

Establishing a Symbiotic Relationship Between Legume Plants and Rhizobial Bacteria

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Summary

Legume crops commonly grown in western Canada have the ability to form an association with a bacterial partner, called *Rhizobium*, and together these partners are capable of “fixing” gaseous nitrogen into a form that is plant available. This symbiotic association is beneficial to both partners. The plant is able to access a form of nitrogen that otherwise is unavailable, and the bacteria receive a ready source of energy from the plant. This remarkable partnership between plant and bacteria is highly organized and coordinated. Indeed, the simplicity of using a commercial rhizobial inoculant to enhance nodulation and ensure adequate nitrogen fixation in commercial legume crop production belies the complexity of the partnership. An examination of the many steps leading to the establishment of a symbiotic N-fixing association helps reveal factors that can contribute to, or prevent, successful nodulation.

Introduction

Nitrogen (N) is essential for all plant life. It is a key component of all amino acids, which are the building blocks of protein, and all nucleic acids including DNA and RNA, which control cellular function and heredity. Nitrogen is a relatively common element yet despite its prevalence it is often one of the most limiting nutrients for plant production. Nitrogen deficiencies occur, in part, because approximately 90% of the N in soil is tied up in the soil organic matter and is only slowly available for plant uptake, as organic matter decomposes. The ultimate source of N is our atmosphere and the air we breathe contains approximately 78% N gas by volume. This N exists as two N atoms (N₂) that share three electrons between the two atoms, forming a very stable triple bond that is not easily broken. Consequently, N₂ gas is considered relatively unreactive, and certainly is unavailable for plant uptake. So how does this unreactive N₂ gas enter the living realm and ultimately support life? Prior to industrialization (i.e., fertilizer N production), approximately 10% of the N entered the living biome through the massive electrostatic energy supplied in a lightning bolt (the weapon of choice of Zeus, the mighty Greek God) that can cleave the triple bond. Remarkably, the remaining 90% of the atmospheric N was transformed to plant available N forms through a process called “biological N fixation”, mediated by a remarkable group of N-fixing prokaryotic bacteria that similarly possess the extraordinary ability to break the triple bond^{8,26}. Zeus and his lightning bolt couldn't hold a candle to these tiny but mighty microbes.

Nitrogen Fixing Microorganisms

Prokaryotes are a diverse group of single-celled organisms that lack a membrane bound nucleus. This group is further subdivided into the bacteria and the archaea, the latter only recognized as a domain of life in the early 1990s. Prokaryotes capable of fixing N share the ability to produce an enzyme called nitrogenase that ultimately catalyzes the reaction that splits the triple bonded N₂ gas into two separate ammonia (NH₃) molecules. Nitrogen fixing prokaryotes are quite diverse and exist in a variety of environments both as free-living microbes, and as organisms that have evolved an associative relationship with various plants. The nitrogenase enzyme is sensitive to oxygen and is irreversibly inactivated in the presence of free oxygen. Consequently, N-fixing prokaryotes have developed a range of strategies to protect nitrogenase from exposure to oxygen. For example, under N limited conditions *Anabaena*, a filamentous cyanobacteria, transform vegetative cells within a filament into specialized cells called heterocysts that are designed specifically to protect the nitrogenase enzyme. Yet another group of N-fixing prokaryotes, collectively called rhizobia, instruct legume roots to form root nodules on their behalf, providing a haven for the rhizobia in which the nitrogenase enzyme is protected, as well as a ready supply of carbohydrates to sustain the rhizobia housed in the nodule (Fig. 1). This relationship between the N-fixing prokaryotes and the legume plants is called a symbiosis, with both partners deriving benefits from the association⁸. Ultimately, the rhizobial partner provides N “fixed” (i.e., captured) from the

atmosphere, and in exchange the plant provides C, which is fixed from atmospheric CO₂ during photosynthesis. This type of mutualistic relationship is

limited to a relatively narrow group of prokaryotic bacteria, including the genera *Rhizobium*, *Mesorhizobium*, *Sinorhizobium*, *Bradyrhizobium* and *Azorhizobium*⁸.



Figure 1. Nodules on faba bean roots formed by indigenous soil rhizobia (Photo courtesy of B. Ta'ran).

Rhizobia live freely in the soil, and even in the absence of a legume host plant, a rhizobial community can persist for several years⁵. As free-living organisms, they live saprophytically, that is, they derive their nourishment from dead or decaying organic matter and thus contribute to decomposition of organic matter and nutrient cycling. Although rhizobia can persist, indigenous strains are not necessarily effective N-fixers, and introduced rhizobia may lose their ability to fix high levels of N over a period of time³. Nitrogen fixing capacity can be lost because the genetic material that provides the codes necessary for nodulation to occur generally exist in small packets of genetic information, called plasmids, floating like tiny “symbiosis islands” within the bacterial cell^{7,8}.

These little islands of information can be lost or transferred from rhizobial cells, resulting in reduced N-fixing capacity. Consequently, although some fields may harbor high populations of indigenous or previously introduced rhizobial strains, there is a tendency for increased symbiotic efficiency, and consequent increases in N accumulation, with the use of effective commercial inoculants³.

Friend or Foe?

That rhizobia can infect and enter legume roots is remarkable. Mother Nature has equipped plants with a suite of mechanisms that *prevent* disease organisms from entering plant tissue, so how do host plants

differentiate between unwanted disease causing organisms, and the friendly N₂-fixing rhizobial invaders? In order to permit infection, a carefully orchestrated biochemical conversation occurs between the host plant roots and the rhizobial bacteria. The conversation involves a multistep, reciprocal recognition of signal molecules released in coordination from both partners¹⁸. As the conversation proceeds, several specific interactions occur, and at each stage of these increasingly complex interactions there is an increasing specificity for the legume-rhizobial pairing⁵.

As with the development of any relationship, the first step is simple recognition. Plant roots excrete a wide range of substances, including a cocktail of phenolic molecules, many of which are called “flavonoids”. Some of these flavonoids can passively diffuse across bacterial cell membranes and are thus sensed by rhizobia²⁷. The flavonoids act as attractants to the rhizobia, and if they are in relatively close proximity, the bacterial cells can use tiny whip-like flagella to follow the concentration gradient towards the most concentrated source of the signal molecule (a process called “chemotaxis”)⁴. Travel for the rhizobia is limited because the cells must move in water films—either on soil particles or roots—and thus movement beyond a few millimeters via the action of the flagella is unlikely². Thus, for these early recognition and attraction events to occur, the rhizobia must be in relatively close contact with the growing root, and in particular, the roots hairs. This has implications in terms of placement of rhizobial inoculants, which must ensure contact between the inoculant and emerging and developing roots. This is easily achieved if the inoculant is applied to the seed coat (as is the case with peat-based powders, liquids and seed coating) because the emerging primary root necessarily comes in contact with the inoculant, and rhizobia attached to the root surface can then spread by passive movement as root

cells grow². Nonetheless, even passive movement along with the growing root is limited and inoculation on the seed coat typically results in a clustering of nodules around the crown region (i.e., in the immediate vicinity of the original seed placement) whereas placement of the inoculant in the soil (i.e., banding of granular or liquid inoculants close to seed row placement) typically leads to enhanced nodulation of lateral roots¹³. *Where the inoculant is thus placed, there shall the nodules develop!*

Early Infection Steps

The soil zone in immediate contact with the root (called the “rhizosphere”) is a welcome environment for invading rhizobia. Along with flavonoids, plant roots secrete a host of compounds, many of which are a ready source of nutrition for the bacteria (Figure 2). Consequently, the rhizobia typically multiply rapidly in the rhizosphere so that a film (called a “biofilm”) of bacteria, comprised of the multiplying rhizobial cells embedded in a self-produced slimy matrix of extracellular polysaccharides, anchors itself to the root and—importantly—to the growing root hairs which are the ultimate points of infection⁵. Root hairs are very thin, hair-like outgrowths from a single epidermal (i.e., surface) cell of the root, and typically are found in the region just behind the growing root tip. These delicate protrusions have very thin walls to facilitate the uptake of water and nutrients. The thin and less cross-linked walls of the root hair also present a less challenging barrier for invading rhizobia⁶.

While flavonoids attract rhizobia, signaling the nearby presence of a suitable host plant, these same plant-derived compounds also cause the activation of a suite of bacterial genes (called *nod* genes) which otherwise remain inactive^{7,27}.

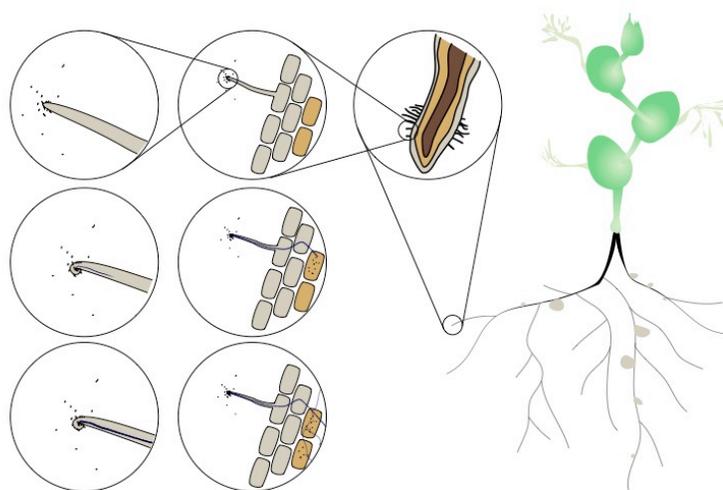


Figure 2. Nodulation events start with the rhizobia recognizing plant signals (flavonoids) emitted by the roots. Rhizobia are attracted to the root hair surface and respond to the plant signal by producing their own signal (Nod factor). The plant root responds by deforming the root hair into a shepherd's crook, trapping the rhizobia. The root hair membrane then grows back within itself, forming an infection tube. Rhizobia travel down the tube, often in single file, by dividing at the front face of the infection tube. The infection thread eventually branches and rhizobia enter nodule meristem cells in the root cortex, ultimately forming a functional N-fixing nodule (illustration by Joel Ens).

The newly active *nod* genes contain the genetic information that encodes for the production of bacterial "Nod factors". Most Nod factor molecules have an oligosaccharide backbone that chemically resembles a fragment of another molecule, called chitin, and thus Nod factors are often called lipo-chitin oligosaccharides, lipo-chitooligosaccharides, or simply "LCOs"²⁴. Rhizobia typically produce a mixture of LCOs²⁴ and LCOs are essential for the development of the symbiotic association in most legumes¹⁹. Different legumes secrete different types of flavonoids, and only certain rhizobia will respond to the specific flavonoid signals by producing rhizobia specific LCOs, thereby establishing a checkpoint for defining the specificity of the relationship between the legume host and the infecting rhizobia²⁷.

The Nod factors (a.k.a. LCOs) produced by the bacteria serve as a signal back to the plant root that an invading army of rhizobia is approaching. Although the Nod factors are chemically similar between different rhizobia (i.e., all share the same basic oligosaccharide molecular structure), Nod factors from different bacteria can have different chemical substituents attached to the oligosaccharide backbone of the

molecule²⁴. These "decorations" are enough for a plant root to recognize a compatible rhizobial invader^{14,27}. Indeed, recognition of the Nod factors occurs only if the plant has specific "Nod factor receptors" (NFRs) compatible with the specific rhizobial Nod factor²⁷. Thus Nod factor receptor recognition of a friendly rhizobial invader (from the perspective of the plant) is yet another checkpoint that further defines the specificity of the host plant-rhizobial partnership. There is some evidence that the quantity of Nod factor that rhizobia produce may be important in early nodulation events⁵. Moreover, it has been suggested that, in some instances, high levels of certain Nod factors can inhibit nodulation, presumably providing a competitive advantage for some rhizobial strains over others¹⁰. Recently, inoculants containing signal molecules, including LCOs, have been introduced for commercial use in Canada. Although the science behind signal molecules is fairly well understood, published studies examining the efficacy of field applications of commercial signal molecule products remains limited, in part because these products are relatively new to the Canadian market.

Assuming that the signals have now been successfully exchanged from plant to rhizobia, and then from rhizobia back to the plant, the scene is set for the bacteria to invade the root, and for nodule initiation to begin deep within the root in anticipation of the arrival of the infecting bacteria (Fig. 1). The most common way for the rhizobia to enter the root involves the entrapment of the bacteria by the tip of the root hair and subsequent formation of an “infection thread”. In response to the Nod factor produced by the rhizobia, the root hair curls into a shepherd's crook, effectively trapping a small number of rhizobia in the bend of the crook, often occurring within six to eight hours⁶. According to Downie⁵, root hair deformations occur in the presence of “astonishingly low concentrations” of Nod factors. As little as 10^{-13} M Nod factor can induce root hair curling¹⁷. From the perspective of the rhizobia that are competing for infection sites, it is clearly a case of being at the right place at the right time, and thus rhizobia that are early and fast colonizers on the surface of the root hair are the winners in the nodulation lottery⁵. The initiation of the infection thread is another significant checkpoint during the infection process; invasion by a non-symbiont opens the plant to infection by ineffective strains, or even pathogens⁵.

Having trapped the rhizobia cells, the root hair then (quite remarkably) begins to grow back into itself, forming a long narrow invagination that becomes a tubular structure composed of plant cell wall components, called an “infection thread”, in which the bacteria enter and travel, following the front edge of the invagination by continuously dividing^{19,27}. Although the rhizobia have now entered the infection thread, technically the bacteria are still external to the plant cell because the infection thread is merely an inversion of the cell wall back into itself. Thus the plant has trapped the bacteria within a plant structure, and thereby maintains strict control of the infection. The infection thread continues to develop from cell to cell, dissolving cells walls in its path but maintaining the tubular infection thread. The infection thread extends into the inner cortex of the root without allowing the bacteria access into the cytoplasm of any of the cells the thread transverses.

The infection thread is very narrow and accommodates only a narrow column of bacteria, usually only a

couple of cells wide⁵. As a consequence, rhizobia that are able to reproduce rapidly within the infection cell typically have an advantage so even if more than one species is initially trapped in the shepherd's crook, the competition for room within the infection thread can be selective for a single strain⁵.

At the same time the infection thread is growing towards the inner portion of the root, the plant has already begun to prepare for the arrival of the rhizobia by transforming normally quiescent root cortex cells into nodule meristems (i.e., a region of actively dividing cells), from which the nodules will develop. Once again, signaling between partners serves to coordinate this stage of the infection process. It is thought that the rhizobia in the infection thread continue to produce Nod factor, and this Nod factor apparently modifies normal plant hormonal signaling within the root, particularly the cytokinin pathway, which subsequently gives rise to the nodule meristems within the cortex^{5,6}. These newly programmed cells form a swelling that will eventually become the nodule structure to house the invading rhizobia.

When the infection thread finally approaches the growing nodule structure, it branches and begins invading the newly formed cells. The bacteria are then “budded off” from the end of the infection thread through a process that keeps the plant cell membrane intact, so the bacteria remain trapped within little packages of plant-derived membrane^{6,8} called “symbiosomes”²⁵. The bacteria continue to divide within the host cells and then undergo significant differentiation, expressing many new genes required to establish the N-fixing enzyme system including the synthesis of nitrogenase (the enzyme required to split the N_2 triple bond) and other proteins required for N fixation, ultimately transforming into morphologically distinct “bacteroids” capable of fixing atmospheric nitrogen⁵. The plant cells of the nodule also begin to differentiate and, in the process, begin producing a range of nodule-specific proteins, collectively called “nodulins”. Significant amongst these nodulins is “leghemoglobin”, a molecule that is chemically and structurally similar to hemoglobin present in red blood cells. Although the plant is responsible for the production of the protein portion of the hemoglobin molecule, rhizobia are capable of producing the “heme” portion, and thus some researchers have argued

that the leghemoglobin molecule might be a truly symbiotic molecule, with both partners contributing molecular building blocks. However, others have argued that the plant host synthesizes both components of the leghemoglobin molecule²². Like the hemoglobin in red blood cells, leghemoglobin regulates oxygen levels. In root nodules, it is essential that oxygen levels are tightly controlled—some oxygen is required to support respiration (the bacteroids themselves need an adequate supply of oxygen to support their energy requirements) but too much oxygen can irreversibly damage the oxygen-sensitive nitrogenase enzyme thereby shutting down the N-fixing system. The presence of leghemoglobin guarantees tightly controlled “micro” aerobic (i.e., very low oxygen) conditions in the nodule, thereby protecting the nitrogenase enzyme. In its active form, plant-produced leghemoglobin has a reddish color, and is responsible for the reddish interior coloration of actively fixing nodules.

How Many Rhizobia Are Needed to Form a Nodule?

In addition to the highly coordinated events between the bacteria and the legume host, there is emerging evidence that there is coordinated sensing (called “quorum” sensing) which occurs between rhizobial bacteria, such that the rhizobia can sense the population density, and coordinate gene expression within the population⁹. Observations that a critical rhizobia population threshold must be reached before nodulation can occur¹ provided early evidence that quorum sensing is likely involved in regulating the expression of some genes, and may regulate one or more stages of the symbiosis⁹. Of particular interest, there have been suggestions that quorum sensing by some rhizobia may be involved in restricting the number of nodules that are formed⁹, preventing the legume from forming too many nodules that would place too heavy a carbohydrate load on the plant, since N fixation is energy expensive.

Interestingly, from a basic science perspective, the early steps in the rhizobial infection process (including recognition of a potential host and chemotaxis towards the host) are very similar to typical pathogenic (i.e., disease-forming) interactions. Moreover, some early plant responses are typical of a plant attempting to defend itself from disease organisms^{21,27}. However,

where successful nodulation occurs, plant host defenses either are not triggered¹⁵ or are suppressed²¹. Wang et al.²⁷ proposed that features of the bacteria, such as the Nod factors²³ and the slimy extracellular polysaccharides produced by the bacteria that help it attach to the plant root during early recognition stages¹¹, may play a role in the suppression of plant defense strategies. Although it is well established that bacterial exopolysaccharides are required for successful nodule invasion, their exact function is still unknown⁹.

There is No Such Thing as a Free Lunch – Nitrogen Fixation is Expensive!

Nitrogen fixation is an energy expensive process, and diverts valuable plant photosynthates to the production of the nodules, N-fixing enzymes and numerous proteins required to support the process. The trade-off between costs and benefits needs to favor improved N supply for the association to be beneficial for the plant. Consequently, the plant needs to have a level of control over the symbiosis, which is achieved by limiting the number of nodules through: 1) systemic autoregulation of nodulation⁶, 2) local hormonal inhibitory regulation²⁰, and 3) elimination of established bacteroids by necrosis of the nodules²¹. Early studies by Nutman¹⁶ revealed that removal of old nodules from red clover roots resulted in the initiation of new nodules, suggesting that the plant recognized the newly acquired capacity to support nodulation. More recently, grafting studies in which shoots and roots from different legume hosts were interchanged, revealed that the shoot exerts control on the level of nodulation in the root²⁰, suggesting a phloem-mediated communication between shoots and roots. This regulation, termed “autoregulation of nodulation”⁶, involves a complex root-to-shoot-to-root signaling loop that reflects the ability of the plant to provide the necessary photosynthates to support N fixation (and therefore, presumably, is moderated by growing conditions). Local hormonal regulation of nodulation within the root itself also occurs. For example, Ryu et al.²⁰ speculate that nutritional conditions, such as availability of soil nitrate N, may be closely connected with hormone biosynthesis and signaling pathways, thereby controlling nodule numbers. They further postulate that legumes likely utilize stress- and growth-related hormones to exert control over nodulation.

Although mechanisms controlling persistence of established nodules remain poorly understood, studies suggest that some legumes are capable of sensing inefficient N-fixing nodules and “punish” these nodules, likely by restricting the permeability of the root cortical cells to oxygen required for energy generation¹².

Conclusion

Inoculation of legumes with efficient N-fixing rhizobial inoculants remains an effective means of enhancing N availability in commercial legume production. The process of establishing nodules and subsequent N-fixation is a highly complex process, and depends on mutual exchange of species specific (i.e., from both partners) molecular signals. Several “checkpoints” exist along the pathway to effective nodulation, and the plant apparently has further controls that can limit photosynthate allocation to the N-fixing process, particularly under stressful environmental conditions that threaten plant growth. Although we have been successful manipulating the N-fixing system by isolating effective N-fixing rhizobia and developing these into commercial inoculants, it remains to be seen if we can further manipulate this complex system and over-ride evolutionary controls already imbedded in this biological process.

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