Biological Nitrogen Fixation

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In agroecosystems, nitrogen is one of the major nutrients limiting plant growth. To meet the increased nitrogen demand in agriculture, synthetic fertilizers have been used extensively in the latter part of the twentieth century, which have led to environmental challenges such as nitrate pollution. Biological nitrogen fixation (BNF) in plants is an essential mechanism for sustainable agricultural production and healthy ecosystem functioning. BNF by legumes and associative, endosymbiotic, and endophytic nitrogen fixation in non-legumes play major roles in reducing the use of synthetic nitrogen fertilizer in agriculture, increased plant nutrient content, and soil health reclamation.

biological nitrogen fixation | nitrogenase | nif genes | legumes and nodules | associative nitrogen fixation | soil microbiome | rhizosphere

1. Introduction

A healthy, functioning soil ensures nutrient cycling for optimum plant growth for agricultural production [1]. However, agricultural productivity is often limited by available soil nutrients, especially nitrogen [2]. Nitrogen is not present in soil parent material despite the fact that nitrogen content in the atmosphere is highest among all the atmospheric gases [3]. Hence, soil nitrogen input for plant nutrition and crop productivity largely depends on organic matter degradation, synthetic fertilizer applications, and biological nitrogen fixation (BNF) via nitrogenase enzyme activity [4][5]. This limited bio-availability of N and the escalating reliance of crop growth on N have created a colossal N-based fertilizer industry worldwide [6][7]. Nitrogenous fertilizer production currently represents a significant expense for the efficient growth of various crops in the developed world. Synthetic N fertilizers are currently used in grain, grass, and fruit productions (about 60% for cereals and 10% with irrigated rice production) [8]. More than 50% of the applied N-based fertilizer is used by the plants and the remaining can be subjected to losses like surface runoff and leaching leading to nitrate contamination of soils and groundwater. In terms of energy efficiency, moreover, manufacturing nitrogen-based fertilizers requires six times more energy than that needed to produce either phosphorous or potassium-based fertilizers [9]. Therefore, reducing dependence on nitrogenous fertilizers in agriculture in the developed world and developing countries may lead to potential gains in an agricultural setting. Biological nitrogen fixation (BNF) in economically important food and forage crops [10] has drawn attention to achieve sustainable agricultural goals in both hemispheres of the world [11]. In livestock production systems in the southeastern USA, strategically planting nitrogen-fixing legumes in cattle pastures has shown to increase the available soil nitrogen [12], thereby reducing the need to apply synthetic nitrogen sources. The diazotrophic microorganisms from bacteria or archaea domains are responsible for BNF and only some prokaryotes are able to

use atmospheric nitrogen through BNF by encoding nitrogenase, an enzyme that catalyzes the conversion of N_2 gas to ammonia (NH_3) [8][13][14]. Despite the phylogenic and ecological diversity among diazotrophic bacteria and their hosts, a synchronized interaction is always a prerequisite between the microbial entities and the host plant to achieve a successful nitrogen fixation system. The importance of this process is enormous as it reduces the dependence on nitrogen fertilizers for plants and thus, for agriculture overall. It has been estimated that worldwide, biological nitrogen fixation produces roughly 200 million tons of nitrogen annually [15][16]. In fact, nearly 50% of the total nitrogen in crop fields is the contribution of BNF by diazotrophic bacteria of the total biosphere nitrogen [17]. Moreover, fixed nitrogen can also be transferred to intercropped non-legumes in the case of mixed cropping systems, such as the soybean–wheat system, or the next season crops in crop rotation [18].

2. Biological Nitrogen Fixation (BNF)

Nitrogen fixation is a dynamic and high-energy demanding process [19]. The pathway for the biological reduction of inert N_2 into the reactive compound NH_3 (ammonia) under micro-aerobic conditions is as follows:

$N_2 + 8H^+ + 8e^- + 16Mg\text{-}ATP \rightarrow 2NH_3 + H_2 + 16Mg\text{-}ADP + 16\ P$ (1)

Free-living diazotrophs correspond to a small fraction of the plant rhizospheres ecosystem, and they belong to alphaproteobacteria (*Rhizobia*, *Bradyrhizobia*, *Rhodobacteria*), betaproteobacteria (*Burkholderia*, *Nitrosospira*), gammaproteobacteria (*Pseudomonas*, *Xanthomonas*), firmicutes, and cyanobacteria [20]. However, their presence, function, and importance can be explained by the “black queen” hypothesis which predicts that in free-living microbial communities, only a few “helpers” that carry the heaviest weight in terms of functions, such as high energy-requiring nitrogen fixation, support the rest of the flora and fauna population or the “beneficiaries” that rely on the “helpers” or the “beneficial” for nitrogen needs [21].

The symbiotic relationship between soil bacteria, collectively known as rhizobia (which includes the genera *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, and *Sinorhizobium*), and legume roots generates nodules (a new differentiated special organ) that fix atmospheric nitrogen through the action of the nitrogenase enzyme [22]. BNF by plants and its bacterial associations represent an important natural system for capturing atmospheric N and processing it into a reactive form of nitrogen through enzymatic reduction. BNF is considered an extremely sensitive process influenced by nutrient and environmental conditions and enables a plant to supply all or part of its requirements through interactions with endo-symbiotic, associative, and endophytic symbionts, thus offering a competitive advantage over any non-nitrogen-fixing plants [15][23][24][25][26]. The highly conserved nitrogenase complex in free-living and symbiotic diazotrophs enables them to participate in various types of associations/interactions with their host plants. BNF by plant–rhizobia symbiotic systems is mediated by endosymbiotic interaction when plants develop root nodules; in legumes and rhizobia, gram-negative alpha proteobacteria are the most common microbial species that associate (endo-symbiotic interaction) with legumes of the Fabaceae (Papilionaceae) family [27][28][29]. Actinomycetes such as the *Parasponia* species (family Cannabaceae) and *Frankia* sp. that associate with a broad spectrum of actinorhizal plants are well documented in nitrogen fixation as well [8]. Cyanobacteria (mainly *Nostoc* sp.) have also been found to colonize different plant organs, either intracellularly in the family Gunneraceae or extracellularly in *Azolla*, *Cycadaceae*, liverworts, and

hornworts. Associative nitrogen fixation (ANF) and/or endophytic symbioses are often observed among diazotrophs, such as *Azospirillum* spp., *Azoarcus* spp. and *Herbaspirillum*, with a wide variety of plant roots including cereals. The nitrogenase protein, as well as the associated proteins and non-proteins forming nitrogenase enzyme, are sensitive to the presence of oxygen [30]. For this extreme sensitivity to oxygen, obligate anaerobes such as *Clostridium pasteurianum* are ideal candidates for nitrogen fixation; however, facultative anaerobes such as *Klebsiella oxytoca* are also capable of fixing nitrogen but only when the oxygen is absent in the system [31]. Obligate aerobes, such as *Azotobacter vinelandii* can also shield nitrogenase from oxygen and perform nitrogen fixation by consuming oxygen via cytochrome oxidases [31][32].

2.1. The Nitrogenase Protein and Nodule Formation

As mentioned earlier, a protein complex called nitrogenase (composed of enzymes with metal co-factors) makes nitrogen fixation possible in plants. The first one is dinitrogenase and the second one is dinitrogenase reductase [33]. According to the active site co-factor binding metal, there exist three types of dinitrogenase in nature. (a) Molybdenum (Mo) nitrogenase; it is most abundant and carries the most significance in the nitrogen-fixing bacterial and archaeal niche and the alternative vanadium (V) and iron-only (Fe) nitrogenases [34]. The molybdenum-dependent dinitrogenase is formed by *nifD* and *nifK* gene products and dinitrogenase reductase is a homodimer of the *nifH* gene product [30][35]. It is well documented that molybdenum nitrogenase is produced in all diazotrophs in nature, while some produce vanadium or iron nitrogenase in addition to Mo-nitrogenase [36][37]. The rhizobium bacteria residing in nodules fix atmospheric nitrogen gas to NH_3, which plants can assimilate via glutamine synthase to form glutamine. In response, the bacteria derive plant carbohydrates, mainly as malate for food and an energy source for nitrogen fixation. Nodules are very complex structures, containing several processes which operate and interact at distinct levels. The process of nodule formation requires a coordinated exchange of signals between the two symbiotic partners [38]. Bacteria had their symbiotic genes first characterized by transposon mutagenesis; this achieved the definition of over 50 nodulation genes (Nod and Nol) in bacteria, and about the same number controlling nitrogen fixation; thus many nod- and fix-bacterial strains exist in many species of rhizobia. Legume–rhizobium symbiosis starts with molecular signaling between the two partners. Early nodulation gene cascades in legumes. Plants release signals such as flavonoids (e.g., the flavone 7,4 dihydroxyflavone and the isoflavone genistein) which are picked up by compatible bacteria in the rhizosphere [39][40] leading to the production of Nod factors (NF) which trigger early events in the nodulation process [41][42]. This triggers the downstream gene cascade including those involved in nucleoporin, cation channels, calcium spiking, early nodule expression, and cytokine signaling leading to cortical and pericyclic cell divisions, and concomitant bacterial infection. Rhizobia are entrapped by root hair curling after the Nod factor has been perceived, which results in initiating the formation of an infection thread (a tubular structure). This infection thread facilitates the penetration of root hair cells and adjacent cortical cells [43]. Cell divisions in cortical and pericycle occur simultaneously resulting in the formation of the nodule primordium. Bacterial cell division facilitates the rhizobial traveling through the infection thread and is eventually freed into the induced nodule primordium cells [44][45]. As nodules mature with time, bacteria are enclosed within the symbiosome membrane, resultant from an inverted plasma membrane of plant origin. In this encapsulated chamber, the bacteria experience a micro-aerobic environment (lower oxygen concentration) and differentiate into bacteroids, fixing diffused nitrogen gas using their nitrogenase enzyme

complex [46][47]. Depending on whether or not the meristem remains active for the life of the nodule, two main types of nodules are formed on the various legume species, (i) indeterminate or (ii) determinate. In the case of determinate nodules, nodular meristematic activity is terminated early and is usually initiated sub-epidermally in the outer cortex, thus giving rise to spherical nodules [48]. In indeterminate nodules, the inner cortex undergoes cell division (anticlinally) followed by periclinal divisions in the pericycle. Here, cylindrical nodules are formed due to more persistent meristems [49][50].

2.2. Genes Encoding Nitrogenase Enzyme

The understanding of the genetic basis of this relationship is of paramount importance and essential for the optimization of nitrogen acquisition rates in legumes themselves. Bacterial *nif* genes are well known to encode the components of the nitrogenase enzyme complex. *nifH*, *nifD*, and *nifK* genes encode the structural subunit of di-nitrogenase reductase and the 2 subunits of di-nitrogenase, respectively. Many rhizobial genes have been fully sequenced, for instance, *Mesorhizobium loti*, *Sinorhizobium meliloti*, and *Bradyrhizobium japonicum* [51][52][53]. These proteins have similar sequences and common structures and functions in many diazotrophs, for instance, *Azotobacter vinelandii*, *Herbaspirillum seropedicae*, *Pseudomonas stutzeri*, and *Bradyrhizobium japonicum* [54][55][56][57]. Furthermore, genetic and biochemical analyses revealed that many additional *nif* genes, including *nifE*, *nifN*, *nifX*, *nifQ*, *nif W*, *nifV*, *nifA*, *nifB*, *nifZ*, and *nifS*, play roles in the regulation of *nif* genes and maturation processes of electron transport and FeMo-cofactor biosynthesis and assembly [58][59]. In addition, the *fixABCX* genes first identified in *Rhizobium meliloti* [60][61] and subsequently in other diazotrophs were reported to encode a membrane complex participating in electron transfer to nitrogenase [62]. The degree of specificity between legumes and rhizobia varies. The Nod factors produced by *Rhizobium etli* and *Rhizobium loti* produce identical Nod factors; however, they have distinct host ranges (*Phaseolus* spp. and *Lotus* spp., respectively) [63]. Moreover, different rhizobia nodulating the same plant may excrete completely different Nod factors. For instance, *Rhizobium tropici* and *R. etli* produce different Nod factors (sulfated and acetylfucosylated, respectively), but both are known to nodulate *Proteus vulgaris* [64]. More examples include *Bradyrhizobium elkanii* and *Bradyrhizobium japonicum*, which have a number of mutual hosts, but their Nod factors differ considerably [65].

2.3. Marker-Assisted Selection of Biological Nitrogen-Fixing Plants

Several studies have identified QTL associated with traits related to biological N fixation (**Table 1** [66][67][68][69]). The QTL markers can be used in marker-assisted selection for breeding plants with better nitrogen fixation attributes. A QTL for the total ureides (acyl derivatives of urea) was identified on chromosome 17 in soybean which explained 13.26% phenotypic variation [70]. Li and the team [71] cloned a candidate gene associated with a major QTL in soybean for increasing nodule size and named it INCREASING NODULE SIZE1 (GmINS1). The overexpression of GmINS1 increased the N content and the biomass of the soybean plant due to an increase in number, biomass, the abundance of infection cells, and nitrogenase activity of large nodules [71]. The result was the opposite when GmINS1 was suppressed by RNA interference [71].

Table 1. Major genomic loci detected for BNF in different legume species [66][67][68][69].

Species	Chromosome Number	QTL or Marker Interval	Plant Response	QTL-Effect, R^2 (%)
Common bean (*Phaseolus vulgaris* L.)	7	*Ndfa7.1*^{DB,SA}	N derived from atmosphere (Ndfa)	14.9
Soybean [*Glycine max* (L.) Merr.]	16	*qBNF-16*	Nodule size & number	15.9–59
Soybean [*Glycine max* (L.) Merr.]	17	*qBNF-17*	Nodule size & number	12.6–18.6
Lotus japonicus	2	TM0550–TM0324	Acetylene reduction activity per plant (ARA/P)	15.1
Lotus japonicus	2	TM0550–TM0002	ARA per nodule number (ARA/NN)	11.1
Lotus japonicus	4	TM0664	ARA per nodule weight (ARA/NW)	10.8
Lotus japonicus	5	TM1417–TM0095	ARA per nodule weight (ARA/NW)	13
Lotus japonicus	3	TM0083	Nodule number (NN)	21.6
Lotus japonicus	1	TM0113–TM0805	Stem length (SL)	13.3
Lotus japonicus	1	TM0027–TM0063	Shoot length without inoculation (SL bac−)	16.7
Lotus japonicus	1	TM0113–TM0805	Shoot length without inoculation (SL bac−)	16
Lotus japonicus	5	TM0095–TM0909	Shoot dry weight without inoculation (SW bac−)	10.7
Cowpea [*Vigna unguiculata* (L.) Walp.]	4 (Likage group)	2_12850/2_54418	Nodule number	48.4
Cowpea [*Vigna unguiculata* (L.) Walp.]	6 (Likage group)	2_11936/2_49231	Nodule fresh weight	21.4

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