**Legume Root Nodules- Evolutionary adaptation for improved survival mechanism**

**Abstract**

Root nodule symbiosis in leguminous plant belonging to Fabaceae family, represents a pivotal evolutionary innovation enabling a mutualistic relationship with nitrogen fixing bacteria. This trait vital for plant growth in nitrogen deficient soils, evolved within the Nitrogen Fixing Clade (NFC), particularly among subfamilies like Papilionoideae and MCC clade, indicating multiple independent origins. Genetic studies emphasizing on the loss of function mutations in the NODULE INCEPTION gene, highlights repeated evolutionary losses of nodulation across lineages. The development of RNS has been profoundly influenced by intricate mechanisms including horizontal gene transfer, reciprocal co-evolutionary dynamics between legumes and rhizobia and selective pressures exerted through the domestication of leguminous species. The translational potential of this research is exemplified through strategic achievements such as enhancing nitrogen use efficiency via genotype driven selection in leguminous crops like cowpea and soyabean and pioneering efforts to engineer root nodule symbiosis in non-leguminous crops like cereals. These applications are pivotal in fostering sustainable agricultural systems, particularly in nitrogen deficient soils. This review emphasis on a comprehensive understanding of the evolutionary and functional dynamics underpinning legume rhizobia symbiosis to guide future innovations aimed at promoting sustainable agriculture and ensuring long term food and nutritional security.

Key words: Root nodule symbiosis (RNS), Nodulation, Nitrogen fixation, Horizontal gene transfer, co-evolution

**Introduction**

Biological nitrogen fixation (BNF) via root nodule symbiosis (RNS) represents a fundamental ecological and agricultural process, particularly among members of the Nitrogen-Fixing Clade (NFC), including legumes, actinorhizal plants, and Parasponia species. This symbiotic interaction, primarily facilitated by nitrogen-fixing bacteria such as *Rhizobium* and *Frankia*, enables host plants to convert atmospheric nitrogen (N₂) into ammonium (NH₄⁺), thereby significantly contributing to the nitrogen economy of terrestrial ecosystems (Werner *et al*., 2014). Root nodules—specialized lateral root structures—create microaerobic environments that support bacterial nitrogenase activity, allowing for efficient nitrogen assimilation by the host plant.

The global agricultural reliance on synthetic nitrogen fertilizers, although effective for yield enhancement, imposes significant environmental costs, including eutrophication and greenhouse gas emissions. Consequently, understanding and enhancing RNS has emerged as a critical avenue for sustainable agriculture (Roy *et al*., 2020). Recent research efforts have focused on characterizing the molecular, genetic, and evolutionary mechanisms underlying RNS, as well as exploring its potential transfer into non-leguminous crops such as maize and wheat. Advancements in these areas hold promise for reducing external nitrogen inputs, improving nitrogen-use efficiency, and fostering climate-resilient cropping systems. This review synthesizes current insights into the evolutionary development, symbiotic mechanisms, and translational potential of RNS, offering a framework to guide future innovations in crop improvement and global nitrogen management strategies.

**Biological Nitrogen Fixation**

Nitrogen forms 78%of earth`s atmosphere, but in unavailable form. The nitrogen present in the atmosphere is only absorbed by plants having bacteria for N fixation in symbiotic relations (O’Brien *et al*., 2016). The ideal growth of plant depends on the adequate availability of fixed nitrogen. The life supporting phenomenon that accounts for most fixed nitrogen is the biological nitrogen fixation. The nitrogenase enzyme complex uses energy in the form of ATP or electrons supplied by respirational process in fixing nitrogen through biological nitrogen fixation. The resulting nitrogen is then readily converted into amides or amino acids by diazotrophic bacteria to produce proteins. When they die, the nitrogen is released into the atmosphere and mineralized and uptaken by some other plants. To quantify the amount of nitrogen fixed biologically is approximated to 150 (million metric tons) of nitrogen all over the world yearly (Herridge *et al*. 2008).



Fig.1 Biological Nitrogen-fixing organisms commonly associated with the nitrogen cycle

From the various modes of BNF as mentioned in Fig.1, when particularly concentrating on the symbiotic relationship between legumes and rhizobia, is a vital process in agricultural and ecological systems. This intricate interaction involves leguminous plants forming nodules on their roots, which house the nitrogen-fixing bacteria-rhizobia. Within these nodules, rhizobia convert atmospheric nitrogen into ammonia, which the plant utilizes for its growth and development. This symbiosis plays a crucial role in nitrogen cycling, providing a sustainable source of nitrogen for plant growth while reducing the use of synthetic fertilizers. Understanding the mechanisms and regulation of this symbiotic relationship is essential for optimizing agricultural productivity and promoting environmental sustainability.

**Legumes - Root nodules**

The biological nitrogen fixation happens through the root nodules of leguminous plants. It houses symbiotic gram-negative bacteria: Rhizobia, which fix atmospheric nitrogen in exchange for carbon from photosynthesis. Nodules are a hallmark of the legume family (Leguminosae or Fabaceae), the third-largest family of flowering plants, with over 19,000 species. However, nodules are not confined to legumes. Rhizobia nodules also occur in the genus Parasponia (Cannabaceae), and members of eight “actinorhizal” families (Rosaceae, Betulaceae, Casuarinaceae, Myricaceae, Rhamnaceae, Elaeagnaceae, Datiscaceae, Coriariaceae) form nodules with Gram-positive bacteria of the genus Frankia (Shen and Bisseling, 2020). Phylogenetic analysis reveals that all nodulating lineages are nested within a single “Nitrogen fixing clade” of Rosides. The scattered taxonomic distribution and structural diversity of nodules across this clade suggest multiple independent origins, underpinned with a shared ancestral predisposition for nodulation (Soltis *et al.,* 1995).

The nodulating (nod) genes in symbiotic bacteria are responsible for host-specific recognition and initiate the production of Nod-factors; the key signaling molecules in establishing root nodule symbiosis. In legumes, theses signal the formation of infection threads, which serve as intracellular and transcellular channels for bacterial entry into the host root. Simultaneously the Nod factor perception induces cortical cell division in the root, giving rise to nodule primordia. The resulting root nodules exhibit intricate structural and physiological integration, reflecting a finely tuned coordination of plant and microbial metabolic processes essential for effective nitrogen fixation (Lodwig *et al*. 2003). Specialized bacteroides within root nodules are compartmentalized in host-derived symbiosomes, where they carry out biological nitrogen fixation. The symbiotic structure reflects a high degree of physiological and metabolic integration between the host plant and its microbial partner to ensure efficient nitrogen supply throughout growth (Sprent and James 2007).

**Plant families in the evolution of root nodules and root nodule symbiosis**

The root nodule symbiosis (RNS) is confined to a single large clade termed as nitrogen-fixing clade (NFC), where nodulation has evolved several times. NFC has nodulation in all four orders: Fabales, Fagales, Cucurbitales, and Rosales. Within this clade, legumes (Fabales) and the non-legume genus Parasponia (Rosales) develop nodules in association with rhizobium bacteria, while actinorhizal plants (Fagales, Cucurbitales, and Rosales) can establish nodules with Frankia bacteria. It's suggested that nodulation independently evolved multiple times within the NFC, occurring eight times with Frankia and twice with rhizobium (Doyle *et al*., 2011).

Fabales are known for their high diversity and abundance of nodulators. Nodulation within legumes likely evolved after the family origin about 60 million years ago. Its occurrence is primarily observed in the monophyletic subfamily Papilionoideae and Mimosoideae-Caesalpinieae-Cassieae (MCC) Clade. The nodulations within these subfamilies observed through scattered distribution and anatomical diversity of nodules suggest that nodulation originated multiple times within NFC (Doyle, 2016; Lui *et al*., 2020).

**Evolution of nodulation**

While nodulation may be the foremost association with legumes for many, it's important to note that nodulation is neither universal among legumes nor exclusive to the family. The distribution of nodulation is intricate and has thus far resisted a straightforward explanation for its origin or origins within or outside the legume family (Doyle 1998). Nodulation symbioses involving either rhizobia or actinorhizal bacteria are observed in nine other plant families beyond Leguminosae, all belonging to the eurosid I group. This suggests that within this group, there exists a factor that facilitates but does not mandate plants to establish nitrogen-fixing symbioses. Consequently, the nitrogen-fixing families within this clade do not seem to share a common ancestor, and nodulation has evidently emerged independently among—and even within—specific families.

Within the legume family, nodulation is prevalent in over 90% of papilionoid genera and just under that percentage in mimosoid genera. However, it is significantly less common, approximately 5%, among the diverse and unrelated genera known as Caesalpinioideae (Sprent 2001). There is no evidence of nodulation at the base of the legume tree: firstly, because none of the Cercideae nodulate, and secondly, because none of the other families within Fabales (Polygalaceae, Surianaceae, or Quillaja) exhibit nodulation. Therefore, it can be reasonably inferred that nodulation originated within the legumes and only after the earliest lineages had diverged. Moreover, the next diverging lineages, regardless of their order of divergence, do not include the ancestors of the majority of nodulating legumes. While the Detarieae and Dialiinae lineages each include a single genus with an unconfirmed report of nodulation, these genera are nested among other taxa that do not nodulate, suggesting the possibility of additional independent origins of nodulation within the family (Bruneau *et al*., 2001).

**Phylogenomics tracing of the evolution of nitrogen-fixing root nodule symbiosis**

For plants to thrive, access to nutrients like nitrogen is essential. A symbiotic relationship called nitrogen-fixing root nodule (NFN) symbiosis benefits legumes and nine other plant families. In this symbiosis, plant roots develop nodules that house nitrogen-fixing bacteria internally. Through this mutually beneficial interaction, bacteria convert atmospheric nitrogen into ammonium, supplying it to the host plant. This enables plants to survive in nitrogen-poor environments on land. In agriculture, NFN symbiosis lessens dependence on nitrogen fertilizers, thereby cutting costs and reducing the ecological footprint and fossil fuel consumption associated with widespread fertilizer use. The collaboration between specific bacteria and their plant hosts provides fixed nitrogen to the plants. Griesmann *et al*. (2018) examined several plant genomes to understand why nitrogen-fixing symbiosis appears sporadically across the evolutionary tree. They discovered remnants of lost pathways in various genomes that could have facilitated nitrogen-fixing symbiosis. This suggests that this symbiotic relationship, which depends on multiple pathways and intricate inter-organism communication, is vulnerable to selection pressures and can gradually disappear over evolutionary periods.

The root nodule symbiosis is restricted to a subset of genera within a single clade of NFC. To explore the genetic basis for the scattered occurrence, Griesmann *et al*. (2018) sequenced the genome of 10 plant species with various root nodule symbiosis characteristics and compared it with 27 other plant species. 13 non-nodulating species mutations were identified in the gene *NODULE INCEPTION*, which is essential for nodule formation and suggests that these species lost their ability to form root nodules independently. This discovery reveals that root nodule symbiosis, which is crucial for global nitrogen cycles and food production has been lost multiple times throughout the evolutionary history in various plant lineages.

**Evolutionary adaptations of root nodules**

The origin of root nodules in legumes is a complex evolutionary process influenced by genetic, physiological, ecological, and environmental factors over millions of years. The specific evolutionary paths may differ among legume species as they follow several proposed pathways and mechanisms like horizontal gene transfer, co-evolution of the legume and rhizobia, and the evolutional diversification alongside domestication.

**a. Horizontal gene transfer and co-evolution:** The horizontal gene transfer has played a pivotal role in the evolution of symbiotic nitrogen fixation, facilitating the dissemination of nodulation genes among rhizobial populations and shaping their capacity to engage with diverse legume hosts. As per the theory, the nodulation and nitrogen fixing genes were exchanged between ancient bacteria and host plants leading to symbiotic relationships. Coevolution contributed to shaping the specificity and efficiency of symbiotic relationships and thereby resulting in diversification in legumes and rhizobia (Provorov, 1997). Although the coevolution of legume rhizobia symbiosis exhibits similarity with gene for gene evolutionary models, its direct association with phytopathogenic systems remains improbable. This symbiotic coevolution is primarily driven by horizontal gene transfer and extensive intergenomic recombination within rhizobial populations which fosters genetic diversity through interactions with hosts (Martinez, 2009).

Legume nodules, specialized structures facilitating nitrogen fixation, likely emerged from the coevolutionary interplay between plants and ancestral rhizobia. Coevolution with rhizobia is a plausible scenario within the evolutionary framework driving legume proliferation and divergence. Alternatively, plants may have continuously exerted selection pressure on bacteria, with bacteria exerting subtle influences on plant evolution, thus meeting the criteria for a coevolutionary hypothesis (Samac and Graham, 2007).

**b. Evolutionary diversification and domestication :** The history of root nodules in legumes has been marked by a great deal of diversification and innovation, leading to a wide variety of nodule types, shapes, and symbiotic relationships. Various legume genera and species have developed distinct strategies for nodulation and specific symbiotic characteristics that suit their ecological environments. This reflects the range of evolutionary paths and environmental pressures they have experienced over time.

Mutch and Young (2004) investigated host-range evolution in Rhizobium leguminosarum by examining strains isolated from nodules of Pisum sativum, Vicia faba, and related wild species cultivated in natural soils. Their analysis of 80 isolates revealed that while only 34% could nodulate V. faba, a markedly higher 89% successfully nodulated the wild taxa, suggesting a reduction in symbiotic promiscuity associated with domestication. Parallel findings have emerged from genomic studies: Greenlon et al. (2019) demonstrated that wild Cicer reticulatum supports a more diverse Mesorhizobium community compared to its domesticated counterpart, Cicer arietinum. Similarly, Chang et al. (2019) reported greater abundance and diversity of Bradyrhizobium in the rhizosphere of wild Glycine soja relative to cultivated Glycine max. Collectively, these studies underscore the impact of domestication on narrowing host-microbe interaction spectra and reducing rhizobial diversity in cultivated legume species**.**

In general, the development of root nodules in legumes is an intriguing demonstration of co-evolutionary adaptation between plants and bacteria, influenced by selective pressures and ecological interactions over long periods. By understanding the evolutionary processes and molecular mechanisms involved in nodulation, researchers can acquire important knowledge about the emergence of symbiotic nitrogen fixation and its importance for plant adaptation, ecosystem dynamics, and agricultural sustainability.

**c. Utilization of root nodules in plant breeding**

Research into legume-rhizobial interactions has uncovered significant variability in nitrogen fixation efficiency across different combinations of plants and rhizobia. Specifically, while a particular bacterial strain may establish a highly effective symbiosis with one host, it might exhibit lower efficiency when paired with another host. Moreover, there isn't a consistent association between specific host genotypes or rhizobial strains and optimal nitrogen fixation performance. Often, the failure or reduced efficiency of the partnership can be attributed to genetic incompatibility, resulting in a lack of coadaptation between the interacting partners. This deficiency negatively impacts bacterial differentiation and survival within nodule cells. Consequently, the host's ability to select the most suitable partners from a pool of compatible rhizobia is crucial for enhancing nitrogen fixation yields.

In legume rhizobial symbiosis, different legume species evolved unique interactions with their rhizobial partners through domestication. Munoz *et al*, (2016) investigated the biological nitrogen fixation (BNF) in cultivated and wild soybean species. The recombinant inbred population developed between a wild and cultivated soybean showed a low level of diversity in SNPs associated with QTLs for total ureides and total nodule fresh weight in cultivated soybean. Results suggested that nitrogen-fixing capacity was enhanced by soybean domestication.

Among the various applications of RNS, enhancing the nitrogen-fixing capacity of crops, characterization of symbiosis traits in wild and cultivated populations, and engineering RNS in non-leguminous crops like rice are given prime importance. The effect of legume rhizobia symbiosis on enhancing nitrogen efficiency has been evaluated by Mekonnen *et al*, (2022). Improved cowpea productivity in nitrogen-deficient environments was identified in cowpea genotypes with high nodulation capacity and root architecture. Through cluster analysis, distinct groups of cowpea genotypes with diverse nodulation potential and grain yield traits were identified. Emphasis on selecting genotypes with compactable interactions with cowpea genotype and rhizobia, aimed at enhancing nitrogen efficiency.

**Conclusion**

In this discussion, we explored various hypotheses regarding the timing of gains/losses of RNS, illustrating how these have contributed to our understanding of legume-root nodule evolution and RNS evolution. Subsequently, enhanced our comprehension of the genetic transformations associated with legume-root nodules and RNS is currently refining our insights into the timing of evolution. In conclusion, understanding the evolution of legume-root nodule and root nodule symbiosis (RNS) and its genetic underpinnings not only sheds light on the historical development of this crucial plant-microbe interaction but also provides insights crucial for modern plant breeding practices. (Lui *et al*., 2020). By leveraging this knowledge, new varieties with enhanced symbiotic nitrogen-fixing capabilities can be developed leading to more sustainable agricultural practices and increased yields.

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