Nitrogen fixation

Symbiotic N2 fixers: *Rhizobium* - Isolation, characteristics, types, inoculum production and field application, legume/pulses plants

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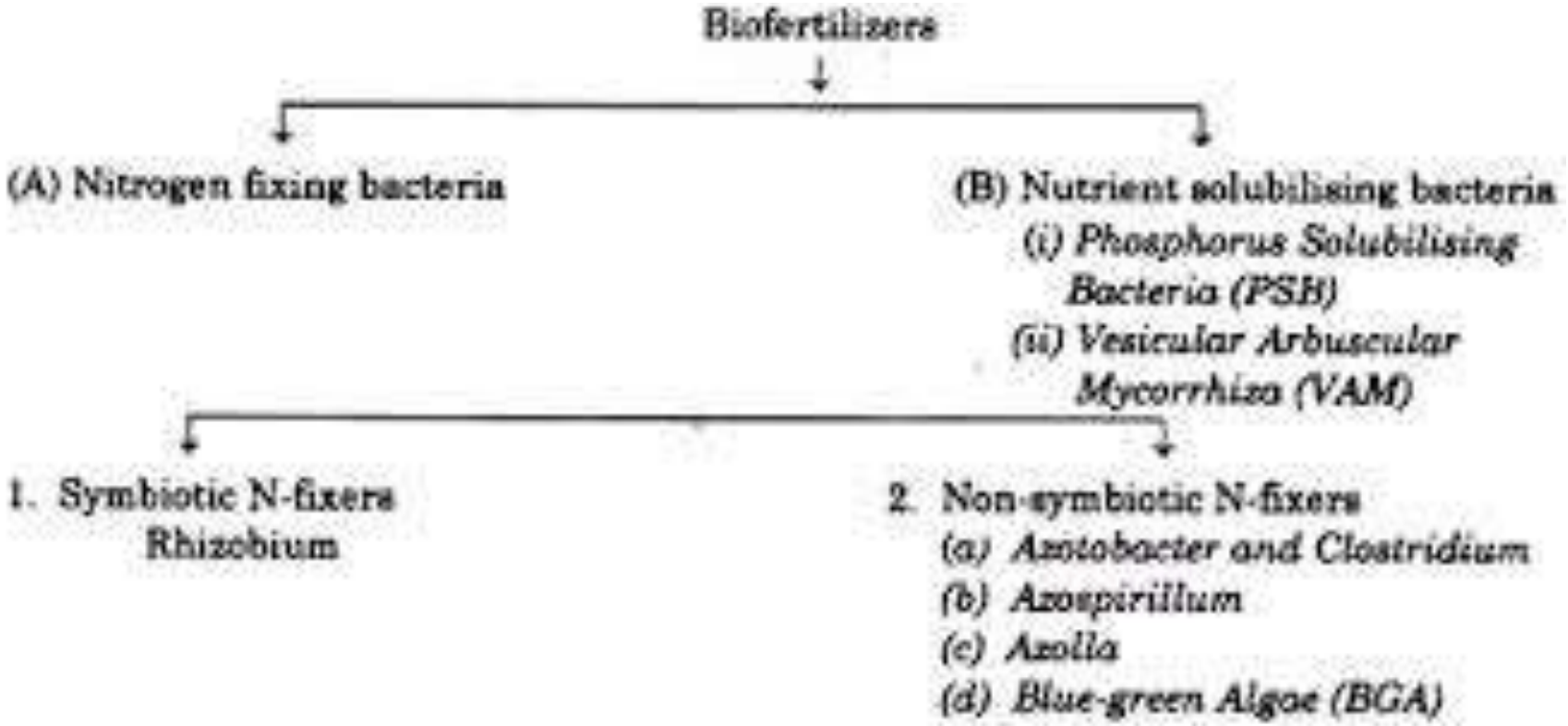
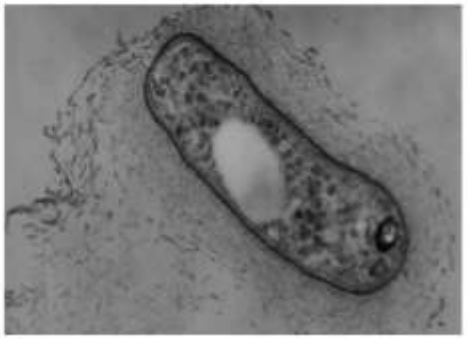

Biofertilizers
(A) Nitrogen fixing bacteria
(B) Nutrient solubilising bacteria
(i) Phosphorus Solubilising Bacteria (PSB)
(ii) Vesicular Arbuscular Mycorrhiza (VAM)
1. Symbiotic N-fixers
Rhizobium
2. Non-symbiotic N-fixers
(a) Azotobacter and Clostridium
(b) Azospirillum
(c) Azolla
(d) Blue-green Algae (BGA)

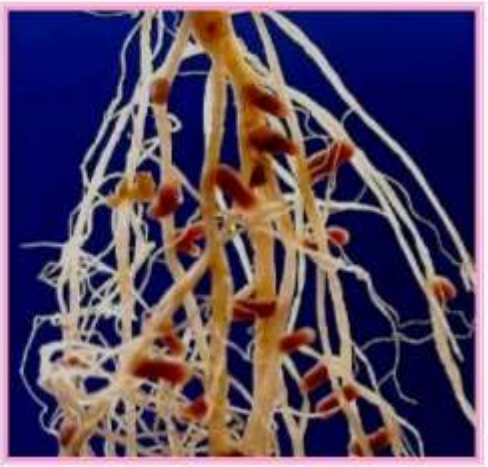
Symbiotic nitrogen fixation :

- Symbiotic nitrogen fixation occurs in plants that harbour nitrogen-fixing bacteria within their tissues
- The best-studied example is the symbiotic association between roots of legumes and bacteria of the genus Rhizobium
- This association results form the root nodules in legumes
- Root nodules – it is a enlarged multicellular structures on roots
- Legume – rhizobium association will fix 25 – 60 kg of molecular nitrogen annually

MORPHOLOGY:

- It belongs to rhizobiaceae family,
- Rhizobium are symbiotic diazotrophs
- It form a endosymbiotic association with legumes.
- Rhizobium is a Gram negative Soil Bacteria.
- They are non sporing bacteria
- Rod shaped cells.





CHARACTERISTICS OF RHIZOBIUM

- Rhizobia invade legume roots through root hairs
- Form effective pink colored nodules in the roots
- Lives symbiotically inside the nodules and fix nitrogen
- Converts atmospheric nitrogen to plant accessible forms
- Most rhizobia are host specific in nature

CROSS INOCULATION GROUPS

- *Rhizobium leguminosarum - Pisum*
- *R. phaseoli - Bean*
- *R. trifoli -Clover*
- *R. meliloti - Medicago (alfa alfa)*
- *R. japonicum - Soyabean*
- *R. lupine - Lupin groups*

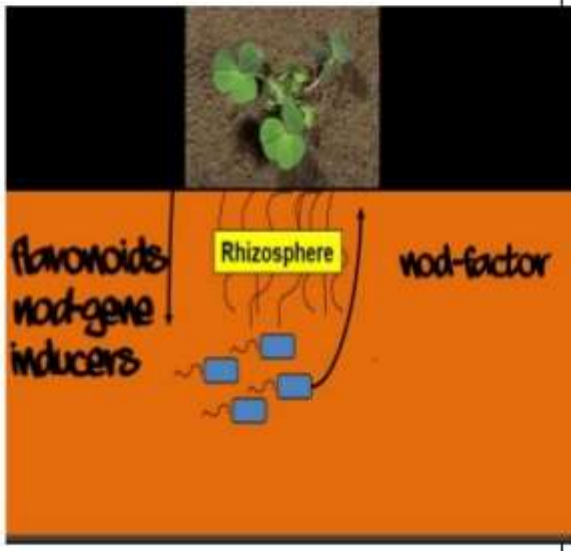
NODULE FORMATION

Step involved in Rhizobium nodulation process

1. Attraction and mulitification of rhizobium
2. Attachment to the root surface
3. Root hair curling
4. Formation of infection thread.
5. Nodule formation

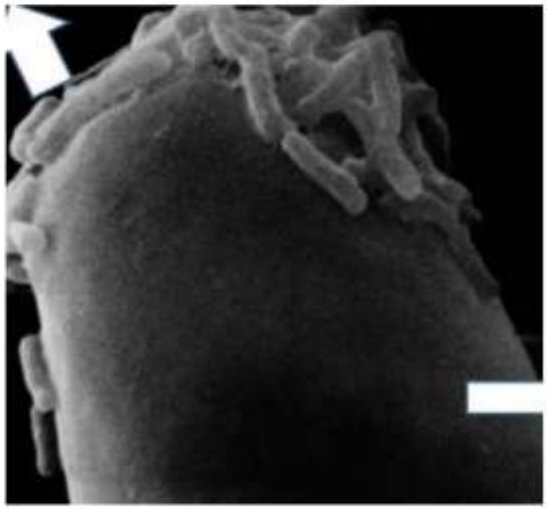
1.Attraction and Multification of Rhizobium

- Intercation between rhizobium and legume is by flavonoids produced and secreated by the legume



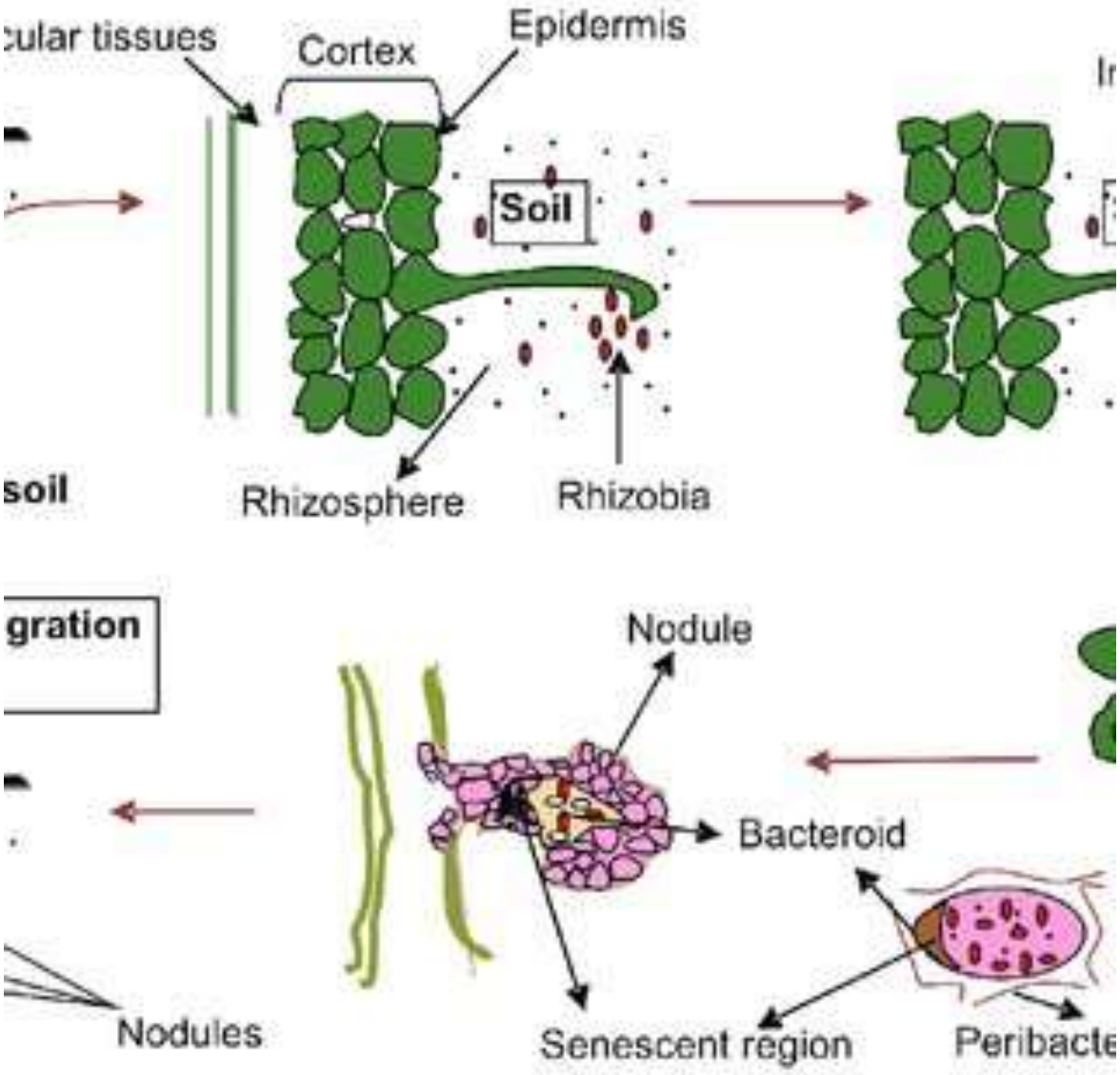
2.Attachment to the root surface

- Exopolysaccharide produced by the rhizobium interact with lectin produced from the plants
- Exopolysaccharide helps to bind rhizobium cells to the root hair.



3.Root hair curling

- Rhizobium produced IAA
- The curling of root hairs is attributed to IAA there fore rhibia become enclosed by the root hair walls
- Root hair curling forms a strucutre called shepherd' crook.


ular tissues
Cortex
Epidermis
Soil
soil
Rhizosphere
Rhizobia
gration
Nodule
Bacteroid
Nodules
Senescent region
Peribacte

The bacteria invade the roots of leguminous plants to form nodules. In the initial process of infection the bacteria accumulate around the root hairs probably because of root exudates and the bacteria release Nod factors in response of which the root hairs curl at their tips. As a result the rhizobia get enclosed in the small coiled compartment formed by the curling.

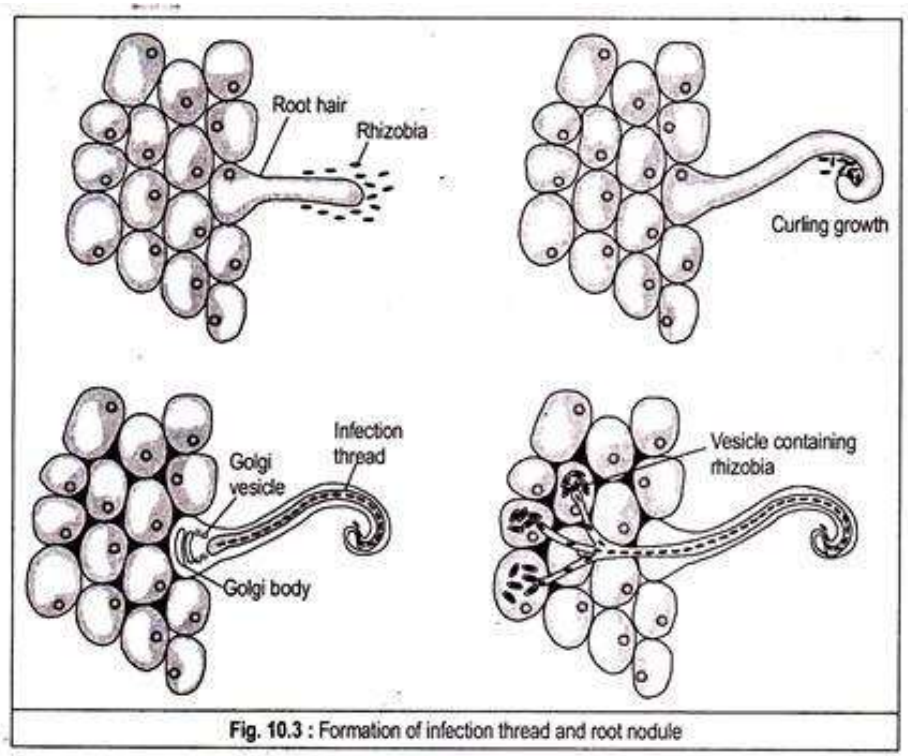
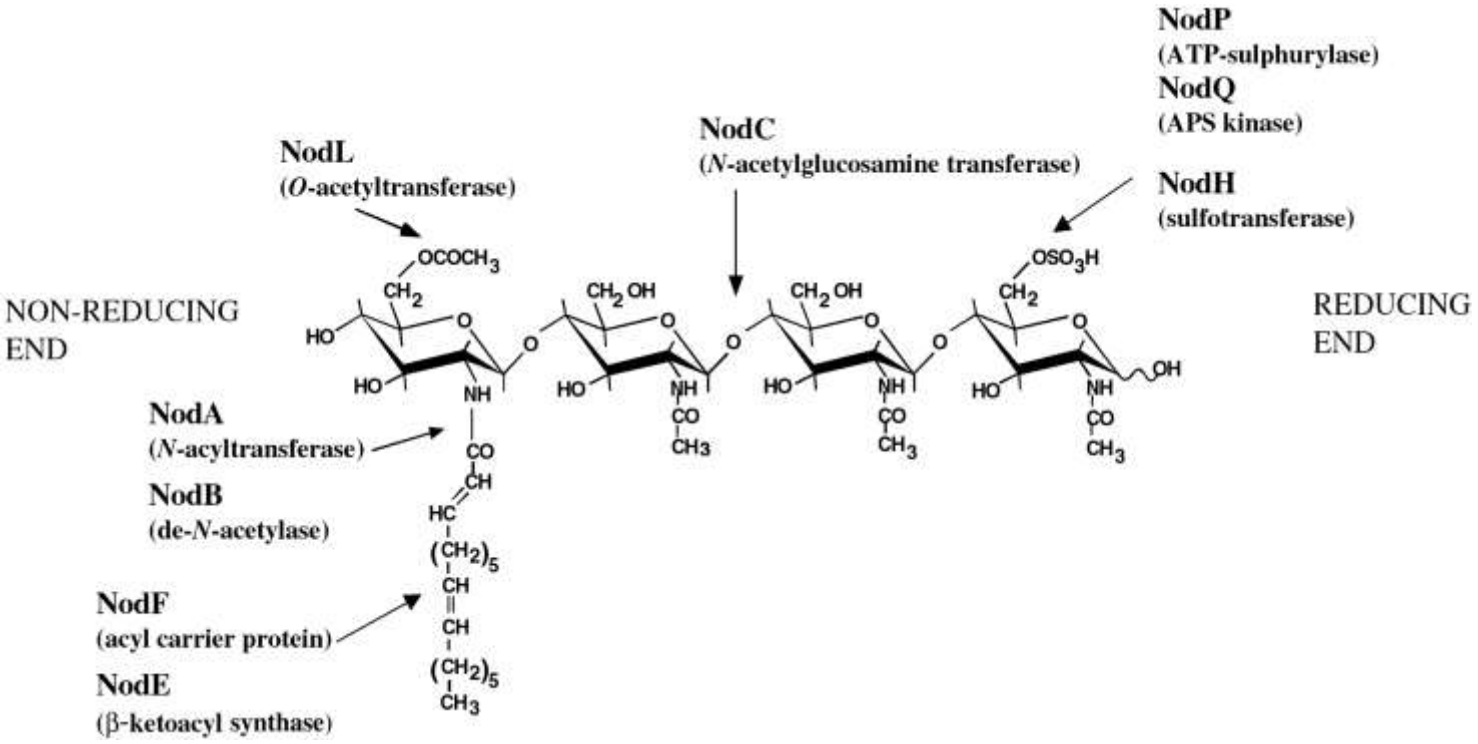


Fig. 10.3 : Formation of infection thread and root nodule

The Nod factors are lipochitin oligosaccharide signal molecules and are the products of three nod genes – nod A nod B and nod C, which are host specific. Legume root hair exudates contain specific sugar-binding proteins called lections that being activated by Nod factors, facilitate attachment of the bacteria to the cell walls of root hairs

The *nod* genes are divided into two categories, common and host specific. The common*nod* genes, *nodABC*, are required for the synthesis of the *N*-acetylglucosamine backbone and attachment of the lipid moiety at the nonreducing end of NF (Fig

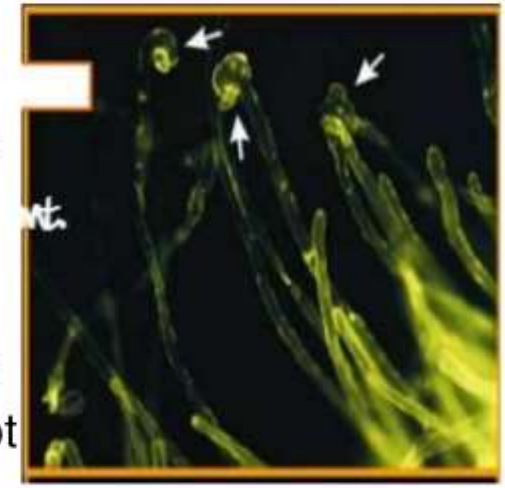


Each Nod protein is encoded by an equivalently named *nod* gene. NodA, NodB, and NodC are common to all rhizobia. The remaining Nod proteins are responsible for the modifications of NF that confer activity on selected legume species

Bacteria either penetrate the soft root tips or invade damaged or broken root hairs and the plant produces infection threads, which are internal tubular extensions of the plasma membrane produced by the fusion of Golgi-derived membrane vesicles at the site of infection.

4.Formation of infection thread.

- Infection thread originates from the tip of the curled protion of root hair ceels.
- It is a tubular structure that carries rhizobia from the root surface to root cortex.
- The nucles of the root hair cell guides the path way of infection thread into the root hair.

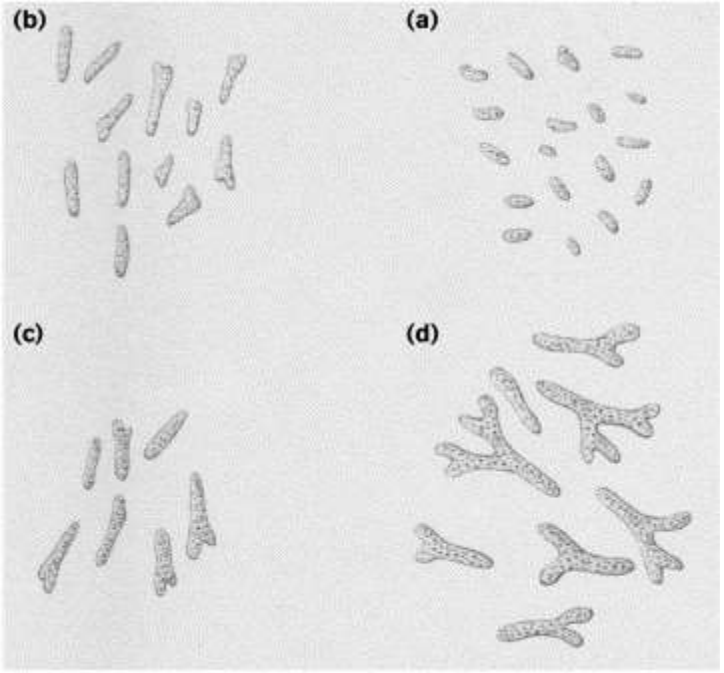


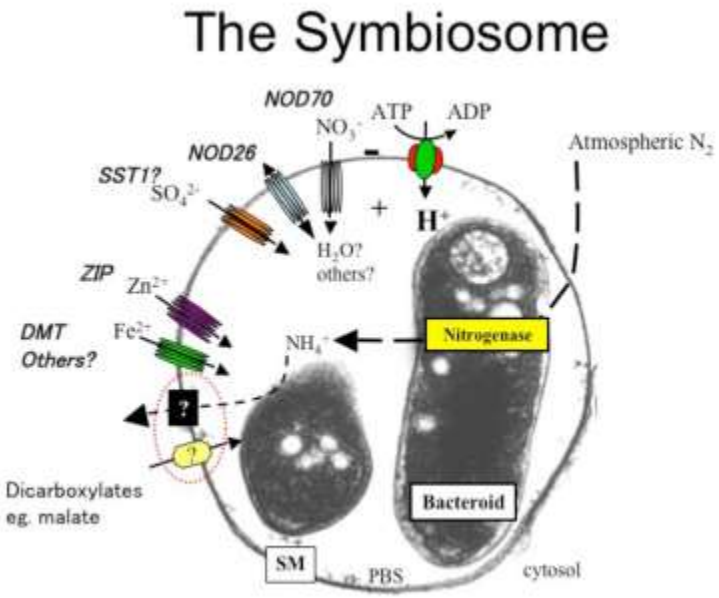
5.Nodule formation

- Infection thread continues to grow behyond the root hair cell and penetrate cortex cell
- On entry into the cortical cells, infection thread branches, rhizobia are released shortly into the cortical cells
- concurrent with the invasion of rhizobia , there occurs as rapid burst of cell division of the host ceels and rhizobia changes there shape to form cells called bactriods
- There fore diploid cells become

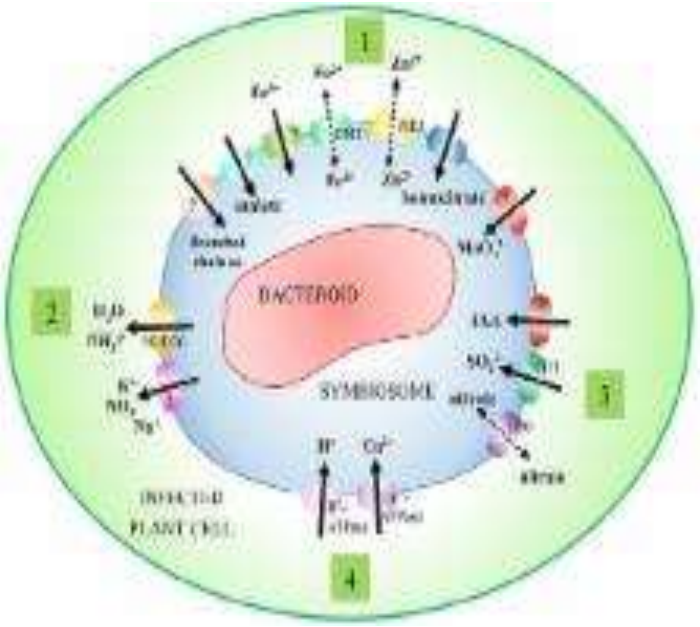
When the infection thread reaches the primordial cells, the tip of the infection thread fuses with the cell membranes of the host cells, releasing the bacteria into them. The bacterial cells are then packaged in a membrane derived from the cell membrane. Branching of the thread enables the bacteria to infect many cells.

Ultimately the bacteria stop growing, begin to enlarge and differentiate into nitrogen fixing endosymbionts called bacteroids. The membrane surrounding the bacteroids is called peribacteroid membrane or symbiosome membrane and are thus separated from the cytoplasm of the host cell in a symbiosome.





The symbiosome membrane (SM) is a physical barrier between the host plant and nitrogen-fixing bacteria in the legume:rhizobia symbiosis, and represents a regulated interface for the movement of solutes between the symbionts that is under plant control. The primary nutrient exchange across the SM is the transport of a carbon energy source from plant to bacteroid in exchange for fixed nitrogen



At a biochemical level two channels have been implicated in movement of fixed nitrogen across the SM and a uniporter that transports monovalent dicarboxylate ions has been characterized that would transport fixed carbon. The aquaporin NOD26 may provide a channel for ammonia. Transport of several other solutes, including calcium and potassium, have been demonstrated in isolated symbiosomes, and genes encoding transport systems for the movement of iron, nitrate, sulfate, and zinc in nodules have been identified.

Carefully uproot some leguminous plants and then wash the root under running water for cleaning it from the soil particles.

Take a sterilizing agent and surface sterilize the nodules. After that wash the root nodules for removing any trace sterilizing agent prior to isolation. 0.1% mercuric chloride or 3-5% hydrogen peroxide can be used as sterilizing agent.

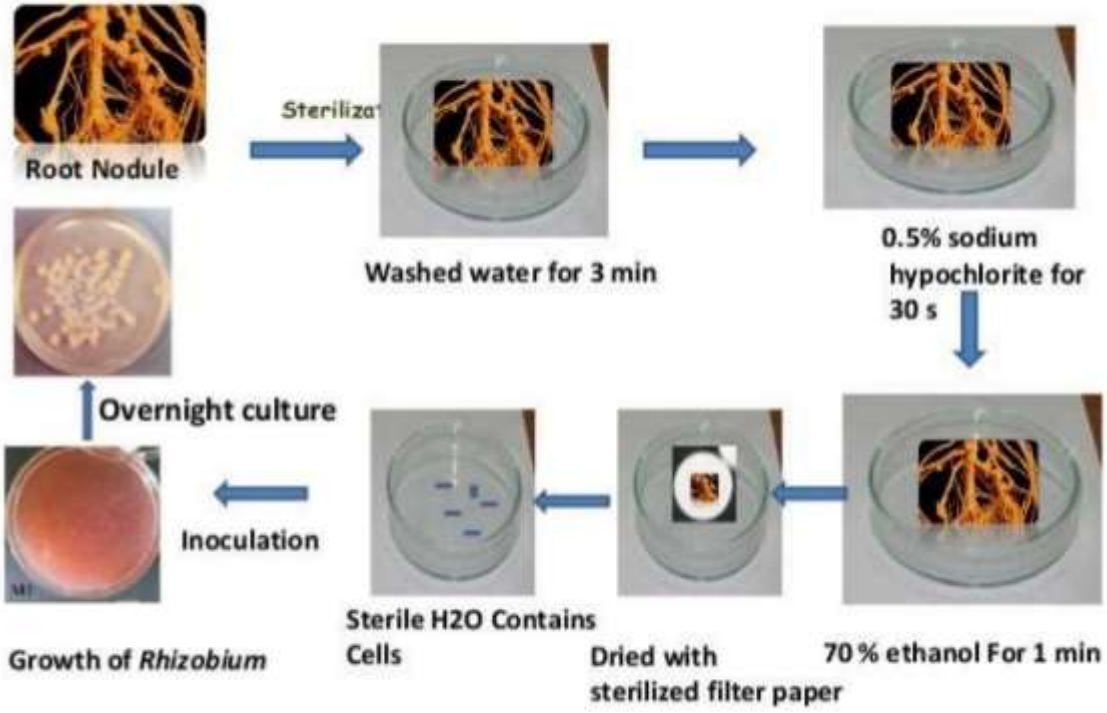
For complete sterilization immer
the nodules in the sterilizing age
about 4-5 minutes and then wash
with distilled water. After that ag
wash it with 70% ethanol and ag
with sterile water.

Wash the nodules in small aliquots of distilled water. Prepare YEMA plates and autoclave it. Then prepare 10-fold dilution of the nodular extract by taking 1 gm of the nodular extract and add it to 10 ml of distilled water. Then mix it well for getting the nodular extract suspension.

Then similarly, 1ml of the nodular extract is added to 9 ml of sterile water for making dilution 10^{-2} and similarly repeat up to 10^{-8}. Then 0.1 ml of suspension from 10^{-3} to 10^{-4} nodular extract are inoculated to sterilized YEMA plates by spread plate method. Then incubate the plate in incubator at 37°C for about 4-7 days.

Rhizobium colonies are see
large, mucoid and elevated
colonies.

ISOLATION OF BACTEIRAL BIOFERTILIZERS



ISOLATION THE RHIZOBIUM FROM LEGUMINOUS PLANT

REQUIREMENTS :

Legume plant roots, sterile distilled water, pipettes, testtubes, YEMA plates, 70% ethanol, 0.1% mercurous chloride solution.

PROCEDURE :

1.Collection of Root Nodules :

- Leguminous plants are carefully uproot
- the rootsystem is washed under runnin to remove the adhesive soil particles.
- The colour of the nodules varies from brown to pink.

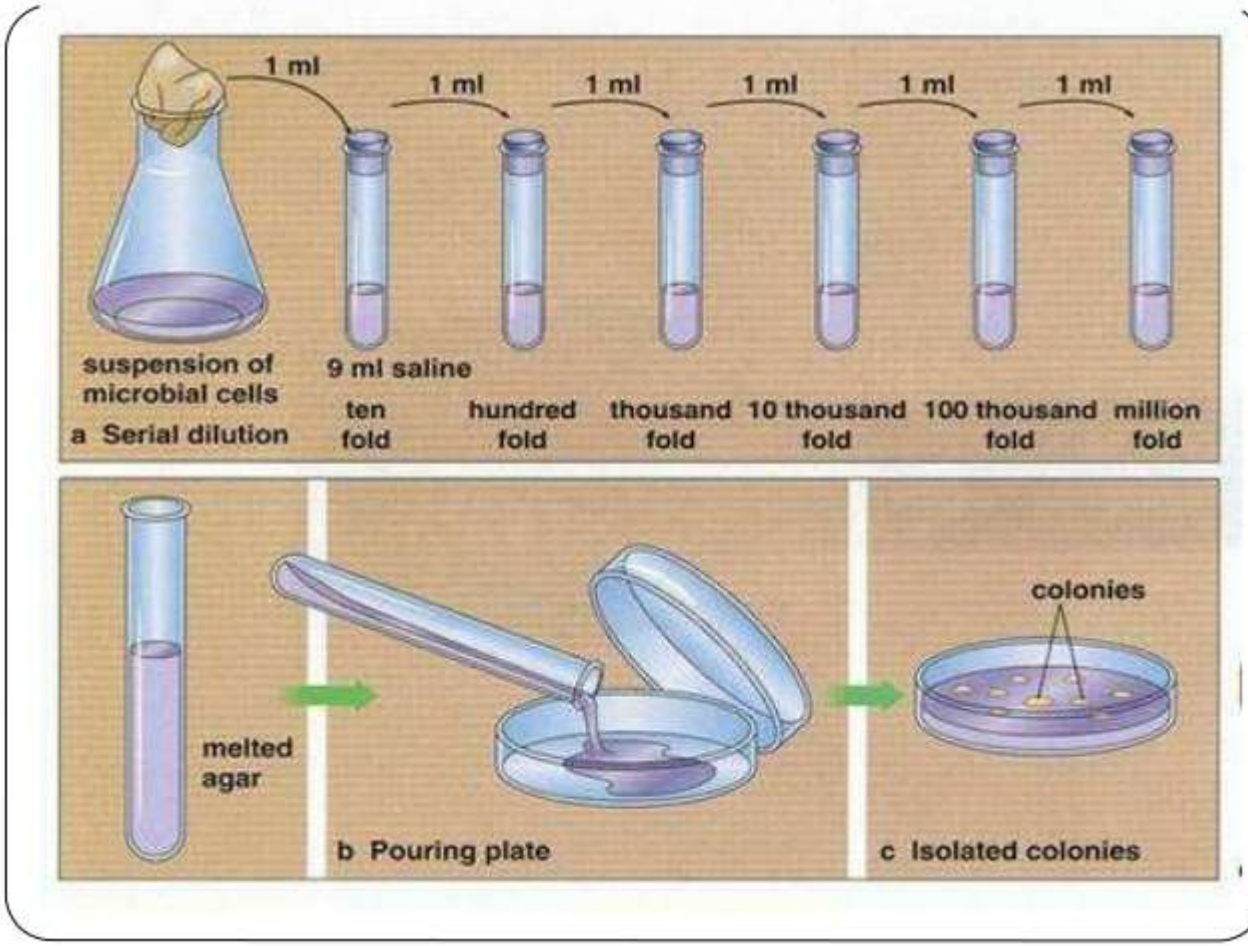
Collection of nodules

2.Surface Sterilisation of Leguminous Root Nodules

- The nodules must be surface sterilised by the sterilising agent and sterilising agent must be washed of from the nodules before they are used for isolation.
- The sterilising agent used us 0.1 % mercuric chloride or 3-5% hydrogen peroxids.
- The nodules are immersed in sterilising agents for 4-5 mints and are washed repeatedly with sterile distilled water.
- Then they are washed in 70% ethylalcohol followed by washing with sterile distilled water.

Isolation of Rhizobium by Serial Dilution Method

- Nodules are washed in a 70% Alcohol and sterile distilled water with the help of a glass rod.
- YEMA plates are prepared and sterilised by autoclave.
- Nodular extract is prepared by taking 1gm of nodular extract into 10 ml of sterile distilled water and mixed well to get suspension.
- 1ml of nodular extract suspension is diluted with 9ml of sterile distilled water making the dilution to 10^{-2},similarly making the dilution upto 10^{-8} are made separately for each nodular extract.
- Suspension 0.1ml of nodular extract suspension from 10 to the power of -3 to -8 dilutions are inoculated into sterile YEMA plates.
- The sample is spread throughout the YEMA plates


1 ml
1 ml
1 ml
1 ml
1 ml
1 ml
suspension of microbial cells
9 ml saline
a Serial dilution
ten fold
hundred fold
thousand fold
10 thousand fold
100 thousand fold
million fold
melted agar
b Pouring plate
colonies
c Isolated colonies

MECHANISM OF NITROGEN FIXATION

The conversion of free nitrogen into nitrogenous salts to make it available for absorption of plants

Basic requirements for Nitrogen fixation

- Nitrogenase and hydrogenase enzyme
- Protective mechanism against Oxygen
- Ferrodoxin
- Hydrogen releasing system or electron donor (Pyruvic acid or glucose/sucrose)
- Constant supply of ATP
- Coenzymes and cofactors - Co A, inorganic phosphate and Mg+2
- Cobalt and Molybdenum

Nitrogenase enzyme

- Active in anaerobic condition
- Made up of two protein subunits
- Non heme iron protein (Fe-protein or dinitrogen reductase)
  - Iron molybdenum protein (Mo Fe-protein or dinitrogenase)
  - Fe protein reacts with ATP and reduces second subunit which ultimately reduces N2 into ammonia
- $N_2 + 6H^+ + 6e^- \rightarrow 2NH_3$

- The reduction of N2 into NH3 requires 6 protons and 6 electrons
- 12 mols of ATP required
- One pair of electron requires 4 ATP
- The modified equation

$$N_2 + 8H^+ + 8e^- \longrightarrow 2NH_3 + H_2$$

- Hydrogen produced is catalyzed into protons and electrons by hydrogenase

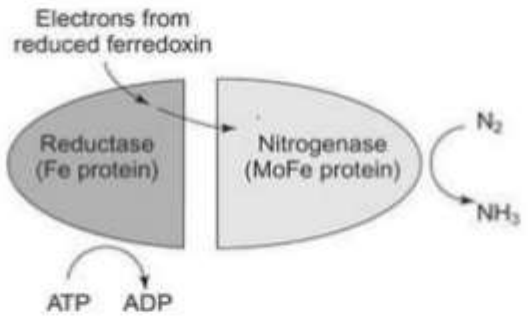
$$H_2 \xrightleftharpoons{\text{hydrogenase}} 2H^+ + 2e^-$$

PATHWAY OF NITROGEN FIXATION IN ROOT NODULES

- Glucose-6-phosphate acts as a electron donor
- Glucose-6-phosphate is converted to phosphogluconic acid

- NADPH donates electrons to ferrodoxin. Protons released and ferrodoxin is reduced
- Reduced ferrodoxin acts as electron carrier. Donate electron to Fe-protein to reduce it. Electrons released from ferrodoxin thus oxidized

- Nitrogenase actually consists of two proteins that work in tandem: the iron (Fe) protein and the molybdenum-iron (MoFe) protein.
- During the catalytic reduction of dinitrogen, the electrons are transferred from the Fe-protein to the MoFe-prote



- Nitrogenase is extremely sensitive to oxygen. Root nodules of nitrogen-fixing plants contain the oxygen-binding protein, leghemoglobin, which protects nitrogenase by binding molecular oxygen.