M.Sc. Botany
Semester V
Plant Physiology
Unit III
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Biological Nitrogen Fixation

Nitrogen is present in many forms in nature. The atmosphere contains 78% by volume molecular nitrogen (N_2). It is an inert gas and cannot be used directly by higher plants.
Acquisition of nitrogen from the atmosphere requires the breaking of an exceptionally stable triple covalent bond with high bond energy between two nitrogen atoms to produce ammonia (NH_3) or nitrate (NO_3^-).These reactions are known as Nitrogen fixation. Thus, Nitrogen Fixation is defined as the conversion of free nitrogen into nitrogenous salts to make it available for absorption by plants.

Depending upon the agency of fixation it may be -
1. Non - biological Nitrogen Fixation
2. Biological Nitrogen Fixation

Non Biological Nitrogen Fixation - It can be
1. Chemical Nitrogen Fixation
2. Physical Nitrogen Fixation

Chemical Nitrogen Fixation

Ammonia is produced industrially by the Haber process, which requires temperatures of 400 to 500^{o}C and nitrogen and hydrogen at pressure of tens of thousands of kilopascals to provide necessary activation energy.

Physical Nitrogen Fixation

It is usually found in rainy season during lightning and thunder storms. During lightening the free nitrogen of atmosphere combine with the oxygen to form nitric oxide. The nitric oxide is then oxidized with O_2 to form nitrogen peroxide. The nitrogen per oxide may combine with water during rains to form nitrous acid and nitric acid. The acids along with the rain water fall on the ground. These acids react with alkaline radicals of the soil to produce water soluble nitrate and nitrite.

Biological Nitrogen Fixation

Approximately, 90% of nitrogen fixation is biological nitrogen fixation, in which prokaryotic organisms are capable of fixing nitrogen. Biological nitrogen fixers are called **Diazotrophs** and nitrogen fixation phenomenon is also called **Diazotrophy.**

Depending on the nature of nitrogen fixers, nitrogen fixation can be categorized into two heads

1. Asymbiotic Biological Nitrogen Fixation
2. Symbiotic Biological Nitrogen Fixation

Asymbiotic Biological Nitrogen Fixation

It is the fixation of free nitrogen of the soil by micro-organisms, living freely outside the plant cell.

Asymbiotic free living N_2 fixers are

Bacteria - They convert free N_2 of the soil into soluble compounds which are absorbed from the soil by the plants. They may be

Aerobes eg. *Azotobacter, Beijerenckia, Derxia*

Anaerobes

Photosynthetic bacteria eg .*Rhodopsedomonas, Rhodospirillum*

Non Photosynthetic bacteria eg. *Clostridium*

Cyanobacteria eg. *Nostoc, Anabaena, Calothrix*

Aerobic nitrogen fixing bacteria such as *Azospirillum* and *Azotobacter*, *Beijerinckia*, *Derxia* are thought to maintain reduced oxygen conditions, through their high level of respiration.

Anaerobic nitrogen fixing bacteria do not have oxygen in their habitat such as *Rhodospirillum* (Photosynthetic) and *Clostridium* (non-Photosynthetic)

In cyanobacteria, such as *Anabaena*, *Calothrix, Nostoc*, anaerobic conditions are created in specialized cells called heterocyst. Heterocysts are thick walled cells which lack photosystem II , the oxygen producing photosystem of chloroplasts, so they do not generate oxygen.

Heterocysts appear to represent an adaptation for nitrogen fixation. Cyanobacteria that lack heterocysts can fix nitrogen only under anaerobic conditions.

Symbiotic Biological Nitrogen Fixation

The fixation of free nitrogen of the soil by micro-organisms living symbiotically inside the plants is called Symbiotic Biological Nitrogen fixation.

Symbiotic nitrogen fixing cyanobacteria which fix nitrogen

eg *Anabaena*, *Nostoc*

They are common symbionts in lichens.

Azolla pinnata, a water fern has *Anabaena azollae* in its fronds.

Cycas contains *Anabaena* or *Nostoc* in the algal zone of corolloid root.

Symbiotic nitrogen fixing bacteria eg *Rhizobium leguminosarum*, *Bradyrhizobium japonicum*, *Franckia sp*.

Associative Symbiotic Nitrogen Fixation

When the bacteria live in close association with the roots of cereals and grasses and fix nitrogen, the association is of loose mutualism type and is called associative symbiosis whereas this nitrogen fixation is called associative symbiotic nitrogen fixation. Here root nodules are not produced. In such association the bacteria live in rhizosphere, a transition zone between soil and root. The bacteria fix nitrogen and supply to the roots and in return the roots provide carbohydrates for the nourishment of bacteria. Eg. *Azotobacter paspali* , a nitrogen fixing bacteria living in the rhizosphere of a tropical grass *Paspalum notatum*

Azospirillum brasilense, a bacteria living in the rhizosphere of cereal roots

Beijerinckia , a bacteria living in the rhizosphere of sugarcane.

SYMBIOTIC NITROGEN FIXATION

Some bacteria can convert atmospheric nitrogen into ammonia. Most of these are free living in the soil. The most common type of symbiosis occurs between members of the plant family Leguminosae and soil bacteria of the genera *Azorhizobium, Bradyrhizobium, Photorhizobium, Rhizobium and Sinorhizobium* collectively known as Rhizobia. Such symbiosis occurs in nodules that are formed on the roots of the plant and contain the nitrogen fixing bacteria.

Nodule

Symbiotic nitrogen fixation occurs in specializes knob like structures known as nodules. Nodules are present in roots of host plants and aerial stems eg. *Sesbania.* Nodules are present on stem by *Azorhizobium*, only when plant faces water logged conditions. When nodules are found on leaves eg. *Psychotria*, the association is called **Phyllosphere**. Bacteria *Klebsiella sp*. is found in the leaf nodules of *Psychotria*. Nodules are formed on the roots of leguminous plants by *Rhizobium.sp*

Steps in Nodule formation (simple outline)

The rhizobia multiply and colonise the surrounding of the roots. The root hair curls up at the tip. Bacteria invade the root hair and enter it. Rhizobia degrade the part of the root hair via enzymes, which produce a thread like structure called Infection thread. Now, bacteria invade infection thread and reaches up to inner cortex of the root. After reaching near the cortex, it stimulates the initiation of formation of nodule.

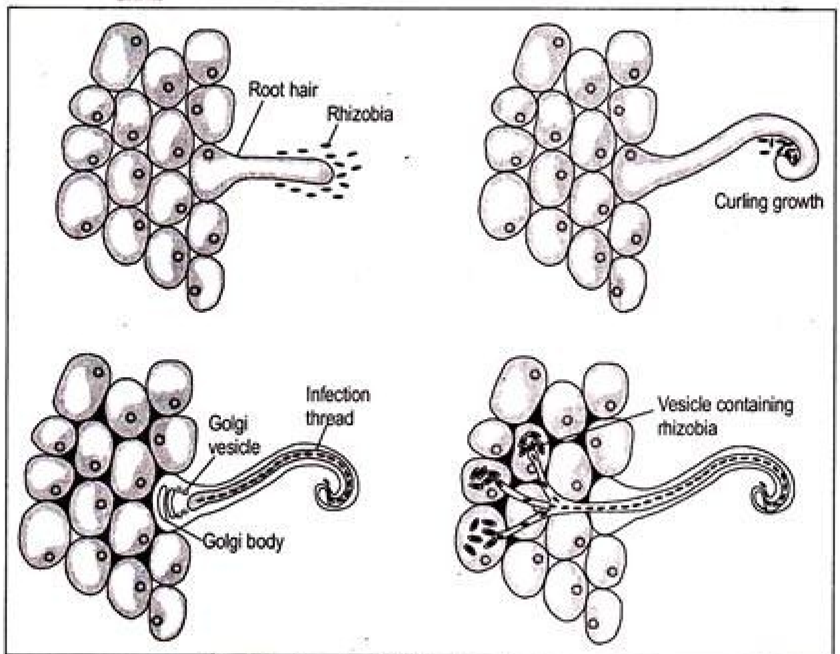


Fig. 10.3 : Formation of infection thread and root nodule

(Image taken from Internet source - Biology Discussion)

Biochemistry of Nodule formation

Establishing Symbiosis requires an Exchange of Signals. The symbiosis between legumes and rhizobia is not obligatory, though under nitrogen limited conditions, it occurs via exchange of signals. This signaling, the subsequent infection process, and the development of nitrogen fixing nodules involve specific genes in both the host and the symbionts.

Plant genes specific to nodules are called **nodulin (*Nod*) genes.**

Rhizobial genes that participate in nodule formation are called **nodulation (*nod*) gene**.

<u>Migration of Bacteria towards the roots of the host plant</u>

Chemotactic movements of free - living soil bacteria towards roots is mediated by chemicals such as **flavonoids** secreted by the roots of the host plant. Interaction with the flavonoids activates rhizobial NodD protein which, induces transcription of nod ABC gene as well as many other nod genes.The activated Nod D protein binds to highly conserved bacterial promoters (at so called nod boxes) and induce the expression of several genes.

Nodulin and nod genes

The nod genes are classified as

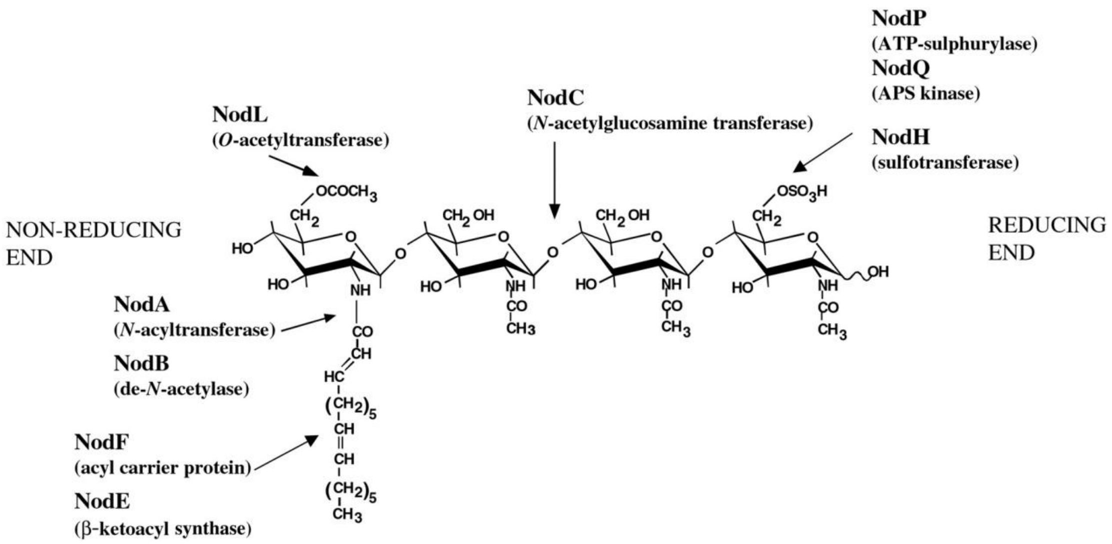
1. Common nod genes - *nod* A, *nod* B, and *nod* C are found in all rhizobial strains.
2. The host specific nod genes - such as *nod* P, *nod* Q, and *nod* H, or *nod* F, *nod* E, and *nod* L differ among rhizobial species and determine the host range.

Many of these genes are involved in the biosynthesis and secretion of signaling molecules called Nod factors. Three of nod genes (nod A, B, C) encode enzymes that are required for synthesis of nodulation factor of nod factor. Nod factor are lipochitn oligosaccharide, signal molecules, which have a β 1-4-, linked N-acetyl D-glucosamine backbone and a fatty acyl chain.

Three of the nod genes (nod A, nod B, and nod C), encode enzymes Nod A, Nod B, and Nod C, respectively

1. Nod A is a N-acyltransferase that catalyzes the addition of fatty acyl chain
2. Nod B is a chitin- oligosaccharide deacetylase that removes the acetyl group from the terminal nonreducing sugar.
3. Nod C is a chitin oligosaccharide synthase that links N-acetyl D glucosamine monomers

A particular legume host responds to a specific Nod factor .The legume receptors for Nod factors are special lectins, produced in root hair. Nod factors activate these lectins, which direct particular rhizobia to appropriate host. This facilitates the attachment of the rhizobia to the cell walls of the root hair.



(Image taken from Internet)

Infection

During the infection process, bacteria that are attached to the root hair release Nod factors that induce curling of the root hair cells.The rhizobia becomes enclosed in the small compartment formed by curling. The cell wall of the root hair degrades in these regions, also in response to Nod factors, allowing the bacterial cells direct access to the outer surface of the plant plasma membrane. After cell wall degradation, the next step is the formation of the **infection thread.** It is an infolded tubular extension of the plasma membrane. The infection thread elongates by fusion with vesicles derived from the golgi apparatus. The thread grows at its tip by the fusion of secretory vesicles to the end of the tube. Deeper into the root cortex, near the xylem , cortical cells dedifferentiate and start dividing , forming a distinct area within the cortex, called a **nodule primordium**, from which the nodule will develop.The nodule primordia form opposite the protoxylem poles of the root vascular bundle. The infection thread filled with proliferating bacteria elongates through the root hair and cortical cell layers, in the direction of the nodule primordium. When the infection thread reaches specialized cells within the nodule, its tip fuses with the plasma membrane of the host cell, releasing bacterial cells that are packaged in a membrane derived from the host cell plasma membrane. Branching of the infection thread inside the nodule enables the bacteria to infect many cells. At first, the bacteria continue to divide, and the surrounding membrane increases in surface area to accommodate this growth by fusing with smaller vesicles. Thereafter, upon an undetermined signal from the plant, the bacteria stop dividing and begin to enlarge and to differentiate into nitrogen fixing endosymbiotic organelles called

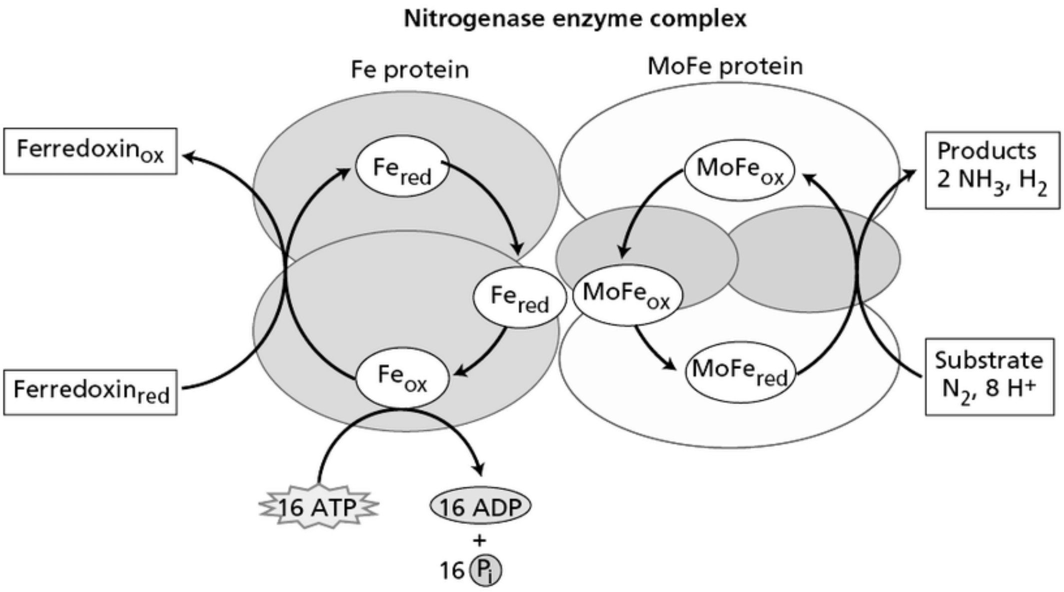
bacteroids. The membrane surrounding the bacteroids is called the **peribacteroid membrane.**

Reduction of nitrogen to ammonia occurs in bacteroids. Nitrogenase enzyme which catalyzes this reaction is very sensitive to oxygen. In root nodules, the oxygen level is regulated by leghemoglobin.

Leghemoglobin

Nodules contain an oxygen binding heme protein called Leghemoglobin. It is present in the cytoplasm of the infected nodule cells, and give the nodule a pink colour. The host plant produces the globin portion of leghemoglobin in response to infection by the bacteria. The bacterial symbiont produces the heme portion. The leghemoglobin has a high affinity for oxygen. Leghemoglobin binds all available oxygen so that it cannot interfere with nitrogen fixation, and efficiently delivers the oxygen to the bacterial electron transfer system.

Nitrogenase Enzyme Complex



(Image taken from Internet)

Biological nitrogen fixation, produces ammonia from molecular nitrogen. The biological process of nitrogen fixation is catalysed by an enzyme complex called Nitrogenase Enzyme Complex.

The reaction for nitrogen fixation

$N_2 + 8 e^- + 8 H^+ + 16 ATP \rightarrow 2NH_3 + 16 ADP + 16 Pi + H_2$

The Nitrogenase Enzyme complex consist of two components

1. The Fe protein
2. The MoFe protein

1. The Fe protein also known as **Dinitrogenase reductase**

It is the smaller of the two components. It is a dimer of two identical 30KDa subunits. It contains a single 4Fe-4S redox centre, bound between the subunits, and can be oxidised and reduced by one electron. It also has two binding sites for ATP/ADP (one on each subunit).

2. The MoFe protein also known as **Dinitrogenase**

It is a tetramer with two copies of two different subunits contains both iron and molybdenum.It's redox centres have a total of 2Mo, 32Fe and 30 S per tetramer.

The genes involved collectively in the synthesis of nitrogenase and catalytic process of nitrogen fixation are called nif genes.

Nitrogen fixation is carried out by a highly reduced form of dinitrogenase and require eight electrons. Six for the reduction of N_2 and two to produce one molecule of H_2 .

Since Nitrogen fixation is a reductive process, it requires electron donor. Reduced ferredoxin acts as a donor for electrons to the Fe protein. Ferredoxin is a small protein containing an Fe - S group. The ultimate source of electrons to reduce ferredoxin is pyruvate.

Atleast 16 molecules of ATP are required for reduction of one molecule of N_2. Dinitrogenase is reduced by the transfer of electrons from Dinitrogenase reductase. The Dinitrogenase tetramer has two binding sites for the reductase. The eight electrons are transferred from reductase to dinitrogenase one at a time. A reduced reductase molecule binds to the dinitrogenase and transfers a single electron, then the oxidized reductase dissociates from dinitrogenase, in a repeating cycle. Each turn of the cycle requires the hydrolysis of ATP molecules by the dimeric reductase.

The production of NH_3 from N_2 and H_2 is an exergonic reaction, as large investment of energy is required to break the triple bond in N_2.

The nitrogenase complex is very sensitive to oxygen. It is irreversibly inactivated by oxygen. Hence the fixation of N_2 must occur under anaerobic conditions. For anaerobic prokaryotic organism , there is no problem.

Facultative prokaryotic organisms such as purple photosynthetic bacteria fix N_2 only in anaerobic conditions.

In aerobic organism such as cyanobacteria, anaerobic conditions are created in specialized cells called Heterocysts.

Symbiotic nitrogen fixing prokaryotes such as *Rhizobium*, maintain a very low concentration of free oxygen in root nodules of leguminous plants by producing leghemoglobin, a homolog of hemoglobin. Leghemoglobin is present in the cytoplasm of infected nodule cells at high concentrations.

NH_3, the primary product of biological nitrogen fixation, is toxic to cells in high concentrations. Thus, it is converted into amides (such as asparagine or glutamine) or ureides (such as allantoin, allantoic acid and citrulline). These organic forms are finally transported to the shoot via the xylem.