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Single or dual inoculation of arbuscular mycorrhizal fungi and rhizobia regulates plant growth and nitrogen acquisition in white clover

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Abstract: The present work aimed to analyse whether and how single or dual inoculation with arbuscular mycorrhizal fungi (*Funneliformis mosseae*, *Paraglomus occultum*, and *Rhizophagus intraradices*) and rhizobia (*Rhizobium trifolii*) improved plant growth and stimulated nitrogen (N) acquisition of white clover. AMF inoculation significantly ($P < 0.05$) increased root nodule number by 117–173%, and additional Rh considerably stimulated mycorrhizal growth. Single AMF or Rh treatment dramatically increased shoot by 36–281% and root biomass by 16–36% than non-inoculated control, and dual inoculation of Rh and *P. occultum* or *R. intraradices* further magnified the positive effect. Leaf and root N content, root total soluble protein content, root nitrogenase activity, and amino acid (e.g., alanine, arginine, asparagine, aspartate, phenylalanine, proline, and tryptophan) concentrations were significantly increased by single or dual inoculation, while dual inoculation of AMF and Rh had significantly superior roles than single corresponding AMF or Rh inoculation. These results suggested that AMF and Rh represented synergetic effects on accelerating N acquisition of white clover to some extent, while the combination of *P. occultum* and Rh was the best.

Keywords: nodulation; nutrient; symbiosis; synergistic effect

Soil microbes are the important driving force on stimulating crop productivity for sustainable agriculture (Bever et al. 2010, He et al. 2019, 2020, Zhang et al. 2020). Soil arbuscular mycorrhizal fungi (AMF) and rhizobia (Rh) are the beneficial microflora to establish symbiosis with host plants. Mycorrhiza or root nodule promotes the nutrient acquisition of plants, including nitrogen (N), and has interactive effects on regulating the allocation of N resource (Larimer et al. 2010, Wu et al. 2019a). As reported by De Oliveira Júnior et al. (2017) in *Piptadenia gonoacantha* plants, AMF stimulated the N_2 fixation of Rh and Rh inoculation increased root AMF colonisation, further hinting the synergy effect. AMF or Rh have a positive effect on legume plants, while whether the dual microbial inoculation confers a synergetic effect on legume plants is unknown.

In soil, the underground mycelium network of mycorrhizas plays an important role in N transfer and distribution of host plants (Hodge 2014). AMF with a great deal of biomass and high N demand represents a significant N pool. AMF extraradical hyphae preferentially utilise ammonium ions (NH_4^+) for the assimilation, as well as a small number of amino acids like asparagine and

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arginine (Jin et al. 2011). These N-metabolites from mycorrhizas would be further transferred to plants for its growth and development. Rh is a gram-negative bacteria that act on biological N_2 fixation with legume plants, about 40 million t per year worldwide (Herridge et al. 2008). Rh colonise in root hairs of legume plants to form nodules, in which Rh synthesize a large amount of nitrogenase and balance the exchange of carbon and N with host plants (Masson-Boivin and Sachs 2018). Each of the two microbes promotes plant N acquisition, while it is not clear whether the dual combination has a synergistic effect on N acquisition. As observed by Hack et al. (2019), in P-deficit soils, AMF did not exhibit any effects on plant nutrition, while in P-sufficient soils, mycorrhizal symbiosis stimulated N acquisition of plants, accompanied by an enhancement of biological N_2 fixation.

White clover (*Trifolium repens* L.) is a high-quality legume plant that produces ~400 kg N/ha/year with biological N_2 fixation (Ledgard et al. 2001). In white clover, AMF and Rh jointly exist in roots, while the relationship between AMF and Rh is unknown. The purpose of the present study was to evaluate the effect of single or dual inoculation with AMF and Rh on plant biomass, soluble protein and N levels, nitrogenase activity, and amino acid concentrations of white clover.

MATERIAL AND METHODS

Culture of AMF and Rh strains. Three AMF species, including *Funneliformis mosseae* (T. H. Nicolson & Gerd.) C. Walker & A. Schüßler, *Paraglomus occultum* (C. Walker) J. B. Morton & D. Redecker, and *Rhizophagus intraradices* (N. C. Schenck & G. S. Sm.) C. Walker & A. Schüßler were provided by the Bank of Glomeromycota in China, Beijing, China. The AMF strains were propagated with isolated spores in pots for three months, and the inocula comprised of AMF-colonised root segments, spores (18 spores/g), and AMF extraradical hyphae.

A rhizobium strain *Rhizobium trifolii* that was provided from the Agricultural Culture Collection of China, Beijing, China, was isolated from white clover. The rhizobium was activated on yeast extract mannitol agar at 28 °C for three days. Subsequently, the growth response of bacterial isolate was assessed using a spectrophotometer at 600 nm, and the bacterial solution was approximately 10^9 cells/mL.

Plant culture. Seeds of white clover were surface-sterilised by alcohol solution (75%) for 5 min and NaOCl solution (5.25%) for 3 min rinsed in sterile water for 5 times, and sown in plastic pots (15 cm top diameter × 10 cm bottom diameter × 12 cm height) containing 1.3 kg autoclaved (0.11 MPa, 121 °C, 2 h) soil and sand mixture (3:1, *v*/*v*). The characteristics of soil were pH 6.2, available nitrogen 230.78 mg/kg, available phosphorus 26.78 mg/kg, and available potassium 130.36 mg/kg. At sowing, 100 g mycorrhizal inoculants were mixed in autoclaved substrates as the AMF treatment, and the non-AMF treatment received autoclaved mycorrhizal inoculants. The 10 mL bacterium solution of Rh was applied into pots as the Rh treatment, and the non-Rh treatment was applied with 10 mL sterile water. After one week, seedlings were thinned to 12 seedlings/pot. All the treated plants were grown in a controlled environment for 100 days with 7000 Lux, 28 °C/23 °C day/night temperature, and 68% relative air humidity. The pots have weekly changed the position to avoid the environmental effect.

Experimental design. The experiment was conducted in a completely randomised blocked design with two factors: inoculations with *F. mosseae*, *P. occultum*, *R. intraradices*, and non-AMF; inoculations with and without Rh. A total of eight treatments were arranged, and each treatment replicated six times, leading to 48 pots.

Variable determinations. At harvest, the plants were divided into shoot and root, which was dried at 80 °C for 48 h and weighted.

The root segment with 1.5 cm length was cleared with a 10% (*w*/*v*) KOH solution at 75 °C for 2 h and stained with 0.05% (*w*/*v*) trypan solution for 1 min (Phillips and Hayman 1970). Root AMF colonisation (%) was expressed as the percentage of AMF-colonised root lengths *versus* total root lengths (Wu et al. 2019b). Soil mycorrhizal hyphal length was determined by the protocol described by Bethlenfalvay and Ames (1987).

Leaf and root soluble protein concentrations were measured by Bradford (1976). N contents of leaf and root were assayed by the Electrochemical Analyser (Smart Chem 200, Scientific Instruments Limited, Weston, USA).

A 0.20-g fresh root sample was ultrasonically extracted with acetonitrile-water for 1 h and centrifuged at 10 000 *g*/min for 5 min. The 1 µL supernatant was analysed by LC-MS system (an LC-20ADXR HPLC system (Shimadzu, Kyoto, Japan) and Q-trap 5500 MASS (Concord, Ontario, Canada)) for amino acid concentrations outlined by Liyanaarachchi et al. (2018), while the standard solution of phenylalanine, alanine, glycine, glutamate, glutamine, arginine,

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proline, aspartate, asparagine, leucine, isoleucine, cystine, methionine, tryptophan, threonine, norvaline, histidine, and ornithine was referred.

A 1-g fresh root sample (20 ± 3 nodules) was incubated with 2 mL acetylene gas in a closed bottle at 30 °C for 30 min, and a 0.5 mL gas from the bottle was collected for analysis of nitrogenase activity with a gas chromatograph (Agilent 7890, Aglient Technologies Inc., Santa Clara, USA). Nitrogenase activity (C_2H_4 mL/g/h) was determined according to the protocol described by Abd-Alla (2014).

Statistical analysis. Data were analysed by one-way analysis of variation, and the significant difference between treatments was compared with Duncan's multiple range test at the 5% level.

RESULTS AND DISCUSSION

Changes in root mycorrhizal colonisation, soil hyphal length, and root nodules. Mycorrhizas were found in roots of white clover inoculated with single AMF and dual AMF and Rh inoculation (Figure 1). We also observed vesicles in root nodules (Figure 1B), indicating the colonisation of AMF in nodules. Root AMF colonisation varied from 78.27% to 97.11% (Figure 2A), and soil mycorrhizal hyphal length was 20.75 to 50.51 cm/g soil (Figure 2B). Rh supplement heavily ($P < 0.05$) promoted root AMF colonisation and soil mycorrhizal hyphal length, irrespectively of AMF species used. Under the condition of the uninoculated and single AMF treatment, white clover still formed root nodules (Figure 1C). It is documented that air and water have a small quantity of Rh (Máthá et al. 2018). Some Rh exists in the seeds of leguminous plants, and they mainly exist in the seed coat and are seldom distributed in cotyledon and embryo (Zhang et al. 2009). Another possibility is that native Rh survived in the autoclaved soil. Compared with the control, single AMF or Rh treatment significantly increased root nodules, and root nodules were significantly higher under single AMF treatment than under single Rh treatment, dependent on AMF species (Figure 2C). In fact, we also found that root biomass was significantly higher in single AMF-inoculated plants than in single Rh-treated plants (Figure 3), indicating that mycorrhizal plants have more developed roots, providing more possibilities for the colonisation of Rh to form nodules. In addition, dual inoculation of AMF and Rh represented significantly ($P < 0.05$) higher root nodule number than single inoculation of AMF or Rh, regardless of AMF species. A similar result was observed in faba bean plants (Abd-Alla et al. 2014). The best mycorrhizal growth and root nodulation were observed by dual inoculation of *P. occultum* and Rh. This result indicated the synergistic effect between AMF and Rh on the symbiotic association of white clover. AMF has no strict compatibility with host plants, whereas the symbiotic system of legume-AMF-Rh has obviously mutual selectivity (Shockley et al. 2004). The positive

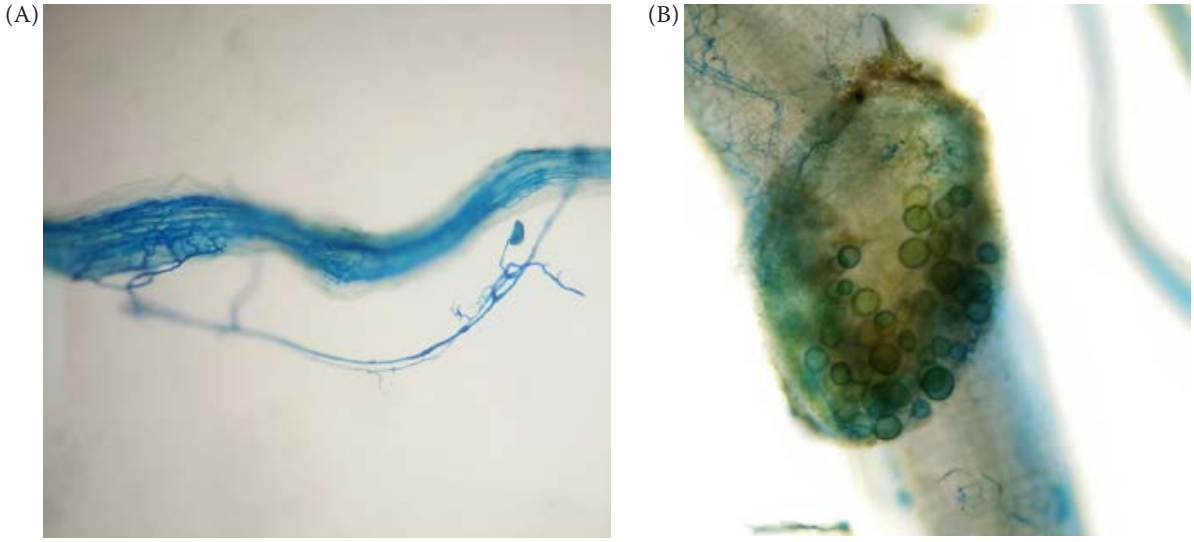

Figure 1. Root mycorrhizal colonisation of white clover plants by (A) single arbuscular mycorrhizal fungi (AMF)-inoculation and (B) dual inoculation of AMF and rhizobia. (A) internal and external mycorrhizal hyphae; (B) internal mycorrhizal hyphae in roots and vesicles in root nodules

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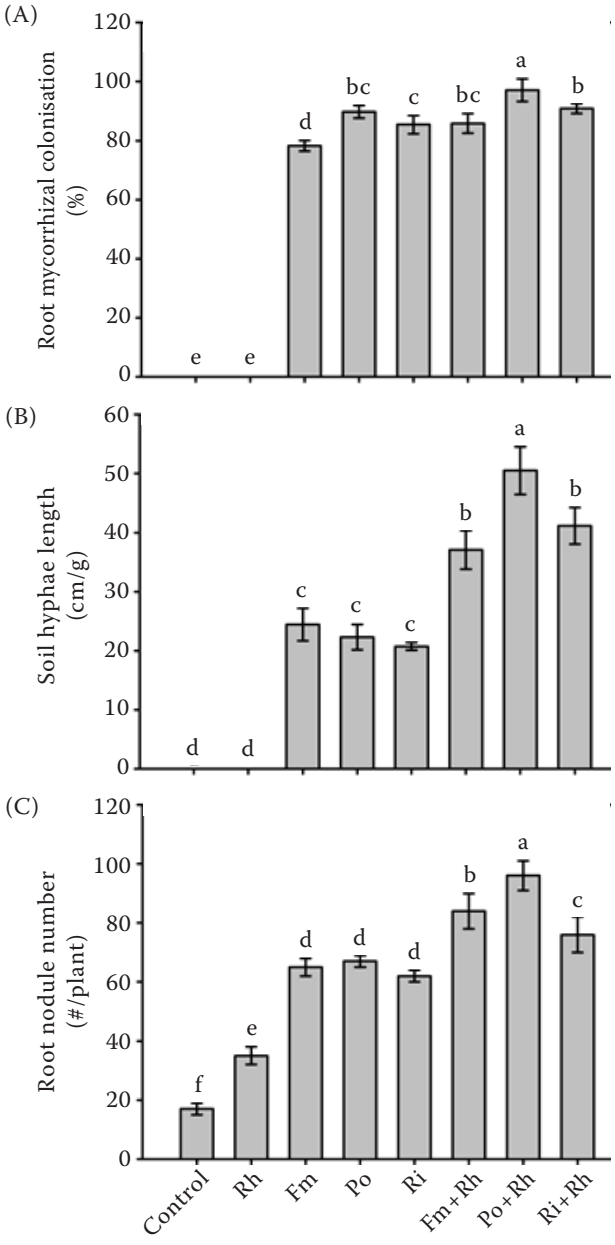

Figure 2. (A) Effects of single or dual inoculation with arbuscular mycorrhizal fungi (AMF) and rhizobia (Rh) on root mycorrhizal colonisation; (B) soil mycorrhizal hyphal length, and (C) root nodule number of white clover. Data (means ± standard deviation, n = 6) with the different letters above bars indicated significant differences between treatments at the 5% level. Control – inoculation without AMF or Rh; Rh – inoculation with *Rhizobium trifolii*; Fm – inoculation with *Funneliformis mosseae*; Po – inoculation with *Paraglomus occultum*; Ri – inoculation with *Rhizophagus intraradices*; Fm + Rh – inoculation with *Funneliformis mosseae* and *Rhizobium trifolii*; Po + Rh – inoculation with *Paraglomus occultum* and *Rhizobium trifolii*; Ri + Rh – inoculation with *Rhizophagus intraradices* and *Rhizobium trifolii*

effect may be due to the fact that AMF and Rh do not compete for each other's colonised sites (Maillet et al. 2011). The enhancement in the nodulation of white clover by AMF inoculation derives from AMF-provided energy for nodule formation and N_2 fixation (Oruru and Njeru 2016).

Changes in plant biomass. Compared with the treatment without Rh or AMF, single Rh or AMF inoculation significantly increased shoot and root biomass of white clover, irrespectively of AMF species (Figure 3). Further, dual inoculation with Rh and *P. occultum* or *R. intraradices* registered a significantly higher shoot and root biomass than corresponding Rh or AMF inoculation. This is in agreement with the findings of Oruru and Njeru (2016) in faba bean. The treatment by both *P. occultum* and Rh represented the best positive effect on biomass production than other treatments, suggesting that the effect was dependent on the combination of AMF and Rh. Inoculated AMF or Rh can significantly increase the biomass of host plants due to enhance P or N uptake (Aliasgharzad et al. 2006).

Changes in soluble protein content. Total soluble protein usually accounts for about 50% of total N in plants, thus, improving nodule function and stimulating symbiosis formation between legume, AMF, and Rh (Cheng et al. 2010). Single or dual inoculation of AMF and Rh collectively increased total soluble protein contents in leaves and roots, compared with

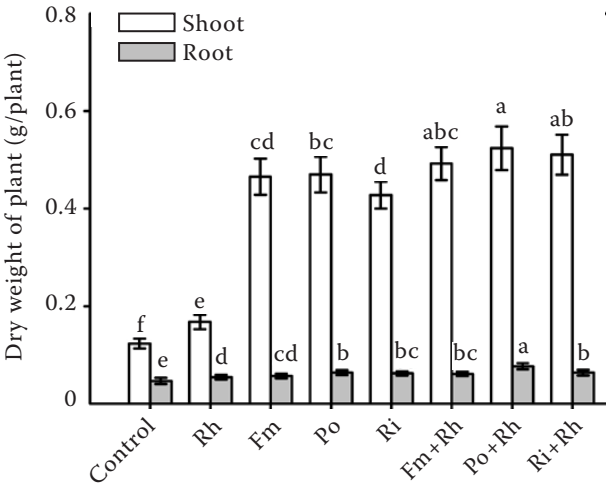

Figure 3. Effects of single or dual inoculation with arbuscular mycorrhizal fungi and rhizobia on the dry weight of white clover. Data (means ± standard deviation, n = 6) with the different letters above bars indicated significant differences between treatments at the 5% level. The abbreviations are the same as in Figure 2

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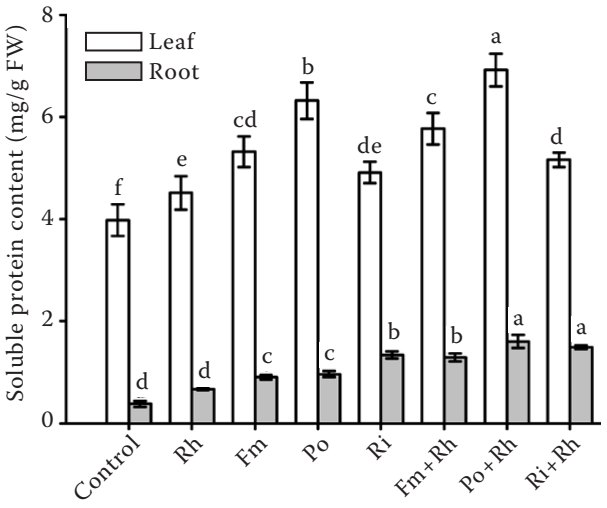

Figure 4. Effects of single or dual inoculation with arbuscular mycorrhizal fungi and rhizobia on total soluble protein levels in leaf and root of white clover. Data (means ± standard deviation, $n = 6$) with the different letters above bars indicated significant differences between treatments at the 5% level. The abbreviations are the same as in Figure 2; FW – fresh weight

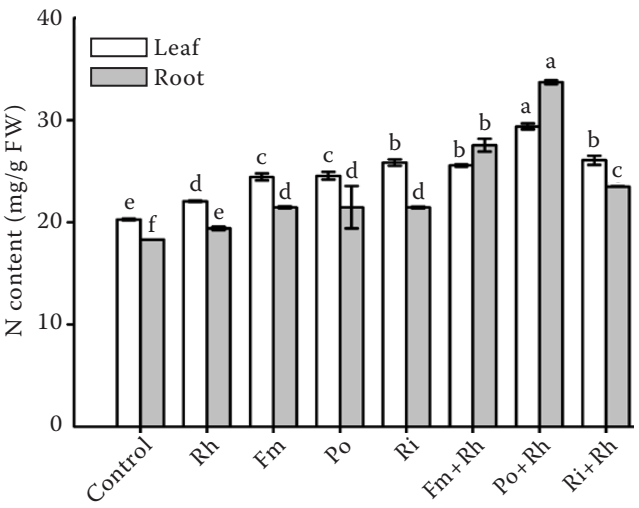

Figure 5. Effects of single or dual inoculation with arbuscular mycorrhizal fungi and rhizobia on nitrogen (N) concentrations in leaf and root of white clover. Data (means ± standard deviation, $n = 6$) with the different letters above bars indicated significant differences between treatments at the 5% level. The abbreviations are the same as in Figure 2; FW – fresh weight

the control treatment (Figure 4). Interestingly, in roots, dual inoculation with Rh and AMF gave markedly higher total soluble protein content than single inoculation with Rh or AMF, irrespective of AMF species. However, in leaves, a significant increase of total soluble protein content was observed in the plants treated by dual *P. occultum* and *R. trifolii versus* by single *P. occultum*. These results indicated that AMF or Rh were easier to stimulate root protein synthesis than dual inoculation. The abundant free amino acids that exist in hyphae of AMF were transferred to root for increasing substrates of protein synthesis (Jin et al. 2011). Furthermore, Rh addition to AMF-inoculated plants further stimulated protein accumulation, indicating more amino acids to be re-used in nodule metabolism (Cheng et al. 2010).

Changes in plant N content. Single or dual inoculation with AMF and Rh collectively increased leaf and root N concentrations, irrespective of AMF species (Figure 5). Moreover, compared with single AMF treatment, dual inoculation with AMF and Rh exhibited higher leaf and root N levels, except for no significant difference of leaf N between dual *R. intraradical* and *R. trifolii* and *R. intraradices*. Improvement of N acquisition in plants by single or dual inoculation played a crucial role in increasing plant growth. An analogous result was reported in green grams (Musyoka et al. 2020) and soybean (Aliasgharzad et al. 2006). AMF-released glomalin-related soil proteins had shown the important contribution of plant and soil N levels (Meng et al. 2020). These results clarify that the effective association between AMF and Rh affects N accumulation to increase plant production and N_2 fixation (Herridge et al. 2008). N accumulation in host plants is influenced by various factors, including plants, symbiotic genotypes, and symbiotic efficiency. The highest N concentration of leaf and root was observed in the plants treated by dual inoculation with *P. occultum* and Rh, suggesting more accumulation of N compounds in the dual-inoculated plants, like amino acids and soluble proteins (Matsubara et al. 2014).

Changes in amino acid content. Amino acids provide nutrient sources for root bacteria. In this work, treatments by single or dual inoculation of AMF and Rh collectively increased root phenylalanine, alanine, glycine, glutamate, arginine, proline, aspartate, asparagine, leucine, isoleucine, cystine, methionine, tryptophan, threonine, norvaline, and histidine content, compared with the non-inoculated control (Table 1). Hereinto, the treatment with both *P. occulum* and Rh gave the highest effect on phenylalanine, alanine, glycine, glutamate, arginine, proline, aspartate, asparagine, leucine, isoleucine, cystine, norvaline, and total amino acid content.

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Table 1. Effect of single or dual inoculation with arbuscular mycorrhizal fungi (AMF) and rhizobia (Rh) on root amino acid levels (μg/g fresh weight) of white clover

	Control	Rh	Fm	Po	Ri	Fm + Rh	Po + Rh	Ri + Rh
Alanine	22.16 ± 1.18^{g}	27.33 ± 1.10^{f}	41.15 ± 1.02^{c}	43.28 ± 1.97^{b}	30.63 ± 0.53^{e}	47.81 ± 1.25^{a}	48.69 ± 1.09^{a}	33.04 ± 1.11^{d}
Arginine	7.36 ± 0.25^{f}	8.89 ± 0.34^{d}	9.03 ± 0.20^{d}	8.20 ± 0.58^{e}	8.42 ± 0.16^{e}	11.33 ± 0.34^{b}	11.83 ± 0.31^{a}	10.02 ± 0.13^{c}
Asparagine	199.85 ± 3.70^{f}	216.65 ± 7.44^{e}	206.57 ± 1.69^{f}	262.60 ± 5.62^{c}	260.01 ± 3.39^{c}	234.54 ± 5.07^{d}	372.22 ± 6.75^{a}	277.83 ± 9.82^{b}
Aspartate	36.67 ± 1.67^{g}	57.33 ± 1.22^{f}	78.85 ± 2.80^{c}	71.27 ± 1.40^{d}	60.87 ± 1.45^{e}	92.75 ± 1.6^{b}	96.65 ± 2.66^{a}	79.89 ± 2.53^{c}
Cystine	0.29 ± 0.02^{e}	0.33 ± 0.02^{d}	0.35 ± 0.02^{cd}	0.40 ± 0.03^{b}	0.33 ± 0.04^{d}	0.39 ± 0.01^{bc}	0.45 ± 0.03^{a}	0.35 ± 0.03^{cd}
Glutamate	50.86 ± 1.73^{d}	51.07 ± 1.07^{d}	57.67 ± 0.93^{c}	69.97 ± 1.94^{b}	68.48 ± 1.45^{b}	49.31 ± 1.31^{d}	75.89 ± 1.90^{a}	75.15 ± 0.26^{a}
Glutamine	18.69 ± 1.20^{a}	13.67 ± 0.71^{b}	12.10 ± 0.53^{c}	9.64 ± 1.02^{e}	6.60 ± 0.33^{f}	9.65 ± 0.43^{e}	10.96 ± 0.54^{d}	8.98 ± 0.23^{e}
Glycine	4.34 ± 0.19^{d}	6.28 ± 0.23^{c}	7.20 ± 0.66^{b}	7.51 ± 0.38^{b}	6.16 ± 0.18^{c}	8.65 ± 0.27^{a}	9.11 ± 0.25^{a}	6.41 ± 0.54^{c}
Histidine	5.77 ± 0.09^{e}	6.24 ± 0.33^{e}	11.01 ± 0.57^{a}	7.09 ± 0.44^{d}	7.84 ± 0.51^{c}	8.23 ± 0.39^{bc}	8.59 ± 0.33^{b}	10.60 ± 0.24^{a}
Isoleucine	12.72 ± 0.21^{f}	18.14 ± 0.46^{e}	28.09 ± 0.75^{c}	27.65 ± 1.41^{c}	25.47 ± 0.65^{d}	30.48 ± 0.72^{b}	32.39 ± 1.25^{a}	27.63 ± 0.95^{c}
Leucine	9.38 ± 0.33^{f}	14.88 ± 0.23^{e}	35.67 ± 0.69^{b}	32.45 ± 1.14^{c}	28.98 ± 0.82^{d}	36.90 ± 1.26^{b}	40.84 ± 0.86^{a}	30.07 ± 1.76^{d}
Methionine	3.37 ± 0.17^{g}	6.20 ± 0.29^{d}	8.31 ± 0.36^{c}	5.89 ± 0.38^{d}	5.20 ± 0.29^{e}	9.46 ± 0.23^{a}	8.88 ± 0.31^{b}	4.37 ± 0.18^{f}
Norvaline	9.35 ± 0.41^{g}	13.52 ± 0.23^{f}	21.52 ± 0.15^{b}	18.49 ± 1.05^{d}	17.44 ± 0.34^{e}	20.51 ± 0.10^{c}	24.83 ± 0.61^{a}	17.89 ± 0.48^{de}
Ornithine	1.49 ± 0.15^{a}	1.53 ± 0.07^{a}	1.27 ± 0.06^{b}	0.72 ± 0.10^{d}	0.52 ± 0.06^{e}	0.75 ± 0.09^{d}	0.67 ± 0.07^{d}	0.94 ± 0.08^{c}
Phenylalanine	5.20 ± 0.14^{h}	8.25 ± 0.23^{g}	21.07 ± 0.48^{c}	19.17 ± 0.81^{d}	15.33 ± 0.23^{f}	21.86 ± 0.60^{b}	24.88 ± 0.79^{a}	17.76 ± 0.40^{e}
Proline	6.47 ± 0.25^{h}	8.11 ± 0.07^{g}	12.54 ± 0.50^{c}	11.73 ± 0.33^{d}	10.36 ± 0.25^{f}	13.82 ± 0.14^{b}	15.76 ± 0.39^{a}	11.24 ± 0.08^{e}
Threonine	8.98 ± 0.33^{f}	14.64 ± 0.60^{e}	22.91 ± 0.60^{c}	22.46 ± 0.99^{cd}	21.42 ± 0.60^{d}	29.13 ± 1.08^{a}	27.71 ± 0.89^{b}	22.14 ± 0.31^{cd}
Tryptophan	5.37 ± 0.1^{5}f	9.61 ± 0.23^{e}	19.82 ± 0.52^{c}	16.83 ± 1.09^{d}	17.22 ± 0.92^{d}	21.39 ± 0.88^{a}	20.06 ± 1.10^{bc}	21.07 ± 0.93^{ab}
Total	408.32 ± 6.11^{f}	482.66 ± 6.34^{e}	595.15 ± 5.41^{d}	635.37 ± 9.09^{c}	591.28 ± 10.06^{d}	646.96 ± 8.30^{bc}	830.43 ± 15.61^{a}	655.38 ± 10.71^{b}

Data (means ± standard deviation, n = 6) followed by different letters indicated significant differences at the 5% level. The abbreviations are the same as in Figure 2

Glutamate accelerates the metabolism of amino acids because glutamate is a vital precursor to synthesize other amino acids. Glutamate was often elevated in spore and hyphae of AMF (Jin et al. 2011). Arginine accounts for more than 90% of total amino acids in hyphae by AMF, and it is an organic N carrier synthesised by AMF after utilisation of N (Craz et al. 2007). Asparagine is the main formation of N transported from nodules to host plants and plays the central role in spore germination of AMF (Jin et al. 2011). Earlier studies indicated that AMF increased glutamate, glycine, alanine, and leucine levels in tomato

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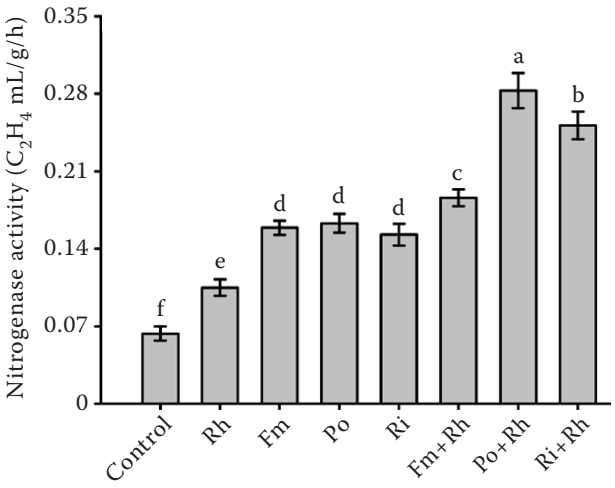

Figure 6. Effects of single or dual inoculation with arbuscular mycorrhizal fungi and rhizobia on root nitrogenase activity of white clover. Data (means ± standard deviation, n = 6) with the different letters above bars indicated significant differences between treatments at the 5% level. The abbreviations are the same as in Figure 2

(Sood 2003); glutamate and serine concentrations in sorghum (Abdel-Fattah and Mohamedin 2000); and glutamine, arginine, aspartate, alanine, and glycine contents in asparagus (Matsubara et al. 2014). Abdel-Fattah and Mohamedin (2000) reported that the degree of increase in amino acids was positively correlated with the level of mycorrhizal colonisation.

Changes in root nitrogenase activity. Nitrogenase, one of the important components in nodulation formation, can use the energy produced by photosynthesis to stimulate N_2 into ammonia. Root nitrogenase activity was notably stimulated by single or dual inoculation with AMF or Rh, irrespective of AMF species (Figure 6). Moreover, compared with single Rh treatment, treatments with single *F. mosseae*, *P. occultum*, and *R. intraradices* showed higher root nitrogenase activity. Similarly, plants with dual inoculation of AMF and Rh had higher root nitrogenase activity than plants with single AMF inoculation. Nitrogenase activity was closely associated with Fe levels, and AMF or Rh inoculation increased Fe contents of plants (Ibiang et al. 2017), thereby, stimulating nitrogenase activity.

REFERENCES

Abd-Alla M.H., El-Enany A.-W.E., Nafady N.A., Khalaf D.M., Morsy F.M. (2014): Synergistic interaction of *Rhizobium leguminosarum* bv. *viciae* and arbuscular mycorrhizal fungi as a plant growth promoting biofertilizers for faba bean (*Vicia faba* L.) in alkaline soil. Microbiology Research, 169: 49–58.

Abdel-Fattah G.M., Mohamedin A.H. (2000): Interactions between a vesicular-arbuscular mycorrhizal fungus (*Glomus intraradices*) and *Streptomyces coelicolor* and their effects on sorghum plants grown in soil amended with chitin of brawn scales. Biology and Fertility of Soils, 32: 401–409.

Aliasgharzad N., Neyshabouri M.R., Salimi G. (2006): Effects of arbuscular mycorrhizal fungi and *Bradyrhizobium japonicum* on drought stress of soybean. Biologia, 61: S324–S328.

Bethlenfalvay G.J., Ames R.N. (1987): Comparison of two methods for quantifying extraradical mycelium of vesicular-arbuscular mycorrhizal fungi. Soil Science Society of America Journal, 51: 834–837.

Bever J.D., Dickie I.A., Facelli E., Facelli J.M., Klironomos J., Moora M., Rilling M.C., Stock W.D., Tibbett M., Zobel M. (2010): Rooting theories of plant community ecology in microbial interactions. Trends in Ecology and Evolution, 25: 468–478.

Bradford M.M. (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72: 248–254.

Cheng Z., McConkey B.J., Glick B.R. (2010): Proteomic studies of plant-bacterial interactions. Soil Biology and Biochemistry, 42: 1673–1684.

Cruz C., Egsgaard H., Trujillo C., Ambus P., Requena N., Martins-Loucao M.A., Jakobsen I. (2007): Enzymatic evidence for the key role of arginine in nitrogen translocation by arbuscular mycorrhizal fungi. Plant Physiology, 144: 782–792.

De Oliveira Júnior J.Q., Jesus E.C., Lisboa F.J., Berbara R.L.L., De Faria S.M. (2017): Nitrogen-fixing bacteria and arbuscular mycorrhizal fungi in *Piptadenia gonoacantha* (Mart.) Macbr. Brazilian Journal of Microbiology, 48: 95–100.

Hack C.M., Porta M., Schäufele R., Grimoldi A.A. (2019): Arbuscular mycorrhiza mediated effects on growth, mineral nutrition and biological nitrogen fixation of *Melilotus alba* Med. in a subtropical grassland soil. Applied Soil Ecology, 134: 38–44.

He J.D., Chi G.G., Zou Y.N., Shu B., Wu Q.S., Srivastava A.K., Kuča K. (2020): Contribution of glomalin-related soil proteins to soil organic carbon in trifoliate orange. Applied Soil Ecology, 154: 103592.

He J.D., Dong T., Wu H.H., Zou Y.N., Wu Q.S., Kuča K. (2019): Mycorrhizas induce diverse responses of root *TIP* aquaporin gene expression to drought stress in trifoliate orange. Scientia Horticulturae, 243: 64–69.

Herridge D.F., Peoples M.B., Boddey R.M. (2008): Global inputs of biological nitrogen fixation in agricultural systems. Plant and Soil, 311: 1–18.

Hodge A. (2014): Interactions between arbuscular mycorrhizal fungi and organic material substrates. Advances in Applied Microbiology, 89: 47–99.

Ibiang Y.B., Mitsumoto H., Sakamoto K. (2017): Bradyrhizobia and arbuscular mycorrhizal fungi modulate manganese, iron,

https://doi.org/10.17221/234/2020-PSE

phosphorus, and polyphenols in soybean (*Glycine max* (L.) Merr.) under excess zinc. Environmental and Experimental Botany, 137: 1–13.

Jin H.R., Jiang D.H., Zhang P.H. (2011): Effect of carbon and nitrogen availability on the metabolism of amino acids in the germinating spores of arbuscular mycorrhizal fungi. Pedosphere, 21: 432–442.

Larimer A.L., Bever J.D., Clay K. (2010): The interactive effects of plant microbial symbionts: a review and meta-analysis. Symbiosis, 51: 139–148.

Ledgard S.F., Sprosen M.S., Penno J.W., Rajendram G.S. (2001): Nitrogen fixation by white clover in pastures grazed by dairy cows: temporal variation and effects of nitrogen fertilization. Plant and Soil, 229: 177–187.

Liyanaarachchi G.V.V., Mahanama K.R.R., Somasiri H.P.P.S., Pumyasiri P.A.N. (2018): Development and validation of a method for direct, underivatized analysis of free amino acids in rice using liquid chromatography-tandem mass spectrometry. Journal of Chromatography A, 1568: 131–139.

Maillet F., Poinsot V., André O., Puech-Pages V., Haouy A., Gueunier M., Cromer L., Giraudet D., Formey D., Niebel A., Martinez E.A., Driguez H., Bécard G., Dénarié J. (2011): Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. Nature, 469: 58–63.

Masson-Boivin C., Sachs J.L. (2018): Symbiotic nitrogen fixation by rhizobia – the roots of a success story. Current Opinion in Plant Biology, 44: 7–15.

Máthá I., Tóth E., Mentes A., Szabó A., Márialigeti K., Schumann P., Felföldi T. (2018): A new *Rhizobium* species isolated from the water of a crater lake, description of *Rhizobium aquaticum* sp. nov. Antonie Van Leeuwenhoek, 111: 2175–2183.

Matsubara Y.I., Okada T., Liu J. (2014): Suppression of fusarium crown rot and increase in several free amino acids in mycorrhizal asparagus. American Journal of Plant Sciences, 5: 235–240.

Meng L.L., He J.D., Zou Y.N., Wu Q.S., Kuča K. (2020): Mycorrhiza-released glomalin-related soil protein fractions contribute to soil total nitrogen in trifoliate orange. Plant, Soil and Environment, 66: 183–189.

Musyoka D.M., Njeru E.M., Nyamwange M.M., Maingi J.M. (2020): Arbuscular mycorrhizal fungi and *Bradyrhizobium* co-inoculation enhances nitrogen fixation and growth of green grams (*Vigna radiata* L.) under water stress. Journal of Plant Nutrition, 43: 1036–1047.

Oruru M.B., Njeru E.M. (2016): Upscaling arbuscular mycorrhizal symbiosis and related agroecosystems services in smallholder farming systems. BioMed Research International, 2016: 4376240.

Phillips J.M., Hayman D.S. (1970): Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Transactions of the British Mycological Society, 55: 158–161.

Shockley F.W., McGraw R.L., Garrett H.E. (2004): Growth and nutrient concentration of two native forage legumes inoculated with *Rhizobium* and mycorrhiza in Missouri, USA. Agroforestry Systems, 60: 137–142.

Sood S.G. (2003): Chemotactic response of plant-growth-promoting bacteria towards roots of vesicular-arbuscular mycorrhizal tomato plants. FEMS Microbiology Ecology, 45: 219–227.

Wu Q.S., He J.D., Srivastava A.K., Zhang F., Zou Y.N. (2019a): Development of propagation technique of indigenous AMF and their inoculation response in citrus. Indian Journal of Agricultural Sciences, 89: 1190–1194.

Wu Q.S., He J.D., Srivastava A.K., Zou Y.N., Kuča K. (2019b): Mycorrhizas enhance drought tolerance of citrus by altering root fatty acid compositions and their saturation levels. Tree Physiology, 39: 1149–1158.

Zhang S.Q. (2009): Study on the distribution and number change of rhizobium on alfalfa plants and seeds. [Master's Thesis]. Lanzhou, Gansu Agricultural University, 51.

Zhang F., Zou Y.N., Wu Q.S., Kuča K. (2020): Arbuscular mycorrhizas modulate root polyamine metabolism to enhance drought tolerance of trifoliate orange. Environmental and Experimental Botany, 171: 103962.

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