

Article

Synergistic Effects of Arbuscular Mycorrhizal Fungi and Rhizobia on Nutrient Acquisition, Growth Promotion, and Protein Profile Optimization in Alfalfa

Xiang Li  and Qianbing Zhang * 

Bingtuan Key Laboratory for Efficient Utilization of Non-Grain Feed Resources, College of Animal Science and Technology, Shihezi University, Shihezi 832003, China

* Correspondence: qbz102@163.com

Abstract

Arbuscular mycorrhizal fungi (AMF) and rhizobia establish mutualistic symbioses with plant roots. This study focused on their effects on alfalfa (*Medicago sativa* L.) growth, root morphology, nutritional quality, photosynthetic characteristics, and protein fractions; eight treatments were established: single inoculation with three AMF strains (*Funneliformis mosseae*, *Claroideoglomus etunicatum*, *Glomus versiforme*) or *Sinorhizobium meliloti* (Sm), dual co-inoculation of each AMF with Sm, and a non-inoculated control (CK). Results showed that all AMF successfully colonized alfalfa roots, with co-inoculation increasing both mycorrhizal colonization rate and nodule number. The *F. mosseae* × Sm treatment achieved the highest colonization (83.3%) and nodule count (76 per plant). Across two years, this treatment significantly increased aboveground biomass, plant height, and stem diameter ($p < 0.05$). *C. etunicatum* × Sm significantly reduced acid detergent fiber content, while dual inoculation markedly improved net photosynthetic rate and light-use efficiency. All inoculations increased rapidly (PB₁) and intermediate-degradable protein (PB₂) but decreased non-protein nitrogen (PA) and bound protein (PC). In conclusion, AMF and rhizobia exhibit significant synergistic effects. Co-inoculation (*F. mosseae* × Sm and *C. etunicatum* × Sm) enhances alfalfa productivity by optimizing root structure, improving photosynthesis, and regulating nitrogen metabolism.

Keywords: arbuscular mycorrhizal fungi; rhizobia; alfalfa; root morphology; nutrient uptake; protein composition



Academic Editor: Jerzy Wielbo

Received: 6 July 2026

Revised: 3 August 2026

Accepted: 4 August 2026

Published: 6 August 2026

Copyright: © 2026 by the authors.

Licensee MDPI, Basel, Switzerland.

This article is an open access article distributed under the terms and conditions of the [Creative Commons Attribution \(CC BY\)](https://creativecommons.org/licenses/by/4.0/) license.

1. Introduction

Alfalfa (*Medicago sativa* L.) is a high-quality perennial leguminous forage [1] characterized by a well-developed root system and rich nutritional value. It is one of the most widely cultivated legume forages globally [2]. Alfalfa is extensively distributed across China, particularly in Xinjiang, which is located in the arid to semi-arid region of Asia. This area experiences low annual precipitation, an arid climate, and high evaporation rates. Owing to its strong nitrogen (N) fixation capacity and remarkable tolerance to drought and cold, alfalfa is widely planted in this region [3]. With the advancement of animal husbandry and the increasing consumer demand for livestock products, the requirement for alfalfa-based products continues to grow. However, studies indicate a substantial gap between alfalfa supply and demand in China, which fails to meet the developmental needs of the forage and livestock industries. Consequently, China's livestock production still faces challenges

such as low international competitiveness and high production costs. Therefore, cultivating high-quality forage crops, enhancing forage yield and quality, and developing alfalfa-based forage-livestock production systems are essential measures to alleviate pressure on grain resources, increase the income of farmers and herders, advance the grass-based livestock industry, and ensure living standards.

Soil microorganisms play a crucial role in regulating ecosystem functions and promoting sustainable development. They directly or indirectly influence ecosystem structure and function by participating in key processes such as nutrient cycling and organic matter decomposition [4]. Among these microorganisms, arbuscular mycorrhizal fungi (AMF) and rhizobia (Rh) are two particularly important groups. They establish symbiotic associations with alfalfa, assisting the plant in acquiring nutrients and water, thereby promoting its growth and development [3].

AMF can form mutualistic symbioses with the roots of approximately 80% of terrestrial plant species, thereby improving plant access to water and nutrients [5]. Following colonization of plant roots, AMF produce extensive extraradical hyphae, which interconnect to form common mycorrhizal networks among different host plants. These networks mediate resource transfer and modulate interspecific plant competition [6]. Furthermore, AMF extraradical hyphae absorb mineral elements such as nitrogen, phosphorus, zinc, and copper, as well as water, in exchange for photosynthetic assimilates (e.g., fatty acids) from the host plant [7]. AMF also exhibit functions such as suppressing root pathogens, regulating phytohormone synthesis, and enhancing plant resistance to abiotic stresses [8]. Rhizobia are Gram-negative bacteria [9] capable of forming symbioses with legumes to fix atmospheric dinitrogen (N_2). The fixed nitrogen is subsequently transferred to the host legume and even to neighboring non-leguminous plants. This pathway represents a critical component of the terrestrial nitrogen cycle and an effective strategy for advancing sustainable agricultural development [10]. Rhizobia form root nodules on leguminous crops, providing the host with nitrogenous nutrients through symbiotic nitrogen fixation. Concurrently, they secrete phytohormones that promote the growth and development of both the legume crop and the bacterial cells themselves [6]. In legume–AMF–rhizobia tripartite symbiosis, the synergistic interaction is thought to operate through bidirectional nutrient exchange: AMF extraradical hyphae enhance phosphorus acquisition, which supports the high energy demand of rhizobial nitrogenase activity, while rhizobia supply nitrogen-rich compounds to the host that are subsequently allocated to AMF, promoting hyphal growth and metabolic activity [11]. This positive feedback loop drives the functional optimization of the dual symbiotic system. Although co-inoculation with AMF and rhizobia has been demonstrated to synergistically promote plant growth and nutrient uptake [5], previous studies have reported that dual inoculation increased legume shoot biomass by up to 88% and significantly enhanced nitrogen and phosphorus contents compared with single inoculation [3]. However, the downstream effects of AMF–rhizobia interactions on host plant protein composition remain inadequately characterized.

The Cornell Net Carbohydrate and Protein System (CNCPS) enables precise fractionation of forage protein components. It serves as a core methodology for systematically evaluating the feeding nutritive value of alfalfa and elucidating the effects of microbial regulation on alfalfa protein quality. Research has shown that nitrogen fertilization can improve forage nutritional quality by increasing non-protein nitrogen (NPN) and soluble protein (SOLP) contents, thereby enhancing total degradable protein and true protein levels [12]. Recent studies have demonstrated that co-inoculation with AMF and rhizobia can significantly enhance alfalfa productivity and nutrient acquisition, particularly under low-input conditions [3]. The synergistic effects of AMF and rhizobia on alfalfa growth, nitrogen fixation, and stress tolerance have been increasingly documented [5]. However,

the mechanisms by which AMF and rhizobial inoculation influence the protein fraction composition of alfalfa remain unclear. Furthermore, the relationships among various alfalfa growth indicators under AMF and rhizobial inoculation conditions are still poorly defined. Therefore, this study investigates the effects of single and dual inoculation with arbuscular mycorrhizal fungi and rhizobia on alfalfa protein fractions. By integrating indicators such as root colonization status, nodule number, agronomic traits, and nutritional quality, this research systematically assesses the potential of these microbial inoculants to enhance alfalfa yield and quality. The findings aim to provide a theoretical basis and data support for improving high-quality forage production and livestock productivity. Based on this, the present study tests the following hypotheses: (1) Co-inoculation with AMF and rhizobia significantly promotes both aboveground and belowground growth of alfalfa; (2) Co-inoculation with AMF and rhizobia synergistically promotes alfalfa growth and nutritional quality by optimizing nitrogen partitioning.

2. Materials and Methods

2.1. Microbial Inoculants

AMF used in this study were *Funneliformis mosseae*, *Claroideoglomus etunicatum*, and *Glomus versiforme* (all procured from the Qingdao Institute of Agricultural Mycorrhizae, Qingdao, China). The inoculum consisted of a sand–soil carrier containing AMF spores, extraradical hyphae, and colonized root fragments of white clover (*Trifolium repens* L.), which served as the propagation host. The spore density of the mixed inoculum was approximately 20–30 spores per gram of inoculum (dry weight basis).

The rhizobial strain used in this study was *Sinorhizobium meliloti* (isolated and screened by Dr. Sun Yanliang from our research group). The test strain was initially propagated on yeast mannitol agar (YMA) plates. Prior to inoculation, the strain was transferred from the plate culture onto slant medium for activation, and subsequently inoculated into Erlenmeyer flasks containing YMA liquid medium to prepare a rhizobial suspension. The concentration of the final *Sinorhizobium meliloti* suspension was adjusted to 1×10^8 CFU mL^{−1} for uniform inoculation.

2.2. Experimental Design and Plant Cultivation

The experiment was arranged in a single-factor completely randomized design comprising eight treatments: single inoculation with *Funneliformis mosseae* (Fm), single inoculation with *Claroideoglomus etunicatum* (Ce), single inoculation with *Glomus versiforme* (Gv), single inoculation with *Sinorhizobium meliloti* (Sm), dual inoculation with *Funneliformis mosseae* and *Sinorhizobium meliloti* (Fm × Sm), dual inoculation with *Claroideoglomus etunicatum* and *Sinorhizobium meliloti* (Ce × Sm), dual inoculation with *Glomus versiforme* and *Sinorhizobium meliloti* (Gv × Sm), and a non-inoculated control (CK). Each treatment was set with twelve biological replicates (12 independent pots). This study conducted continuous monitoring on the same batch of potted alfalfa across two consecutive growing seasons. No pots, cultivation substrates, or plant seedlings were replaced throughout the two-year experiment.

The pot experiment was conducted using plastic pots with dimensions of 23 cm × 17 cm × 15 cm (top diameter × bottom diameter × height). Prior to use, the pots were immersed in 75% ethanol for 20 min for surface disinfection and then set aside. The experimental soil was collected from the forage trial field at the Experimental Station of Shihezi University (44°20' N, 86°30' E). The soil samples were air-dried naturally, passed through a 1 cm sieve to remove stones and residual plant root fragments, and subsequently analyzed for basic physicochemical properties. The sieved soil was sterilized by autoclaving

at 121 °C under moist heat for 30 min prior to use. The physical and chemical properties of the soil are shown in Table 1.

AMF were inoculated using the hole-sowing method. After sterilization and thorough mixing, 3 kg of the soil mixture composed of sterilized soil and perlite at a ratio of 3:1 was placed into each pot. For the single AMF inoculation treatments, 10 g of inoculum was added per pot, whereas for the dual inoculation treatments, 5 g of AMF inoculum was applied. The non-inoculated control received an equivalent amount of sterilized (inactivated) inoculum to maintain a consistent microbial background. Finally, the inoculum was covered with a 200 g layer of topsoil.

Alfalfa seeds were surface-sterilized with 75% ethanol for 30 s, followed by disinfection with 5% sodium hypochlorite for 10 min. The seeds were then rinsed thoroughly with sterile water and sown into the pots at a depth of 1–2 cm, with one seed per sowing hole and seven seeds per pot. At the three-leaf stage, seedlings were thinned, retaining five uniform and vigorously growing alfalfa seedlings per pot. Pots were arranged in a completely randomized layout. Fourteen days after alfalfa seedling emergence, rhizobial inoculation was performed. At this growth stage, alfalfa seedlings have developed primary lateral roots, which are the optimal sites for rhizobial infection and nodulation. This time interval also alleviates early root niche competition between AMF and rhizobium, favoring their stable co-colonization and synergistic effects. For the single rhizobial inoculation treatments, 10 mL of rhizobial suspension was injected into the soil adjacent to the taproot of each plant using a syringe (Jiangsu Zhengkang Medical Apparatus Co., Ltd., Changzhou, China). For the dual inoculation treatments, 5 mL of rhizobial suspension was applied. The non-rhizobial-inoculated controls received an equivalent volume of sterilized (inactivated) rhizobial suspension. Inoculation was repeated every 7 days, for a total of three injections.

All pots were placed under open-air outdoor conditions throughout the two consecutive growing seasons. Daily meteorological data during the experimental period were recorded by the automatic weather station at the Shihezi University Experimental Station. The daily average ambient temperature ranged from 16 °C to 34 °C, and the daily relative humidity fluctuated between 35% and 72% across the two growing seasons. Under natural field environments, alfalfa flowers underwent complete open pollination mediated by wild insect pollinators, and no artificial pollination or pollination isolation treatment was applied in this study. For irrigation management, all pots received uniform manual surface watering with a consistent regime: watering was conducted every 2–3 days, and the frequency was moderately adjusted according to local rainfall events to avoid drought stress or waterlogging. Soil water content was maintained at 60–70% of field water capacity across all treatments to eliminate water availability as a confounding factor.

Table 1. Physical and chemical properties of the tested soil.

Organic Matter (g·kg ⁻¹)	Bulk Density (g·cm ⁻³)	Total Nitrogen (g·kg ⁻¹)	Total Phosphorus (g·kg ⁻¹)	Alkaline Nitrogen (mg·kg ⁻¹)	Available Phosphorus (mg·kg ⁻¹)	Available Potassium (mg·kg ⁻¹)
23.60	1.47	1.55	0.22	70.50	18.10	135.20

2.3. Measurements and Analytical Methods

2.3.1. Aboveground Biomass

At the early flowering stage (10% bloom), the aboveground parts of alfalfa from three pots per treatment showing uniform growth were manually harvested by cutting at 2 cm above the soil surface. The harvested fresh samples were weighed immediately to obtain fresh weight. The fresh forage samples were then oven-dried at 105 °C for 30 min to

deactivate enzymes, after which the temperature was adjusted to 65 °C. Samples were dried to a constant weight, and the dry weight was recorded as aboveground biomass, expressed as the mean value (g/pot).

2.3.2. Plant Height and Stem Diameter

Concurrent with the determination of aboveground biomass, ten uniformly growing alfalfa plants were selected from each treatment. Plant height was measured by aligning a ruler parallel to the main stem and recording the distance from the stem base to the apex; the mean value was expressed in centimeters (cm). Stem diameter was measured using a digital vernier caliper at a position 5 cm above the soil surface on the corresponding stems of the ten plants; the mean value was expressed in millimeters (mm).

2.3.3. The Mycorrhizal Colonization Rate and Nodule Number

Three intact alfalfa root systems were randomly selected from each treatment and rinsed thoroughly with tap water. Mycorrhizal colonization rate was determined following the method described by Phillips and Hayman [13]. Root segments (1 cm in length) were excised and cleared in 10% KOH solution at 90 °C for 2 h, followed by bleaching in 10% H₂O₂ for 15 min. The root segments were then acidified in 0.2 mol/L HCl solution for 10 min and stained with acid fuchsin in lactic acid for 30 min. Subsequently, the stained root segments were examined under a microscope, and AMF colonization rate was calculated as the percentage of colonized root length relative to the total root length observed.

2.3.4. Root System Architecture

Alfalfa root systems were scanned into digital images using a root scanner (Epson Scanner) (Seiko Epson Corporation, Suwa, Nagano, Japan). The images were subsequently processed and analyzed digitally with Win-RHIZO 2017a software to measure and characterize morphological traits including total root length, number of lateral roots, root surface area, and root volume.

2.3.5. Nutritional Quality

Plant nitrogen (N) content was determined using the Kjeldahl method [14]. Plant phosphorus (P) content was measured using the molybdenum-antimony anti-colorimetric method [15]. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were determined according to the Van Soest method [16].

2.3.6. Photosynthetic Parameters

Prior to alfalfa harvest (at the early flowering stage), photosynthetic parameters were measured on a clear, cloudless day between 11:00 and 14:00 Beijing Time using a CI-340 portable photosynthesis system (CID Bio-Science, Inc., Camas, WA, USA). For each treatment, three healthy and intact leaves from the upper one-third of the alfalfa canopy were selected. Photosynthetically active radiation (PAR) was maintained at approximately 1400 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Net photosynthetic rate (P_n), transpiration rate (Tr), stomatal conductance (G_s), and intercellular CO₂ concentration (C_i) were recorded. Water use efficiency (IWUE) was calculated as P_n/Tr , and light use efficiency (LUE) was calculated as P_n/PAR .

$$IWUE = P_n/Tr \quad (1)$$

2.3.7. Analysis for Protein Fractions (CNCPS)

Soluble protein (SOLP) concentration was determined according to the method of Bradford [17] with minor modifications. A phosphate-buffered solution (pH 6.7–6.8) was prepared by dissolving 43.5 g Na₂HPO₄·12H₂O and 56.5 g NaH₂PO₄·2H₂O in distilled

water and making up to 1 L. Ground and dried samples (0.05 g) were placed in 15 mL centrifuge tubes, and 10 mL of phosphate-buffered solution and 1 mL of sodium azide solution were added. After incubation at 20–25 °C for 3 h, the mixture was centrifuged at 4000 rpm for 10 min. The supernatant was collected, and the SOLP concentration was determined spectrophotometrically at 595 nm after reaction with Coomassie Brilliant Blue G-250 reagent, using bovine serum albumin (BSA) as the standard. Concentrations of NPN, NDIP, and ADIP were determined following the procedures described by Licitra et al. [18]. For NPN determination, 0.5 g of sample was placed into a 250 mL conical flask, mixed with 50 mL of distilled water, and allowed to stand for 30 min. Subsequently, 10 mL of 10% (*w/v*) trichloroacetic acid (TCA) was added, and the mixture was incubated for another 30 min. The solution was then filtered, and the residue was rinsed twice with TCA solution. The filter paper containing the residue was dried and digested for nitrogen determination to quantify the NPN fraction. For NDIP and ADIP determinations, the residues obtained after neutral detergent fiber (NDF) and acid detergent fiber (ADF) extractions, respectively, were dried and subjected to Kjeldahl digestion to determine residual nitrogen content. Based on the Cornell Net Carbohydrate and Protein System (CNCPS) [19], crude protein (CP) was partitioned into five fractions: non-protein nitrogen (PA, Fraction A), rapidly degradable true protein (PB₁, Fraction B1), intermediately degradable true protein (PB₂, Fraction B2), slowly degradable true protein (PB₃, Fraction B3), and bound protein (PC, Fraction C). The concentrations of these fractions were calculated using the following equations:

$$PA (\%CP) = NPN (\%CP) \times 0.01 \times SOLP (\%CP) \quad (2)$$

$$PB_1 (\%CP) = SOLP (\%CP) - PA (\%CP) \quad (3)$$

$$PC (\%CP) = ADIP (\%CP) \quad (4)$$

$$PB_3 (\%CP) = NDIP (\%CP) - ADIP (\%CP) \quad (5)$$

$$PB_2 (\%CP) = 100 - PA (\%CP) - PB_1 (\%CP) - PB_3 (\%CP) - PC (\%CP) \quad (6)$$

2.4. Statistical Analysis

Data were organized using Microsoft Excel 2021, and graphs were generated using Origin 2021. Statistical analyses were performed with SPSS 26.0. A one-way analysis of variance (ANOVA) was conducted on the two-year dataset, followed by Pearson correlation analysis to examine relationships among indicators. Mantel tests were employed to assess the correlations between alfalfa growth parameters and nutritional quality, photosynthetic characteristics, and protein fractions. The objective was to determine the effects of arbuscular mycorrhizal fungi and rhizobia interactions on alfalfa agronomic traits, nutritional quality, nodulation and root colonization, and protein composition.

3. Results

3.1. Changes in Alfalfa Growth Under Different Inoculation Treatments

Both single and dual inoculation treatments promoted aboveground and belowground growth of alfalfa relative to the CK treatment, with the growth-promoting effects of dual inoculation generally surpassing those of single inoculation (Figure 1). The dual inoculation treatments *Fm* × *Sm*, *Ce* × *Sm*, and *Gv* × *Sm* significantly increased aboveground biomass, plant height, and stem diameter across the two experimental years ($p < 0.05$). Specifically, compared with the CK group, the *Fm* × *Sm* treatment increased aboveground biomass by 34.09–47.35%, plant height by 21.20–31.32%, and stem diameter by 23.00–26.27% over the two years, with statistically significant differences ($p < 0.05$) (Figure 1a–c). In compar-

ison with the CK group, single inoculation with *Gv* and *Sm* did not significantly affect aboveground biomass ($p > 0.05$), although a certain degree of improvement was observed.

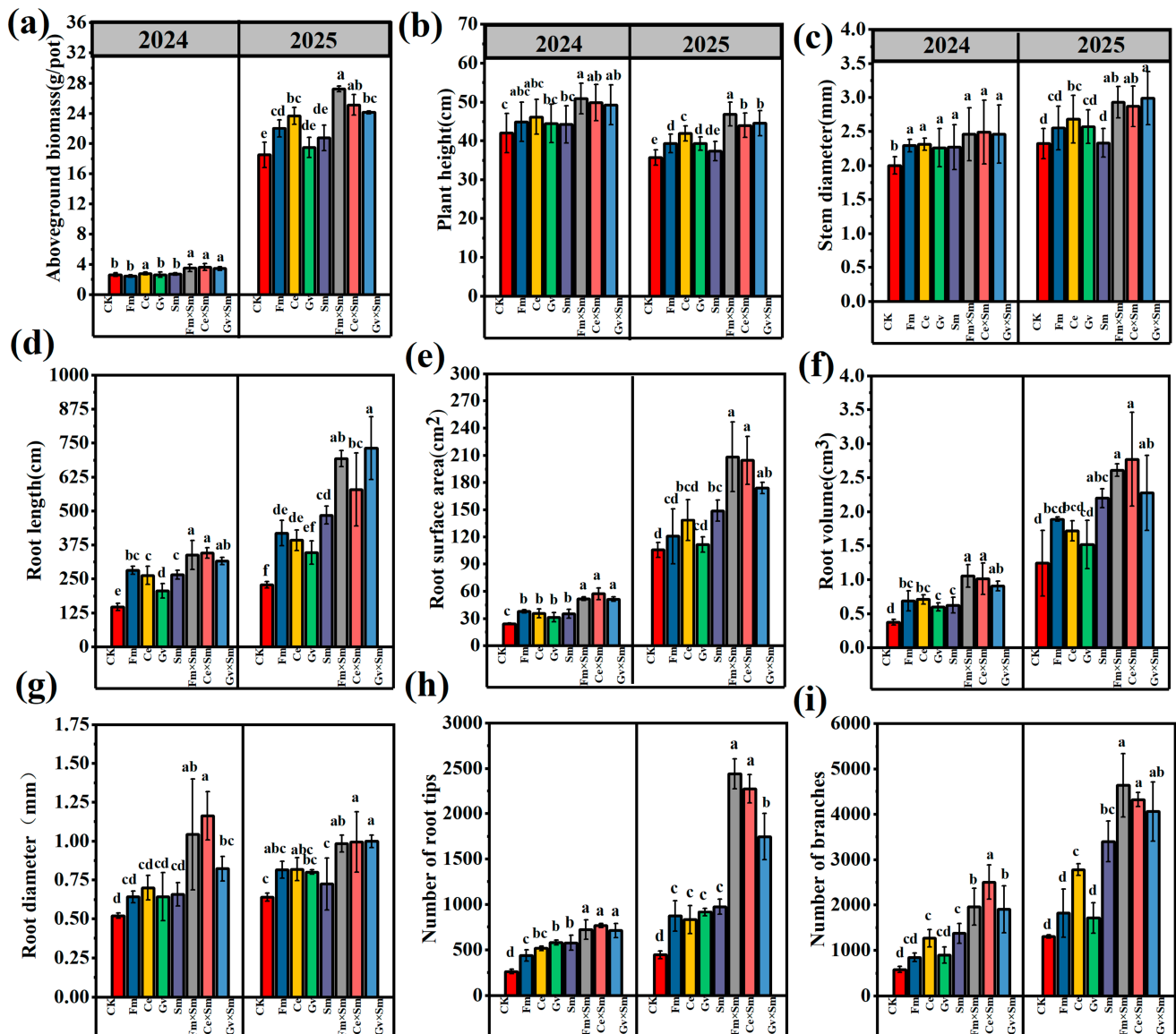


Figure 1. Growth of alfalfa under different inoculation treatments. Data are presented as mean ± standard error. Different lowercase letters indicate significant differences at $p < 0.05$. Panels (a–i) represent aboveground biomass, plant height, stem diameter, total root length, root surface area, root volume, average root diameter, number of root tips, and number of root forks, respectively. Data are means ± SE ($n = 8$).

Compared with the CK group, single inoculation with *Fm*, *Ce*, *Gv*, and *Sm* significantly increased total root length, root surface area, root volume, and number of root tips in alfalfa ($p < 0.05$), whereas no significant differences were observed in average root diameter ($p > 0.05$). Dual inoculation treatments (*Fm* × *Sm*, *Ce* × *Sm*, and *Gv* × *Sm*) significantly enhanced total root length, root surface area, root volume, average root diameter, number of root tips, and number of root forks during both years of alfalfa growth ($p < 0.05$). Over the two-year period, compared with the CK group, single inoculation with *Fm* increased root length by 82.88–91.92%, root surface area by 14.34–57.89%, root volume by 52.25–84.14%, average root diameter by 23.51–27.34%, number of root tips by 65.86–95.87%, and number of root forks by 39.54–45.54%. Compared with single inoculation with *Fm*,

the dual inoculation treatment $Fm \times Sm$ further increased root length by 19.86–65.83%, root surface area by 36.22–72.74%, root volume by 38.23–52.85%, average root diameter by 20.61–62.56%, number of root tips by 65.18–180.23%, and number of root forks by 132.18–155.57% (Figure 1d–i). Both single and dual inoculation treatments promoted alfalfa growth, with dual inoculation exhibiting superior overall efficacy.

3.2. Changes in Nodule Number and Mycorrhizal Colonization Rate of Alfalfa Under Different Inoculation Treatments

Across the two experimental years, both the single inoculation with Sm and the dual inoculation treatments significantly increased nodule number in alfalfa ($p < 0.05$). Compared with the single Sm inoculation treatment, the dual inoculation treatments enhanced nodule number by 60.84–83.59% and 49.48–58.13%, respectively (Figure 2a,b).

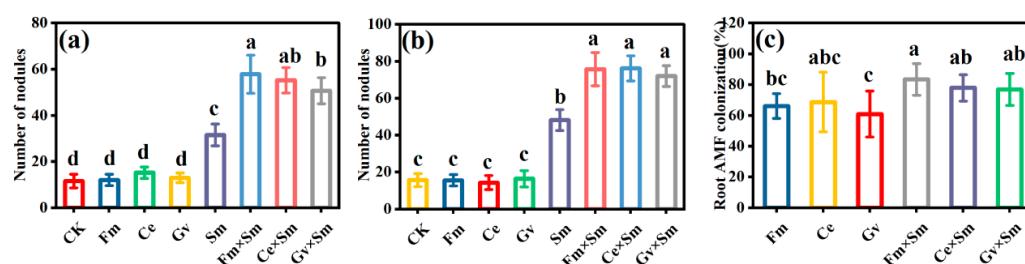


Figure 2. Nodule number and mycorrhizal colonization rate of alfalfa under different inoculation treatments. Data are presented as mean $\pm$ standard error. Different lowercase letters indicate significant differences at $p < 0.05$. Panels (a–c) represent nodule number in 2024, nodule number in 2025, and mycorrhizal colonization rate in 2025, respectively. Data are means $\pm$ SE ($n = 8$).

No mycorrhizal colonization was observed in alfalfa plants not inoculated with AMF, whereas all AMF-inoculated treatments exhibited substantial root colonization (Figure 2c). Among these, the dual inoculation treatments displayed higher colonization rates than the single AMF inoculation treatments. Notably, the $Fm \times Sm$ treatment achieved the highest colonization rate, which was significantly greater than that of the single Fm and Gv inoculation treatments ($p < 0.05$).

3.3. Changes in Fiber, Nitrogen, and Phosphorus Contents of Alfalfa Under Different Inoculation Treatments

Both single and dual inoculation treatments reduced the neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents of alfalfa to varying degrees, while increasing plant nitrogen (N) and phosphorus (P) contents. Across the two experimental years, compared with the CK group, the dual inoculation treatment $Ce \times Sm$ significantly reduced the ADF content of alfalfa ($p < 0.05$), whereas single inoculation treatments did not significantly alter ADF or NDF contents ($p > 0.05$) (Figure 3a,b).

Nitrogen and phosphorus are essential macronutrients for plant growth and development. Over the two-year period, compared with the CK group, all dual inoculation treatments significantly increased total nitrogen content in alfalfa plants ($p < 0.05$). Single inoculation with Fm , Ce , and Gv increased nitrogen content by 9.88–12.20%, 12.44–19.04%, and 12.52–17.86%, respectively, relative to CK. Notably, the $Ce \times Sm$ dual inoculation treatment achieved the highest nitrogen content, which was 27.15–31.53% greater than that of CK, with statistically significant differences ($p < 0.05$). Compared with single inoculation of either AMF or rhizobia, dual inoculation significantly enhanced total phosphorus content in alfalfa ($p < 0.05$), with increases ranging from 9.39% to 60.92% (Figure 3c,d).

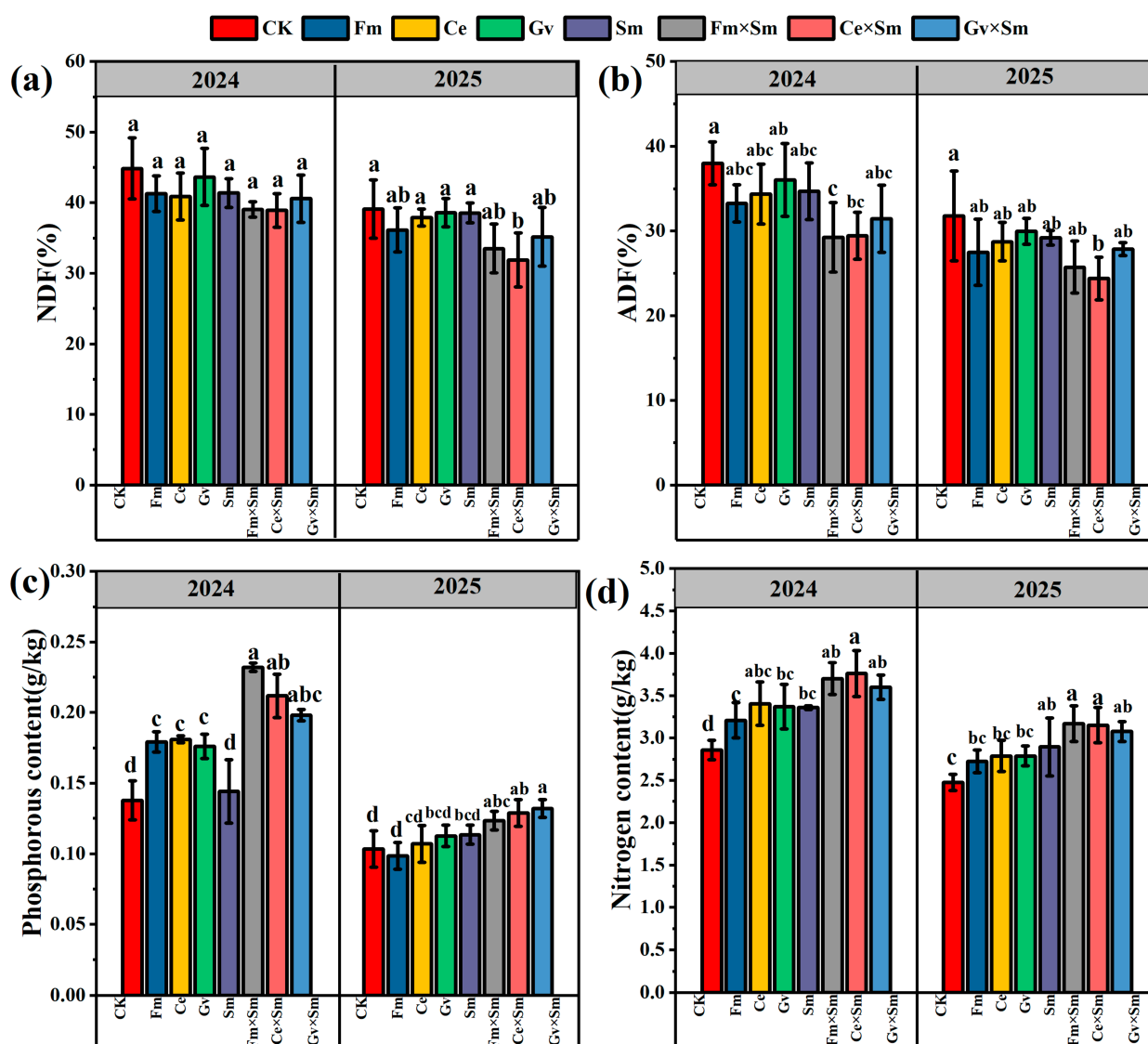


Figure 3. Fiber, nitrogen, and phosphorus contents of alfalfa under different inoculation treatments. Data are presented as mean $\pm$ standard error. Different lowercase letters indicate significant differences at $p < 0.05$. Panels (a–d) represent NDF content, ADF content, phosphorus (P) content, and nitrogen (N) content, respectively. Data are means $\pm$ SE ($n = 3$).

3.4. Changes in Leaf Photosynthetic Characteristics of Alfalfa Under Different Inoculation Treatments

All inoculation treatments enhanced leaf net photosynthetic rate (P_n), transpiration rate (Tr), and stomatal conductance (G_s) of alfalfa to varying degrees, while reducing intercellular CO_2 concentration (C_i) (Figure 4). Over the two-year period, compared with the CK treatment, the dual inoculation treatments significantly increased alfalfa net photosynthetic rate by 56.87–71.47% in 2024 and by 40.95–56.79% in 2025 ($p < 0.05$). Concurrently, the dual inoculation treatments significantly decreased intercellular CO_2 concentration by 32.65–33.43% ($p < 0.05$). In contrast, single inoculation treatments did not significantly affect net photosynthetic rate, transpiration rate, stomatal conductance, or intercellular CO_2 concentration relative to CK ($p > 0.05$).

All inoculation treatments effectively improved leaf light use efficiency (LUE) in alfalfa. Notably, compared with the CK treatment, the dual inoculation treatments significantly enhanced LUE ($p < 0.05$). However, leaf instantaneous water use efficiency (WUE) did not differ significantly between single and dual inoculation treatments ($p > 0.05$) (Figure 5).

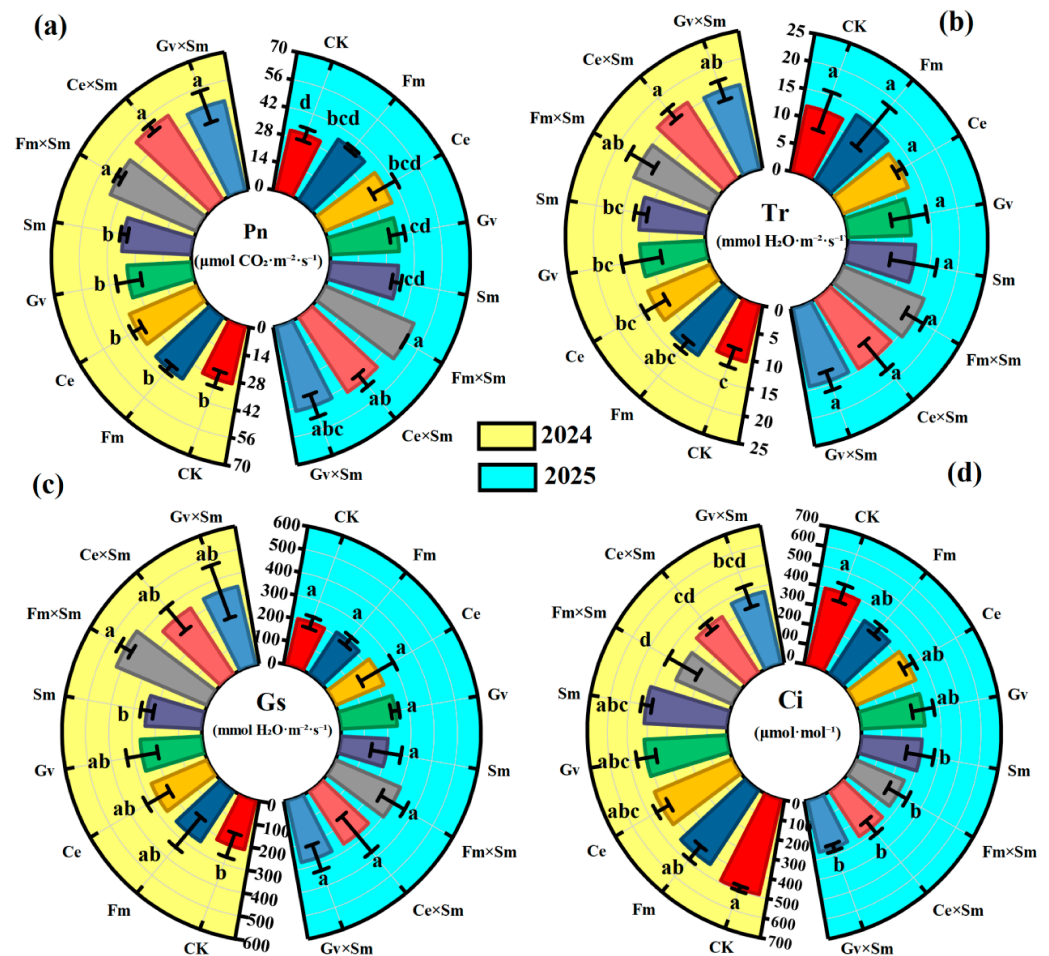


Figure 4. Leaf photosynthetic parameters of alfalfa under different inoculation treatments. Data are presented as mean $\pm$ standard error. Different lowercase letters indicate significant differences at $p < 0.05$. Panels (a–d) represent net photosynthetic rate (Pn), transpiration rate (Tr), stomatal conductance (Gs), and intercellular CO₂ concentration (Ci), respectively. Data are means $\pm$ SE ($n = 3$).

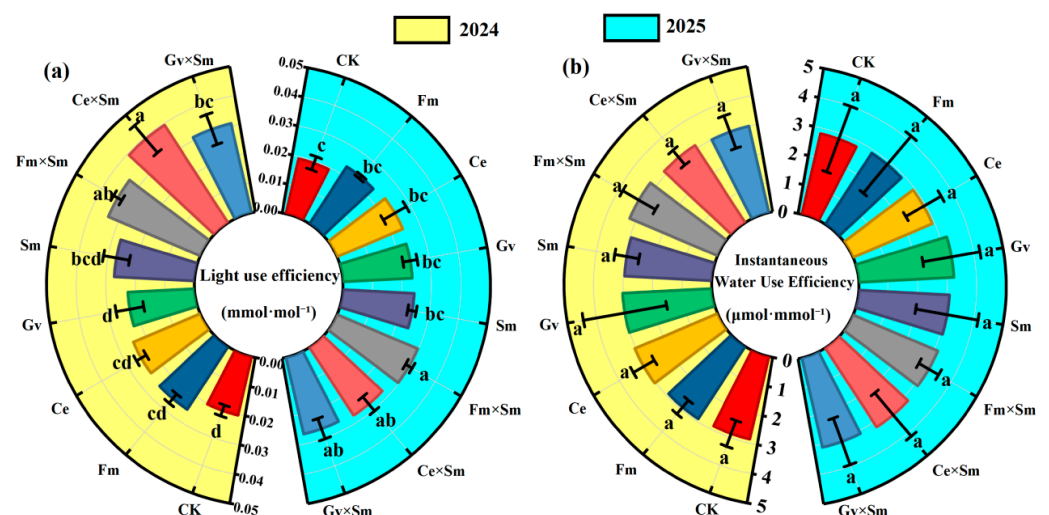


Figure 5. Light use efficiency and leaf instantaneous water use efficiency of alfalfa under different inoculation treatments. Data are presented as mean $\pm$ standard error. Different lowercase letters indicate significant differences at $p < 0.05$. Panel (a) represents light use efficiency (LUE); panel (b) represents leaf instantaneous water use efficiency (IWUE). Data are means $\pm$ SE ($n = 3$).

3.5. Changes in Nitrogen Partitioning of Alfalfa Under Different Inoculation Treatments

Inoculation treatments significantly affected crude protein (CP), soluble protein (SOLP), and non-protein nitrogen (NPN) contents in alfalfa ($p < 0.05$), but had no significant effect on acid detergent insoluble protein (ADIP) (2024–2025) or neutral detergent insoluble protein (NDIP) (2025) contents ($p > 0.05$). During 2024–2025, inoculation treatments significantly increased CP and SOLP contents and decreased NPN content in alfalfa, while no significant changes were observed in ADIP and NDIP contents. Compared with the CK group, dual inoculation treatments significantly decreased NPN content by 7.60–12.23% ($p < 0.05$), whereas single inoculation treatments did not significantly affect NPN content ($p > 0.05$). Relative to the single *Sm* inoculation treatment, the dual inoculation treatments *Fm* $\times$ *Sm*, *Ce* $\times$ *Sm*, and *Gv* $\times$ *Sm* increased CP content over the two years by 29.42% and 27.79%, 31.53% and 27.15%, and 29.88% and 24.10%, respectively. Compared with the CK group, the dual inoculation treatments significantly increased alfalfa SOLP content ($p < 0.05$), with increases ranging from 75.29% to 105.91% over the two-year experimental period (Figure 6).

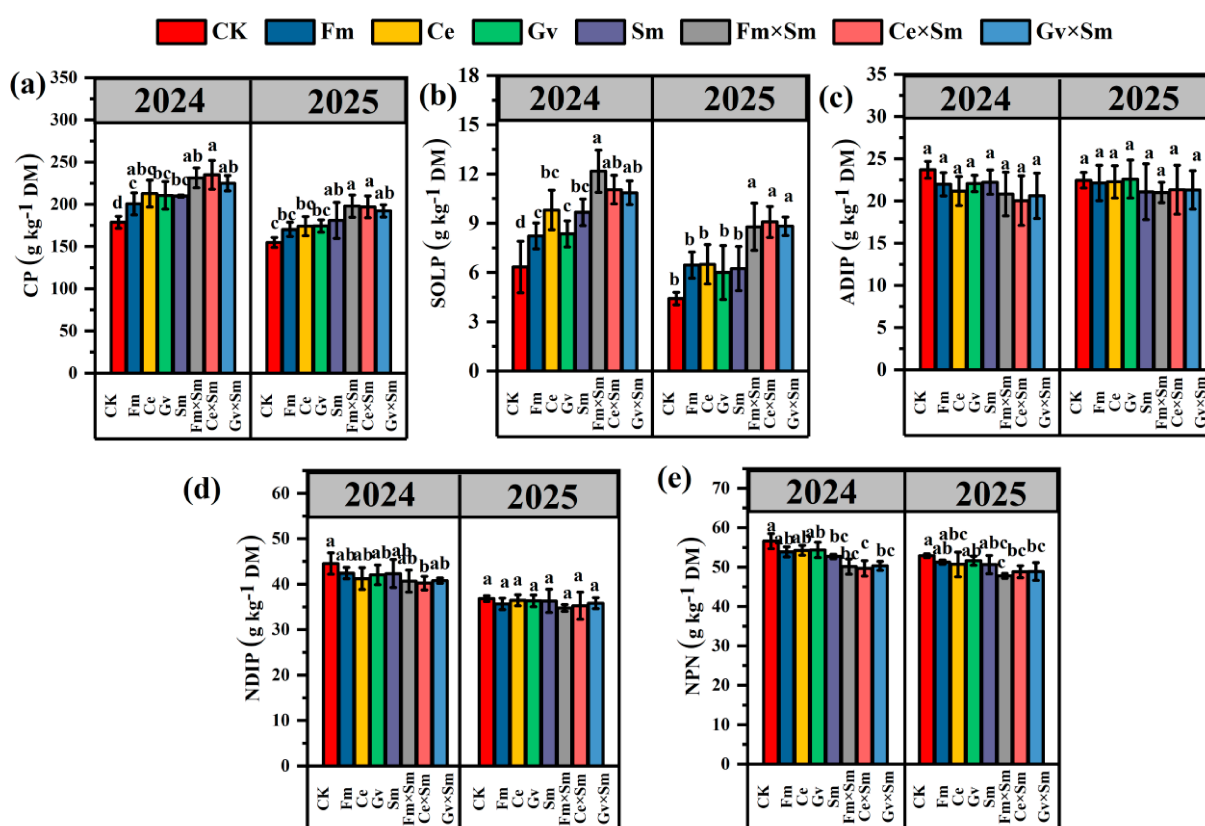


Figure 6. Nitrogen partitioning in alfalfa under different inoculation treatments. Data are presented as mean $\pm$ standard error. Different lowercase letters indicate significant differences at $p < 0.05$. Panels (a–e) represent crude protein (CP), soluble protein (SOLP), neutral detergent insoluble protein (NDIP), acid detergent insoluble protein (ADIP), and non-protein nitrogen (NPN), respectively. Data are means $\pm$ SE ($n = 3$).

3.6. Changes in Protein Fractions of Alfalfa Under Different Inoculation Treatments

All inoculation treatments increased the contents of rapidly degradable true protein (PB₁) and intermediately degradable true protein (PB₂), while decreasing the contents of non-protein nitrogen (PA) and bound protein (PC) in alfalfa during the 2024–2025 period. Compared with the CK group, all inoculation treatments significantly reduced PA content ($p < 0.05$). Notably, the dual inoculation treatments *Fm* $\times$ *Sm*, *Ce* $\times$ *Sm*, and *Gv* $\times$ *Sm* reduced PA content by 19.32–19.75%, 14.69–17.29%, and 13.50–14.14%, respectively, relative

to the single AMF inoculation treatments, with statistically significant differences ($p < 0.05$). In comparison with the CK group, all inoculation treatments significantly increased PB_2 content ($p < 0.05$). Specifically, the dual inoculation treatments $Fm \times Sm$, $Ce \times Sm$, and $Gv \times Sm$ enhanced PB_2 content by 37.21–39.08%, 34.07–42.95%, and 31.01–37.30%, respectively, compared with CK. In 2024, the dual inoculation treatments $Fm \times Sm$ and $Ce \times Sm$ significantly reduced the PB_3 content of alfalfa by 25.90% to 26.63% compared with the CK group ($p < 0.05$). In 2025, however, no significant differences in PB_3 content were observed among any of the treatment groups (Figure 7).

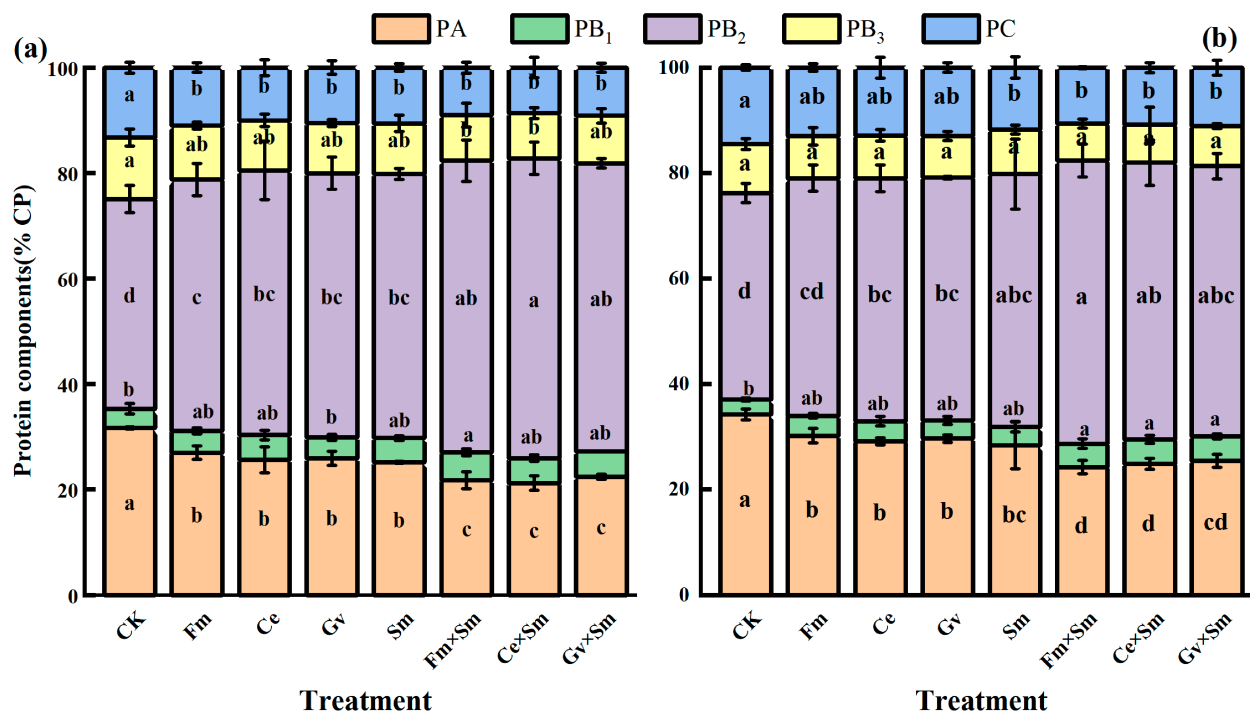


Figure 7. Protein fractions of alfalfa under different inoculation treatments. Data are presented as mean $\pm$ standard error. Different lowercase letters indicate significant differences at $p < 0.05$. Panel (a) represents protein fractions in 2024; panel (b) represents protein fractions in 2025. Data are means $\pm$ SE ($n = 3$).

3.7. Correlation Analysis

To further elucidate the relationships among protein fractions, photosynthetic performance, nutritional quality, and aboveground biomass of alfalfa across the two experimental years, Pearson correlation analysis and Mantel tests were conducted. Aboveground biomass was significantly positively correlated with instantaneous water use efficiency (IWUE) and phosphorus (P) content ($p < 0.05$), and highly significantly positively correlated with nitrogen (N) content, transpiration rate (Tr), stomatal conductance (Gs), and light use efficiency (LUE) ($p < 0.01$). Conversely, aboveground biomass exhibited a significant negative correlation with acid detergent fiber (ADF) content ($p < 0.05$) and a highly significant negative correlation with neutral detergent fiber (NDF) content and intercellular CO₂ concentration (Ci) ($p < 0.01$) (Figure 8). Mantel test results further revealed highly significant positive correlations ($p < 0.01$) between all measured root morphological parameters (total root length, root surface area, root volume, average root diameter, number of root tips, and number of root forks) and aboveground growth indicators (plant height, stem diameter, and aboveground biomass). Furthermore, root morphology exhibited highly significant positive correlations ($p < 0.001$) with photosynthetic characteristics, nutritional quality, and protein fractions (Figure 9).

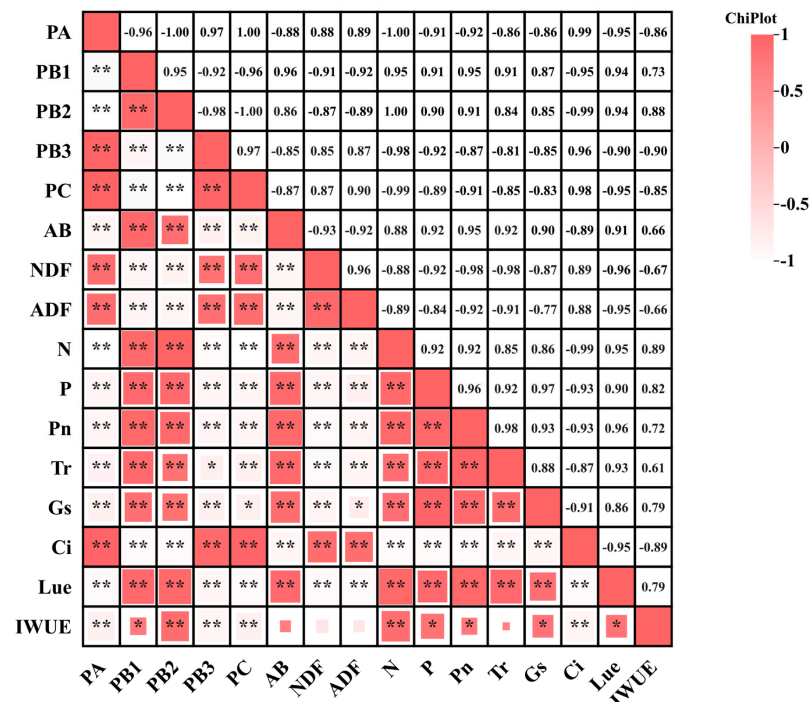


Figure 8. Correlation analysis of protein fractions, photosynthetic performance, nutritional quality, and aboveground biomass in alfalfa under different inoculation treatments. Square size indicates the level of significance, and square color denotes positive (white) or negative (red) correlation. * indicates significance at $p < 0.05$, and ** indicates significance at $p < 0.01$. Abbreviations used in the figure are as follows: PA, non-protein nitrogen (Fraction A); PB₁, rapidly degradable true protein; PB₂, intermediately degradable true protein; PB₃, slowly degradable true protein; PC, bound protein (unavailable protein, Fraction C); AB, aboveground biomass; NDF, neutral detergent fiber; ADF, acid detergent fiber; N, nitrogen content; P, phosphorus content; Pn, net photosynthetic rate; Tr, transpiration rate; Gs, stomatal conductance; Ci, intercellular CO₂ concentration; LUE, light use efficiency; IWUE, instantaneous water use efficiency.

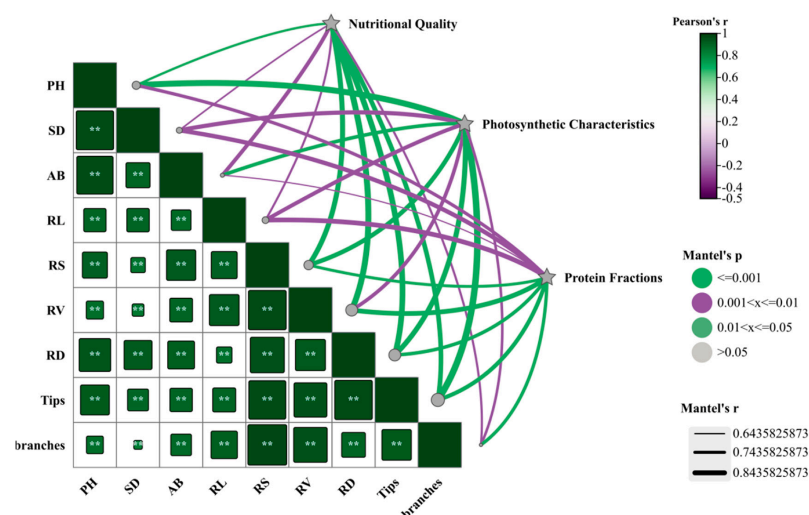


Figure 9. Correlation analysis of nutritional quality, protein fractions, and photosynthetic performance in alfalfa under different inoculation conditions. Line thickness indicates the coefficient of determination (R^2) level, square size denotes the significance level, and square color represents positive (green) or negative (purple) correlation. ** indicates significance at $p < 0.01$. Abbreviations used in the figure are as follows: PH, plant height; SD, stem diameter; AB, aboveground biomass; RL, total root length; RS, root surface area; RV, root volume; RD, average root diameter; Tips, number of root tips; Branches, number of root forks.

4. Discussion

4.1. Effect of Inoculation on Alfalfa Growth

The rhizosphere of leguminous plants has evolved a tripartite symbiotic association involving rhizobia, legumes, and AMF over long-term coevolution. This tripartite symbiosis has become a widespread phenomenon in natural ecosystems and plays a crucial role in both ecological functioning and agricultural production [11]. The present study demonstrated that, compared with the non-inoculated control, single inoculation with AMF, single inoculation with rhizobia, and co-inoculation with AMF and rhizobia all promoted both aboveground and belowground growth of alfalfa. Notably, the co-inoculation treatments exhibited superior growth-promoting effects, indicating a significant synergistic interaction between AMF and rhizobia ($p < 0.05$). Similar synergistic effects have been reported in lotus and white clover [5,20]. Previous research has shown that the synergistic action of AMF and rhizobia significantly enhances mung bean biomass and promotes its growth [21]. This synergy may be attributed to the fact that the nitrogen fixation process in rhizobia is highly dependent on adenosine triphosphate (ATP) supply, which requires adequate phosphorus as a substrate for ATP synthesis. The extraradical hyphal network of AMF substantially expands the spatial reach and efficiency of phosphorus acquisition by alfalfa roots, thereby alleviating phosphorus limitation for the efficient operation of rhizobial nitrogenase. Concurrently, rhizobia convert atmospheric dinitrogen into ammonia through symbiotic nitrogen fixation; the synthesized nitrogenous compounds are transported downward to the roots via the phloem, providing an ample nitrogen source for AMF hyphal extension and metabolism. Consequently, the symbiotic system under dual inoculation achieves more efficient material and energy fluxes, ultimately driving significant biomass accumulation [6].

The root system is the most active nutrient-absorbing organ in plants, and root morphology directly influences the plant's capacity for soil anchorage and the spatial extent of nutrient acquisition [22]. A well-developed root architecture facilitates enhanced uptake of nutrients and water, thereby promoting plant growth and yield improvement [23]. The present study demonstrated that dual inoculation with AMF and rhizobia promoted alfalfa root growth, expanded the spatial distribution of the root system, and increased the absorptive surface area for nutrients and water. This finding is consistent with results reported by Ramírez-Flores et al. [24] in maize. Following root colonization, AMF secrete auxin-like compounds and strigolactones, which induce the initiation and elongation of lateral root primordia, thereby modifying alfalfa root architecture as evidenced by increased root length and expanded root surface area. Rhizobia, through the formation of root nodules, contribute numerous highly efficient nitrogen-fixing “functional nodes” on the root system. Under dual inoculation, the extraradical hyphal network of AMF proliferates through the soil matrix, generating pores and exudates that improve aeration and aggregate structure within the rhizosphere microenvironment, thereby creating more favorable conditions for nodule formation. The mutual facilitation between AMF and rhizobia drives alfalfa growth and development into a positive feedback loop [25]. Therefore, in agricultural practice, the adoption of dual inoculation technology with AMF and rhizobia represents an effective strategy for fully realizing the biological nitrogen fixation potential of legumes, reducing dependency on chemical fertilizers, and promoting the sustainable development of animal husbandry.

4.2. Effect of Inoculation on Nodule Number and Mycorrhizal Colonization Rate of Alfalfa

Root nodules are specialized organs formed following the establishment of an obligate symbiotic relationship between rhizobia and leguminous plants, and they represent the exclusive sites for biological nitrogen fixation. Within physiological thresholds, nod-

ule number is positively correlated with the nitrogen fixation potential of the plant; a higher nodule count generally reflects a more robust symbiotic nitrogen fixation capacity, thereby substantially enhancing the nitrogen nutritional status of the host [26]. Research has demonstrated that nodule formation is a highly coordinated molecular interaction process involving the recognition and exchange of specific signaling molecules. Successful nodulation signifies the establishment of an effective mutualistic symbiosis between the plant and rhizobia [27]. Mycorrhizal colonization rate is a key quantitative indicator for assessing the extent of symbiotic establishment between arbuscular mycorrhizal fungi and host plants [28]. It exhibits a significant positive correlation with the plant's efficiency in acquiring relatively immobile nutrients such as phosphorus. A higher mycorrhizal colonization rate typically denotes a more extensive extraradical hyphal network. These hyphae can extend into soil microsites that are inaccessible to roots, thereby greatly expanding the plant's capacity to explore the soil matrix and facilitating the mobilization and uptake of mineral elements including phosphorus, zinc, and copper [29].

The results of the present study indicate that co-inoculation with AMF and rhizobia exerted a significant synergistic promotional effect on the symbiotic nitrogen fixation system of alfalfa. All three AMF species successfully colonized alfalfa roots, and co-inoculation of AMF with rhizobia yielded the most pronounced enhancement in both nodule number and colonization rate. This finding is consistent with the conclusions reported by Ben Laouane et al. [30]. The emergence of this synergistic effect stems from the intimate interaction between the two symbionts at both signaling and nutritional levels. At the signaling level, the hyphal network established by AMF can facilitate the long-distance transport of host recognition signals such as flavonoids, constituting a “navigation system” that guides the directional migration of rhizobia toward the host root system, thereby markedly improving early nodule formation [31]. At the nutritional level, under conditions of nitrogen excess, co-inoculation—compared with single inoculation or non-inoculation—has been shown to significantly promote the growth of black locust (*Robinia pseudoacacia*) in cadmium-contaminated soil and to enhance both mycorrhizal colonization rate and nodule number [32]. This is because AMF, through their extensive extraradical hyphal networks, effectively expand the root absorptive area within the soil and significantly improve host plant acquisition of key nutrients such as phosphorus and zinc. Phosphorus, as a core element in cell division and energy metabolism, is an essential prerequisite for nodule formation and the exertion of nitrogen fixation capacity [33]. Therefore, AMF indirectly promote rhizobial infection and colonization by enhancing root vitality and ensuring an adequate phosphorus supply, ultimately leading to a significant increase in nodule number. Concurrently, the nitrogen supplied by rhizobia through biological nitrogen fixation optimizes the plant's carbon–nitrogen metabolic balance, enabling the host to allocate more abundant photosynthate carbon to the AMF symbiont, thereby further enhancing mycorrhizal colonization rate and promoting the expansion of the hyphal network [34].

Evidently, AMF and rhizobia do not function independently within leguminous plants; rather, they constitute a tightly integrated functional consortium. Through bidirectional nutrient exchange and signaling cross-talk, this consortium establishes a positive feedback loop that jointly drives the functional optimization of the dual “mycorrhiza-nodule” symbiotic system [35]. By examining this interaction from an integrated “signaling–nutrition” perspective, the present study deepens our understanding of the synergistic mechanisms between AMF and rhizobia and provides a robust theoretical foundation for the development of ecological agricultural technologies based on multifunctional microbial co-inoculation.

4.3. Effect of Inoculation on Nutritional Quality and Nitrogen and Phosphorus Contents of Alfalfa

The nutritional quality of alfalfa is a critical determinant of its feeding value and economic worth [36]. Nitrogen (N), phosphorus (P), neutral detergent fiber (NDF), and acid detergent fiber (ADF) are core indicators for evaluating its nutritional value and digestive characteristics. Nitrogen content in alfalfa plants serves as the most direct measure of protein nutritional status, as protein constitutes the material basis for rumen microbial proliferation and the physiological requirements of the host animal [37]. Phosphorus is a key constituent of nucleic acids, ATP, and phospholipids, participating in energy metabolism, genetic information transfer, and cell membrane construction [38]. The results of the present study demonstrate that the interaction between arbuscular mycorrhizal fungi (AMF) and rhizobia significantly optimizes alfalfa nutritional quality. All inoculation treatments markedly increased plant nitrogen and phosphorus contents while concurrently reducing NDF and ADF concentrations. Among the treatments, the co-inoculation treatment (*Ce* × *Sm*) exhibited the most favorable outcome. Previous research has indicated that co-inoculation with AMF and rhizobia enhances the capacity of alfalfa to acquire nitrogen (N) and phosphorus (P), and these microorganisms are key partners in plant–microbe interactions [3]. AMF can fulfill a substantial portion of host phosphorus requirements via the mycorrhizal pathway while receiving a significant allocation of photosynthetic carbon from the host, thereby driving carbohydrate metabolism and redistribution [39]. Under conditions of adequate nitrogen and phosphorus supply, plants may adjust their carbon metabolism patterns, allocating more photosynthate carbon toward the synthesis of non-fibrous components such as proteins and soluble sugars, while relatively reducing the proportion directed toward structural cell wall constituents (e.g., cellulose, hemicellulose, and lignin). This metabolic shift consequently leads to decreased ADF and NDF contents [40]. Fiber content is a key index for evaluating forage digestibility. Reductions in NDF and ADF contents signify a decrease in cell wall components and an increase in the proportion of digestible organic matter, thereby substantially improving forage quality and animal intake efficiency. This alteration may be associated with AMF-mediated enhancement of host phosphorus nutrition, promotion of carbohydrate metabolism, and accumulation of cellular contents [41]. The present study found that all inoculation treatments reduced alfalfa NDF and ADF contents to varying degrees, with co-inoculation exhibiting superior efficacy. This outcome is primarily attributable to the mutually beneficial relationship established between AMF and rhizobia, which synergistically promotes biological nitrogen fixation and nutrient acquisition [29]. The results of this study further corroborate this mechanism: AMF hyphae and rhizobial nitrogen fixation jointly enhance nitrogen uptake, while the increase in phosphorus content relies predominantly on the capacity of AMF to expand the absorption zone and mobilize sparingly soluble soil phosphorus. The interaction between AMF and rhizobia reduces fiber content and elevates nitrogen and phosphorus nutrient levels [42], thereby significantly optimizing the nutritional value and feeding quality of alfalfa. This provides an effective microbial management strategy for environmentally sustainable forage production.

4.4. Effect of Inoculation on Photosynthetic Characteristics of Alfalfa

Photosynthesis is a fundamental determinant of crop productivity. As leaves serve as the primary site for photosynthetic activity, effectively enhancing their photosynthetic rate facilitates the accumulation and partitioning of photoassimilates, thereby promoting crop yield improvement [43]. Photosynthetic parameters such as net photosynthetic rate, stomatal conductance, transpiration rate, and intercellular CO₂ concentration directly reflect the variation characteristics of plant photosynthesis. Net photosynthetic rate directly governs the biomass accumulation capacity and growth status of plants, while transpiration

rate reflects the intensity of water loss through stomata; together, these two processes constitute the core of gas exchange between the plant and the environment [44]. The key variable linking these two processes is stomatal conductance, which directly regulates the diffusive flux of CO₂ and water vapor between the leaf and the atmosphere, thereby determining both the substrate supply for photosynthesis and the water loss associated with transpiration [45].

The results of the present study demonstrate that, compared with the CK group, the dual inoculation treatments significantly enhanced net photosynthetic rate, transpiration rate, and stomatal conductance ($p < 0.05$), exhibiting superior overall effects. Concurrently, the dual inoculation treatments significantly reduced the intercellular CO₂ concentration of alfalfa ($p < 0.05$). A plausible explanation for these findings is that AMF, through their extensive extraradical hyphal network, substantially expand the spatial range and mobilization capacity of the root system for soil phosphorus acquisition. Phosphorus is a core element constituting ATP, NADPH, and intermediates of photosynthetic carbon metabolism [46]. An adequate phosphorus supply ensures the efficient progression of photophosphorylation, thereby providing ample energy for carbon assimilation reactions. Simultaneously, rhizobia supply the plant with abundant nitrogen through biological nitrogen fixation; nitrogen is an essential raw material for the synthesis of chlorophyll molecules and reaction center proteins of the photosystems [47]. Consequently, co-inoculation synergistically promotes a marked increase in chlorophyll content, elevates stomatal conductance, and reduces intercellular CO₂ concentration as a result of enhanced CO₂ assimilation. This leads to an increase in the availability of substrates for photosynthesis and a corresponding elevation in net photosynthetic rate, ultimately enhancing overall photosynthetic performance.

4.5. Effect of Inoculation on Nitrogen Partitioning and Protein Fractions of Alfalfa

Nitrogenous compounds constitute 40–50% of the dry matter of protoplasm and are integral components of the amino acids that form proteins [3]. Total nitrogen in plant tissues can be further fractionated into distinct functional components based on solubility and chemical structure; this fractionation process provides profound insights into the nutritional metabolic status and utilization value of nitrogen. The results of the present study demonstrate that inoculation with AMF and rhizobia significantly increased the contents of crude protein (CP), soluble protein (SOLP), and non-protein nitrogen (NPN) in alfalfa across the two experimental years ($p < 0.05$). These findings indicate that the tripartite symbiotic system comprising AMF, rhizobia, and alfalfa optimizes host nitrogen metabolism and partitioning through synergistic interactions. Previous research has shown that the interaction between AMF and rhizobia significantly enhances leaf nitrogen content and nitrogen assimilation capacity in white clover, suggesting that dual inoculation exerts a synergistic effect on nitrogen assimilation in leguminous plants [48], which is consistent with the results of the present study. Concurrently, dual inoculation led to synchronous increases in both soluble protein and non-protein nitrogen contents. Soluble protein serves as a critical indicator of plant physiological status. The functional core of soluble protein comprises key enzymes involved in photosynthetic carbon assimilation (e.g., Rubisco) [49] and their associated structural components, key enzymes of the GS/GOGAT cycle responsible for nitrogen assimilation [50], as well as various defense-related proteins including antioxidant enzymes and ferredoxin [51]. A coordinated increase in protein fractions typically signifies that the plant is in a vigorous growth state characterized by active nitrogen metabolism, efficient photosynthesis, and robust stress tolerance. Non-protein nitrogen primarily includes free amino acids, amides (e.g., glutamine, asparagine), and nucleic acids, representing both a precursor pool for protein synthesis and the principal form of nitrogen transport within the plant [52]. In the present study, dual inoculation enhanced nitrogen

fixation and uptake, thereby optimizing nitrogen transport and transformation processes within the plant. This improvement is largely attributable to the upregulation of numerous nitrogen metabolism-associated proteins during the symbiotic nitrogen fixation process [53]. Neutral detergent insoluble protein (NDIP) and acid detergent insoluble protein (ADIP) predominantly represent cell wall-bound proteins or highly denatured proteins, which exhibit extremely low digestibility and constitute nutritionally undesirable components in forage [11]. The results of the present study suggest that the interaction between AMF and rhizobia promotes the preferential allocation of nitrogen toward the synthesis of highly active functional proteins and metabolic intermediates, rather than its deposition as structurally recalcitrant proteins of limited availability. This preferential allocation enhances the proportion of total nitrogen that is readily utilizable by ruminants, thereby improving the biological value of the forage—an attribute of paramount importance for the quality of alfalfa as a premium forage crop. These findings provide a robust theoretical foundation for the utilization of microbial symbionts in the cultivation of high-quality forage and the development of sustainable agricultural technologies.

As a leguminous forage, the protein content and composition of alfalfa directly influence feed quality and nutritional value. AMF and rhizobia, two key groups of soil microorganisms, establish mutualistic symbiotic associations with alfalfa and regulate its nutrient acquisition and metabolic processes [3]. The Cornell Net Carbohydrate and Protein System (CNCPS) protein fractionation scheme provides an intuitive representation of the utilization patterns of individual protein fractions by ruminants. True protein fractions are extensively degraded in the rumen, contributing to the nitrogen supply for rumen microorganisms and being incorporated into carbon skeletons [54]. The results of the present study demonstrate that, compared with the CK group, inoculation treatments—particularly dual inoculation—increased the contents of rapidly degradable true protein (PB₁) and intermediately degradable true protein (PB₂) in alfalfa during the 2024–2025 period, while concurrently reducing the contents of non-protein nitrogen (PA) and bound protein (PC). The dual inoculation treatments exhibited a synergistic effect superior to that of single inoculation, indicating complementary mechanisms between AMF and rhizobia in promoting nutrient uptake and metabolism in alfalfa. Co-inoculation with AMF and rhizobia represents an effective biological strategy for enhancing alfalfa protein quality and holds considerable potential for practical application. This approach provides a viable biotechnological pathway for developing high-quality protein forage and improving the production efficiency of animal husbandry.

5. Conclusions

AMF and rhizobia exhibit synergistic promotional effects on alfalfa. Inoculation with AMF effectively colonized alfalfa roots and established functional symbiotic associations. Co-inoculation with AMF and rhizobia (specifically *Fm* × *Sm* and *Ce* × *Sm*) synergistically enhanced alfalfa growth and increased biomass accumulation by optimizing root system architecture, augmenting photosynthetic performance, and modulating nitrogen metabolism. However, these findings are based on controlled pot experiments and a two-year time-frame, which may limit their direct applicability to field conditions. Despite this, the dual inoculation strategy shows promise as a biofertilizer approach to reduce chemical fertilizer dependence in alfalfa production. Future research should focus on field validation, long-term sustainability assessment, and elucidation of the underlying molecular mechanisms governing the observed synergies.

Author Contributions: Conceptualization, X.L. and Q.Z.; methodology, X.L. and Q.Z.; validation, X.L.; formal analysis, X.L.; investigation, X.L.; resources, Q.Z.; data curation, X.L.; writing—original draft preparation, X.L.; writing—review and editing, Q.Z.; visualization, X.L.; supervision, Q.Z.;

project administration, Q.Z.; funding acquisition, Q.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Xinjiang Uygur Autonomous Region Key R&D Task Special Project (NO. 2023B02031), Science and Technology Program of XPCC (2026DB001) and National Natural Science Foundation of China (NO. 32260347).

Data Availability Statement: The raw experimental data supporting the conclusions of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Abbreviations

The following abbreviations are used in this manuscript:

ADF	acid detergent fiber
AMF	arbuscular mycorrhizal fungi
CNCPS	Cornell Net Carbohydrate and Protein System
CP	crude protein
<i>Fm</i>	<i>Funneliformis mosseae</i>
Gs	stomatal conductance
<i>Gv</i>	<i>Glomus versiforme</i>
LUE	light use efficiency
N	nitrogen
NDF	neutral detergent fiber
NPN	non-protein nitrogen
P	phosphorus
PA	non-protein nitrogen fraction
PB ₁	rapidly degradable true protein
PB ₂	intermediately degradable true protein
PB ₃	slowly degradable true protein
PC	bound protein
Pn	net photosynthetic rate
SOLP	soluble protein
<i>Sm</i>	<i>Sinorhizobium meliloti</i>
Tr	transpiration rate
WUE	water use efficiency
<i>Ce</i>	<i>Claroideoglomus etunicatum</i>
Ci	intercellular CO ₂ concentration

References

- Li, F.; Li, Y.Z. Effect of alfalfa cultivars and ages on the occurrence of verticillium wilt caused by verticillium alfalfae. *Plant Dis.* **2021**, *106*, 496–503. [\[CrossRef\]](#) [\[PubMed\]](#)
- An, X.X.; Liu, J.Y.; Liu, X.S.; Ma, C.H.; Zhang, Q.B. Optimizing phosphorus application rate and the mixed inoculation of arbuscular mycorrhizal fungi and phosphate-solubilizing bacteria can improve the phosphatase activity and organic acid content in alfalfa soil. *Sustainability* **2022**, *14*, 11342. [\[CrossRef\]](#)
- Sun, Y.H.; Kang, X.L.; Li, R.X.; Cui, Y.N.; Long, M.X.; He, S.B. Arbuscular mycorrhizal fungi and rhizobia synergistically enhance alfalfa productivity and alleviate nutrient limitations, particularly under low-input conditions. *Plant Physiol. Biochem.* **2025**, *229*, 110353. [\[CrossRef\]](#) [\[PubMed\]](#)
- Bauer, J.T.; Blumenthal, N.; Miller, A.J.; Ferguson, J.K.; Reynolds, H.L. Effects of between-site variation in soil microbial communities and plant-soil feedbacks on the productivity and composition of plant communities. *J. Appl. Ecol.* **2017**, *54*, 1028–1039. [\[CrossRef\]](#)
- Liu, X.Q.; Xie, M.M.; Hashem, A.; Abd-Allah, E.F.; Wu, Q.S. Arbuscular mycorrhizal fungi and rhizobia synergistically promote root colonization, plant growth, and nitrogen acquisition. *Plant Growth Regul.* **2023**, *100*, 691–701. [\[CrossRef\]](#)

6. Qiao, X.; Bei, S.K.; Li, H.G.; Christie, P.; Zhang, F.S.; Zhang, J.L. Arbuscular mycorrhizal fungi contribute to overyielding by enhancing crop biomass while suppressing weed biomass in intercropping systems. *Plant Soil* **2016**, *406*, 173–185. [[CrossRef](#)]
7. Smith, S.E.; Smith, F.A. Roles of arbuscular mycorrhizas in plant nutrition and growth: New paradigms from cellular to ecosystem scales. *Annu. Rev. Plant Biol.* **2011**, *62*, 227–250. [[CrossRef](#)] [[PubMed](#)]
8. Thilagar, G.; Bagyaraj, D.J.; Rao, M.S. Selected microbial consortia developed for chilly reduces application of chemical fertilizers by 50% under field conditions. *Sci. Hortic.* **2016**, *198*, 27–35. [[CrossRef](#)]
9. Msimbira, L.A.; Jaiswal, S.K.; Dakora, F.D. Identification and characterization of phages parasitic on bradyrhizobia nodulating groundnut (*Arachis hypogaea* L.) in south africa. *Appl. Soil Ecol.* **2016**, *108*, 334–340. [[CrossRef](#)] [[PubMed](#)]
10. Ferreira, N.S.; Matos, G.F.; Meneses, C.H.S.G.; Reis, V.M.; Rouws, J.R.C.; Schwab, S.; Baldani, J.I.; Rouws, L.F.M. Interaction of phytohormone-producing rhizobia with sugarcane mini-setts and their effect on plant development. *Plant Soil* **2020**, *451*, 221–238. [[CrossRef](#)]
11. Gorgia, P.; Tsikou, D. Tripartite symbiosis between legumes, arbuscular mycorrhizal fungi and nitrogen fixing rhizobia: Interactions and regulation. *Plant Cell Environ.* **2025**, *49*, 3789–3807. [[CrossRef](#)] [[PubMed](#)]
12. Zhao, J.T.; Huang, R.Z.; Wang, X.Z.; Ma, C.H.; Li, M.; Zhang, Q.B. Effects of combined nitrogen and phosphorus application on protein fractions and nonstructural carbohydrate of alfalfa. *Front. Plant Sci.* **2023**, *14*, 1124664. [[CrossRef](#)] [[PubMed](#)]
13. Phillips, J.M.; Hayman, D.S. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Trans. Br. Mycol. Soc.* **1970**, *55*, 158–161. [[CrossRef](#)]
14. Kjeldahl, J. Neue Methode zur Bestimmung des Stickstoffs in organischen Körpern. *Z. Anal. Chem.* **1883**, *22*, 366–382. [[CrossRef](#)]
15. Murphy, J.; Riley, J.P. A modified single solution method for the determination of phosphate in natural waters. *Anal. Chim. Acta* **1962**, *27*, 31–36. [[CrossRef](#)]
16. Van Soest, P.J.; Robertson, J.B.; Lewis, B.A. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* **1991**, *74*, 3583–3597. [[CrossRef](#)] [[PubMed](#)]
17. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [[CrossRef](#)] [[PubMed](#)]
18. Licitra, G.; Hernandez, T.M.; Van Soest, P.J. Standardization of procedures for nitrogen fractionation of ruminant feeds. *Anim. Feed Sci. Technol.* **1996**, *57*, 347–358. [[CrossRef](#)]
19. Sniffen, C.J.; O'Connor, J.D.; Van Soest, P.J.; Fox, D.G.; Russell, J.B. A net carbohydrate and protein system for evaluating cattle diets: II. Carbohydrate and protein availability. *J. Anim. Sci.* **1992**, *70*, 3562–3577. [[CrossRef](#)] [[PubMed](#)]
20. Tsikou, D.; Nikolaou, C.N.; Tsiknia, M.; Papadopoulou, K.K.; Ehaliotis, C. Interplay between rhizobial nodulation and arbuscular mycorrhizal fungal colonization in lotus japonicus roots. *J. Appl. Microbiol.* **2023**, *134*, 1xac010. [[CrossRef](#)] [[PubMed](#)]
21. Gough, E.C.; Owen, K.J.; Zwart, R.S.; Thompson, J.P. Arbuscular mycorrhizal fungi acted synergistically with bradyrhizobium sp. to improve nodulation, nitrogen fixation, plant growth and seed yield of mung bean (*Vigna radiata*) but increased the population density of the root-lesion nematode *Pratylenchus thornei*. *Plant Soil* **2021**, *465*, 431–452. [[CrossRef](#)]
22. Concha, C.; Doerner, P. The impact of the rhizobia–legume symbiosis on host root system architecture. *J. Exp. Bot.* **2020**, *71*, 3902–3921. [[CrossRef](#)] [[PubMed](#)]
23. Garcia, J.; Schmidt, J.E.; Gidekel, M.; Gaudin, A.C.M. Impact of an antarctic rhizobacterium on root traits and productivity of soybean (*Glycine max* L.). *J. Plant Nutr.* **2021**, *44*, 1818–1825. [[CrossRef](#)]
24. Ramírez-Flores, M.R.; Bello-Bello, E.; Rellán-Álvarez, R.; Sawers, R.J.H.; Olalde-Portugal, V. Inoculation with the mycorrhizal fungus rhizophagus irregularis modulates the relationship between root growth and nutrient content in maize (*Zea mays* ssp. *mays* L.). *Plant Direct* **2019**, *3*, e00192. [[CrossRef](#)] [[PubMed](#)]
25. Wang, W.X.; Shi, J.C.; Xie, Q.J.; Jiang, Y.N.; Yu, N.; Wang, E.T. Nutrient exchange and regulation in arbuscular mycorrhizal symbiosis. *Mol. Plant.* **2017**, *10*, 1147–1158. [[CrossRef](#)] [[PubMed](#)]
26. Zhang, W.Q.; Liu, W.Y.; Wang, K.; Cheng, H.P.; Bai, X.L.; Zhang, J.H.; Wei, G.H.; Chen, J. Persulfidation of host NADPH oxidase RbohB by rhizobial 3-mercaptopyruvate sulfurtransferase maintains redox homeostasis and promotes symbiotic nodulation in soybean. *Mol. Plant.* **2025**, *18*, 1843–1863. [[CrossRef](#)] [[PubMed](#)]
27. Zanetti, M.E. FER meets the Nod factor pathway. *Nat. Plants* **2023**, *9*, 1581–1582. [[CrossRef](#)] [[PubMed](#)]
28. Cheng, H.Q.; Giri, B.; Wu, Q.S.; Zou, Y.N.; Kuča, K. Arbuscular mycorrhizal fungi mitigate drought stress in citrus by modulating root microenvironment. *Arch. Agron. Soil Sci.* **2022**, *68*, 1217–1228. [[CrossRef](#)]
29. Jiang, Z.; Thakur, M.P.; Liu, R.Q.; Zhou, G.Y.; Zhou, L.Y.; Fu, Y.L.; Zhang, P.P.; He, Y.H.; Shao, J.J.; Gao, J.; et al. Soil P availability and mycorrhizal type determine root exudation in sub-tropical forests. *Soil Biol. Biochem.* **2022**, *171*, 108722. [[CrossRef](#)]
30. Ben Laouane, R.; Meddich, A.; Bechtaoui, N.; Oufdou, K.; Wahbi, S. Effects of arbuscular mycorrhizal fungi and rhizobia symbiosis on the tolerance of medicago sativa to salt stress. *Gesunde Pflanz.* **2019**, *71*, 135–146. [[CrossRef](#)]
31. He, J.; Van Dingenen, J.; Goormachtig, S.; Calon-Salmon, M.; Declerck, S. Legume-specific recruitment of rhizobia by hyphae of arbuscular mycorrhizal fungi. *ISME J.* **2025**, *19*, wrf100. [[CrossRef](#)] [[PubMed](#)]

32. Zou, D.C.; Du, H.X.; Zhang, F.S.; Gao, L.; Xiong, B.C.; Guo, P.; Ma, M. Responses of robinia pseudoacacia co-inoculated with rhizobia and arbuscular mycorrhizal fungi to cadmium under nitrogen excess condition. *Plant Soil* **2025**, *508*, 909–924. [[CrossRef](#)]
33. Huang, Y.M.; Zhai, L.M.; Zhou, Y.; Lv, J.H.; Liu, Y.; Wu, T.; Zhang, X.Z.; Han, Z.H.; Wang, Y. MdPHR2 and MdARF6-4 synergistically regulate arbuscular mycorrhizal symbiosis and the transcription of MdPHT1;13, enhancing phosphorus uptake in apple rootstocks. *Plant J.* **2025**, *121*, e70070. [[CrossRef](#)] [[PubMed](#)]
34. Tschitschko, B.; Esti, M.; Philippi, M.; Kidane, A.T.; Littmann, S.; Kitzinger, K.; Speth, D.R.; Li, S.; Kraberg, A.; Tienken, D.; et al. Rhizobia–diatom symbiosis fixes missing nitrogen in the ocean. *Nature* **2024**, *630*, 899–904. [[CrossRef](#)] [[PubMed](#)]
35. Hnini, M.; Oubohssaine, M.; Rabeh, K.; Idriss, H.R. Mechanisms of bacterial and fungal mediation in plant health and their ecological and agricultural significance. *Discov. Sustain.* **2025**, *6*, 1055. [[CrossRef](#)]
36. Ma, J.X.; Huangfu, W.K.; Yang, X.; Xu, J.Y.; Zhang, Y.; Wang, Z.C.; Zhu, X.Y.; Wang, C.Z.; Shi, Y.H.; Cui, Y.L. “King of the forage”—Alfalfa supplementation improves growth, reproductive performance, health condition and meat quality of pigs. *Front. Vet. Sci.* **2022**, *9*, 1025942. [[CrossRef](#)] [[PubMed](#)]
37. Gao, H.; Yang, D.H.; Yang, L.; Han, S.Y.; Liu, G.Y.; Tang, L.; Chen, J.X.; Wang, D.; Guo, C.G. Co-inoculation with sinorhizobium meliloti and enterobacter ludwigii improves the yield, nodulation, and quality of alfalfa (*Medicago sativa* L.) under saline-alkali environments. *Ind. Crops Prod.* **2023**, *199*, 116818. [[CrossRef](#)]
38. Cao, S.M.; Li, T.T.; Shao, Y.X.; Zhang, L.Y.; Lu, L.; Zhang, R.J.; Hou, S.S.; Luo, X.G.; Liao, X.D. Regulation of bone phosphorus retention and bone development possibly by related hormones and local bone-derived regulators in broiler chicks. *J. Anim. Sci. Biotechnol.* **2021**, *12*, 88. [[CrossRef](#)] [[PubMed](#)]
39. Li, Y.W.; Chen, H.W.; Gu, L.; Wu, J.W.; Zheng, X.T.; Fan, Z.L.; Pan, D.J.; Li, J.T.; Shu, W.S.; Rosendahl, S.; et al. Domestication of rice may have changed its arbuscular mycorrhizal properties by modifying phosphorus nutrition-related traits and decreasing symbiotic compatibility. *New Phytol.* **2024**, *243*, 1554–1570. [[CrossRef](#)] [[PubMed](#)]
40. Cao, L.N.; Zhang, S.; Cao, J.Y.; Chang, R.H.; Qu, C.P.; Li, C.M.; Yan, J.X.; Quan, X.K.; Xu, Z.R.; Liu, G.J. Nitrogen modifies wood composition in poplar seedlings by regulating carbon and nitrogen metabolism. *Ind. Crops Prod.* **2024**, *219*, 119118. [[CrossRef](#)]
41. Sheteiwy, M.S.; El-Sawah, A.M.; Alyafei, M.; Sulieman, S.; Ali, B.; Joško, I.; Feculak, M.; El-Keblawy, A.; Ulhassan, Z.; Hamoud, Y.A.; et al. Sugar metabolism and metabolite profiling of maize grains are largely improved by the combination of arbuscular mycorrhizal fungi and humic acid. *Ind. Crops Prod.* **2025**, *234*, 121546. [[CrossRef](#)]
42. Mpongwana, S.; Manyevere, A.; Mupangwa, J.; Mpendulo, C.T.; Mashamaite, C.V. Improving soil fertility through dual inoculation with arbuscular mycorrhizal fungi and rhizobium on a eutric cambisol cultivated with forage legumes in a semi-arid region. *Heliyon* **2024**, *10*, e24817. [[CrossRef](#)] [[PubMed](#)]
43. Amthor, J.S.; Bar-Even, A.; Hanson, A.D.; Millar, A.H.; Stitt, M.; Sweetlove, L.J.; Tyerman, S.D. Engineering strategies to boost crop productivity by cutting respiratory carbon loss. *Plant Cell* **2019**, *31*, 297–314. [[CrossRef](#)] [[PubMed](#)]
44. Hatfield, J.L.; Dold, C. Water-use efficiency: Advances and challenges in a changing climate. *Front. Plant Sci.* **2019**, *10*, 103. [[CrossRef](#)] [[PubMed](#)]
45. Gallagher, B.K.; Gergeoura, S.; Fraser, D.J. Effects of climate on salmonid productivity: A global meta-analysis across freshwater ecosystems. *Glob. Change Biol.* **2022**, *28*, 7250–7269. [[CrossRef](#)] [[PubMed](#)]
46. Zhang, Y.; Yang, Y.; Ma, Y.C.; Wang, D.F.; Wang, X.Q.; Zhang, L.D.; Meng, L.L.; Xu, K.D.; Li, X.L.; Shanguan, X.X.; et al. A mycorrhiza-induced phosphate transporter taPT31-7a regulating inorganic phosphate uptake, arbuscular mycorrhiza symbiosis, and plant growth in wheat. *J. Agric. Food Chem.* **2025**, *73*, 16262–16275. [[CrossRef](#)] [[PubMed](#)]
47. Gamalero, E.; Glick, B.R. Rhizobia and non-rhizobial nodule bacteria with ACC deaminase increase both nodulation and stress resistance. *Front. Microbiol.* **2025**, *16*, 1662592. [[CrossRef](#)] [[PubMed](#)]
48. Xie, M.M.; Chen, S.M.; Zou, Y.N.; Srivastava, A.K.; Rahman, M.M.; Wu, Q.S.; Kuča, K. Effects of rhizophagus intraradices and rhizobium trifolii on growth and N assimilation of white clover. *Plant Growth Regul.* **2021**, *93*, 311–318. [[CrossRef](#)]
49. Shimakawa, G.; Demulder, M.; Flori, S.; Kawamoto, A.; Tsuji, Y.; Nawaly, H.; Tanaka, A.; Tohda, R.; Ota, T.; Matsui, H.; et al. Diatom pyrenoids are encased in a protein shell that enables efficient CO₂ fixation. *Cell* **2024**, *187*, 5919–5934. [[CrossRef](#)] [[PubMed](#)]
50. Yu, B.Y.; Zhou, Y.H.; Shi, Y.F.; Wang, S.S.; Pang, Q.; Yang, C.; Roitsch, T.; Hu, W.M.; Wang, Y.Z.; Liu, F. Arabidopsis Vacuolar protein sorting 9a (VPS9a) is required for glutamine synthetase/glutamate synthase (GS/GOGAT) cycle and autophagy under nutrient starvation. *Plant J.* **2025**, *123*, e70289. [[CrossRef](#)] [[PubMed](#)]
51. Mao, X.; Yang, H.B.; Ji, M.M.; Xu, P.L.; Fan, G.Q. Comprehensive identification, stress responses and protein-protein interactions of the ferredoxin gene family in the fast-growing tree species Paulownia fortunei. *BMC Plant Biol.* **2025**, *25*, 975. [[CrossRef](#)] [[PubMed](#)]
52. Tarakhovskaya, E.; Gulk, E.; Zamyatkina, E. Non-proteinogenic amino acids from marine macroalgae: Diversity, functional roles, and biological activity. *Phycologia* **2025**, *64*, 366–388. [[CrossRef](#)]

53. Lyu, X.C.; Sun, C.Y.; Zhang, J.; Wang, C.; Zhao, S.H.; Ma, C.M.; Li, S.; Li, H.Y.; Gong, Z.P.; Yan, C. Integrated proteomics and metabolomics analysis of nitrogen system regulation on soybean plant nodulation and nitrogen fixation. *Int. J. Mol. Sci.* **2022**, *23*, 2545. [[CrossRef](#)] [[PubMed](#)]
54. Shakeri, P.; Riasi, A.; Tahmasbi, R. The effect of pistachio by-product extracts treatment in protecting soybean meal and canola meal protein from rumen microbial degradation. *J. Sci. Food Agric.* **2020**, *100*, 5222–5229. [[CrossRef](#)] [[PubMed](#)]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.