RAMSADAY COLLEGE

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E-material for 4th semester general

SUDIP MONDAL

GUEST TEACHER IN DEPT OF BOTANY

3.6.2 Nitrogen cycle

In the environment, nitrogen exists in both inorganic (e.g. nitrogen, ammonia, nitrate) and organic (e.g. amino acid, nucleic acids) forms. These forms undergo different transformations, changing from one form to another by physical and biological processes. The major transformations of nitrogen are biological nitrogen fixation, nitrification, denitrification and ammonification.

Biological nitrogen fixation

It is the biosynthetic process of reduction of molecular nitrogen into ammonia. This process is performed by nitrogen fixing organisms. All nitrogen-fixing organisms are prokaryotes.

Ammonification

Most of the nitrogen in the soil exists in organic forms. When organic nitrogen is converted into ammonium ions by bacteria and fungi, it is called *ammonification*.

Bacillus ramosus, proteus vulgaris, clostridium sp

Nitrification

The oxidation of ammonium ions to nitrite and subsequent oxidation of nitrite to nitrate is called *nitrification*. It is carried out exclusively by prokaryotic organisms. The first step in nitrification is the oxidation of ammonium ions to nitrite. This step is carried out by nitrifying bacteria of genera *Nitrosomonas, Nitrosospira* and *Nitrosococcus*. The second step in nitrification is the oxidation of nitrite to nitrate. This step is carried out by nitrifying bacteria of genera *Nitrospira, Nitrobacter* and *Nitrococcus*.

$$\underset{\text{Ammonium ion}}{NH_4^+} \xrightarrow{\text{Nitrosomonas}} \underset{\text{Nitrite ion}}{NO_2^-} \xrightarrow{\text{Nitrobacter}} \underset{\text{Nitrate}}{NO_3^-}$$

Denitrification

The process of conversion of NO_3^- into N_2 by anaerobic denitrifying bacteria is called *denitrification*. These bacteria use nitrate rather than oxygen as an electron acceptor during respiration. This causes nitrate to be reduced, producing inturn NO_2^-, NO, N_2O and finally N_2. Denitrification can only be performed under anaerobic conditions. The process is performed primarily by heterotrophic denitrifying bacteria (such as *Paracoccus denitrificans* and various pseudomonads), although autotrophic denitrifiers have also been identified (e.g. *Thiobacillus denitrificans*).

$$2NO_3^- \longrightarrow 2\,NO_2^- \longrightarrow 2\,NO \longrightarrow N_2O \longrightarrow N_2$$

Anammox

Anammox (an abbreviation for ANaerobic AMMonium OXidation) is a recently discovered bacterial process. In this biological process, nitrite and ammonium ions are converted directly into nitrogen gas.

3.6.3 Nitrogen assimilation

Living matter contains a large amount of nitrogen incorporated in proteins, nucleic acids and many other biomolecules. For plant growth, the nitrogen demand for the formation of cellular matter is met by inorganic nitrogen in two alternative ways:

1. Assimilation of the nitrate or ammonia contained in water or soil.
   In soil, ammonia is formed as an end product due to the degradation of organic matter (*ammonification*) and is further oxidized to nitrate again by *nitrifying bacteria* present in the soil (*nitrification*).
2. Fixation of molecular nitrogen from air.
   Nitrogen fixing prokaryotes are able to fix molecular nitrogen from the air (termed *nitrogen fixation*). Some plants develop a symbiotic association with these N_2-fixing bacteria, which supply them organic nitrogen.

Assimilation of the nitrate or ammonia

Plants obtain the bulk of their nitrogen from soil in the form of either nitrate or ammonium ions. The transport of nitrate into the root cells is mediated by active process. A proton gradient across the plasma membrane, generated by a H^+-ATPase, drives the uptake of nitrate against a concentration gradient. Assimilation of mineral nitrogen into organic molecules is a complex process. Nitrate is first reduced to ammonium ion before it can be assimilated by plants. The reduction of nitrate to ammonia by higher plants is accomplished in two steps:-

In the *first step,* nitrate is reduced to nitrite by the enzyme *nitrate reductase.* This reaction takes place in the cytosol. Nitrate reductases are composed of two identical subunits, each containing three prosthetic groups – FAD, heme (cyt-b_{557}) and a Mo containing organic molecule called *pterin*. It requires reducing agent NAD(P)H.

In the *second step,* nitrite is reduced to ammonia by the enzyme *nitrite reductase*. Nitrite reductase contains two prosthetic groups, Fe_4S_4 and siroheme. Siroheme is a cyclic tetrapyrrole with one Fe-atom in the centre. This reaction takes place in proplastids (of the root) or chloroplasts (of the shoot).

$$NO_3^- \xrightarrow[\textit{Nitrate reductase}]{\text{NAD(P)H}} NO_2^- \xrightarrow[\textit{Nitrite reductase}]{\text{Ferredoxin (Red)}} NH_4^+$$

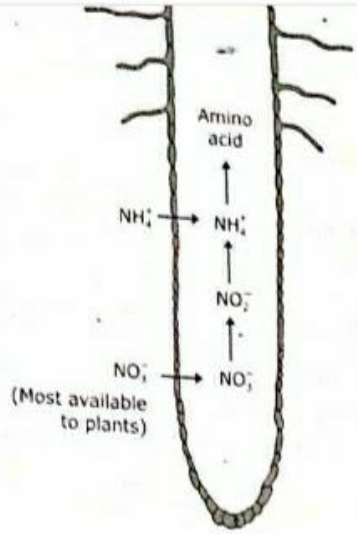
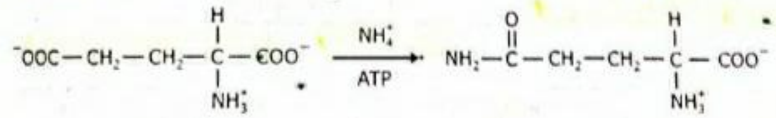


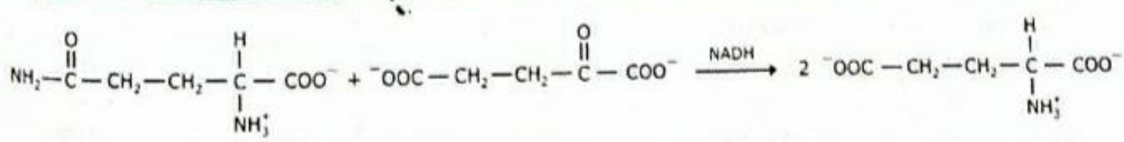
Figure 3.11 Nitrate is the most abundant form of nitrogen in the soils and is most available to plants. Plants also take nitrogen from soil in form of ammonium ions. In the plant cell the nitrate is converted into nitrite ion, then to ammonium ion, and finally into the amide nitrogen of glutamine. Plants such as legumes also form symbiotic relationships with nitrogen-fixing bacteria to convert molecular nitrogen into ammonium ion (not shown in the figure).

Plant cells avoid ammonium toxicity by rapidly converting the ammonium generated from nitrate assimilation into amino acids. The conversion of ammonia into amino acid involves the sequential action of two enzymes *glutamine synthetase* and *glutamate synthase*. Glutamine synthetase combines ammonium ion with glutamate to form glutamine. This reaction requires ATP and divalent cations as a cofactor.



Glutamate Glutamine

Glutamine is then converted back to glutamate by the transfer of the amide group to a molecule of α-ketoglutarate. It is catalyzed by glutamate synthase (glutamine-2-oxoglutarate amino-transferase, GOGAT) and requires reducing potential in the form of NADH. Plants contain two types of GOGAT; one accepts electrons from NADH; the other accepts electrons from ferredoxin. The reaction gives rise to two molecules of glutamate.



Glutamine α-Ketoglutarate Glutamate

Ammonium ions can be assimilated *via* an alternative pathway called *reductive amination*. Glutamate dehydrogenase catalyzes the reductive amination of α-ketoglutarate and forms glutamate. This enzyme is unusual in being able to utilize either NAD^+ or $NADP^+$. An NADH-dependent form of glutamate dehydrogenase is found in mitochondria, and an NADPH-dependent form is localized in the chloroplasts.

Once assimilated into glutamine and glutamate, nitrogen is incorporated into other amino acids via transamination reactions. The enzymes that catalyze these reactions are known as *aminotransferases*.

3.6.4 Biological nitrogen fixation

Nitrogen is present in many forms in the biosphere. The atmosphere contains about 78% (by volume) molecular nitrogen. Acquisition of nitrogen from the atmosphere requires the breaking of an exceptionally stable triple covalent bond between two nitrogen atoms to produce ammonia (NH_3) or nitrate (NO_3^-). Conversion of molecular nitrogen to nitrate or ammonia is termed as *nitrogen fixation*, which can be accomplished by both industrial and natural processes. Natural processes of nitrogen fixation includes *lightning, photochemical reactions* and *biological nitrogen fixation*. Approximately 90% of nitrogen fixation is biological nitrogen fixation, in which prokaryotic organisms fix molecular nitrogen into ammonium ions. It is a reductive biosynthetic process. Few prokaryotic organisms (termed as nitrogen fixing organisms or *diazotroph*) are capable of biological nitrogen fixation only. Eukaryotic organisms are unable to fix nitrogen. The biological reaction of nitrogen fixation generates at least one mole of H_2 in addition to two moles of NH_3 for each mole of nitrogen molecule. Hence, total eight electrons are required in reduction of one mole of nitrogen to two moles of NH_3.

$$N_2 \xrightarrow{8e^- + 8H^+} 2NH_3 + H_2$$

However, the actual reduction of N_2 occurs in three discrete steps, each involving an electron pair:

$$N{\equiv}N \xrightarrow[2H^+ + 2e^-]{} NH{=}NH \xrightarrow[2H^+ + 2e^-]{} H_2N{-}NH_2 \xrightarrow[2H^+ + 2e^-]{} 2NH_3 + H_2$$

The biological process of nitrogen fixation is catalyzed by an enzyme complex called **nitrogenase complex**. There are three different forms of nitrogenase that differ in their requirement for molybdenum, vanadium, or iron as a critical metallic component. Most of the nitrogenases that have been studied contain a Mo cofactor. Nitrogenase consists of two proteins: a *dinitrogenase reductase* and *dinitrogenase*. The *dinitrogenase reductase* (also called the *Fe protein*) is a dimer of identical 30 kDa subunits bridged by a 4Fe-4S cluster. *Dinitrogenase* is a tetramer with two copies of two different subunits. It contains both Fe and Mo. Because molybdenum is present in this cluster, the dinitrogenase component is also called the *molybdenum-iron protein (MoFe protein)*. The MoFe cofactor is the site of nitrogen fixation. The genes involved collectively in the synthesis of nitrogenase and the catalytic process of N_2 fixation are called *nif* genes. Accessory genes are called *fix* genes, and they are also necessary for the function and regulation of nitrogenase in aerobic nitrogen-fixing bacteria.

Since nitrogen fixation is a reductive process so it requires electron donor. In most nitrogen-fixing microorganisms, *reduced ferredoxin*, acts as a donor for electrons. Ferredoxin is a small (14 to 24 kDa) protein containing an Fe-S group. At least 16 molecules of ATP are required for reduction of one molecule of N_2.

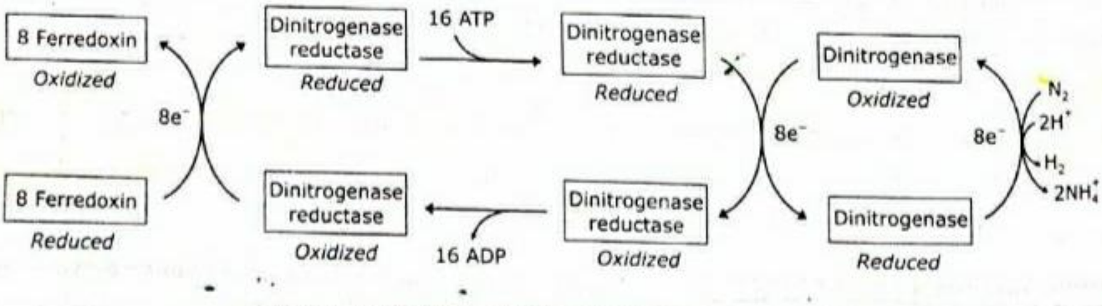


Figure 3.12 Nitrogen fixation by nitrogenase complex.

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 organisms, 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*. 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 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.

Nitrogen fixing prokaryotes

Nitrogen-fixing prokaryotes can be divided into those that carry out biological nitrogen fixation in a symbiotic or a commensalistic relationship with a eukaryote and those that fix nitrogen in a free-living state. Most of the nitrogen-fixing prokaryotes are free-living in the soil. They include photosynthetic as well as non-photosynthetic genera. Only few nitrogen fixing prokaryotes form symbiotic associations with higher plants.

Examples of free-living nitrogen-fixing prokaryotes

Aerobic nitrogen-fixing bacteria : *Azospirillum*, *Azotobacter*

Anaerobic nitrogen-fixing bacteria : photosynthetic (e.g. *Rhodospirillum*), non-photosynthetic (e.g. *Clostridium*).

Symbiotic nitrogen-fixing prokaryotes may or may not form nodules. The most common form of symbiotic association results in the formation of enlarged and multicellular structures called *nodules* on the root (or occasionally the stem) of the host plant. In case of Leguminous plants (such as peas, beans, clover, alfalfa, soybean), the symbiont is a bacterium of genera *Rhizobium, Bradyrhizobium* and *Azorhizobium*. Although most leguminous plants form nodules on their roots, a few leguminous plants bear nodules on their stems. One common example of a stem-nodulated leguminous plant is *Sesbania*.

Nodules formation also occurs in certain nonleguminous plants such as *Alnus, Myrica and Casuarina*. However, the symbiont in these nonleguminous nodules is actinomycetes of genus *Frankia*. Both *Rhizobium and Frankia* live freely in the soil, but fix nitrogen only when in symbiotic association with an appropriate host plant. Some symbiotic associations do not form nodules. *Anabaena azollae*, a nitrogen-fixing cyanobacterium, lives in pores on the fronds of a water fern called *Azolla*.

Table 3.3 Examples of nitrogen fixing bacterial and archaeal genus and their lifestyle

Genus	*Phylogenetic affiliation*	*Lifestyle*
Nostoc, Anabaena	Bacteria	Free-living, aerobic, photolithotrophic
Pseudomonas, Azotobacter	Bacteria	Free-living, aerobic, chemotroph
Thiobacillus	Bacteria	Free-living, aerobic, chemotroph
Methanococcus	Archaea	Free-living, anaerobic, chemotroph
Chromatium, Chlorobium	Bacteria	Free-living, anaerobic, phototroph
Desulfovibrio, Clostridium	Bacteria	Free-living, anaerobic, chemotroph
Rhizobium, Frankia	Bacteria	Symbiotic, aerobic, chemotroph

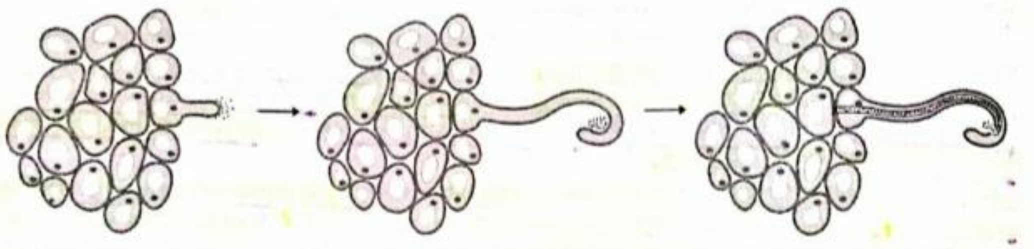
Rhizobium - legume symbiosis

Rhizobium is gram-negative motile rod-shaped proteobacteria that can grow free-living in soil or can infect leguminous plants and establish a symbiotic existence. Infection of the roots of a legume with species of *Rhizobium* genera leads to the formation of *root nodules*.

Chemotactic movement of free-living soil bacteria towards roots is mediated by chemicals such as flavonoids, homoserine, secreted by the roots. A specific adhesion protein called *rhicadhesin* is present on the surfaces of *Rhizobium* species. Rhicadhesin, a calcium-binding protein, plays rôle in plant-bacterium attachment. Root exudates also induce the *nodulation* (*nod*) genes responsible for nodule formation. Rhizobia secrete NodD, which is a protein

... the flavonoids secreted by the plants. Interaction with the flavonoids activates NodD, then NodD returns to the rhizobium to induce the transcription of *nodABC* genes as well as many other nod genes.
The *nod* genes are classified as common *nod* genes or host-specific *nod* genes. The common *nod* genes - *nodA*, *nodB* and *nodC* - are found in all rhizobial strains; the host-specific *nod* genes-such as *nodP*, *nodQ* and *nodH*; or *nodF*, *nodE* and *nodL*-differ among rhizobial species and determine the host range. Three of the *nod* genes (*nodA*, *nodB* and *nodC*) encode enzymes that are required for synthesis of *nodulation factors* or **nod factors**. Nod factors are *lipo-chitooligosaccharide*, the derivatives of chitin. Nod factors of all studied rhizobia are composed of a β-1,4-linked N-acetyl-D-glucosamine. The non-reducing terminal sugar moiety is substituted with a fatty acid whose structure varies between different rhizobial species. NodA (product of gene nodA) is an *N*-acyltransferase that catalyzes the addition of a fatty acyl chain. NodB (product of gene nodB) is a chitin-oligosaccharide deacetylase that removes the acetyl group from the terminal nonreducing sugar. NodC (product of gene nodC) is a chitin-oligosaccharide synthase that links *N*-acetyl-D-glucosamine monomers.

Nod factors induce root hair curling and trigger plant cell division. The process of nodule development begins with the infection of a root hair by a *Rhizobium* bacterium. During the infection process, rhizobia that are attached to the root hairs release Nod factors that induce curling of the root hair cells and also sends mitogenic signals that stimulate cell division in cell cortex. Cell divisions lead to formation of *root nodule*. Nod factors also induce several nodule-specific plant genes called **nodulin** genes. During development of the root nodules the nodulin genes are differentially expressed.



Attachment of the *Rhizobia* to root hairs in response to chemical attractants released by plant.

Excretion of Nod factors by the bacterium. Nod factor promotes root hair curling and plant cortical cell division.

Formation of infection thread (cellulosic tube) and invasion into the cortical cells.

Figure 3.13 The infection process during nodule formation.

Rhizobia invade the root by digesting the root hair cell wall. 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 infection thread filled with dividing rhizobia elongates through the root hair into the root cortex. Finally the tip of infection thread fuses with the plasma membrane of the cortical cell, releasing bacterial cells that are packaged in a membrane derived from the host cell plasma membrane. In the infected cells, the rhizobia divide and enlarge to form *bacteroids*, which are separated from the plant cytosol by 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*. It gives legume nodules a pink color. The globin protein is encoded by the host plant, whereas the heme group is primarily synthesized by the bacterium and transported into the host cytoplasm. Leghemoglobin is localized in the cytosol of the infected plant cells and not inside the peribacteroid membrane.

Assimilation of ammonium ions (ammonium assimilation)

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. Allantoin is synthesized in peroxisomes from uric acid, and allantoic acid is synthesized from allantoin in the endoplasmic retuculum.