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Investigation of Nitrogen Fixation Efficiency in Diverse Alfalfa Varieties Utilizing *Sinorhizobium meliloti* LL2

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Abstract: To investigate the precise and efficient symbiosis between *Sinorhizobium meliloti* LL2 and different alfalfa varieties, we conducted experiments using eight alfalfa varieties along with the *S. meliloti* LL2. Our objective was to identify highly effective symbiotic combinations by analyzing differences in nodulation, nitrogen fixation, and biomass accumulation. The results revealed that Gannong NO.9 had higher values for single effective root nodule weight (1.30 mg) and the number of infected cells in root nodules (2795) compared to other varieties ($p < 0.05$). Additionally, Gannong NO.9 exhibited the highest nitrogenase activity (0.91 $\mu mol \cdot g^{-1} \cdot h^{-1}$), nitrogen fixation percentage (67.16%), and amount of nitrogen fixation (18.80 mg/pot). Moreover, there was a significant 26.50% increase in aboveground tissue nitrogen accumulation compared to the control check (CK) ($p < 0.05$). Furthermore, underground tissue showed excellent values for nitrogen accumulation (35.68 mg/plant) and crude protein content (17.75%) when compared with other treatments. The growth of plants was demonstrated by the combined impact of nodulation and nitrogen fixation. The distribution of biomass after nitrogen fixation was compared to the control group ($p < 0.05$) to investigate accumulation. The eight combinations of symbiotic nitrogen fixation (SNF) were classified into six distinct types based on their significantly different biomass growth rates compared to CK. ① Aboveground accumulation type: Gannong NO.9 (there was a 24.31% increase in aboveground dry weight); ② aboveground and underground accumulation type: Qingshui (the aboveground dry weight increased by 135.94%, while the underground dry weight grew by 35.26%); ③ aboveground accumulation, underground depletion type: Gannong NO.5 ( ); ④ zero-growth type (there was no significant difference in dry weights, both above and below ground, compared to CK): WL168HQ, WL319HQ and Longzhong; ⑤ aboveground and underground depletion type: WL298HQ (the aboveground dry weight decreased by 29.29%, while the underground dry weight decreased by 20.23%); ⑥ underground depletion type: Gannong NO.3 (the underground dry weight showed a decrease of 34.49%); no type with aboveground consumption and underground accumulation was found. The study clarified the optimal combination of LL2 and Gannong NO.9, finding that biomass accumulation after symbiotic nitrogen fixation is variety-dependent.

Keywords: alfalfa; nitrogen accumulation; rhizobia; symbiotic nitrogen fixation system; variety effect

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1. Introduction

Over the years, extensive research has been conducted on the correlation between mineral elements and crop growth and development, particularly nitrogen. Nitrogen is a fundamental mineral element essential for plant nutrition, playing a pivotal role not only in plant structure but also in regulating various physiological and metabolic processes [1]. Notably, nitrogen exhibits high mobility within soil particles, posing challenges in controlling its distribution while presenting multiple pathways for loss and multidirectional

movement towards different destinations [2]. The excessive use of nitrogen fertilizers since the 1990s has resulted in a range of environmental issues, including soil, water, and air pollution, as well as ecosystem-wide nitrogen enrichment [3,4]. Consequently, enhancing biological nitrogen fixation becomes imperative.

Alfalfa (*Medicago sativa* L.), commonly referred to as the king of forage grasses, is a perennial herbaceous plant renowned for its exceptional attributes, including high yield, superior quality, strong resistance, and versatile applications. In China, alfalfa has a rich cultivation history that spans from its initial introduction to its widespread adoption over the years. Its perennial growth habit and characteristics of growth root nodules make significant contributions to soil improvement, conservation of soil and water resources, as well as environmental protection in today's fragile ecological landscape [5,6].

Rhizobia can establish a symbiotic relationship with alfalfa, forming root nodules to fix and convert atmospheric inorganic nitrogen into organic nitrogen, thereby providing essential nutrients for the plant. Rhizobia exhibit remarkable adaptability by thriving in soil and colonizing all parts of plants, effectively enhancing both biotic resistance, such as disease resistance, and abiotic resistance, such as salt tolerance of the host [7–10]. The symbiotic nitrogen fixation between legumes and rhizobia not only enhances soil fertility but also improves the ecological environment while simultaneously elevating yield and forage quality, such as crude protein content of legume crops [11]. Previous studies have demonstrated that inoculating alfalfa with rhizobia resulted in a significant increase of 38.7% in plant height, 44.2% in aboveground biomass, and an impressive 99.7% boost in underground biomass compared to its control check (CK). Moreover, the number, weight, and activity of root nodules exhibited substantial increments of 76.05%, 224%, and 34.57% respectively when compared to CK [12].

The symbiotic system of alfalfa and rhizobia demonstrates a robust host specificity, which plays a pivotal role in enhancing alfalfa yield. Inoculating alfalfa with compatible rhizobia is among the most effective approaches to augment symbiotic nitrogen fixation and achieve increased yield. Rhizobia strains exhibit selectivity towards different varieties of alfalfa, resulting in varying symbiotic effects when simultaneously inoculated with different varieties [13]. The symbiotic variations between rhizobia and different combinations of alfalfa varieties are determined by comprehensive studies, which have demonstrated the profound impact of alfalfa varieties, rhizobia strains, and their mutual compatibility. Remarkably higher yields are achieved from exceptional combinations of high-efficiency rhizobia and carefully selected alfalfa varieties compared to suboptimal pairings [14,15]. For example, research findings showed that Gannong NO.3 exhibited superior seedling growth when inoculated with strain 17581, while Longdong did not demonstrate similar advantages under identical conditions [16]. Therefore, it is crucial to screen for synergistic matches between specific varieties of alfalfa along with compatible rhizobia and promote their application in practical production to enhance both economic and ecological benefits within the alfalfa industry. Previous studies have indicated that *S. meliloti* LL2 significantly enhances growth in both Gannong NO.3 and Gannong NO.9 through positive symbiotic [17].

Currently, the primary methods for comprehensive plant trait evaluation include fuzzy membership function method, principal component analysis (PCA), cluster analysis and so on [18,19]. The membership function enables a more scientific and rational assessment of varieties by comprehensively considering multiple factors; for example, the heat treatment was conducted on various cultivars of alfalfa, and a fuzzy membership function was employed to screen for varieties exhibiting resistance or sensitivity to heat stress [20]. The fuzzy membership function, however, presents certain limitations: when dealing with high-dimensional data, it becomes crucial to consider the interrelationships and interactions among multiple variables, thereby rendering the calculations more intricate. PCA is a multivariate statistical technique that linearly transforms multiple variables to select fewer significant variables while retaining as much original information as possible [21]. The principal component analysis aims to reduce the dimensionality of high-dimensional data,

extract essential information and characteristic data, and then combine it with the fuzzy membership function method. This combination enhances the clarity of complex data structures and improves the accuracy and reliability of evaluations. Consequently, it has become a prominent methodology for investigating plant stress resistance as well as screening and evaluating variety germplasms; for instance, the evaluation and screening of peanut varieties for salt tolerance [22], the evaluation and screening of wild *Leymus sibiricus* L. germplasm resources under salt stress [23], and the investigation of cold tolerance among different varieties of alfalfa were conducted [24]. Although there have been studies on evaluating salt tolerance and cold tolerance in legumes, few studies have reported on the comprehensive evaluation of symbiotic nitrogen fixation effects in alfalfa inoculated with rhizobia using PCA and the affiliation function.

Therefore, based on the team's previous research findings, eight alfalfa varieties were selected for the purpose of classifying the growth-promoting effects resulting from symbiotic nitrogen fixation (SNF) between different alfalfa varieties and *S. meliloti* LL2. The comprehensive assessment of the SNF effect, incorporating PCA and fuzzy membership function, plays a pivotal role in guiding the application of precise nitrogen fixation technology by identifying and selecting optimal combinations for efficient symbiotic nitrogen fixation.

2. Materials and Methods

2.1. Alfalfa Varieties and Rhizobia Strains

Eight domestic and foreign alfalfa varieties widely planted in Northwest China were selected as the materials, with details shown in Table 1. The rhizobia strain utilized in the experiment was *Sinorhizobium meliloti* LL2 (LL2), which is currently preserved at the Key Laboratory of Grassland Ecosystem, Ministry of Education, Gansu Agricultural University.

Table 1. Test alfalfa varieties.

Code	Scientific Name	Habitat	Source
WL168	*M. sativa* WL168HQ	America	Beijing Rytway Ecotechnology Co., Ltd. (Beijing, China)
WL298	*M. sativa* WL298HQ		
WL319	*M. sativa* WL319HQ		
QS	*M. sativa* Qingshui	China	Prataculture of Gansu Agricultural University
LZ	*M. sativa* Longzhong		
G9	*M. sativa* Gannong No.9		
G3	*M. sativa* Gannong No.3		
G5	*M. sativa* Gannong No.5		

2.2. The Field Experiment

2.2.1. Site Overview

The experiment was conducted at the Forage Experimental Station of Anning District, Gansu Agricultural University, situated in northwest Lanzhou City (105°41′ E, 34°05′ N, mean altitude 1595 m). The region exhibits a temperate semi-arid continental climate with an average annual temperature of 9.7 °C, average annual precipitation of 451.6 mm, annual evaporation rate of 1664 mm, sunshine duration of 2446 h, and an annual frost-free period lasting for 210 days. The terrain is characterized by flat topography with homogeneous soil fertility consisting predominantly of loess soil.

2.2.2. Seed Preparation

A total of 400 healthy and uniform alfalfa seeds were carefully selected and placed in sterilized 50 mL Erlenmeyer flasks on a sterile operating table. Each variety's seeds underwent a series of meticulous disinfection procedures, including a 5 min soaking in iodophor followed by rinsing with sterile water. Subsequently, the seeds were soaked for one minute in sodium chloride–Tween solution (ST: a sodium chloride–Tween solution

consisting of 0.9% sterile sodium chloride solution and 0.5% Tween 80) to enhance the surface activity of the disinfectant before being rinsed again with sterile water [25]. Finally, any excess water was absorbed using sterile filter paper prior to storage for future use.

2.2.3. Seedling Cultivation

In this experiment, a single-factor randomized block design was employed using eight different varieties of alfalfa and LL2 as materials. Nutrient soil (purchased from GanSu LvNeng Agricultural science and Technology Co., Ltd., Wuwei, China) was subjected to high-temperature sterilization and loaded into flowerpots (diameter 18 cm, height 13 cm, volume 3.3 L). Each pot received 1.4 g of ammonium dihydrogen phosphate as the base fertilizer (N content per unit area < 5). The growth conditions and experimental parameters were meticulously controlled to account for variations within each block as experimental errors. Eight alfalfa varieties were placed in each plot, with different plots treated with rhizobia solution. Sterile water was used as CK, and four replicates were performed for each condition. Plant samples were collected at the squaring stage.

2.2.4. Rhizobia Inoculation

The rhizobia strains preserved at −80 °C were activated by inoculating them onto Tryptone yeast (T.Y.) medium followed by cultivation in a biochemical incubator set to maintain temperature at 28 °C for a duration of 24 h. A solitary colony was subsequently chosen and transferred into a triangular flask comprising 400 mL of yeast mannitol agar (YMA) liquid medium. The culture underwent agitation under conditions of constant shaking (180 rpm) and maintained temperature (28 °C) over an 18 h, with bacterial concentrations reaching up to 1.0×10^9 cfu·mL^{-1}. After this step, the bacteria were centrifuged at 25 °C and 10,000 rpm for 10 min to facilitate the removal of the supernatant layer through sedimentation forces generated during the centrifugation process. Finally, equal volumes of sterilized water were utilized for thorough rinsing of the bacteria with vigorous agitation facilitated by vortex oscillators [26].

On the 15th day of seedling growth (upon emergence of the first true leaf), rhizobia inoculation was conducted in the evening. Each flowerpot received 40 mL of rhizobia solution, while an equal volume of sterile water was used as a control. The bacteria were inoculated every four days for a total of three cycles, ensuring that the inoculation amount in each pot exceeded 1.0×10^9 cfu·mL^{-1}.

2.3. Indoor Sand Culture Experiment

The experiment was carried out in a sand culture chamber (light 28 °C/14 h, dark 20 °C/10 h, light intensity 260~350 mol·m^{-2}·s^{-1}, relative humidity 60%~70%). The seeds, prepared as described before, were sown to a culture cup with sterilized sand of 9 cm in diameter and 12 cm in height. Each cup was sown with 20 seeds, 15 seedlings were preserved, and each six cups were placed in a hydroponic box. Rhizobia solution was prepared and inoculated as described in Section 2.2.4. There were eight treatments in total, and each treatment was repeated six times. Before inoculation, nitrogen-free nutrient solution was added to each hydroponic basin once, and distilled water was added to supplement water.

When the first true leaf appeared, 200 mg ^{15}N (i.e.,200 mg ^{15}N·pot^{-1}) was accurately added to each culture cup. The nitrogen source utilized in this research was urea CO($^{15}NH_2$)$_2$, with a 10% abundance of ^{15}N urea provided by Shanghai Yuanye Bio-Technology Co., Ltd., Shanghai, China, and subjected to shading for a duration of 24 h. After a culture period of 35 days, samples were collected for the determination of relevant indexes.

2.4. Parameter Determination

2.4.1. Root Nodule Indexes

After rinsing the plants, the number of pink nodules per plant was determined by randomly selecting ten plants for each inoculation treatment. (The nodules were placed under a stereomicroscope for observation, and the pale pink nodules were the effective root nodules [27]).

After rinsing the plants, a total of 10 plants were randomly selected from each treatment to quantify the number of pink root nodules and subsequently weighed using an analytical balance to determine the fresh weight of individual pink root nodules. Each treatment was replicated three times.

The fully developed and efficient root nodules were preserved in a 50% formalin-aceto-alcohol (FAA) solution for a specified period, subsequently embedded in paraffin to create sections. These sections were later stained with toluidine blue dye and examined under a Lycra biological microscope for scanning and photography purposes. The quantity of infected root nodule cells across various symbiotic combinations was measured utilizing ImageJ 1.5g (National Institutes of Health, Bethesda, MD, USA) software.

2.4.2. Nitrogen Fixation Indexes

The nitrogenase activity was determined using the acetylene reduction method [28].

$$C_2H_4 \text{ levels } \left(\mu mol \cdot g^{-1} \cdot h^{-1}\right) = \frac{C \times hx \times V}{hs \times 1000 \times 22.4 \times t \times m} \times 10^6 \quad (1)$$

In the formula, C represents the standard concentration of C_2H_4 ($nmol \cdot mL^{-1}$), hx denotes the peak area of the sample, V indicates the volume of the reaction gas (mL), hs signifies the peak area of the standard C_2H_4, 22.4 represents the molar volume of C_2H_4 ($L \cdot mol^{-1}$), t refers to the reaction time (h), and m corresponds to root nodules' weight (g).

The plants were subjected to drying at a consistent temperature of 70 °C, followed by measurement of their dry weight utilizing an electronic balance. The ^{15}N atomic percentage of each sample was determined using the ^{15}N isotope analyzer (DELTA V Advantage), and the nitrogen fixation percentage and nitrogen fixation amount were calculated utilizing the formula.

Nitrogen content of the sample $\%N$: Kjeldahl determination [29]

$$\text{Nitrogen fixation percentage } \%N_{daf} = \left(1 - \frac{\%^{15}N_{dfF}}{\%^{15}N_{dfNF}}\right) \times 100 \quad (2)$$

$^{15}N_{dfF}$ represents the ^{15}N atomic percentage in the sample, $^{15}N_{dfNF}$ represents the ^{15}N atomic percentage in nature.

$$\text{Total Nitrogen content } N_t = \%N \times \text{Sample dry weight } \left(g \cdot pot^{-1}\right) \quad (3)$$

$$\text{Nitrogen fixation amount } N_{fixed} = N_t \times \%N_{dfa} \quad (4)$$

The aboveground dry weight (ADW), underground dry weight (UDW), aboveground nitrogen content (ANC), and underground nitrogen content (UNC) of treatment were detected, respectively. The methodology for the dry weight of aboveground and underground components was elucidated via the Section 2.4.3. Nitrogen content determination was performed using the Kjeldahl method.

$$ANA = ANC \times ADW \quad (5)$$

$$UNA = UNC \times UDW \quad (6)$$

The nitrogen content was determined using the Kjeldahl method, whereas the crude protein content was calculated by multiplying the nitrogen content by a factor of 6.25.

2.4.3. Growth Indexes

After rinsing the plants, remaining water was absorbed using filter paper. Then, the aboveground and underground tissue of 10 plants were separated for each treatment, and the fresh samples were subjected to kill out at 105 °C for a duration of 15 min, followed by drying at 75 °C to determine their dry weight. Each treatment was replicated three times.

The height of each individual plant was measured using a ruler, with 10 plants randomly selected and assessed in each treatment.

After rinsing the plants, remaining water was absorbed using filter paper. Then, the stem and leaf of 10 plants were separated for each treatment, and the fresh samples were subjected to kill out at 105 °C for a duration of 15 min, followed by drying at 75 °C to determine their dry weight. Each treatment was replicated three times.

$$\text{Stem–Leaf ratio} = \text{Leaf dry weight/stem dry weight} \quad (7)$$

2.5. Statistic Analysis

The data collation and calculation of indicators and coefficient of variation were conducted using Microsoft Office 2019 software, while the SPSS 27.0 (IBM Corporation, Armonk, NY, USA) software was utilized for statistical analysis, including analysis of variance ($p < 0.05$), analysis of correlation, PCA, and fuzzy membership function method. Additionally, the Origin 2024b (OriginLab, Northampton, MA, USA) software was used for data visualization.

2.5.1. Principal Component Analysis

The dimensionality and complexity of the data for evaluating the symbiotic nitrogen fixation effect in different varieties of alfalfa after inoculation with rhizobia were reduced using PCA in SPSS 27.0. We calculated the eigenvector value of each index in each principal component and the contribution rate of each principal component.

2.5.2. Membership Function

The first four eigenvalues of principal components I and II in principal component analysis were utilized to select a total of eight indexes, which were then comprehensively evaluated using the membership function method to assess the symbiotic nitrogen fixation effect of different varieties of alfalfa inoculated with rhizobia. The membership function value for each index was calculated based on the provided formula, and the comprehensive evaluation value was determined by considering the membership function values for all selected indexes [22].

The equation for computing the value of the membership function is as follows:

$$U(X_{ij}) = (X_{ij} - X_{j\ \min}) / (X_{j\max} - X_{j\ \min}) \quad (8)$$

The membership function value, denoted as $U(X_{ij})$, in the formula represents the evaluation index value of the i-th variety for the j-th criterion. $X_{j\ \max}$ denotes the maximum value of this index, while $X_{j\ \min}$ represents its minimum value.

The weight calculation formula is:

$$W_j = P_j / \sum_{j=1}^{n} P_j \quad (9)$$

In the formula, W_j represents the weight of the j-th evaluation index, while P_i represents the coefficient of variation of the j-th evaluation index.

The calculation formula of comprehensive evaluation is:

$$D = \sum_{\substack{i=1 \\ j=1}}^{n} \left[U(X_{ij} \times W_j) \right] \quad (10)$$

The D value in the formula represents the comprehensive assessment of the symbiotic nitrogen fixation efficacy between different alfalfa varieties and LL2. A higher D value indicates a more robust symbiotic nitrogen fixation effect and enhanced adaptability of alfalfa varieties to LL2.

3. Results

3.1. SNF Ability Variations Among Different Alfalfa Varieties and Rhizobia Combinations

3.1.1. The Nodulation Ability of Different SNF Combinations Varies

After inoculation with LL2, there were no significant differences in the number of effective root nodules per plant among various alfalfa varieties, as depicted in Figure 1a. Among them, WL168 exhibited the highest root nodule number (8.78), while WL298 had the lowest number at only 6.59. Significant differences were observed in single effective root nodule weight among varieties (Figure 1b). Notably, G9 displayed a significantly higher weight of individual effective root nodules at 1.30 mg compared to other varieties. Conversely, the single effective root nodule weight of WL319 (0.30 mg) was significantly lower than that of other varieties ($p < 0.05$).

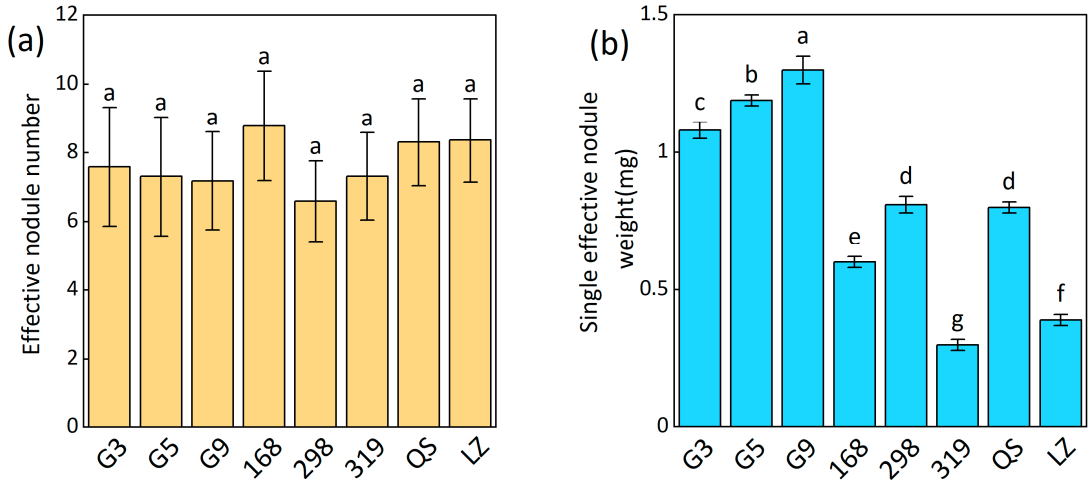

Figure 1. The nodulation ability of different symbiotic combinations was different. (**a**) The number of effective root nodules per plant in various alfalfa varieties inoculated with LL2; (**b**) the weight of the single effective root nodule across different alfalfa varieties inoculated with LL2. The lowercase letters in the figure indicate the statistical significance of differences between various treatments for the same variety, as determined by Tukey's HSD test ($p < 0.05$). In the diagram, G3 represents Gannong NO.3; G5 corresponds to Gannong NO.5; G9 denotes Gannong NO.9; 168 signifies WL168HQ; 298 indicates WL298HQ; and 319 represents WL319HQ; QS stands for Qingshui, while LZ is an abbreviation for Longzhong. The same below.

3.1.2. Microstructure Characteristics of Root Nodules in Different SNF Combinations

To investigate the differences in rhizobia infection during the symbiotic process, paraffin sections of root nodules formed by various alfalfa varieties were examined following inoculation with LL2. As illustrated in Figure 2a, G9 exhibited a higher degree of toluidine blue staining within the root nodules, displaying more intense coloration. The count of infected root nodule cells in this variety was 2795 (Figure 2b), significantly surpassing that of other varieties ($p < 0.05$). This finding suggests that LL2 effectively infected and colonized

G9, resulting in a greater number of infected root nodule cells and enhanced efficiency of symbiotic nitrogen fixation due to their closer arrangement. In contrast, WL298 had loosely arranged cells within its root nodules and lighter toluidine blue staining (Figure 2a). The number of infected root nodule cells in WL298 was only 928, which was significantly lower than that observed in other varieties ($p < 0.05$) (Figure 2b).

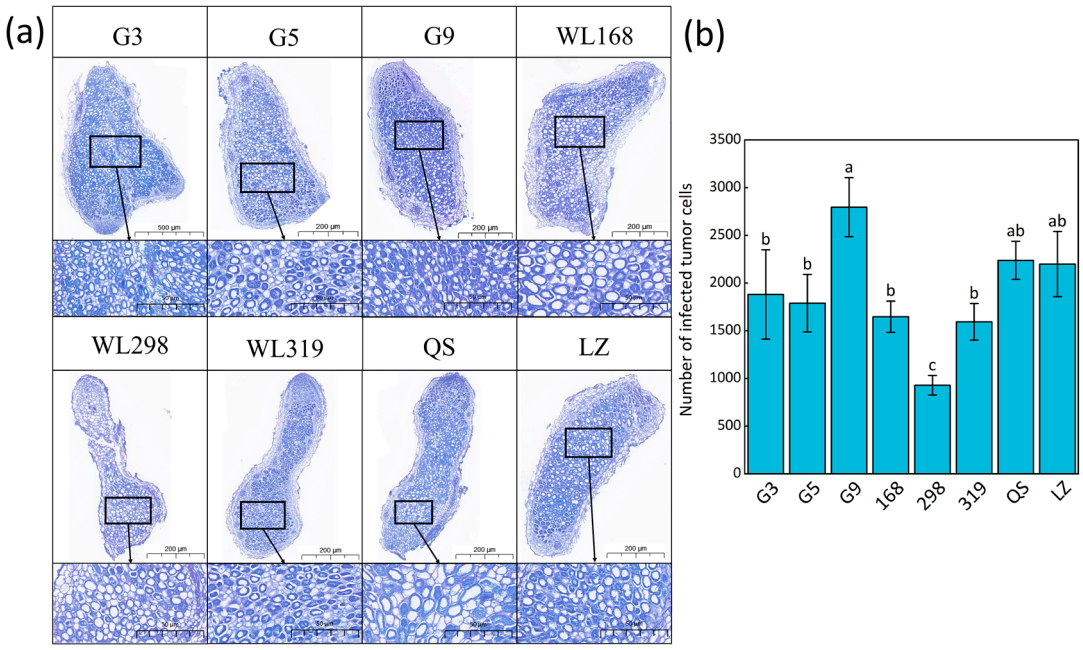

Figure 2. Root nodule sections and the number of root nodule cells infected by rhizobia LL2 in different alfalfa–rhizobia combinations. (**a**) The toluidine blue staining sections of root nodules of different symbiotic combinations; (**b**) the number of infected cells in root nodules of different symbiotic combinations. The lowercase letters in the figure indicate the statistical significance of differences between various treatments for the same variety, as determined by Tukey's HSD test ($p < 0.05$).

3.2. Variations in the Nitrogen Fixation Capacity Among Different Alfalfa Varieties and Rhizobia Combinations

3.2.1. Nitrogen Fixation Ability

To investigate the variations in nitrogen fixation among different symbiotic combinations, we quantified the nitrogenase activity, nitrogen fixing percentage, and nitrogen fixation amount of eight symbiotic combinations. The nitrogenase activity and nitrogen fixation percentage of alfalfa varieties exhibited significant difference ($p < 0.05$) upon inoculation with LL2. Notably, G9 exhibited superior levels of nitrogenase activity (0.91 $\mu mol \cdot g^{-1} \cdot h^{-1}$) (Figure 3a), nitrogen fixation percentage (67.16%) (Figure 3c), and nitrogen fixation amount (18.80 $mg \cdot pot^{-1}$) (Figure 3d), which all significantly surpassed those of other treatments ($p < 0.05$). Conversely, LZ displayed markedly lower levels of nitrogenase activity compared to other treatments ($p < 0.05$), measuring only 0.09 $\mu mol \cdot g^{-1} \cdot h^{-1}$ (Figure 3a); however, its nitrogen fixation percentage exceeded 61% (Figure 3c). Although QS ranked second in terms of nitrogenase activity (0.59 $\mu mol \cdot g^{-1} \cdot h^{-1}$) (Figure 3a), its nitrogen fixing percentage (33.68%) (Figure 3c) and nitrogen fixation amount (5.56 $mg \cdot pot^{-1}$) (Figure 3d) were significantly lower than those observed for other varieties ($p < 0.05$). The total plant N content ($^{14}N + {}^{15}N$) was highest for G3, which was significantly greater than that found in other varieties, reaching a value as high as 3.83% (Figure 3b).

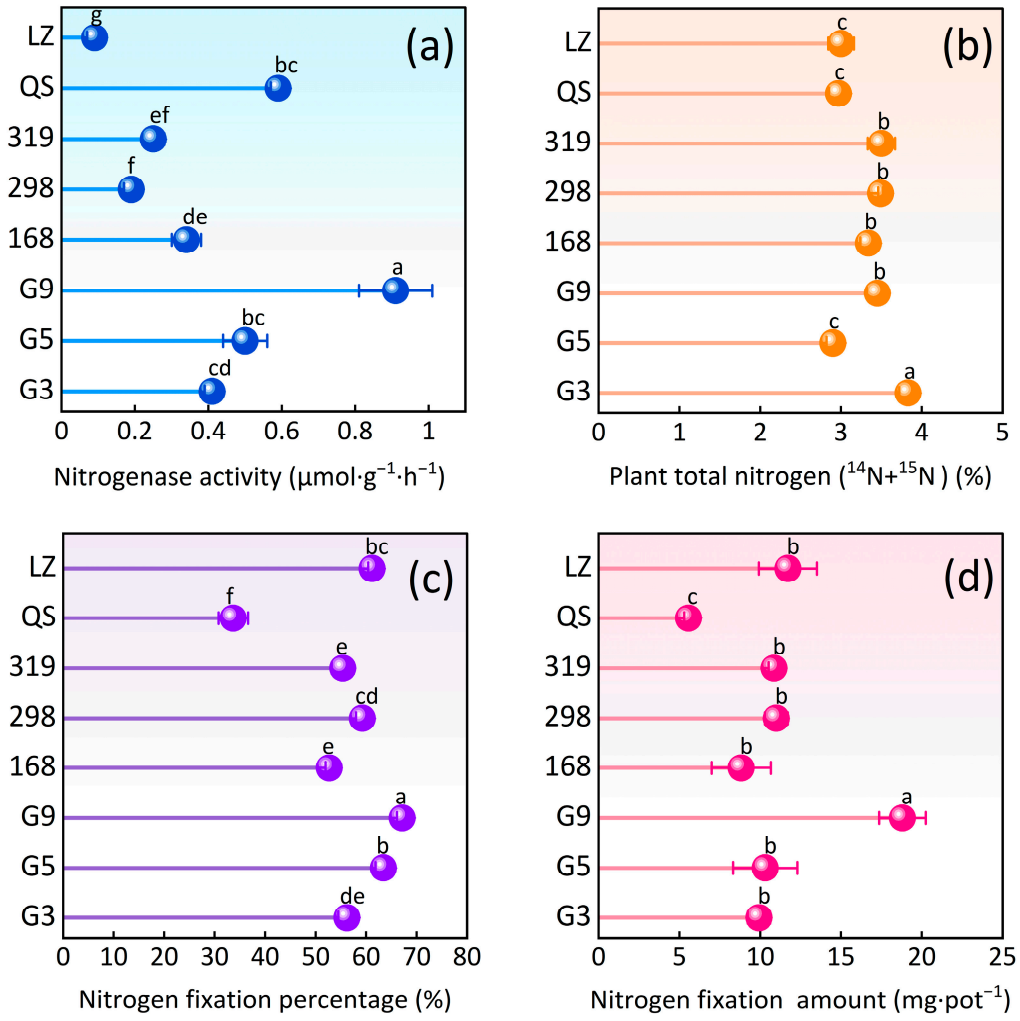

Figure 3. The difference of nitrogen fixation ability in different alfalfa–rhizobia combinations. (**a**) The nitrogenase activity of various symbiotic combinations; (**b**) the total nitrogen content in different symbiotic combinations following ^{15}N treatment (in indoor sand culture); (**c**) the nitrogen fixation percentage of diverse symbiotic combinations; (**d**) the amount of nitrogen fixation in distinct symbiotic combinations. The lowercase letters in the figure indicate the statistical significance of differences between various treatments for the same variety, as determined by Tukey's HSD test ($p < 0.05$).

3.2.2. The Nitrogen Accumulation in Aboveground and Underground Tissue

The disparity in nitrogen accumulation between the aboveground and underground parts was pronounced following inoculation with LL2. The aboveground tissue nitrogen accumulation in QS, G5, and G9 was significantly higher than that of the other treatments and their CK ($p < 0.05$). The QS inoculation treatment was the highest (155.41 mg·plant^{-1}) (Figure 4a), which was increased by 173.48% compared with CK ($p < 0.05$). The inoculated treatments of G5 and G9 showed increases of 56.35% and 26.50% (Figure 4c), respectively, compared to CK ($p < 0.05$). In contrast, the LZ inoculated treatment demonstrated only a marginal increase of 0.02% compared to CK, with no significant difference observed. Other varieties exhibited lower aboveground tissue nitrogen accumulation than CK. Notably, the aboveground tissue nitrogen accumulation in the G3 and WL319 inoculated treatments was significantly lower than that in CK by 21.81% and 28.86%, respectively ($p < 0.05$). Furthermore, the underground tissue nitrogen accumulation in the G9 inoculated treatment (35.68 mg·plant^{-1}) and CK (35.36 mg·plant^{-1}) (Figure 4b) was significantly higher than that of other treatments ($p < 0.05$), with no significant difference between the two. Both G5

and WL298 showed significantly lower values ($p < 0.05$), with WL298 demonstrating the most significant decrease at 26.42% (Figure 4c).

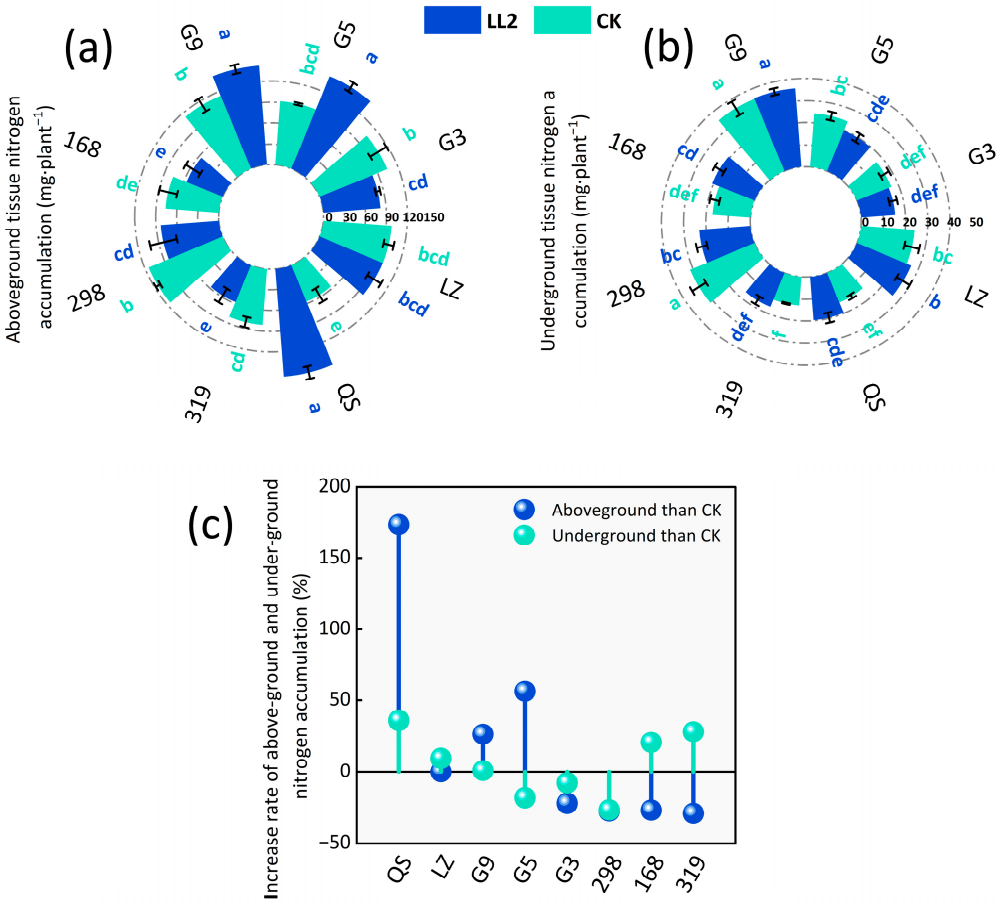

Figure 4. The nitrogen accumulation differences in aboveground and underground tissue among different symbiotic combinations. (**a**) The aboveground nitrogen accumulation in various alfalfa cultivars subjected to inoculated treatment and CK; (**b**) the underground nitrogen accumulation in diverse alfalfa cultivars subjected to inoculated treatment and CK; (**c**) the increase rate of both aboveground and underground nitrogen accumulations in the inoculation treatment, relative to CK. In the abscissa of (**c**), the same type (the increase rate of aboveground and underground tissue nitrogen accumulation) varieties were ranked together. The lowercase letters in the figure indicate the statistical significance of differences between various treatments for the same variety, as determined by Tukey's HSD test ($p < 0.05$).

3.2.3. Crude Protein Content

The crude protein content of all tested alfalfa varieties ranged from 14.70% to 17.75%, as illustrated in Figure 5a. Among these varieties, the G9 inoculated treatment exhibited a significantly higher crude protein content (17.75%) compared to other inoculated treatments ($p < 0.05$). Conversely, QS displayed a significantly lower crude protein content than other varieties in the inoculated treatment ($p < 0.05$), while G9 and QS did not differ significantly from CK. Moreover, G5 and LZ demonstrated an increase in crude protein content of 12.92% and 7.46%, respectively, compared to CK ($p < 0.05$). In contrast, WL319 exhibited a significant decrease in crude protein content compared to CK ($p < 0.05$), showing a reduction of 4.34% (Figure 5b).

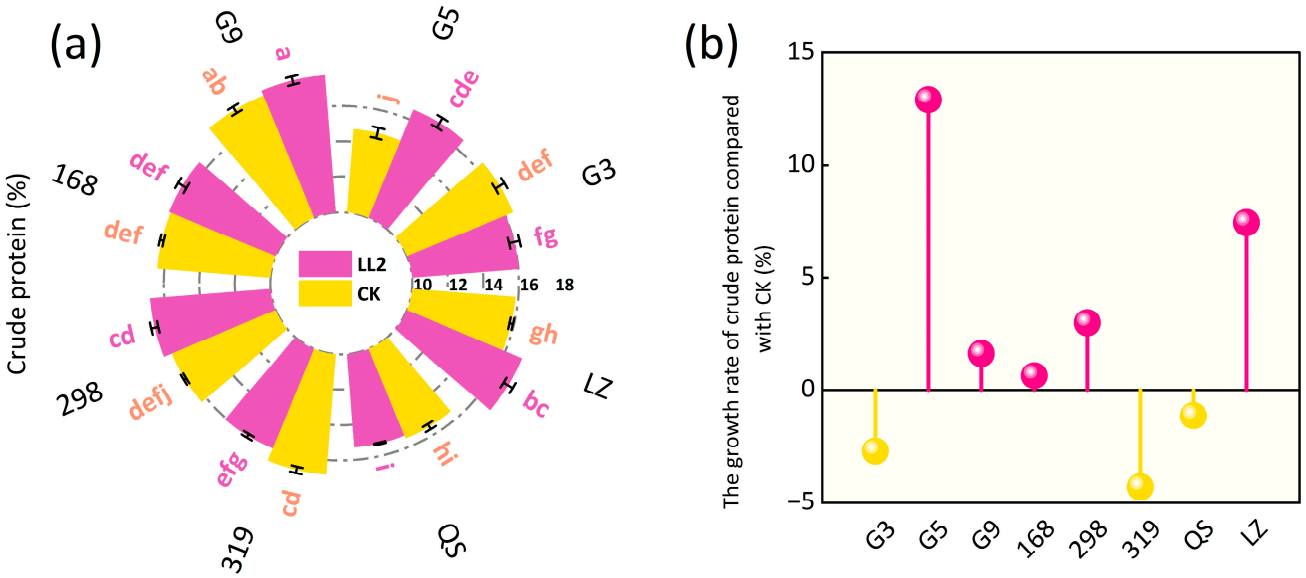

Figure 5. The difference of crude protein content in different symbiotic combinations. (**a**) represents the crude protein content of different varieties of alfalfa in both the inoculated treatment and CK; (**b**) represents the growth rate of crude protein content in the inoculation treatment compared with CK. The lowercase letters in the figure indicate the statistical significance of differences between various treatments for the same variety, as determined by Tukey's HSD test ($p < 0.05$).

3.3. Growth Characteristics Among Different Alfalfa Varieties and Rhizobia Combinations

3.3.1. Plant Height

The plant heights of various alfalfa varieties exhibited significant differences following inoculation with LL2 ($p < 0.05$), as illustrated in Figure 6a. Among them, the QS displayed the highest plant height at 75.32 cm, which was significantly greater than that of CK by 20.35%. G3, WL319, and LZ also demonstrated significantly higher plant heights compared to CK, with increases of 16.47%, 8.78%, and 17.03%, respectively. WL168 exhibited a significantly lower plant height than CK ($p < 0.05$), showing a decrease of 14.73%. The other varieties exhibited no statistically significant differences compared to CK (Figure 6b).

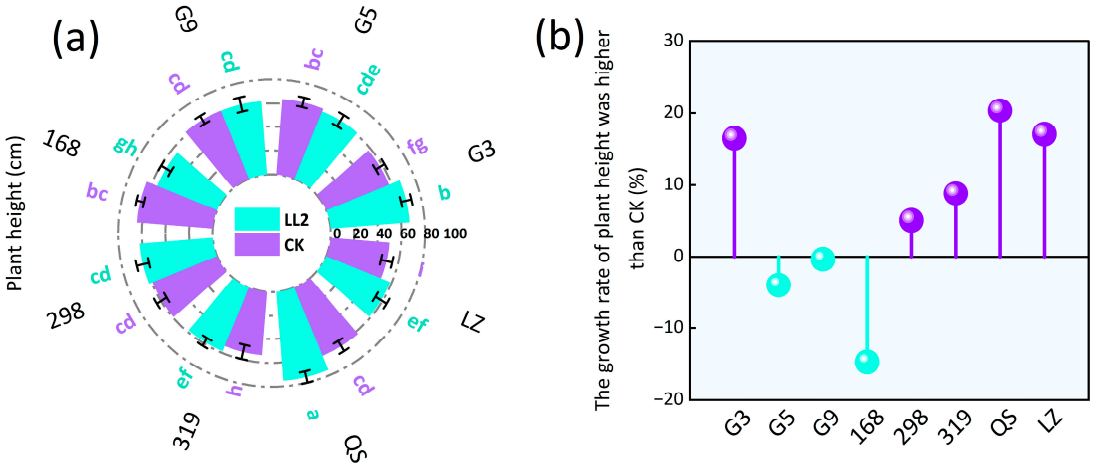

Figure 6. The variations in plant height across in different symbiotic combinations. (**a**) represents the plant height of different varieties of alfalfa in both the inoculated treatment and CK; (**b**) represents the growth rate of plant height in the inoculation treatment compared with CK. The lowercase letters in the figure indicate the statistical significance of differences between various treatments for the same variety, as determined by Tukey's HSD test ($p < 0.05$).

3.3.2. Aboveground and Underground Biomass

The biomass of different varieties and treatments exhibited significant variations ($p < 0.05$) (Figure 7a). QS inoculated with LL2, demonstrated the highest aboveground dry weight (6.35 g·plant^{-1}), which was 135.94% higher than that of CK and significantly distinct from other varieties ($p < 0.05$). Moreover, G5 and G9 displayed substantial increases of 38.52% and 24.31%, respectively, compared to CK ($p < 0.05$). Inversely, the aboveground dry weight of other varieties decreased relative to CK, with G3 exhibiting a significant reduction of 19.51% ($p < 0.05$). Furthermore, significant differences in underground dry weight were observed among treatments of different varieties ($p < 0.05$). QS (1.25 g·plant^{-1}) and WL168 (1.18 g·plant^{-1}) exhibited substantial increases of 29.67% and 35.87%, respectively, in their underground dry weight after inoculation with LL2 compared to CK ($p < 0.05$). Conversely, G3 and G5 experienced reductions of 34.26% and 28.70%, respectively, with G3 displaying the lowest underground dry weight among the varieties under LL2 inoculation, and this difference was statistically significant (Figure 7b).

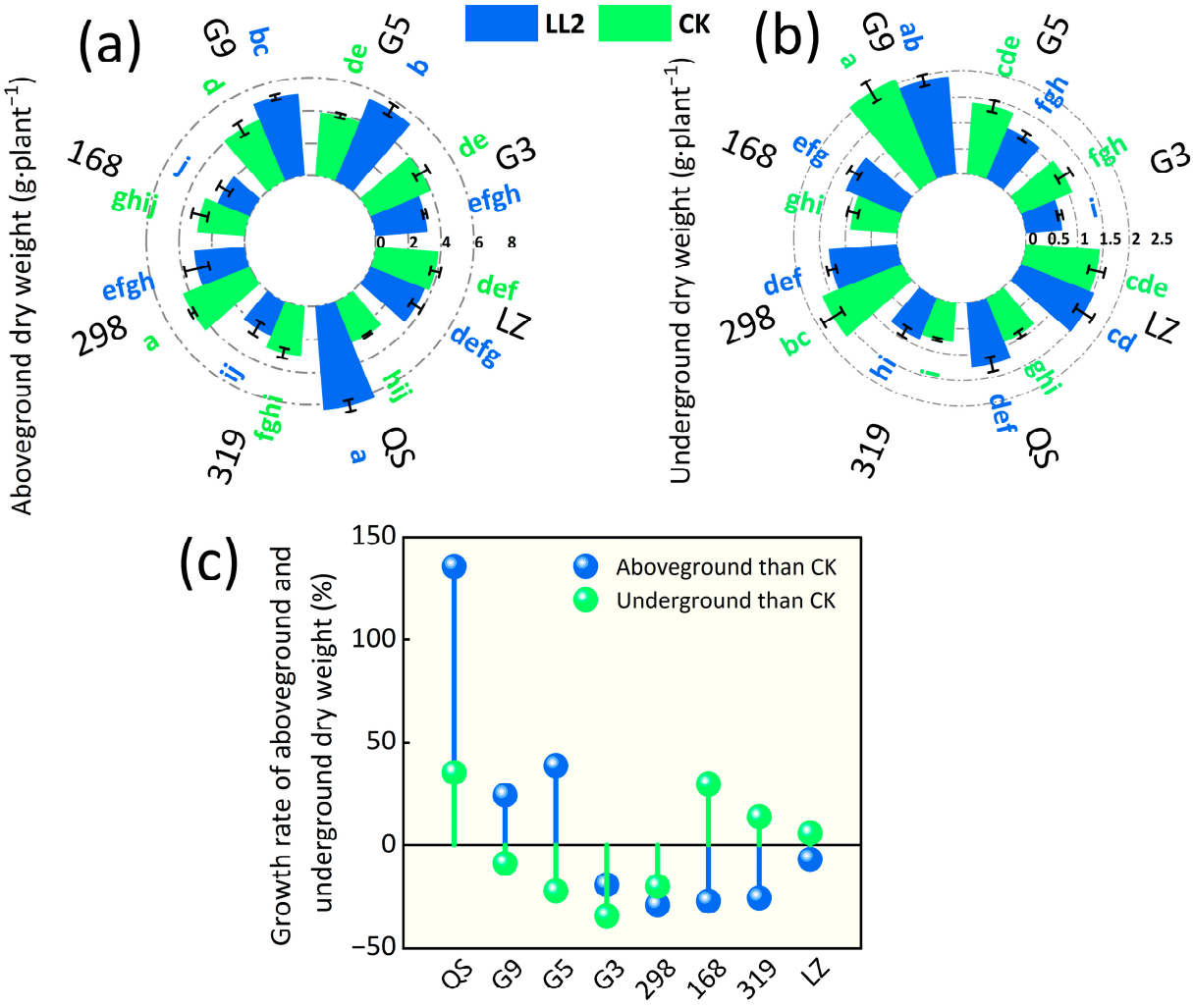

Figure 7. The disparities in the aboveground and underground biomass in different symbiotic combinations. (**a**) The aboveground dry weight in various alfalfa cultivars subjected to inoculated treatment and CK; (**b**) the underground dry weight in diverse alfalfa cultivars subjected to inoculated treatment and CK; (**c**) the increase rate of both aboveground and underground dry weight in the inoculation treatment, relative to CK. In the abscissa of (**c**), the same type (the increase rate of aboveground and underground dry weight) varieties were ranked together. The lowercase letters in the figure indicate the statistical significance of differences between various treatments for the same variety, as determined by Tukey's HSD test ($p < 0.05$).

3.3.3. Stem–Leaf Ratio

The different varieties display significant variations upon inoculation with LL2. WL298 exhibited the highest stem–leaf ratio, reaching 1.23. In comparison to CK, G3, G5, G9, and WL168 displayed an increase in their stem–leaf ratios, with WL168HQ showing a statistically significant difference ($p < 0.05$). The remaining varieties exhibited lower stem–leaf ratios compared to CK (Figure 8a).

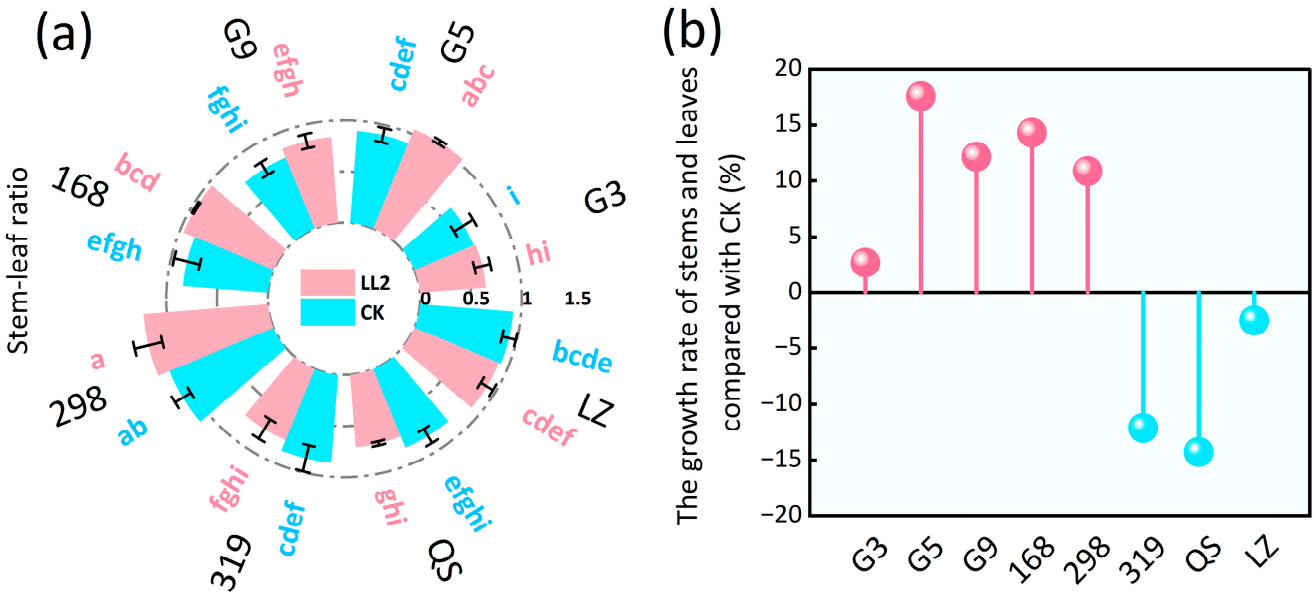

Figure 8. The ratio of stem and leaf varies in different symbiotic combinations. (**a**) The stem–leaf ratio of different varieties of alfalfa in both the inoculated treatment and CK; (**b**) the growth rate of stem–leaf ratio in the inoculation treatment compared with CK. The lowercase letters in the figure indicate the statistical significance of differences between various treatments for the same variety, as determined by Tukey's HSD test ($p < 0.05$).

The growth rate of the stem–leaf ratio for G5, exhibited the most substantial increase (17.54%). In comparison to CK, this variety demonstrated the most significant rise in the stem–leaf ratio, while QS displayed a negative growth rate with the largest recorded decrease at 14.27% (Figure 8b).

3.4. Effect of SNF on Variation Coefficient

Under the condition of LL2 inoculation, the coefficients of variation for 14 indicators of symbiotic nitrogen fixation effects were calculated for eight symbiotic combinations. The coefficients of variation for these indicators ranged from 5.25% to 59.40%. Notably, nitrogenase activity exhibited the highest variability with a coefficient of variation of 59.40%, followed by single effective root nodule weight, aboveground dry weight, and aboveground tissue nitrogen accumulation with coefficients of variation at 42.09%, 37.34%, and 36.50% respectively. In contrast, crude protein displayed minimal variability with a coefficient of variation as low as only 5.25% (Table 2). These findings suggest that LL2 inoculation exerted a significant impact on nitrogenase activity while demonstrating high specificity. However, it had negligible influence on crude protein levels indicating low specificity.

The coefficients of variation for different indicators between the inoculated treatments and CK of the eight varieties exhibited significant differences (Table 3). Notably, QS inoculated with LL2 demonstrated a substantial impact on aboveground nitrogen accumulation (65.69%) and aboveground dry weight (57.23%). In contrast, LZ displayed minimal variation in aboveground tissue nitrogen accumulation (0.02%), while G9 showed only a slight difference in plant height between the inoculated treatment and CK (0.24%). A smaller

coefficient of variation indicates a lesser disparity between inoculated and uninoculated samples. The crude protein content among all eight varieties ranged from 1.96% to 7.75%, suggesting that inoculation had negligible effects on this index.

Table 2. Coefficient of variation of 14 indexes of symbiotic nitrogen fixation effect index of eight kinds of alfalfa.

Index	Average	Standard Deviation	Standard Deviation (%)
Number of effective root nodules per plant	7.678	0.688	8.96
Single effective root nodule weight (mg)	0.808	0.340	42.09
Number of infected root nodule cells	1883.625	514.603	27.32
Nitrogenase activity ($\mu mol \cdot g^{-1} \cdot h^{-1}$)	0.409	0.243	59.40
Aboveground tissue nitrogen accumulation ($mg \cdot plant^{-1}$)	102.4	37.375	36.50
Underground tissue nitrogen accumulation ($mg \cdot plant^{-1}$)	22.83	6.030	27.18
Nitrogen fixation percentage (%)	56.121	9.533	16.99
Nitrogen fixation amount ($mg \cdot pot^{-1}$)	0.311	0.062	19.81
Plant total nitrogen ($^{14}N + {}^{15}N$) (%)	3.310	0.304	9.19
Crude protein	16.488	0.866	5.25
Plant height	62.554	5.872	9.39
Aboveground dry weight ($g \cdot plant^{-1}$)	3.881	1.449	37.34
Underground dry weight ($g \cdot plant^{-1}$)	1.229	0.351	28.55
Stem–leaf ratio	0.886	0.181	20.38

Table 3. Coefficient of variation of nitrogen fixation and growth indexes of different alfalfa varieties comparing the inoculation and CK.

Alfalfa Varieties	Coefficient of Variation (%)						
	Aboveground Tissue Nitrogen Accumulation	Underground Tissue Nitrogen Accumulation	Crude Protein	Plant Height	Aboveground Dry Weight	Underground Dry Weights	Stem–Leaf Ratio
G3	17.32	5.72	1.96	10.76	15.29	29.47	1.88
G5	31.09	14.01	8.03	2.88	22.84	17.88	11.40
G9	16.55	0.64	3.61	0.24	15.33	6.69	8.10
WL168	21.75	13.27	7.75	11.24	22.42	18.27	9.44
WL298	22.32	21.52	5.91	3.47	24.26	15.92	7.30
WL319	23.85	17.50	2.69	5.95	20.95	9.13	9.13
QS	65.69	21.68	4.55	13.06	57.23	21.20	10.86
LZ	0.02	6.41	2.47	11.10	5.15	4.06	1.81

3.5. Differential Evaluation Concerning Effects of Rhizobia Strain LL2 on the SNF Efficiency of Different Varieties

3.5.1. Correlation Analysis

The nitrogen fixation effect of the alfalfa–rhizobia symbiotic system cannot be fully captured by a single index. Therefore, this study conducted a correlation analysis on the symbiotic nitrogen fixation effect indicators of different alfalfa types inoculated with LL2. The aboveground tissue nitrogen accumulation and aboveground dry weight, underground tissue nitrogen accumulation and underground dry weight, nitrogen fixation percentage and nitrogen fixation amount were highly significantly positively correlated, and the single effective root nodule weight was significantly positively correlated with nitrogenase activity; the number of effective root nodules per plant was significantly negatively correlated with crude protein, and all other indicators had no significant correlation. Among them, the nitrogen fixation percentage and nitrogen fixation amount had no significant correlation with nitrogenase activity. This indicated that nitrogenase activity could only indicate

nitrogen fixation potential. At that time, the strength of nitrogen fixation ability was also affected by other factors. Among them, the nitrogen fixation percentage and nitrogen fixation amount had no significant correlation with nitrogenase activity. This indicated that nitrogenase activity could only indicate nitrogen fixation potential. At that time, the strength of nitrogen fixation ability was also affected by other factors (Figure 9).

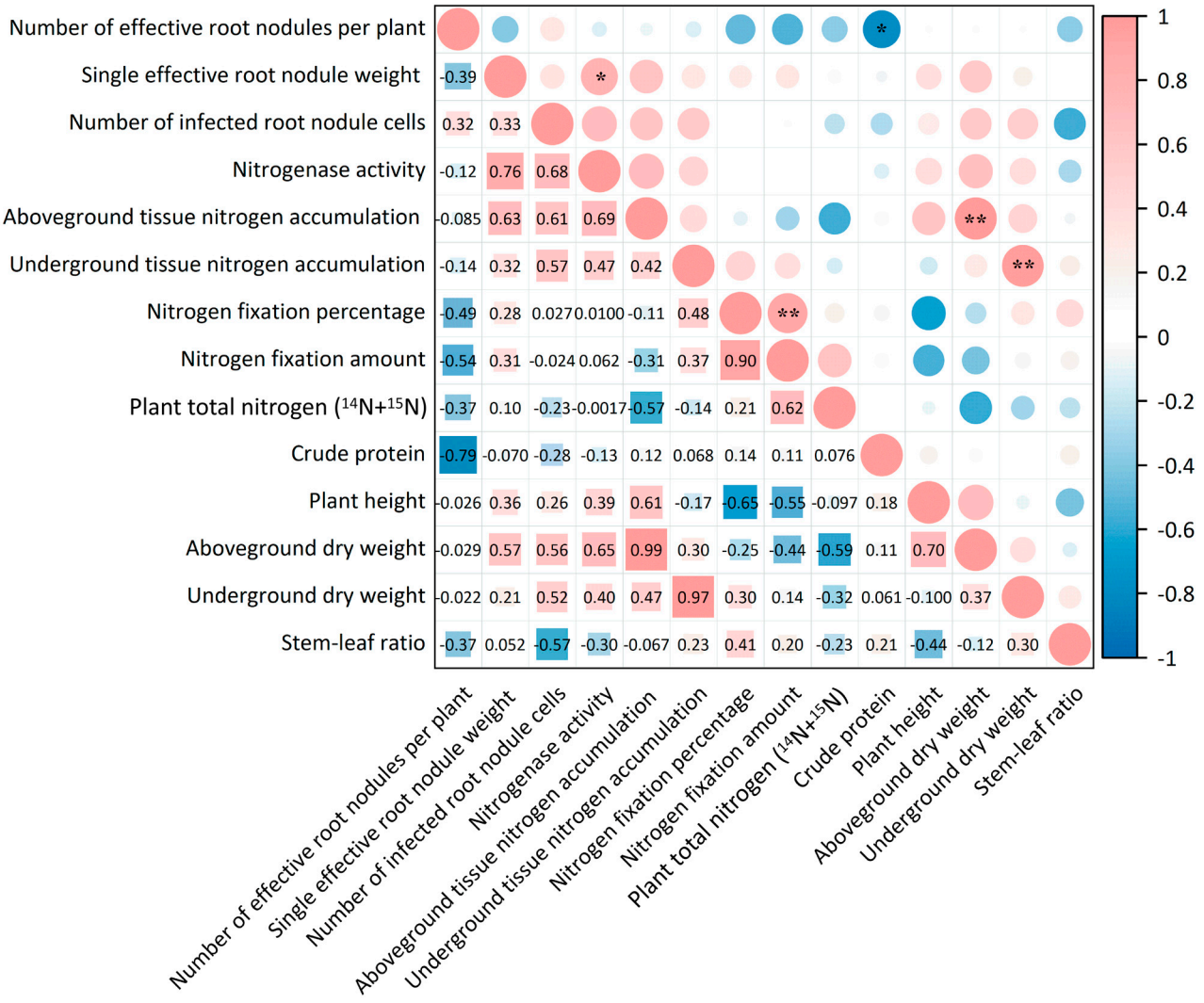

Figure 9. The correlation between symbiotic nitrogen fixation efficacy indices of alfalfa inoculated with LL2. * and ** represent significant differences at 0.05 and 0.01 level, respectively.

3.5.2. Screening of Main Indicators

Principal component analysis was performed on each indicator of the test materials, and the principal components were extracted with the cumulative contribution rate reaching 85% as the threshold. The results showed that the cumulative contribution rate of the first four principal components reached 87.894%, and the contribution rates were 33.740%, 25.662%, 15.002%, and 13.490%, respectively (Table 4). The total contribution rate of principal components I and II reached 59.402%. The loading matrix of each component showed that the contribution rate of principal component I was the largest, which was mainly determined by aboveground tissue nitrogen accumulation, aboveground dry weight, nitrogenase activity, and the number of infected root nodule cells. The greater the number of infected cells, the better the nitrogenase activity and the greater the symbiotic nitrogen fixation potential. The principal component II was mainly determined by underground tissue nitrogen accumulation, underground dry weight, nitrogen fixation percentage, and

nitrogen fixation amount. The higher the nitrogen fixation percentage and nitrogen fixation amount, the stronger the nitrogen fixation ability, which has a certain influence on biomass and nitrogen accumulation.

Table 4. The eigenvalues and contribution rates of the four principal components and load matrix of each factor were extracted by principal component analysis.

Factor	Principal Component			
	I	II	III	IV
Eigenvalue	4.724	3.593	2.100	1.889
Contribution rate/%	33.740	25.662	15.002	13.490
Accumulative contribution rate/%	33.740	59.402	74.404	87.894
Number of effective root nodules per plant	0.012	−0.183	−0.349	0.057
Single effective root nodule weight	0.131	0.115	0.154	0.159
Number of infected root nodule cells	0.163	0.01	−0.187	0.194
Nitrogenase activity	0.171	0.059	0.035	0.21
Aboveground tissue nitrogen accumulation	0.202	0.002	0.076	−0.103
Underground tissue nitrogen accumulation	0.111	0.185	−0.191	−0.065
Nitrogen fixation percentage	−0.033	0.252	−0.067	0.021
Nitrogen fixation amount	−0.059	0.243	−0.003	0.199
Plant total nitrogen ($^{14}N + {}^{15}N$)	−0.096	0.085	0.147	0.367
Crude protein	−0.009	0.084	0.322	−0.216
Plant height	0.126	−0.134	0.264	0.053
Aboveground dry weight	0.198	−0.037	0.1	−0.106
Underground dry weights	0.118	0.141	−0.208	−0.161
Stem–leaf ratio	−0.048	0.134	−0.001	−0.378

3.5.3. The Comprehensive Assessment

Through principal component analysis, the eight indexes of the top four eigenvalues in principal component I and II were screened out, which were aboveground tissue nitrogen accumulation, aboveground dry weight, nitrogenase activity, number of infected root nodule cells, underground tissue nitrogen accumulation, underground dry weight, nitrogen fixation percentage, and nitrogen fixation amount, respectively. The coefficient of variation ranged from 59.40% to 16.99%. The coefficient of variation was large, indicating that these indicators had a large degree of dispersion of data, which could reasonably reflect the differences in nodulation, nitrogen fixation, and growth promotion effects of different symbiotic combinations. Therefore, these eight indexes were used as the key traits to evaluate the nitrogen fixation and growth promotion of symbiotic combinations, and the membership function method was used to comprehensively evaluate the nodulation and nitrogen fixation effects of eight groups of symbiotic combinations. It can be seen from Table 5 that the symbiotic nitrogen fixation effect of alfalfa and LL2 was ranked as follows: G9 > QS > G5 > LZ > G3 > WL298 > WL168 > WL319. Among these, the comprehensive evaluation value of G9-LL2 combination was 0.938, while the comprehensive evaluation value of WL319-LL2 combination was only 0.211.

Table 5. Comprehensive evaluation results of membership function.

Treatment	The D Value	Rank
G9-LL2	**0.937902821**	**1**
QS-LL2	0.585020043	2
G5-LL2	0.575772789	3
LZ-LL2	0.426585897	4

Table 5. *Cont.*

Treatment	The D Value	Rank
G3-LL2	0.334872736	5
WL298-LL2	0.316787268	6
WL168-LL2	0.268595499	7
WL319-LL2	0.210707858	8

4. Discussion

4.1. Differences in SNF Between Different Rhizobia–Alfalfa Combinations

Biological nitrogen fixation is the most important way for leguminous plants to absorb nitrogen during the whole growth and development process, and nodules are an important structure for biological nitrogen fixation. The number and weight of nodules are two important indexes for evaluating the nodulation ability of rhizobia [30], and an important characteristic of alfalfa rhizobia symbiotic systems is symbiotic specificity. If different alfalfa varieties are inoculated with the same strain at the same time, and the same alfalfa varieties are inoculated with several strains respectively, there will be different symbiotic effects, and great differences between each other. Previous studies have shown that different varieties of alfalfa would produce different nodulation effects after inoculation with the same rhizobia [17]. For example, research found that alfalfa variety Giant 201 had certain differences in nodulation effect after being inoculated with different rhizobia strains [31]. The present study demonstrated significant variation in the single effective root nodule weight among eight alfalfa varieties inoculated with LL2 (Figure 1b). G9 exhibited the highest single effective root nodule weight, accompanied by the largest number of colonized and infected root nodule cells as well as the highest nitrogenase activity, which significantly differed from the values of other varieties. Conversely, WL298 displayed the lowest number of infected root nodule cells and low nitrogenase activity. Nitrogenase activity and the number of infected cells were identified as key indicators for determining principal component I (Table 4), indicating a close relationship between these two indexes and symbiotic nitrogen fixation efficiency. In summary, the symbiotic effect of G9 and LL2 exhibited superior performance with the highest nitrogen fixation potential. In contrast, the combination of WL298-LL2 demonstrated a weak symbiotic effect and low nitrogen fixation potential. The ^{15}N isotope dilution method was employed in this experiment to visually assess the nitrogen fixation ability of eight alfalfa varieties in conjunction with LL2 (Figure 3). The G9-LL2 combination significantly outperformed other combinations in terms of both nitrogen fixation percentage and amount. Results from root nodule index and nitrogenase activity further confirmed that the G9-LL2 symbiotic combination showcased exceptional nitrogen fixation ability. The nitrogenase activity of QS ranked second; however, its nitrogen fixation percentage and amount were significantly lower compared to those of other combinations. Conversely, LZ exhibited the lowest nitrogenase activity but higher nitrogen fixation percentage and amount. The findings of multiple studies have consistently demonstrated that while nitrogenase activity can serve as an indirect indicator for evaluating the effectiveness of legume rhizobia symbiotic system in fixing nitrogen, it should not be solely relied upon. Due to the variability of nitrogenase activity throughout different periods, a high level of activity at a specific time does not necessarily indicate a correspondingly high amount of nitrogen fixation. Moreover, the acetylene reduction method can only assess the nitrogenase activity in plant nodules during certain stages of growth and development [32,33]. Considering the number of infected root nodules in the LZ-LL2 combination and the morphology of nodule sections, it is evident that the nitrogen fixation potential is not low. Therefore, there seems to be a discrepancy between the actual amount of nitrogen fixation and the measured nitrogenase activity in this symbiotic combination. This could potentially be attributed to factors such as small nodule volume during sampling or loss/low activity of nitrogenase due to water loss within LZ root nodules over time. The root nodule indexes and nitrogen fixation percentage of

the QS-LL2 combination did not align with the observed level of nitrogenase activity. This discrepancy may be attributed to high root nodule activity during the sampling period, but weak early-stage nitrogen fixation ability resulting in low nitrogen fixation outcomes. In conclusion, it is evident that nitrogenase activity alone cannot fully determine nitrogen fixation capability. The coefficient of variation for nitrogenase activity reached 59.40%, significantly higher than other symbiotic nitrogen fixation indexes. Correlation analysis also revealed a significant association between nitrogenase activity and single root nodule weight, suggesting limited regulation of other indexes by variations in nitrogenase activity, indicating that additional factors influence overall nitrogen fixation ability.

4.2. Differences in Growth-Promoting Effects Between Different Rhizobia–Alfalfa Combinations

The findings demonstrated that different alfalfa varieties exhibited varying responses to rhizobia inoculation under identical soil conditions. Strains that demonstrate exceptional performance on one variety may only elicit secondary reactions on another variety [34]. This interaction between the two organisms emphasizes the importance of establishing a compatible symbiotic relationship between rhizobia and their host plants. In the process of symbiotic nitrogen fixation, nitrogen undergoes transformation within the root nodules and is subsequently transported from the roots to the stems and leaves of the host plant, providing energy for its growth [35]. Considering dry weight and nitrogen content as indicators of nitrogen accumulation, significant correlations were observed between 13 indexes in alfalfa inoculated with LL2. Notably, there were highly significant associations between aboveground nitrogen accumulation and aboveground dry weight, as well as underground tissue nitrogen accumulation and underground dry weight. Based on the significant differences observed in aboveground and underground tissue nitrogen accumulation, as well as aboveground and underground dry weight among different varieties inoculated with rhizobia and uninoculated in this experiment, it can be concluded that these two factors exhibit similar patterns of change. Biomass serves as a crucial metric for evaluating production performance in alfalfa while also being a key determinant of variety quality [36,37]. In this study, different varieties of alfalfa inoculated with LL2 had significant differences in plant biomass as the final basis for the classification of the combination (Figure 4), and the growth-promoting effects of the symbiotic nitrogen fixation combination after inoculation were divided into six types (Table 6).

Table 6. The change types of aboveground and underground biomass in different symbiotic combinations.

Type	Feature	Symbiotic Combinations
Aboveground accumulation type	The aboveground dry weight exhibited a significant increase, while no statistically significant difference was observed in the underground dry weight.	G9-LL2
Aboveground and underground accumulation type	The dry weight of both aboveground and underground biomass exhibited an increase.	QS-LL2
Aboveground accumulation, underground depletion type	The aboveground biomass demonstrated a significant increase, whereas the underground biomass exhibited a substantial decrease.	G5-LL2
Zero-growth type	The aboveground and underground dry weights did not exhibit any statistically significant disparity.	WL168-LL2, WL319-LL2, LZ-LL2
Aboveground and underground depletion type	The aboveground and underground biomass demonstrated a substantial reduction.	WL298-LL2
Underground depletion type	The aboveground biomass did not exhibit any significant difference, whereas a notable decrease was observed in the underground biomass.	G3-LL2

Nitrogen is a crucial component of plant chlorophyll, proteins, nucleic acids, and hormones, playing a pivotal role in the growth and development of legumes [38,39]. Yield enhancement results from the accumulation of assimilates, while quality improvement arises from their conversion into various substances [40]. Consequently, rhizobia inoculation may facilitate the biomass accumulation of alfalfa. G5, QS, and G9 demonstrated aboveground accumulation characteristics, exhibiting increased levels of aboveground nitrogen accumulation and crude protein compared to CK. This enhancement can be attributed to rhizobia infection in alfalfa roots, leading to nodule formation for symbiotic nitrogen fixation. As a result, a significant amount of converted nitrogen is translocated to the aboveground tissues for energy storage, ensuring ample nitrogen supply for alfalfa plants [26]. These modifications in growth and physiological metabolism of alfalfa plants facilitate nutrient accumulation, resulting in a substantial increase in dry weight of aboveground tissues. However, QS exhibited significant increases in plant height, aboveground and underground dry weight, and aboveground nitrogen accumulation; nevertheless, there was a decrease in crude protein content. The reason may be attributed to the recognition of rhizobia as pathogens by the roots of leguminous plants, which leads to a series of short-term defense responses [41,42]. QS is a recently domesticated wild variety that demonstrates stronger defense responses compared to other varieties [43], which may impede its efficient binding with rhizobia. Consequently, various physiological metabolic activities are stimulated in the plant, promoting biomass accumulation and stem growth while increasing fiber content and subsequently decreasing crude protein content. It should be noted that this study solely focused on symbiotic nitrogen fixation and growth promotion indicators, and thus further exploration is required to elucidate the molecular mechanisms underlying this phenomenon. The aboveground and underground dry weight of WL168, WL319, and LZ did not show any significant alteration. The reason may be that the plant consumes plant energy to maintain the life activities of the nodule itself while transforming nitrogen, resulting in no significant effect on its biomass and nitrogen accumulation, which is classified as zero-growth type. In contrast, WL298 and G3 exhibited consumptive behavior due to the high-energy demand associated with the nitrogen fixation process mediated by nitrogenase [44]. In cases where there is poor symbiotic interaction between varieties and rhizobia, excessive nodular consumption may occur without corresponding benefits for plant growth or effective nitrogen fixation, ultimately exerting negative effects on plant biomass and substance accumulation. The findings of this study contradict previous research that showed LL2 had a beneficial growth-promoting effect on G3 [17]. The variation in the impact of root nodules on plants may be due to differences in sampling time. In the previous study, samples were taken during the peak phase of symbiotic nitrogen fixation when plants were smaller and root nodule activity was higher. The substances produced through symbiotic nitrogen fixation were enough to support both their own growth and that of plants, resulting in positive biomass accumulation. However, over time, there was a gradual decline in root nodule aging and nitrogen fixation capacity. Nonetheless, the root nodule itself continued to perform vital life activities, depleting plant energy without efficient nitrogen fixation. As a result, this negatively affected material accumulation in plants. These factors may contribute to the discrepancy between this study and previous findings.

In this study, we assessed the symbiotic nitrogen fixation and growth-promoting effects of eight alfalfa varieties, with a specific focus on the final outcomes of eight combinations of symbiotic nitrogen fixation. However, it is important to note that there are intricate regulatory mechanisms and pathways within the symbiotic system formed by alfalfa and rhizobia that have the potential to influence the ultimate outcomes. Therefore, further exploration and research are warranted.

5. Conclusions

The efficiency of symbiotic nitrogen fixation varied among rhizobia strains and alfalfa varieties. The nitrogen fixation effect is primarily influenced by the phenotype and structural characteristics of efficient root nodules, as well as the number of infected root nodules. When alfalfa varieties and rhizobia strains have high adaptability, it enhances nitrogen fixation ability and positively impacts biomass accumulation. Conversely, low adaptability leads to reduced nitrogen fixation ability and negatively affects biomass accumulation. Rhizobia can only obtain a nitrogen source for alfalfa, and its allocation to different tissues and organs is regulated by the host plant. These findings are significant for regulating nitrogen fixation in the legume–rhizobia symbiosis.

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