Fixing Life – The Rhizobium-Legume Symbiosis

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1 Introduction

Nitrogen is an essential – often limiting – nutrient involved in the growth of plants. While N_2 gas makes up 78% of Earth's atmosphere, plants cannot metabolise this directly and require N_2 to be first "fixed" into either ammonia (NH_3) or nitrate (NO_3^-) [17].

This process requires enormous amounts of energy – the formation of artificial nitrogen fertilisers accounts for nearly 50% of the fossil fuel use in agriculture [3]. Furthermore, agricultural run-off can lead to nitrate-tainted drinking water and the eutrophication of lakes and rivers [30].

Biological nitrogen fixation (BNF), while less environmentally taxing, remains limited to a small set of prokaryotic "diazotrophs" [30]. While many crops can form loose, associative symbioses with diazotrophs in the soil, much of the nitrogen fixed in these relationships never makes it to the plant [12]. Nodule-forming symbioses, however, allow for the more complete transfer of fixed nitrogen [9]. While a number of nodulating symbioses exist, the rhizobium-legume symbiosis is both the best-studied and most relevant – responsible for the majority of BNF world-wide [9, 30].

This paper details the establishment of the rhizobium-legume symbiosis while highlighting common themes and linking processes back to host-rhizobium specificity. A primer providing some biological context will precede a step-by-step discussion of the events leading up to symbiosis.

2 Background

2.1 The Benefits of Symbiosis

Rhizobial symbiosis ultimately results in diazotrophic bacteria colonizing plant root nodules and intracellularly fixing N_2 – the host receives NH_3 while the rhizobium receives sugars and protection from predators [15]. Additionally, many rhizobia secrete growth stimulating metabolites such as lumichrome and riboflavin [5, 15]. These molecules benefit nearly all plants, not just legumes. A recent review found that the growth of 11 non-legume crops was accelerated by the presence of rhizobia [18]. In nutrient poor conditions, the effects of rhizobial inoculation on plant growth can be especially dramatic (Fig. 1).

2.2 Nitrogen Fixation

The N≡N bond of N_2 is extraordinarily stable, having a bond energy of $940\,\mathrm{kJ\,mol^{-1}}$; however, all nitrogen fixing reactions must eventually break this bond. The nitrogenase enzyme responsible for this contains both reductase (Fe) and nitrogenase (FeMo) subunits (Fig. 2A) [2].

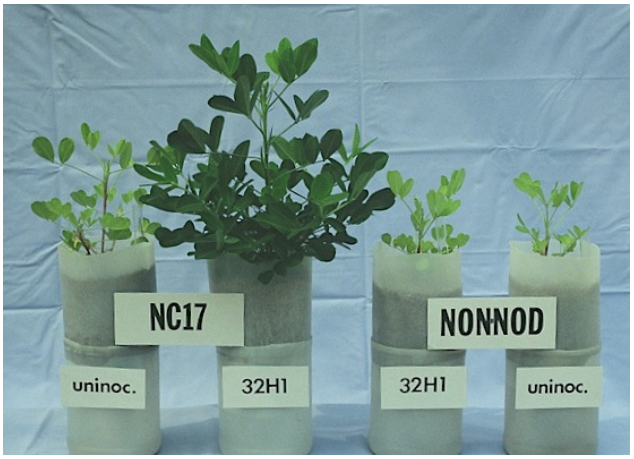


Figure 1: Symbiotic Rhizobia Improve Peanut Growth NC17 (wild-type) and NONNOD (non-nodulating) peanuts were grown with and without inoculation by *Bradyrhizobium* strain 32H1. Photo by the Nature Knowledge Project [27].

The reductase protein strips electrons from transporters such as ferredoxin and provides them with a high reducing power. The hydrolysis of ATP (two per e^-), transfers these electrons to the nitrogenase protein where they are used in the step-wise reduction of N_2 (Fig. 2B) [2]. This transformation also results in the formation of H_2, yielding the overall reaction:

$$N_2 + 8\,e^- + 8\,H^+ \xrightarrow[ATP]{16} 2\,NH_3 + H_2$$

Complicating things is the sensitivity of the nitrogenase complex to oxygen. Even brief exposure results in the rapid oxidation of metal cofactors within the enzyme, leading to its irreversible deactivation. The reductase protein has a particularly short half-life of just 45 seconds [10].

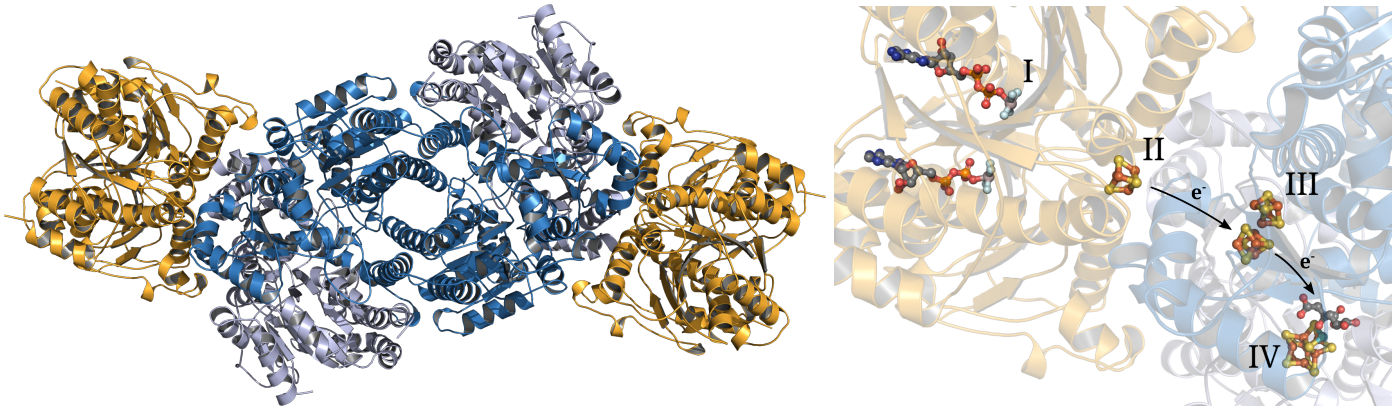
3 Establishing Symbiosis

3.1 Rhizobium Recruitment

3.1.1 Flavonoid Secretion

The first step in establishing any symbiosis is attracting a compatible partner. In this case, as nitrogen availability begins to dwindle, the host attracts rhizobia by secreting flavonoids into the rhizosphere (Fig. 3) [14]. These compounds act as chemoattractants; by following the flavonoid gradient, rhizobia make their way towards legumes in need [3].

In addition to attracting rhizobia, flavonoids play a critical role in determining host specificity: different rhizobia respond differently to different flavonoids [12]. For example, the *nod* genes of *S. meliloti* – vital to symbiosis – are



(A) Cartoon Representation of the Nitrogenase Complex **(B)** Electron Transfer within the Nitrogenase Complex

Figure 2: Structure and Function of the Nitrogenase Complex
(A) The nitrogenase complex is composed of three proteins: one nitrogenase (shown in blue and silver) and two reductases (shown in orange). The nitrogenase protein is a tetramer containing two α chains (silver) and two β chains (blue). Each reductase protein is composed of two identical γ chains (orange). **(B)** The stepwise reduction of N_2 is performed via a number of cofactors. First, two molecules of ATP (I) are hydrolysed by the reductase protein. Note that this structure contains ADP + AlF_4^- as a stable ATP mimic. The energy released by ATP hydrolysis drives the transfer of stored electrons from the 4Fe-4S cluster of the reductase (II) to the P-cluster of the nitrogenase (III). Finally, electrons from the P-cluster are used to progressively reduce the N_2 in complex with the FeMo-cluster (IV). This unusual FeMo cofactor is stabilised by an additional molecule of homocitrate (grey and red) [2]. Graphics produced using PyMol and structural data from PDB entry 1N2C [25].

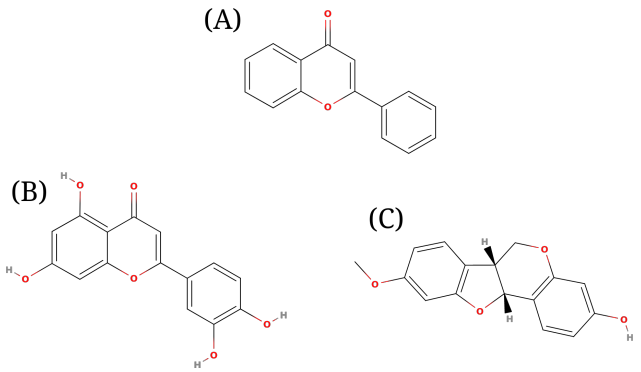


Figure 3: Structural Diversity of Flavonoids
(A) The general structure of a flavonoid. All flavonoids contain two benzene rings linked through a third pyran or pyrone ring [12]. **(B)** The structure of luteolin, capable of inducing rhizobial *nod* genes. **(C)** The structure of medicarpin, a flavonoid derivative that acts as a phytoalexin. Produced using MolView and structural data from PubChem [24].

promoted by flavonoids like luteolin (Fig. 3B) but repressed by others like medicarpin (Fig. 3C) [3].

While medicarpin might not induce *nod* genes, it still plays an important role in host specificity: medicarpin doubles as a phytoalexin. While the medicarpin produced by *M. truncatula* kills most rhizobia, its symbiont, *S. meliloti*, is resistant [14]. Producing phytoalexins allow hosts to quickly select many non-compatible rhizobia out of the rhizosphere.

3.1.2 Nod Factor Induction

On a molecular level, most rhizobia bind flavonoids via the nodD receptor [3, 13]. NodD then undergoes a conformational change allowing it to bind regulatory *nod* boxes within the genome. This promotes the production of signalling molecules called Nod factors. While many flavonoids can bind nodD, only some are capable producing the correct DNA-binding conformation [13].

3.1.3 Root Hair Adhesion

Finally, in preparation for infection, rhizobia weakly bind the Ca^{2+} localised at the tips of growing root hairs via the rhicadhesin protein [9]. This binding is then reinforced by cellulose fibrils from the host, fimbria from the rhizobium, and lectin interactions. These lectins provide yet another host specificity checkpoint, selectively linking compatible rhizobial exopolysaccharides to the plant cell wall [9].

3.2 Initiation of Infection

3.2.1 Nod Factor Perception

The Nod factors (NFs) produced by rhizobia are lipochitooligosaccharides: chitin oligomer backbones containing a 16–19 carbon fatty acid and a range of additional, host-specific modifications [3, 15, 16, 23]. *M. truncatula*, for example, only responds to NFs containing a C-6 sulphation of the reducing terminus (Fig. 4) [15, 23].

The adhesion of rhizobia to root hairs concentrates NFs and triggers the plant's lysin motif receptor-like kinases (LysM-RLKs), including: the NFP protein (responsible for nodulation) and the LYK3 + NFP complex (responsible for initiating infection) [3, 8, 9]. Notably, some rhizobia can initiate nodulation, but *not* infection; therefore, it is presumed that NFP is a more general receptor than the LYK3 + NFP complex [3, 8].

3.2.2 Calcium Signalling

The activation of host LysM-RLKs initiates a signalling cascade opening a number of ion channels in the cell (Fig. 5A).

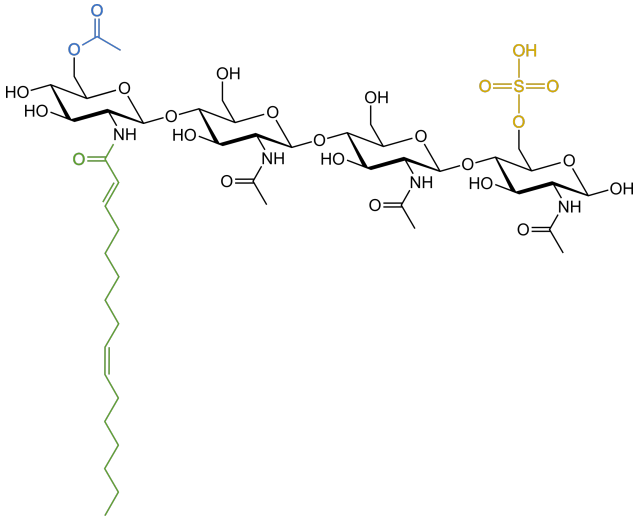


Figure 4: *S. meliloti* Produces Sulphated NFs
S. meliloti Nod factor is composed of four chitin monomers (black) and a 16 carbon fatty acid (green). Additionally, an acetyl (blue) and sulphate (yellow) group are present. This sulphate group is required to initiate symbiosis with *M. truncatula*. Graphic adapted from Wikimedia Commons [29].

Depending on the LysM-RLKs activated, two distinct outcomes are possible: a Ca^{2+} flux and / or Ca^{2+} spiking [3, 8, 22].

A Ca^{2+} flux is characterised by a rapid rise in cytoplasmic Ca^{2+} levels followed by widespread membrane depolarisation [26]. This flux occurs almost immediately following NF exposure and leads to root hair curling and rhizobial infection [3, 8, 26].

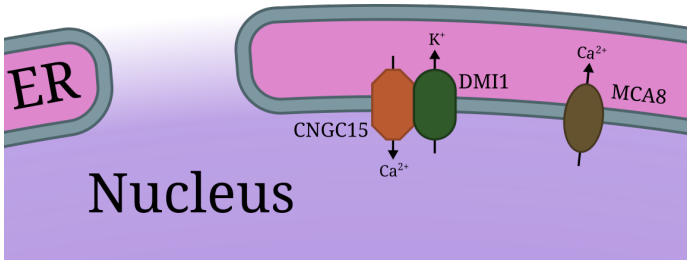
Ca^{2+} spiking, on the other hand, is a slower reaction – lagging NF exposure by around 10 minutes. A feedback loop between Ca^{2+} regulated ion channels leads to long-lasting, recurring peaks in nuclear Ca^{2+} concentrations (Fig. 5B) [3, 8]. These Ca^{2+} signals are "decoded" by the calcium and calmodulin-dependent protein kinase (CCaMK) which subsequently initiates nodule formation via the common symbiotic signalling pathway (CSSP) [3, 26].

3.2.3 Root Hair Curling

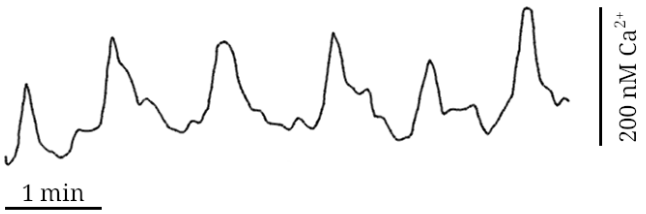
While rhizobia can colonise hosts in a number of ways, the most common method involves the infection of developing root hairs (Fig. 6A). Under normal circumstances, these root hairs maintain a Ca^{2+} gradient that encourages the polar growth of actin filaments; however, the membrane depolarisation following a Ca^{2+} flux disrupts this gradient [22]. The result is the curling or branching of root hairs – often forming "shepherd's crooks", each containing a small "focus" of trapped rhizobia (Fig. 6B) [1, 22].

3.2.4 Infection Thread Formation

These sealed foci allow for the localised degradation of the host cell wall and the initiation of an infection thread (IT). This IT, a pocket travelling back through the length of the root hair (Fig. 6C), is the result of both continued rhizobial growth and a Ca^{2+} induced rearrangement of the root hair cytoskeleton [22].



(A) Ion Channels Involved in *M. truncatula* Ca^{2+} Signalling



(B) *M. truncatula* Ca^{2+} Spiking Following NF Exposure

Figure 5: NF Exposure Triggers Host Ca^{2+} Signalling
(A) NF binding results in the activation of cyclic nucleotide gated channel 15 (CNGC15) and does not make infections 1 (DMI1). Additional Cl^- channels are present in *L. japonicus* (not shown). CNGC15 allows Ca^{2+} ions to flood the nucleus (from the ER) while DMI1 allows for the reverse flow of K^+. Afterwards, MCA8 pumps Ca^{2+} ions back across the membrane, reestablishing the original gradient [3, 8, 22]. **(B)** A plot of Ca^{2+} concentration over time shows repeated spikes in nuclear calcium levels (the result of CNGC15 + DMI1) followed by a more gradual decline (the work of MCA8). Data from Capoen et al. [4].

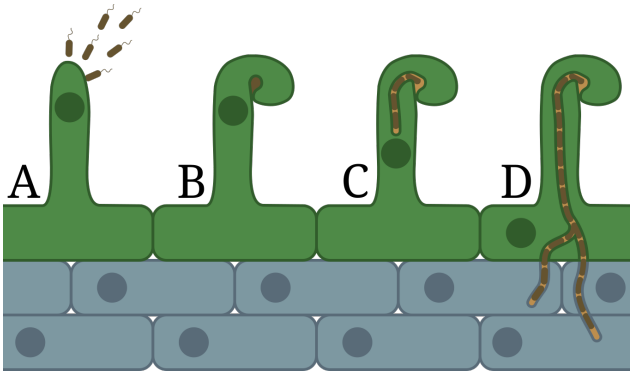


Figure 6: Rhizobia Infect Hosts via Root Hairs
(A) Secreted flavonoids attract rhizobia, which then adhere to the root hair. **(B)** The perception of rhizobial NFs leads to root hair curling. **(C)** An infection thread (IT) begins to form within the root hair. The host nucleus – previously located near the tip of the root hair – guides the IT back towards the root. **(D)** ITs eventually leave the epidermis (green) and continue into the cortex (blue) where they begin to branch and infect host cells.

The rhizobia, however, aren't in the clear yet – many hosts require the rhizobia to present specific exopolysaccharides or face destruction by the plant's defences. When symbionts of *M. truncatula* lack succinoglycan, ITs are aborted and uninfected nodules result [3, 22].

If the IT isn't aborted, it eventually exits the distal wall of the epidermal cell where it continues deeper into the root towards the developing nodule (Fig. 6D) [3, 22].

3.3 Nodule Formation

3.3.1 The Common Symbiotic Signalling Pathway

While ITs have been working their way through the epidermis, nodules have been growing within the root cortex. The common symbiotic signalling pathway, previously set in motion by nuclear Ca^{2+} spiking, prepares the host for symbiosis before producing a number of nodule-forming proteins – among them, hormone receptors such as MtCRE1 [3, 22].

3.3.2 Hormonal Changes

Nodule organogenesis, like all plant growth, involves a number of hormonal signals – particularly those concerning cytokinin and auxin. However, *in contrast* to the way that most plant growth is initiated, nodulation is triggered by *low auxin* and *high cytokinin* conditions [3, 22]. Because cytokinin naturally represses auxin-transporting PIN proteins, these hormones interact antagonistically; therefore, both introducing cytokinin and artificially inhibiting auxin transport had the same nodule-forming effect in *M. truncatula* [3].

3.3.3 Nodule Differentiation

Depending on the legume, nodules can either grow continuously (resulting in indeterminate nodules), or to a fixed size (forming determinate nodules) [3, 9, 16]. In contrast to normal roots that start in the pericycle, these nodules develop from cortical cells (Fig. 7) [8, 12].

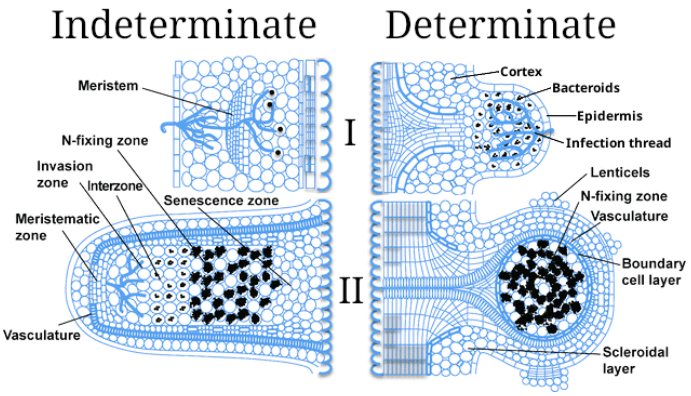


Figure 7: The Two Types of Leguminous Nodules
(I) Rhizobia leave the IT and begin colonising the developing nodule. In the determinate nodule, a large number of cells are infected at this stage. Comparatively few cells are infected in the indeterminate nodule. **(II)** Nodules reach maturity and begin fixing nitrogen. The determinate nodule has stopped growing and all of its bacteroids are mature. The indeterminate nodule grows indefinitely, meaning new cells are constantly being infected and bacteroids display a developmental gradient. Graphic adapted from Ferguson et al. [8].

Additionally, developing nodules express CCS52A, which degrades mitotic cyclins and allows for gene replication without mitosis [16]. The result is endoreduplication, yielding highly polyploid cells, each 80× their original size and capable of containing ∼50,000 rhizobia [16].

3.4 Nodule Colonisation & Specialisation

3.4.1 Symbiosome Formation

Upon reaching the root cortex, the rhizobia-containing ITs begin to ramify, branching to infect the nodule [3]. As these ITs penetrate individual host cells, they shed rhizobia-containing "infection droplets". These membrane-enveloped rhizobia then develop into specialised organelles called symbiosomes, acquiring many unique symbiotic proteins in the process (Fig. 8) [3, 16].

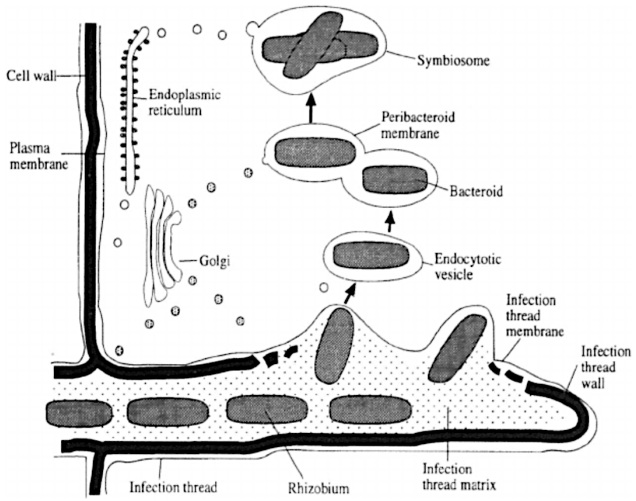


Figure 8: Rhizobia Escape ITs to Form Symbiosomes
Within a nodule cell, the wall surrounding ITs can degrade, allowing rhizobia to be endocytosed by the host. These endosomes can sometimes merge, resulting in vesicles containing several rhizobia. While symbiosomes retain a number of proteins from the plasma membrane, they receive many new, symbiosis-specific molecules from the ER and Golgi. Graphic from Whitehead and Day [28].

3.4.2 Bacteroid Differentiation

These endocytosed rhizobia soon develop into "bacteroids", undergoing – depending on the host – partial or irreversible differentiation [16, 22]. Generally, legumes with *indeterminate* nodules will yield *terminally* differentiated bacteroids that cannot be recultured as free-living bacteria [13].

Legumes like *M. truncatula* produce more than 700 nodule-specific cysteine-rich (NCR) peptides capable of driving terminal bacteroid differentiation [3, 13, 16, 22]. These peptides serve to increase membrane permeability and disrupt binary fission, but also represent a final host-specificity check. Many NCR peptides double as antimicrobial defensins; therefore, if the rhizobium is to survive, it must degrade just enough of these peptides to strike the balance between differentiation and death [3].

Just as disrupting mitosis led to endoreduplication in the host, the NCR-mediated disruption of binary fission leads to the formation of enlarged, highly polyploid bacteroids. NCR247 in particular can prevent rhizobial division by inhibiting Z-ring and septum formation [16].

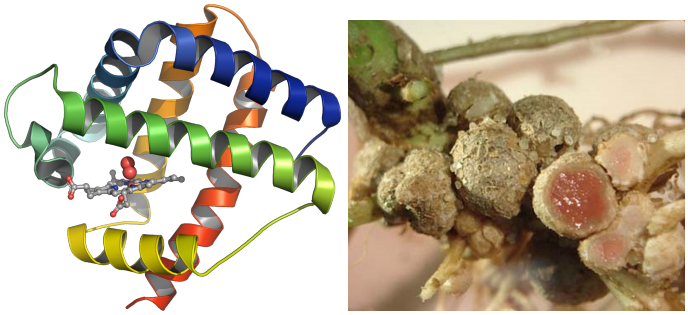
Finally, as oxygen levels within the nodule begin to drop, the rhizobial FixLJ operon triggers dramatic metabolic changes: aggressively down-regulating most genes while selectively up-regulating many nitrogen fixing ones [3, 13, 15].

3.4.3 Host Contributions to Fixation

As a result of differentiation, many bacteroids lose the ability to synthesize their own biomolecules in a phenomenon known as "symbiotic auxotrophy". Consequentially, these bacteroids often need to import a number of metabolites from the host [22].

Surprisingly, most rhizobia have also lost the NifV gene required to form the homocitrate cofactor of nitrogenase; instead, this essential component is supplied by the host via a plant-specific homocitrate synthase [21, 22].

Finally, to avoid damage to the nitrogenase complex, oxygen levels within the root nodule must be kept as low as possible. The host accomplishes this via the formation of physical diffusion barriers as well as the production of oxygen-binding leghaemoglobin (Fig. 9) [7, 19]. Leghaemoglobin buffers the cytoplasmic O_2 concentration between 7nM and 11nM, maintaining a reservoir of oxygen for ATP synthesis while keeping O_2 away from the nitrogenase. This helps create the high-energy, low-oxygen environment required for nitrogen fixation [7].



(A) Cartoon Representation of Oxyleghaemoglobin **(B)** Cross-section of a Root Nodule Containing Leghaemoglobin

Figure 9: Leghaemoglobin Keeps O_2 Levels Low
(A) Leghaemoglobin is a relatively small protein (16 kDa) containing a haem group (grey) which binds O_2 (red spheres). It closely resembles myoglobin, an oxygen binding protein found in animal muscles, but has a 20× greater affinity for O_2 [7]. Produced using PyMol and PDB entry 2GDM [11] **(B)** The oxidised Fe^{3+} within leghaemoglobin gives the protein a red colour. This bloody pigmentation can be seen here in a freshly-cut root nodule. Image from Penn State University [20].

4 Conclusion

When it comes to the rhizobium-legume symbiosis, there exists an intimidating amount of diversity; nevertheless, a number of common themes have emerged – particularly, the pervasiveness and redundancy of host-specificity checks. Whether they be host-required NF modifications or the presence of particular exopolysaccharides, these play a vital role in the constant battle against parasitic, "cheating" rhizobia. Without them, there would be nothing to stop unhelpful, *non-fixing* rhizobia from colonising the host and stealing plant nutrients [6].

While a high-level overview of the symbiosis is starting to come together, many of the molecular details remain obscure. For instance, how Ca^{2+} signals are propagated from the epidermis to the site of nodule development is still uncertain [8, 22]. Additionally, most studies have remained limited to the model legumes *M. truncatula* and *L. japonicus*, so it is unclear how much of this research is transferrable.

The extension of biological nitrogen fixation to non-legume crops could be the next leap forward for agriculture. Modern genetic tools have enabled numerous novel approaches to this – including the direct transplantation of rhizobial fixation machinery into plant mitochondria [19]. Whether via this method or another, replacing artificial nitrogen fertilisers with biological nitrogen fixation would empower agriculture to feed Earth's ever-expanding population without leaving such a muddy footprint on Mother Nature's doorstep.

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