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Rhizobia in legumes: A boon to low-nitrogen soil

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Abstract

Biological nitrogen fixation serves as the cornerstone for nitrogen input in agricultural soils, especially in arid regions. The most impactful nitrogen-fixing systems are symbiotic, and they played a pivotal role in enhancing the fertility and productivity of nitrogen-deficient soils. Among these systems, legume symbioses have garnered significant attention and have been the focus of extensive research. In these powerful partnerships, the bacteria *Rhizobium* efficiently fix nitrogen within all leguminous plants by forming specialized structures known as nodules. These nodules are essential for effective biological nitrogen fixation. Within the Rhizobium-legume symbiosis, the plant supplies a carbon source. At the same time, the bacteria convert atmospheric nitrogen into ammonium, an unviable process that enables legumes to flourish in nitrogen-poor soils. This remarkable symbiotic relationship is not just beneficial but essential for the advancement of sustainable agriculture. It mitigates the environmental consequences associated with traditional farming practices and conserves vital nitrogen fertilizers. The Rhizobium-legume symbiosis, encompassing both herbaceous and tree legumes, emerges as a premier solution for enhancing soil fertility and rehabilitating arid landscapes. Therefore, it stands as a critical focus for future research and agricultural innovation.

Keywords: BNF, symbioses, *rhizobium,* legume, nodules

Introduction

The primary source of nitrogen (N) input in the ecosystem is biological nitrogen fixation (BNF) (Vitousek and Field, 2001) [60]. BNF plays a crucial role in climate preservation and sustainable agriculture by producing an estimated 200 Tg of organic nitrogen annually (Galloway *et al*., 2003; Peoples *et al*., 2009) [49, 55]. In legumes, a symbiotic process known as symbiotic nitrogen fixation (SNF) occurs within the host roots, where a group of bacteria, specifically rhizobia, convert atmospheric nitrogen into an organic form enzymatically. Grain legumes, which rank second globally in crop production, constitute 27% of this total. Plant-based proteins account for 60% of the world's total human protein needs, with legumes contributing approximately 50% of that (Smýkal *et al*., 2015) [59].

An evaluation of global protein sustainability indicates a shift from animal-based to plant-based proteins (Chaudhary *et al*., 2018; Leinonen *et al*., 2019) [47, 53]. Legumes are essential in agriculture for two main reasons. Firstly, their high protein content (20-25% of dry weight) serves as an important source of nutrition, especially for those who cannot afford animal-based products. Secondly, legumes produce less environmental waste than non-legume crops because they can fix atmospheric nitrogen, which lessens the need for synthetic fertilizers (MacWilliam *et al*., 2014; Romeiko, 2019) [54, 57]. While the precision of life cycle assessments (LCA) may differ, they are utilized to evaluate greenhouse gas (GHG) emissions from all phases of crop production (Caffrey and Veal, 2013) [46]. To grasp the significance of legumes in sustainable agriculture, one must consider the environmental implications of synthetic nitrogen usage. Of the 120 Tg of chemically manufactured nitrogen required each year (FAO, 2017), almost half is applied to fertilize rice, maize, and wheat (Ladha *et al*., 2016) [52]. Wheat, for instance, has an estimated nitrogen-use efficiency of only 33%, with the remaining nitrogen either escaping as nitrous oxide (N_2O) emissions or contributing to water pollution (Raun and Johnson, 1999) [56].

According to the U.S. Environmental Protection Agency (EPA), nitrous oxide (N_2O) has a greenhouse gas effect that is 294 times more potent than that of carbon dioxide on a per-mass basis. Furthermore, fossil fuels play a significant role in the production of nitrogen fertilizers, with CO_2 emissions from burning fossil fuels estimated between 1.10 and 3.37 tons per ton.

By 2050, the chemical synthesis of nitrogen fertilizers is projected to represent 2% of global energy consumption (Glendining *et al*., 2009) [51]. Agriculture, which includes both crop farming and livestock raising, is responsible for approximately one-third of global greenhouse gas emissions (Gilbert, 2012) [50]. Concerns regarding the sustainability of this sector arise from the carbon footprint associated with agricultural practices and the environmental effects of fertilizer runoff.

The Intergovernmental Panel on Climate Change (IPCC) has stressed the critical need to address climate change and lower greenhouse gas emissions. In this particular situation, growing legumes offers a hopeful approach to reducing agriculture's carbon footprint by expanding legume cultivation and enhancing the efficiency of the symbiotic nitrogen fixation (snf) process. However, the affordability and widespread availability of synthetic nitrogen fertilizers have historically taken precedence over snf. Efforts to improve legume crops have primarily focused on agronomic factors such as yield, disease resistance, and other beneficial traits. The lack of focus on improving symbiotic nitrogen fixation efficiency in legume breeding programs can be attributed to its complexity, as it requires the integration of multiple gene combinations in both the host plant and its symbiotic partner. The widespread availability of chemical fertilizers also contributed to the preference for synthetic nitrogen sources (roy *et al*., 2020) [30]. Unlike genes related to plant growth and development, genes involved in symbiotic relationships are more difficult to map on chromosomes and evaluate their heritability in breeding programs. Additionally, optimizing symbiotic efficiency requires a deep understanding of environmental interactions and effective deployment strategies, making trait stability across different conditions a crucial factor. Since native rhizobial strains are well-adapted to specific ecological niches, introducing genetically modified or improved strains poses significant challenges. Developing elite rhizobial strains tailored for specific crops is essential to maximizing efficiency, yet this area has not received sufficient research attention (Sessitsch *et al*., 2002) [58]. In recent years, increased awareness of rhizobia's ecological role has led to significant advancements in understanding the nitrogen-fixing process. This paper provides an overview of the key factors influencing symbiosis and nitrogen fixation efficiency, highlighting snf as a potential breeding trait. By addressing existing research gaps, this work aims to foster greater interest in strengthening this beneficial relationship for a more sustainable future.

Current problems and future perspectives on legume productivity

Legume productivity in the semi-arid tropics is severely affected by drought, a prolonged period of inadequate rainfall that depletes soil moisture and reduces water availability in plant tissues. This water deficit prevents crops from achieving their expected yields, leading to substantial declines in production and negatively impacting national economies. Grain legumes that rely on rainfall during their vegetative and reproductive growth stages are particularly vulnerable to intermittent drought stress, with subsequent rainfall playing a crucial role in recovery. Terminal drought stress, which occurs during the pod-filling stage, is especially detrimental to rainfed crops, further exacerbating yield losses. This is even more critical for crops that rely on stored soil moisture and are cultivated in post-rainy seasons. Key leguminous crops in the semi-arid tropics, such as groundnut, chickpea, and pigeon pea, are highly susceptible to water shortages and extreme temperatures during flowering and seed development, resulting in major production losses (Sankareswari R Uma & Thirumalaikumar R, 2017) [31].

To maintain sustainable agriculture in drought-prone areas, farmers need to implement targeted strategies. This includes analyzing weather data from the past decade to optimize sowing times, adopting pressurized micro-irrigation systems with advanced soil moisture sensors, and introducing slow-release encapsulated fertilizers and pesticides to improve soil and crop resilience. Additionally, pre-treating leguminous crops like black gram with drought-tolerant Rhizobium strains (Sankareswari R Uma *et al*., 2017) [31], maintaining permanent soil organic cover, and employing conservation techniques such as minimum tillage and zero tillage can help mitigate the effects of drought.

Food and nutritional insecurity remain prevalent in semi-arid regions, necessitating a balance between increasing food production, managing water scarcity, and ensuring adequate nutrition. Given their high protein and micronutrient content, grain legumes play a crucial role in enhancing food security for rural communities. Expanding the variety of cultivated legumes, including minor grain legumes, could serve as a buffer against the adverse impacts of climate change on major crops. At the Year of Pulses - ICRISAT 2016 conference, Ramasamy advocated for a shift in pulse research towards increasing farmer incomes rather than solely focusing on productivity. Additionally, biofortification should be explored as a means to address nutritional deficiencies, particularly among children in India.

Nitrogen Fixation

Nitrogen fixation is the biological process that transforms atmospheric nitrogen (N_2) into ammonia (NH_3). The N_2 molecule is stable due to its strong triple bond, which makes abiotic conversions infrequent. High-energy events, such as lightning, provide the necessary activation energy for nitrogen to form reactive compounds. For more than a century, the Haber-Bosch process has been constructed to convert N_2 into ammonia under conditions of high temperature and pressure, facilitating the large-scale production of fertilizers.

Rhizobia

Rhizobia, the bacterium that fixes nitrogen as well as colonizes the root and stem nodules of leguminous plants. As Gram-negative Proteobacteria, they are phylogenetically diverse and can thrive in soil independently of their host plants. Despite their metabolic diversity, rhizobia share traits with soil-dwelling pseudomonad bacteria.

Nodule formation starts with Nod factors, signaling molecules that stimulate development. Rhizobia enter the plant through infection threads that form in coiled root hairs, establishing a symbiotic relationship. Notably, some less-studied legume species exhibit significant deviations from this typical model, and the effects of these variations on rhizobial traits are largely unexplored.

In specialized rhizobia known as bacteroids, nitrogen fixation occurs in the central zone of nodules, driven by dicarboxylic Krebs cycle molecules for microaerobic respiration. The expression of the most important genes, *viz*., *nif* and *fix* genes, is activated by low oxygen levels in the bacteroid environment. As noted by Noel (2009) [26], current rhizobial genera have evolved through horizontal gene transfer among Proteobacteria, with mobile genetic elements playing a key role in their symbiotic adaptations.

Biogeochemical Process: The Nitrogen Cycle

The biogeochemical process of nitrogen transforms atmospheric nitrogen into forms that are biologically usable for plants and animals. The most required element is nitrogen, but it was present in an unavailable form that cannot be taken as such by living organisms.

The nitrogen cycle involves several key processes:

- **Nitrogen fixation:** Nitrogen present in the atmosphere is converted into ammonia by Rhizobia, the nitrogen fixers
- **Nitrification:** Ammonia is converted into nitrites and nitrate and making it useful to plants.
- **Denitrification:** Denitrifying bacteria are involved in the conversion of nitrates back into atmospheric nitrogen (N_2).
- **Decay and putrefaction:** Organic forms of nitrogen derived from dead organisms and waste products are degraded by decomposers, releasing nitrogen to the soil.

Nitrogen exists in both organic (within living organisms) and inorganic (atmospheric and soil) forms. It cycles through ecosystems via food chains and microbial transformations, maintaining ecological balance. The marine environment harbors the most intricate biogeochemical pathways, which play a pivotal role in regulating nitrogen availability across diverse aquatic microbiomes.

Nitrogen Fixation Process

Nitrogen Fixation: The First Stage of the Nitrogen Cycle

The nitrogen cycle begins with nitrogen fixation, the process by which inert atmospheric nitrogen (N_2) is reduced to ammonia (NH_3), making it accessible for plant uptake. This process occurs through natural mechanisms such as precipitation, biological fixation, and industrial fixation. Atmospheric nitrogen is deposited into soils from surface waters and the air, particularly through rainfall.

Biological nitrogen fixation (BNF) is primarily facilitated by Rhizobium and Azotobacter bacteria, which contain the enzyme nitrogenase. This enzyme enables the reaction between gaseous nitrogen and hydrogen, producing ammonia. Nitrogen fixation can occur through.

- **Atmospheric fixation:** Natural phenomena like lightning provide the energy to convert nitrogen into usable compounds.
- **Industrial fixation:** Nitrogen fixation at the industrial scale is achieved via the Haber-Bosch process, which synthesizes ammonia under elevated temperature and pressure, thereby sustaining global fertilizer production
- **Physical and biological fixation:** While physicochemical methods contribute 10% of natural nitrogen fixation, the remaining 90% is attributed to biological processes (Vitousek & Field, 2001) [60].

Nitrogen's Role in Plants and Ecosystems

Nitrogen is the most essential macroelement next to carbon, hydrogen, and oxygen. It comprises the following derived components

1. Amino acids and proteins - the fundamental units of cellular structure and function.
2. Nucleic acids (DNA & RNA) - carriers of genetic information.
3. Chlorophylls - pigments crucial for photosynthesis.
4. Phytohormones - regulators of plant growth and development.
5. Enzymes and vitamins - catalysts and cofactors driving metabolic processes.

Plants primarily absorb nitrogen in the form of nitrate (NO_3^-) and ammonium (NH_4^+). Nitrate predominates in well-aerated, neutral soils, whereas ammonium is more abundant in acidic or waterlogged conditions. Additional nitrogen sources include amino acids released from decomposing organic matter, urea derived from animal excreta, and chemical fertilizers. Despite the abundance of atmospheric nitrogen (N_2), higher plants cannot directly utilize it, making biological nitrogen fixation indispensable for plant nutrition. Symbiotic nitrogen fixation (SNF), particularly in legumes, involves rhizobial bacteria colonizing root nodules and enzymatically converting atmospheric nitrogen into organic forms that plants can assimilate. This process represents the primary natural input of nitrogen into ecosystems, contributing approximately 200 Tg of organic nitrogen annually (Galloway *et al*., 2003; Peoples *et al*., 2009) [49, 55]."

Legumes and Global Protein Sustainability

Legumes constitute the second-largest crop group after cereals, contributing about 27% of total global crop production. Plant-based proteins account for nearly 60% of the global human protein intake, with legumes supplying almost half of this fraction (Smýkal *et al*., 2015) [59]. Recent global assessments highlight the need to transition from animal-based to plant-based protein sources, with legumes playing a central role in advancing sustainable food systems (Chaudhary *et al*., 2018; Leinonen *et al*., 2019) [47, 53]."

Legumes play a pivotal role in agriculture owing to their high protein content (20-25% dry weight) and their low environmental footprint, largely attributable to biological nitrogen fixation. In contrast to synthetic fertilizers—which are major contributors to greenhouse gas emissions and water pollution—legumes naturally enrich soils with nitrogen, thereby reducing the carbon footprint of agricultural systems (MacWilliam *et al*., 2014; Romeiko, 2019) [54, 57].

Despite their benefits, synthetic nitrogen fertilizers remain widely used due to affordability and accessibility, overshadowing the potential of symbiotic nitrogen fixation (SNF). Research on legume improvement has largely focused on yield and disease resistance, with SNF efficiency receiving less attention due to its genetic complexity.

Drought stress, particularly in semiarid tropics, hampers legume productivity and economic stability. To ensure sustainable agriculture, strategies such as micro-irrigation, slow-release fertilizers, drought-tolerant Rhizobium strains, and conservation practices are essential (Sankareswari R Uma *et al*., 2017) [31].

Expanding minor grain legumes can enhance food security by diversifying protein sources and mitigating climate risks. Boosting SNF efficiency and supporting legume research are critical steps toward sustainable agriculture, reduced greenhouse gas emissions, and improved farmer livelihoods.

Nitrogen cycle

The nitrogen cycle is a biogeochemical process through which nitrogen is transformed into various forms, passing consecutively from the atmosphere to the soil to organisms and back into the atmosphere. This cycle involves several key processes, including nitrogen fixation, nitrification, denitrification, decay, and putrefaction. Nitrogen exists in both organic and inorganic forms. Organic nitrogen is found in living organisms and is passed through the food chain via the consumption of other organisms. Inorganic nitrogen, on the other hand, is abundant in the atmosphere.

Symbiotic bacteria play a crucial role in this process by converting inert nitrogen into forms that plants can use, such as nitrites and nitrates. These transformations are essential for maintaining the ecosystem. The marine nitrogen cycle is particularly complex and is present in various biomes.

Nitrogen fixation Process

Nitrogen fixation is the first stage of the nitrogen cycle. This process converts atmospheric nitrogen (N2), which is mostly inert, into ammonia (NH3), which can be utilized by living organisms. Nitrogen gas enters the soil from surface waters and the atmosphere through precipitation during nitrogen fixation. Bacteria such as Rhizobium and Azotobacter are key players in this process; they contain an enzyme called nitrogenase, which enables them to react with hydrogen and gaseous nitrogen to produce ammonia. Nitrogen can be fixed through natural processes, such as lightning (atmospheric fixation), or artificially, through industrial methods that create ammonia under extreme pressure and temperature.

There are two types of nitrogen fixation: physical and biological. Nitrogen is one of the most essential macronutrients for living organisms, following carbon, hydrogen, and oxygen. It is crucial for the synthesis of amino acids, proteins, nucleic acids, cytochromes, chlorophylls, alkaloids, phytohormones, and several vitamins. Microbes and plants compete for the limited supply of nitrogen in the soil. Plants primarily absorb nitrogen in the form of nitrate (NO3-) or ammonium ions (NH4+). Nitrate is more prevalent in well-oxygenated, non-acidic soils, while ammonium is dominant in acidic or waterlogged conditions.

Other sources of available soil nitrogen include amino acids from decaying organic matter, animal excreta (urea), and chemical fertilizers, which plants can absorb directly. The atmosphere serves as the primary source of nitrogen for plants, existing as free diatomic (N2) molecules. This gas is highly inert and must be fixed, as higher plants cannot utilize it directly. Nitrogen fixation is the process through which free nitrogen (both molecular and elemental) is converted into nitrogenous compounds that plants can absorb. Around 10% of natural nitrogen fixation occurs through physicochemical methods, while approximately 90% is achieved through biological methods.

Physical Nitrogen Fixation

(i) Natural Nitrogen Fixation

When thunder and lightning produce an electric discharge in the clouds, N2 and Nitric oxide (NO) is created when airborne O_2 reacts. Nitrogen peroxide (NO_2) is created when the nitric oxides undergo further oxidation with oxygen

The reactions are as follows:

N_2 + O_2 Lightning → Thunder 2N0 (Nitric Oxide); 2NO + $O_2 \rightarrow 2NO_2{}^{\text{Oxidation}}$ (Nitrogen peroxide)

During the rains, NO_2 combines with rain water to form nitrous acid (HNO_2) and nitric acid (HNO_3). The acids fall on the soil along with rain water and react with the alkaline radicals to form water soluble nitrates (NO_3-) and nitrites ($N0_2$-).

$2NO_2 + H_2O \rightarrow HNO_2 + HNO_3$; HNO_3 + Ca or K salts → Ca or K nitrates

The nitrates are soluble in water and are directly absorbed by the roots of the plants.

(ii) Industrial Nitrogen Fixation:

Nitrogen and hydrogen (obtained from water) are directly combined at high temperatures and pressures to make ammonia in an industrial setting. It is subsequently transformed into a variety of fertilizers, including urea

Biological Nitrogen Fixation:

Biological nitrogen fixation is the process through which living organisms convert atmospheric nitrogen into nitrogenous compounds. This process primarily involves two categories of microorganisms: those that have symbiotic relationships with plants and those that are free-living (non-symbiotic).

Atmospheric nitrogen is converted into ammonia in the presence of the enzyme nitrogenase, a process known as biological nitrogen fixation (BNF). Only a few bacteria, such as the symbiotic Rhizobium and Frankia, along with free-living species like Azospirillum and Azotobacter, are known to produce nitrogenase, which acts as a biological catalyst. Nitrogen constitutes over 80% of the Earth's atmosphere as inert di-nitrogen (N_2), which cannot be utilized directly by most plants. The di-nitrogen molecule is made up of two nitrogen atoms held together by a triple covalent bond. Breaking this triple bond requires approximately 225 kilocalories of energy, making the process quite challenging.

Nitrogen Fixers

Among Earth's organisms, only certain prokaryotes, such as bacteria and cyanobacteria, can fix atmospheric nitrogen. These organisms are referred to as nitrogen fixers or diazotrophs and are responsible for fixing about 95% of the total nitrogen that is naturally converted each year, which amounts to approximately 200 million metric tons. Diazotrophs can be classified into two categories: asymbiotic (free-living) and symbiotic, as outlined below: Diazotrophs may be asymbiotic (free living) or symbiotic such as given below:

Nitrogen-Fixing Microorganisms

1. Free-Living Nitrogen-Fixing Bacteria

Several saprophytic bacteria contribute to nitrogen fixation in soils. Aerobic species include *Azotobacter* and *Beijerinckia*, while anaerobic species include *Clostridium*. *Desulfovibrio* represents a chemotrophic nitrogen-fixing bacterium, whereas photoautotrophic nitrogen-fixing bacteria include *Rhodopseudomonas*, *Rhodospirillum*, and *Chromatium*. Collectively, these bacteria contribute approximately 10-25 kg of nitrogen per hectare annually.

2. Free-Living Nitrogen-Fixing Cyanobacteria

Numerous cyanobacteria (formerly referred to as blue-green algae) are capable of nitrogen fixation. These include *Anabaena*, *Nostoc*, *Aulosira*, *Cylindrospermum*, and *Trichodesmium*. They are particularly significant in waterlogged soils where denitrifying bacteria are active. *Aulosira fertilissima* is recognized as the most effective nitrogen fixer in rice fields, while *Cylindrospermum* shows activity in sugarcane and maize fields. Cyanobacteria contribute approximately 20-30 kg of nitrogen per hectare annually.

3. Symbiotic Nitrogen-Fixing Cyanobacteria

Certain cyanobacteria establish symbiotic associations with plants. *Anabaena* and *Nostoc* are common symbionts in lichens, *Anthoceros* (a bryophyte), *Azolla* (a water fern), and cycads. The fronds of *Azolla pinnata* harbor *Anabaena azollae*, which has been widely used as a biofertilizer in rice cultivation due to its nitrogen-fixing capacity.

4. Symbiotic Nitrogen-Fixing Bacteria

Leguminous plants (Papilionaceae) form root nodules that harbor gram-negative, aerobic bacteria of the genus *Rhizobium*. Related genera such as *Aerorhizobium* colonize both root and stem nodules, e.g., in *Sesbania rostrata*. Another significant symbiont, *Frankia alni*, occurs in the root nodules of actinorhizal plants such as *Alnus* and *Casuarina*. Additionally, symbiotic nitrogen-fixing associations have been reported in members of *Rubiaceae* and *Myrsinaceae*, where *Xanthomonas* and *Mycobacterium* occur as endosymbionts.

Although many *Rhizobium* species are present in soils as free-living bacteria, they are unable to fix nitrogen independently. Nitrogen fixation occurs only when they form symbiotic associations with legumes. Similarly, *Frankia* can exist freely in soil but fixes nitrogen exclusively in symbiosis.

Symbiotic Nitrogen Fixation

Symbiotic nitrogen fixation in legumes involves a series of coordinated molecular and cellular interactions between *Rhizobium* spp. and the host plant roots. The process can be described in the following steps:

1. Nodule Formation

Nodule development requires specific signaling events between the legume host and compatible rhizobia.

- **Host specificity:** The rhizosphere surrounding young legume roots contains diverse microorganisms. To recruit compatible rhizobia, host plants secrete root exudates such as flavonoids and betaines. In response, specific *Rhizobium* strains synthesize signaling molecules called Nod factors (lipochitooligosaccharides).
- **Root hair response:** Nod factors are recognized by lectin-like receptor proteins on root hair surfaces, triggering root hair deformation and curling. This morphological change traps the rhizobia, enabling entry into the root hair.
- **Infection thread formation:** Rhizobia penetrate the root hair through an invagination of the plasma membrane called the infection thread. As the thread elongates, dividing bacteria advance toward the root cortex.
- **Bacterial release and differentiation:** Rhizobia are released from the infection thread into cortical cells, either singly or as membrane-bound clusters. Once inside, they differentiate into nitrogen-fixing forms known as bacteroids. Bacteroids lose their ability to divide, undergo cell wall modifications, and become surrounded by a plant-derived membrane called the peribacteroid membrane, together forming the symbiosome.
- **Nodule organogenesis:** Concurrently, cortical cells undergo division and differentiation, giving rise to root nodules that house the bacteroids and establish the site of biological nitrogen fixation.

2. Mechanism of nitrogen fixation

The root nodules provide the specialized microaerobic environment required for nitrogen fixation. They contain all of the essential components, including leghemoglobin (a plant hemoprotein that regulates oxygen concentration) and the nitrogenase enzyme complex, which catalyzes the reduction of atmospheric nitrogen.

The nitrogenase complex consists of two metalloproteins:

- **Fe-protein (dinitrogenase reductase; azoferredoxin):** a homodimer containing a [4Fe-4S] cluster, responsible for electron transfer using ATP hydrolysis.
- **MoFe-protein (dinitrogenase; molybdoferredoxin):** a heterotetramer containing molybdenum-iron cofactors (FeMo-co), which serves as the catalytic site for nitrogen reduction.

The overall reaction catalyzed by nitrogenase is:

The overall equation is

$$N_2 + 8e^- + 8H + 16\ ATP \xrightarrow[\text{Nitrogenase}]{\underline{Mg_2^-}} 2NH_3 + H_2 + 16\ ADP + 16P_i$$

Nitrogenase

Atmospheric dinitrogen (N_2), stabilized by a triple covalent bond, is reduced stepwise to form ammonia (NH_3), the first stable product of biological nitrogen fixation. The ammonia produced is rapidly assimilated into organic compounds, primarily through the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway, leading to the synthesis of amino acids and other nitrogenous metabolites

The nitrogenase has a high sensitivity to oxygen. Nodules contain an oxygen scavenger known as leghaemoglobin (Lb), a reddish-pink pigment, to protect these enzymes. Leghemoglobin is thought to be either outside the peribacteroid membrane or in between bacteroids, according to two different theories. The free di-nitrogen initially binds to the MoFe protein during nitrogen fixation and is not released until it has been fully reduced to ammonia. Di-nitrogen reduction is a stepwise reaction that produces NH_3

(ammonia), which is then protonated at physiological pH to produce NH4+. This process involves ferredoxin serving as an electron donor to the Fe-protein (nitrogenase reductase), which hydrolyze ATP and reduces the MoFe protein, which in turn reduces the substrate N_2.

$$\underset{\text{Dinitrogen}}{N\equiv N} \xrightarrow{2e-\ 2H^+} \underset{\text{(Hydrazine)}}{HN{=}NH} \xrightarrow{2e-\ \ 2H-} \underset{\text{(Diamide)}}{H_2N=NH_2} \xrightarrow{2e-\ \ 2H} \underset{\text{(Ammonia)}}{+\,2NH_3}$$

Intermediate of Nitrogen fixation

Assimilation of Ammonia: Nitrogenase instantly protonates the ammonia it produces to create ammonium ions (NH4+). Near the site of generation, NH4+ is promptly employed to manufacture amino acids because it is poisonous to plants. Three processes are used to synthesize amino acids: transamination, catalytic amination, and reductive animation.

1. Reductive amination

Glumate dehydrogenase (GDH) catalyzes the synthesis of glutamic acid in this process

$$\alpha\text{-Ketoglutaric acid} + NH_4^+ + NADPH \rightleftharpoons \text{Glutamate} + H_2O + NAD(P)$$

2. Catalytic amidation

It is a twostep process catalyzed by glutamine synthetase (GS) and glutamate synthetase (glutamine-2-oxyglutarate amino transferase,or GOGAT).

$$\text{Step:1 Glutamate} + NH_4 - + ATP \xrightarrow{\text{Glutamate Synthetase}} \text{Glutamine} + ADP + Pi$$

$$\text{Step 2: Glutamate} + 2^{-}\ \text{Oxyglutarate} + NADH + H^+\ \xrightarrow{\text{GOGAT}}\ \text{Glutamate} + NAD$$

Out of the two glutamates produced one returns to GS while the other is exported to the plant.

3. Transamination

The primary amino acid from which transamination yields other amino acids is glutamate or glutamic acid. All of these processes are catalyzed by the enzyme aminotransferases (= transaminases). Transferring an amino group from one amino acid to the keto group of a keto acid is known as transamination

Glutamate (amino donor) + Oxaloacetate (amino acceptor) → Aspartate (amino acid) + 2 oxyglutarate

The fixed nitrogen in nitrogen-fixing plants is transferred from the nodules to other plant components through the xylem in the form of ureides (allantoin, allantoic acid, and citrulline) and amides (asparagines and glutamine). Amides are created by substituting another NH2-radicle for the -OH portion of two amino acids, glutamic acid and aspartic acid. As a result, amides are structural components of most proteins and have higher nitrogen content than amino acids.

Nitrate Assimilation

Plants cannot use nitrate in their natural state. Prior to being integrated into organic molecules, it is first reduced to ammonia. Nitrate is reduced in two stages

1. Reduction of nitrate to nitrite

Nitrate reductase, an inducible enzyme, is responsible for it. The molybdoflavoprotein is the enzyme. It needs the reduced coenzyme NADH or NADPH to function, and FAD or FMN brings it into contact with nitrate.

$$NO_3^- + NAD(P)H + H^+ \rightleftharpoons NO_2^- + H_2O + NAD(P)^+$$

2. Reduction of nitrate

Nitrite reductase is the enzyme responsible for reducing nitrite. Iron and copper are found in the metallo flavoprotein that is the enzyme. In leaf cells and other cells' leucoplasts, it takes place inside the chloroplast. Nitrite reductase needs power to reduce. It's NADH (NADPH in cells with light) and NADPH. Ferredoxin is also necessary for the reduction process and is found in the green tissues of higher plants. Nitrite is thought to be translocated to leaf cells in higher plants, or another electron donor, such as FAD, is thought to function in cells that are not lit. ammonia's by-product of nitrite reduction.

$$2NO_2^- + 7NAD(P)H + 7H^+ \xrightarrow{\text{Nitrate reductase}} 2NH_3 + 4H_20 + 7NAD(P)^+$$

Ammonia thus produced combines with organic acids to produce amino acids. Amino acids form protein by the process of translation.

The above data were retrieved from www.biology discussion.com

Scientific evidence on nitrogen fixation

The study explores the role of legumes in improving soil fertility and nitrogen availability in both natural and agricultural ecosystems (Schob *et al*., 2018; Yu *et al*., 2021; Furey & Tilman, 2021) [35, 11, 44]. In cropping systems like maize intercropping and oilseed rape rotation, legumes boosted peanut biomass, root nodulation, and N_2 fixation in the soil (Li, 2021) [19]. The findings suggest that maize positively influences peanut nitrogen fixation by enhancing nodulation.

Researchers also investigated the factors driving improved nodulation and nitrogen absorption in peanut roots and the rhizosphere. Peanuts cultivated in diverse cropping systems accumulated flavonoids, coumarins, and related metabolites, which were influenced by environmental conditions such as light exposure, UV radiation, temperature changes, and drought. These compounds had a greater effect on bacterial beta diversity than alpha diversity in the rhizosphere.

The study further highlights nitric oxide (NO) as a key molecule in plant stress responses and development (Corpas *et al*., 2008, 2013; Signorelli *et al*., 2013, 2019) [6, 38, 37, 38]. NO plays a role in hormone signaling and regulates processes like ripening, branching, flowering, germination, and root growth (Lozano-Juste & Leon, 2011; Sanz *et al*., 2014; Klessig *et al*., 2000) [15, 34, 16].

At different stages of this process, NO also affects the BNF.

- No metabolism in the nodule
- No sources
- Effects on BNF, nodule senescence, and nodule formation

Nodule development

The Cellular Origin of Roots and Nodules Are Different

Nodules and roots originate from distinct cellular processes. According to Madsen *et al*. (2010) [20], root nodules form through two coordinated processes: organogenesis, which shapes the nodule structure, and infection, which enables bacterial colonization. When rhizobia detect flavonoid secretion, a host-symbiont signal exchange begins, leading to the production of Nod factors (NFs)—lipochitooligosaccharides that initiate nodule formation. The detailed figure (Fig 1.) is shown below.

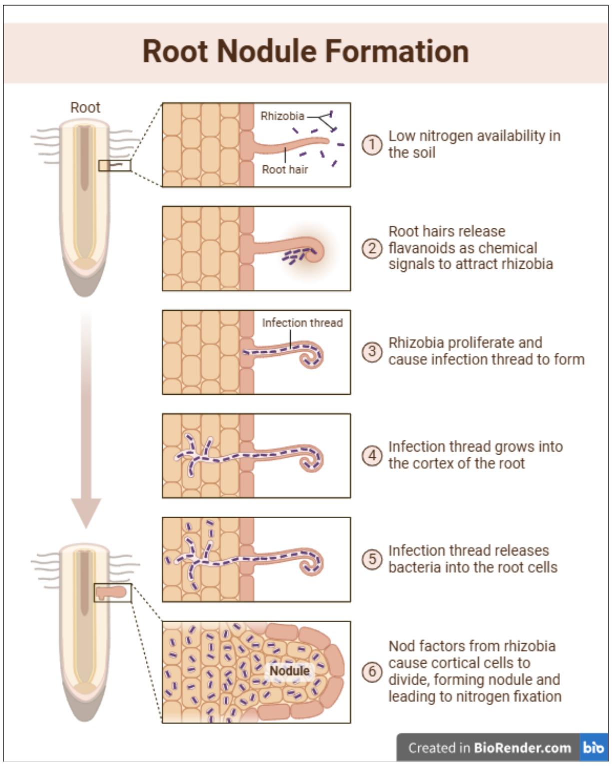

Fig 1: Root nodule formation

Molecular Basis of Rhizobium-Legume Symbiosis

In legumes such as *Lotus* and *Medicago truncatula*, rhizobial recognition and infection are tightly coordinated processes. LysM receptor-like kinases located on the root epidermis perceive rhizobial Nod factors (NFs), enabling host plants to discriminate between compatible and incompatible strains (Radutoiu *et al*., 2003, 2007; Madsen *et al*., 2011) [28, 29]. This specificity ensures that each legume species is nodulated by its cognate rhizobia.

Following recognition, infection is initiated in the susceptible root zone, where root hairs undergo curling to entrap rhizobial microcolonies. The bacteria penetrate through an infection thread (IT)—a tubular invagination of the plant cell wall—which extends toward the root cortex. Concurrently, cortical cells dedifferentiate to form a nodule primordium (Held *et al*., 2010) [13].

Once the IT reaches the primordium, rhizobia are released into membrane-bound compartments known as symbiosomes, where they differentiate into nitrogen-fixing bacteroids. Nodule morphology varies among legumes:

- *Medicago* forms indeterminate nodules, elongated structures with persistent meristematic activity.
- Soybean, Lotus, and common bean form determinate nodules, spherical structures with transient meristem activity.

  Indeterminate nodules typically arise from the inner cortex, while determinate nodules originate in the middle or outer cortex.

Nodule development is orchestrated by the interplay between rhizobial infection and organogenesis signaling pathways (Penmetsa *et al*., 2003) [41]. Nod factor signaling promotes root cell growth and IT progression (Geurts & Bisseling, 2002) [12], while phytohormones such as auxin (AUX) and cytokinin (CK) modulate infection and primordium formation (Roy *et al*., 2020) [30].

Within nodules, bacteroids drive biological nitrogen fixation (BNF) via the nitrogenase enzyme complex, reducing atmospheric nitrogen (N_2) to ammonium (NH_4^+), which is assimilated into amino acids and other nitrogenous compounds (Masson-Boivin *et al*., 2009) [22]. This symbiosis allows legumes to thrive in nitrogen-deficient soils, reducing dependence on synthetic fertilizers and contributing to sustainable agriculture and food security.

Role of Nitric Oxide (NO) in Symbiosis

Nitric oxide (NO) is a versatile signaling molecule in plants and animals (Cueto *et al*., 1996) [8]. In plants, it regulates growth and development, mediates biotic interactions (Yamasaki & Cohen, 2006), and modulates responses to abiotic stresses such as salinity, osmotic stress, and high temperature (Mathieu *et al*., 1998) [23]. NO also functions in plant defense, where it induces programmed cell death, defense gene expression, and reactive oxygen species (ROS) production (Leach *et al*., 2010; Zemojtel *et al*., 2006) [17, 45].

During the establishment of nitrogen-fixing symbiosis, NO is transiently produced in legume nodules (Crawford *et al*., 2006) [7]. Unlike pathogenic microbes, rhizobia establish a mutualistic association with legumes, undergoing differentiation into bacteroids within nodules. Because nitrogenase is highly oxygen-sensitive, nodules maintain a microoxic environment regulated by leghemoglobin. In this context, NO serves as a regulatory molecule influencing nodule development, bacteroid differentiation, and the balance between plant defense and symbiosis (Cohen & Yamasaki, 2003; Sudhamso & Crane, 2009) [5, 39].

Hydrogen in Symbiotic Nitrogen Fixation

Initially considered an energy waste, hydrogen (H_2) is a byproduct of the nitrogenase reaction. Schubert & Evans (1976) [36] assessed nitrogenase efficiency, while Dixon (1978) [9] noted hydrogenase activity in pea root nodules. Further studies confirmed that some rhizobial strains contain uptake hydrogenase (Hup), which may recycle electrons and support nitrogenase respiration.

Research on R. leguminosarum hydrogenases (Leyva *et al*., 1987) identified at least 17 common hup genes (Baginsky *et al*., 2002) [2], regulated by the NifA protein (Brito *et al*., 1997) [4]. These genes are present in Azorhizobium, Bradyrhizobium, and Rhizobium species. Genome sequencing of Rlv UPM791 revealed that hup gene presence is highly strain-specific, with diverse evolutionary origins across species.

Energy supply to nitrogenase: the O_2 paradox

Oxygen Regulation and Nitrogen Fixation in Rhizobia

Rhizobia, as obligate aerobes, require oxygen for energy metabolism, yet nitrogenase enzymes (FeMoCo, Fe protein, and MoFe protein) are oxygen-sensitive (Mortenson & Thorneley, 1979) [25]. To balance this, the nodule inner cortex functions as a diffusion barrier, regulating oxygen supply with the help of leghaemoglobin, which facilitates controlled oxygen transport to nitrogenase (Vance & Heichel, 1991) [40].

Under microaerobic conditions, plants redirect glycolysis to malate and later succinate, enabling ATP production in bacteroids through a high-affinity terminal oxidase. Since leghaemoglobin concentration is stable in nitrogen-fixing cells, it is often used as an indicator of nitrogen fixation capacity (Virtanen *et al*., 1955; Bergersen, 1961) [42, 3]. Appleby (1984) [1] provided extensive evidence that leghaemoglobin aids in rhizobial respiration, maintaining an optimal oxygen tension (~10 nm in soybean nodules). The necessity of hemoglobin for nitrogenase activity was later confirmed in 2005.

Impact of Rhizobium Strains on Nodulation and Yield

Sankareswari R. Uma & Ilamurugu K. (2017) [31] categorized acid-tolerant and intolerant rhizobial strains based on growth behavior in low pH media (5.5-6.0). UV-induced mutants MB 1 (Blackgram) and MG 1 (Greengram) exhibited temperature resistance (35°C-50°C) and acid adaptation, with MG 1 growing at pH 5.5 but slightly reduced compared to pH 6.0. After 72 hours at 45°C, MG 1 population increased from 7.65 to 8.02 $\log_{10}$ cfu/ml before declining.

High aluminum (Al^{3+}) levels hindered nodulation by suppressing root hairs in blackgram, soybean, cowpea, and greengram (Sankareswari R Uma & Ilamurugu, 2017a) [31]. In cowpea, microbial populations dropped from 8.75 $\log_{10}$ cfu/ml (25 μM Al^{3+}) to 8.68 $\log_{10}$ cfu/ml (50 μM Al^{3+}), while greengram populations declined from 9.20 $\log_{10}$ to 9.07 $\log_{10}$ cfu/ml under similar conditions. However, Blackgram (Bcp1) isolates at pH 5.5 showed significant growth 15 days post-incubation at 0 μM Al^{3+}.

Conclusion

Future Prospects of *Rhizobium* in Sustainable Agriculture

Rhizobium-based inoculants offer a cost-effective and sustainable alternative to chemical fertilizers. Further research on scaling production, enhancing shelf-life, and reducing costs could improve accessibility for farmers in developing countries, aiding food security. Increased policy support, funding, and regulations could drive innovation and widespread adoption.

A growing interest in molecular communication between Rhizobium and plants highlights the need to explore signaling mechanisms that enable root invasion and nitrogen-fixing nodule formation. Research on strain specificity and its optimization for different legumes could enhance agricultural productivity.

The future of Rhizobium lies in biotechnology, microbiome interactions, and integrated farming technologies, reinforcing its role in sustainable agriculture, environmental conservation, and climate change mitigation.

Footnotes

Biological Nitrogen fixation

https://www.biologydiscussion.com/ecosystem/biological-nitrogen-cycle/biological-nitrogen-cycle-with-diagram-ecosystem/75459

Nitrogen fixation types

https://www.biologydiscussion.com/nitrogen-fixation/types-nitrogen-fixation/nitrogen-fixation-types-physical-and-biological-nitrogen-fixation-with-diagram/14969

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Contribution

RU wrote the manuscripts and acquired title-related information; PJ made revisions to the paper. All the author have read and given their approval.

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