no root barrier, 0.387%, 0.391%, 0.798%, and 0.512% with a nylon net barrier, and 0.372%, 0.372%, 0.731%, and 0.520% with a plastic sheet barrier, respectively. Under the N1 treatment, the ^{15}N abundances in maize and alfalfa shoots and roots were 0.387%, 0.390%, 0.789%, and 0.511% with no root barrier, 0.383%, 0.382%, 0.796%, and 0.565% with a nylon net barrier, 0.372%, 0.371%, 0.774%, and 0.544% with a plastic sheet barrier, respectively (Table 4).

When maize as ^{15}N -donor plant, the ^{15}N abundances in shoots and roots of alfalfa were significantly affected by the N levels but were not significantly affected by the root separation method and the interaction between N levels and root separation method (Table 3). Under the N0 treatment, the ^{15}N abundances in the alfalfa shoots and roots were 0.369% and 0.368% with no root barrier, 0.370% and 0.369% with a nylon net barrier, and 0.369% and 0.369% with a plastic sheet barrier, respectively. Under the N1 treatment, the ^{15}N abundance in the alfalfa shoot and root was 0.368% and 0.368% with no root barrier, 0.368% and 0.367% with a nylon net barrier, 0.369% and 0.368% with a plastic sheet barrier, respectively (Table 4).

Table 3. Results of the repeated measures ANOVA for shoot and root ^{15}N abundance of maize and alfalfa, with nitrogen level (N) and root separation method (RS) as the independent variables and year (Y) as the repeated measure.

Factors	Df	^{15}N Abundance (Alfalfa as ^{15}N -donor Plant)				^{15}N Abundance (Maize as ^{15}N -donor Plant)	
		Maize Shoot	Maize Root	Alfalfa Shoot	Alfalfa Root	Alfalfa Shoot	Alfalfa Root
Y	1	0.26 ns	3.69 ns	68.27 **	374.08 **		
N	1	0.68 ns	26.42 **	1.30 ns	13.58 **	6.94 *	24.14 **
RS	2	47.13 **	221.05 **	2.44 ns	5.16 *	1.63 ns	2.71 ns
Y × N	1	2.50 ns	13.59 **	8.09 **	5.94 *		
Y × RS	2	3.00 ns	12.73 **	4.94 *	5.20 *		
N × RS	2	1.18 ns	9.47 **	0.63 ns	6.68 **	3.34 ns	1.86 ns
Y × N × RS	4	2.11 ns	1.73 ns	1.68 ns	3.35 *		

Values are the means. Analysis of variance (ANOVA) p values and symbols are defined as follows: * $p < 0.05$; ** $p < 0.01$; and NS: $p > 0.05$. The other symbols are the same as in Table 1.

Table 4. ^{15}N abundance (%) in shoots and roots of maize and alfalfa under different conditions.

Years	Treatment	¹⁵ N Abundance (Alfalfa as ¹⁵ N-donor Plant)				¹⁵ N Abundance (maize as ¹⁵ N-donor Plant)	
		Maize Shoot	Maize Root	Alfalfa Shoot	Alfalfa Root	Alfalfa Shoot	Alfalfa Root
2015	N0	NS	0.386 ± 0.003ab	0.392 ± 0.003a	0.918 ± 0.071a	0.601 ± 0.062ab	
		NNS	0.384 ± 0.006ab	0.387 ± 0.005b	0.879 ± 0.099ab	0.576 ± 0.004b	
		PSS	0.372 ± 0.005c	0.371 ± 0.001d	0.783 ± 0.096b	0.583 ± 0.006b	
	N1	NS	0.391 ± 0.006a	0.394 ± 0.005a	0.844 ± 0.037ab	0.571 ± 0.024b	
		NNS	0.382 ± 0.011b	0.381 ± 0.004c	0.856 ± 0.022ab	0.637 ± 0.035a	
		PSS	0.372 ± 0.001c	0.371 ± 0.001d	0.794 ± 0.077b	0.577 ± 0.009b	
2016	N0	NS	0.388 ± 0.003a	0.393 ± 0.003a	0.627 ± 0.052c	0.432 ± 0.001c	0.369 ± 0.008a
		NNS	0.390 ± 0.002a	0.396 ± 0.003a	0.717 ± 0.027ab	0.448 ± 0.001b	0.370 ± 0.010a
		PSS	0.372 ± 0.000c	0.373 ± 0.000c	0.679 ± 0.055bc	0.457 ± 0.001a	0.369 ± 0.007a
	N1	NS	0.382 ± 0.001b	0.387 ± 0.001b	0.734 ± 0.048ab	0.452 ± 0.000c	0.368 ± 0.015a
		NNS	0.384 ± 0.004b	0.384 ± 0.003b	0.735 ± 0.012ab	0.493 ± 0.001b	0.368 ± 0.011a
		PSS	0.374 ± 0.002c	0.371 ± 0.000c	0.754 ± 0.033a	0.511 ± 0.017a	0.369 ± 0.007a
Control (2015)		0.372 ± 0.004	0.371 ± 0.010	0.373 ± 0.006	0.370 ± 0.007		
Control (2016)		0.370 ± 0.003	0.371 ± 0.006	0.370 ± 0.009	0.368 ± 0.006		

Values are means ± SD (n = 4). Analysis of variance (ANOVA) ^{15}N abundance values and symbols are defined as: * $p < 0.05$; ** $p < 0.01$; and NS: $p > 0.05$. PSS, NNS, and NS represent complete root separation with a plastic sheet, partial root separation with nylon nets, and no root separation, respectively. Different lowercase letters indicate significant difference by N levels and root barrier method within the same root separation treatment ($p = 0.05$).

3.4. N Transfer

The N transfer rate and amount were significantly affected by N level, root separation pattern, and year (Table 5). Under the N0 treatment, the rate and the amount of N transfer from alfalfa to maize was 15.05% and 204.56 mg pot^{−1} with no root barrier and 9.30% and 165.12 mg pot^{−1} with a nylon net barrier (Table 5). The N transfer accounted for 4.72% and 10.81% of the total N that accumulated in maize and alfalfa with no root barrier and 4.48% and 6.86% of that accumulated in maize and alfalfa with a nylon net barrier, respectively. Under the N1 treatment, the rate and the amount of N transfer from alfalfa to maize was 12.47% and 197.70 mg pot^{−1} with no root barrier and 7.02% and 139.04 mg

pot⁻¹ with a nylon net barrier in 2015 and 2016. The N transfer accounted for 3.64% and 13.32% of the total N that accumulated in maize and alfalfa with no root barrier and 2.36% and 5.86% of that accumulated in maize and alfalfa with a nylon net barrier, respectively. However, there was no N transfer in the plastic sheet separation (PSS) treatment since the plastic sheet (0.5 mm) was used to completely separate the roots and the soil.

Table 5. Nitrogen transferred from ¹⁵N-labeled plants to intercropped plants under different experimental conditions.

Years	Treatment		Rate of N Transferred (%)	Amount of N Transferred (mg pot ⁻¹)	N Transferred as of Maize N (%)	N Transferred as of Alfalfa N (%)
2015	N0	NS	14.52 ± 1.01a	167.92 ± 13.82a	3.53 ± 0.40a	8.93 ± 0.12a
		NNS	7.48 ± 2.06b	119.89 ± 34.31b	3.18 ± 0.43a	5.09 ± 0.32b
	N1	NS	11.86 ± 1.48a	165.83 ± 25.60a	4.41 ± 0.26a	16.55 ± 0.45a
		NNS	5.14 ± 0.14b	90.40 ± 1.99b	1.96 ± 1.04b	4.89 ± 0.36b
2016	N0	NS	15.58 ± 2.29a	241.19 ± 30.45a	5.90 ± 0.64a	12.69 ± 1.85a
		NNS	11.12 ± 1.30b	210.36 ± 27.94a	5.78 ± 0.65a	8.62 ± 1.07b
	N1	NS	13.07 ± 1.00a	229.56 ± 8.32a	2.87 ± 0.12a	10.09 ± 0.68a
		NNS	8.89 ± 0.39b	187.67 ± 4.17b	2.75 ± 0.08a	6.82 ± 0.24b
2 year average	N0	NS	15.05 ± 1.51a	204.56 ± 18.79a	4.72 ± 0.42a	10.81 ± 1.42a
		NNS	9.30 ± 0.71b	165.12 ± 15.97b	4.48 ± 0.22a	6.86 ± 0.33b
	N1	NS	12.47 ± 0.93a	197.70 ± 12.76a	3.64 ± 0.26a	13.32 ± 0.77a
		NNS	7.02 ± 1.70b	139.04 ± 11.97b	2.36 ± 0.60b	5.86 ± 1.50b

The data represent the means of four replicates; values followed by different letters in the same column are significantly different at the $p < 0.05$ probability level. The other symbols are the same as in Table 4.

3.5. Correlation Analysis

The maize biomass (2015: $r^2 = 0.5649$, $p < 0.0001$, 2016: $r^2 = 0.5696$, $p < 0.0001$), the N uptake (2015: $r^2 = 0.5572$, $p < 0.0001$, 2016: $r^2 = 0.2508$, $p = 0.0127$), and the N utilization (2015: $r^2 = 0.6361$, $p = 0.0019$, 2016: $r^2 = 0.3584$, $p = 0.0397$) were significantly and positively correlated with the amount of N transferred in the system between maize and alfalfa (Figure 4A,C,E). However, the alfalfa biomass (2015: $r^2 = 0.5726$, $p < 0.0001$, 2016: $r^2 = 0.8407$, $p < 0.0001$), the N uptake (2015: $r^2 = 0.4234$, $p = 0.0006$, 2016: $r^2 = 0.7907$, $p < 0.0001$), and the N utilization (2016: $r^2 = 0.4370$, $p = 0.0134$) were negatively correlated with the amount of N transferred (Figure 4B,D,F).

Figure 4. Correlations between N transfer and biomass and between N uptake and N utilization of maize (A,C,E) and alfalfa (B,D,F) in 2015 and 2016.

4. Discussion

4.1. Crop Biomass and N Uptake in the Intercropping System of Maize and Alfalfa

Previous studies found that root interactions generate yield advantages in different legume–cereal intercropping systems, e.g., peanut/rice, faba bean/wheat, and faba bean/maize intercropping systems [7,32,33]. Consistent with these studies, our data indicated that maize with no root barrier had the highest biomass. However, the opposite was true for alfalfa, in which greater biomass was achieved with the nylon net or plastic separation than with no root separation (Figure 2). In our experiment, we also found that the average values of aggressivity of maize relative to alfalfa (Ama) in the two year experiment were higher than one in all treatments. The N competitive ratio of maize relative to alfalfa (NCRma) values were also higher than one in each treatment in 2015 and 2016 (Table A1). Therefore, the competitiveness of maize was greater than that of alfalfa in the maize/alfalfa intercropping system, and the increased biomass in the maize/alfalfa intercropping was attributed to the significant increase in maize biomass, which is consistent with previous field trial studies [5,20]. The maize biomass was 32.37% higher with a nylon net barrier than with a plastic sheet barrier, which could be related to mycorrhizae and hyphae [8,31] or to the movement of water and nutrients from the alfalfa side of the pots in response to the much higher demands of the maize. In addition, the maize biomass with no root barrier was 48.69% higher than that with a plastic sheet barrier and 12% higher than that with a nylon net barrier, which could be due to the extra N from the alfalfa side, the other nutrients, or the soil that was available. Therefore, our data demonstrated that root contact stimulated the growth of non-legumes in a maize/alfalfa intercropping system. However, the alfalfa biomass was reduced by 40.94% with no root barrier compared with that under the plastic sheet barrier treatment and by 19.76%

compared with that under the nylon net barrier treatment. This finding may be due to the maize plants being taller than the alfalfa plants in the intercropping system (under the N0 treatment, the plant heights of maize and alfalfa were 238.93 cm and 78.14 cm, respectively. Under N1 treatment, the plant heights of maize and alfalfa were 250.62 cm and 69.53 cm, respectively), which limited the growth of alfalfa because of a shading effect [34] (Figure A1). The significant difference between 2015 and 2016 may be related to the low atmospheric temperature from July to September 2015 (Tables 1 and 2, Figure A2), which affected the growth of maize during the tassel initiation and silking stages [5,20]. Although water shortages are a key factor limiting crop growth and nutrient availability [35,36], in our pot experiment, sufficient water was applied throughout the growth period; therefore, water was not a limiting factor for plant growth.

Based on the averages of 2015 and 2016, under the N0 treatment, the whole plant N concentration for maize and alfalfa was 0.59% and 2.68%, respectively. Under N1 treatment, the whole plant N concentration for maize and alfalfa was 0.99% and 2.85%, respectively. Several studies have shown that intercropping can increase N uptake and utilization through niche complementarity and facilitation processes [8,21,33]. Consistent with these studies, our data indicated that root contact enhanced the N uptake of intercropped maize; a significant increase was observed, especially under low N conditions, indicating that root interaction was more conducive to the acquisition of plant nutrients under nutrient stress (Figure 3). In addition, the higher biomass and N uptake in maize than in alfalfa indicated that maize was more competitive for N than alfalfa; the root system of maize grew faster and wider than that of alfalfa (maize root length density: 1.41–2.14 cm cm⁻³; alfalfa root length density: 0.07–0.12 cm cm⁻³).

4.2. N Transfer between Maize and Alfalfa

The transfer of N from legumes to cereal plants by root interactions is an important strategy for the improvement of N utilization in intercropping systems. Previous studies have found that N transfer occurs in most cereal/legume intercropping systems and that the N transfer rates range from 3.1% to 15% [7,21]. Consistent with these findings, the ¹⁵N foliar feeding results clearly showed that the transfer rate was 7.02–15.05%, which falls well within an acceptable range (Table 5). Three important and distinct pathways of N transfer from legumes to non-legumes are (1) decomposition of legume root tissues; (2) root exudation; and (3) mycorrhizae [31,37]. Although root decomposition plays an important role in N transfer, it is generally a slow process [37]. Therefore, the root decomposition pathway may not contribute greatly to short-term N transfer. In our study, the amount of N transferred from alfalfa to maize under the nylon net barrier treatment was 165.13 mg pot⁻¹. This amount may have been mainly transferred through the root exudation or the mycorrhizal pathways in response to the much higher N demand by the maize, since the maize and the alfalfa roots were not in direct contact. In addition, the amount of N transferred without a root barrier was 1.24–1.42 times higher than that with a nylon net barrier regardless of the N level applied (Table 5). The main reason for the high nitrogen transfer with no N barrier may be that the close root contact of maize and alfalfa can stimulate the plants to release more N-containing compounds or generate more mycelial connections between the legume and the cereal plants [21]. Moreover, root contact reduces the travel distance of N compounds through mass flow [31], which efficiently promotes N transfer in soybean/maize intercropping systems [14]. In addition, root morphology and biomass influence the uptake and the utilization of soil water and nutrients and permit subsequent N transfers between cereals and legumes [23]. The maize root length density and root biomass with no root barrier were significantly higher than those with the nylon net barrier (data not shown). The higher maize root length density and root biomass with no root barrier may have facilitated the acquisition of N compounds from alfalfa. Overall, the N transfer contributed 3.64–4.72% of the total maize N uptake with no root barrier and 2.36–4.48% of the total maize N uptake with the nylon net barrier (Figure 4), demonstrating that N transfer from alfalfa to maize improves the N uptake and the N utilization in an alfalfa/maize intercropping system. Consistent with previous studies [38–40], we found that N addition significantly restricted N transfer (Table 5), which may be related to the greater likelihood of maize using N from soil

and fertilizer than biologically fixed N from alfalfa in N-rich soils [7,22,41]. Our other research data also proved this problem regarding the effect of different nitrogen application levels and root separation methods on nitrogen fixation ability of alfalfa in a maize/alfalfa intercropping system (Tables A2 and A3, Figures A3 and A4).

Overall, the maize/alfalfa intercropping system has the potential to improve total biomass and N uptake compared to monoculture. This result is consistent with many other studies and demonstrates the advantages of intercropping [5,42]. In addition, the total biomass and the N uptake with no root barrier were significantly higher than those with the nylon net barrier. In conclusion, the root contact plays an important role in intercropping [14]. However, the maize/alfalfa intercropping system has no commercial applicability. This is mainly due to the following reasons. First, with the increase of China's maize storage and market competition after China's accession to the WTO, the farmers' enthusiasm for agricultural production has severely reduced since the price of maize fell sharply in 2016 [43]. Second, the key limiting factor for large-scale production of intercropping is the mechanization of intercropping [44]. This problem is becoming more and more acute due to rising labor costs and the gradual intensification of rural labor shortages. To realize the mechanization of intercropping production, on the one hand, it requires agronomically standardized, high-yield, and efficient inter-species arrangement patterns and row spacing arrangements; on the other hand, it has corresponding sowing, fertilizer application, and harvest machinery. Obviously, compared to a monoculture system, the management intercropping system is more complicated and inconvenient. In order to simplify the management of this intercropping system, it will be necessary to integrate multidisciplinary knowledge to develop efficient machinery. Third, the crop combination and the proper variety selection in intercropping are the keys to success, but little attention has been paid to this. Appropriate crop species or variety combinations can enhance positive interspecific interactions between crops, reduce competition between crops, and increase productivity. Therefore, it is a problem worthy of attention to breed crops with certain crop characteristics that are conducive to inter-species promotion and reduce competition [45].

Finally, further research is required to assess the long-term benefits of the composite crop population and its responses to rainfall, planting years, and environmental stresses (pest, disease, and freezing injury) in order to avoid agronomic risks and economic loss. Therefore, the dissemination of efficient intercropping technologies and expert technical guidance as well as financial support of government will necessarily play important roles in putting alfalfa/maize intercropping into practice in agricultural and pastoral areas in Northeast China [5].

5. Conclusions

The two year pot experiments demonstrated that N transfer between the two plants occurred mainly from alfalfa to maize. The N transfer rate was 7.02–15.05%, which accounted for 2.36–4.72% of the total N uptake. Root contact can improve N transfer, which can increase total biomass, total N uptake, and N utilization in maize. Our results highlight the importance and the relevance of root contact for increasing of N transfer and utilization in maize/alfalfa intercropping systems. Maize/alfalfa intercropping has obvious advantages in terms of crop biomass and N uptake, which can ensure food security and provide superior forage. In addition, N fixation and transfer from alfalfa to maize can improve soil fertility and reduce chemical fertilizer application. In conclusion, the promotion and the application of maize/alfalfa are determined as the optimal strategy, which will be beneficial to further development for intercropping.

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Appendix A

Figure A1. Schematic diagram of the maize/alfalfa intercropping pot experiment.

Figure A2. Air temperature (curve) of the experimental site in 2015 and 2016.

Table A1. Aggressivity and competitiveness of maize relative to alfalfa for the nitrogen level (N) and root separation method (RS) between 2015 and 2016.

Years	Treatment	A _{ma}	NCR _{ma}
2015	N0	NS	2.30
		NNS	1.53
		PSS	2.90
	N1	NS	1.48
		NNS	0.55
		PSS	1.65
2016	N0	NS	2.07
		NNS	1.46
		PSS	3.88
	N1	NS	2.26
		NNS	1.59
		PSS	2.03

A_{ma}: aggressivity of maize relative to alfalfa; NCR_{ma}: nitrogen competitive ratio of maize relative to alfalfa. A_{ma}: aggressivity of maize relative to alfalfa; NCR_{ma}: nitrogen competitive ratio of maize relative to alfalfa. If A_{ma} is greater than 0, the competitive ability of maize exceeds that of maize in intercropping; if NCR_{ma} is greater than 1.0, the N competitive ability of maize will be greater than that of alfalfa in intercropping.

Table A2. Results of repeated measures ANOVA about effects of year (Y), nitrogen levels (N), and root separation method (RS) on nodule number and dry weight of alfalfa.

Factors	Nodule Number		Dry Weight	
Y	106.39	<0.001 **	16.44	<0.001 **
N	191.63	<0.001 **	285.27	<0.001 **
RS	82.74	<0.001 **	41.94	<0.001 **
Y × N	3.02	0.091	3.14	0.085ns
Y × RS	1.59	0.218	2.90	0.068ns
N × RS	3.74	0.033 *	6.25	0.005 **
Y × N × RS	2.59	0.089	1.27	0.293ns

Note: * $p < 0.05$; ** $p < 0.01$; and ns: $p > 0.05$.

Figure A3. Effects of nitrogen levels and root separation patterns on nodule number and dry weight of alfalfa. Different lowercase letters show significant difference between root separation patterns under the same nitrogen levels at $p \leq 0.05$.

Table A3. Results of repeated measures ANOVA about effects of year (Y), nitrogen levels (N), and root separation method (RS) on %Ndfa and Ndfa of alfalfa.

Factors	Alfalfa %Ndfa		Alfalfa Ndfa	
Y	7.42	<0.001 **	74.15	<0.001 **
N	239.16	<0.001 **	54.60	<0.001 **
RS	339.12	<0.001 **	44.47	<0.001 **
Y × N	4.77	0.036 *	7.39	<0.001 **
Y × RS	3.33	0.047 *	2.30	0.114ns
N × RS	24.55	<0.001 **	5.28	0.009 **
Y × N × RS	3.28	0.049 *	1.15	0.328ns

Note: * $p < 0.05$; ** $p < 0.01$; and ns: $p > 0.05$.

Figure A4. Effects of year, nitrogen levels and root separation method on %Ndfa (A,B) and Ndfa (C,D) of alfalfa. Different lowercase letters show significant difference between root separation method under the same nitrogen levels at $p \leq 0.05$.

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