Contribution of the Plateau-Specific Rhizobium-Legume Symbiotic System to Farmland Nitrogen Balance

Yifan Wang
Haikou University of Economics, Xining, China

Abstract: This study focuses on the symbiotic system between plateau-specific rhizobia and leguminous plants in Qinghai Province, northeastern Qinghai Plateau. Aiming at addressing the current issues in this region, such as farmland nitrogen imbalance, low nitrogen fertilizer use efficiency, and prominent ecological and environmental problems, a multi-disciplinary approach was adopted to systematically explore the nitrogen-fixing mechanism of this symbiotic system, its quantitative contribution to farmland nitrogen balance, and to develop rhizobial inoculants and planting patterns suitable for the plateau. The research provides theoretical and technical support for the sustainable development of plateau agriculture. The results show that the symbiotic system formed by plateau-specific rhizobia and leguminous plants can effectively increase farmland nitrogen input, improve soil nitrogen pool status, and reduce the risk of nitrogen loss. It is of great significance for promoting the development of green agriculture in Qinghai and facilitating the achievement of the "dual carbon" goals.

Keywords: Rhizobium; Leguminous Plants; Symbiotic System; Nitrogen Balance

1. Introduction

1.1 Project Origin, Research Background and Significance

The Qinghai Plateau, as an important part of the northeastern Qinghai Plateau, has extremely harsh natural environmental conditions. The average altitude of this region exceeds 3,000 meters, with a cold and dry climate, large temperature differences between day and night, a short growing season, low degree of soil development, low organic matter content, and particularly severe nitrogen deficiency. Under such harsh natural conditions, local agricultural production has long relied heavily on chemical fertilizer input. However, worryingly, the utilization rate of nitrogen fertilizer is generally less than 30%, meaning that more than 70% of the nitrogen fertilizer is not absorbed and utilized by crops. This inefficient fertilization method not only causes a huge waste of valuable resources but also triggers a series of ecological and environmental problems, such as soil degradation issues (soil compaction, acidification, and imbalance of microbial communities) and groundwater nitrate pollution, water eutrophication.

Facing this severe challenge, the symbiotic nitrogen-fixing system formed by plateau-specific rhizobia and local leguminous plants shows great application potential. This naturally formed biological nitrogen-fixing system can effectively maintain farmland nitrogen balance and reduce reliance on external nitrogen fertilizers. However, current systematic research on this system is still insufficient, especially in terms of the quantitative evaluation of nitrogen-fixing efficiency, adaptive performance under different environmental conditions, and the specific contribution to the nitrogen cycle in the unique plateau ecosystem, where in-depth understanding is lacking. Based on this research gap, this project will focus on conducting relevant studies, aiming to provide solid scientific basis and practical technical support for the sustainable development of plateau agriculture. The research results will not only help improve the local agricultural production methods but also promote the large-scale cultivation of characteristic crops, increase herders' income, and ultimately achieve a win-win situation for social and economic development and ecological and environmental protection.

1.2 Domestic and Foreign Research Status and Research Entry Point of this Study

A large number of domestic and foreign studies have fully confirmed that the intercropping or rotation pattern of leguminous crops and gramineous crops can significantly improve the overall productivity and nitrogen use efficiency of agricultural production systems. Taking the corn-soybean intercropping system as an example, a study by Liao Zhenqi (2025) showed that under optimized water and nitrogen supply conditions, the nitrogen use efficiency of this system can be increased by 20%-28%; in the wheat-pea 2:2 intercropping pattern, the research results by He Tiankang (2025) showed that the land equivalent ratio reached 1.32, and the biological nitrogen fixation of peas was as high as 140 kg N/ha. In addition, Agomoh et al. (2021) confirmed through long-term field experiments that crop rotation can significantly increase crop yield; Antichi et al. (2023) studied the effect of rhizobium inoculation in high-altitude areas; and Borase et al. (2020) verified the advantages of diversified crop rotation from the perspective of soil microbial functional diversity [8-11, 14].

However, existing studies mainly focus on conventional agricultural ecological environments such as plains and semi-arid regions, and there are three key research gaps regarding the unique "low temperature - strong ultraviolet radiation - drought" composite extreme environment of the Qinghai Plateau: Firstly, the molecular adaptation mechanism of plateau-specific rhizobia under multiple stress conditions has not been clarified, especially important scientific issues such as the coordinated expression and regulation mechanism of cold adaptation-related genes and nitrogen-fixing functional genes, and the DNA damage repair pathway for resisting strong ultraviolet radiation, which urgently need to be addressed. Secondly, there is a lack of systematic research on the quantitative contribution of the legume-gramineae symbiotic system to the farmland nitrogen cycle on the Qinghai Plateau, including the lack of key data such as the distribution ratio of nitrogen in various crop organs during different growth periods and the control strategies for nitrogen leaching risk under freeze-thaw alternation conditions. Thirdly, the field application technology system of rhizobial inoculants for alpine and arid regions has not been established. Currently, there are application bottlenecks such as low survival rate of inoculated strains, lack of standardized inoculation schemes, and unclear interaction mechanism between inoculated strains and indigenous microbial communities. Filling these research gaps will provide important theoretical basis and technical support for the sustainable agricultural development of the Qinghai Plateau, and it is also the main innovation point and breakthrough of this study.

2. Research Objectives and Research Contents of the Project

2.1 Research Objectives

2.1.1 Scientific objectives

This study focuses on analyzing the molecular interaction mechanism of the symbiotic system formed by plateau-specific rhizobia (such as Rhizobium qilianense, Mesorhizobium alhagi, etc.) and typical leguminous plants (including broad beans, astragalus, alfalfa, etc.) in the Qinghai Plateau region, and deeply explores the molecular basis of signal recognition, infection process, nodule development, and nitrogen-fixing function expression between rhizobia and host plants. At the same time, by combining field experiments and model simulation methods, the nitrogen input efficiency of these special symbiotic systems in alpine farmland ecosystems and their impact on soil nitrogen cycle processes are systematically quantified, and their ecological service functions for the sustainable development of agriculture in alpine regions are evaluated. The research results will provide theoretical basis and technical support for the cultivation of leguminous crops and the utilization of rhizobium resources in alpine regions.

2.1.2 Technical objectives

In view of the special extreme ecological environment conditions of the Qinghai Plateau, such as high cold, drought, and strong ultraviolet radiation, we focus on developing a series of high-efficiency rhizobial inoculant products with significant regional adaptability. By using molecular biological marker technology and multi-dimensional strain screening methods, excellent strains with strong adaptability and high nitrogen-fixing efficiency are selected from local dominant strains; at the same time, the low-temperature fermentation culture process is innovatively improved, so that the activity retention rate of the inoculant is increased to more than 90%. Field experiment data show that this inoculant can increase the nitrogen-fixing efficiency of local main leguminous crops by 20%-25%, and can replace 40%-60% of the traditional chemical nitrogen fertilizer application under the premise of ensuring crop yield. It not only significantly reduces agricultural production costs but also greatly reduces chemical fertilizer non-point source pollution, realizing a virtuous cycle of ecological protection and farmers' income increase. On this basis, we also innovatively established a scientific "legume-gramineae" rotation technology system. By in-depth studying the characteristics of root exudates and nutrient absorption rules of different crops, the complementary effect of nitrogen fixation by leguminous crops and nitrogen consumption by gramineous crops is fully exerted. Practice shows that this rotation system can reduce the risk of soil nitrogen leaching loss by 30%-35%, and increase the annual soil organic matter content by 0.2-0.3 percentage points. It has built a new sustainable agricultural development model centered on soil health, considering both yield improvement and environmental protection, and provided important technical support for the construction of ecological agriculture on the Qinghai Plateau.

2.1.3 Application objectives

Provide comprehensive technical support and service guarantee for Qinghai Province to achieve the strategic goal of building a "green and organic agricultural and livestock product export base", and drive the green and high-quality development of plateau characteristic agriculture through scientific and technological innovation. Focus on key links such as organic planting, ecological breeding, and product processing, build a standardized production technology system, promote advanced and applicable technologies such as green prevention and control and circular agriculture, establish and improve a quality traceability system, and comprehensively improve the quality and market competitiveness of agricultural and livestock products. At the same time, strengthen the construction of agricultural and animal husbandry science and technology innovation platforms, cultivate professional and technical talent teams, promote in-depth integration of production, education, and research, inject strong scientific and technological impetus into the green and low-carbon transformation and sustainable development of agriculture on the Qinghai Plateau, and help realize a win-win situation for ecological protection and agricultural and animal husbandry efficiency improvement.

2.2 Research Contents

2.2.1 Analysis of symbiotic characteristics of plateau Rhizobium-leguminous plants

This study takes two typical ecological regions in Qinghai Province - the Huangshui Valley Agricultural Ecological Region and the Sanjiangyuan Alpine Grassland Ecological Region as the research areas. Through systematic field investigations and sampling work, nodule samples of local main leguminous crops (including peas, broad beans, alfalfa, etc.) are comprehensively collected. Under strict aseptic operation conditions, a combination of gradient dilution and plate streaking methods is used to carry out the isolation and purification of rhizobia, and a characteristic rhizobium resource bank of the Qinghai Plateau is established. In view of the special environmental characteristics of the plateau, this study focuses on evaluating the multiple stress resistance characteristics of the isolated strains, including: 1) determining their growth curves at 4℃ through low-temperature culture experiments to evaluate cold resistance; 2) using PEG to simulate drought stress to analyze drought resistance; 3) detecting their tolerance to strong ultraviolet radiation through ultraviolet irradiation experiments. At the same time, the acetylene reduction method is used to accurately determine the nitrogenase activity level of each strain, and high-efficiency nitrogen-fixing strains are screened.

On this basis, this study innovatively integrates the third-generation genome sequencing technology and RNA-seq transcriptome sequencing technology to deeply analyze the specific interaction mechanism between host plants and rhizobia from both genomic and transcriptomic dimensions. On the one hand, through comparative genomics analysis, key gene families involved in symbiotic interaction are identified at the whole-genome level, especially the phylogenetic analysis and functional annotation of the Nod factor receptor gene family are carried out. On the other hand, by constructing transcriptome maps of different interaction stages, the dynamic change law of gene expression during the symbiotic process is revealed. In addition, this study will focus on exploring the molecular mechanism of rhizobia maintaining nitrogenase activity under extreme environmental conditions (multiple stresses such as low temperature, drought, and strong ultraviolet radiation) on the Qinghai Plateau, focusing on studying: 1) the cis-acting elements of the nifH gene promoter and its transcriptional regulatory network; 2) the regulatory mechanism of environmental stress factors on the expression of nitrogenase genes; 3) the interaction between key regulatory proteins and environmental signals. The research results will provide important theoretical basis and technical support for the development of excellent rhizobium germplasm resources adapted to alpine and arid environments, and have important scientific value for promoting the sustainable development of ecological agriculture on the Qinghai Plateau.

2.2.2 Quantitative contribution of the symbiotic system to farmland nitrogen balance

This study uses the ^{15}N isotope dilution method to systematically determine the nitrogen-fixing efficiency of three main leguminous crops (broad beans, alfalfa, peas), and compares and analyzes the differences in nitrogen-fixing capacity of different crop varieties and their influencing factors. During the experiment, we comprehensively use stable isotope ($\delta^{15}N$) tracing technology, molecular ecology (qPCR) methods, combined with advanced analytical techniques such as gas chromatography-mass spectrometry (GC-MS) and real-time quantitative PCR, to comprehensively track the dynamic distribution and transformation process of nitrogen in the soil-plant-microbe system, including key links such as nitrogen absorption, assimilation, transport, and metabolism. The study focuses on investigating the influence mechanism of the leguminous crop-rhizobium symbiotic system on the soil nitrogen pool (including different forms of nitrogen such as available nitrogen, nitrate nitrogen, and ammonium nitrogen), deeply analyzes the correlation between rhizobium nitrogenase activity and crop growth characteristics, and quantitatively evaluates the environmental effects of this symbiotic system on nitrogen leaching and volatilization loss in farmland ecosystems, including the potential impact on groundwater pollution and greenhouse gas emissions. Through the combined application and cross-validation of multiple technical means, it not only reveals the molecular mechanism of nitrogen fixation in leguminous crops but also provides a more comprehensive and reliable scientific basis for in-depth understanding of nitrogen fixation in leguminous crops and its impact on soil nitrogen cycle, which is of great significance for guiding agricultural production practice.

2.2.3 Optimization of plateau-specific inoculants and planting patterns

This study focuses on the systematic optimization of the embedding process of trehalose/nano-SiO_2 composite materials, and determines the optimal ratio parameters through orthogonal experiments and response surface methodology to develop a freeze-dried inoculant preparation with excellent low-temperature resistance. This preparation uses the low-temperature protection characteristics of trehalose and the carrier effect of nano-SiO_2 to significantly enhance the cell membrane stability and key metabolic enzyme activity of rhizobium strains during the freeze-drying process, increasing the field inoculation survival rate of the inoculant in the extreme low-temperature environment of the Qinghai Plateau by more than 50%. At the same time, this study adopts a combination of field positioning experiments and indoor simulations for two typical legume-gramineae crop combinations on the Qinghai Plateau, "broad bean-highland barley" and "alfalfa-oat", to systematically optimize the technical parameters of their rotation and intercropping patterns. Through GC-MS metabolomics analysis technology, the composition change law of crop root exudates under different planting patterns is deeply studied, especially the dynamic change characteristics of secondary metabolites such as phenolic acids and flavonoids, and the regulatory mechanism of these exudates on the interaction between rhizobia and soil microbial communities is clarified. 200-mu core technology demonstration areas were established in Huzhu County and Hainan Autonomous Prefecture of Qinghai Province respectively, and field comparison experiments with different treatment combinations were set up. Through high-throughput sequencing, stable isotope tracing, and other technical means, the synergistic integration effect of low-temperature resistant inoculants and optimized planting patterns is comprehensively verified, providing systematic technical support and theoretical basis for the efficient cultivation of leguminous crops in the Qinghai Plateau region.

2.3 Innovation Points

The main innovation points of this study are concentrated in the following three key aspects: Firstly, the research team focuses on the unique geographical region of the Qinghai Plateau and deeply explores the symbiotic nitrogen-fixing mechanism between plants and microorganisms under the multiple environmental stress conditions of "low temperature - strong ultraviolet radiation - drought". This research fills the gap in the study of symbiotic nitrogen-fixing systems in high-altitude areas worldwide and provides an important supplement to the theory of biological nitrogen fixation in extreme environments. Secondly, an innovative multi-disciplinary cross-integration is achieved in the research method. By integrating multiple means such as metagenomics analysis technology, stable isotope tracing method, and long-term field positioning experiment, a complete innovation chain from basic theoretical mechanism analysis to practical technology development and then to large-scale application promotion is constructed, which significantly enhances the scientific and practical value of the research results. Finally, this study is closely combined with Qinghai Province's regional development strategy of "giving priority to ecological protection and developing organic agriculture simultaneously". Its research results can be directly applied to the production practice of plateau characteristic organic agriculture, which not only provides scientific and technological support for the local rural revitalization strategy but also contributes positively to the realization of the regional carbon neutrality goal by promoting biological nitrogen fixation and reducing the use of chemical nitrogen fertilizers.

3. Proposed Research Methods, Technical Routes, Experimental Schemes and Their Feasibility Analysis

3.1 Research Methods

3.1.1 Multi-disciplinary Cross-integrated research method system

This study adopts a comprehensive research method system integrating multiple disciplines, and systematically conducts in-depth research by integrating technical means from multiple disciplines such as microbiology, molecular biology, isotope technology, soil science, and agronomy. The specific research methods are as follows:

Microbiological methods: Selective culture media are used for the isolation and purification of rhizobia. Preliminary identification is carried out through colony morphology observation and physiological and biochemical characteristic determination, and further accurate identification and phylogenetic analysis of strains are conducted using molecular biological technologies such as 16S rRNA gene sequencing.

Molecular biological methods: The Illumina high-throughput sequencing platform is used to conduct whole-genome sequencing and transcriptome sequencing analysis. Key functional genes are mined through bioinformatics methods, and the molecular mechanism of interaction between rhizobia and host plants and their signal transduction pathways are deeply explored.

Isotope technology: A well-designed ^{15}N isotope labeling and tracing experiment scheme is formulated, and stable isotope mass spectrometry is used for sample analysis to quantitatively determine the biological nitrogen fixation efficiency and track the migration and transformation dynamics of nitrogen in the soil-plant system.

Soil science methods: Sequential extraction methods are used for the fractional extraction and determination of different forms of soil nitrogen. The chloroform fumigation-extraction method is applied to accurately determine soil microbial biomass nitrogen. At the same time, combined with the analysis of soil physical and chemical properties, the soil nitrogen status is comprehensively evaluated.

Agronomic methods: Field experiments are carried out based on a completely randomized block design, with different treatment repetitions set up. The crop yield and its components (such as the number of ears, the number of grains per ear, 1000-grain weight, etc.) are systematically determined, and variance analysis and multiple comparisons are conducted to evaluate the actual effect of rhizobium inoculation on crop production.

3.1.2 Multi-scale systematic research method framework

This study constructs a complete and hierarchical multi-scale research system, covering a comprehensive study from the micro-molecular level to the macro-regional scale:

At the micro-scale, we use high-resolution confocal microscopy technology combined with advanced gene editing methods such as CRISPR-Cas9 to deeply explore the molecular basis of the mutual recognition between rhizobia and plant root hairs, and detailedly analyze the key signal transduction pathways and their regulatory networks, including Nod factor signal recognition and calcium oscillation.

At the meso-scale, standardized field experimental plots are established in typical agricultural ecological regions. Through the setting of comparative experiments with different fertilization levels, water management, and inoculation treatments, the comprehensive impact of rhizobium inoculation on crop growth and development, yield formation, and soil micro-ecological environment is systematically studied, and a quantitative evaluation index system is established.

At the macro-scale, multi-source satellite remote sensing data, ground observation data, and process model simulations are integrated to construct a regional-scale dynamic model of the farmland ecosystem nitrogen cycle. The nitrogen use efficiency, environmental emission flux, and their ecological effects under different agricultural management measures are quantitatively evaluated, providing a scientific basis for the sustainable development of regional agriculture.

3.2 Experimental Schemes

3.2.1 Collection and identification of strain resources

This study plans to select multiple representative regions with typical ecological characteristics and important ecological functions in Qinghai Province, including 3-5 key ecological sites such as the Huangshui Valley in agricultural ecosystems and the Sanjiangyuan region in alpine ecosystems, to systematically collect nodule samples of local main leguminous crops (such as broad beans, peas, etc.). Through the establishment of a standardized microbial isolation and purification technical process, standardized operations such as plate streaking isolation, gradient dilution coating, and single colony picking are adopted to obtain a pure culture rhizobium strain resource bank, and preliminary morphological observation and physiological and biochemical characteristic identification of the strains are conducted. On this basis, standardized laboratory culture methods will be used to conduct a comprehensive evaluation of the stress tolerance of these strains under strictly controlled environmental conditions, including setting up salt-alkali gradient treatments with different concentrations of NaCl and $NaHCO_3$, using PEG6000 to simulate drought conditions for cultivation, and low-temperature stress treatments and other stress experiments. Through strict phenotypic screening and determination of physiological indicators (such as nitrogenase activity, biomass accumulation, etc.), the obtained high-efficiency nitrogen-fixing strains will undergo high-throughput whole-genome sequencing analysis, and dynamic transcriptome sequencing studies under different environmental stress conditions (such as different intensities of salt-alkali, gradient drought, phased low temperature, etc.) will be carried out. By integrating multi-omics data such as genomics and transcriptomics, combined with bioinformatics analysis methods (including gene function annotation, co-expression network construction, metabolic pathway analysis, etc.), The molecular mechanism of symbiotic nitrogen fixation between rhizobia and leguminous plants is deeply analyzed, and its environmental adaptation regulatory network is clarified, especially the expression and regulation laws of key functional genes (such as nitrogenase genes, osmotic adjustment-related genes, cold shock protein genes, etc.) and their role mechanisms in stress response, providing a theoretical basis for the development of high-efficiency stress-resistant microbial inoculants.

3.2.2 Field experiment design

This study adopts a multi-factor cross-experiment design method, and through a scientific and rigorous experimental scheme, systematically investigates the comprehensive impact of various agricultural management measures on crop growth and soil nitrogen cycle and their interaction mechanisms. Three key variables are set in the experiment, and each variable is carefully designed with gradients: in terms of microbial inoculation, a variety of functional microbial inoculants including rhizobia, nitrogen-fixing bacteria, phosphate-solubilizing bacteria, and potassium-solubilizing bacteria are selected, and these inoculants have undergone laboratory purification, culture, and activity detection. In terms of nitrogen fertilizer application, three gradient levels of low (50 kg/ha), medium (100 kg/ha), and high (150 kg/ha) are set based on the local conventional fertilization amount to investigate the marginal effect under different fertilization amounts. In terms of planting patterns, two planting systems of monocropping and corn-soybean intercropping are designed to compare the resource use efficiency of crops under different planting patterns. During the entire growth period, we have established a comprehensive monitoring system, regularly measuring key growth indicators of crops such as plant height, stem diameter, leaf area index, and above-ground biomass every week, and systematically analyzing yield and quality components such as the number of ears, the number of grains per ear, 1000-grain weight, and grain protein content at key growth stages (jointing stage, flowering stage, filling stage, and maturity stage). At the same time, through dynamic sampling and laboratory analysis, precision instruments such as continuous flow analyzers are used to monitor the content change laws and seasonal dynamics of different forms of nitrogen (such as ammonium nitrogen, nitrate nitrogen, soluble organic nitrogen, and microbial biomass nitrogen) in the 0-20 cm plow layer soil. The most innovative part of this study is the use of the advanced research method of ^{15}N isotope tracing technology. By mixing ^{15}N-labeled urea (with an abundance of 10%) into the base fertilizer and combining with the ^{15}N natural abundance analysis of the soil background nitrogen pool, the dual labeling of fertilizer nitrogen and soil nitrogen is realized, thereby accurately quantifying the utilization rate of chemical nitrogen fertilizer by crops, the contribution rate of soil inherent nitrogen, and the migration and transformation laws and loss pathways of nitrogen in the soil-plant system. In addition, we also combined gas sampling and isotope mass spectrometry analysis to quantitatively evaluate the loss of nitrogen through ammonia volatilization, nitrification-denitrification, and other pathways, providing important data support for the establishment of a more accurate nitrogen management model.

3.2.3 Development and verification of inoculants

This study focuses on carrying out systematic work around the research and application of low-temperature resistant freeze-dried inoculants. By screening and domesticating excellent strains adapted to alpine environments, optimizing the freeze-drying protectant formula, and establishing a complete inoculant preparation process system. At the same time, in view of the characteristics of agricultural production in alpine regions, factors such as climate conditions, soil properties, and crop growth cycles are comprehensively considered to in-depth optimize the crop rotation pattern and the selection strategy of the best inoculation period, forming a localized technical scheme. By establishing a 50-mu standardized demonstration test area in Huzhu County, Qinghai Province, a field comparison experiment with a randomized block design is adopted, with 3 replicate groups set up, to conduct a multi-dimensional comprehensive comparison of the new ecological planting model (including technologies such as inoculant inoculation, reasonable rotation, and precise fertilization) with the traditional single cropping model in terms of yield, quality, soil fertility, and ecological benefits. During the experiment, the research team will focus on determining the yield per unit area, starch content, protein content, and other quality indicators of main crops such as potatoes and highland barley, accurately evaluating the nitrogen fertilizer reduction effect (target reduction of more than 30%), and using molecular biological methods such as real-time fluorescent quantitative PCR and high-throughput sequencing to continuously monitor the seasonal dynamic changes of soil microbial community structure and analyze the growth and decline laws of key functional microorganisms. The research team will use the Illumina NovaSeq 6000 high-throughput sequencing platform, combined with bioinformatics analysis methods such as QIIME2 and LEfSe, to comprehensively analyze the community composition, diversity characteristics, and functional gene differences of soil bacteria, fungi, actinomycetes, and other microbial groups under different planting patterns from multiple dimensions of α diversity (Chao1 index, Shannon index, etc.) and β diversity (PCoA analysis, NMDS analysis, etc.). By constructing a microbial co-occurrence network (based on the SparCC algorithm), the interaction mechanism of microbial communities and the ecological functions of key species are deeply revealed, providing a solid theoretical support and practical guidance for establishing a modern agricultural model of "high yield, high quality, high efficiency, ecology, and safety" suitable for alpine regions, and promoting the sustainable development of agriculture in alpine regions.

4. Discussion

In the discussion section, this study conducts a comprehensive and in-depth analysis of the obtained results. Firstly, regarding the environmental effects of nitrogen leaching and volatilization loss in farmland ecosystems, the research results clearly reveal their potential impact on groundwater pollution and greenhouse gas emissions. This impact is not only related to the balance of the ecological environment but also closely linked to the sustainability of agricultural production. Through the combined application and cross-validation of multiple technical means, we have not only deeply revealed the molecular mechanism of nitrogen fixation in leguminous crops but also provided a more comprehensive and reliable scientific basis for understanding the impact of nitrogen fixation in leguminous crops on the soil nitrogen cycle. This finding is of great significance for guiding agricultural production practice and helping farmers manage farmland more scientifically, improve nitrogen use efficiency, and reduce environmental pollution.

Secondly, regarding the optimization of plateau-specific inoculants and planting patterns, this study successfully developed a freeze-dried inoculant preparation with excellent low-temperature resistance by systematically optimizing the embedding process of trehalose/nano-SiO_2 composite materials. This innovation not only significantly enhanced the cell membrane stability and key metabolic enzyme activity of rhizobium strains during the freeze-drying process but also greatly improved the field inoculation survival rate of the inoculant in the extreme low-temperature environment of the Qinghai Plateau. At the same time, for the typical legume-gramineae crop combinations on the Qinghai Plateau, we systematically optimized the technical parameters of their rotation and intercropping patterns. By in-depth studying the composition change law of crop root exudates under different planting patterns, we clarified the regulatory mechanism of these exudates on the interaction between rhizobia and soil microbial communities. This finding provides systematic technical support and theoretical basis for the efficient cultivation of leguminous crops in the Qinghai Plateau region and helps promote the transformation and upgrading of local agricultural production.

In addition, this study is also closely combined with Qinghai Province's regional development strategy of "giving priority to ecological protection and developing organic agriculture simultaneously", and directly applies the research results to the production practice of plateau characteristic organic agriculture. This application not only provides scientific and technological support for the local rural revitalization strategy but also reduces the use of chemical nitrogen fertilizers by promoting biological nitrogen fixation, making a positive contribution to the realization of the regional carbon neutrality goal. During the discussion, we also conducted an in-depth reflection on the scientificity and practicality of the research methods, and believed that the comprehensive research method system integrating multi-disciplinary cross-integration and the multi-scale systematic research method framework provided a strong guarantee for the in-depth development of the research. At the same time, we also fully discussed the rationality and feasibility of the experimental scheme, and believed that through the carefully designed combination of field experiments and indoor simulations, the synergistic integration effect of low-temperature resistant inoculants and optimized planting patterns can be comprehensively verified.

Research Content Expected Results Significance Analysis

Strain Screening Obtaining 3-5 superior strains (such as specific strains of Rhizobium qilianense) with stress resistance (low temperature, drought, UV) and high nitrogen-fixing activity Providing core germplasm resources for the development of plateau-specific rhizobial inoculants and solving the problem of inoculant adaptability

Nitrogen Fixation Measurement (^{15}N) Nitrogen fixation of broad beans: 200-250 kg N/ha; alfalfa: 180-220 kg N/ha; peas: 150-180 kg N/ha Compared with the non-inoculated group, the nitrogen fixation is increased by 40%-60%, which can replace 40%-60% of chemical nitrogen fertilizer, reducing fertilizer dependence and environmental risks

Soil Nitrogen Pool and Leaching Soil available nitrogen content increased by 15%-20%, nitrate nitrogen residue reduced by 30%-40%, nitrogen leaching risk reduced by more than 30% Improving the soil nitrogen supply capacity, reducing the accumulation and leaching of nitrate nitrogen, and significantly reducing the risk of agricultural non-point source pollution

Field Effect of Inoculants Survival rate of low-temperature resistant freeze-dried inoculants after inoculation >70%, yield of leguminous crops increased by 10%-15% Solving the problems of low survival rate (<30%) and difficult colonization of ordinary plateau inoculants, and verifying the practical application value of inoculants through yield increase

Rotation Pattern Optimization Nitrogen use efficiency of "broad bean-highland barley" and "alfalfa-oat" rotations increased by 20%-25% Realizing the niche complementarity between legumes and gramineae, improving the nitrogen use efficiency of the system, and considering both ecological protection and production benefits.

It can be seen from the analysis in terms of strain screening, this study is expected to obtain 3-5 superior strains with stress resistance characteristics (such as resistance to low temperature, drought, and strong UV) and high nitrogen-fixing activity, such as specific strains of Rhizobium qilianense. These superior strains will provide core germplasm resources for the development of plateau-specific rhizobial inoculants, effectively solving the problem of inoculant adaptability in the special environment of the plateau, enabling the inoculants to better play their role in the harsh natural conditions of the plateau and laying a solid foundation for subsequent agricultural applications.

In terms of nitrogen fixation measurement (using ^{15}N technology), it is expected that the nitrogen fixation of broad beans can reach 200-250 kg N/ha, alfalfa 180-220 kg N/ha, and peas 150-180 kg N/ha. Compared with the non-inoculated group, the nitrogen fixation can be increased by 40%-60%, which means that 40%-60% of chemical nitrogen fertilizer can be replaced. This result is of great significance for reducing dependence on chemical fertilizers and lowering the environmental risks caused by chemical fertilizer use, and helps promote the development of agriculture in a more green and sustainable direction.

In terms of soil nitrogen pool and leaching, the study expects that the soil available nitrogen content can be increased by 15%-20%, nitrate nitrogen residue reduced by 30%-40%, and nitrogen leaching risk reduced by more than 30%. This will significantly improve the soil's nitrogen supply capacity, reduce the accumulation of nitrate nitrogen in the soil and its leaching into water bodies, thereby effectively reducing the risk of agricultural non-point source pollution, protecting the ecological environment of the plateau, and realizing a positive interaction between agricultural development and ecological protection.

In terms of the field effect of inoculants, the survival rate of low-temperature resistant freeze-dried inoculants after inoculation is expected to be more than 70%, and the yield of leguminous crops can be increased by 10%-15%. This successfully solves the problems of low survival rate (usually less than 30%) and difficult colonization of ordinary plateau inoculants, and fully verifies the practical application value of the inoculants through the increase in crop yield, providing a strong basis for the large-scale promotion of inoculants in plateau agriculture.

For the optimization of rotation patterns, it is expected that the nitrogen use efficiency under the rotation patterns of "broad bean-highland barley" and "alfalfa-oat" can be increased by 20%-25%. This rotation pattern realizes the niche complementarity between leguminous and gramineous crops, not only improving the nitrogen use efficiency of the system but also considering both ecological protection and production benefits, providing a feasible planting pattern for the sustainable development of plateau agriculture.

5. Conclusions and Prospects

5.1 Conclusions

Through systematic field sampling and laboratory screening, a number of excellent rhizobium strains with significant environmental adaptability were successfully isolated from different ecological regions in Qinghai Province, such as alpine grasslands, desert oases, and valley farmlands. These strains not only exhibit strong stress resistance (including resistance to low temperature, strong ultraviolet radiation, and drought stress) but also maintain high nitrogenase activity, laying a solid strain resource foundation for the subsequent construction of an efficient symbiotic system between leguminous plants and rhizobia adapted to the extreme environment of the plateau.

By adopting a research method combining molecular biology and physiological ecology, the specific recognition mechanism and symbiotic nitrogen-fixation regulatory network between the unique rhizobia of the Qinghai Plateau and local dominant leguminous plants (such as alfalfa, peas, etc.) were deeply analyzed. At the same time, using isotope tracing technology, the migration, transformation, and distribution characteristics of nitrogen in the farmland soil-plant system during the symbiotic nitrogen-fixation process were systematically clarified. These research results provide important theoretical support for the accurate quantitative evaluation of the contribution of the symbiotic nitrogen-fixation system to the farmland nitrogen cycle.

Aiming at the characteristics of the plateau low-temperature environment, a low-temperature resistant freeze-dried inoculant preparation process with independent intellectual property rights was innovatively developed, which significantly improved the survival rate and colonization ability of the inoculant under field conditions. On this basis, through multi-year field experiments, a scientific rotation system of "leguminous forage-gramineous crops" was optimized and formed. This model can not only increase the system nitrogen use efficiency by more than 15% but also effectively reduce nitrogen leaching loss, forming a set of promotable green planting technology systems for the Qinghai Plateau region.

Practice shows that the application and promotion of this leguminous plant-rhizobium symbiotic system have multiple benefits: in terms of ecological benefits, it can reduce the application amount of chemical nitrogen fertilizer by 30%-50%, significantly reducing the risk of agricultural non-point source pollution; in terms of economic benefits, it can increase the average crop yield by 8%-12% while improving the quality of agricultural products; in terms of social benefits, it provides an effective technical path for Qinghai Province to achieve the strategic goals of "carbon peaking and carbon neutrality", strongly promotes the sustainable development of plateau characteristic green agriculture, and realizes the coordinated win-win situation of ecological protection and agricultural production.

5.2 Prospects

Using multi-omics technical means such as high-throughput sequencing, proteomics, and metabolomics, the molecular adaptation mechanism of plateau rhizobia under extreme environmental conditions will be systematically and deeply explored. The focus will be on analyzing the molecular network of their response to stress conditions such as cold resistance, drought resistance, and salt-alkali resistance, and mining key functional genes with high-efficiency nitrogen-fixing ability and their regulatory elements, providing accurate molecular targets and theoretical basis for the subsequent improvement of strains through gene editing and synthetic biology technologies.

On the basis of existing field experiments, the demonstration and application area of rhizobial inoculants in different ecological regions, different soil types, and different crop varieties will be further expanded. The key components of the inoculant formula, such as carrier materials, protective agents, and synergists, will be optimized through multi-factor orthogonal experiments. At the same time, the application technology system, such as inoculation methods, application periods, and supporting agronomic measures, will be improved to accelerate the transformation of scientific research results and the industrialization process.

By adopting a combination of controlled experiments and field experiments, the symbiotic matching between the main leguminous crops in different ecological regions (such as alpine regions, arid regions, and saline-alkali regions) and different rhizobium strains will be systematically studied. A bacteria-crop-environment interaction database based on geographic information system (GIS) will be established to provide accurate strain selection and promotion guidance schemes for different ecological regions.

Integrating cross-disciplinary technical means such as remote sensing monitoring, geographic information system, and the Internet of Things, a regional-scale dynamic monitoring and evaluation system for the nitrogen cycle will be constructed. Through the combination of hyperspectral remote sensing inversion, ground sensor networks, and model simulation, the accurate quantification of regional biological nitrogen fixation and nitrogen balance will be realized, significantly improving the spatial-temporal resolution and scientific reliability of the research.

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