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# Expanding agricultural potential through biological nitrogen fixation: Recent advances and diversity of diazotrophic bacteria

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**Abstract:** Biological nitrogen fixation (BNF) by diazotrophic bacteria is one of the oldest and most crucial processes in nature. In this process, bacteria form symbiotic associations with plants, capturing atmospheric nitrogen and making it readily available to them. The diversity of nitrogen-fixing bacteria is vast. Recent advancements in molecular biology techniques have enabled the identification of new genera and species capable of fixing nitrogen and providing other types of nutrients for plants. From an agronomic perspective, this process is fundamental in increasing crop productivity sustainably and -cost-effectively. This review aims to categorize the most recent updates on the diversity of nitrogen-fixing bacteria and showcase the main advances in the genetic improvement of legumes for this characteristic. Recent research has revealed a wide diversity of species applicable to various crops of agronomic interest, and many of these bacteria have been used either alone or in consortium with other microorganisms. This study demonstrates the agricultural potential of these new discoveries and the vast possibilities for expanding research into the diversity of microorganisms responsible for BNF in agriculture.

**Keywords:** Biological nitrogen fixation, Crop productivity, Eco-friendly agriculture, Molecular biology techniques, Microbial diversity.

**Abbreviations:** Biological Nitrogen Fixation (BNF), Crop Productivity (CP), Eco-friendly Agriculture (EA), Molecular Biology Techniques (MBT), Microbial Diversity (MD)

## Introduction

Biological nitrogen fixation (BNF) is one of the most efficient and ancient biological processes in nature (Al-Tawaha et al., 2022). Diazotrophic bacteria, which have the ability to perform this process in legumes, possess protein machinery known as nitrogenase. Through this complex of protein subunits, the bacterium converts atmospheric nitrogen into ammonia, making it directly available to the plant. The nitrogenase enzyme consists of two components (component I and II), which can be subdivided into several protein subunits. Different species of bacteria possess varying subunits. However, they all share a common characteristic: the ability to fix nitrogen (Sharma et al., 2023).

Recent advancements in molecular biology have rendered the discovery of novel bacteria strains and enzyme complexes both challenging and promising. These discoveries open avenues for innovative biological products in agriculture. Concurrently, elucidating the mechanisms of biological nitrogen fixation has emerged as an imperative, driven by the global agriculture mandate or sustainability and enhanced productivity (Threatt and Rees, 2023). Consequently, numerous international studies have focused

on identifying molecular markers linked to biological nitrogen fixation. These investigations facilitate genetic enhancement programs aimed at cultivating commercially significant species like soybeans - *Glycine max* L. and common beans - *Phaseolus vulgaris* L.*, which may exhibit improved* symbiosis with nitrogen-fixing bacteria. Furthermore, this research paves the way for novel technological advancements in both the identification and discovery of promising new bacterial strains and the breeding of cultivars with a heightened capacity for nitrogen fixation.

With the global population on the rise and food demands increasing, ensuring a stable food supply has become a paramount challenge. Leveraging biological nitrogen fixation through diazotrophic bacteria presents a sustainable and economical strategy to boost crop yields and strengthen food security. Realizing sustainable agricultural practices necessitates an in-depth knowledge of the diversity and potential for genetic enhancement of nitrogen-fixing bacteria, highlighting the crucial role these microorganisms play in addressing contemporary agricultural and food security challenges. This study contributes to the existing knowledge base by methodically organizing recent advancements in the field, underscoring the significance of nitrogen-fixing bacteria across different crops, and pinpointing directions for future research. This approach not only enriches our comprehension of these critical microorganisms but also opens up new possibilities for enhancing crop productivity and sustainability through the exploitation of biological nitrogen fixation. These findings have significant implications for sustainable agriculture and food security, and provide a framework for the development of effective and environmentally responsible agricultural practices. This literature review endeavors to aggregate and analyze contemporary insights into on the diversity of nitrogen-fixing bacteria and the recent progress in legume genetic enhancement for this attribute.

***Molecular insights into nitrogen fixation and nodulation in legumes***

Biological nitrogen fixation (BNF) is a critical component of the soil nitrogen cycle. This process is facilitated by a complex ensemble of enzymes found within Bacteria and Archaea (prokaryotes), known as nitrogenase. These prokaryotic organisms, termed diazotrophs, span several phyla including actinomycetes, cyanobacteria, and proteobacteria, as well as the archaeal domain. Diazotrophs are bifurcated into two principal categories: non-symbiotic, capable of autonomous nitrogen fixation in natural settings; and symbiotic, which establish symbiotic associations with plants for nitrogen fixation. Symbiotic diazotrophs partake in symbiosis with a broad array of plant species, encompassing ferns, woody plants, and notably, legumes. The symbiotic bond between members of the *Rhizobiaceae* family and leguminous plants (Fabaceae family) originated approximately 60 million years ago, with current investigations into this symbiosis holding substantial agronomic and commercial significance due to the pivotal role of many crops within this extensive family.

In the context of symbiosis with leguminous plants, nodules are formed on the roots, serving as specialized microenvironments or "biofactories" for the conversion of $N_2$ to $NH_3$. Each nodule houses around $10^9$ bacterial cells. Within these nodules, bacteria are supplied with carbon and oxygen in a controlled environment and, in return, supply the host plant with reduced, bioavailable nitrogen. The initiation of this process is triggered by the plant's release of flavonoids, which stimulates the bacterial expression of a lysine transcriptional regulator (*LysR*). This, in turn, activates the expression of nodulation genes (nod, nol, and noe), encoding enzymes known as Nod Factors (NF). These Nod Factors are recognized by the plant's LysM RLK receptor, leading to the activation of a leucine-rich receptor (LRR RLK) in the root. This triggers a signaling cascade that activates genes involved in nodule formation, thereby inducing root nodulation. These bacteria possess the unique capability to transform atmospheric nitrogen into ammonium, facilitated by the nitrogenase enzyme.

The preceding section offers an overview of the initial nodulation process in leguminous plants. This process, however, unfolds with greater complexity, characterized by the differential expression of an array of genes and transcription factors. Advances in molecular biology have incrementally unveiled the complex dynamics of nodulation and the intricate signaling interactions between plants and bacteria. A study by Dalla Via et al. (2015) identified 2,606 genes in Phaseolus vulgaris that exhibited differential expression during the initial stages of interaction with Rhizobium etli. Many of these genes were transcription factors influenced by Nod Factor (NF), Exopolysaccharide (EPS), and Lipopolysaccharide (LPS) genes, alongside small RNAs and genes associated with the circadian clock, which adapt to variations in daylight duration at the root level.

It is essential to acknowledge that different species, and even diverse strains within the same bacterial species, may not induce identical responses across all leguminous plants. The variance in plant and bacterial genetics, environmental conditions, and additional factors can introduce significant diversity in the nodulation process, complicating any generalization of the interactions between legumes and *Rhizobium* species. The role of EPS produced by Rhizobium, in both free-living and symbiotic states, exemplifies this point. EPS play a pivotal role in enhancing microbial tolerance under adverse environmental conditions and predominantly influence pre-infection stages in the plant-bacteria interaction. However, the necessity of EPS varies markedly across different legume-bacteria symbioses. For instance, the symbiosis between *Sinorhizobium fredii* HH103 and *Glycyrrhiza uralensis* does not strictly require EPS, whereas in the symbiosis with *Mesorhizobium tianshanense*, EPS are essential. Similarly, EPS are not critical for nodulation in beans by R. tropici CIAT 899, but are necessary in R. leguminosarum symbiosis.

The symbiotic relationship between leguminous plants and nitrogen-fixing bacteria holds significant agronomic and commercial interest, given the pivotal role of many such crops in the global food supply. The nodulation process is far more complex than initially perceived, involving the differential expression of numerous genes and transcription factors, as well as the production of exopolysaccharides that affect pre-infection events. Nonetheless, the variability in responses elicited by different leguminous species and bacterial strains underscores the challenge in generalizing the interactions between legumes and Rhizobium species. Continued research is imperative to fully decipher the mechanisms of this vital symbiosis and to devise strategies for enhancing crop yields while reducing environmental impacts.

***Nitrogenase enzyme***

Biological nitrogen fixation (BNF) is a critical component of soil fertility, facilitated by an enzyme complex known as

nitrogenase. Nitrogenase is divided into components I and II, with Component I existing as three isoenzymes. These isoenzymes enable atmospheric nitrogen fixation through distinct mechanisms: Nitrogenase 1 (FeMo-nitrogenase) requires molybdenum (Mo) and iron (Fe); Nitrogenase 2 requires vanadium (V) or Fe; and Nitrogenase 3 operates solely with Fe. Bioinformatics analysis by Zhang et al. (2021) revealed a highly conserved core structure among these enzymes, with Nitrogenase 1, crucial for plant symbiosis, being the most thoroughly studied and understood in terms of its role in nitrogen fixation.

The nitrogenase structure comprises six subunits. The Mo-dependent enzyme complex (Component I) forms a heterotetramer, consisting of four distinct subunits derived from the NifDK genes. This complex harbors an active site known as the FeMo cofactor. Component II is a homodimer, made up of two identical NifH subunits. The assembly of the nitrogenase enzyme necessitates two metal complexes, the 8Fe-7S and 7Fe-9S-C, serving as enzymatic cofactors in the NifDK and FeMo subunits, respectively. A third metal complex, the 4Fe4S, is situated within the NifH proteins. These cofactors and metallocenters are notably sensitive to oxygen exposure, with the described structure pertaining to the Mo-dependent nitrogenase enzyme.

Nitrogenases in non-symbiotic bacteria exhibit unique characteristics, including additional subunits like vnfG and anfG, alongside distinct active sites for varying cofactors. The N2 to NH3 reduction process within nitrogenase involves the hydrolysis of multiple ATP molecules. Specifically, an Fe protein bound to 2 ATPs attaches to a MoFe protein subunit, triggering a series of reactions that facilitate electron transfer, ATP hydrolysis, pyrophosphate release, and dissociation of the oxidized Fe protein. This process requires 16 ATP molecules for the reduction of one N2 molecule, denoting significant energy expenditure for the cell.

Recent research elucidates the ATP hydrolysis mechanism, electron transfer, and energy transduction within this process. Key findings include: ATP hydrolysis post-electron transfer; the reaction's rate-limiting step being the release of pyrophosphates; electron flow from a 4Fe-4S cluster to an 8Fe-7S group (the P group), then to the FeMo cofactor; and the MoFe subunits' operation on negative cooperation, decreasing ligand binding affinity as saturation occurs.

In summary, the nitrogenase enzyme is pivotal for biological nitrogen fixation, essential for soil fertility and global food security. The Mo-dependent Nitrogenase 1 is the best-known enzyme within this group, playing a significant role in nitrogen fixation. Nonetheless, the formation and functioning of nitrogenase involve complex requirements, including specific metal complexes and a high-energy reduction process. Advances in molecular biology have provided deeper insights into nitrogen fixation mechanisms, highlighting the importance of continued research to optimize agricultural productivity and minimize environmental impacts.

***Genetic diversity of nitrogen-fixing bacteria***

As highlighted in the previous section, the nitrogenase protein comprises multiple subunits, with the nifH gene, encoding one of these subunits, serving as a widely utilized biomarker by researchers to identify microorganisms capable of atmospheric nitrogen fixation. Despite the absence of a universally specific primer for nitrogenase genes applicable across all diazotrophic bacteria, significant insights have been garnered through the use of degenerate primers alongside transcriptomic, metagenomic, and metatranscriptomic approaches. These methodologies have been instrumental in uncovering new genes and, consequently, novel nitrogen-fixing bacteria. The degenerate primers (5'GCIWTYTAYGGIAARGGIGG'3) and (5'AAICCRCCRCAIACIACRTC'3), derived from aligning numerous *nif* genes, facilitated the discovery of 23 novel nitrogen-fixing bacteria in rice roots, demonstrating the enduring relevance of this primer set in contemporary scientific investigations.

Recent endeavors using degenerate primers have unveiled alternative nitrogenase enzymes in bacteria lacking molybdenum (Mo) in their habitat, with notable findings in the phyla *Firmicutes* and *Chloroflexi*. Advanced metagenomic analyses have identified 10 new genera and 25 new species harboring alternative nitrogenase enzymes. A notable study evaluated 930 genomes from the *Paenibacillaceae* family, revealing 160 genomes across four genera possessing Mo-nitrogenase, with 23 genomes also containing alternative nitrogenases. This research underscores the diverse distribution and variety of nitrogenase enzymes within the *Paenibacillaceae* family.

The advent of high-throughput sequencing technologies has significantly enhanced the capacity to analyze genomic data, revealing genes and enzymes pivotal to nitrogen fixation. Bioinformatics, particularly in metagenomic studies, has enabled detailed examinations of microbial communities in various environments, facilitating the identification of novel genes and mechanisms crucial for nitrogen fixation. For instance, bioinformatics tools have been utilized to predict genes in metagenomics analysis, broadening the search for bacteria with the nifH gene. Moreover, the integration of bioinformatics and amplicon sequencing has assessed numerous primers for the nifH gene, identifying a wide array of sequences related to the nifH gene across different components of the sugarcane ecosystem.

However, reliance solely on the nifH gene for identifying and characterizing nitrogen-fixing microbes may lead to oversight, as up to 20% of nifH-containing genomes lack nifD and nifK, essential for nitrogenase subunits. Therefore, incorporating nifD and nifK as markers or studying multiple genes is recommended for a more comprehensive analysis.

In Brazil, studies on diazotrophic bacteria of significant economic and environmental interest have been conducted, particularly those involved in nodulating crops such as soybeans, peanuts, Acacia, common beans, and fava beans. Investigations into the genetic diversity of *Bradyrhizobium* strains and diazotrophic bacteria isolated from various crops and natural vegetation have revealed high levels of intraspecies diversity and potential co-evolution among symbionts, showcasing the importance of these studies in understanding the complex interactions within microbial communities and their impact on agriculture.

***Molecular markers related to biological fixation in plants***

In recent years, there have been significant strides in understanding the molecular underpinnings of biological nitrogen fixation (BNF) by diazotrophic bacteria across various plant species. This progress is driven by the critical role of nitrogen fixation in global agriculture and ecosystem nitrogen cycling. Grasping the molecular basis of BNF is crucial for developing plants that can fix nitrogen more efficiently, offering substantial economic and social benefits.

The common bean (*Phaseolus vulgaris*) plays a vital role in food security as a major source of vegetable protein for many populations. However, its biological nitrogen fixation

**Table 1.** Recent reported N-fixing bacteria and their related plant species.

| Species/ phyla | Plant related | Reference |
|---|---|---|
| *R. etli* | *P. vulgaris* | Dalla Via et al. 2015 |
| *S. meliloti* | *Medicago sativa* L | Kang et al. 2020 |
| *R. phaseoli*, *R. trifolii, R. grahamii* and *S. americanum* | *P. vulgaris* | Peralta et al. 2016 |
| *Azotobacter, Vibrio, Klebsiella, Enterobacteriaceae, Enterobacter, Burkholderia, Pleomorphomonas, Sphingomonas, Rhodospirillaceae, Sinorhizobium, Azorhizobium, Leptolyngbya sp. JSC-1, Xanthobacteraceae, Amorphomonas, Bradyrhizobium, Bradyrhizobiaceae, Methylocapsa, Heliobacteriaceae* and *Clostridium* | *S. officinarum* L. | Gaby et al. 2018 |
| *Bradyrhizorium, Azospirillum, Hyphomicrobium, Gluconacetobacter* and *Geobacter* spp | Temperate forest soil | Lladó et al. 2017 |
| *Cyanobacteria, Firmicutes, Proteobacteria, Spirocheates, Verrucomicrobia, Bradyrhizobium, Methylobacterium, Desulfovibrio* and *Proteobaceia* | Acid forest soil | Meng et al. 2019 |
| *Azotobacter* spp.; *Agrobacterium tumefaciens, Methylococcus capsulatus, Geobacter bemidjiensis, Dechloromonas aromática, Burkholderia xenovorans, Xanthobacter autotrophicus* and *Sideroxydans lithotrophicus* | | Soni et al. 2016 |
| *Bacillus, Stenotrophomonas, Agrobacterium, Cellulomonas, Paenibacillus* | *Cenchrus ciliares, Sorghum bicolor, Cynodon* spp | Antunes et al. 2019 |
| | *Bertholletia excelsa* | Chalita et al. 2019 |
| *Bradyrhizobium, Agrobacterium* and *Rhizobium* | *Phaseolus lunatus* | Chibeba et al. 2020 |
| *B. diazoefficiens* sp USDA 110 and *S.* (Ensifer) *fredii* CCBAU45436, | *G. max* L. | Munoz et al. 2016 |
| *Bradyrhizobium* (NKS4, NKM2 and NKTG2) | *G. max* | Nguyen et al. 2020 |
| *Bacillus mojavensis, Pseudomonas aeruginosa, Alcaligenes faecalis, P. syringae* and *B. cereus* | *G. max* | Ezaka et al. 2020 |
| *Pseudomonas protegens* Pf-5 x940 | *Zea mays* L. and *Triticum aestivum* L. | Fox et al. 2016 |
| *A. brasilense* (Ab-V5 and Ab-V6) | *Zea mays* and *Triticum aestivum* | Fukami et al. 2016 |
| *Lysinibacillus sphaericus, Klebsiella pneumoniae* and *Bacillus cereus* | *Oryza sativa* L. | Shabanamol et al. 2018 |
| *Enterobacter cloacae* RCA25, *Klebsiella varicola* RCA 26, *Herbaspirillum seropedicae* z67 and *Sinorhizobium fredii* NGR234 | *O. sativa* | Defez et al. 2017 |
| *Pseudomonas stutzeri* A15 | *O. sativa* | Saelao et al. 2017 |
| *Azospirillum* sp. | *Brachiaria* | Hungria et al. 2021; Leite et al. 2019 |
| *Roseateles terrae* and *Burkholderia gladioli* | *S. officinarum* | Muthukumarasamy et al. 2017 |
| *Streptomyces chartreusis*, strain WZS021 | *S. officinarum* | Wang et al. 2017 |
| *Enterobacter roggenkampii* ED5 | *S. officinarum, S. barberi, S. robustum, S. spontaneum and S. sinense* | Guo et al. 2020 |
| *Bradyrhizobium japonicum* SAY3-7, *Bradyrhizobium elkanii* BLY3-8 and *Streptomyces griseoflavus* | *Vigna radiata* L. and *G. max* | Htwe et al. 2019 |
| *B. megaterium* and the *Rhizobium* IITA-PAU 987 | *G. max* | Korir et al. 2017 |
| *B. japonicum* and *A. brasilense* | *G. max* | De Sousa et al. 2020; Hungria et al. 2015 |
| *B.diazoefficiens* USDA 110 and *Bacillus velezensis* S141 | *G. max* | Sibponkrung et al. 2020 |
| *A. brasilense* Ab-V6 and *R. tropici* CIAT 899 | *Zea mays* | Fukami et al. 2018 |
| *B. japonicum* USDA 110 and *Pseudomonas putida* TSAU1 | *G. max* | Egamberdieva et al. 2017 |
| *Trichoderma harzianum* with *Bradyrhizobium* spp | *Arachis hypogaea* L | Neelipally et al. 2020 |

rates are notably lower compared to other legumes, such as soybeans (*Glycine max*), prompting ongoing research into its nitrogen-fixing mechanisms. A recent study highlighted that only 20% of the isolates identified as classical rhizobia were effective in nitrogen fixation in common beans, with several species *of Rhizobium phaseoli, R. trifolii, R. grahamii*, and *S. americanum* showing successful colonization capabilities.

Comparative genomic analyses have revealed significant homology among these species, enhancing our understanding of their symbiotic relationships.

Genome-wide association studies (GWAS) in common beans have pinpointed multiple single nucleotide polymorphism (SNP) markers associated with nitrogen fixation efficiency, identifying crucial genes linked to this process. Notably,

SNPs on chromosomes Pvo3, Pvo7, and Pvo9, and the identification of Leucine-rich repeat receptor-like protein kinases (LRR-RLK) genes, have been associated with enhanced nitrogen fixation capabilities. The identification of candidate genes and quantitative trait loci (QTLs) through GWAS offers promising avenues for molecular marker-assisted breeding programs aimed at improving nitrogen fixation.

Soybeans, given their significant economic value, have been the focus of extensive studies investigating QTLs related to nitrogen fixation under various environmental conditions. These studies have uncovered QTLs that account for a substantial portion of the phenotypic variation observed in the field, linking root architecture to BNF efficiency and identifying genomic regions associated with nodule and shoot dry weight. Such insights are invaluable for breeding programs targeting enhanced BNF.

The integration of transcriptomics studies and bioinformatics approaches has facilitated the identification of differential gene expression and SNPs associated with BNF in soybeans and other legumes. These efforts are advancing our understanding of the complex interactions between legumes and nitrogen-fixing bacteria, contributing to increased legume productivity, food security, and the sustainable use of agricultural resources.

***Nitrogen fixing bacteria in soybeans***

Soybean, being the most extensively researched legume in terms of biological nitrogen fixation (BNF), is scrutinized not only for potential productivity gains through BNF inoculation but also for the optimal methods of inoculant application and the impact of pesticides on diazotrophic bacteria development. The exploration into the physiology and interactions between plants and microorganisms is expanding, with some cultivars demonstrating superior BNF efficiency. Recent research posits that soybean domestication may have been influenced by BNF efficiency, with domesticated cultivars showing significantly better BNF performance after inoculation with *Bradyrhizobium diazoefficiens* sp. USDA 110 and *S. fredii* CCBAU45436 compared to their wild counterparts. This aligns with findings that inoculated plants yield considerably more than those reliant on mineral nitrogen.

Excessive mineral nitrogen can adversely affect BNF, with studies recommending that soybean fertilization at sowing should not surpass 20 kg ha-1 of N, as higher doses could inhibit nodulation and potentially decrease yields. Certain *Bradyrhizobium* strains, however, have shown resilience, forming active nodules even in higher nitrate conditions, suggesting promising avenues for enhancing soybean nodulation and yields.

The effect of pesticides on BNF inoculation has also been a recent focus, with findings indicating that glyphosate, for example, can inhibit *rhizobia* growth and thus BNF, particularly in glyphosate-resistant soy varieties. Other studies have observed that varying glyphosate levels can impact critical functions such as indole acetic acid production and nutrient solubilization, further influencing nitrogen fixation.

Advancements in understanding the genetics behind BNF have identified key genes, such as GmVTL1a/b, essential for symbiosis due to its role in iron transportation within nodules. Another significant discovery is the UPS1 gene, whose insertion into soybeans has been shown to enhance nitrogen assimilation from nodules, underscoring the importance of efficient nitrogen export mechanisms from nodules to other plant parts.

These investigations into soybeans are critical not just for the crop's economic value but also because soybeans serve as a model organism for studying BNF in legumes. Insights gained from soybean research often extend to other vital legumes, including beans and peanuts, enhancing our understanding of BNF across a wider spectrum of agriculturally important crops.

***Nitrogen-fixing bacteria in grasses***

Plant growth-promoting rhizobacteria (PGPR) represent a pivotal group of bacteria capable of establishing a basic form of nitrogen-fixing symbiosis with plants. PGPR interact with plants through root exudates without typically invading plant tissues, thereby enhancing nutritional support, including nitrogen, in various crops like wheat, maize, ferns, sugarcane, and rice. This support is crucial for both human and animal nutrition.

Research has demonstrated the efficacy of different PGPR genera and species in benefiting cultivated plants. For instance, *Pseudomonas protegens* Pf-5 x940 significantly increased nitrogen supply and biomass accumulation in maize and wheat. Similarly, inoculation with *Azospirillum brasilense* strains reduced the need for nitrogen fertilizers by 25% in maize and wheat. In rice, diazotrophic endophytic bacteria such *as Lysinibacillus sphaericus, Klebsiella pneumoniae,* and *Bacillus cereus* were identified, showing biocontrol and nitrogen-fixation activity. Specific bacteria like *Enterobacter cloacae RCA25* and *Klebsiella varicola* RCA 26 significantly boosted nitrogen supply in inoculated rice plants.

Pasture areas, often challenged by low fertility and degradation, have seen promising results from PGPR inoculation. This method provides a cost-effective and sustainable approach to enhance growth, productivity, and nutritional status of forages. Inoculations with *Azospirillum* sp. in *Brachiaria* pastures have demonstrated a reduction in nitrogen fertilization needs by 20%, without impacting key production components.

Sugarcane, a critical commodity for producing alcohol and sugar, receives significant attention in agricultural research to enhance productivity and industrial returns. Studies focus on identifying new PGPR strains to improve sugarcane growth and nutrient uptake. Notably, strains like *Roseateles terrae* and *Burkholderia gladioli* were isolated from sugarcane varieties, showing capabilities in nitrogen fixation and nutrient solubilization. Additionally, *Streptomyces chartreusis* strain WZS021 was found to significantly increase shoot and root dry matter, as well as nitrogen content in sugarcane.

However, results on the effectiveness of PGPR inoculation in sugarcane have been mixed, with some studies showing non-significant outcomes and others reporting modest increases in yield and nitrogen content. Despite these variations, a notable study reported significant increases in total nitrogen in the aerial tissue of Brazilian sugarcane cultivars and in soil nitrogen after inoculation with BNF strains.

These findings underscore the significant role of nitrogen-fixing bacteria in supporting grass crops, pointing to sustainable ways to improve nitrogen availability for both soil and plant, thereby contributing to the agricultural ecosystem's overall health and productivity.

***Co-inoculation and its effect on biological nitrogen fixation***

The concept of co-inoculation, involving the simultaneous application of multiple microorganisms to enhance plant growth, has seen a significant rise in research interest. This approach has been recognized for its potential to improve plant development, stress tolerance, and atmospheric nitrogen capture across various crops. Highlighting this, several recent studies across different cultures have provided insights into the benefits of co-inoculation.

For instance, a study on mung bean and soybean demonstrated that co-inoculation with *Bradyrhizobium japonicum* SAY3-7, *B. elkanii* BLY3-8, and *Streptomyces griseoflavus* led to increased plant growth, nodulation, nitrogen fixation, nutrient absorption, and seed yield. Similarly, co-inoculating *Bacillus megaterium* with *Rhizobium strain* IITA-PAU 987 in soybean resulted in enhanced shoot dry weight and a 31% increase in nitrogen uptake compared to treatments with *Rhizobium* alone.

In soybeans, the foliar application of *B. japonicum* and *Azospirillum brasilense* during the vegetative phase proved to be an effective strategy for increasing nodulation, plant height, and grain yield, even under severe drought conditions. Studies under varied soil and climate conditions have shown that co-inoculating soybean with *B. japonicum* and *A. brasilense* can boost productivity without additional fertilizer use, even in soils with existing rhizobia populations. Another soybean study highlighted the benefits of co-inoculating *B. diazoefficiens* USDA 110 with *Bacillus velezensis* S141, which resulted in larger nodules and increased nitrogen fixation, with *B. velezensis* contributing to indole-3-acetic acid (IAA) biosynthesis.

In maize, co-inoculation with *A. brasilense* Ab-V6 and *Rhizobium tropici* CIAT 899 was found to alleviate salt stress, evidenced by reduced expression of stress-related genes, upregulation of antioxidant genes, and decreased proline production. Soybeans subjected to salt stress showed improved growth and nutrient content when co-inoculated with *B. japonicum* USDA 110 and *Pseudomonas putida* TSAU1. Additionally, in peanuts, the co-cultivation of *Trichoderma harzianum* with *Bradyrhizobium* spp. enhanced nodule formation, biomass, and chlorophyll content.

These findings underscore the effectiveness and often superior outcomes of co-inoculation compared to single-strain inoculation, highlighting its potential as a sustainable agricultural practice to enhance plant growth and productivity.

**Conclusions**

This literature review concludes that molecular research has substantially enhanced our comprehension of the processes involved in biological nitrogen fixation. These investigations have revealed a broad array of microorganisms with the capability to fix nitrogen across different plant species. It's deduced that molecular-based approaches, such as next-generation sequencing (NGS) and molecular markers, are crucial for unraveling scientific knowledge and its subsequent practical application in utilizing nitrogen-fixing bacteria within agriculture. Such insights are pivotal for moving agriculture towards more sustainable and productive practices, reducing reliance on chemical fertilizers—a limited resource—and maximizing the use of biological components, which hold vast, yet untapped potential.

The burgeoning interest in these strategies is evident among biological product companies, driven by the accruing benefits and the opportunity for product differentiation. The market for biological products is on a rapid growth trajectory, with both companies and farmers seeking sustainable tools and alternatives to enhance productivity and manage pests and diseases effectively. This body of research is instrumental in fostering an agriculture that is increasingly sustainable, aligning with broader environmental and food security goals.

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