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Research Article

Effects of Arbuscular Mycorrhizal Fungi and Rhizobia on Physiological Activities in White Clover (*Trifolium repens*)

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Abstract

Background and Objective: Arbuscular mycorrhizal fungi (AMF) and rhizobia stimulated biological N-fixing in leguminous plants. This work was aimed to analyze the effects of single and dual inoculation with AMF and rhizobia on growth and mineral nutritional acquisition (especially N) in white clover (*Trifolium repens* L.). **Materials and Methods:** An arbuscular mycorrhizal fungus *Rhizoglomus intraradices* and a rhizobia *Rhizobium trifolii* were inoculated into rhizosphere of potted white clover and plant growth, soluble sugar, soluble protein, leghemoglobin, mineral nutrition and soil glomalin concentrations were analyzed after 100 days of inoculations. **Results:** *Rhizobium trifolii* inoculation positively promoted root mycorrhizal colonization. Single AMF or rhizobia treatment significantly increased the number of stolon joints and root biomass, which was related with symbiont-stimulated increases of soluble sugar and soluble protein. A higher root leghemoglobin concentration was found in -AMF+Rh plants than in non-inoculated plants. The AMF or rhizobia inoculation mainly promoted leaf nutrition acquisition including N. **Conclusion:** So, it was concluded that AMF and rhizobia jointly stimulated plant growth, root soluble sugar and soluble protein accumulation in white clover, while AMF did not show a cooperative effect on N fixation of rhizobia.

Key words: *Trifolium repens*, *Rhizobium trifolii*, leghemoglobin, mycorrhizal fungi, N-fixing, rhizobia, white clover

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

White clover (*Trifolium repens* L.) is a kind of excellent leguminous grasses, which is widely planted for pasture and afforest in the world. In the rhizosphere of white clover, there are lots of soil beneficial micro-organisms, including arbuscular mycorrhizal fungi (AMF) and rhizobia, which have an important roles in biological nitrogen (N)-fixing. It was documented that soil AMF formed the mutualistic symbiosis with 80% of terrestrial plants, namely, arbuscular mycorrhizas (AMs)[1]. The symbiosis was characterized by the reciprocal exchange of nutrients and carbohydrates between AMs and host plants[2]. The AMF had many advantages in host plants such as; improving crop growth, increasing nutrient acquisition, stabilizing soil structure and physical-chemical properties, increasing the survival rate of micro-propagated plants and enhancing the tolerance to abiotic stresses[3]. In addition, AMF enlarged the absorption area of roots in host plants, thus enhancing the absorption of nutrients, such as; P, N, Cu and Zn[2,4]. Earlier studies indicated that white clover could be colonized by native AMF species and the inoculation with *Funneliformis mosseae* and *Paraglomus occultum* significantly increased N, P, K and Cu absorption in white clover under ample water and drought stress conditions[5].

Rhizobia that is the Gram-negative bacteria in the soil is a kind of symbiotic N-fixation bacteria in nodules of legume plants. In general, leguminous plants obtained N by rhizobia for plant growth[6]. Rhizobia strongly stimulated leguminous plants to develop root nodules for establishing the metabolic cooperation such as ; rhizobia changed N_2 into ammonia to the plant for assimilation and the plant translocated carbohydrates to the root bacteria use for the growth[7]. In fact, in the rhizosphere microecosystem of leguminous plants, a large number of AMF and rhizobia are co-existing[8]. In the long-term evolutionary process, the relationship among AMF, rhizobia and plants was a complex[9]. The AMF and rhizobia collectively had the capability to increase nutrient acquisition and to protect plants from the infection of root pathogens[10]. It is not clear whether the two soil beneficial micro-organisms co-work in plants. In general, mycorrhizal fungi and rhizobia collectively existed in rhizosphere of leguminous plants and it was not clear how the two soil micro-organisms affect plant growth and physiological responses of leguminous plants. The objectives of the present study were to analyze the changes in plant growth, mycorrhizal colonization, soluble sugar, soluble protein, leghaemoglobin, soil glomalin and tissue mineral nutrition acquisition in a leguminous plant, white clover, after inoculated with AMF and/or rhizobia in rhizosphere and also to evaluate the effect on N acquisition of plants.

MATERIALS AND METHODS

Experimental design: The experiment was conducted in the Yangtze University, Jingzhou, China between June, 1- September, 8, 2017. The experiment was arranged in four treatments with completely randomized block design: (1) White clover was not inoculated with AMF and rhizobia (-AMF-Rh), (2) White clover was inoculated with AMF, but not rhizobia (+AMF-Rh), (3) White clover was inoculated with rhizobia, but not AMF (-AMF+Rh) and (4) White clover was inoculated with both AMF and rhizobia (+AMF+Rh). Each treatment had 6 replicates, with the total of 24 pots (12 seedlings/pot).

Plant culture: The seeds of white clover were soaked in distilled water for 8 h, disinfected with 95% alcohol for 5 min and rinsed with sterilized water. *Rhizoglomus intraradices* and *Rhizobium trifolii* were provided by the Bank of Glomeromycota in China (BGC) and the Agricultural Culture Collection of China, respectively. The AM fungal strain was propagated with *Trifolium repens* in pots for 16 weeks. The *R. trifolii* strain was activated by liquid culture of rhizobia AGAR-1.

A total of 30 seeds were sown into a 2.5 L pot, where 80 g mycorrhizal inoculums (23 spores/g) were mixed with autoclaved substrates of soils and sands (4:1, v/v). Before sowed, the seeds were soaked with 4.27×10^8 CFU mL^{-1} of *Rhizobium trifolii* for 30 min. One month after inoculations, the seedlings were thinned to 12 seedlings per pot. All treated plants were placed in a greenhouse of Yangtze University campus (Jingzhou, China) with 982 µmol m^{-2} sec^{-1} photosynthetic photon, 25-30°C and 80% relative air humidity.

Plant harvest and estimation of root mycorrhizal colonization: After 100 days of seeding planting, stolon joint number, adventitious root number and root biomass were measured. Fresh root systems were cut into 1 cm long root segments, which were stained with 0.05% trypan blue for 3 min in terms of the protocol described by Phillips and Hayman[11]. Root mycorrhizal colonization was estimated as the percentage of mycorrhiza-colonized root lengths against total observed root lengths.

Determinations of soluble sugar, soluble protein and leghemoglobin levels: Soluble sugar content was determined by anthrone colorimetry as described by Wang *et al*.[12]. Soluble protein concentration was measured by Bradford assay[13]. Leghemoglobin was measured as the procedure outlined by Wilson and Reisenauer[14].

Determinations of mineral nutrition: Leaf and root samples were dried, sieved by 1 mm mesh and digested with H_2O_2-H_2SO_4. The mineral nutrient (N, P, K, Ca, Mg, Fe and Mn) concentrations in leaves and roots were followed by the Plasma Emission Spectrometer (IRIS Advantage, American Thermoelectric Company, USA).

Determinations of soil GRSP: Rhizospheric substrates adhering on root surface were collected, mixed, air-dried and sieved (2 mm). Determinations of easily extractable glomalin-related soil protein (EE-GRSP) and difficultly extracted glomalin-related soil protein (DE-GRSP) concentrations in the substrate were done according to Wu *et al.*[1]. Total glomalin-related soil protein (T-GRSP) was the sum of EE-GRSP and DE-GRSP.

Statistical analysis: Data were analyzed by one-way variance (ANOVA) in the SAS software. Significant differences between all the treatments were compared with the Duncan's multiple range tests at p<0.05.

RESULTS

Root mycorrhizal colonization of white clover: The root mycorrhizas were observed only in the +AMF-Rh and +AMF+Rh treated white clover seedlings (Table 1). Addition of rhizobia could significantly promote root mycorrhizal colonization.

Plant growth of white clover: Compared with -AMF-Rh plants, single inoculation with either rhizobia or AMF and double inoculation with AMF and rhizobia (+AMF+Rh) significantly increased stolon joint number and root biomass (Table 1). In addition, compared with -AMF-Rh treatment, +AMF-Rh treatment did not change adventitious root number, while +AMF+Rh and -AMF+Rh treatments significantly increased adventitious root number and the highest adventitious root number was found in the +AMF+Rh inoculated seedlings.

Soluble sugar and soluble protein levels of white clover: Compared with -AMF-Rh plants, -AMF+Rh, +AMF-Rh and +AMF+Rh inoculated plants recorded significantly higher root soluble sugar concentrations, respectively (Fig. 1a). In leaf, only dual inoculation with AMF and rhizobia significantly increased soluble sugar concentration than non-inoculated treatment. The -AMF+Rh, +AMF-Rh and +AMF+Rh treatments significantly increased root and leaf soluble protein concentrations (Fig. 1b).

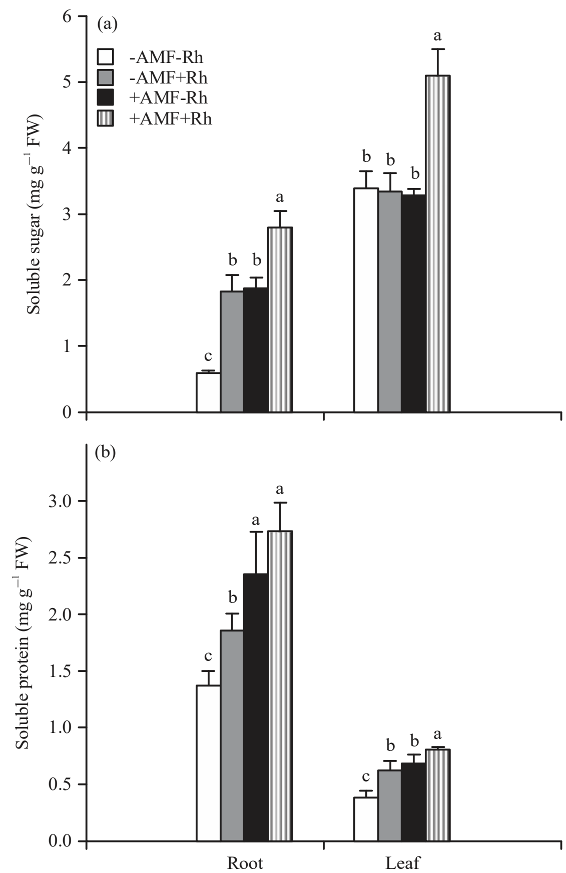

Fig. 1(a-b): Effects of AMF (*Rhizoglomus intraradices*) and rhizobia (*Rhizobium trifolii*) on (a) Soluble sugar and (b) Soluble protein concentrations in leaf and root of potted white clover (*Trifolium repens*)
Data (Mean±SD, n = 4) follow by different letters above the bars showed significant (p<0.05) differences between treatments

Table 1: Effects of AMF (*Rhizoglomus intraradices*) and rhizobia (*Rhizobium trifolii*) on plant growth and root mycorrhizal colonization of potted white clover (*Trifolium repens*)

Treatments	Stolon joint number (number/pot)	Root biomass (g FW/plant)	Adventitious root number (number/plant)	Root mycorrhizal colonization (%)
-AMF-Rh	22.2±1.9^{d}	3.05±0.62^{b}	4.20±0.45^{c}	0±0^{c}
-AMF+Rh	33.6±3.3^{b}	6.80±0.63^{a}	7.80±0.84^{b}	0±0^{c}
+AMF-Rh	28.8±2.7^{c}	7.16±0.97^{a}	3.80±0.45^{c}	86±4^{b}
+AMF+Rh	42.6±3.2^{a}	7.12±0.73^{a}	13.40±1.14^{a}	94±4^{a}

Data (Means±SD, n = 4) follow by different letters showed significant (p<0.05) difference between treatments

Table 2: Effects of AMF (*Rhizoglomus intraradices*) and rhizobia (*Rhizobium trifolii*) on mineral nutrition concentrations in leaves and roots of potted white clover (*Trifolium repens*)

Treatments	N (%)	P (mg g^{-1})	K (mg g^{-1})	Ca (mg g^{-1})	Mg (mg g^{-1})	Mn (mg kg^{-1})	Fe (mg g^{-1})
Leaf							
-AMF-Rh	1.06±0.09^{c}	2.61±0.20^{b}	6.64±0.40^{c}	3.72±0.21^{b}	3.31±0.23^{c}	99.1±5.2^{c}	2.81±0.21^{b}
-AMF+Rh	1.67±0.06^{a}	3.26±0.17^{a}	9.47±0.81^{b}	4.96±0.33^{a}	3.69±0.15^{b}	108.2±9.4^{bc}	2.27±0.16^{c}
+AMF-Rh	1.41±0.08^{b}	3.47±0.21^{a}	9.17±1.25^{b}	4.92±0.63^{a}	3.69±0.23^{b}	113.6±5.8^{b}	3.76±0.30^{a}
+AMF+Rh	1.45±0.09^{b}	3.38±0.26^{a}	11.82±0.75^{a}	4.92±0.29^{a}	4.41±0.29^{a}	151.1±9.0^{a}	3.72±0.21^{a}
Root							
-AMF-Rh	2.36±0.18^{b}	2.86±0.16^{ab}	13.54±1.01^{b}	16.72±1.72^{b}	3.76±0.25^{c}	101.0±5.6^{d}	0.43±0.03^{c}
-AMF+Rh	2.52±0.30^{b}	2.38±0.14^{c}	12.47±0.75^{b}	18.24±0.92^{ab}	4.04±0.15^{bc}	154.8±10.9^{b}	0.37±0.03^{d}
+AMF-Rh	3.03±0.21^{a}	2.73±0.22^{b}	12.70±1.09^{b}	16.37±1.49^{b}	4.08±0.21^{b}	118.9±3.1^{c}	0.50±0.03^{b}
+AMF+Rh	3.03±0.16^{a}	3.02±0.11^{a}	19.43±1.23^{a}	20.05±1.46^{a}	4.96±0.16^{a}	168.8±8.8^{a}	0.61±0.04^{a}

Data (Mean±SD, n = 4) follow by different letters in the same line showed significant (p<0.05) difference between treatments

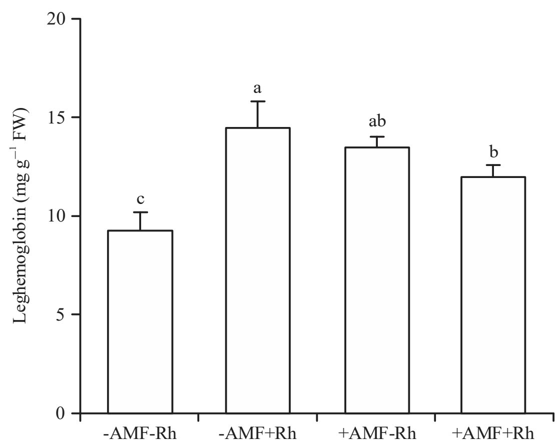

Fig. 2: Effects of AMF (*Rhizoglomus intraradices*) and rhizobia (*Rhizobium trifolii*) on root leghemoglobin content in potted white clover (*Trifolium repens*)

Data (Mean±SD, n = 4) follow by different letters above the bars showed significant (p<0.05) differences between treatments

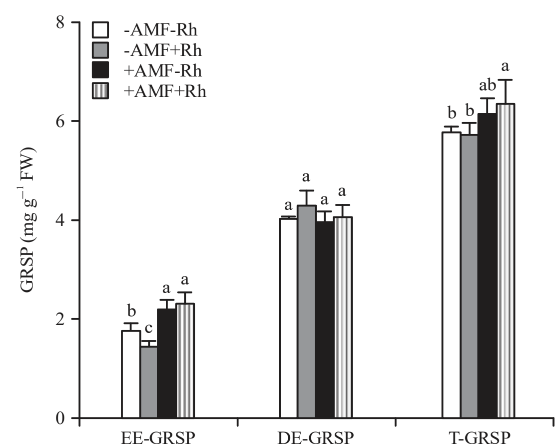

Fig. 3: Effects of AMF (*Rhizoglomus intraradices*) and rhizobia (*Rhizobium trifolii*) on rhizospheric EE-GRSP, DE-GRSP and T-GRSP concentrations in potted white clover (*Trifolium repens*)

Data (Mean±SD, n = 4) follow by different letters above the bars showed significant (p<0.05) differences between treatments

Leghemoglobin contents of white clover: All the inoculated treatments collectively increased root leghemoglobin content, as compared with -AMF-Rh treatment (Fig. 2). The highest root leghemoglobin content was found in -AMF+Rh treated seedlings among the four treatments.

Soil GRSP fraction levels in rhizosphere of white clover: Single rhizobia treatment did not alter soil DE-GRSP and T-GRSP concentrations, while significantly reduced soil EE-GRSP concentrations as compared with the un-inoculated treatment (Fig. 3). Single AMF application significantly increased soil EE-GRSP levels, but did not affect DE-GRSP and T-GRSP levels. Dual inoculation with AMF and rhizobia further strengthened the positive effect on soil T-GRSP levels.

Nutritional acquisition of white clover: Compared with -AMF-Rh control, -AMF+Rh, +AMF-Rh and +AMF+Rh treatments significantly enhanced leaf N, P, K, Ca, Mg and Mn levels, respectively (Table 2). The inoculated plants with single AMF, double rhizobia and AMF significantly enhanced root N, compared with non-inoculated plants. Compared with -AMF-Rh and +AMF+Rh increased root K, Ca and Mg concentrations. Compared with non-inoculated plants, single inoculation with rhizobia or AMF and double inoculation with both rhizobia and AMF improved root Mg and Mn concentrations.

DISCUSSION

The present study indicated a positive effect of single AMF inoculation and rhizobia inoculation on improving root growth of white clover, which is in agreement with tea plants after colonized by AMF[15]. Symbiont-induced root alteration might be linked to an improved uptake of nutrients and the endogenous hormone balance[15,16]. In addition, dual AMF and rhizobia inoculation further strengthened the positive effect on plant growth of white clover, which indicated that the two beneficial soil symbiotic strains had the synergistic work in stimulating growth of host plants[17], because a considerably higher root mycorrhizal colonization was found in +AMF+Rh treatment vs. +AMF-Rh treatment.

The present study showed that single AMF or rhizobia significantly increased root soluble sugar contents but did not alter leaf soluble sugar contents. Possibly, both mycorrhizas and root nodules need host plants to provide carbohydrates from leaves to roots for their growth[2,6]. Moreover, dual inoculated with AMF and rhizobia further increased soluble sugar accumulation in leaves and roots of white clover, indicating that the two symbionts collectively stimulated the photosynthates accumulation of host plants for sustaining more energy in tissues for symbiotic growth.

Compared with non-inoculated treatment, single inoculation with AMF or rhizobia significantly increased leaf, root soluble protein concentrations and dual inoculation further enlarged the positive effect, especially in leaf. This suggested that new proteins were induced under AMF and/or rhizobia inoculation conditions. A similar result was reported by Wu *et al.*[18] in micro-propagated trifoliate orange seedlings colonized by *Glomus mosseae* and *Glomus versiforme*. Soluble protein was likely to be of particular importance in N nutrition as it usually constitutes around 50% of total N. In addition, single rhizobia and/or AMF inoculation also increased root leghemoglobin contents of white clover. This was in agreement with Garg and Manchanda[19] in *Cajanus cajan* plants. It seemed that AMF treatment or rhizobia increased N accumulation through stimulating the production of root leghemoglobin concentrations.

CONCLUSION

Single inoculation with AMF or rhizobia considerably improved plant growth performance of white clover and dual inoculation with AMF and rhizobia further strengthened the positive effect, which was closely associated with symbiont-stimulated soluble sugar and soluble protein levels. In addition, rhizobia inoculation accelerated root leghemoglobin levels to increase N-fixing, thus, resulting the transfer of N from root to leaf. The AMF did not show a cooperative effect on N-fixing of rhizobia in white clover.

SIGNIFICANCE STATEMENT

In this study, it found a positive effect of rhizobia (*Rhizobium trifolii*) on mycorrhizal colonization of *Rhizophagus intraradices*. The AMF and rhizobia showed a synergic effect on stimulating plant growth, soluble sugar and soluble protein. However, AMF and rhizobia exhibited a competitive role in biological N-fixation, where rhizobia were a main path. Such results provide a significant relationship between AMF and rhizobia in leguminous plants.

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