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Lesson: Nitrogen metabolism

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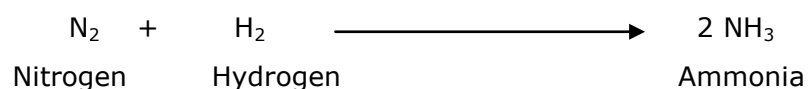
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Introduction

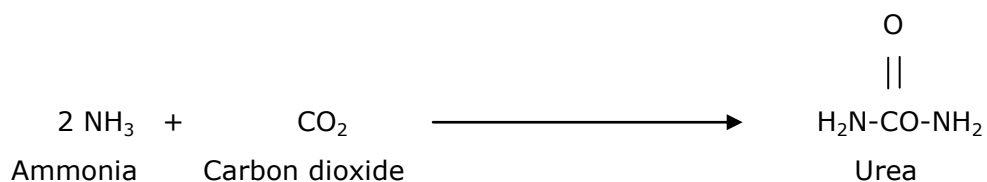
Nitrogen is present in many forms in the biosphere. Although vast quantities of molecular nitrogen is present in the atmosphere (77% by volume), it is not available for direct use by living organisms. Acquisition of this molecular nitrogen and its conversion to ammonium (NH_3) and nitrate (NO_3^-) accounts for nitrogen fixation. Conversion of inorganic to organic nitrogen is carried out by plants mainly. Nucleotides and amino acids (found in nucleic acids and proteins) mainly have nitrogen as their principal moiety constituting the prominent biochemical compounds in plant cells.

The process of nitrogen fixation requires a large input of energy because it accounts to the production of NH_3 and NO_3^- from nitrogen molecule by breakage of the stable triple covalent bond present in the dinitrogen molecule. Both natural and industrial processes contribute to the process of nitrogen fixation.

- 1. Industrial Nitrogen Fixation:** This type of fixation contributes to 20% of total nitrogen fixed. Nitrogen in combination with hydrogen forms ammonia in the presence of high temperature (about 200°C) and high pressure (about 200 atmospheres) and a metal catalyst (like iron). High activation energy of such a condensation reaction is overcome by such extreme conditions i.e. high temperature and high pressure.



This reaction is called **Haber-Bosch process** and serves as an initial trigger for the production of industrial and agricultural goods, as illustrated in the following equation:

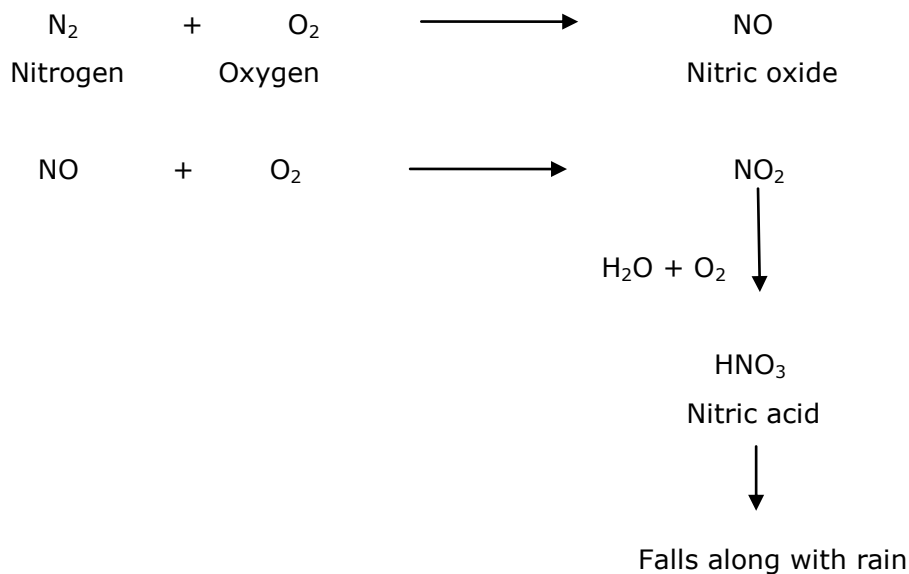


- 2. Atmospheric nitrogen fixation:** This can occur by any of the following two methods:

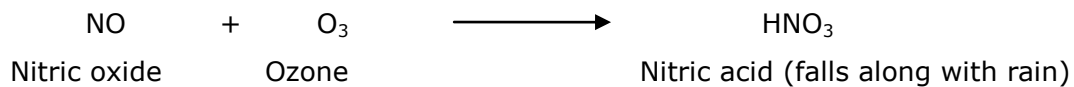
- i) Lightning:** This form of nitrogen fixation amounts to about 8% of the total nitrogen fixed. In the presence of lightning, highly reactive OH^\bullet , H and O are formed from water vapour

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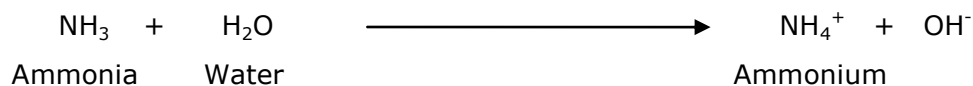
and oxygen. These free H and O atoms subsequently form nitric acid (HNO_3) when they come in contact with N_2 . HNO_3 thus formed, falls along with rain on earth surface.



ii) **Photochemical reactions:** This contributes to about 2% of the total nitrogen fixed. This kind of reaction leads to the production of nitric acid that subsequently falls along with rain.



3. Biological nitrogen fixation: This contributes to about 70% of the total nitrogen fixed. This process is carried out mainly by prokaryotic microorganisms like bacteria or blue green algae (cyanobacteria) which fix nitrogen into ammonia (NH_3). This ammonia dissolves in water to form ammonium:



This process of nitrogen fixation is very critical from agricultural point of view because nitrogen fertilizers produced through industrial means hardly ever meet agricultural requirements.

This process can occur either asymbiotically or symbiotically which has been discussed in detail later in the chapter.

Historical aspects of nitrogen fixation

- The history of nitrogen fixation is as old as the history of agriculture when crop rotation involving legumes was practised. However, it was in 1838 that Boussingault proposed that legumes increase the nitrogen content of the soil at the expense of atmospheric nitrogen.
- It was in 1888 that Beijerinck isolated *Rhizobium* for the first time from root nodules. He was later honoured by naming a bacterium after his name i.e. *Beijerinckia*. Before this discovery root nodules were thought to be insect galls.
- Nitrogen fixation by free living bacteria like *Clostridium pasteuriarum* was first shown by Winogradsky in 1893.
- The first cell free nitrogen fixation was done by Carnahan in 1960 using the enzyme Nitrogenase.
- However, it was in 1972 that Dixon and Postgate mapped the *nif* genes in *Klebsiella pneumoniae* and transferred them to *E. Coli*.

Biological Nitrogen Fixation

As stated earlier, this kind of fixation mechanism provides a major means of the entrance of atmospheric nitrogen into the nitrogen cycle by converting atmospheric nitrogen to ammonium. The role of organisms in nitrogen fixation is of great importance to the food chain in forests, freshwater and marine environments and even arctic regions. The activities of the roots of nitrogen-fixing plants benefit the roots of surrounding plants either through excretion of nitrogen from nodules or through decomposition of nodules or even whole plants. This contribution is important in agriculture in which mixed legumes and grasses are often used as pastures. About 15% of nearly 20,000 species in family fabaceae have been examined for nitrogen fixation and approximately 90% of these have root nodules in which fixation occurs. Important non-legumes that fix nitrogen are *Alnus*, *Myrica*, *Hippophae*, *Casaurina*. It can occur by two means:

1. Asymbiotic nitrogen fixation

This includes nitrogen fixation by a free living diazotroph bacteria or blue-green algae. These prokaryotes generally live in the soil independent of any other organism. The enzyme nitrogenase which fixes nitrogen requires anaerobic conditions. However, free-living

bacteria that are capable of fixing nitrogen are either, aerobic, facultative or anaerobic. They have adopted different strategies for maintain the requisite anaerobic conditions.

- i) **Aerobic nitrogen-fixers:** Bacteria like *Azotobacter* maintain increased respiration levels and thus, create low oxygen concentrations in an attempt to fix nitrogen.
- ii) **Facultative nitrogen fixers:** They generally fix nitrogen under anaerobic conditions although they are capable of growing under both aerobic and anaerobic conditions.
- iii) **Anaerobic nitrogen fixers:** Bacteria can be either photosynthetic (*Rhodospirillum*) or nonphotosynthetic (*Clostridium*). Oxygen is not present in their habitat and therefore, they don't require to create anaerobic conditions for fixing nitrogen unlike the above two fixers.

Table citing the various examples of nitrogen-fixing organisms:

Type	Nitrogen fixing genera
Obligate aerobes	<i>Azotobacter</i> <i>Azospirillum</i> <i>Beijerinckia</i> <i>Azotococcus</i>
Obligate anaerobes	<i>Clostridium pasteuriarum</i> <i>Methanococcus</i>
Facultative aerobes	<i>Klebsiella pneumonia</i> <i>Bacillus</i>
Photosynthetic bacteria	<i>Rhodospirillum rubrum</i> <i>Chromatium</i>
Chemosynthetic bacteria	<i>Thiobacillus ferrooxidus</i>
Cyanobacteria (Blue-green algae)	<i>Nostoc</i> <i>Anabaena</i> <i>Oscillatoria</i> <i>Spirulina</i> <i>Syctonema</i>

Source: Author

2. **Symbiotic nitrogen fixation**

Nitrogen fixation occurring as a result of symbiotic relationship between the prokaryote and higher host plant where the prokaryote directly provides the host plant with fixed nitrogen in exchange for other nutrients and carbohydrates. However, the enzyme **nitrogenase** that catalyze the reactions for fixing nitrogen is produced only by prokaryotic organisms.

Table citing the examples of prokaryotic and eukaryotic symbionts:

Nitrogen fixing symbiont (Prokaryotic)	Host plant (Eukaryotic symbiont)
<i>Azorhizobium</i> <i>Photorhizobium</i> <i>Bradyrhizobium</i>	Leguminous plants
<i>Rhizobium sp.</i>	<i>Parasponia</i> (non-leguminous plant)
<i>Frankia</i>	Actinorhizal plants: <i>Casuarina</i> <i>Alnus</i> <i>Zygophyllum</i> <i>Podocarpus</i> <i>Myrica</i>
<i>Nostoc</i>	Thalli of <i>Anthoceros</i> Stem nodules in <i>Gunnera</i> (angiosperm)
<i>Anabaena</i>	<i>Azolla</i> (water fern) Coralloid roots of <i>Cycas</i>
<i>Azospirillum lipoforum</i>	Roots of C ₄ grasses: <i>Miscanthus</i> Sugarcane

Source: Author

A very frequent form of symbiotic association is seen to occur between members of the plant family fabaceae (Leguminous plants) and soil bacteria of the genus *Azorhizobium*, *Bradyrhizobium*, *Photorhizobium*, *Rhizobium* (collectively called RHIZOBIA). *Rhizobium* has a large number of species and strains that infect a particular species of legume. This is thus, a strict symbiont-symbiont relationship. Some species of *Rhizobium* are thus given a generic status as well.

Table citing the associations of host plants with different species of *Rhizobium*

Plant host	Rhizobial symbiont
Soybean (<i>Glycine max</i>)	<i>Bradyrhizobium japonicum</i> (slow-growing type) <i>Sinorhizobium fredii</i> (fast-growing type)

Alfalfa (<i>Medicago sativa</i>) <i>Melilotus</i>	<i>Sinorhizobium meliloti</i>
Sesbania (aquatic)	<i>Azorhizobium</i> (forms both stem and root nodules)
Bean (<i>Phaseolus</i>) Pea (<i>Pisum sativum</i>)	<i>Rhizobium leguminosarum</i> bv. <i>Phaseoli</i> <i>Rhizobium leguminosarum</i> bv. <i>Viciae</i>
Aeschynomene (aquatic)	<i>Photorhizobium</i> (photosynthetically active rhizobia that form stem nodules, probably associated with adventitious roots)

Source: Author



Figure: Root nodules in *Medicago*.

Source:

http://upload.wikimedia.org/wikipedia/commons/b/b3/Medicago_italica_root_nodules_2.JPG (CC)



Figure: Stem nodules in *Sesbania rostrata*.

Source:

http://4.bp.blogspot.com/_bBqysXGhPys/TQZKp1XZQdl/AAAAAAAAACQU/aBCpwt3ti8w/s400/SLIP+3.1.Fig.+3.5.B.+SESBANIA+STEM+VII+SHOWING+NODULES.JPG

Nitrogen fixation requires anaerobic conditions

The enzyme nitrogenase that catalyze these reactions of nitrogen fixation have sites that facilitate the high energy exchange of electrons (because nitrogen fixation involves the expenditure of large amounts of energy). Oxygen can damage these sites (being a strong electron acceptor) and irreversibly inactivate nitrogenase. Therefore, anaerobic conditions are required for fixing nitrogen. All the organisms fixing nitrogen either through asymbiotic or symbiotic means function under natural anaerobic conditions or create an internal, local anaerobic environment in the presence of oxygen.

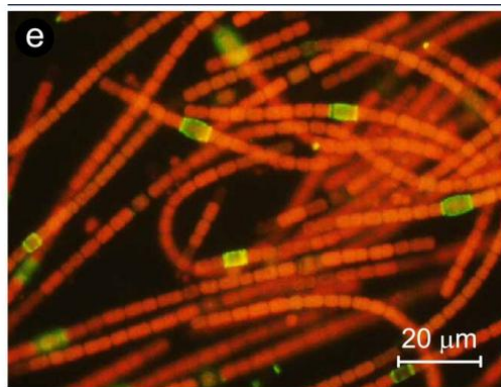


Figure: Filament of *Anabaena* showing heterocysts.

Source: http://www.mdpi.com/2075-1729/4/4/988/htm#fig_body_display_life-04-00988-f002 (CC)

Specific cells called **Heterocysts** present in cyanobacteria to create anaerobic conditions. These thick-walled cells are distinguished only when ammonium deprived conditions are faced by these organisms. Absence of photosystem II i.e. the oxygen producing photosystem of chloroplasts further helps in creating anaerobic conditions. These structures represent an adaptation for nitrogen fixation as they appear commonly among aerobic cyanobacteria that fix nitrogen.

To maintain an adequate supply of nitrogen in the soil of flooded rice fields (as in asian countries), these microorganisms (both heterocyst and non-heterocyst types) are a major means. These microorganisms fix nitrogen when the fields are flooded and die as the fields dry, releasing fixed nitrogen to the soil.

Another important source of available nitrogen in flooded rice fields is the *Azolla-Anabaena* association which fixes around 0.5 Kg of atmospheric nitrogen per hectare per day (a rate that is sufficient to attain moderate rice yields).

Process of nodulation in legumes

The symbiotic interaction between leguminous plant and *Rhizobia* leading to root nodule formation is not obligatory. The seedlings of leguminous plants may germinate as such without any association with rhizobia and they may not seek any interaction throughout their life cycle. Also, *Rhizobium* strains frequently occur as free-living bacteria in the soil. However, the two organisms develop a symbiotic association only under nitrogen deficient or limited conditions through some signal exchange mechanisms. This signalling mechanism, the corresponding infection process and the subsequent formation of nodules requires the presence of specific genes in both the symbionts.

1. Nodulins (*Nod*) genes: These are genes present in the **plant symbiont**
2. Nodulation (*nod*) genes: These are genes present in the **rhizobial symbiont** that participate in the formation of nodules.

The *nod* genes might be present commonly or they might be host-specific. Like, the *nodA*, *nodB*, *nodC* occurring in almost all the rhizobial strains are categorised under common *nod* genes. However, *nodP*, *nodQ* and *nodH*; or *nodF*, *nodE* and *nodL* genes are classified as host-specific genes that are unique to rhizobial species and thus help in deciding the host range (i.e. the plants that can be infected by a particular rhizobial strain). Only one of the genes, the regulatory *nodD* is constitutively expressed, and its product (NodD) regulates the transcription of other *nod* genes.

Both the plant nodulins and rhizobial nodulation genes produce some specific compounds that help in accomplishing the process of nitrogen fixation in legumes. Let's have a look at what are the compounds produced by both these characteristic genes.

Nodulins (*Nod*) genes: These genes produce

1. Flavonoids:
 - They are secreted in the rhizosphere.
 - They help to attract rhizobia towards root hairs.

- They are known to activate nodulation genes of the bacterium.
2. Lectins:
- These are sugar binding proteins.
 - They are present on membrane of root hairs.
 - They help in recognition of a particular strain of *Rhizobium*.
3. Globulins:
- This constitutes the globin part of leghemoglobin (oxygen-binding heme protein).

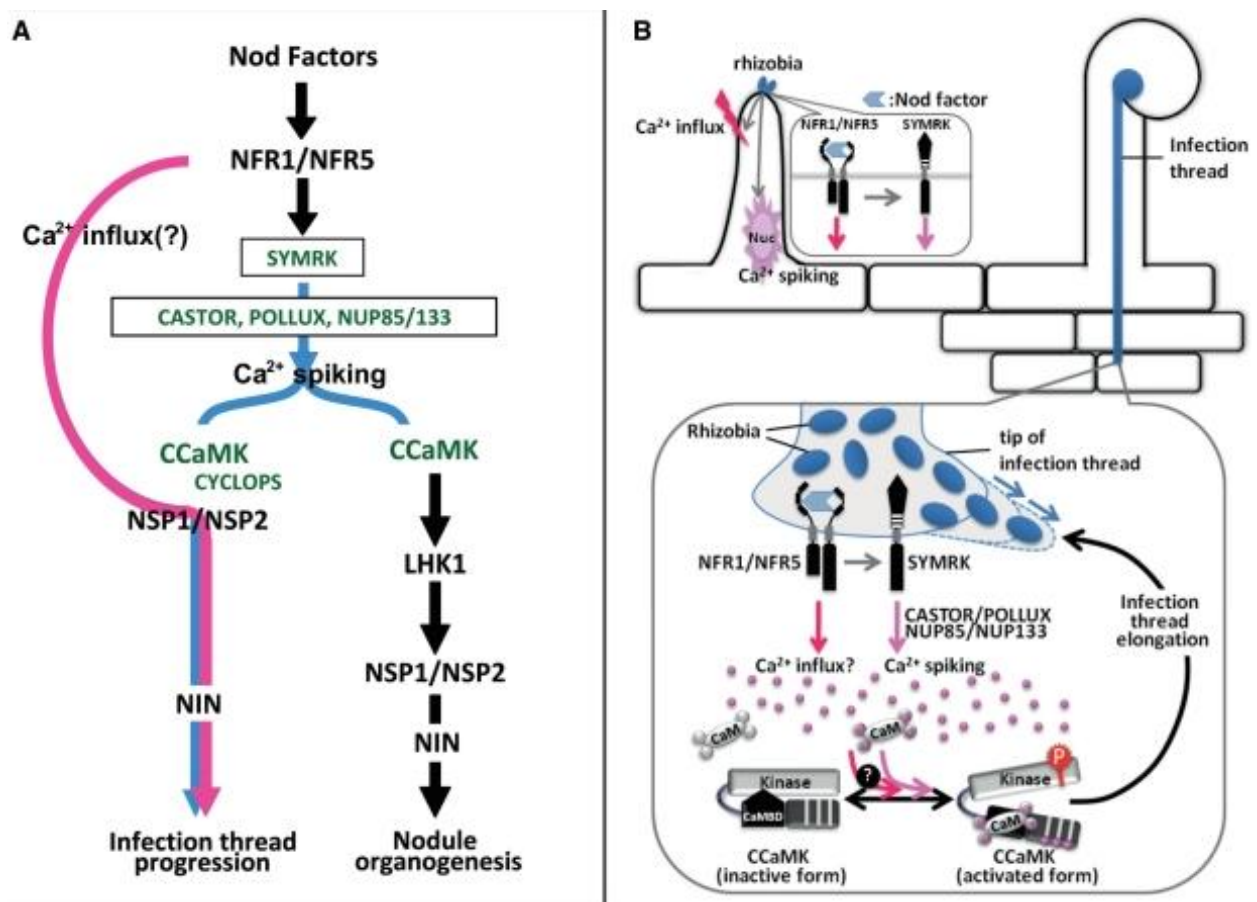


Figure: A,B. Illustration of the interaction of Nod factors to initiate nodule organogenesis in plant roots.

Source: http://openi.nlm.nih.gov/detailedresult.php?img=2938637_pcq107f2&req=4 (CC)

Nodulation (nod) genes:

These genes of *Rhizobium* perform the following functions:

1. *nodD* gene produces a NodD protein which activates other *nod* genes.

2. Lipochitin:

- This is an oligosaccharide composed of 3-6 units of monosaccharides.
- These are host specific.
- These bind to lectins present on the roots.

3. Heme group of leghemoglobin.

There are approximately 17 *nif* genes present only in prokaryotes.

Two processes i.e. root infection and nodule organogenesis involved in the formation of nodules are found to occur simultaneously. *Rhizobium* is a rod-shaped soil bacterium which lives on root exudates rich in vitamins, sugars and amino acids.

The first response resulting in the migration of rhizobia towards roots of plant symbiont is a chemotactic one. Rhizobia are attracted to the root hairs under the influence of chemical attractants (isoflavonoids and betaines) released by roots of legumes. This leads to an increased population of rhizobia in the rhizosphere of legumes.



These chemical attractants i.e. isoflavonoids and betaines turn on the NodD protein found in rhizobial strains. This protein then triggers the transcription of other *nod* genes.



Tryptophan secreted by the root hairs is converted to Indole acetic acid (IAA) by an enzyme of *Rhizobium*. IAA plays a role in the infection process.



IAA triggers curling and deformation of the root hairs. The rhizobia become enclosed in the small compartment formed by curling.



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Cellulases and pectinases released by the rhizobia results in the degradation of cell wall in these regions leading to softening of the cell walls. The plant plasma membrane thus comes in direct contact with the bacterial cells.

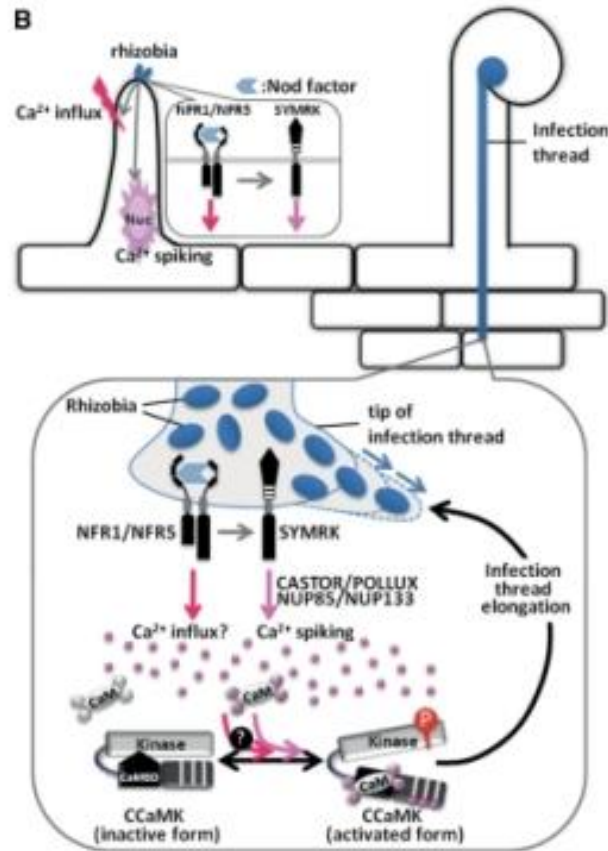


Figure: Infection process during nodule organogenesis.

Source: http://openi.nlm.nih.gov/detailedresult.php?img=2938637_pcq107f2&req=4 (CC)

Legume-*Rhizobium* recognition occurs with the help of lectins (proteins on the membranes of root hairs of legumes) and oligosaccharides (present in the cell wall of rhizobia) so that only a particular strain of rhizobia enters the root hair.



Next step is the formation of **infection thread** (an internal tubular extension of the plasma membrane that is produced by the fusion of golgi derived membrane vesicles at the site of infection that surrounds a stream of rhizobia). Fusion of secretory vesicles to the end of the tube results in the growth of infection thread at its tip.



