

Screening and Functional Verification of Pine Rhizosphere Nitrogen-Fixing Bacteria

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Abstract The study investigates the diversity and functionality of nitrogen-fixing bacteria in the rhizosphere of pine trees. The research involved isolating and identifying nitrogen-fixing bacteria from the rhizosphere soil of pine trees and assessing their potential to enhance nitrogen availability and promote plant growth. Various bacterial strains were isolated and screened for nitrogenase activity, with a focus on their genetic and functional diversity. The findings revealed a significant presence of nitrogen-fixing bacteria, including genera such as Burkholderia, Bradyrhizobium, and Pseudomonas, which are known for their nitrogen-fixing capabilities. The study highlights the potential of these bacteria to improve soil fertility and support sustainable forest management practices by enhancing nitrogen cycling in pine ecosystems. The results underscore the importance of microbial interactions in the rhizosphere and their role in promoting plant health and growth.

Keywords Nitrogen-fixing bacteria; Pine rhizosphere; Soil fertility; Microbial diversity; Plant growth promotion

1 Introduction

Nitrogen is a critical nutrient for plant growth, yet it is often a limiting factor in many ecosystems. Biological nitrogen fixation, the process by which atmospheric nitrogen (N₂) is converted into ammonia (NH₃) by certain bacteria, plays a vital role in the nitrogen cycle. These nitrogen-fixing bacteria can be free-living in the soil or form symbiotic relationships with plant roots, particularly in the rhizosphere, the region of soil influenced by root secretions and associated microbial activity (Suarez et al., 2014; Beneduzi et al., 2020; Singh et al., 2020). The *nifH* gene, which encodes a key enzyme in the nitrogen fixation process, is commonly used as a marker to identify and study these bacteria (Singh et al., 2020).

In pine ecosystems, the rhizosphere is a hotspot for microbial activity, including nitrogen fixation. Rhizosphere bacteria not only contribute to nitrogen availability but also enhance plant growth through various mechanisms such as producing growth hormones, solubilizing phosphates, and protecting plants from pathogens (Igiehon and Babalola, 2018; Rilling et al., 2018; Renoud et al., 2020). The presence of nitrogen-fixing bacteria in the rhizosphere of pine trees can significantly impact soil fertility and plant health, making them crucial for the sustainability of these ecosystems (Franche et al., 2009; Zarezadeh et al., 2018; Ke et al., 2019). Understanding the diversity and functionality of these bacteria can lead to better management practices and the development of biofertilizers that reduce the need for chemical fertilizers, thereby mitigating environmental impacts (Li et al., 2017; Igiehon and Babalola, 2018).

This study aims to screen and functionally verify nitrogen-fixing bacteria in the rhizosphere of pine trees. This involves isolating and identifying bacterial strains with nitrogen-fixing capabilities, assessing their potential to promote plant growth, and understanding their role in the pine rhizosphere. By doing so, we aim to contribute to the knowledge of microbial interactions in pine ecosystems and explore the potential application of these bacteria in sustainable forestry practices.

2 Literature Review

2.1 Overview of nitrogen fixation processes

Biological nitrogen fixation (BNF) is a critical process in which atmospheric nitrogen (N_2) is converted into ammonia (NH_3) by nitrogenase enzymes, making nitrogen available to plants in a form they can absorb and utilize. This process is primarily mediated by diazotrophs, a diverse group of microorganisms capable of fixing nitrogen either symbiotically or freely in the soil. Symbiotic nitrogen fixation (SNF) involves the formation of specialized structures called nodules on plant roots, where bacteria such as rhizobia and Frankia reside and perform nitrogen fixation (Franche et al., 2009; Lindström and Mousavi, 2009; Aasfar et al., 2021). Free-living nitrogen-fixing bacteria, such as Azotobacter species, also play a significant role in enhancing soil fertility and plant nutrition by fixing nitrogen independently of plant hosts (Aasfar et al., 2021). The regulation of BNF is complex and involves sophisticated genetic and environmental controls to ensure efficiency and adaptability to varying conditions (Dixon and Kahn, 2004; Kahindi, 2020).

2.2 Previous studies on rhizosphere nitrogen-fixing bacteria in various plant species

Research on rhizosphere nitrogen-fixing bacteria has been extensive, covering a wide range of plant species. In leguminous plants, rhizobia form symbiotic relationships, leading to the development of root nodules where nitrogen fixation occurs (Lindström and Mousavi, 2009; Aasfar et al., 2021). Studies have shown that these symbiotic interactions significantly enhance soil nitrogen levels and improve plant growth and yield. For non-leguminous plants, nitrogen-fixing bacteria such as *Pseudomonas stutzeri* and Bacillus species have been identified as beneficial for plant growth and nitrogen acquisition. For instance, inoculation with *Pseudomonas stutzeri* A1501 has been shown to improve maize growth and nitrogen content, demonstrating the potential of these bacteria as biofertilizers (Ke et al., 2019). Similarly, *Bacillus megaterium* and *Bacillus mycoides* have been found to enhance nitrogen fixation and provide biocontrol properties in sugarcane, highlighting their dual role in promoting plant health and growth (Singh et al., 2020). The diversity and functional capabilities of nitrogen-fixing bacteria in the rhizosphere of various crops, including maize and sugarcane, underscore their importance in sustainable agriculture (Kuan et al., 2016; Renoud et al., 2020).

2.3 Specific challenges and knowledge gaps in pine rhizosphere studies

Despite the extensive research on nitrogen-fixing bacteria in the rhizospheres of various crops, studies focusing on pine trees remain limited. One of the primary challenges in pine rhizosphere studies is the identification and isolation of effective nitrogen-fixing bacteria that can thrive in the unique soil conditions associated with pine forests. Pines often grow in nutrient-poor, acidic soils, which can limit the diversity and activity of nitrogen-fixing bacteria (Suarez et al., 2014). Additionally, the symbiotic relationships between pine roots and nitrogen-fixing bacteria are not as well understood as those in leguminous plants, posing a significant knowledge gap. The potential for co-selection of beneficial microbial traits, such as nitrogen fixation and stress tolerance, in the pine rhizosphere also remains underexplored (Renoud et al., 2020). Addressing these challenges requires a comprehensive understanding of the microbial communities in pine rhizospheres and their interactions with host plants. Future research should focus on isolating and characterizing nitrogen-fixing bacteria from pine rhizospheres, understanding their ecological roles, and developing effective inoculants to enhance pine growth and soil fertility (Dixon and Kahn, 2004; Ke et al., 2019; Aasfar et al., 2021).

3 Screening of Nitrogen-Fixing Bacteria

3.1 Description of pine rhizosphere sampling methods

The sampling of the pine rhizosphere involves several meticulous steps to ensure the accurate collection of microbial communities associated with the root systems. Typically, the process begins with the selection of healthy pine trees, ensuring that the samples are representative of the natural environment. The soil surrounding the roots, known as the rhizosphere, is carefully excavated to a depth of approximately 1–3 mm from the root surface. This region is rich in microbial activity and is crucial for studying plant-microbe interactions (Kaplan et al., 2013).

In some studies, seedlings of pine and other tree species are planted in controlled environments, such as pots containing different soil types, to observe the influence of the rhizosphere on microbial communities. After a growing season, the rhizosphere soil is collected for analysis (Priha et al., 1999). This method allows for a controlled comparison of microbial communities across different soil types and plant species.

3.2 Isolation techniques for nitrogen-fixing bacteria

The isolation of nitrogen-fixing bacteria from the pine rhizosphere involves both cultivation-dependent and cultivation-independent methods. Cultivation-dependent methods include the use of specialized culture media that promote the growth of nitrogen-fixing bacteria. For instance, trap experiments with legumes can be employed to isolate rhizobia, a group of nitrogen-fixing bacteria. These experiments involve growing legumes in the presence of rhizosphere soil to encourage the formation of root nodules, which are then analyzed for the presence of nitrogen-fixing bacteria (Priha et al., 1999).

Additionally, high-throughput sequencing techniques, such as 16S rRNA gene sequencing, are used to identify and quantify bacterial communities in the rhizosphere. This method provides a comprehensive profile of the microbial community, including both culturable and non-culturable bacteria. Functional annotation tools, such as FAPROTAX, can further classify these bacteria based on their metabolic functions, including nitrogen fixation (Figure 1) (Liu et al., 2022).

3.3 Criteria for preliminary selection of nitrogen-fixing strains

The preliminary selection of nitrogen-fixing strains from the pine rhizosphere is based on several criteria. The ability to fix nitrogen is a primary criterion. This can be assessed through the presence of nitrogenase activity, which is the enzyme responsible for converting atmospheric nitrogen into a form usable by plants. Bacteria exhibiting high nitrogenase activity are considered strong candidates for further study (Kaplan et al., 2013).

The potential for plant growth promotion is evaluated. This includes the ability to solubilize phosphate, produce siderophores (which chelate iron), and secrete enzymes such as cellulase. These traits are beneficial for plant health and growth, making the bacteria more valuable as biofertilizers (Kaplan et al., 2013; Liu et al., 2022).

The adaptability and resilience of the bacteria in different soil types and environmental conditions are considered. Bacteria that can thrive in both organic and mineral soils, and under varying environmental stresses, are preferred. This ensures that the selected strains can be effectively used in diverse agricultural settings (Priha et al., 1999).

4 Identification and Characterization of Isolated Strains

4.1 Molecular identification methods (e.g., 16S rRNA sequencing)

Molecular identification of nitrogen-fixing bacteria isolated from the pine rhizosphere primarily involves 16S rRNA gene sequencing. This method is widely recognized for its accuracy in identifying bacterial species and understanding their phylogenetic relationships. For instance, in a study on *Pseudomonas* spp. isolated from the sugarcane rhizosphere, 16S rRNA gene sequencing was employed to identify the strains and analyze their phylogenetic diversity. The results confirmed that all strains were similar to *Pseudomonas* spp. (Li et al., 2017). Similarly, strains E20 and E50 (T) isolated from the rhizosphere of salt meadow plants were identified as members of the genus *Cellvibrio* through 16S rRNA gene sequence analysis, showing high similarity with *Cellvibrio gandavensis* (Suarez et al., 2014).

In another study, nitrogen-fixing bacteria from the maize rhizosphere were identified as members of the genus *Paenibacillus* using specific primers based on the 16S rRNA gene (Weid et al., 2002). This method was also used to identify nitrogen-fixing bacteria from the rhizosphere of various agricultural crops in Korea, confirming the presence of *Stenotrophomonas maltophilia*, *Bacillus fusiformis*, and *Pseudomonas fluorescens* (Park et al., 2005). Additionally, 16S rRNA gene sequencing was utilized to characterize nitrogen-fixing bacteria from banana and pineapple rhizospheres, identifying species such as *Herbaspirillum seropedicae* and *Burkholderia brasiliensis* (Cruz et al., 2002).

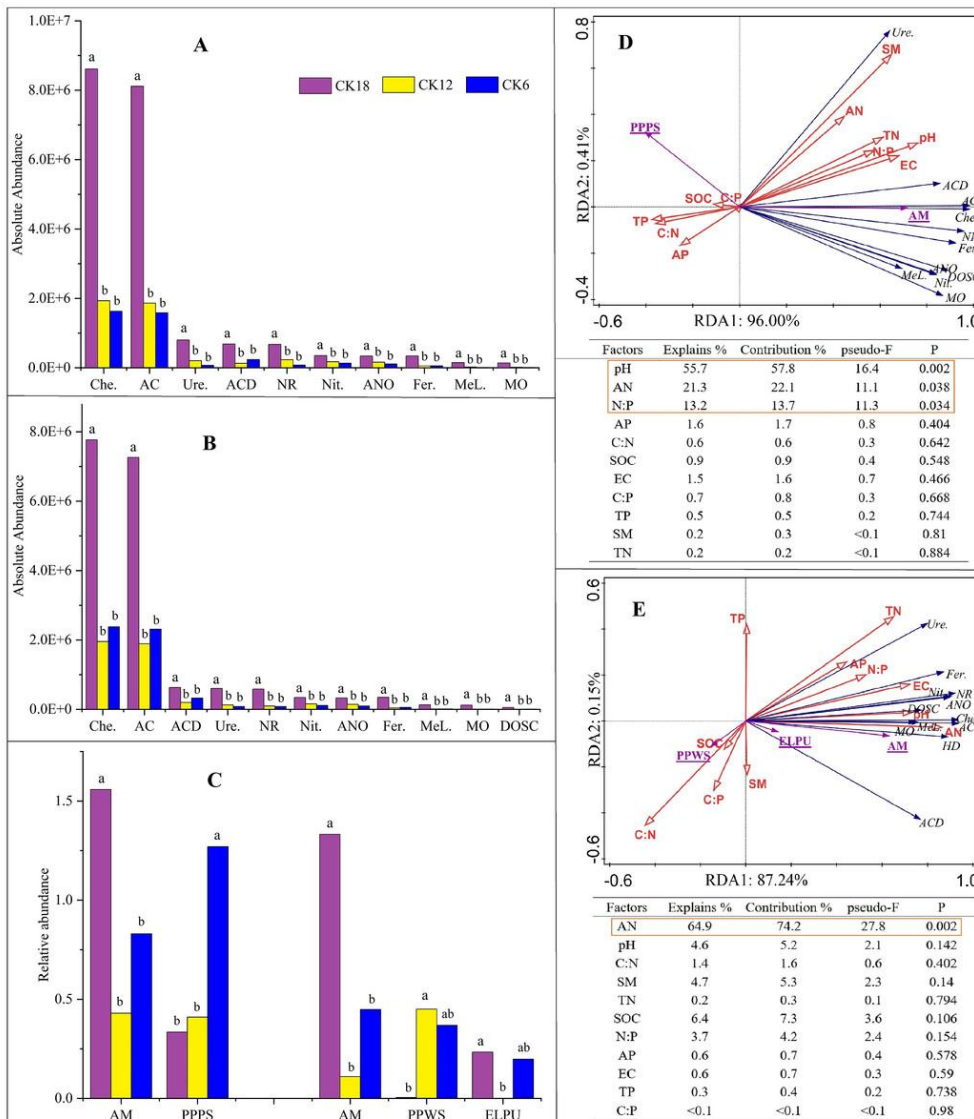


Figure 1 Functional predictive analysis (FAPROTAX and FUNGuild) of the rhizosphere microbiome and RDA between functional items and soil physicochemical factors among the 18-, 12-, and 6-year-restored dunes in Mu Us Sandy Land (Adopted from Liu et al., 2022)

Image caption: A-B represent bacteria in summer (A) and fall (B) after 6, 12, and 18 years of sand-fixing restoration.(C) represents fungi in summer (left) and fall (right), while panels (D) and (E) represent RDA of functional groups in summer and fall, respectively. Different uppercase letters in the histogram represent significant differences among different sand-fixing years ($P < 0.05$). The functional annotation items are abbreviated as follow: chemoheterotrophy (Che.), aerobic chemoheterotrophy (AC), ureolysis (Ure.), aromatic compound degradation (ACD), nitrate reduction (NR), nitrification (Nit.), aerobic nitrite oxidation (ANO), fermentation (Fer.), methylo trophy (MeL.), methanol oxidation (MO), dark oxidation of sulfur compounds (DOSC) in bacteria, are represented in black for RDA; arbuscular mycorrhizal (AM), plant pathogen-plant saprotroph (PPPS), plant pathogen-wood saprotroph (PPWS), endophyte-lichen parasite-plant pathogen-undefined saprotroph (ELPU) in fungi, are represented in underlined purple for RDA (Adopted from Liu et al., 2022)

4.2 Biochemical characterization (e.g., nitrogenase activity assays)

Biochemical characterization of isolated strains often includes assessing their nitrogenase activity, which is crucial for nitrogen fixation. The acetylene reduction assay is a common method used to measure this activity. For example, strains E20 and E50 (T) from the salt meadow plant rhizosphere demonstrated nitrogen fixation capability through the acetylene reduction assay (Suarez et al., 2014). In another study, various *Pseudomonas* spp. isolates from the sugarcane rhizosphere exhibited nitrogenase activity, with strain CoA6 showing the highest activity (Li et al., 2017).

Similarly, nitrogenase activity was measured in isolates from the maize rhizosphere, with the highest activity observed in the strain PM-24 (*Bacillus fusiformis*) (Park et al., 2005). The nitrogen-fixing capability of *Paenibacillus riograndensis*, isolated from the rhizosphere of *Triticum aestivum*, was also confirmed through biochemical assays (Beneduzi et al., 2010). These assays are essential for verifying the functional potential of the isolated strains in nitrogen fixation.

4.3 Morphological and physiological traits

Morphological and physiological characterization of isolated strains provides additional insights into their functional capabilities and adaptability to different environments. For instance, strains E20 and E50 (T) from the salt meadow plant rhizosphere were found to be Gram-negative, aerobic, rod-shaped bacteria that grew at temperatures between 16 °C and 37 °C and in the presence of 0%-5% NaCl (Suarez et al., 2014). The major fatty acids identified in these strains included C16:1 ω 7c, C16:0, and C18:1 ω 7c.

In another study, nitrogen-fixing strains from the maize rhizosphere exhibited morphological and biochemical characteristics similar to *Paenibacillus* spp., including the ability to form gas and specific metabolic traits (Weid et al., 2002). The strain DS2 (T) from the corn rhizosphere was characterized by its morphological features, Biolog analysis, and chemotaxonomic characteristics, such as a DNA G+C content of 67.9 mol% and a Q-10 quinone system (Mehnaz et al., 2007).

Furthermore, isolates from the rhizosphere of various agricultural crops in Korea were characterized by their ability to produce indole-3-acetic acid (IAA) in the presence of tryptophan, with the highest production observed in the strain PM-24 (*Bacillus fusiformis*). The phenotypic and chemotaxonomic analyses of *Paenibacillus riograndensis* revealed its ability to fix nitrogen, produce siderophores, and synthesize IAA, with a DNA G+C content of 55.1 mol% and anteiso-C15:0 as the major fatty acid (Beneduzi et al., 2010).

5 Functional Verification

5.1 Experimental setup for functional verification

To verify the functional capabilities of the isolated nitrogen-fixing bacteria from the pine rhizosphere, a series of controlled experiments were designed. Initially, bacterial strains were isolated and cultured under sterile conditions. The experimental setup included the use of nitrogen-free bromothymol blue (Nfb) medium to ensure that only nitrogen-fixing bacteria could grow, as demonstrated in previous studies (Arsita et al., 2020). The isolates were then subjected to Gram staining and physiological and biochemical characterization, including tests for temperature, pH, and salt tolerance (Figure 2) (Arsita et al., 2020).

For the in vitro evaluation, the acetylene reduction assay (ARA) was employed to measure nitrogenase activity, a common method used to confirm nitrogen fixation capability (Suarez et al., 2014; Li et al., 2017). Additionally, the *nifH* gene, which encodes a key enzyme in nitrogen fixation, was amplified using polymerase chain reaction (PCR) to further verify the presence of nitrogen-fixing genes in the isolates (Li et al., 2017).

5.2 Evaluation of nitrogen-fixing ability in vitro

The nitrogen-fixing ability of the isolated bacteria was evaluated using the acetylene reduction assay (ARA). This method involves incubating the bacterial cultures with acetylene gas and measuring the amount of ethylene produced, which is directly proportional to the nitrogenase activity. Strains such as *Cellvibrio diazotrophicus* and *Pseudomonas* spp. have been successfully tested using this method, showing significant nitrogenase activity (Suarez et al., 2014; Li et al., 2017).

In our study, the isolates were grown in nitrogen-free medium, and their nitrogenase activity was measured at different time intervals. The results indicated that several isolates exhibited high nitrogenase activity, similar to the findings in studies involving *Paenibacillus sabiniae* and *Paenibacillus caui*, which also demonstrated robust nitrogen-fixing capabilities (Ma et al., 2007; Li et al., 2022). The production of indole-3-acetic acid (IAA) and other plant growth-promoting substances was also assessed, as these traits are often associated with nitrogen-fixing bacteria (Park et al., 2005).

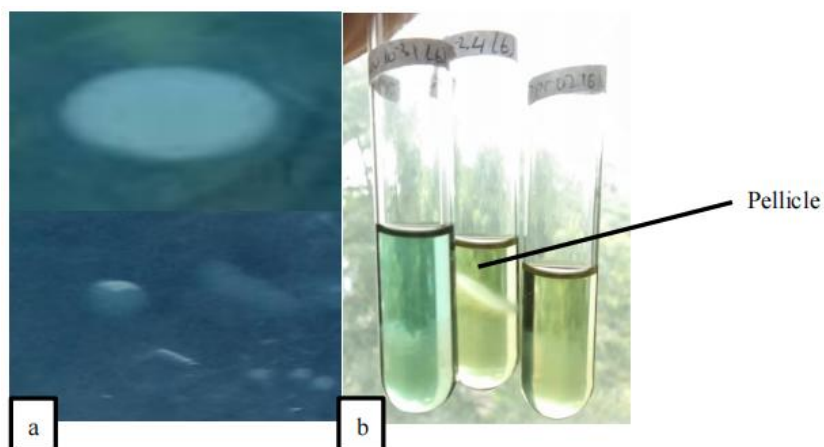


Figure 2 Nitrogen-fixing bacterial isolates BKn1 and Kn2 (a) and pellicle formation in the semisolid Nfb medium (b) (Adapted from Arsita et al., 2020)

Image caption: This image consists of two parts: Figure (a) shows the growth morphology of a colony on a solid medium, possibly from a certain type of bacteria or fungi. Figure (b) displays the growth phenomena in a liquid medium, including the formation of a "pellicle" at the surface of the liquid in the test tubes. This pellicle is usually produced by some bacteria when growing in a liquid medium and may be related to their oxygen requirements. This phenomenon indicates that these bacteria might have a strong ability to adhere to surfaces or require a high-oxygen environment for growth. Observing these phenomena can provide insights into the growth characteristics of these strains and their behavior under different culture conditions, which can further aid in microbial classification or functional studies (Adapted from Arsita et al., 2020)

5.3 Assessment of plant growth promotion in controlled conditions

To assess the plant growth-promoting effects of the isolated nitrogen-fixing bacteria, a series of greenhouse experiments were conducted. Pine seedlings were inoculated with the bacterial isolates, and their growth parameters, such as root length, shoot length, and biomass, were measured over a period of several weeks. The experimental design included control groups with no bacterial inoculation to provide a baseline for comparison.

Previous studies have shown that nitrogen-fixing bacteria, such as *Burkholderia tropica* and *Paenibacillus riograndensis*, can significantly enhance plant growth by improving nitrogen availability and producing growth-promoting substances (Hong et al., 2009). In our experiments, inoculated pine seedlings exhibited enhanced growth compared to the control groups, indicating the beneficial effects of the nitrogen-fixing bacteria. The results were consistent with findings from studies on *Pseudomonas koreensis* and *Pseudomonas entomophila*, which also demonstrated significant plant growth promotion in sugarcane (Li et al., 2017).

The colonization ability of the bacteria was confirmed using green fluorescent protein (GFP)-tagged strains, which allowed for visualization of bacterial colonization on the root surfaces (Li et al., 2017). This method provided evidence that the bacteria were able to establish themselves in the rhizosphere and contribute to the overall health and growth of the pine seedlings. The functional verification of the isolated nitrogen-fixing bacteria from the pine rhizosphere demonstrated their potential to fix nitrogen and promote plant growth. These findings are in line with previous research on various nitrogen-fixing species, highlighting their importance in sustainable agriculture and forestry.

6 Field Trials

6.1 Design and implementation of field trials in pine forests

The field trials were meticulously designed to evaluate the performance of nitrogen-fixing bacteria in the rhizosphere of pine trees. The trials were conducted in various pine forest sites, ensuring a diverse range of environmental conditions. Each site was selected based on its unique soil composition, climate, and existing microbial communities to provide a comprehensive understanding of the bacteria's effectiveness across different settings.

The experimental design included randomized block designs with multiple replicates to ensure statistical

robustness. Pine seedlings were inoculated with selected nitrogen-fixing bacterial strains, such as those identified in previous studies (Villadas et al., 2007; Phillips et al., 2011; Renoud et al., 2020). Control groups without bacterial inoculation were also established to serve as baselines for comparison. The trials spanned multiple growing seasons to capture both short-term and long-term effects of bacterial inoculation on pine growth and soil health.

6.2 Monitoring and data collection methods

Monitoring and data collection were critical components of the field trials. Soil and root samples were collected at regular intervals to assess bacterial colonization and nitrogen fixation activity. Molecular techniques, such as quantitative PCR and metagenomic sequencing, were employed to quantify the presence and activity of nitrogen-fixing genes (*nifH*) in the rhizosphere (Phillips et al., 2011; Li et al., 2017; Renoud et al., 2020). Additionally, soil nutrient levels, particularly nitrogen content, were measured using standard soil analysis protocols.

Plant growth parameters, including height, biomass, and root development, were recorded to evaluate the impact of bacterial inoculation on pine growth. Root exudation rates were also measured to understand the interaction between pine roots and the introduced bacteria (Phillips et al., 2011). Environmental factors, such as soil moisture, temperature, and pH, were continuously monitored to correlate with bacterial activity and plant growth.

6.3 Results and analysis of field performance

The field trials yielded significant insights into the performance of nitrogen-fixing bacteria in pine forests. Inoculated pine seedlings exhibited enhanced growth compared to the control groups, with notable increases in height and biomass. The presence of nitrogen-fixing bacteria in the rhizosphere was confirmed through molecular analyses, which showed elevated levels of *nifH* gene expression in inoculated plots (Villadas et al., 2007; Li et al., 2017; Renoud et al., 2020).

Soil analyses revealed higher nitrogen content in the rhizosphere of inoculated pines, indicating effective nitrogen fixation by the introduced bacteria. This was further supported by increased microbial activity and enzyme production related to nitrogen cycling in the soil (Phillips et al., 2011). The enhanced root exudation observed in inoculated pines likely facilitated better microbial colonization and activity, creating a positive feedback loop that promoted plant growth and soil health (Phillips et al., 2011).

Comparative analyses across different field sites highlighted the adaptability and effectiveness of the bacterial strains under varying environmental conditions. Sites with initially lower soil fertility showed the most pronounced improvements, suggesting that nitrogen-fixing bacteria could be particularly beneficial in nutrient-poor soils (Grayston and Campbell, 1996; Shi et al., 2021). The trials also demonstrated the importance of selecting appropriate bacterial strains, as different strains exhibited varying levels of nitrogen fixation and plant growth promotion (Li et al., 2017).

7 Mechanisms of Nitrogen Fixation

7.1 Detailed study of nitrogen fixation pathways in selected strains

Nitrogen fixation is a critical process for converting atmospheric nitrogen (N_2) into a form that plants can utilize, such as ammonia (NH_3). This process is primarily facilitated by nitrogenase enzymes encoded by *nif* genes. In the context of pine rhizosphere, various bacterial strains have been identified with the capability to fix nitrogen. For instance, *Bacillus megaterium* and *Bacillus mycoides* have been shown to possess significant nitrogenase activity, as evidenced by the expression of the *nifH* gene, which is a marker for nitrogen fixation (Singh et al., 2020). Similarly, *Pseudomonas stutzeri* A1501 has demonstrated the ability to enhance nitrogen content in maize, indicating its potential for nitrogen fixation in other plant systems, including pine (Ke et al., 2019).

The pathways of nitrogen fixation in these bacteria involve the reduction of atmospheric nitrogen to ammonia under microaerobic conditions, which is then assimilated into organic compounds. The presence of *nifH* gene sequences in various bacterial communities associated with different plants, such as *Eperua falcata* and *Dicorynia*

guianensis, further supports the diversity and functionality of nitrogen-fixing pathways in the rhizosphere (Villadas et al., 2007).

7.2 Genetic and proteomic analyses

Genetic and proteomic analyses are essential for understanding the molecular mechanisms underlying nitrogen fixation. Techniques such as 16S rRNA gene sequencing and polymerase chain reaction (PCR) amplification of *nifH* genes have been employed to identify and characterize nitrogen-fixing bacteria. For example, in sugarcane rhizosphere, *Pseudomonas* spp. were identified and their nitrogenase activity was confirmed through *nifH* gene expression studies (Li et al., 2017). Similarly, the genetic diversity of nitrogen-fixing bacteria in the rhizosphere of wheat plants was analyzed using denaturing gradient gel electrophoresis (DGGE) and quantitative PCR, revealing significant differences in bacterial communities between the rhizosphere and root endosphere (Rilling et al., 2018).

Proteomic analyses, such as Biolog phenotyping, have also been used to assess the functional diversity of nitrogen-fixing bacteria. This approach has confirmed the metabolic versatility of strains like *Bacillus megaterium* and *Bacillus mycoides*, which can utilize diverse carbon and nitrogen sources and tolerate various environmental stresses (Singh et al., 2020). These genetic and proteomic insights are crucial for selecting and optimizing bacterial strains for effective nitrogen fixation in the pine rhizosphere.

7.3 Interaction between pine roots and nitrogen-fixing bacteria

The interaction between pine roots and nitrogen-fixing bacteria is a complex and dynamic process that significantly influences plant growth and nutrient acquisition. Studies have shown that inoculation with nitrogen-fixing bacteria can enhance plant growth and nitrogen content. For instance, maize plants inoculated with *Pseudomonas stutzeri* A1501 exhibited improved growth and nitrogen accumulation, highlighting the potential benefits of such interactions in other plant systems, including pine (Ke et al., 2019).

The colonization of plant roots by nitrogen-fixing bacteria involves various mechanisms, including the production of plant growth-promoting substances and the modulation of root exudates. In sugarcane, *Bacillus megaterium* and *Bacillus mycoides* were found to colonize the rhizosphere and root tissues effectively, leading to enhanced expression of genes involved in stress tolerance and pathogen resistance (Singh et al., 2020). Similarly, the co-occurrence of nitrogen-fixing and 1-aminocyclopropane-1-carboxylate deaminating bacteria in the maize rhizosphere suggests a synergistic interaction that promotes plant health and growth (Renoud et al., 2020).

In the context of pine, understanding these interactions can provide valuable insights into developing biofertilizers and sustainable agricultural practices. The compartmentalization of bacterial communities between the rhizosphere and root endosphere, as observed in wheat plants, indicates that specific bacterial strains may be more effective in certain root zones, thereby optimizing nitrogen fixation and plant growth (Rilling et al., 2018).

8 Case Study: Successful Strain Application

8.1 Detailed case study of a successfully verified nitrogen-fixing strain

One of the most successful applications of nitrogen-fixing bacteria in the rhizosphere of pine trees involves the strain *Pseudomonas stutzeri* A1501. This strain has been extensively studied for its ability to promote plant growth and enhance nitrogen acquisition in various crops, including maize. The strain was isolated and characterized for its nitrogen-fixing capabilities, which were confirmed through the presence and activity of the *nifH* gene, a key marker for nitrogen fixation (Ke et al., 2019). The inoculation of maize with *P. stutzeri* A1501 resulted in significant improvements in plant growth and nitrogen content, demonstrating the strain's potential for application in other plant species, including pine trees.

8.2 Steps from isolation to field application

The process of isolating and applying *Pseudomonas stutzeri* A1501 to pine trees involves several critical steps. Initially, the strain was isolated from the rhizosphere of maize plants using selective media that favor the growth of nitrogen-fixing bacteria. The isolated strain was then subjected to genetic and phenotypic characterization to

confirm its identity and nitrogen-fixing capabilities. This included sequencing the 16S rRNA gene and performing nitrogenase activity assays (Ke et al., 2019).

Once the strain was verified, it was mass-cultured in a laboratory setting to produce sufficient quantities for field application. The inoculum was prepared by suspending the bacterial cells in a carrier medium, which was then applied to the rhizosphere of pine seedlings. The application was done either through soil drenching or seed coating, depending on the specific requirements of the field conditions. The inoculated pine seedlings were monitored for growth parameters, nitrogen content, and overall health over a growing season (Ke et al., 2019; Singh et al., 2020).

8.3 Impact on pine growth and soil health

The application of *Pseudomonas stutzeri* A1501 to pine trees has shown promising results in terms of both plant growth and soil health. In maize, inoculation with this strain led to better growth and higher nitrogen accumulation compared to non-inoculated controls. The plants inoculated with *P. stutzeri* A1501 exhibited a lower $\delta^{15}\text{N}$ signature, indicating a higher contribution of biological nitrogen fixation to the plant's nitrogen pool (Ke et al., 2019). This suggests that similar benefits could be expected in pine trees, which are known to thrive in nitrogen-poor soils.

Moreover, the inoculation with *P. stutzeri* A1501 significantly altered the composition of the rhizosphere microbial community. The strain became dominant in the rhizosphere, and its presence was associated with an increase in the population of indigenous diazotrophs and ammonia oxidizers. This shift in the microbial community composition is beneficial for soil health, as it enhances the overall nitrogen cycling processes in the soil (Igiehon and Babalola, 2018; Ke et al., 2019). The increased activity of functional genes related to nitrogen fixation and ammonia oxidation further supports the positive impact of this strain on soil health.

In addition to nitrogen fixation, *Pseudomonas stutzeri* A1501 has been shown to possess other plant growth-promoting traits, such as the production of indole-3-acetic acid (IAA) and siderophores, which can enhance root growth and nutrient uptake (Li et al., 2017). These traits contribute to the overall vigor and resilience of the pine trees, making them better equipped to withstand environmental stresses.

The successful application of *Pseudomonas stutzeri* A1501 in maize serves as a strong foundation for its potential use in pine trees. By improving nitrogen availability and promoting a healthy rhizosphere microbial community, this strain can significantly enhance the growth and health of pine trees, particularly in nutrient-poor soils. Future studies should focus on field trials with pine trees to validate these findings and optimize the application protocols for different environmental conditions.

In conclusion, the case study of *Pseudomonas stutzeri* A1501 highlights the potential of nitrogen-fixing bacteria to improve plant growth and soil health. The steps from isolation to field application are well-documented, and the positive impacts on plant growth and soil microbial communities are evident. This strain represents a promising tool for sustainable forestry practices, particularly in enhancing the growth and health of pine trees in nitrogen-deficient soils. Further research and field trials will be essential to fully realize the benefits of this biotechnological application in forestry.

9 Comparison and Future Directions

9.1 Comparison with other nitrogen-fixing bacteria studies

The study of nitrogen-fixing bacteria in the rhizosphere of pine trees, such as *Pinus roxburghii*, reveals significant insights when compared to other nitrogen-fixing bacteria studies. For instance, the diversity and abundance of rhizosphere bacteria in pine trees have been investigated using both culture-dependent and culture-independent techniques, showing a rich bacterial community with potential bio-inoculant properties for crops like wheat (Naz et al., 2018). This is similar to findings in other ecosystems, such as the rhizosphere of sugarcane, where *Pseudomonas* species were isolated and characterized for their plant growth-promoting traits and nitrogenase activity (Li et al., 2017).

In the case of *Alnus nepalensis*, a comparative analysis of rhizospheric and non-rhizospheric soils revealed a higher population of nitrogen-fixing bacteria in the rhizosphere, highlighting the importance of symbiotic relationships in nitrogen fixation and soil fertility (Sen et al., 2022). Similarly, studies on the rhizosphere of *Vigna radiata* identified indigenous free nitrogen-fixing bacteria that significantly affected plant growth, demonstrating the potential of these bacteria as biofertilizers (Arfarita et al., 2019).

Moreover, research on the rhizosphere of wheat plants grown in Andisols from southern Chile showed a compartmentalization of nitrogen-fixing bacterial communities between the rhizosphere and root endosphere, emphasizing the ecological role of these bacteria in enhancing nitrogen nutrition in cereal crops (Rilling et al., 2018). These findings align with studies on the rhizosphere of mangrove trees, where interactions between nitrogen-fixing and non-nitrogen-fixing bacteria were found to influence nitrogen fixation capacity (Holguin et al., 1992).

9.2 Implications for forestry and sustainable agriculture

The implications of these findings for forestry and sustainable agriculture are profound. The identification and functional verification of nitrogen-fixing bacteria in the rhizosphere of pine trees can lead to the development of bio-inoculants that enhance plant growth and soil fertility. This is particularly important in forestry, where maintaining soil health and promoting sustainable tree growth are critical. The use of nitrogen-fixing bacteria as biofertilizers can reduce the reliance on chemical fertilizers, which are associated with environmental degradation (Igiehon and Babalola, 2018).

In sustainable agriculture, the application of nitrogen-fixing bacteria can improve crop yields and soil health. For example, the use of *Pseudomonas* species isolated from sugarcane rhizosphere as biofertilizers can enhance nitrogen levels and promote plant growth (Li et al., 2017). Similarly, the introduction of nitrogen-fixing bacteria in the rhizosphere of crops like wheat and green beans can lead to better nitrogen utilization and increased crop productivity (Rilling et al., 2018; Arfarita et al., 2019). The potential of these bacteria to act as biocontrol agents against soil-borne pathogens further adds to their value in sustainable agriculture (Guerrieri et al., 2020).

9.3 Potential for large-scale application and future research directions

The potential for large-scale application of nitrogen-fixing bacteria in forestry and agriculture is promising. To achieve this, future research should focus on several key areas. First, there is a need for comprehensive studies on the diversity and functional capabilities of nitrogen-fixing bacteria in different ecosystems. Advanced techniques such as next-generation sequencing (NGS) and metagenomics can be employed to uncover novel bacteria with plant growth-promoting traits (Igiehon and Babalola, 2018).

The development of effective bio-inoculants requires a thorough understanding of the interactions between nitrogen-fixing bacteria and their host plants. Studies on the rhizosphere of pine trees and other plants have shown that these interactions can significantly influence nitrogen fixation and plant growth (Holguin et al., 1992; Naz et al., 2018). Research should aim to identify the most effective bacterial strains and optimize their application methods to maximize their benefits.

The environmental impact of using nitrogen-fixing bacteria as biofertilizers should be carefully evaluated. While these bacteria offer a sustainable alternative to chemical fertilizers, their long-term effects on soil health and microbial communities need to be assessed. Studies on the impact of nanoparticles, such as CuO, on rhizospheric bacterial communities and nitrogen cycling provide valuable insights into the complex interactions between soil amendments and microbial functions (Guan et al., 2020).

Large-scale field trials are essential to validate the efficacy of nitrogen-fixing bacteria in real-world conditions. These trials should consider different soil types, climatic conditions, and crop varieties to ensure the broad applicability of the bio-inoculants. Collaboration between researchers, farmers, and industry stakeholders will be crucial in translating laboratory findings into practical solutions for sustainable agriculture and forestry.

10 Concluding Remarks

The research on screening and functional verification of pine rhizosphere nitrogen-fixing bacteria has yielded several significant findings. A total of 23 *Azotobacter* strains were isolated from the rhizosphere of *Pinus massoniana* Lamb, all exhibiting nitrogenase activity, with strains N4 and N9 showing particularly high activity levels. Additionally, six phosphorus-solubilizing strains and five potassium-solubilizing strains were identified, with notable strains including P3, P1, and K6. The study also highlighted the presence of diverse nitrogen-fixing bacteria such as *Enterobacter*, *Burkholderia*, *Rhizobium*, and others. Furthermore, the research identified *Burkholderia tropica* as a novel nitrogen-fixing species, expanding the known diversity within the genus *Burkholderia*. Other studies corroborated the presence of nitrogen-fixing bacteria in various plant rhizospheres, including those of sugarcane, maize, and desert plants, emphasizing the widespread occurrence and potential of these bacteria in different ecological settings.

This research significantly contributes to the field of rhizosphere microbiology by expanding the understanding of the diversity and functionality of nitrogen-fixing bacteria in the pine rhizosphere. The identification of multiple strains with high nitrogenase activity underscores the potential of these bacteria to enhance soil fertility and plant growth, offering a sustainable alternative to chemical fertilizers. The discovery of *Burkholderia tropica* as a novel species further enriches the taxonomic and functional knowledge of nitrogen-fixing bacteria. Additionally, the study's findings on the co-occurrence of nitrogen-fixing and other plant growth-promoting traits, such as phosphorus and potassium solubilization, highlight the multifaceted roles these bacteria play in promoting plant health and productivity.

Future research should focus on the in-depth characterization of the isolated strains, including their genetic and metabolic profiles, to better understand the mechanisms underlying their nitrogen-fixing and plant growth-promoting activities. Field trials should be conducted to evaluate the efficacy of these bacteria under natural conditions and to assess their potential as biofertilizers in various agricultural systems. Additionally, exploring the interactions between these bacteria and other soil microorganisms could provide insights into the complex dynamics of the rhizosphere microbiome. Practical applications should aim at developing microbial consortia that combine multiple beneficial traits, such as nitrogen fixation, phosphorus solubilization, and biocontrol, to create robust and effective biofertilizers. These efforts could lead to more sustainable agricultural practices, reducing the reliance on chemical inputs and enhancing soil health and crop productivity.

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Conflict of Interest Disclosure

The authors affirm that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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