

Discipline: Applied Botany

Paper: Plant Metabolism

Lesson: Nitrogen metabolism

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Nitrogen Metabolism

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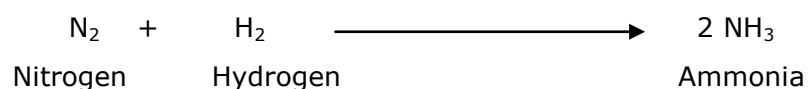
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Introduction

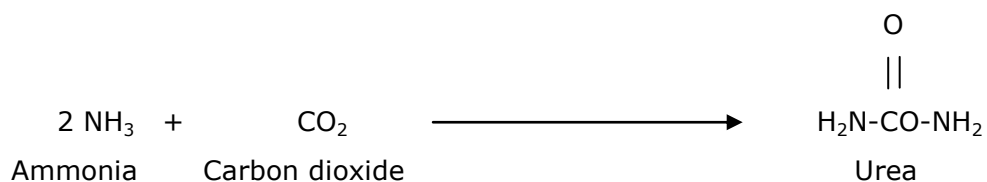
Nitrogen is present in many forms in the biosphere. Although vast quantities of molecular nitrogen is present in the atmosphere (77% by volume), it is not available for direct use by living organisms. Acquisition of this molecular nitrogen and its conversion to ammonium (NH_3) and nitrate (NO_3^-) accounts 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:

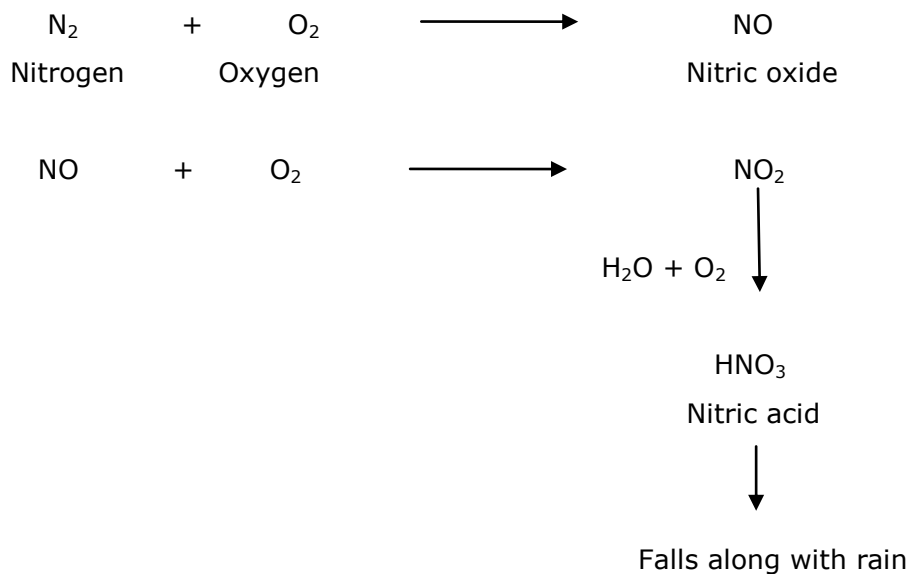


- 2. Atmospheric nitrogen fixation:** This can occur by any of the following two methods:

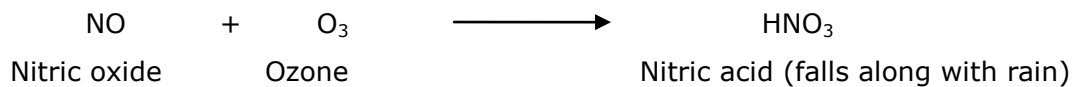
- i) Lightning:** This form of nitrogen fixation amounts to about 8% of the total nitrogen fixed. In the presence of lightning, highly reactive OH^\cdot , H and O are formed from water vapour

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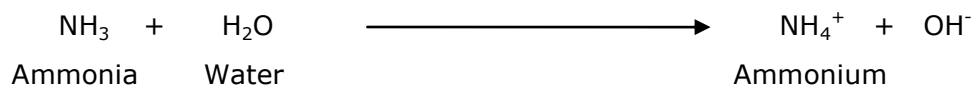
and oxygen. These free H and O atoms subsequently form nitric acid (HNO_3) when they come in contact with N_2 . HNO_3 thus formed, falls along with rain on earth surface.



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.

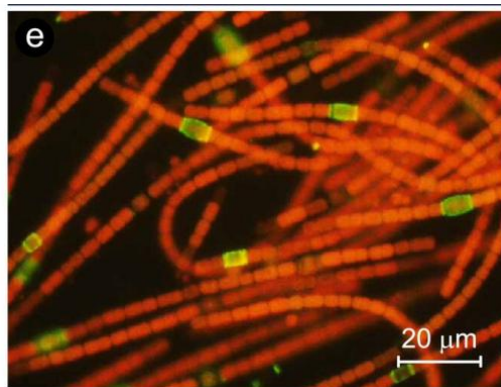


Figure: Filament of *Anabaena* showing heterocysts.

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).

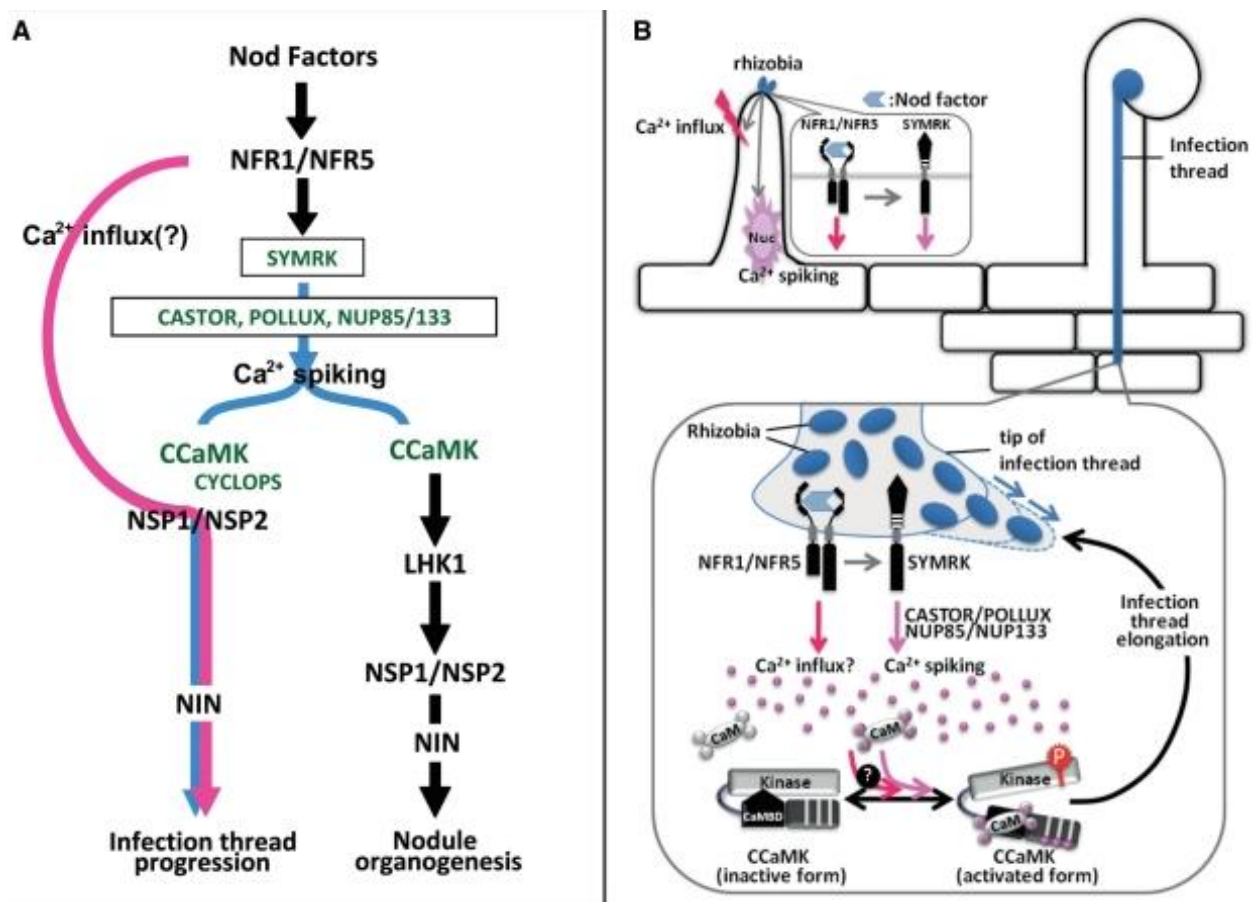


Figure: A,B. Illustration of the interaction of Nod factors to initiate nodule organogenesis in plant roots.

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.

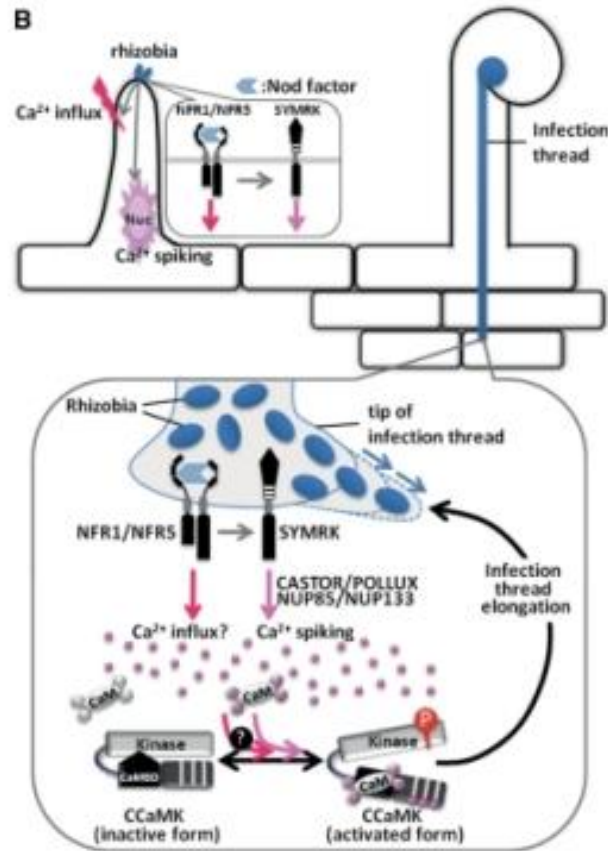


Figure: Infection process during nodule organogenesis.

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.



A distinct area called a **nodule primordium** is formed within the deep cortex, near the xylem (which serves as a trigger for the development of nodule) through the division of cortical cells. Stimulation for cell division is provided by a cytokinin (isopentenyl adenine) produced by rhizobia. This cell division occurs even before the infection thread reaches there.



The infection thread filled with proliferating rhizobia elongates through the root hair and cortical cell layers in the direction of the nodule primordium.



The tip of the infection thread fuses with the plasma membrane of the plant cell once the thread reaches specialized cells within the nodule. The bacterial cells are thus released and packaged in the membrane originated from plant cell plasma membrane. These bacteria have ability to infect many cells inside the nodule through branching of the infection thread.



At first the bacteria continue to divide, and the surrounding membrane increases in surface area to accommodate this growth by fusing with smaller vesicles. Soon after this division, upon an undetermined signal from the host plant, the bacteria stop dividing and begin to enlarge and differentiate into **bacteroids** (nitrogen-fixing endosymbiotic organelles). A bacteroid is larger, rounded and is able to synthesize **nitrogenase** enzyme. A group of bacteroids is surrounded by a membrane called **peribacteroidal membrane** (contains leghaemoglobin).



The nodule as a whole develops vascular system (which facilitates the exchange of fixed nitrogen produced by bacteroids for the nutrients contributed by the plant) and a layer of cells (to exclude oxygen from the root nodule interior).

Temperate legumes like peas have a nodule meristem and thus their nodules are extended and cylindrical unlike the spherical nodules in tropical legumes like soybean and peanuts (because they lack a persistent meristem).

Nitrogen Metabolism

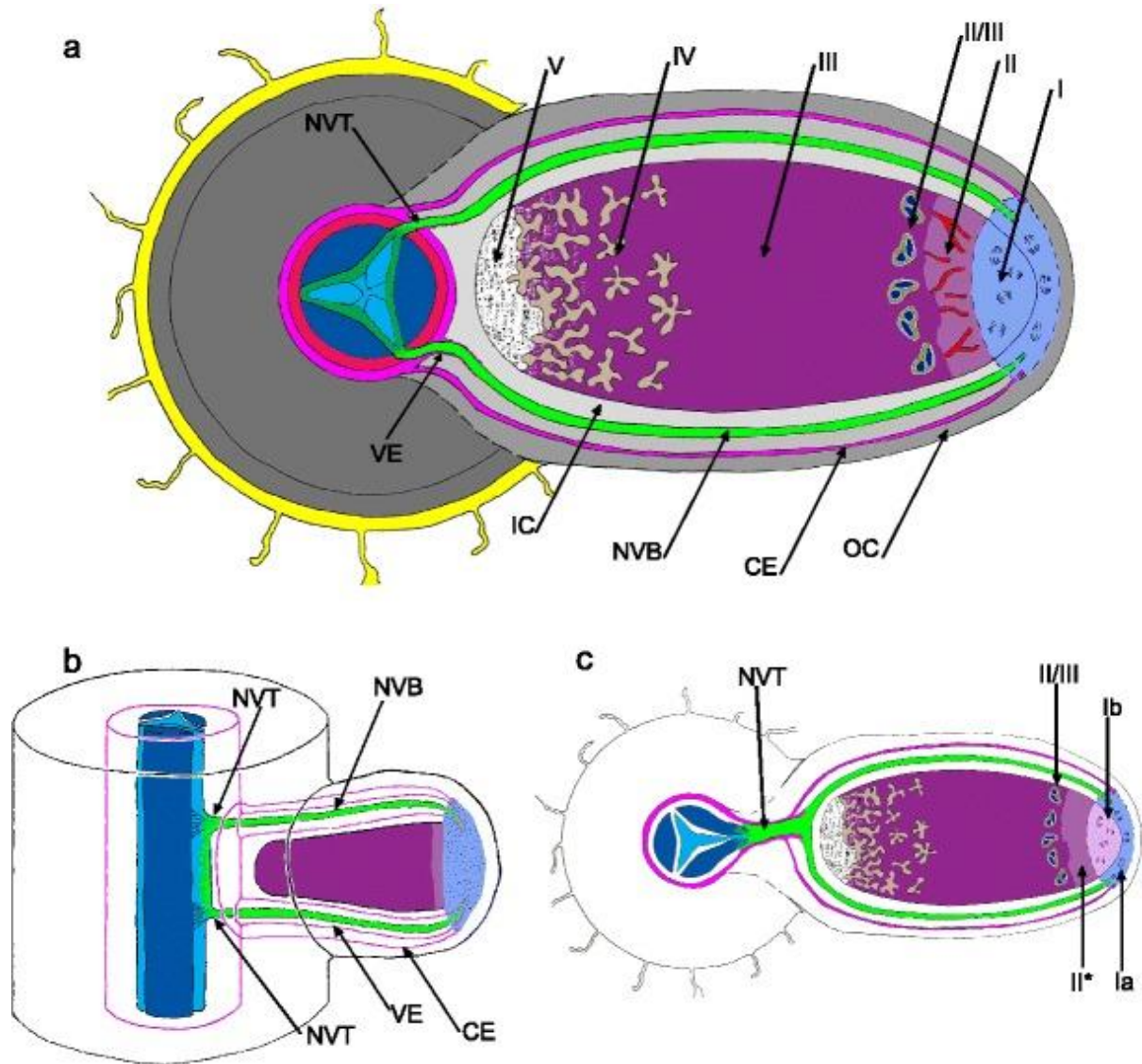


Figure: Depiction of a complete root nodule showing the development of vascular system in the nodule.

I uninfected nodular meristem; II penetration zone; III Nitrogen fixation; IV senescence; V saprophytic. OC outer cortex; CE cortical epidermis; IC inner cortex; NVT nodular vascular trace; NVB nodular vascular bundle; VE vascular endodermis;

Source: <https://www.scienceopen.com/document/vid/80f5734e-f922-440b-857e-80b2de2542ef;jsessionid=eKyBQJf+9hPdKWv7rcvBqYww.master:so-app1-prd?0> (CC)

Leghemoglobin

- It is a heme protein capable of binding to oxygen in nodules.
- It occurs at a high concentration in the infected cells.
- It is present in the cytoplasm of infected cells and gives the nodules a pink color.

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- This heme protein has a very high affinity for oxygen since the K_m value is approximately $0.01 \mu\text{M}$. This provides about 10 times higher affinity than even β chain of human haemoglobin.
- The host plant produces the globin portion of leghemoglobin in response to infection by bacteria and the bacterial symbiont produces the heme portion.
- Gas permeability is regulated in the nodules of legumes and actinorhizal plants. Although this mechanism helps in attaining a level of oxygen that is sufficiently low (to avoid inactivation of nitrogenase but supporting respiration as well), but it is not fully deciphered. It might involve potassium fluxes into and out of the infected cells.
- Earlier this protein was considered to provide a buffer for nodule oxygen, but recently it has been proved to store oxygen enough to maintain respiration in nodules for a very short span of time. It functions mainly to transport oxygen to the respiring bacterial cells, analogous to the way Hb transports oxygen to respiring tissues in animals. To continue aerobic respiration under such conditions, the bacteroid uses a specialised electron transport chain in which the terminal oxidase has an affinity for oxygen even higher than that of leghemoglobin.

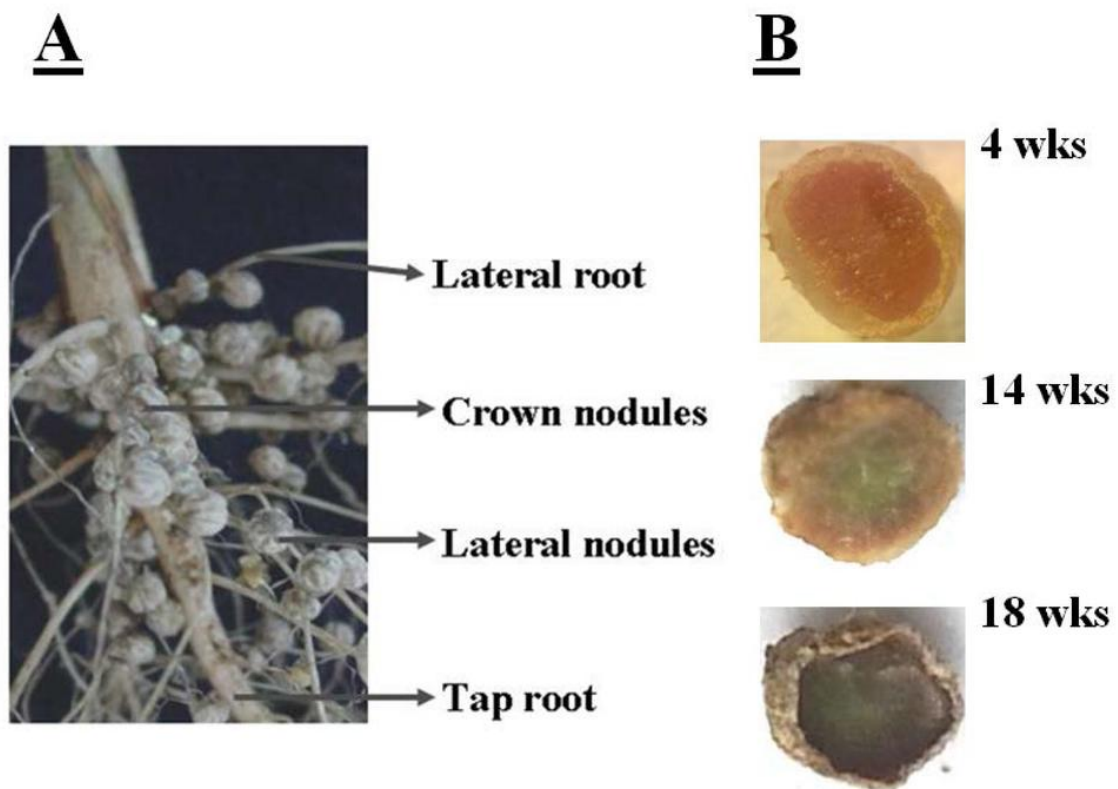


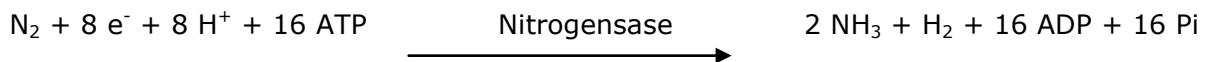
Figure: Soyabean root nodule cut open to show the pinkish interiors containing leghemoglobin.

Source: <http://www.mdpi.com/2073-4395/3/3/550/htm> (CC)

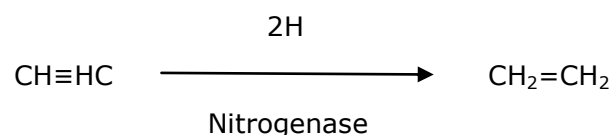
Nitrogenase enzyme complex

General characteristics

Like industrial nitrogen fixation, biological nitrogen fixation also produces ammonia from molecular nitrogen. What we have learnt till now is how the legume-rhizobia interaction occurs, but still we need to learn the mechanism of nitrogen fixation through enzyme nitrogenase.



- The enzyme nitrogenase is an oxidoreductase (reduces dinitrogen to ammonia). In this reaction, hydrogen is evolved by coupling the reduction of nitrogen to ammonia (a 6 electron transfer) to the reduction of two protons.
- It is produced by *nif* genes.
- It is produced only by prokaryotes.
- It is highly sensitive to oxygen as both the components of nitrogenase are inactivated by oxygen.
- It is a very expensive enzyme as 16 ATP and 4 NADPH₂ are needed to convert one mole of nitrogen to 2 moles of ammonia. ΔG° for the overall reaction (stated above) of biological nitrogen fixation is about -200 KJ/mol. Since the overall reaction is highly exergonic, ammonium production is limited by the slow operation of the nitrogenase complex (about 5 nitrogen molecules are reduced per second). To compensate for this slow turnover time, bacteroid synthesizes large amounts of nitrogenase (upto 20% of the total protein in the cell).
- This enzyme is not exactly substrate specific. It can also add H to triple bond across several compounds. One of the reactions catalyzed by nitrogenase is used in estimating nitrogenase activity i.e. the reduction of acetylene to ethylene.



Structure

Nitrogenase complex is a multiprotein complex which has two separate components:

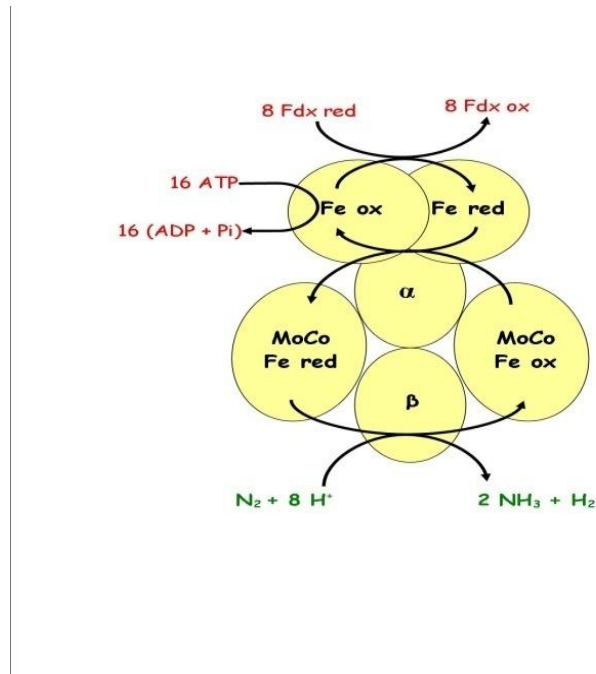


Figure: Nitrogenase enzyme structure showing the two components.

Source: <http://upload.wikimedia.org/wikipedia/commons/2/2a/Nitrogenase.jpg> (CC)

1. Azoferreredoxin or Fe protein

- Smaller of the two units.
- It is present as a dimer.
- It has two indistinguishable subunits of 30-72 kDa each (depending on the life form).
- There is a presence of a Fe- S²⁻ cluster in each of the two subunits (4 iron and 4 sulfur). These clusters take a part in oxidation-reduction reactions that are needed for the conversion of N₂ to NH₃.
- It is very sensitive to oxygen and is therefore, irreversibly inactivated by oxygen.
- Receives electrons from ferredoxin and transfers them to the other component.
- Also binds to ATP and Mg²⁺. Binding and subsequent hydrolysis of ATP molecule brings about a conformational change in Fe protein that facilitates the redox reactions.

2. Azomolybdoferredoxin or Mo-Fe protein

- Larger of the two units.
- There are 4 subunits in this protein amounting to a molecular mass of 180-235 kDa depending on the species involved.
- There are two molybdenum-iron-sulfur clusters present in each of the four subunits.
- Oxygen can also inactivate MoFe protein just like Fe protein.
- This component receives electrons from azoferreredoxin and transfers them to N_2 which gets reduced to NH_3 .

Considering the overall nitrogen reduction reaction, Ferredoxin provides electron to Fe protein, which in turn hydrolyzes ATP and causes the reduction of MoFe protein. MoFe protein can then reduce numerous substrates, although under natural conditions, it reacts only with N_2 and H^+ .

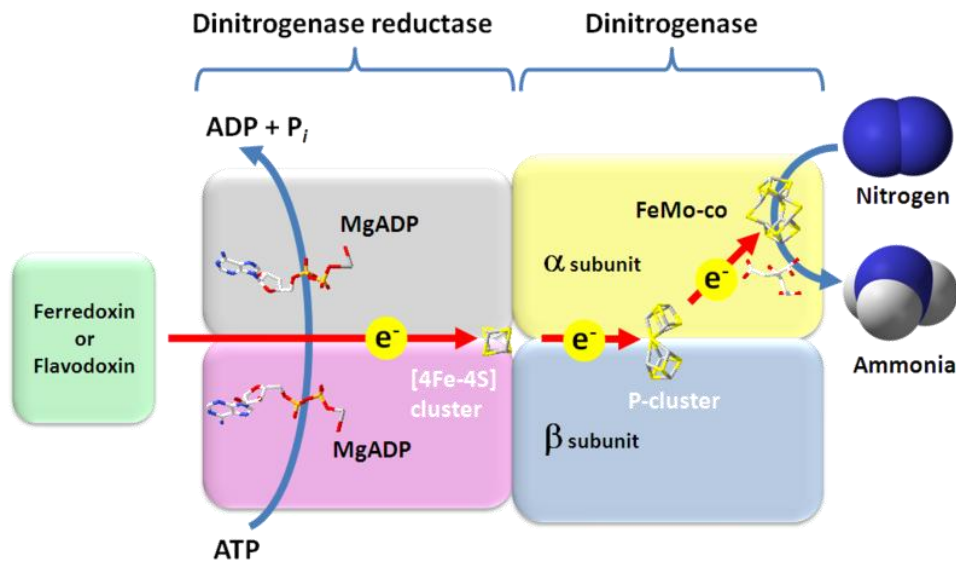
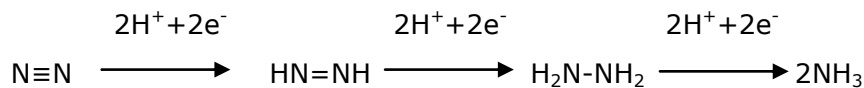


Figure: Reaction mechanism illustrating the action of enzyme nitrogenase.

Source: http://commons.wikimedia.org/wiki/File:Nitrogenase_reaction.PNG (CC)

Regulation of Nitrogenase

The enzyme nitrogenase is regulated by several factors:

1. Ammonia: Exogenous ammonia switches off more than 90% of nitrogenase activity.
2. Nitrate: High levels of nitrate inhibit nitrogenase activity. It does not inhibit this enzyme directly instead, nitrite produced from nitrate damages nitrogenase.
3. Oxygen: Since both the units of nitrogenase are sensitive to oxygen, oxygen also regulates its activity.

4. Molybdenum

5. ATP

6. Hydrogenase: This promotes the activity of nitrogenase because it removes hydrogen from the site of nitrogen fixation. Reduction of H^+ to hydrogen gas under natural conditions can compete with N_2 reduction for electrons from nitrogenase. In rhizobia, 30 to 60% of the energy supplied to nitrogenase may be lost as H_2 . This diminishes the efficiency of nitrogen fixation. Some rhizobia however, contain enzyme hydrogenase that can split H_2 formed and generate electrons for N_2 reduction, thus improving efficiency of nitrogen fixation.

Cell free nitrogen fixation can also be seen when cell free extracts of *Clostridium* (containing nitrogenase), ATP, Mg^{2+} , a strong reducing agent (like sodium dithioate) and oxygen deficiency are provided.

Unassimilated ammonium or nitrate may be dangerous

Once fixed into ammonia or nitrate, nitrogen enters a biogeochemical cycle and passes through several organic or inorganic forms before it eventually returns to molecular oxygen. Ammonium and nitrate generated through fixation or released through decomposition of organic matter become the object of intense competition among plants and microorganisms. Plants have thus, developed mechanisms for scavenging these ions rapidly from the soil solution. Under elevated soil concentration, ammonium or nitrate may accumulate within plant tissues since the capacity of a plant to assimilate these NH_4^+ or NO_3^- ions may be quite less as compared to the absorption of these ions by roots. This unassimilated ammonium or nitrate may be dangerous.

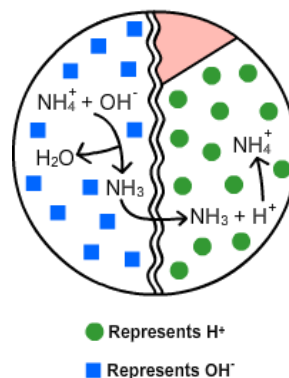


Figure: Illustration of the phenomenon of ammonium toxicity dissipating ammonium gradients.

Source: ILLL in-house

Ammonium, if it accumulates to high levels in living tissues, can prove lethal to plants and animals. Ammonium disturbs transmembrane H^+ gradients (as shown in the above figure) that are required for electron transport (in both photosynthesis and respiration) and also in the vacuole for metabolite sequestration. In contrast to ammonium, increased amounts of nitrate can be stored by plants or translocated from tissue to tissue without detrimental effects. However, humans or livestock may suffer from methemoglobinemia if they consume plant material rich in nitrate wherein haemoglobin when combined with nitrite (formed from nitrate in liver) is no longer able to bind to oxygen.

Plants can store high levels of nitrate, in contrast to ammonium or they can even translocate it from tissue to tissue without deleterious effects. However, if humans or livestock consume plant material rich in nitrate, they may suffer from methemoglobinemia (liver reduces nitrate to nitrite which combines with haemoglobin and it is no longer able to bind to oxygen). Nitrate may also be converted to nitrosamines by humans and other animals, which act as potent carcinogens.

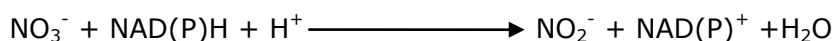
In the coming sections of chapter, we shall discuss in detail the process by which plants assimilate nitrate into organic compounds via a two step process: reduction of NO_3^- to NO_2^- and then to NH_4^+ and finally to amino acids through the action of different enzymes.

Nitrate assimilation

Through several high and low-affinity nitrate-proton cotransporters, plant roots assimilate most of the nitrate into organic compounds after effectively absorbing nitrate from the soil solution.

Nitrate reductase

First step in the process of nitrate assimilation is carried out in the cytosol with the help of enzyme nitrate reductase by catalyzing the reduction of nitrate to nitrite.



where $NAD(P)H$ indicates $NADH$ or $NADPH$. $NADH$ is commonly used as an electron donor by almost all the nitrate reductases except some found in roots that have the potential to use $NADH$ or $NADPH$.

Structure

Nitrogen Metabolism

Nitrate reductase of higher plants is known to be formed from two identical subunits and each subunit is composed of 3 prosthetic groups:

1. Flavin adenine dinucleotide (FAD)
2. Heme
3. Pterin (complex of molybdenum atom and an organic molecule)

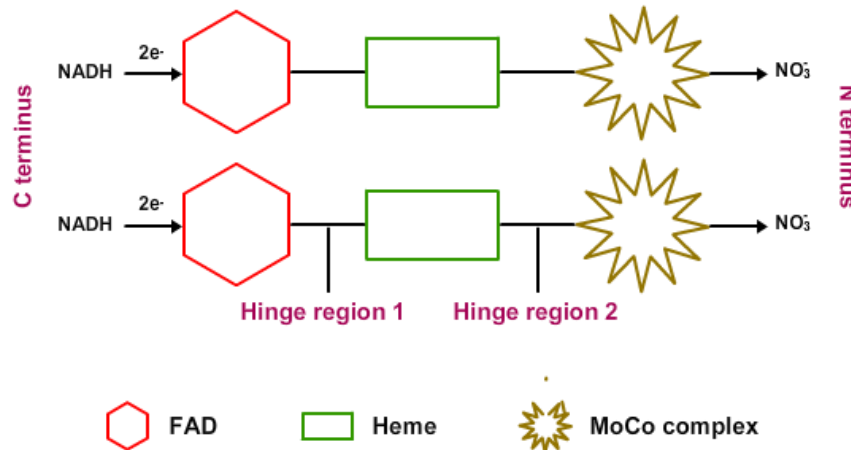


Figure: Model of Nitrate reductase dimer.

Source: ILLI in-house

The nitrate reductase dimer model illustrates three binding domains whose polypeptide sequences are similar in eukaryotes. As illustrated above in figure 11, binding of NADH at the C-terminus triggers a 2 e⁻ transfer from the FAD-binding region of the two subunits to the amino terminus of the nitrate reductase dimer with the intermediary e⁻ transfer components. Reduction of NO₃⁻ occurs at the N terminus through the last component of the dimer i.e. MoCo complex. Hinge regions formed by the polypeptide sequences account for the variability among species.

Regulation

Light, carbohydrates and nitrate manipulate the synthesis of nitrate reductase at the levels of RNA and protein synthesis. Additionally, the enzyme is also regulated at post-translational level (involving a reversible phosphorylation).

Light, carbohydrate and other environmental factors/darkness and Mg²⁺



Stimulate a protein phosphatase/protein kinase



Dephosphorylates/phosphorylates a key serine residue in hinge 1 region (between Mo complex and heme-binding domains) of nitrite reductase

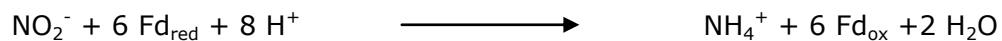


This thus, activates/deactivates (after the interaction of serine residue with a 14-3-3 inhibitor protein) the enzyme nitrate reductase.

Nitrite reductase

Nitrite, being a highly reactive and potentially toxic ion, is immediately transported by plant cells from cytosol (after nitrate reduction) to chloroplasts and plastids in leaves and roots respectively.

- Here, the enzyme **nitrite reductase** catalyzes the reduction of nitrite to ammonium in accordance with the following reaction (a transfer of six electrons):



where Fd_{red} and Fd_{ox} stands for the reduced and oxidized forms of ferredoxin respectively. In chloroplasts and nongreen tissues, ferredoxin is derived from photosynthetic electron transport and from NADPH generated by oxidative pentose phosphate pathway respectively.

- Nitrite reductase in both chloroplasts and root plastids consist of only one polypeptide and it contains the following 3 components:

1. 2 prosthetic groups
2. One iron-sulfur cluster (Fe_4S_4)
3. A specialized heme

Action of these three groups together results in reduction of nitrite to ammonium. The electron flow through these three groups can be represented as follows:

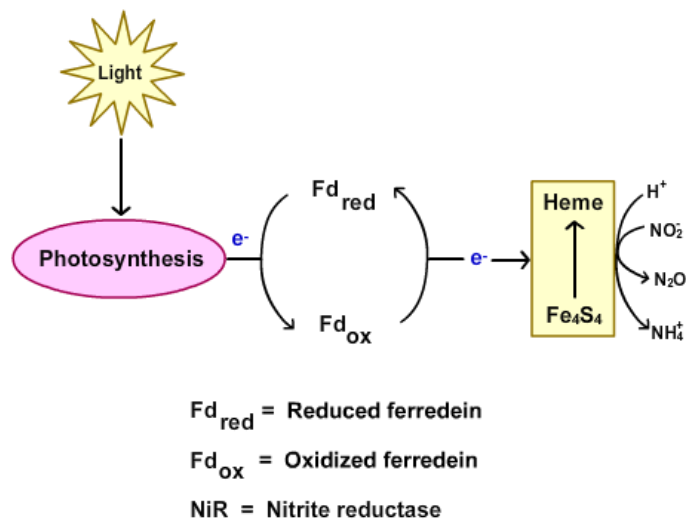


Figure: Model illustrating the reduction of nitrite by nitrite reductase.

Source: ILLI in-house

- Nitrite reductase is targeted to the plastids since it has an N-terminal transit peptide although the site of its coding (i.e. nucleus) and synthesis (i.e. cytoplasm) are different.
- The transcription of nitrite reductase mRNA is induced upon elevated concentrations of nitrate or exposure to light.
- Both roots and shoots are involved in nitrate assimilation depending on the ratio of NR activity in both roots and shoots or upon comparison of nitrate and reduced nitrogen concentrations. In many plants, when roots receive small amounts of nitrate, reduction occurs primarily in the roots. But, as the supply of nitrate increases, a greater proportion of absorbed nitrate is translocated to shoot and assimilated there. Even under conditions of similar nitrate supply, variation among species is noticed when considering the balance between root and shoot nitrate metabolism. Example, nitrate metabolism can occur either in roots as in white lupine (*Lupinus albus*) or in shoots in some other plants like in cocklebur (*Xanthium strumarium*).

Ammonium assimilation

Introduction

Ammonium assimilation is a very rapid process because

1. Ammonium inhibits nitrogen fixation.
2. Ammonium inhibits ATP synthesis in both chloroplasts and mitochondria by acting as an uncoupling agent.
3. Ammonium is a very toxic compound.

Therefore, its necessary for the plant cells to rapidly convert the ammonium (generated from nitrate assimilation or photorespiration or through direct absorption from soil or produced through energy-dependent nitrogen fixation) into amino acids so that it does not accumulate anywhere in the plant. Perhaps, all the ammonium seems to be converted first into amide group of glutamine except for traces of ammonium lost to the atmosphere as ammonia. The sequential action of the two enzymes i.e. glutamine synthetase and glutamate synthase is primarily responsible for this conversion.

Two alternative pathways

1. Reductive amination by GDH

It involves incorporation of ammonia by **glutamate dehydrogenase (GDH)** in the following manner:

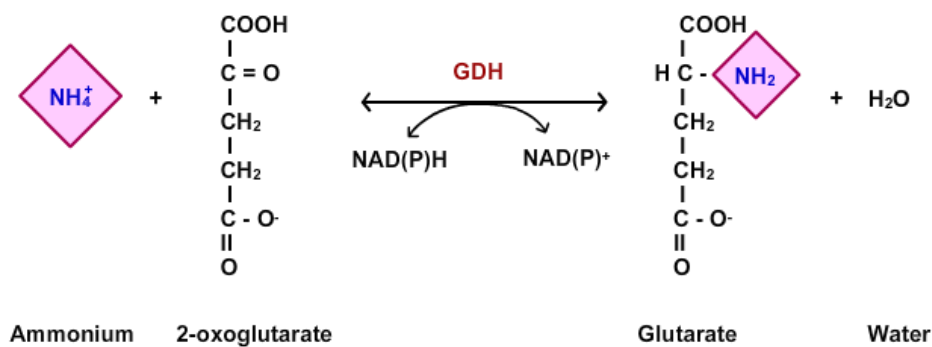


Figure: Action of GDH showing conversion of 2-oxoglutarate to glutamate.

Source: ILLL in-house

This process is considered to be of little importance as GDH has lower affinity to ammonium (has higher K_m for ammonium).

2. Ammonia incorporation by glutamine synthetase (GS)

- Glutamate when combined with ammonium forms glutamine in the presence of GS.

- GS has higher affinity for ammonium (lower K_m for ammonium) than GDH.
- GS catalyzed reaction requires 1. An ATP hydrolysis
 2. A Cofactor (a divalent cation like Mg^{2+} , Mn^{2+} or Co^{2+})
- GS is inhibited by methionine sulfoximine.
- There are two classes of GS in plants:
 1. In cytosol: These forms of GS are found to express in seeds undergoing germination or in root and shoot vasculature (produce glutamine for intracellular nitrogen transport).
 2. In root plastids or shoot chloroplasts: Amide nitrogen is generated for local consumption in root plastids through GS while photorespiratory ammonium is reassimilated in shoot chloroplasts.
- There is a little effect on the cytosolic forms with the change in light and carbohydrate levels although they can have a pronounced effect on the expression of plastid forms of GS.

GOGAT or glutamate synthase

The activity of this enzyme **glutamine 2-oxoglutarate aminotransferase** is stimulated upon an increase in the levels of glutamine present in plastids. Two molecules of glutamate are produced upon the transfer of amide group from glutamine to 2-oxoglutarate in the presence of GOGAT.

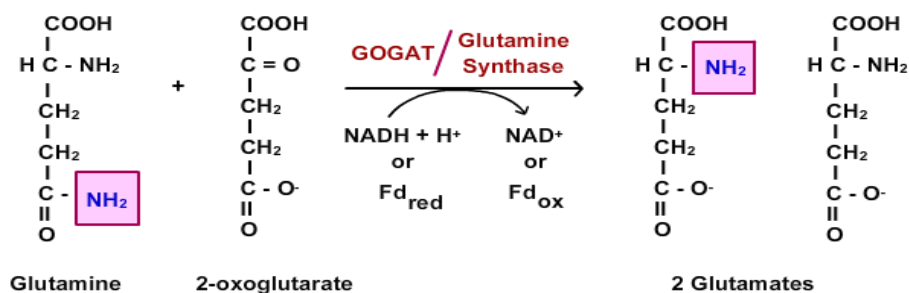
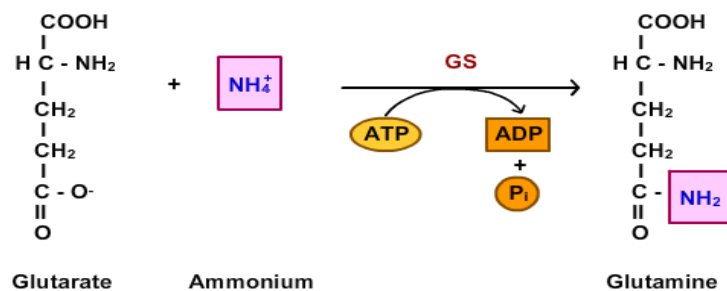


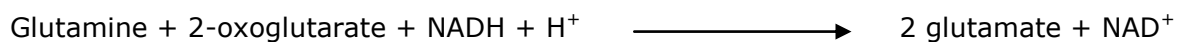
Figure: Action of the enzymes glutamine synthetase and glutamate synthase.

Source: ILLI in-house

The following two types of GOGAT are found in plants:

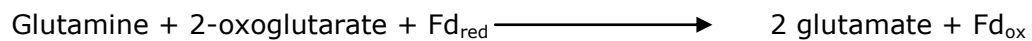
1. NADH-GOGAT

- NADH type of the enzyme (NADH-GOGAT) accepts electrons from NADH.
- It is located in tissues which do not undergo photosynthesis like roots or vasculature of developing leaves.
- NADH-GOGAT present in roots is primarily involved in the assimilation of NH_4^+ absorbed from the rhizosphere.
- In vascular bundles of developing leaves, it assimilates glutamine translocated from roots or senescing leaves.



2. Fd-GOGAT

- Ferredoxin type of enzyme (Fd-GOGAT) accepts electrons from ferredoxin:
- It is present in chloroplasts and plays a role in photorespiratory N_2 metabolism.
- An increase in the quantity and activity of this enzyme has been found with increase in the levels of light.
- Glutamine generated during assimilation of nitrate is incorporated primarily through this class of enzyme present in root plastids.



Transamination Reactions

Once assimilated into glutamine and glutamate, transamination reactions operate to incorporate nitrogen into other amino acids.

Aminotransferases

- Transamination reactions are catalyzed by a class of enzymes categorised as aminotransferases.

Nitrogen Metabolism

- Example: Through **aspartate aminotransferase**, amino group of glutamate is transferred to carboxyl group of aspartate to form another amino acid and α -ketoglutarate is set free:

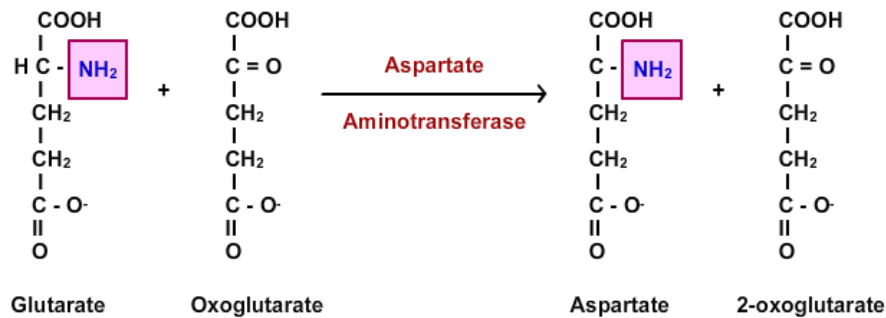


Figure: Action of aspartate aminotransferase illustrating transamination reaction.

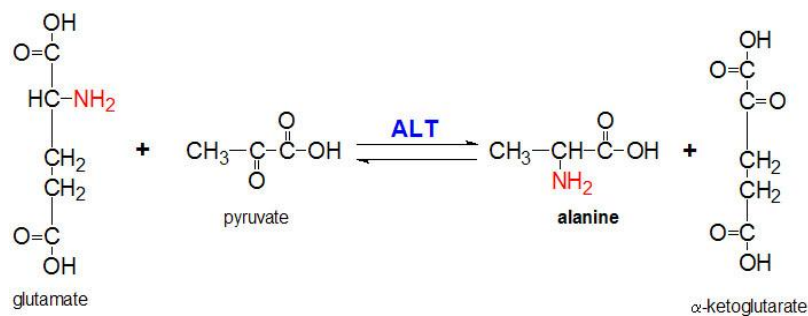
Source: ILLL in-house

- A cofactor i.e. pyridoxal phosphate (vitamin B₆) is required in all reactions catalyzed by aminotransferases. It helps to transfer amino group from amino acid to ketoacid and forms a complex with the enzyme.
- Aminotransferases are found in the:
 - Chloroplasts
 - Mitochondria
 - Glyoxysomes
 - Peroxisomes

Aminotransferases found in chloroplasts have a significant role in amino acid biosynthesis.

Similar other transamination reactions are:

- Glutamate + pyruvate \longrightarrow alanine + 2-oxoglutarate



2. Glutamate + hydroxypyruvate \longrightarrow serine + 2-oxoglutarate
3. Glutamate + phenylpyruvate \longrightarrow phenylalanine + 2-oxoglutarate

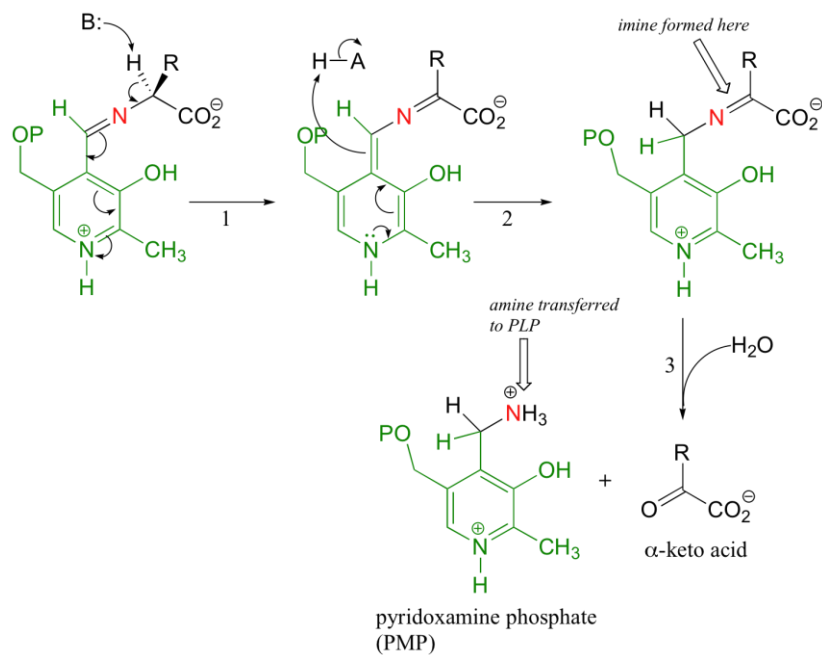


Figure: Depiction of the role of pyridoxal phosphate as a cofactor during transamination reaction.

Source:

http://chemwiki.ucdavis.edu/Organic_Chemistry/Organic_Chemistry_With_a_Biological_Emphasis/Chapter_14%3A_Reactions_with_stabilized_carbanion_intermediates%2C_part_II/Section_14.4%3A_Pyridoxal_phosphate_-_an_electron_sink_cofactor (CC)

Amino acid biosynthesis

Certain amino acids like histidine, isoleucine, leucine, lysine, methionine, phenylalanine, tryptophan, threonine and valine are not synthesized by humans and most animals and thus young humans obtain most of these so called essential amino acids from their diet. However, plants synthesize all amino acids that are common in proteins. As discussed in the previous section, nitrogen-containing amino group derives from transamination reactions with glutamine or glutamate. Amino acids derive its carbon skeleton from the products of glycolysis like 3-phosphoglycerate, phosphoenolpyruvate or pyruvate, or from products of citric acid cycle like 2-oxoglutarate or oxaloacetate.

Summary

- Acquisition of nitrogen from the atmosphere is a crucial part of nitrogen fixation and plants form a crucial part in this exchange of inorganic to organic nitrogen.
- The process of nitrogen fixation can be accomplished by three processes i.e. industrial, atmospheric and biological, of which biological nitrogen fixation contributes maximum (70%) to the total nitrogen fixed.
- Biological nitrogen fixation can occur either asymbiotically or symbiotically utilizing the enzyme nitrogenase synthesized by the prokaryotic symbiont always.
- Nitrogenase can be irreversibly inactivated by oxygen and thus organisms capable of fixing nitrogen either work under anaerobic conditions present naturally or in some cases like in cyanobacteria, heterocysts are formed for creating anaerobic environments.
- The most common nitrogen fixation mechanism occurs between leguminous plants and *Rhizobium*, involving the interaction of products of nodulins genes (produced by plant) and nodulation genes (produced by rhizobia) during the process of nodulation.
- Root nodules contain bacteroids enveloped in peribacteroidal membrane (containing oxygen binding heme protein leghemoglobin) and also develop vascular system facilitating the exchange of nitrogen for the nutrients contributed by the plant.
- The enzyme nitrogenase is an oxidoreductase catalyzing the reduction of dinitrogen to ammonia and consists of two components: azoferredoxin and azomolybdoferredoxin.
- Ammonium dissipates transmembrane proton gradients, inhibits ATP synthesis, inhibits nitrogen fixation, if it accumulates to high levels in plant tissues, and therefore must be rapidly assimilated.
- Nitrate assimilation occurs in cytosol with the help of an enzyme called nitrate reductase (composed of FAD, heme, pterin) and nitrite reductase in chloroplasts or root plastids (composed of two prosthetic groups, iron-sulfur cluster and specialized heme).
- Two alternative pathways exist for ammonium assimilation i.e. reductive amination by GDH and ammonia incorporation by GS, followed by the action of GOGAT.
- Transamination reactions then operate through the action of aminotransferases to incorporate nitrogen into other amino acids, thus providing a significant role in amino acid biosynthesis.

Exercises

State whether true or false:

1. Nitrogen fixation requires aerobic conditions.
2. Anaerobic conditions are created in specialized cells called heterocysts in cyanobacteria.
3. Migration of bacteria towards roots of host plant is a chemotactic response.
4. Indole-acetic acid plays no role in the infection process during root nodule formation.
5. Glutamine synthetase has lower affinity for ammonium than glutamate dehydrogenase.
6. Aminotransferases catalyze transamination reactions.

Correct answer/option:

1. False
2. True
3. True
4. False
5. False
6. True

Justification/Feedback for the correct answer:

1. Nitrogen fixation requires anaerobic conditions for the smooth functioning of the nitrogen-fixing enzyme i.e. nitrogenase, otherwise this enzyme could be irreversibly inactivated.
2. This statement is true because heterocysts are specialized thick walled cells to create anaerobic conditions in cyanobacteria for the functioning of the enzyme nitrogenase.
3. This statement is true because rhizobia are attracted towards roots of host plant under the influence of chemical attractants (isoflavanoids and betaines) released by roots of legumes.
4. IAA plays a role in the infection process since IAA triggers curling and deformation of root hairs. (IAA is formed from tryptophan secreted by root hairs in response to an enzyme of rhizobia).
5. Glutamine synthetase has higher affinity for ammonium than glutamate dehydrogenase because it has lower K_m for ammonium.
6. Aminotransferases are group of enzymes that catalyze the transfer of amino group from one compound to another. Example: aspartate aminotransferase transfers amino group of glutamate to carboxyl group of aspartate to form another amino acid and 2-oxoglutarate is set free.

Fill in the blanks:

1. ----- isolated *Rhizobium* for the first time from root nodules.
2. Important non-legumes that fix nitrogen are -----, ----- and -----.
3. Nodulins genes produce -----, ----- and -----.
4. Nodulation genes produce -----, ----- and -----.
5. Enzymes released by rhizobia resulting in degradation of cell wall of roots of host plant are - ----- and -----.
6. ----- gives the root nodules a pink color.
7. The three prosthetic groups present in each of the subunits of nitrate reductase are ----- -----, ----- and -----.
8. ----- and ----- are the two types of GOGAT present in plants.
9. All transamination reactions require ----- as a cofactor.

Write short notes on:

1. Nitrogenase
2. Heterocyst
3. Transamination reactions
4. Haber-bosch process
5. Nodulins genes
6. Leghemoglobin
7. GOGAT

Glossary

Aminotransferases: Enzymes catalyzing the transamination reactions i.e. incorporating nitrogen into other amino acids from glutamine or glutamate.

Bacteroids: Large, round, nitrogen fixing endosymbiotic organelle synthesizing enzyme nitrogenase and differentiated from the dividing bacteria during nodulation in legumes.

Heterocyst: Thick walled cells that specifically differentiate under conditions of ammonium deprivation in filamentous cyanobacteria (like *Nostoc*, *Anabaena*) thus, representing an adaptation for nitrogen fixation in aerobic cyanobacteria.

Leghemoglobin: A high-affinity oxygen binding heme protein present in the peribacteroidal membrane imparting a pink color to the infected nodules.

Nitrate reductase: An enzyme catalyzing the reduction of nitrate to nitrite in the cytosol during nitrate assimilation.

Nitrite reductase: An enzyme catalyzing the reduction of nitrite to ammonium in either chloroplasts or root plastids during the process of nitrate assimilation.

Nitrogen fixation: Conversion of molecular nitrogen (atmospheric) into ammonium and nitrate as a result of the breaking of a stable covalent bond in the dinitrogen molecule.

Nitrogenase: An oxidoreductase present in prokaryotes that catalyzes the reduction of nitrogen to ammonia and is produced by *nif* genes.

Nodulation genes: Rhizobial genes participating in nodule formation and producing NodD protein, lipochitin and heme portion of leghemoglobin.

Nodulins: Plant genes specific to root nodules producing flavanoids, lectins and globulins during nodulation in legumes.

Peribacteroidal membrane: A membrane surrounding the group of bacteroids containing the pigment leghemoglobin in the nodule.

Transamination reactions: Reactions catalyzing the transfer of amino group from glutamine or glutamate to incorporate nitrogen into other amino acids.

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Weblinks

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