Effects of Arbuscular Mycorrhizal Fungi and Rhizobia on Nitrogen Assimilation in White Clover: Postprint

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Abstract

To reveal the roles of arbuscular mycorrhizal fungi (AMF) and rhizobia in nitrogen (N) assimilation of white clover, white clover was inoculated singly or jointly with Paraglomus occultum and Rhizobium trifolii, and the effects on growth, photosynthesis, leaf N and amino acid contents, and activities of N assimilation-related enzymes were analyzed. The results showed that: (1) Single inoculation with AMF or rhizobia, as well as combined inoculation with AMF and rhizobia, all significantly increased plant height, stolon length, leaf number, aboveground biomass, total biomass, chlorophyll b and total chlorophyll contents, steady-state quantum efficiency, and leaf N content of white clover, with the enhancement effect following the order of combined inoculation > single AMF > single rhizobia > uninoculated control. (2) Combined inoculation with AMF and rhizobia also significantly increased the contents of alanine, arginine, asparagine, aspartic acid, glutamine, glutamic acid, and histidine in white clover leaves, while significantly enhancing the activities of leaf N assimilation-related enzymes such as nitrate reductase, nitrite reductase, glutamine synthetase, glutamate synthase, glutamate dehydrogenase, asparagine synthetase, and aspartate aminotransferase, and significantly promoting AMF colonization in white clover roots. This study demonstrates that combined inoculation with AMF and rhizobia effectively promotes N assimilation by activating the activities of N assimilation-related enzymes, producing more amino acids, and further promoting the growth of white clover plants. The article indicates that AMF and rhizobia have a synergistic effect, effectively promoting N assimilation in white clover.

Full Text

Effects of Arbuscular Mycorrhizal Fungi and Rhizobia on Nitrogen Assimilation in White Clover

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Abstract

To elucidate the roles of arbuscular mycorrhizal fungi (AMF) and rhizobia in nitrogen (N) assimilation in white clover (*Trifolium repens* L.), we conducted single or combined inoculations with *Paraglomus occultum* and *Rhizobium trifolii* under potted conditions. The effects on plant growth, photosynthesis, leaf N and amino acid contents, and activities of N assimilation-related enzymes were analyzed. The results showed that: (1) Single inoculation with AMF or rhizobia, as well as combined inoculation, significantly increased plant height, stolon length, leaf number, shoot biomass, total biomass, chlorophyll b and total chlorophyll contents, steady-state light quantum efficiency, and leaf N content. The enhancement followed the order: combined inoculation > single AMF > single rhizobia > non-inoculated control. (2) Combined inoculation significantly increased contents of alanine, arginine, asparagine, aspartate, glutamine, glutamic acid, and histidine in white clover leaves, while markedly enhancing activities of nitrate reductase, nitrite reductase, glutamine synthetase, glutamate synthetase, glutamate dehydrogenase, asparagine synthetase, and aspartate aminotransferase. Additionally, rhizobium inoculation significantly promoted AMF colonization of white clover roots. These findings demonstrate that combined inoculation of AMF and rhizobia effectively promotes N assimilation in white clover by activating N assimilation-related enzymes, generating more amino acids, and further enhancing plant growth. The study reveals synergistic effects between AMF and rhizobia in promoting N assimilation in white clover.

Keywords: arbuscular mycorrhizal fungi, rhizobia, white clover, nitrogen (N) assimilation, amino acids

Introduction

Arbuscular mycorrhizal fungi (AMF) are beneficial microorganisms widely distributed in soils that form extraradical hyphae on root surfaces after establishing symbiosis with plants, thereby facilitating plant acquisition of nitrogen (N),

phosphorus (P), and other nutrients. AMF assist host plants in absorbing various forms of soil N, with ammonium (NH_4^+) being the primary form absorbed by AMF extraradical hyphae (Xie et al., 2022). AMF also increase the accumulation of free amino acids and other N sources in host plants, with hyphal contributions to plant N reaching up to 74% (Zhang and Yang, 2018). Thus, AMF play a crucial role in host plant N uptake.

Rhizobia are common Gram-negative bacteria in soils that colonize legume root hairs to form nodules, establishing a symbiotic system for biological nitrogen fixation and aiding plant N acquisition (Masson-boivin & Sachs, 2018). Previous studies have shown that combined inoculation with AMF and rhizobia promotes biological nitrogen fixation and enhances N levels in legumes more effectively than single inoculation, while also improving soil uranium removal efficiency and demonstrating stronger phytoremediation potential (Ren et al., 2019). However, other studies have reported that combined inoculation can even inhibit N uptake in peas and mung beans (Saxena et al., 1997; Blilou et al., 1999). These inconsistent results reveal that the effects of combined AMF and rhizobia inoculation on host N uptake are complex and require further investigation. In particular, whether this combination can promote N assimilation in legumes such as white clover (*Trifolium repens* L.) remains unclear.

Plants primarily acquire nitrate (NO_3^-) and ammonium (NH_4^+) from soil, but these inorganic forms must be assimilated into organic nitrogen compounds such as amino acids and proteins before they can be utilized. This assimilation process requires the participation of multiple enzymes. Plant-absorbed NO_3^- is first reduced to nitrite (NO_2^-) by nitrate reductase (NR) and then converted to NH_4^+ by nitrite reductase (NiR). NH_4^+ is subsequently incorporated into glutamine (Gln) under the action of glutamine synthetase (GS) and ATP, and then catalyzed into glutamate (Glu) by glutamate synthetase (GOGAT). The GS/GOGAT pathway assimilates NH_4^+ into organic nitrogen, accounting for up to 95% of NH_4^+ assimilation in plants (Hirel & Gadal, 1980). Glutamate dehydrogenase (GDH) serves as a supplementary pathway to GS/GOGAT, functioning primarily when NH_4^+ concentrations are high to catalyze Glu synthesis (Hodges, 2002). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) convert Glu formed via the GS/GOGAT pathway into aspartate (Asp) and alanine (Ala). Asparagine (Asn), one of the main organic N forms transported in plant phloem, is synthesized under the influence of asparagine synthetase (AS) (Xue, 2018). Currently, it remains unclear whether combined inoculation with AMF and rhizobia affects the levels of host N assimilation products.

White clover, a perennial legume forage, exhibits good compatibility with both rhizobia and AMF. It is characterized by stoloniferous growth, strong expansion capacity, rapid regeneration, and high crude protein content, making it a primary species for green space construction and a cost-effective quality forage (Zhao et al., 2004). The objective of this experiment was to analyze the effects of single or combined inoculation with AMF and rhizobia on white clover

growth, photosynthesis, N content, amino acid composition, and activities of N assimilation-related enzymes, thereby elucidating their influence on N assimilation in white clover.

Materials and Methods

1.1 Experimental Materials

White clover seeds were purchased from the Hubei Provincial Seed Station. Based on previous experimental results from Xie et al. (2020), the AMF strain *Paraglomus occultum* was selected, obtained from the Bank of Glomeromycota in China (BGC) and propagated in pot culture with white clover for three months at the Root Biology Institute of Yangtze University. Spore density determination confirmed 20 spores per gram of AMF inoculum. The test rhizobium strain *Rhizobium trifolii* was obtained from the Agricultural Culture Collection of China. It was activated in yeast mannitol liquid medium and cultured as single colonies. A single colony was transferred to 40 mL liquid medium and cultured at 220 $r \cdot min^{-1}$ and 28 °C for 18 h. Then 1 mL of this culture was transferred to 20 mL fresh liquid medium and cultured under the same conditions for an additional 3 h. The culture was centrifuged at 8,000 ×g for 2 min, the supernatant was discarded, and the pellet was resuspended in sterile water to a concentration of 4.27$×10^{8}$ CFU $\cdot$ mL^{-1} (OD_{600} = 0.3).

The cultivation substrate consisted of soil and sand at a 3:1 ratio (V/V), sterilized by autoclaving (0.11 MPa, 121 °C, 2 h) to inactivate indigenous fungal spores. The soil was collected from the deciduous fruit tree base of Yangtze University, and the sand was river sand with diameter < 4 mm. Plastic pots measured 15 cm top diameter, 10 cm bottom diameter, and 12 cm height, each filled with 1.3 kg of substrate.

1.2 Experimental Design

The experiment consisted of four treatments: (1) non-inoculated control, (2) inoculation with rhizobia (Rh), (3) inoculation with AMF, and (4) combined inoculation with AMF and rhizobia. Each treatment had eight replicates arranged randomly, totaling 32 pots.

1.3 Plant Cultivation

Before sowing, white clover seeds were surface-sterilized with 95% ethanol and 0.525% sodium hypochlorite for 5 min each, then rinsed five times with sterile water. Seeds were sown at 30 seeds per pot in plastic pots containing the cultivation substrate. Three weeks after sowing, seedlings were thinned to 12 plants per pot. Inoculation treatments were applied at sowing time. For single rhizobium inoculation, each pot received 10 mL of *R. trifolii* suspension, and seeds were pre-soaked in the suspension for 30 min. For single AMF inoculation, 100 g of *P. occultum* inoculum was mixed into the cultivation substrate.

For combined inoculation, 100 g of *P. occultum* inoculum was mixed into the substrate, seeds were pre-soaked in *R. trifolii* suspension for 30 min, and 10 mL of the suspension was added to the substrate. The non-inoculated control received equal amounts of sterilized *P. occultum* inoculum and *R. trifolii* suspension, with seeds soaked in sterilized suspension for 30 min. Plants were grown in a controlled growth chamber with light intensity of 900 Lux, temperature of 28 °C/23 °C (day/night), and relative humidity of 68%. Pot positions were regularly changed to avoid environmental effects. No additional nutrients were supplied during cultivation. Plants were watered daily with 100 mL at 17:00. The experiment was terminated after 12 weeks.

1.4 Measurement of Plant Growth and Physiological Indicators

At harvest, plants were separated into shoots and roots. Growth parameters including plant height, leaf number, stolon length, and biomass were measured manually. Chlorophyll fluorescence parameters were measured in vivo using a FluorCam chlorophyll fluorescence imaging system.

AMF colonization was determined using the method of Phillips and Hayman (1970), with colonization rate calculated as the percentage of AMF-infected root segments relative to total observed root segments. Chlorophyll content was measured using the method described by Wang (2016). Leaf N content was analyzed using a discrete chemistry analyzer (Autochem 1200) after leaf digestion. Leaf amino acid composition was extracted with acetonitrile-water via ultrasonication, centrifugation, and microfiltration (Liyanaarachchi et al., 2018), then analyzed by high-performance liquid chromatography-mass spectrometry (Shimadzu LC-20ADXR and Applied Biosystems Sciex Q-trap 5500).

Leaf nitrate reductase activity was measured using the sulfanilamide colorimetric method (Cervilla et al., 2009). Nitrite reductase activity was determined according to Ogawa et al. (1999). Glutamate synthetase activity was measured following Singh and Srivastava (1986). Glutamate dehydrogenase activity was determined using the method of Liu et al. (2007). Asparagine synthetase activity was measured according to Shifrin et al. (1974). Alanine aminotransferase and aspartate aminotransferase activities were determined using the method described by Liang et al. (2013). Glutamine synthetase activity was measured with slight modifications to the method of Husted et al. (2002): 0.2 g fresh sample was ground in 3 mL of 50 mmol $\cdot$ L^{-1} Tris-HCl buffer (containing 0.1 mol $\cdot$ L^{-1} Tris, 2 mmol $\cdot$ L^{-1} $MgSO_4$, 2 mmol $\cdot$ L^{-1} dithiothreitol, and 40 mmol $\cdot$ L^{-1} sucrose), then centrifuged at 10,000 ×g for 15 min at 4 °C. To 0.7 mL supernatant, 1.6 mL reaction solution (80 mmol $\cdot$ L^{-1} $MgSO_4$, 20 mmol $\cdot$ L^{-1} L-Na-glutamate, and 20 mmol $\cdot$ L^{-1} L-cysteine) and 0.7 mL ATP solution were added, mixed, and incubated at 37 °C for 30 min. Then 1 mL color developer (0.2 mol $\cdot$ L^{-1} trichloroacetic acid, 0.37 mol $\cdot$ L^{-1} $FeCl_3$, and 0.6 mol $\cdot$ L^{-1} HCl) was added, mixed, and after 10 min color development, centrifuged at 5,000 ×g for 10 min. The supernatant absorbance was measured at 540 nm.

1.5 Statistical Analysis

Data were analyzed using SAS® software (version 9.1.3; SAS Institute Inc., Cary, NC, USA). Analysis of variance was performed, and multiple comparisons were conducted using Duncan's new multiple range test.

Results

2.1 Effects of Single or Combined Inoculation on AMF Colonization of White Clover Roots

No mycorrhizal colonization was observed in roots of non-AMF-inoculated plants, while mycorrhizal structures were visible in AMF-inoculated plants (Fig. 1 [Figure 1: see original paper]). The AMF colonization rate was (77.9$±7.9)±$5.8)% in plants with combined AMF and rhizobia inoculation, indicating that rhizobium inoculation promoted AMF colonization of white clover roots.

2.2 Effects of Single or Combined Inoculation on White Clover Growth

All inoculation treatments promoted white clover growth (Table 1). Compared with the non-inoculated control, single rhizobium inoculation significantly increased plant height, stolon length, leaf number, shoot biomass, and total biomass by 9.3%, 49.4%, 14.5%, 19.9%, and 18.2%, respectively, with no significant effect on root biomass. Single AMF inoculation and combined inoculation significantly increased all growth parameters: plant height, stolon length, leaf number, shoot biomass, root biomass, and total biomass increased by 12.1%, 48.7%, 34.7%, 32.9%, 22.6%, and 31.0% with AMF alone, and by 22.5%, 202.6%, 54.9%, 74.1%, 30.2%, and 66.3% with combined inoculation, respectively. Moreover, the growth-promoting effect of combined inoculation was significantly superior to single inoculations, while single AMF inoculation was more effective than single rhizobium inoculation for leaf number and biomass.

2.3 Effects of Single or Combined Inoculation on Chlorophyll Content

As shown in Fig. 2 [Figure 2: see original paper], compared with the non-inoculated control, single rhizobium inoculation significantly increased chlorophyll b and total chlorophyll contents by 42.5% and 20.8%, respectively, without significantly affecting chlorophyll a. Both single AMF inoculation and combined inoculation significantly increased chlorophyll a, b, and total chlorophyll contents by 30.4%, 72.6%, and 38.3% (AMF alone) and 41.6%, 102.6%, and 53.1% (combined), respectively. The promoting effect of combined inoculation on chlorophyll content was significantly superior to either single inoculation.

2.4 Effects of Single or Combined Inoculation on Chlorophyll Fluorescence Parameters

Fig. 3 [Figure 3: see original paper]A-C shows that compared with the non-inoculated control, single AMF or rhizobium inoculation had no significant effect on maximum quantum yield (QY_{max}), while combined inoculation significantly increased QY_{max} by 31.4%. All inoculation treatments significantly increased steady-state light quantum efficiency (QY_{Lss}) and significantly decreased steady-state non-photochemical quenching (NPQ_{Lss}).

2.5 Effects of Single or Combined Inoculation on Leaf N Content

Compared with the non-inoculated control, leaf N content in white clover significantly increased by 9.6%, 18.8%, and 30.3% with single rhizobium, single AMF, and combined inoculation, respectively (Fig. 4 [Figure 4: see original paper]). The enhancement effect of combined inoculation was significantly higher than that of either single inoculation.

2.6 Effects of Single or Combined Inoculation on Leaf Amino Acid Contents

Compared with the non-inoculated control, single rhizobium inoculation significantly increased Ala and Gln contents by 27.5% and 38.8%, respectively, significantly decreased Orn content by 48.3%, and had no significant effect on Arg, Asn, Asp, Glu, or His contents. Both single AMF and combined inoculation significantly increased Ala, Arg, Asn, Asp, Gln, Glu, and His contents by 80.8%, 104.5%, 115.4%, 34.1%, 99.5%, 64.7%, and 103.1% (AMF alone) and 98.9%, 227.0%, 114.4%, 56.8%, 101.4%, 45.5%, and 154.7% (combined), respectively, while significantly decreasing Orn content by 28.6% and 39.5% (Table 2). Combined inoculation showed more pronounced promotion of leaf amino acid contents.

2.7 Effects of Single or Combined Inoculation on Activities of N Assimilation-Related Enzymes

Compared with the non-inoculated control, single rhizobium inoculation significantly increased GS, GOGAT, GDH, AS, and AST activities by 25.0%, 13.6%, 25.5%, 26.9%, and 36.0%, respectively. Single AMF inoculation significantly increased NR, NiR, GOGAT, GDH, AS, and AST activities by 29.3%, 33.6%, 33.7%, 26.0%, 44.1%, and 36.8%, respectively. Combined inoculation significantly increased NR, NiR, GS, GOGAT, GDH, AS, and AST activities by 64.3%, 85.5%, 39.8%, 58.1%, 51.7%, 68.2%, and 57.1%, respectively (Table 3). The enhancement effect of combined inoculation on N assimilation-related enzyme activities was markedly higher than that of single inoculations.

Discussion and Conclusion

3.1 Discussion

In this study, rhizobium inoculation significantly promoted AMF colonization of white clover roots, suggesting that rhizobium introduction facilitates arbuscular mycorrhiza formation in roots. This occurs because rhizobia meet AMF N demands through nitrogen fixation, favoring AMF establishment in roots (Xavier & Germida, 2003). Additionally, while rhizobia increase N levels in legumes, the efficiency of biological nitrogen fixation largely depends on P supply to maintain internal N-P balance. AMF facilitate P uptake, prompting legumes to provide sufficient carbon to AMF, which promotes mycorrhizal formation (Liu et al., 2020) and subsequently enhances P acquisition.

The synergistic effect of combined AMF and rhizobium inoculation on white clover growth was significantly greater than single inoculations, consistent with findings in faba bean by Talaat and Abdallah (2008). This synergism arises because rhizobium introduction not only enhances nitrogen fixation efficiency and total N fixed but also promotes AMF colonization rates in white clover roots, leading to better growth of both plant roots and AMF and expanding the absorption area for water and nutrients.

Single inoculation with either AMF or rhizobia significantly increased chlorophyll b and total chlorophyll contents, with combined inoculation further enhancing these effects. Moreover, combined inoculation significantly increased QY_{max} and QY_{Lss} while decreasing NPQ_{Lss}. QY_{max} and QY_{Lss} are sensitive indicators of photosynthetic performance, whereas NPQ_{Lss} represents dissipation of excess excitation energy as heat (Huang et al., 2022). Combined inoculation improved light energy conversion efficiency and reaction center electron transport activity while reducing thermal energy dissipation, thereby maximizing photosynthate accumulation. Consequently, plants with combined inoculation exhibited higher photosynthetic capacity, and the abundant photosynthates provided resources for growth of plant roots, AMF, and rhizobia.

Single inoculation with rhizobia or AMF significantly increased leaf N content, with combined inoculation showing even greater enhancement. Legume nitrogen fixation efficiency is closely related to P supply, as P is essential for ATPase synthesis required for nitrogen fixation and for nodule formation. The extensive extraradical hyphal network of AMF facilitates P acquisition, providing P needed for nodule formation and thereby enhancing symbiotic nitrogen fixation (Xie et al., 2022). Arginine (Arg) is the primary amino acid transported in AMF hyphae, comprising 90% of total hyphal amino acids (Jin, 2008), making it crucial for mycorrhizal N transfer. This study confirmed that AMF inoculation significantly increased Arg content, which also serves as a precursor for messenger molecules like polyamines that promote cell division (Yang & Gao, 2007). The significantly higher Arg content in combined inoculation plants, consistent with results in mung bean by Diao et al. (2014), indicates that AMF and

rhizobia synergistically increase Arg to promote shoot cell division and plant growth.

Asparagine (Asn) is the main form of N compound transported from nodules to the host plant (Xue, 2018), which explains why single rhizobium inoculation increased Asn content more than single AMF inoculation. The synergistic effect of combined inoculation significantly increased multiple amino acid contents, demonstrating that this treatment promotes N assimilation.

AMF inoculation significantly increased NR and NiR activities in white clover leaves, while rhizobium inoculation had no effect, indicating that AMF facilitates NO_3^- to NH_4^+ conversion, consistent with previous studies in alfalfa (Tian et al., 2020). Combined inoculation further enhanced the stimulatory effects of single AMF inoculation on NR and NiR activities, suggesting synergistic enhancement of NO_3^- to NH_4^+ conversion rates in mycorrhizal plant leaves.

Single or combined inoculation increased GOGAT, GDH, and GS activities, demonstrating that these treatments promote N assimilation into amino acids by regulating enzyme activities in the GS/GOGAT and GDH pathways, with combined inoculation showing more pronounced effects. Rhizobium inoculation was particularly effective in promoting NH_4^+ conversion to Gln. Both single and combined inoculation significantly enhanced AS and AST activities, promoting Asn and Asp accumulation. These results indicate that combined inoculation effectively activates N assimilation-related enzymes and increases amino acid production.

3.2 Conclusion

Combined inoculation with AMF and rhizobia exhibits synergistic effects that effectively promote white clover plant growth, significantly enhance photosynthetic capacity and leaf N content, increase contents of alanine, arginine, asparagine, aspartate, glutamine, glutamate, and histidine, and markedly elevate activities of key N assimilation enzymes including nitrate reductase, nitrite reductase, glutamine synthetase, glutamate synthetase, glutamate dehydrogenase, asparagine synthetase, and aspartate aminotransferase. Additionally, combined inoculation significantly promotes AMF colonization of white clover roots. These findings demonstrate that the synergistic interaction between AMF and rhizobia effectively enhances N assimilation in white clover.

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