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# A Small Amount of Nitrogen Transfer from White Clover to Citrus Seedling via Common Arbuscular Mycorrhizal Networks

**Linfa Fang [1], Xinhua He [1], Xueliang Zhang [1], Yehua Yang [1], Rui Liu [1], Songmei Shi [1], Xiaojun Shi [1,2,3] and Yuting Zhang [1,2,3,*]**

[1] College of Resources and Environment, Southwest University, Chongqing 400715, China; fanglfswu@163.com (L.F.); xinhua.he@uwa.edu.au (X.H.); z15023735317@163.com (X.Z.); 15286385656@sina.cn (Y.Y.); liurglor@163.com (R.L.); shismei@email.swu.edu.cn (S.S.); shixj@swu.edu.cn (X.S.)
[2] Key Laboratory of Efficient Utilization of Soil and Fertilizer Resources, Chongqing 400715, China
[3] State Cultivation Base of Eco-Agriculture for Southwest Mountainous Land, Southwest University, Chongqing 400715, China
* Correspondence: zyt2018@swu.edu.cn

**Abstract:** Few studies have examined if perennial leguminous cover crops are able to transfer nitrogen (N) via common mycorrhizal networks (CMNs) to neighboring fruit trees; the gradient of such N transfer could affect the N nutrition of both plants. Using separated three-column chambers to grow plants in a greenhouse, 99 atom% $^{15}N$ as $(^{15}NH_4)_2SO_4$ was applied to leaves of white clover (*Trifolium repens* L.) and $^{15}N$ was then traced in neighboring citrus (*Citrus sinensis* (L.) Osbeck) seedlings interconnected by an arbuscular mycorrhizal fungus (AMF, *Rhizophagus intraradices*). A range of 66.85–68.74% mycorrhizal colonization in white clover (mycorrhizal and/or *Rhizobium trifolii* inoculated) and 19.29–23.41% in citrus (non-mycorrhizal inoculated) was observed after 12 months of AMF inoculation in the white clover, indicating a successful CMN linkage was established between these two plant species. This CMN establishment resulted in significant increases in biomass, N accumulation, and $^{15}N$ content of citrus when accompanied with nodulated and mycorrhizal fungus colonized white clover. N transfer from white clover to citrus was significantly greater under nodulation plus mycorrhization (46.23 mg N per pot, 1.71% of N transferred) than under non-inoculated control (4.36 mg N per pot, 0.21% of N transferred), and higher than sole mycorrhization (36.34 mg N per pot, 1.42% of N transferred). The percentage of N in citrus derived from white clover under nodulated/mycorrhization was 1.83–1.93%, and was highest in leaves (3.31%), moderate in stems (2.47%), and lowest in roots (0.41%) of citrus. In summary, results from this experiment demonstrated that nearly 2.0% of N transferred from white clover to citrus via CMN. Further studies are needed to quantify N transfer between white clover and citrus by other routes, including soil or root exudation pathways.

**Keywords:** $^{15}N$; *Citrus sinensis* (L.) Osbeck; *Rhizobium trifolii*; *Rhizophagus intraradices*; *Trifolium repens* L.



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## 1. Introduction

Citrus is the second widest cultivated fruit tree in the world, covering a planted area of 6.4 million ha in 2017 [1]. The rapid development of citrus plantations or orchards has induced a large demand for sources of external nitrogen (N). The excessive N inputs in citrus orchards could result in nutrient loss to the environment, which will inevitably lead to environmental pollution [2,3]. Leguminous cover crops, which have the capacity to symbiotically fix atmospheric $N_2$, could fix 110–227 kg N $ha^{-1}$ $year^{-1}$ in agroecosystems [4,5]. Intercropping fruit trees with leguminous cover crops has been considered an environmentally friendly way for sustainable agriculture while reducing the use of N fertilizers [6,7]. Previous studies found that leguminous cover crops made significant contribution to N nutrient in different orchard systems [8–12]. For instance, in a grapevine

production system, 14–19% of grapevine total N was transferred from $N_2$-fixing subterranean clover (*Trifolium subterraneum* L.) and burr medic (*Medicago polymorpha* L.) [10]. In an apple orchard, nearly 50% of the total N in apple trees was provided by a mix of leguminous cover crops, including subterranean clover, burr medic, black medic (*Medicago lupulina* L.), birdsfoot trefoil (*Lotus corniculatus* Linn.), and colonial bentgrass (*Agrostis tenuis* Sibth.) [13]. About 30% of N in leguminous cover crops (*Flemingia macrophylla* (Willd.) Prain, *Desmodium intortum* (Mill.) Urb., *Leucaena leucocephala* (Lam.) de Wit) was transferred to the coffee plants in a coffee plantation [14–16]. However, limited information is available for the N contribution of leguminous cover crops to citrus.

The possible mechanisms of N transfer from leguminous cover crops to adjacent plants involve both aboveground and belowground routes. Aboveground N transfer via leaf absorption of volatilized N-containing compounds is one way of N transfer [17]. Belowground N transfer via soil mass flow and diffusion, and/or mycorrhizas may be the main pathways from perennial leguminous cover crops to fruit trees [18,19]. Previous studies have shown that mycorrhizal fungi could form mutualistic symbiosis associating with the roots of more than 80% of terrestrial plant species [12,20–23]. One or more mycorrhizal fungi can colonize two or more plants, forming common mycorrhizal networks (CMNs) between different plant species [21,24]. Numerous studies have demonstrated different percentages of N transfer to other plants via CMNs, such as 1.3–3.5% from ectomycorrhizal fungus (ECM) linked *Casuarina cunninghamiana* Miq. to *Eucalyptus maculata* Hook.f. [20,24], and 5.5–24.8% from AMF linked *Medicago polymorpha* L. to grapevines [11]. Previous studies have also observed that CMNs could be formed between citrus and white clover roots [12,25]. However, the quantity and dynamics of N transferred from perennial leguminous cover crops to citrus trees are still unknown.

In addition, the N transfer via CMNs is based on the assumption that N flows from the N-rich legume to the N-poor non-legume [26]. Nitrogen gradients between N-rich donors and N-limited receivers may be a driving force for the N transfer via CMNs [20,24]. Previous studies found that because of an effective $N_2$-fixation by legume crops, N transfer was significantly increased to neighboring grapevines [11], rice [27], grasses [28], and *Dichanthium aristatum* (Poir.) C. E. Hubb. [19]. Generally, the N concentration of white clover (3.3–3.8%, leaf) is higher than citrus (2.5–2.7%, leaf; 1.17–1.42%, root) [29], which may drive the flow of N from the former to the latter plant. Thus, is N transfer from white clover to citrus improved by $N_2$-fixation?

In the present study, a citrus seedling and white clover were grown in separated three-column chambers in a greenhouse, and $(^{15}NH_4)_2SO_4$ was used as a $^{15}N$ tracer to address the possible N movement from white clover to citrus. Our objectives were to test if (a) N in white clover could be transferred to citrus via a CMN and if (b) N transfer could be improved by enhanced $N_2$ fixation of white clover.

## 2. Materials and Methods

### *2.1. Experimental Pots and Plant Growth Conditions*

This experiment was conducted in a greenhouse at Southwest University, Chongqing, China, from April 2018 to April 2019. Citrus used in this study was Tarocco (*Citrus sinensis* (L.) Osbeck) grafted on trifoliate orange rootstock (*Poncirus trifoliata* (L.) Raf.), while white clover was *Trifolium repens* L. Citrus and white clover were grown in a three-column chambered pot as shown in Figure 1. The experimental pots were stainless steel boxes (500 mm × 400 mm × 300 mm), which were separated by two perforated stainless steel plates (10 mm wide) to form a 10 mm air gap for preventing water and solute movement between two chambers. Each perforated plate had 390 holes (8 mm in diameter). On both sides of the plate, there was a 37 μm hardware cloth that allowed hyphal connections between two chambers but prevented roots.

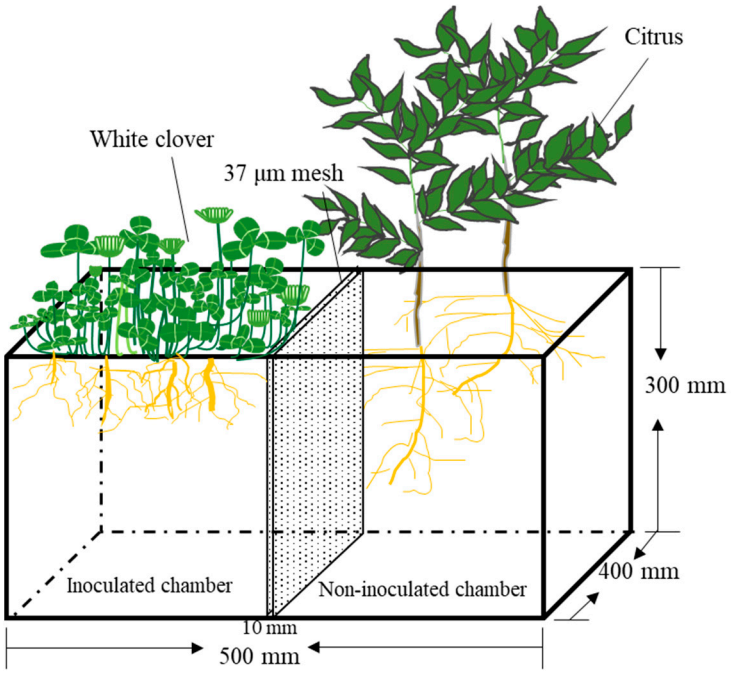


**Figure 1.** Schematic view of a three-column chamber pot. One chamber held 50 white clover seedlings that were inoculated with or without rhizobium and/or mycorrhizal fungus, and the other chamber was for two non-inoculated citrus seedlings.

The growth medium consisted of a 5:1 sterilized (121 °C, 0.11 Mpa, 30 min) mixture of field soil (Eutric Regosol [30], collected from a citrus orchard of Southwest University campus) and fine sand (50 kg dry soil, 10 kg dry fine sand). This soil/sand mixture contained 10.53 g/kg soil organic matter, 19.6 mg/kg soil $NH^+_4$-N, 32.8 mg/kg soil Olsen P, 180 mg/kg soil available K, and had pH 7.5. The moisture of the growth medium in the pot was maintained at 60–65% of maximum field capacity. In order to maintain soil moisture while avoiding water movement between the two chambers, 0.5% (*w*/*w*) high-water-holding capacity crystals (SOCO®, Polymer, Qingdao, China) were mixed with the growth media.

The experimental pot contained two citrus seedlings in one chamber and 50 white clover seedlings in another chamber. Nine-month-old citrus seedlings, obtained from the Citrus Research Institute (Chinese Academy of Agricultural Sciences, Beibei, China), were transplanted into pots in the greenhouse. Before transplanting into the pots, the whole citrus seedlings, particularly the roots, were washed five times with distilled water. The white clover seeds were surface sterilized by 5% NaClO for 5 min, rinsed with sterile water, and germinated on sterilized plates for 24 h, and then sown in the pots in the greenhouse. Rhizobial suspension from *Rhizobium trifolii* was added to the soil surface near the top root of white clovers at weeks 1, 2, and 3 (50 mL each time) after transplanting. The *Rhizobium trifolii* was purchased from the China Agriculture Microbiological Culture Collection Center, China. For the AMF inoculation, 180 g of inoculum (~54 million spores) of *Rhizophagus intraradices* (Monterey Frontage Road, Gilroy, CA, USA) was placed at 10 cm depth in the growth media in the chamber at the time of transplanting.

### *2.2. Experimental Design*

Four treatments were examined: (1) no rhizobial and no arbuscular mycorrhizal inoculation as the control (CK), (2) inoculated with a rhizobium *Rhizobium trifolii* only (+Rhi), (3) inoculated with an AMF *Rhizophagus intraradices* (+AMF) only, and (4) inoculated with both *Rhizobium trifolii* and AMF (+Rhi + AMF). The white clover parts, as N supplier, were inoculated with or without *Rhizobium trifolii* and AMF (*Rhizophagus intraradices*). The citrus seedlings as N receiver were inoculated with neither *Rhizobium trifolii* nor AMF.

Each treatment had four replicates, for a total of 16 pots in a completely randomized arrangement. To minimize possible environmental effects, the position of pots was shifted once a week over the growth period.

### 2.3. $^{15}N$ Labeling

The $^{15}N$ isotope dilution method, the $^{15}N$ natural abundance method and the $^{15}N$ leaf-labeling method were generally used to measure N transfer. The leaf-labeling technique, the most effective and straight-forward method, has been successfully used between mycorrhizal plants, according to Lu et al. [28], Meng et al. [31] and He et al. [20,24,32,33]. In particular, successful N transfer from white clover to neighboring grasses has been demonstrated by the $^{15}N$ foliar-labeling method [34]. After 12 months of growth, white clovers were labeled with $^{15}N$. One leaf of white clover was inserted into a 50 mL centrifuge tube filled with 2 mL 1% $^{15}N$ solution (99 atom% $^{15}N$ as $(^{15}NH_4)_2SO_4$) and wrapped on a bamboo stick near the clover. Six white clover leaves were labeled in each pot. To avoid spillage and evaporation, the centrifuge tubes were sealed using Parafilm (Menasha, WI, USA) after wrapping.

### 2.4. Plant Harvest

The labeled white clover and citrus leaves were collected the day after 14-day labeling. A few white clover leaves were collected before $^{15}N$ labeling for the estimation of $N_2$ fixation [20]. White clover and citrus were destructively separated into different components: leaves and roots for white clover; leaves, stems and roots for citrus. A portion of fresh root sample was used for estimation of mycorrhizal colonization. Roots were cut into segments about 1 cm long and mixed, then each sample was cleared with 10% KOH at 90 °C (white clover roots, 30 min; citrus roots, 60 min) and stained with 0.05% trypan blue in lactoglycerol to test the root colonization, according to Phillips and Hayman (1970) [35]. Root mychorrhizal colonization was illustrated as the proportion of AMF-colonized root length against total root length. The plant components were washed, oven-dried, ground in a ball-mill (QM100s, Beijing, China), and analyzed for total N and $^{15}N$ (vario PYRO Cube-IsoPrime 100, Elementar-Isoprime Ltd., Cheadle Hulme, UK).

### 2.5. Calculation and Statistical Analysis

Since the white clover in the first harvest was not labeled with enriched $^{15}N$, the percentage of plant nitrogen derived from fixation ($\%N_{fix}$) was calculated according to the $^{15}N$ natural abundance method [36,37]:

$$\%N_{fix} = \frac{\delta^{15}N_{nonfixing\ plant} - \delta^{15}N_{fixing\ plant}}{(\delta^{15}N_{nonfixing\ plant} - B)} \times 100 \quad (1)$$

where B is the $\delta^{15}N$ value of non-inoculated $N_2$-fixing plant grown without N supplement.

The amount of N in white clover derived from fixation ($N_{fix}$) was calculated [38]:

$$N_{fix} = Ncontent_{white\ clover} \times \frac{\%N_{fix}}{100} \quad (2)$$

N transfer was calculated as %N transfer, amount N transfer (mg per pot), and %N in citrus derived from transfer (%NDFT), using the following formulas [24,28].

The percentage of N transfer in citrus root, stem, and leaf (%N transfer):

$$\%N_{transfer} = \frac{^{15}Ncontent_{citrus\ organs}}{^{15}Ncontent_{citrus} + ^{15}Ncontent_{white\ clover}} \times 100 \quad (3)$$

where

$$^{15}Ncontent_{plant} = \frac{Atom\ \%^{15}N\ excess_{plant} \times total\ N_{plant}}{Atom\ \%^{15}N\ excess_{labeled\ N}} \quad (4)$$

Amount of N transferred (mg per pot):

$$N_{transfer} = \frac{\%N_{transfer} \times \text{total } N_{white\ clover}}{100 - \%N_{transfer}} \quad (5)$$

Percent of N in citrus organs derived from transfer (% NDFT):

$$\%NDFT = \frac{N_{transfer}}{\text{Total } N_{citrus}} \times 100 \quad (6)$$

Using SPSS 20.0 (SPSS, Inc., Chicago, IL, USA), data (mean ± SE, $n$ = 4) were subjected to a one-way variance (ANOVA) analysis and differences between treatments were compared (Duncan's multiple comparison test) and considered significant at $p < 0.05$.

## 3. Results

### *3.1. Biological Nitrogen Fixation in White Clover and Formation of Mycorrhizas*

The $\delta^{15}N$ value in +Rhi white clover was ~0.71‰, and $N_2$-fixation supplied ~31.28% of total N requirement in white clover (Table 1). The weights of nodule averaged 2.27–2.38 g per pot and were similar between +Rhi and +Rhi + AMF treatments, but nodule numbers under +Rhi + AMF were significantly higher than under +Rhi. Root mycorrhizal colonization was less than 2% in the control (Table 2, Figure S1), which was significantly lower than when inoculated with AMF treatments in both citrus (19.29–23.41%) and white clover (66.85–68.74%), and white clover root colonization was greater than citrus under both +AMF and +Rhi + AMF treatments.

**Table 1.** $N_2$-fixation capacity of nodulated white clover grown in pots.

| Treatments | Shoot $\delta^{15}N$(‰) | Nodule Numbers | Nodule Fresh Weight (g) | $\%N_{fix}$ | $N_{fix}$ (mg/pot) |
|---|---|---|---|---|---|
| CK | 1.25 ± 0.14 a | – | – | – | – |
| +Rhi | 0.71 ± 0.13 b | 126 ± 10 b | 2.27 ± 0.02 a | 31.28 ± 5.43 a | 710.44 ± 100.19 a |
| +AMF | 1.19 ± 0.09 a | – | – | – | – |
| +Rhi + AMF | 0.703 ± 0.11 b | 168 ± 12 a | 2.38 ± 0.02 a | 34.85 ± 6.94 a | 923.38 ± 167.40 a |

Data are means ± SE ($n$ = 4). Values followed by a different letter are significantly different ($p < 0.05$). Percent $N_{fix}$ and $N_{fix}$ were calculated by Equations (1) and (2), respectively. Abbreviation: CK, no rhizobium and no arbuscular mycorrhizal inoculation; +Rhi, inoculated with rhizobium; +AMF, inoculated with mycorrhizal fungus.

**Table 2.** Mycorrhizal colonization of white clover and citrus grown in pots.

| Treatments | White Clover Root (%) | Citrus Root (%) |
|---|---|---|
| CK | 1.48 ± 0.14 b | 1.35 ± 0.45 b |
| +Rhi | 1.52 ± 0.75 b | 1.41 ± 0.10 b |
| +AMF | 66.85 ± 0.71 a | 19.29 ± 4.91 a |
| +Rhi + AMF | 68.74 ± 6.70 a | 23.41 ± 4.82 a |

Data are means ± SE ($n$ = 4). Values followed by different letters indicate significant differences between treatments for the same plant species (a, b) ($p < 0.05$). Abbreviations: CK, no rhizobium and no arbuscular mycorrhizal inoculation; +Rhi, inoculated with rhizobium; +AMF, inoculated with mycorrhizal fungus.

### *3.2. Plant Biomass*

Aboveground biomass production of both white clover and citrus had the similar pattern: +AMF ≈ +Rhi + AMF > +Rhi > CK (Figure 2a,d). Significantly higher (1.30–1.35 times) belowground biomass in citrus was under +Rhi + AMF than under +Rhi and CK (Figure 2e). In addition, the plant total biomass production was similar between treatments in white clover (Figure 2c), while showing a significant pattern as +Rhi + AMF > +AMF ≈ +Rhi ≈ CK, and also CK < +AMF in citrus (Figure 2f).

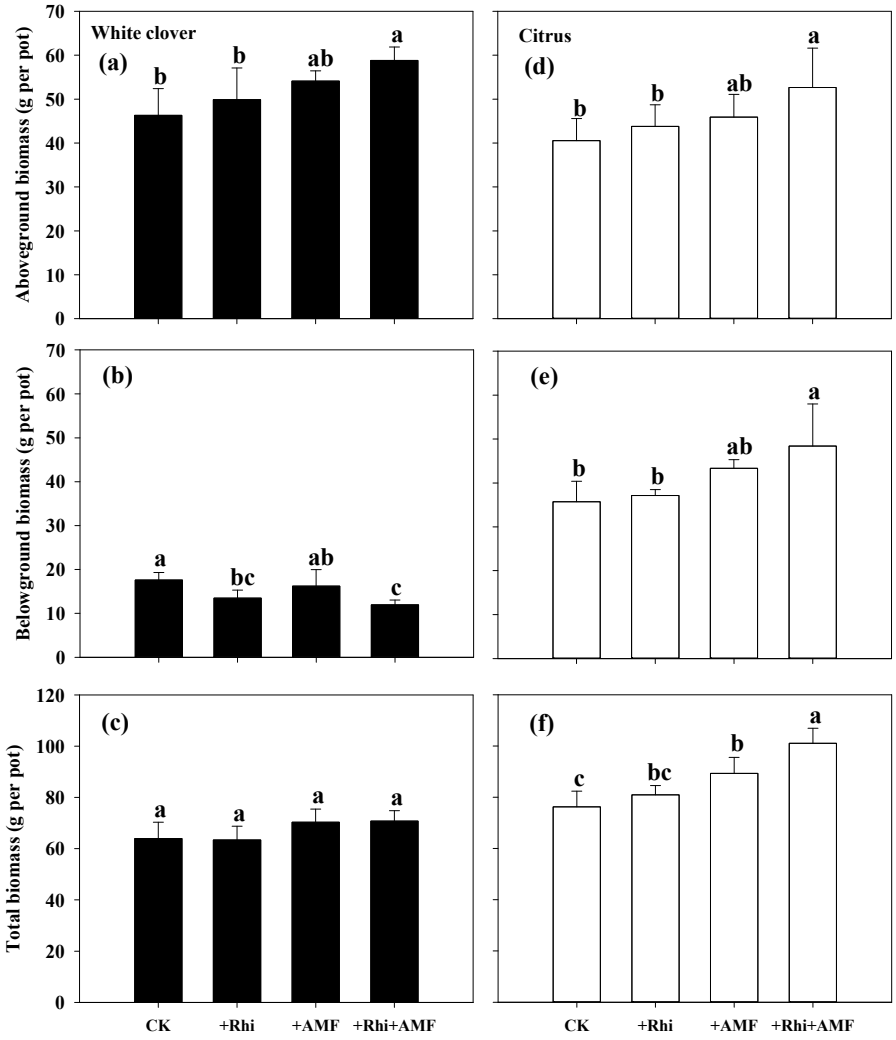


**Figure 2.** Aboveground (**a**,**d**), belowground (**b**,**e**), and total biomass (**c**,**f**) (g per pot) for white clover and citrus affected by rhizobia and mycorrhizal. Different letters above columns designate significant differences ($p < 0.05$) between treatments. Abbreviations: CK, no rhizobium and no arbuscular mycorrhizal inoculation; +Rhi, inoculated with rhizobium; +AMF, inoculated with mycorrhizal fungus.

### *3.3. Plant N Concentration and Content*

Either aboveground or belowground N concentrations of white clover have no significantly differences (Table 3). Aboveground N concentrations in citrus were significantly greater under both +AMF and +Rhi + AMF than under CK. Belowground N concentration of citrus was greater under both +AMF and +Rhi + AMF treatments than CK. Tissue N concentrations were generally greater in white clover than in citrus irrespective of aboveground or belowground parts.

Leaf N content in white clover under +Rhi + AMF was 1.22–1.33 times higher than under both +Rhi or CK, and citrus was 1.48–1.57 times greater under +Rhi + AMF than under +Rhi or CK (Table 5). Citrus roots N content in +Rhi + AMF treatment was higher (1.37–1.55 times) than +Rhi and CK treatments. There were no significant differences in stem N content of citrus between treatments. Total N content of white clover and citrus was significantly (1.16–1.17 times, white clover; 1.36–1.50 times, citrus) greater under +Rhi + AMF than under +Rhi and CK. In both plant species, the biomass production (aboveground, belowground, or total) was positively correlated with plant N content (Figure 3, $r^2 = 0.94$–$0.99$).

**Table 3.** N concentration (%) in various parts of white clover and citrus grown in pots.

| Treatments | White Clover | | Citrus | |
|---|---|---|---|---|
| | Aboveground | Belowground | Aboveground | Belowground |
| CK | 3.78 ± 0.07 a | 2.61 ± 0.14 a | 2.31 ± 0.03 b | 1.81 ± 0.03 b |
| +Rhi | 3.88 ± 0.09 a | 2.65 ± 0.06 a | 2.30 ± 0.10 b | 1.93 ± 0.21 ab |
| +AMF | 3.92 ± 0.31 a | 2.52 ± 0.11 a | 2.53 ± 0.05 a | 1.88 ± 0.08 ab |
| +Rhi + AMF | 3.99 ± 0.18 a | 2.65 ± 0.08 a | 2.59 ± 0.08 a | 2.034 ± 0.07 a |

Data are means ± SE ($n$ = 4). Values followed by different letters indicate significant differences between treatments for the same plant species (a, b) ($p < 0.05$). Abbreviations: CK, no rhizobium and no arbuscular mycorrhizal inoculation; +Rhi, inoculated with rhizobium; +AMF, inoculated with mycorrhizal fungus.

### *3.4. $^{15}N$ Atom Excess and $^{15}N$ Content*

After labeling for 14 days, leaf $^{15}N$ atom excess was 0.38–0.49% and root excess was 0.22–0.29% in white clover (Table 5). In the leaf and stem part of citrus, +AMF and +Rhi + AMF treatments had similar low $^{15}N$ atom excess (<0.00075%), while $^{15}N$ atom excess with +AMF and +Rhi + AMF treatments was higher than +Rhi and CK treatments. There were no statistical differences in citrus root $^{15}N$ atom excess between the four treatments.

The $^{15}N$ content was much greater in white clover than in citrus (Table 5) and were similar between treatments in white clover (leaf and root, or total). In contrast, significantly greater tissue (leaf, stem, root, and total) $^{15}N$ content between treatments +Rhi + AMF > +AMF > +Rhi > CK was observed in citrus, but not between +AMF and +Rhi + AMF in roots.

### *3.5. N Transfer from White Clover to Citrus*

For citrus, %N transfer under +AMF and +Rhi + AMF was very low in both stems and roots (<0.3%), and was relatively higher in leaves (1.04–1.27%) (Figure 4). The total %N transfer in citrus was greater under +AMF and +Rhi + AMF (1.42% and 1.71%) than under CK and +Rhi (0.21% and 0.26%).

Similar to %N transfer, leaf and stem N transfer amounts in citrus were 15.1–26.0 and 6.9–9.8 times higher under +AMF and +Rhi + AMF than under +Rhi and CK (Table 4). The total amounts of N transfer from white clover to citrus were 36.34–46.23 mg per pot.

**Table 4.** Amount of nitrogen transfer in various parts of citrus.

| Treatments | Root | Stem | Leaf | Total |
|---|---|---|---|---|
| CK | 1.82 ± 0.51 b | 0.78 ± 0.27 b | 1.75 ± 0.70 b | 4.36 ± 0.75 b |
| +Rhi | 2.31 ± 0.57 ab | 0.83 ± 0.07 b | 1.31 ± 0.22 b | 4.81 ± 0.79 b |
| +AMF | 4.03 ± 0.54 a | 5.74 ± 0.93 a | 26.58 ± 3.86 a | 36.34 ± 5.11 a |
| +Rhi + AMF | 4.10 ± 0.71 a | 7.78 ± 1.09 a | 34.34 ± 5.21 a | 46.23 ± 5.79 a |

Data are means ± SE ($n$ = 4). Values followed by different letters indicate significant differences ($p < 0.05$) between treatments. Abbreviations: CK, no rhizobial and no arbuscular mycorrhizal inoculation; +Rhi, inoculated with rhizobium; +AMF, inoculated with mycorrhizal fungus.

The percentage of N in citrus roots derived from white clover plants were very low (≤0.5%) (Figure 5). Leaf, stem, and total plant %NDFT were significantly higher (6.5–7.2, 7.0–11.76, and 12.16–18.4 times) under +AMF and +Rhi + AMF than under +Rhi and CK.

**Table 5.** N content (mg per pot), $^{15}N$ atom % excess, $^{15}N$ content (mg per pot) in various parts of white clover and citrus grown in pots. White clovers were labeled with $(^{15}NH_4)_2SO_4$ for 14 days before harvest.

| Treatments | White Clover | | | Citrus | | | |
|---|---|---|---|---|---|---|---|
| | **Leaf** | **Root** | **Total** | **Leaf** | **Stem** | **Root** | **Total** |
| | | | N content (mg per pot) | | | | |
| CK | 1756.96 ± 139.98 b | 473.77 ± 6.72 a | 2273.72 ± 134.21 b | 670.24 ± 32.79 b | 272.15 ± 24.23 a | 643.92 ± 44.92 b | 1586.32 ± 72.54 c |
| +Rhi | 1923.91 ± 145.58 b | 363.66 ± 13.32 bc | 2287.57 ± 138.57 b | 711.76 ± 91.02 b | 302.27 ± 13.55 a | 716.34 ± 49.76 b | 1730.36 ± 114.77 bc |
| +AMF | 2119.29 ± 74.23 ab | 406.35 ± 41.17 ab | 2525.64 ± 93.24 ab | 870.92 ± 24.52 ab | 288.85 ± 28.11 a | 876.74 ± 37.79 ab | 1976.52 ± 82.21 b |
| +Rhi + AMF | 2341.23 ± 37.15 a | 315.75 ± 9.45 c | 2656.97 ± 42.68 a | 1048.12 ± 14.78 a | 322.88 ± 25.14 a | 990.05 ± 12.51 a | 2361.06 ± 53.60 a |
| | | | $^{15}N$ atom % excess | | | | |
| CK | 0.49 ± 0.10 a | 0.29 ± 0.03 a | – | 0.00075 ± 0.00012 c | 0.00027 ± 0.00014 c | 0.0012 ± 0.0007 a | – |
| +Rhi | 0.43 ± 0.11 a | 0.26 ± 0.05 ab | – | 0.00072 ± 0.00022 c | 0.00005 ± 0.00003 c | 0.0013 ± 0.0006 a | – |
| +AMF | 0.38 ± 0.11 a | 0.22 ± 0.03 c | – | 0.010 ± 0.00067 b | 0.0066 ± 0.0007 b | 0.0016 ± 0.0001 a | – |
| +Rhi + AMF | 0.41 ± 0.03 a | 0.27 ± 0.03 ab | – | 0.013 ± 0.0029 a | 0.0095 ± 0.0031 a | 0.0016 ± 0.0002 a | – |
| | | | $^{15}N$ content (mg per pot) | | | | |
| CK | 8.67 ± 2.63 a | 0.79 ± 0.16 a | 9.45 ± 2.80 a | 0.0077 ± 0.0067 c | 0.0030 ± 0.0015 c | 0.0073 ± 0.0035 c | 0.018 ± 0.006 c |
| +Rhi | 8.13 ± 1.24 a | 0.80 ± 0.18 a | 8.92 ± 1.39 a | 0.0050 ± 0.0012 c | 0.0032 ± 0.0007 c | 0.0099 ± 0.0050 ab | 0.018 ± 0.003 c |
| +AMF | 8.12 ± 2.39 a | 0.65 ± 0.18 a | 8.78 ± 1.93 a | 0.087 ± 0.009 b | 0.019 ± 0.006 b | 0.013 ± 0.0006 ab | 0.12 ± 0.01 b |
| +Rhi + AMF | 9.38 ± 0.68 a | 0.88 ± 0.16 a | 10.27 ± 0.61 a | 0.13 ± 0.04 a | 0.030 ± 0.008 a | 0.016 ± 0.005 a | 0.18 ± 0.04 a |

Data are means ± SE ($n$ = 4). Values followed by different letters indicate significant differences between treatments for the same plant species (a, b, c) ($p < 0.05$). Abbreviations: CK, no rhizobium and no arbuscular mycorrhizal inoculation; +Rhi, inoculated with rhizobium; +AMF, inoculated with mycorrhizal fungus.

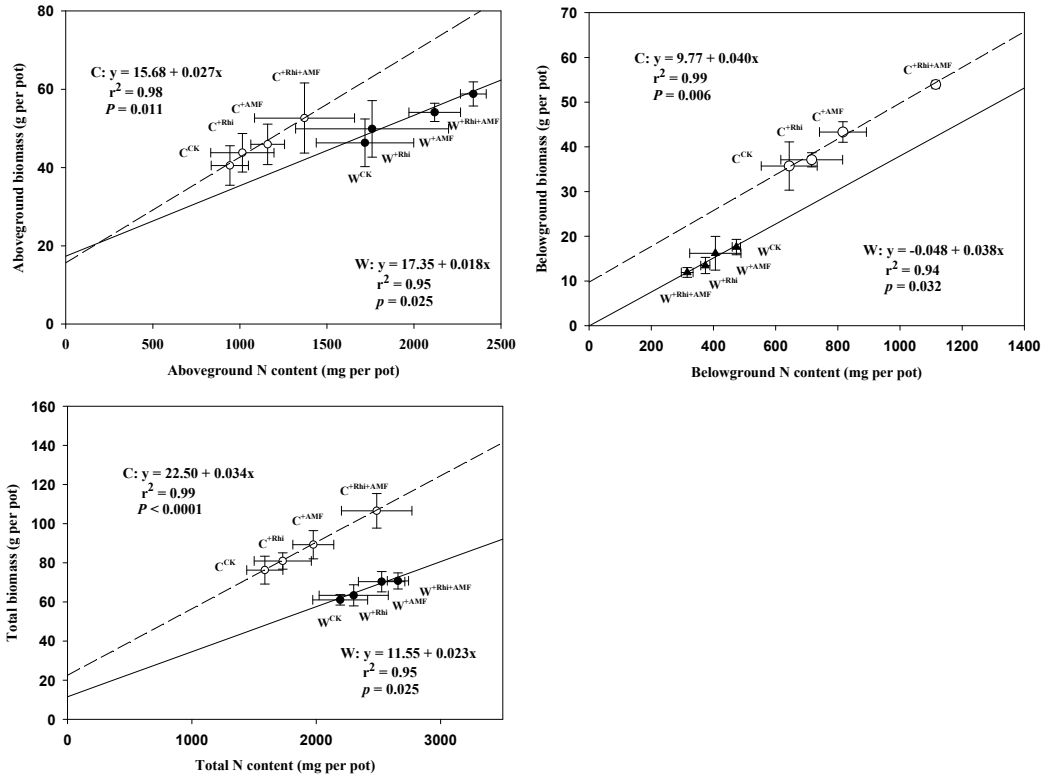


**Figure 3.** Relationship between biomass and N content of various parts of white clover and citrus. Data are mean ± SE ($n$ = 4) and white clover are indicated by solid symbols and citrus by open symbols. Regressions are shown for white clover (solid lines) and for citrus (dashed lines). Abbreviations: W, white clover; C, citrus; CK, no rhizobium and no arbuscular mycorrhizal inoculation; +Rhi, inoculated with rhizobium; +AMF, inoculated with mycorrhizal fungus.

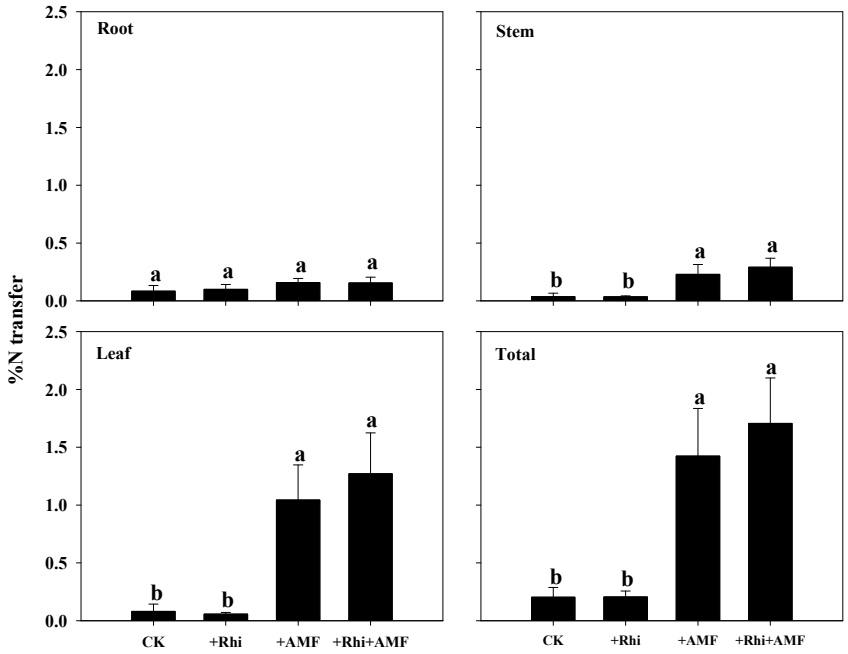


**Figure 4.** Percentage of nitrogen transfer in various parts of citrus affected by rhizobial and mycorrhizal inoculation. Data are means ± SE ($n$ = 4). Different letters above columns indicate significant differences ($p < 0.05$) between treatments. Abbreviations: CK, no rhizobium and no arbuscular mycorrhizal inoculation; +Rhi, inoculated with rhizobium; +AMF, inoculated with mycorrhizal fungus.

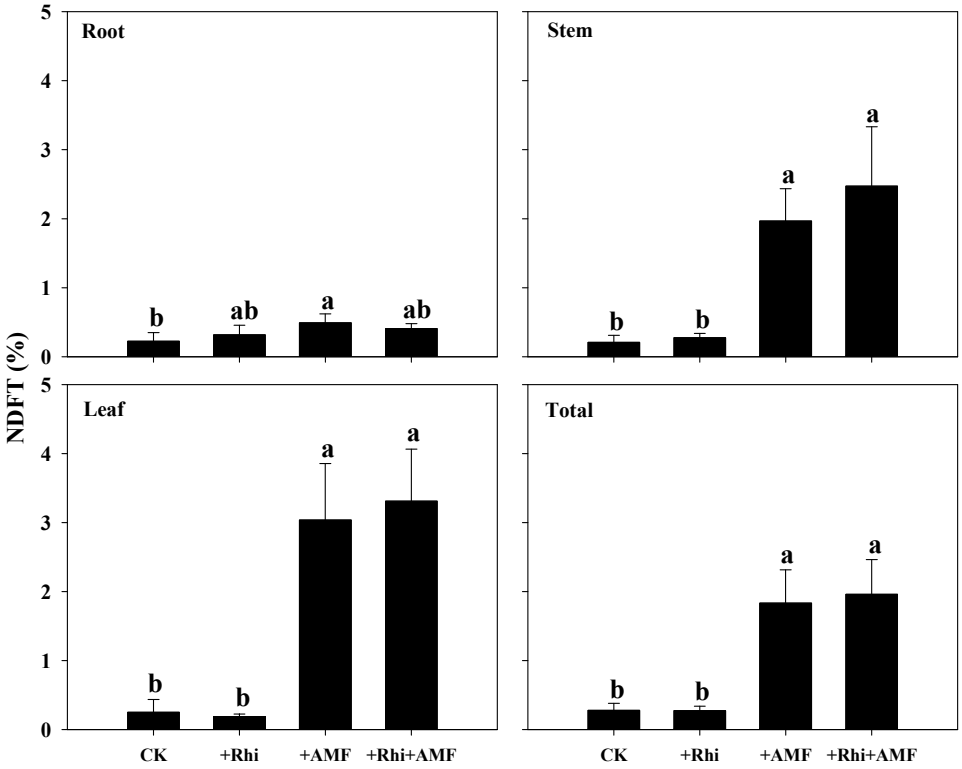


**Figure 5.** Percentage of N derived from transfer (NDFT) in various parts of citrus affected by rhizobial and mycorrhizal inoculation. Data are means ± SE ($n$ = 4). Different letters above columns indicate significant differences ($p < 0.05$) between treatments. Abbreviations: CK, no rhizobial and no arbuscular mycorrhizal inoculation; +Rhi, inoculated with rhizobium; +AMF, inoculated with mycorrhizal fungus.

## 4. Discussion

Our study used the $^{15}N$ foliar-labeling method to determine N transfer from white clover to citrus. The results obtained validated our hypothesis that N in white clover had been transferred to citrus via CMNs and the amount of N transferred was improved by $N_2$-fixing of white clover, particularly under doubled nodulation and mycorrhization (Table 4 and Figure 4).

### *4.1. Biological Nitrogen Fixation and Mycorrhizal Colonization*

We used the rhizobium strain *Rhizobium trifolii* that is highly effective in the nodule formation on white clover [16]. $N_2$-fixation by white clover was 31.28% in this study, which was comparable to the data obtained in an earlier study (39%) [38,39]. In addition, the rest of the plant's N was derived from the growth media or nutrient, seed and root N exudation, etc. [40]. This 31.28% $N_2$-fixation was different from 50–96% in 3–6-year-old [41], 70–87% in 2–3-year-old [42] and 39–82% in 7–8-year-old white clovers [39]. These differences may be attributed to several factors. Firstly, their field studies were conducted for more than 2 years [39,42], but the present greenhouse study only lasted for 1 year. Secondly, we used the $^{15}N$ natural abundance to estimate $N_2$-fixation and the non-inoculated white clover as the reference plant, rather than the perennial ryegrass in other studies [42,43]. Compared with perennial ryegrass, the white clover differs in the capacity of N uptake and root soil exploration [43].

In this study, the percent of root mycorrhizal colonization in pot-growing white clover was comparable with that in other pot and field studies (60–90%) [44,45]. In contrast, mycorrhizal colonization in citrus was lower (19.29–23.41%) than that in other studies (50–80%) [8,46,47]. Several possible factors could contribute to these differences in mycor-

rhizal colonization. Firstly, previous studies inoculated with AMF in the citrus rhizosphere side directly, which increased the opportunities for mycorrhizal fungi to infect citrus roots [46,47]. In this present study, AMF was only inoculated in the white clover rhizosphere. Wu et al. (2017b) [12] recently inoculated with AMF to white clover rhizosphere but not to the citrus rhizosphere in a split chamber, and the 23.6% AMF colonization observed in the citrus was comparable with the 31.28% in citrus for this present study. Secondly, there are differences in mycorrhizal infection rate between citrus genotypes. A greenhouse study showed higher mycorrhizal colonization for five citrus genotypes: Volkamer lemon, Sour orange, Swingle citrumelo, Carrizo citrange, and Trifoliate orange, which were 83%, 77%, 73%, 61%, and 53%, respectively [47]. Thirdly, different AMF species may have differential effects on AMF colonization [48]. For instance, *Glomus intraradic* was used in Graham et al. (1997) [47], but *Diversispora spurca* was applied in Wu et al. (2017b) [12]. Additionally, legumes often strongly depend on mycorrhizal fungi for nutrient acquisition [49,50], as demonstrated by the higher mycorrhizal colonization in white clover (66.85–68.74%) than in citrus (19.29–23.41%), as shown in this study (Table 2).

### *4.2. Response of Plant Growth Inoculated with Rhizobium and Arbuscular Mycorrhizal Fungus*

Nodulation plus mycorrhizal colonization, i.e., nodulation/mycorrhization, in donor plants can increase N transfer to receiver plants [12,20,28]. Our results were consistent with these findings since white clover that was inoculated with both rhizobium and AMF enhanced biomass production and N accumulation of citrus (Figures 2 and 3, Table 5). It may be related to an increased root biomass and the capacity of nutrient acquisition, which is supported by the facts that citrus root mycorrhizal colonization, biomass, and N content were higher under +AMF or +Rhi + AMF than under CK (Figure 2, Tables 2 and 5). Compared with the +AMF treatment, the higher total N content in citrus under +Rhi + AMF suggested that rhizobia did also have an effect on N accumulation. This result was supported by studies focusing on $N_2$-fixing and non-$N_2$-fixing woody perennials [20,24,28,33]. A physiological benefit could occur to receivers from such N transfers [20].

### *4.3. N Transfer from White Clover to Citrus*

Our result demonstrated that a small amount of N transfer from white clover to citrus occurred via a CMN (Figures 4 and 5). The N transfer hypothesis states that N flows from an N-rich legume donor (N source) to an N-poor non-legume receiver (sink) [20]. In this study, N concentrations of white clover were higher than citrus in either plant aboveground or belowground parts (Table 3), which enabled N transfer from white clover to citrus. A study had partitioned N transfer from cover crop to grapevine; the proportion of N transfer in various parts of grapevines ranged from 12% to 56% [10], which was generally higher from white clover to citrus as was noted in this study. This might be because their field experiment had no barrier to prevent movement of soil solution between the different plants; thus, it could not determine if the labeled N in the clover was actually passed by CMNs, root exudates, or soil solution. In our study, the air gap between the two chambers of the growth chamber prevented this. In general, N transfers through CMNs were lower than other routes (soil medium, root exudation) of N transfer from a leguminous cover crop of *Medicago polymorpha* L. to grapevine [11], and from a leguminous tree *Gliricidia sepium* (Jacq.) Kunth ex Walp. to fodder grass *Dichanthium aristatum* (Poir.) C.E. Hubb. [49]. A study also found that %N transfer from leguminous cover crop (*Medicago polymorpha* L.) to grapevine via CMNs was 5.5% [11], which was comparable with data in this present study (Figure 4).

Similar with %N transfer, total plant N derived from clover to citrus via AMF was as low as 1.83–1.96%. It could be contributed to several factors. Firstly, a short 14-day time of $^{15}N$ transfer must be considered. Similar N transfer results (%NDFT was 2.64%) were observed after 15 days of $^{15}N$ labeling [51]. Several studies showed that %NDFT could be up to 40% of total N in the facilitated plant over several months [24,52,53]. Secondly, the citrus root could also take up N from a soil pathway, except through a CMN

with AMFs. Previous studies found that about 26–36% of total N in citrus came from N fertilizer [54,55]. Thirdly, the mobilization of N from reserve organs (root) could satisfy most N requirement of citrus and decrease N uptake from soil media, including AMF. Indeed, the citrus roots could export 30–35% of reserve N to other plant parts of citrus [56]. In this study, percentages of NDFT were lower in roots than in leaves in citrus while N contents in roots were comparable with those in leaves (Tables 4 and 5), indicating a flow of N from root to leaves.

Although there was very low %N transfer and amounts of N transfer to AMF colonized citrus, N transfer in rhizobium-inoculated treatment was higher than no rhizobium-inoculated treatment (Figures 4 and 5), indicating that white clover with effective $N_2$-fixation enhanced N fluxes to citrus. Similar results were observed between *Casuarina cunninghamiana* Miq and *Eucalyptus maculata* Hook.f. interconnected by ectomycorrhizal fungi [24,32], and subterranean clover and grapevines interconnected by AMF [10]. Moreover, white clover intercropped in fruit trees has been steadily increasing in recent years due to the development of organic soil management. Thus, field N fertilization strategy on citrus orchards should be changed if it was mixed with an $N_2$-fixing cover crop.

In summary, our experiment was designed to make N transfer from clover to citrus only possible via mycorrhizae. Even if citrus' AMF colonization had been three or four times greater than we observed, and more similarity to citrus growing under normal conditions, it seems likely that %NDFT would have been <10%. This strongly suggests that transfers via other mechanisms are relatively important mechanisms under normal agricultural conditions. The most likely mechanism might be the exudation of N and natural death of clover roots and nodules, followed by a conversion of organic N to mineral forms by soil organisms. Mineral N would then enter the soil solution and become potentially available for uptake by citrus. The extent of uptake would depend on numerous factors, including the size of the citrus root system and soil moisture. Our result suggests that N transfer via CMNs is an N transfer mechanism, but perhaps not the major one.

## 5. Conclusions

This study demonstrated that $N_2$-fixation supplied around 30% of total N in white clover, and an enhanced N accumulation was displayed in both white clover and adjacent citrus when the combined inoculation of rhizobium and AMF was employed to white clover. The N transfer from white clover to citrus via a CMN of AMF accounted for 1.42–1.71% of the citrus total N after 14 days of $^{15}N$ labeling. The combined nodulation/mycorrhization enhanced 27.2% of N transfer from white clover compared to only mycorrhization. To demonstrate how N transfer through other pathways could affect plant N utilization and hence plant biomass production, further studies are needed to quantify N transfer through soil solution, root exudates, or dead plant tissues (e.g., roots, leaves, nodules).

**Supplementary Materials:** The following are available online at https://www.mdpi.com/2073-4395/11/1/32/s1, Figure S1: Mycorrhizal colonization of white clover roots and citrus roots

**Author Contributions:** Conceptualization, Y.Z., X.H., L.F., and X.S.; methodology, X.H.; software, L.F.; validation, Y.Z., X.H., X.Z., Y.Y., R.L., and S.S.; formal analysis, L.F.; resources, X.H., S.S., and X.S.; writing—original draft preparation, L.F.; writing—review and editing, Y.Z., X.H., and X.S.; supervision, X.S.; project administration, X.S.; funding acquisition, X.S. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** The data presented in this study are available on request from the corresponding author. The data are not publicly available because the raw data used in this study are a small part of database which is preparing for another manuscript.

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