M.Sc. Botany

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MBOTCC-7: Physiology & Biochemistry

Unit –II

BIOLOGICAL NITROGEN FIXATION

(Non-symbiotic and symbiotic)

Nitu Bharti

Assistant Professor

Department of Botany

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Nitrogen is one of the major limiting nutrients in plant growth. Nitrogen fixation refers to the conversion of atmospheric nitrogen to ammonia and then to nitrogen containing organic compounds that can available to all forms of life, it requires the breaking of an exceptionally stable triple covalent bond between two nitrogen atoms $(N{\equiv}N)$ to produce ammonia (NH_3) or nitrate (NO^{3-}). Nitrogen can be fixed by non-biological processes, such as lightening or the Haber-Bosch process used to produce fertilizer products such as urea. Biological nitrogen fixation (about 90 % of the total nitrogen fixed) is carried out only by prokaryotes , which are belonging to diverse groups. The overall reaction of biological fixation is

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

Hence, biological nitrogen fixation requires three components:

I. a strong reducing agent,
II. ATP to transfer hydrogen atoms to dinitrogen (N≡N), and
III. the enzyme systems.

The reducing agent (FAD) and ATP are provided by photosynthesis and respiration.

Nitrogen fixing microbes (Diazotrophs)

Diazotrophs are generally active in rhizosphere.They are classified according to their mode of fixation.

I. Free living nitrogen fixers
II. Associative nitrogen fixers
III. Endophytic nitrogen fixers
} Non-symbiotic nitrogen fixers
IV. Symbiotic nitrogen fixers

TABLE 12.2
Examples of organisms that can carry out nitrogen fixation

Host plant	N-fixing symbionts
Symbiotic nitrogen fixation	
Leguminous: legumes, *Parasponia*	*Azorhizobium, Bradyrhizobium, Photorhizobium, Rhizobium, Sinorhizobium*
Actinorhizal: alder (tree), *Ceanothus* (shrub), *Casuarina* (tree), *Datisca* (shrub)	*Frankia*
Gunnera	*Nostoc*
Azolla (water fern)	*Anabaena*
Sugarcane	*Acetobacter*
Free-living nitrogen fixation	
Type	**N-fixing genera**
Cyanobacteria (blue-green algae)	*Anabaena, Calothrix, Nostoc*
Other bacteria	
Aerobic	*Azospirillum, Azotobacter, Beijerinckia, Derxia*
Facultative	*Bacillus, Klebsiella*
Anaerobic	
Nonphotosynthetic	*Clostridium, Methanococcus* (archaebacterium)
Photosynthetic	*Chromatium, Rhodospirillum*

Nitrogen Fixation Requires Anaerobic Conditions

Because oxygen irreversibly inactivates the nitrogenase enzymes involved in nitrogen fixation, nitrogen must be fixed under anaerobic conditions. Thus each of the nitrogen fixing organisms either functions under natural anaerobic conditions or can create an internal anaerobic environment in the presence of oxygen.

I. Free living nitrogen fixers:- They are capable of fixing atmospheric nitrogen independently of other living organism. Free-living bacteria that are capable of fixing nitrogen are aerobic, facultative, or anaerobic.

- Obligate aerobic - Azotobacter, thought to maintain reduced oxygen conditions (microaerobic conditions) through their high levels of respiration.
  Gloeothece, evolve O_2 photosynthetically during the day and fix nitrogen during the night.
  Cyanobacteria- Anabaena cylindrica, Gloeocapsa, Nostoc muscorum
- Anaerobic - oxygen does not pose a problem, because it is absent in their habitat.
  These anaerobic organisms can be either photosynthetic (e.g., Rhodospirillum), or nonphotosynthetic (e.g., Clostridium)
- Facultative anaerobic - Bacillus polymyxa, Facultative organisms, which are able to grow under both aerobic and anaerobic conditions, generally fix nitrogen only under anaerobic conditions.

II. Associative nitrogen fixers :-Johannah Dobreiner observed a loose association of Azospirillum lipoferum , a nitrogen fixer, with roots of certain Brazilian grasses and maize in 1975. These are widespread in the soils of tropical, subtropical and temperate regions. Azospirillum bacteria are aerobic non-fermentative chemoorganotrophic vibroid to S-shaped containing polyhydroxyalkanoate granules (PHA).

III. **Endophytic nitrogen fixers:-**Endophytes multiply and spread within plant tissues without causing damage . e.g. *Gluconacetobacter diazotrohicus*: an acetic acid bacterium first isolated from sugarcane plants.

IV. **Symbiotic nitrogen fixers:-**This is mutually beneficial relationship between microbes and plant. e.g.

- **Rhizobium** (Rhizobium-legume association)
- **Bradyrhizobium** (Bradyrhizobium-soybean association)
- **Azorhizobium** (Azorhizobium – *Sesbania rostrata* association) - Form stem nodule along with root nodule
- **Actinomycetes**
  e.g.Frankia (Frankia-Casuarina association)
- **Cyanobacteria:** Cyanobacteria are one of very few groups of organisms that can convert inert atmospheric nitrogen into an organic form, such as nitrate or ammonia. In cyanobacteria, anaerobic conditions are created in specialized cells called heterocysts . **Heterocysts** are thick-walled cells that differentiate when filamentous cyanobacteria are deprived of $NH4^+$. These cells lack photosystem II, the oxygen-producing photosystem of chloroplasts, so they do not generate oxygen. Heterocysts appear to represent an adaptation for nitrogen fixation, in that they are widespread among

aerobic cyanobacteria that fix nitrogen. Cyanobacteria that lack heterocysts can fix nitrogen only under anaerobic conditions such as those that occur in flooded fields.

- Lichens (Cyanobacteria-fungus association)
- Bryophytes (Anabaena-Anthoceros)
- Pteridophytes (*Anabaena azollae-Azolla*)-Azolla is a small fast growing aquatic fern. *Anabaena azollae* , a cyanobacterium lives in cavities of Azolla leaves. It fixes nitrogen from the air and excretes the nitrogenous compounds into the leaf cavity of fern. *A.pinnata* is an excellent biofertiliser for rice.
- Gymnosperms (Nostoc-Cycads), Coralloids root
- Angiosperms (Nostoc-Gunnera)

Legume Rhizobium symbiosis:- Rhizobium is predominant symbiotic nitrogen fixing bacterium. It is rod shaped, motile, gram-negative, non-spore forming bacterium and utilize organic acid salts as carbon sources. The bacteria are mostly rhizospheric microorganisms, despite its ability to live in soil for long period of time. Each bacterial sps.infects only certain sps. of plants.

Nodule Formation: Two processes—infection and nodule organogenesis ,occur simultaneously during root nodule formation.

1. During the infection process, rhizobia that are attached to the root hairs release Nod factors that induce a pronounced curling of the root hair cells. 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.
2. The next step is formation of the 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.
3. 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. Ethylene is synthesized in the region of the pericycle, diffuses into the cortex, and blocks cell division.
4. 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 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.

The nodule as a whole develops features as a vascular system (which facilitates the exchange of fixed nitrogen produced by the bacteroids for nutrients contributed by the plant) and a layer of cells to exclude O_2 from the root nodule interior.

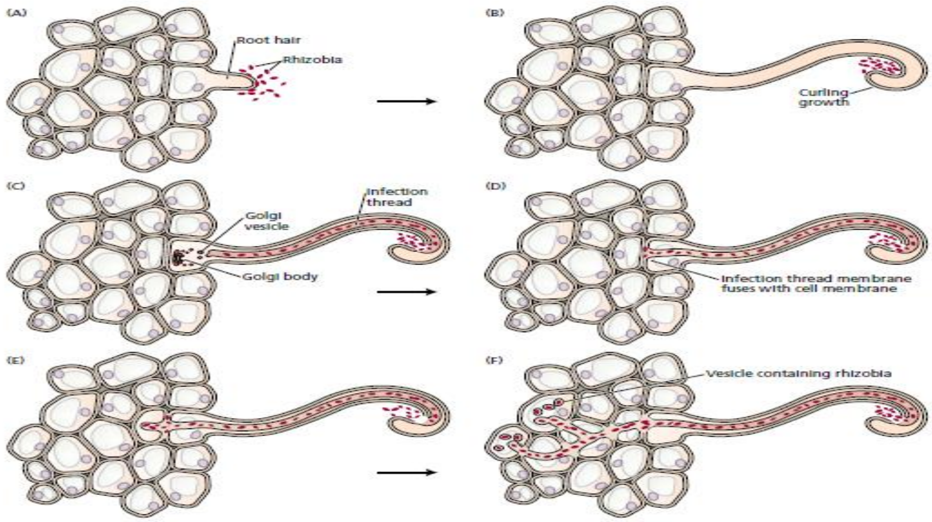

Fig: The infection process during nodule organogenesis. (A) Rhizobia bind to an emerging root hair in response to chemical attractants sent by the plant. (B) In response to factors produced by the bacteria, the root hair exhibits abnormal curling growth, and rhizobia cells proliferate within the coils. (C) Localized degradation of the root hair wall leads to infection and formation of the infection thread from Golgi secretory vesicles of root cells. (D) The infection thread reaches the end of the cell, and its membrane fuses with the plasma membrane of the root hair cell. (E) Rhizobia are released into the apoplast and penetrate the compound middle lamella to the subepidermal cell plasma membrane, leading to the initiation of a new infection thread, which forms an open channel with the first. (F) The infection thread extends and branches until it reaches target cells, where vesicles composed of plant membrane that enclose bacterial cells are released into the cytosol

Nitrogenase: The biological nitrogen fixation is catalyzed by a multimeric enzyme complex nitrogenase. The enzyme nitrogenase is synthesized by the bacteria. It consists of two conserved proteins: an iron (Fe) containing dinitrogenase reductase and a molybdenum iron (Mo-Fe) dinitrogenase, neither of which has catalytic activity by itself. Other substrate for nitrogenase are N_2O,N_3, C_2H_2 etc.

1. The **Fe protein** is the smaller of the two components and has two identical subunits of 30 to 72 kDa each, depending on the organism. Each subunit contains an iron–sulfur cluster (4 Fe and 4 S^{2-}). The Fe protein is irreversibly inactivated by O_2 with typical half-decay times of 30 to 45 seconds
2. The **MoFe protein** has four subunits, with a total molecular mass of 180 to 235 kDa, depending on the species. Each subunit has two Mo–Fe–S clusters. The

Mo-Fe protein is also inactivated by oxygen, with a half-decay time in air of 10 minutes.

Leghemoglobin:- A red iron-containing oxygen-binding heme protein similar to blood hemoglobin is found in the cytoplasm of infected nodule cells at high concentrations and gives the nodules a pink color.

i. The prosthetic group protoheme is synthesized by the bacteroids, whilst the sysnthesis of the protein part (globin) involves the plant cells.
ii. It acts as a oxygen scavenger for nitrogenase enzyme and facilitates the diffusion of oxygen to symbiotic bacteroids in order to promote nitrogen fixation.

Recently, it has been known that an excessive use of chemical fertilizer (nitrogenous fertilizer) induces environmental pollution, Therefore, concerns on biological nitrogen fixation, a natural nitrogen supplying source are increasing. Further they are eco-friendly and are compatible with long term sustainability.