Biological Nitrogen Fixation in the Era of Microbiome Engineering: Opportunities for Sustainable Agriculture

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***Abstract*—Biological nitrogen fixation (BNF) has long been recognized as a cornerstone of sustainable agriculture, particularly through the legume-rhizobium symbiosis. A proof-of-concept breakthrough has now demonstrated that large clusters of nitrogen-fixing and symbiosis-related genes—collectively termed "symbiosis islands"—can be transferred horizontally from rhizobia into non-nitrogen-fixing bacterial backgrounds, enabling some recipient strains to colonize plant hosts and perform BNF. This development is not novel for BNF per se, which has been well-characterized through rhizobia, Azospirillum, Azotobacter, endophytes, and diverse plant growth-promoting rhizobacteria (PGPR); rather, the novelty resides in the demonstration that a large, functional gene cluster governing symbiotic nitrogen fixation can be transplanted into new bacterial taxa via horizontal gene transfer (HGT). This review places these findings in the context of the scientific history of BNF and PGPR research, examines the molecular architecture of symbiosis islands, evaluates the ecological and biosafety dimensions of such transfers, and assesses pathways toward engineering cereal-associated microbiomes. Remaining barriers—including field performance, host specificity, regulatory acceptance, and ecological stability—are critically evaluated. The overarching message is that the future of sustainable crop production will depend critically on the intelligent deployment of beneficial microbes, microbiome management, and evidence-based biological agriculture innovation.**

***Index Terms*—biological nitrogen fixation, symbiosis island, horizontal gene transfer, plant growth-promoting rhizobacteria, PGPR, rhizobia, sustainable agriculture, microbiome engineering, BioAgriculture, Azospirillum, Azotobacter, non-legume crops**

I. Introduction

Nitrogen is the most growth-limiting macronutrient for crop plants in virtually all agricultural ecosystems. The Haber–Bosch process, developed in the early twentieth century, enabled the industrial synthesis of ammonia from atmospheric dinitrogen (N_2) and transformed global food production. Today, synthetic nitrogen fertilizers support the nutrition of roughly half of the world's population (Erisman et al. 2008). However, their manufacture is energy-intensive—consuming approximately 1–2% of global fossil fuel energy—and their excessive application drives greenhouse gas emissions, nitrate leaching, eutrophication, and disruption of terrestrial nitrogen cycles (Tilman et al. 2002; Fowler et al. 2013). The imperative to develop more sustainable alternatives has never been more acute.

Biological nitrogen fixation (BNF) represents the enzymatic conversion of atmospheric N_2 to ammonia catalyzed by the nitrogenase complex in diazotrophic microorganisms. Globally, BNF contributes an estimated 100–140 million metric tonnes of reactive nitrogen annually to terrestrial ecosystems (Fowler et al. 2013; Ladha et al. 2022). Among the most agriculturally significant diazotrophs are the rhizobia—a phylogenetically diverse but functionally cohesive group of Alpha- and Betaproteobacteria that form specialized nitrogen-fixing root nodules on leguminous plants. The legume-rhizobium symbiosis has been

exploited in agriculture for well over a century, and commercial rhizobial inoculants are now widely used worldwide (Giller 2001; Peoples et al. 2009).

Beyond rhizobia, a broad range of plant growth-promoting rhizobacteria (PGPR), including free-living diazotrophs such as *Azotobacter vinelandii*, associative species such as *Azospirillum brasilense,* and diverse endophytes contribute fixed nitrogen to non-legume crops including rice, maize, sugarcane, and wheat (Dobbelaere et al. 2003; Baldani and Baldani 2005; Bashan and de-Bashan 2010). These organisms have been the subject of intensive research and commercial development within the biological agriculture (BioAg) sector.

A recent proof-of-concept study demonstrated that a large cluster of nitrogen-fixing and symbiosis-related genes (a "symbiosis island") can be horizontally transferred from rhizobial bacteria into previously non-nitrogen-fixing strains, enabling some recipients to colonize plant hosts and acquire BNF capabilities (Montoya et al. 2026). This work provides experimental evidence that key symbiotic functions can be transferred as integrated genetic modules and re-established in novel bacterial backgrounds, expanding our understanding of the evolutionary and synthetic biology potential of BNF. While not the first demonstration of horizontal gene transfer (HGT) of symbiosis genes among rhizobia (Sullivan and Ronson 1998; Sullivan et al. 1995), what distinguishes the current work is the functional conversion of genuinely non-diazotrophic bacteria via transfer of a large, integrated gene cluster into distinct phylogenetic groups.

This review is organized as follows. Section 2 provides context on the history and significance of BNF in agriculture and the PGPR research field. Section 3 examines the molecular architecture of symbiosis islands and the mechanisms of their horizontal transfer. Section 4 evaluates the recent proof-of-concept breakthrough and its scientific novelty. Section 5 considers implications for engineering crop-associated microbiomes with emphasis on cereals. Section 6 addresses the principal barriers to field deployment. Section 7 discusses the broader alignment with the BioAg research agenda, and Section 8 offers conclusions and a forward perspective.

II. Biological Nitrogen Fixation in Agriculture: History and Current Status

2.1 The Legume–Rhizobium Paradigm

The nodulation symbiosis between legumes and rhizobia constitutes the most productive biological nitrogen-fixing system known in terrestrial agriculture. Fixation rates can reach 20–300 kg N ha^{-1} yr^{-1} in well-managed legume systems, with commercial soybean cultivation supported by *Bradyrhizobium japonicum* inoculants achieving some of the highest values (Peoples et al. 2009; Ladha et al. 2022). The molecular dialogue underpinning this symbiosis is extraordinarily refined: plant-exuded flavonoids activate rhizobial NodD transcription factors, which in turn induce biosynthesis of lipochitooligosaccharide Nod factors, triggering root hair curling, infection thread formation, cortical cell division, and nodule organogenesis (Oldroyd et al. 2011; Geurts et al. 2016). Within mature nodules, rhizobial bacteroids express the oxygen-sensitive nitrogenase complex under microaerobic conditions maintained in part by leghemoglobin, reducing N_2 to NH_3 for plant assimilation.

Approximately 70% of the roughly 19,300 species in the Fabaceae are capable of nodulating with rhizobia (Warwick and Lewis 2003). Yet the world's three most important cereal crops—rice, wheat, and maize—do not form root nodules and depend almost entirely on external nitrogen inputs for maximum productivity (Guo et al. 2022; Ladha et al. 2022). This fundamental asymmetry between legume and cereal nitrogen nutrition has been a central driver of chemical fertilizer dependence and the long-standing aspiration to extend nodulation capacity beyond the legume family.

2.2 Free-Living and Associative Diazotrophs: PGPR and Beyond

Beyond nodule-forming rhizobia, a taxonomically diverse array of free-living and plant-associated diazotrophs contribute to BNF in agricultural soils. *Azotobacter vinelandii* and related species fix nitrogen aerobically in the rhizosphere, though their per-hectare contribution is generally more modest than nodule symbioses (Watanabe and Cabrera 1979). *Azospirillum* species, particularly *A. brasilense* and *A. irakense*, form loose associations with root surfaces and internal tissues of grasses and cereals, contributing fixed nitrogen alongside multiple additional growth-promoting mechanisms including phytohormone biosynthesis, phosphate solubilization, and induction of systemic resistance (Bashan and de-Bashan 2010; Dobbelaere et al. 2003).

Endophytic diazotrophs, including *Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae*, *Burkholderia* spp., and *Azoarcus* spp., colonize internal plant tissues and can make substantial contributions to the nitrogen nutrition of sugarcane, rice, and other crops under specific conditions (Baldani and Baldani 2005; James et al. 2002). The iconic case of Brazilian sugarcane varieties achieving high yields with minimal nitrogen fertilization—attributable in part to endophytic BNF—remains a proof-of-principle for the agronomic potential of associative and endophytic diazotrophs (Boddey et al. 1995; Urquiaga et al. 2012).

The broader PGPR concept encompasses not only diazotrophs but any rhizosphere-colonizing bacteria that promote plant growth through mechanisms including phosphate solubilization, siderophore production, induced systemic resistance, volatile organic compound emission, and phytohormone biosynthesis (Kloepper and Schroth 1978; Bashan and Holguin 1998; Vessey 2003; Ehinmitan et al. 2024). The integration of BNF with these ancillary PGPR functions has been a major focus of inoculant development and continues to underpin the commercial BioAg sector.

2.3 Commercial BioAg and Inoculant Sector

The market for biological crop inputs—including microbial inoculants, biostimulants, and biopesticides—has grown substantially over the past two decades, driven by regulatory pressure on synthetic inputs, farmer demand for sustainable solutions, and advances in microbial formulation technology (Backer et al. 2018; Ehinmitan et al. 2024). Commercial rhizobial inoculants for soybean alone account for a multi-billion-dollar global market, and the PGPR inoculant space for cereals continues to expand. Nevertheless, field performance of PGPR inoculants remains inconsistent, constrained by soil-plant-microbe-environment interactions that synthetic fertilizers bypass by sheer chemical mass action (Bashan et al. 2014). Improving the reliability, host specificity, and longevity of microbial inoculants in complex field environments remains a central challenge for the BioAg research agenda.

Table 1 (see below) provides a comparative overview of major biological nitrogen-fixing systems and their relevance to sustainable agriculture, plant growth promotion, and next-generation microbiome engineering approaches, highlighting differences in nitrogen-fixation strategy, host association, agronomic advantages, limitations, and future potential for BioAg applications.

III. Symbiosis Islands: Architecture, Ecology, and Horizontal Gene Transfer

3.1 Molecular Architecture of Symbiosis Islands

The genetic determinants of nodulation and BNF in rhizobia are encoded on mobile genetic elements (MGEs)—either large symbiotic plasmids (pSym) or chromosomally integrated genomic islands termed "symbiosis islands" (Sullivan and Ronson 1998; Wardell et al. 2022). The seminal demonstration that a 500-kb chromosomal symbiosis element of *Mesorhizobium loti* ICMP3153 integrates site-specifically into a phenylalanine-tRNA gene and encodes a phage P4-family integrase established the paradigm for symbiosis

island biology (Sullivan et al. 1995). This island carries nod genes governing biosynthesis and secretion of Nod factors, nif genes encoding nitrogenase subunits and associated regulatory proteins, fix genes required for microaerobic respiration and nitrogen fixation within nodules, and an array of additional genes supporting the symbiotic lifestyle including vitamin biosynthesis loci (Sullivan and Ronson 1998; Guo et al. 2022).

The classification of symbiosis gene MGEs has been refined into integrative and conjugative elements (ICEs), which excise from the chromosome, transfer via conjugation, and re-integrate in recipient cells, and symbiotic plasmids, which are transferred without chromosomal integration (Wardell et al. 2022; Hill et al. 2021). The distinction is significant for understanding transfer frequency, stability, and potential for ecological spread. In either case, the gene content is functionally modular: nod and nif gene clusters can be transferred together or separately, and the recipient genomic background can influence post-transfer gene expression and phenotypic manifestation (Okubo et al. 2016).

3.2 Mechanisms and Ecology of Horizontal Transfer

HGT of rhizobial symbiosis genes is now understood to be ecologically widespread and evolutionarily significant (Young and Haukka 1996; Wernegreen and Riley 1999; Parker 2012). Phylogenetic incongruence between core genomic loci (e.g., 16S rRNA, recA) and symbiosis gene loci (nifH, nodC) in field-isolated rhizobia provides robust evidence that symbiosis genes have a history of horizontal movement distinct from chromosomal evolution of their bacterial hosts (Parker 2012; van Cauwenberghe et al. 2014). Transfer is facilitated by flavonoid signals from plant roots—the same compounds that activate Nod factor biosynthesis in established rhizobia—which can induce excision and transfer of the symbiosis island, creating a chemical signalling loop linking plant rhizosphere chemistry to bacterial gene exchange (Ling et al. 2016).

The ecological consequence of HGT in field soils can be rapid and dramatic. Nonsymbiotic mesorhizobia naturally occurring in a field site where *Mesorhizobium loti* ICMP3153 was introduced as inoculant were subsequently recovered as effective legume symbionts, having acquired the symbiosis island from the inoculant strain in situ (Sullivan et al. 1995; Sullivan and Ronson 1998). This observation—that local soil bacteria can be converted to functional nitrogen-fixing symbionts by acquiring a transferable gene island—is the conceptual precursor to the broader proof-of-concept now being explored for non-rhizobial bacteria. The practical implications extend to both the efficacy of inoculant programs and to ecological risk assessment of engineered strains.

3.3 Constraints on Transfer and Functional Expression

The successful conversion of a non-symbiotic bacterium to a functional symbiont by HGT of a symbiosis island is far from guaranteed. Multiple barriers exist. First, the symbiosis island must be transferred, integrated, and maintained in the recipient background. Second, the nod and nif gene products must be expressed at appropriate levels; this depends on regulatory compatibility between island-encoded activators and the recipient's transcriptional machinery. Third, the recipient must be capable of the complex developmental transformations required for host infection, nodule invasion, and differentiation into nitrogen-fixing bacteroids. Fourth, the converted strain must compete effectively for root colonization in complex soil microbiomes (Parker 2012; Guo et al. 2022). Each step presents a potential bottleneck, and the frequency of successful functional conversion has historically been low even among closely related mesorhizobia.

Recipient genome modifications—including substitutions and rearrangements at the chromosomal level—have been identified as important facilitators of symbiotic gene expression following HGT (Remigi et al. 2016). Error-prone DNA polymerases that accelerate adaptation to symbiosis after HGT have been implicated in the rapid evolutionary refinement of newly acquired symbiotic traits, suggesting that natural selection and genomic plasticity together govern the ecological trajectory of converted strains.

IV. The Proof-of-Concept Breakthrough: What Is New and Why It Matters

4.1 Nature of the Breakthrough

The proof-of-concept demonstration advances the HGT paradigm in a significant direction: transfer of a large, integrated symbiosis island from rhizobia not merely to other mesorhizobia or closely related strains, but into genuinely non-nitrogen-fixing bacterial backgrounds representing distinct phylogenetic groups. Some recipient strains, following acquisition of the gene cluster, were found capable of colonizing plant hosts and fixing atmospheric nitrogen, fulfilling the most basic functional criteria of a nitrogen-fixing symbiont (Montoya et al. 2026). This represents a conceptual expansion of the "conversion" concept beyond the pre-adapted mesorhizobial recipients studied in earlier work.

The scientific novelty is not in BNF itself—known and studied for more than a century—nor in HGT of nod-nif genes among related rhizobia, which has been documented for decades (Sullivan et al. 1995; Parker 2012; Wardell et al. 2022). The novelty lies in demonstrating that the symbiotic phenotype is sufficiently modular and portable that it can be functionally reconstituted in a new bacterial chassis via a single large-scale HGT event, without the extensive co-evolutionary history that characterizes established rhizobial lineages. This has profound implications for our understanding of the evolutionary origins of nitrogen-fixing symbiosis and for synthetic biology approaches to microbiome engineering.

4.2 Scientific Significance in Evolutionary Context

From an evolutionary perspective, the rhizobial clade arose through HGT of key symbiotic genes into ancestral soil bacteria, with subsequent elaboration by mutation, recombination, and selection within the new symbiotic context (Remigi et al. 2016). Nitrogen fixation genes (nifHDK and nifENB) are conserved across 14 bacterial and archaeal phyla, suggesting an ancient origin predating the evolution of nodulation-specific nod genes, which are restricted to Alpha- and Betaproteobacteria (Dos Santos et al. 2012; Okubo et al. 2016). The new work recapitulates in laboratory time what evolution accomplished over geological time, with the important qualification that modern molecular tools provide both greater control and greater capacity for rational design than did natural evolutionary processes.

4.3 Implications for PGPR Research

For the PGPR research community, this proof-of-concept validates a long-standing conceptual framework: that the genes governing nitrogen fixation and symbiotic competence are accessible, transferable, and in principle not permanently restricted to established rhizobial lineages. This opens the possibility of asking whether PGPR strains already well-adapted to cereal rhizospheres—that colonize roots efficiently, survive in soil, promote plant growth, and interact productively with the host immune system—could be engineered to add nitrogen fixation to their functional repertoire. This is fundamentally different from simply applying nitrogen-fixing rhizobia to non-legume crops, an approach that has achieved only modest results due to poor colonization, host incompatibility, and absence of the nodulation machinery in cereals.

V. Engineering Crop-Associated Microbiomes for BNF: Pathways and Prospects

Recent advances in synthetic biology and microbial engineering have stimulated interest in transferring complete symbiotic gene clusters into well-adapted PGPR chassis organisms. Rather than engineering nitrogen fixation directly into plants, this approach seeks to exploit naturally efficient rhizosphere colonizers that can be endowed with biological nitrogen-fixation capacity through horizontal transfer of symbiosis islands or synthetic nitrogen-fixation modules. The conceptual framework underlying this strategy is illustrated in Figure 1.

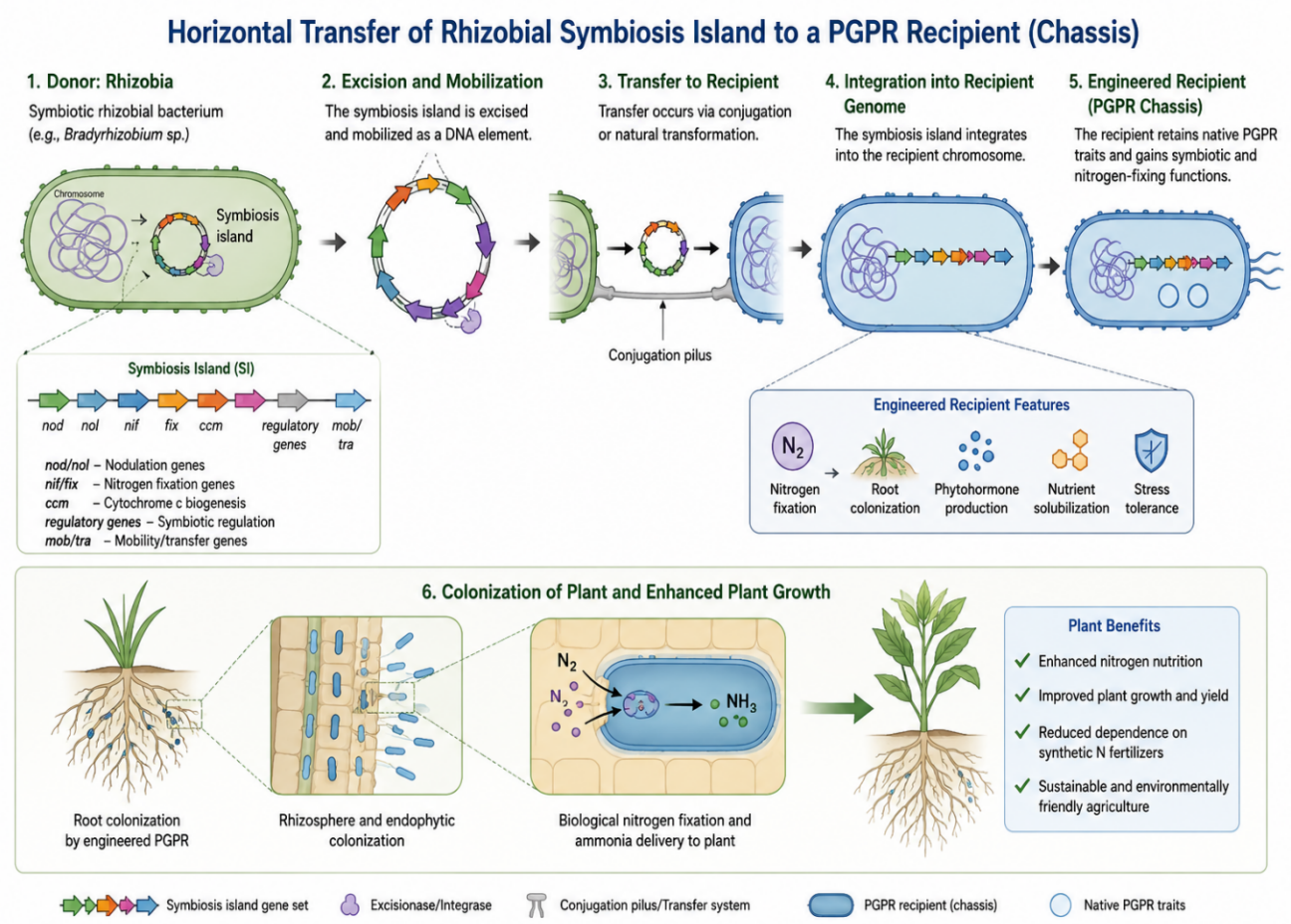

Figure 1. Horizontal transfer of rhizobial symbiosis islands into plant growth-promoting rhizobacteria (PGPR) chassis for next-generation biological nitrogen fixation.

Symbiosis islands containing nodulation (*nod*), nitrogen fixation (*nif*), fixation (*fix*), and regulatory genes are excised from donor rhizobia and transferred through horizontal gene transfer mechanisms to recipient PGPR strains. Following chromosomal integration, recipient bacteria retain their native plant growth-promoting traits while acquiring nitrogen-fixing capabilities. Engineered strains subsequently colonize the rhizosphere or internal plant tissues, contributing biologically fixed nitrogen and enhanced plant growth. This strategy represents a promising approach for microbiome engineering in cereal crops such as rice, wheat, and maize.

5.1 Target Crops and Target Organisms

The three crops most urgently targeted for BNF engineering are rice, wheat, and maize, which together account for the majority of global caloric intake and nitrogen fertilizer consumption (Guo et al. 2022; Ladha et al. 2022). Sugarcane, sorghum, and other cereals or grasses represent secondary targets. The two broad conceptual strategies are: (i) introduction of nitrogen fixation genes directly into the plant nuclear or plastid genome (endogenous fixation); and (ii) engineering of plant-associated microbiomes to support BNF by cereal-adapted microbes (microbiome engineering). While endogenous plant engineering has received significant research investment (Rogers and Oldroyd 2014; Guo et al. 2022), the inherent challenges of expressing and regulating the oxygen-sensitive nitrogenase complex in photosynthetically active plant cells remain formidable.

Microbiome engineering, of which the symbiosis island HGT approach is one variant, offers an alternative route: direct manipulation of the bacterial partners rather than the plant. Target organisms must satisfy several criteria—they should be effective rhizosphere colonizers of the target crop, metabolically compatible with nitrogen fixation, capable of stable maintenance of transferred genetic elements, and safe for environmental

release (Geddes et al. 2019; Ladha et al. 2022). Candidate PGPR strains with established track records in cereal rhizospheres, such as *Pseudomonas, Bacillus, Herbaspirillum*, and *Azospirillum* species, are among those considered most promising.

5.2 Synthetic Biology Approaches

Advances in synthetic biology have enabled increasingly sophisticated approaches to nitrogen fixation engineering in diverse bacterial backgrounds. The transfer of minimal nif gene sets sufficient for nitrogenase assembly and activity has been demonstrated in non-native hosts including Escherichia coli and various Gram-positive bacteria (Rogers and Oldroyd 2014; Geddes et al. 2019). Key technical challenges include ensuring adequate reducing power and ATP supply for nitrogenase function, protecting nitrogenase from oxygen damage, engineering nitrogen sensing and regulatory circuits to prevent wasteful fixation when combined nitrogen is available, and maintaining genetic stability of inserted gene clusters under field conditions (Geddes et al. 2019; Guo et al. 2022).

5.3 Host Specificity and Rhizosphere Competence

A critical dimension often underappreciated in laboratory proof-of-concept studies is that effective BNF in the field requires not merely that a bacterium possesses nitrogen fixation genes, but that it colonizes the right niche within the plant root system in sufficient numbers for a sufficient duration during the crop growing season. The ecology of rhizosphere colonization is complex, involving competition with indigenous microbiota, specificity of root exudate recognition, chemotaxis toward appropriate carbon and oxygen gradients, and compatibility with plant innate immune responses (Buée et al. 2009; Bulgarelli et al. 2013; Hacquard et al. 2015).

The advantage of working with established PGPR strains as chassis organisms is that colonization, survival, and plant compatibility have already been selected for. The added challenge is ensuring that insertion of a large symbiosis island does not disrupt these existing traits. Careful genomic site selection for island integration, attention to metabolic interactions, and phenotypic characterization of engineering outcomes in the target crop rhizosphere will all be essential.

VI. Barriers to Field Deployment: Ecological, Biosafety, and Regulatory Dimensions

6.1 Ecological Stability and Persistence

Genetically modified or engineered microorganisms introduced into agricultural soil must demonstrate ecological stability—that the modified trait persists for agronomically meaningful periods, does not impose fitness costs that select for phenotype loss, and does not spread uncontrollably into non-target environments. The history of engineered microbial inoculants provides sobering precedent: many strains perform well in controlled conditions but are outcompeted by indigenous microbiota in the complex, variable soil environment (Bashan et al. 2014). Symbiosis island elements that can transfer by conjugation may spread beyond engineered strains to non-target bacteria, with unpredictable ecological consequences (Wardell et al. 2022). Genetic containment strategies—including chromosomal insertion to reduce conjugative transfer, conditional lethality systems, and privatized synthetic regulatory circuits—will be important tools for risk management.

6.2 Biosafety and Regulatory Acceptance

Regulatory frameworks for the deliberate environmental release of genetically modified microorganisms vary significantly among jurisdictions and have evolved considerably in recent years, though the pace of regulatory modernization has not always kept pace with the rate of synthetic biology innovation. In major agricultural economies including the United States, European Union, Brazil, India, and China,

engineered nitrogen-fixing microorganisms will face requirements for environmental risk assessment, efficacy data, contained field trials, and product registration as biological pesticides or biofertilizers, depending on the jurisdiction's classification framework (Berg et al. 2020; Ehinmitan et al. 2024). The societal dimensions of releasing novel nitrogen-fixing organisms—including farmer perception, supply chain integration, and intellectual property structures—are no less important than the scientific and regulatory dimensions.

6.3 Field Performance and Farmer-Level Reliability

Ultimately, any BNF technology must deliver reliable, measurable yield or nitrogen benefit to farmers across diverse agronomic environments. The track record of PGPR inoculants demonstrates that laboratory promise rarely translates directly to consistent field performance without extensive agronomic optimization, including attention to formulation, application timing, compatibility with agrochemicals, soil type, and variety interactions (Bashan et al. 2014; Ehinmitan et al. 2024). Enhanced BNF strains will face the same challenges, compounded by the additional metabolic complexity and potential fitness costs of nitrogen fixation. A systems-level approach integrating microbiology, agronomy, soil science, and crop physiology will be essential for translating molecular proof-of-concept into farmer-level solutions.

VII. Alignment with the BioAg Research Agenda

For researchers working in PGPR, BioAg, and the broader field of beneficial plant-microbe interactions, the proof-of-concept demonstration of functional symbiosis island transfer represents simultaneously a validation and a call to action. It validates the modular, transferable nature of BNF, confirming what decades of HGT research in rhizobia have implied: the nitrogen-fixing phenotype is not irreversibly embedded in a narrow evolutionary lineage, but is in principle accessible to engineering in diverse bacterial backgrounds. At the same time, it underscores the gap between laboratory demonstration and field deployment that PGPR researchers have navigated—with mixed success—for more than four decades.

The research community is well-positioned to contribute to the next phase of this work. Expertise in rhizosphere ecology and colonization biology is essential for understanding which bacterial chassis organisms are most promising and how to maintain their rhizosphere competence after genetic modification. Experience with inoculant formulation and field trial design provides frameworks for moving from laboratory to agricultural settings. Long-standing advocacy for science-based BioAg—including rigorous efficacy evaluation, ecologically sound deployment strategies, and integration of beneficial microbes into whole-farm nitrogen management—provides the ethical and practical framework within which this technology must be developed.

The future of sustainable agriculture will not be built on a single technological solution. Biological nitrogen fixation in all its forms—from classical rhizobial inoculants to PGPR consortia to next-generation engineered diazotrophs—must be integrated with soil health management, crop rotation, cover cropping, and reduced but optimized synthetic nitrogen use. The goal is not to eliminate synthetic nitrogen from agriculture overnight, but to progressively expand the biological contribution in ways that are reliable, affordable, and ecologically sound.

VIII. Conclusions and Forward Perspective

The demonstration that a large cluster of nitrogen-fixing and symbiosis-related genes can be horizontally transferred from rhizobia into previously non-nitrogen-fixing bacteria, enabling some recipients to colonize plant hosts and perform BNF, represents a significant proof-of-concept advance in microbiome engineering and agricultural biotechnology. Its importance lies not in the discovery of BNF itself—long

exploited through rhizobial inoculants, Azospirillum, Azotobacter, endophytic diazotrophs, and other PGPR—but in demonstrating that complex symbiotic functions can be transferred as integrated genetic modules into novel microbial backgrounds. This finding reinforces the concept that nitrogen-fixing capacity is not confined to a limited evolutionary lineage but can potentially be extended to microbial chassis already adapted to agriculturally important crops.

The successful transfer of symbiosis islands provides a new framework for developing next-generation microbial inoculants capable of combining efficient rhizosphere colonization, plant growth promotion, and biological nitrogen fixation. Such advances could contribute to reducing dependence on synthetic nitrogen fertilizers, improving nutrient-use efficiency, and mitigating the environmental impacts associated with intensive fertilizer use, including greenhouse gas emissions, nitrate leaching, and ecosystem degradation. In particular, the prospect of engineering cereal-associated microbiomes for crops such as rice, wheat, and maize offers an attractive pathway toward more sustainable and climate-resilient agricultural production systems.

Nevertheless, substantial scientific, ecological, and regulatory challenges remain. Future research must address the optimization of post-transfer gene expression, maintenance of symbiotic functionality and ecological fitness, long-term genetic stability, rhizosphere competence, host specificity, and potential risks associated with HGT in natural environments. Equally important will be the development of robust biosafety assessment frameworks, transparent regulatory pathways, and comprehensive field-validation programs capable of evaluating performance across diverse agroecological conditions.

The convergence of microbial ecology, synthetic biology, genomics, microbiome engineering, and precision agriculture is creating unprecedented opportunities to redesign beneficial plant-microbe interactions for enhanced agricultural sustainability. However, technological innovation alone will not ensure success. Long-term adoption will depend on the development of safe, reliable, economically viable, and farmer-friendly solutions that deliver consistent benefits under real-world farming conditions. If achieved, the integration of engineered BNF, advanced PGPR technologies, and microbiome-based crop management could become a transformative pillar of sustainable agriculture, contributing significantly to global food security, environmental stewardship, and climate resilience in the twenty-first century.

IX. Acknowledgments

The authors gratefully acknowledge the Head, Department of Biological Sciences, Rani Durgawati University, Jabalpur, Madhya Pradesh, India, for providing institutional facilities and support. The financial assistance received from the Council of Scientific and Industrial Research (CSIR), Government of India, is also sincerely acknowledged.

Table 1. *Comparative characteristics of major biological nitrogen-fixing and plant growth-promoting bacterial systems relevant to sustainable agriculture and next-generation microbiome engineering.*

Microbial Group	Representative Organisms	N_2-Fixation Strategy	Host Association	Additional PGPR Functions	Advantages	Major Limitations	BioAg Engineering Potential
Rhizobia	*Bradyrhizobium japonicum,*	Symbiotic BNF within	Legumes	Phytohormone production,	Highest known agricultura	Limited primarily to legume	Source of symbiosis islands

	Rhizobium leguminosarum, Mesorhizobium loti	root nodules		nutrient mobilization, stress tolerance	l BNF efficiency (20–300 kg N/ha/yr); proven commercial inoculants	hosts; nodulation absent in cereals	and nitrogen-fixation genes for future engineering
Azospirillum spp.	*A. brasilense, A. lipoferum*	Associative N_2 fixation in rhizosphere and root tissues	Cereals, grasses, maize, wheat, rice	Auxin production, root stimulation, phosphate solubilization, stress mitigation	Excellent root colonization and commercial success in cereals	Nitrogen contribution generally lower than rhizobial symbiosis	Promising chassis for engineered nitrogen-fixing microbiomes
Azotobacter spp.	*A. vinelandii, A. chroococcum*	Free-living aerobic N_2 fixation	Rhizosphere-associated	Vitamins, phytohormones, siderophores, biopolymers	Broad environmental adaptability and high nitrogenase activity	High energy demand; inconsistent field performance	Suitable model for synthetic biology and nitrogenase engineering
Endophytic Diazotrophs	*Gluconacetobacter diazotrophicus, Herbaspirillum seropedicae, Azoarcus* spp.	Endophytic N_2 fixation within plant tissues	Sugarcane, rice, maize, grasses	Growth promotion, stress tolerance, nutrient acquisition	Close proximity to plant tissues enhances nitrogen transfer efficiency	Host specificity and variable colonization success	Strong candidates for microbiome engineering of non-legume crops
Engineered Symbiosis-Island Recipients	Non-diazotrophic bacterial strains receiving transferred symbiosis islands	Acquired N_2 fixation via HGT	Potentially legumes and non-legume crops	Depends on recipient chassis; may retain native PGPR traits	Demonstrates portability of symbiotic phenotype; expands engineering possibilities	Regulatory concerns, ecological stability, gene-expression compatibility, field validation required	Next-generation platform for engineering cereal-associated N_2-fixing microbiomes
Engineered PGPR	*Pseudomonas, Bacillus,*	Synthetic or	Rice, wheat,	Retains established	Combines strong	Technology still	High potential

Chassis (Future)	*Azospirillum, Herbaspirillum* with nif/symbiosis modules	transferred BNF pathways	maize, sorghum	PGPR functions while adding BNF capacity	rhizosphere competence with biological N_2 fixation	experimental; requires biosafety validation and field testing	for reducing synthetic fertilizer dependence in future BioAg systems

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