Biological Nitrogen fixation –Part-2

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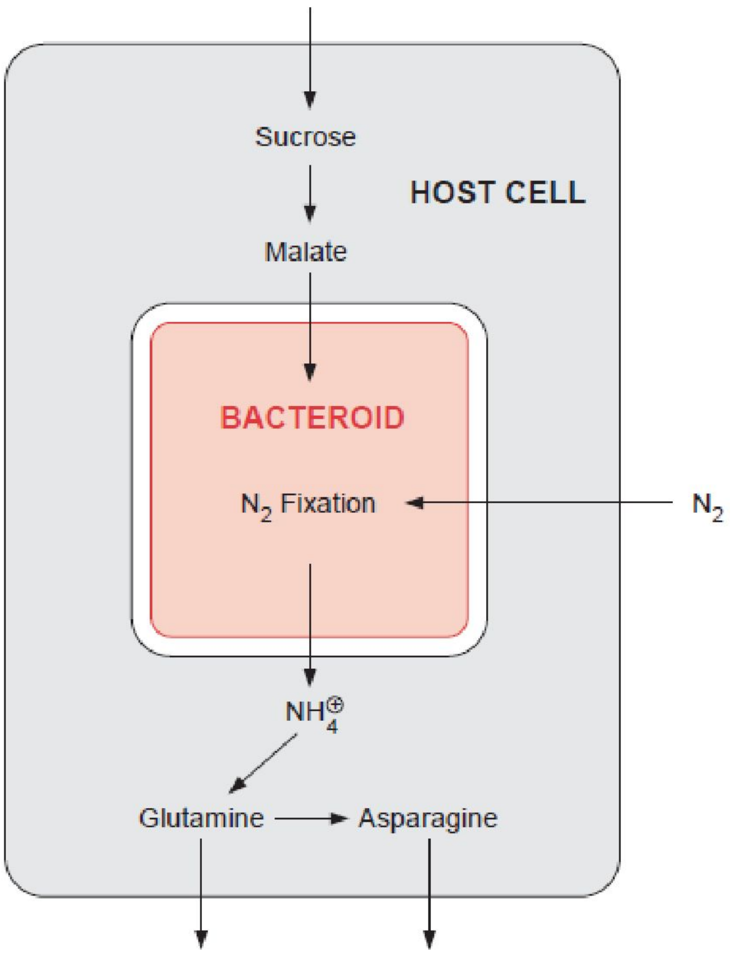
Metabolic products are exchanged between bacteroids and host cells.

Malate – provided by host cells to bacteroids.

- The main substrate provided by the host cells to the bacteroids is **malate,** formed from sucrose, which is delivered by the sieve tubes.
- Sucrose is metabolized by sucrose synthase, degraded by glycolysis to phosphoenolpyruvate, which is carboxylated to oxaloacetate, and latter is reduced to malate. Nodule cells contain high activities of phosphoenolpyruvate carboxylase. NH4+ is delivered as a product of N2 fixation to host cell, where it is then converted mainly into **Glutamine** and **asparagine** and then transported via xylem vessels to other plant parts.

- The nodules of some plants (e.g., those of soybean) export the fixed nitrogen as ureides (urea degradation products), especially **allantoin** and **allantoic Acid.**

- Malate taken up into the bacteroids is oxidized by the citrate cycle. The reducing equivalents thus generated are the fuel for fixation of N2.



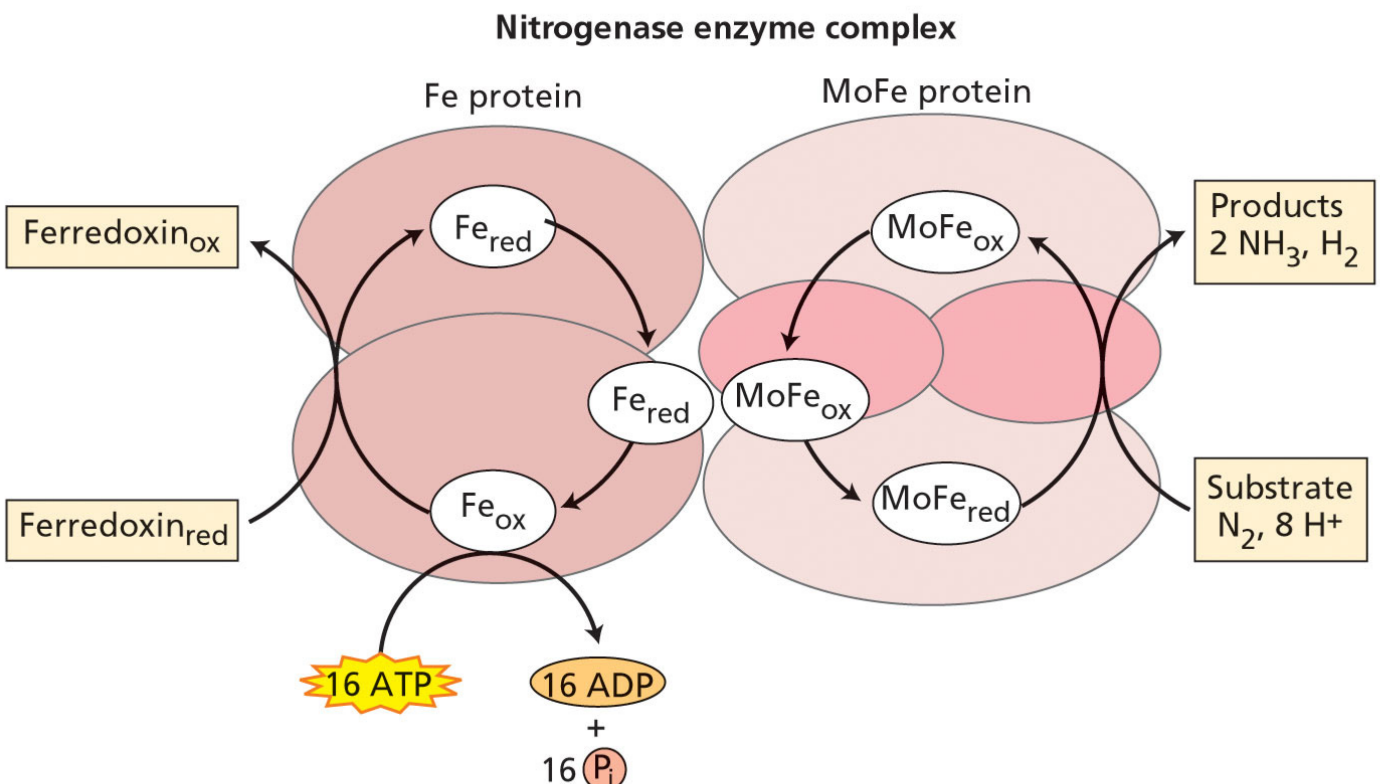
Metabolism of infected cells in a root nodule. Glutamine and asparagine are formed as the main products of N_2 fixation

Nitrogenase Reductase delivers electrons for nitrogenase reaction

Nitrogen fixation is catalyzed by the **nitrogenase complex**, a highly complex system with nitrogenase reductase and nitrogenase as the main components. This complex is highly conserved and is present in the cytoplasm of the bacteroids.

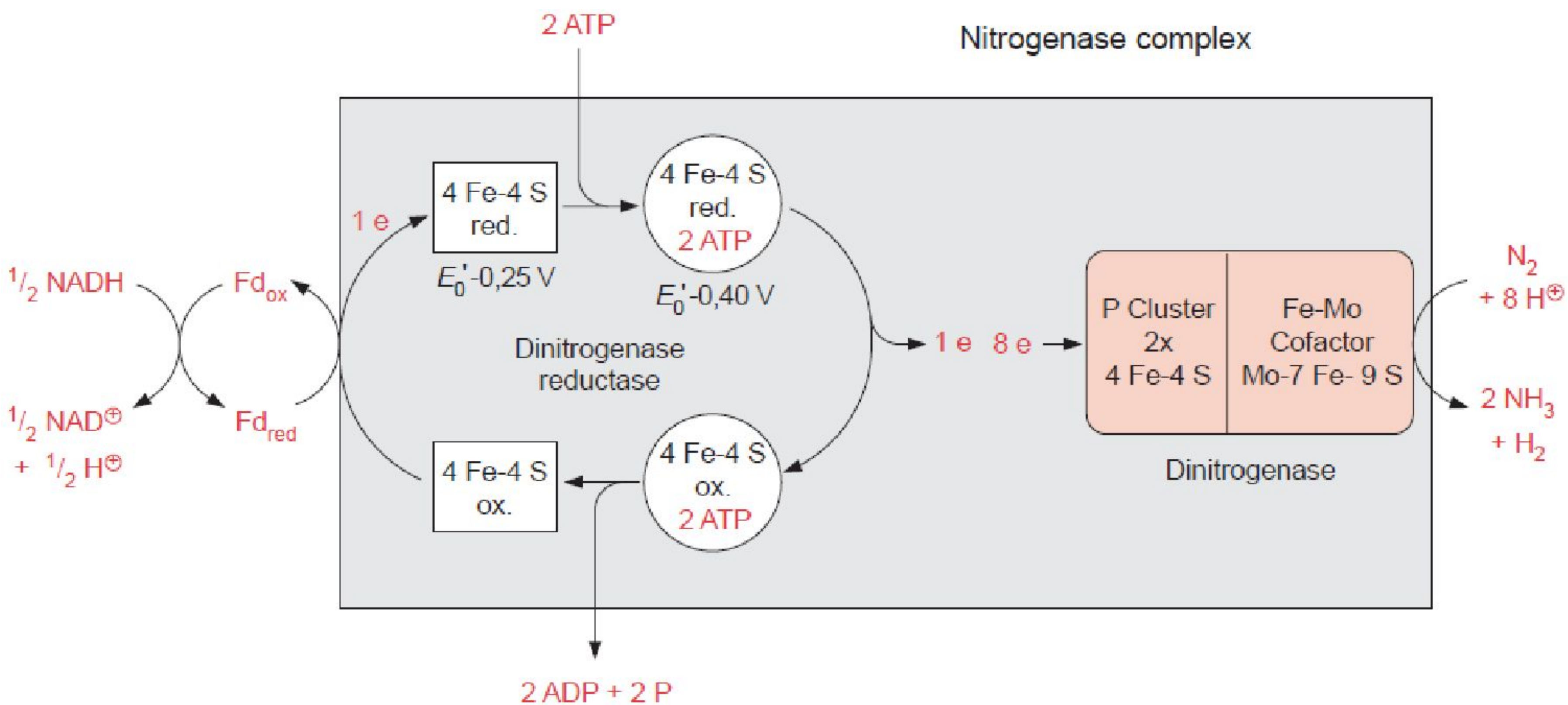
Nitrogenase complex

Bacteria that fix N2 possess enzyme complex nitrogenase, a highly conserved complex in cytoplasm of bacteroids. Has 2 components (enzs, neither of which is active without the other) . Component 1 is **nitrogenase** (k/a Mo-Fe protein, as it has Mo & Fe) and component 2 is **nitrogenase reductase**, a comparatively smaller mol. k/a Fe-protein. Both mol contain S and may contain **Fe-S centres.**



Nitrogenase Reductase delivers electrons for nitrogenase reaction: From NADH formed in citrate cycle, electrons are transferred via soluble ferredoxin to **nitrogenase reductase,** a 1-electron carrier, consisting of 2 identical subunits, which together form a **4Fe-4S cluster** and contain 2 binding sites for ATP.

After reduction of nitrogenase reductase, 2 molecules of ATP bind to it, resulting in a conformational change, by which the redox potential of the 4Fe-4S cluster is raised from -0.25 to -0.40 V. Following transfer of an electron to nitrogenase, 2 ATP molecules bound to protein are hydrolyzed to ADP and Pi, and then released from protein. As a result, the conformation with lower redox potential is restored and enzyme is again ready to take up 1 electron from ferredoxin. Thus. with consumption of 2 ATP molecules, 1 electron is transferred from NADH to nitrogenase by nitrogenase reductase



N2 as well as H+ are reduced by nitrogenase

Nitrogenase is an $\alpha_2\beta_2$ tetramer. The α and β subunits have a similar size and are similarly folded. The tetramer contains two catalytic centers, probably reacting independently of each other, and each contains a so-called P cluster, consisting of two **4Fe-4S clusters** and an iron molybdenum cofactor (**FeMoCo**). FeMoCo is a large redox center made up of Fe4S3 and Fe3MoS3, which are linked to each other via three inorganic sulfide bridges. N2 molecule is bound in the cavity of the FeMoCo center and that the electrons required for N2 fixation are transferred by the P cluster to the FeMoCo center.

Nitrogenase is able to reduce other substrates beside N2 (e.g., protons, which are reduced to molecular hydrogen):

$$2H^+ + 2e^- \xrightarrow{\textit{Nitrogenase}} H_2$$

During N2 fixation at least one molecule of hydrogen is formed per N2 reduced:

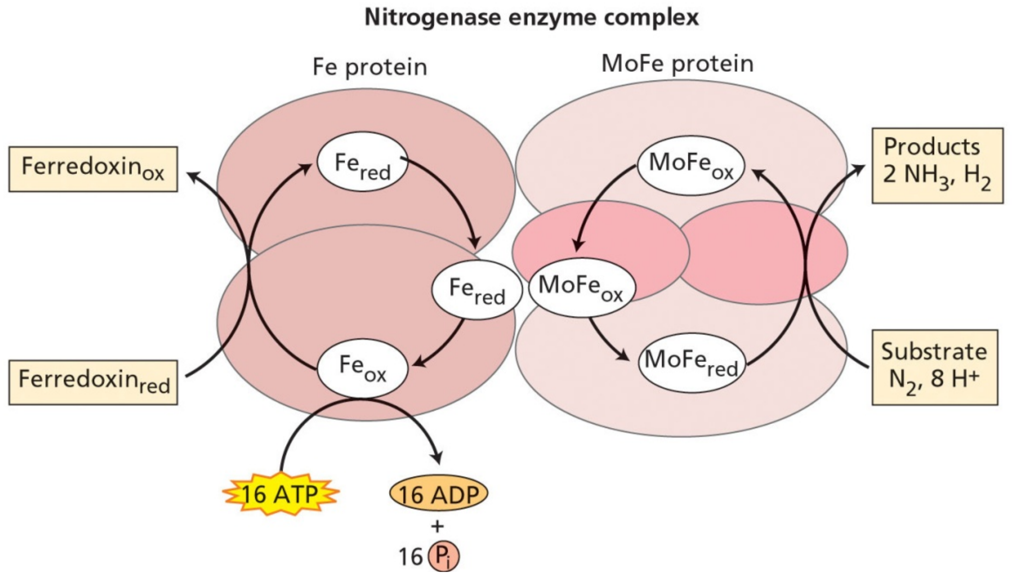
$$8H^+ + 8e^- + N_2 \xrightarrow{\textit{Nitrogenase}} 2NH_3 + H_2$$

Thus the balance of N2 fixation is at least:

$$N_2 + 4NADH + 4H^+ + 16ATP \longrightarrow 2NH_3 + H_2 + 4NAD^+ + 16ADP + 16P$$

For activity the nitrogenase complex requires:

1. A strong reducing agent like Ferridoxin or flavodoxins
2. ATP
3. A regulating system for NH3 production & utilization
4. A system which protects the N-fixing system from inhibition by molecular O2



Electrons are supplied via Ferredoxin first to Nitrogenase reductase, & then with the consumption of 16 mole ATP per mole N to nitrogenase. It is the Mo protein that catalyses actual reduction of N2. At the same time 2 H+ are reduced to H2.

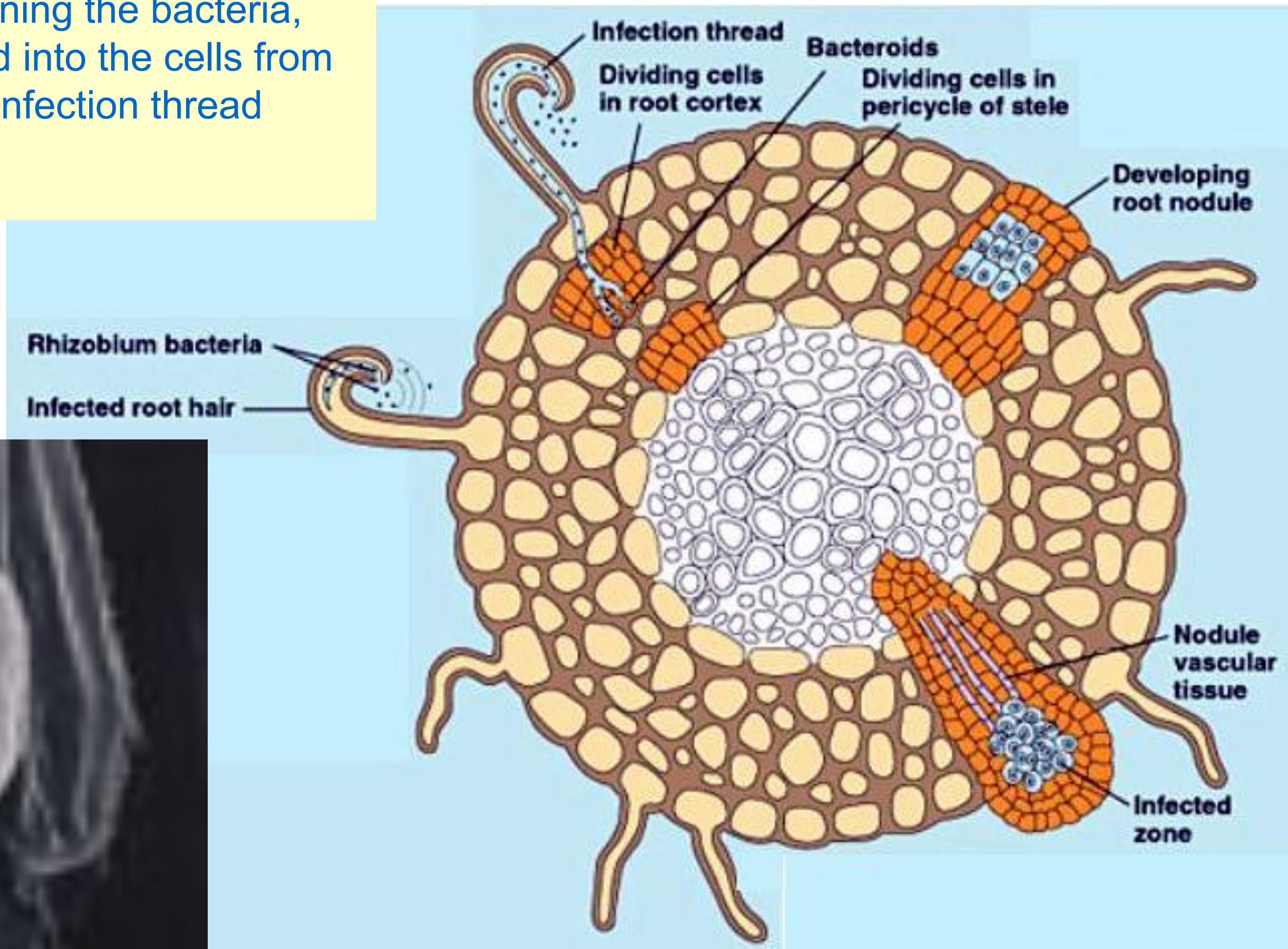
$N_2 + 8H^+ + 8e^- + 16\ ATP$ □ $2NH_3 + H_2 + 16ADP + 16\ Pi$

- In biological nitrogen fixation 2 moles of ammonia are produced from 1 mole of N_2 gas, using 16 moles of ATP and a supply of electrons and protons (H^+ ions). (Biological N fixation is energetically expensive)
- Nitrogenase & Nitrogenase reductase are located in cyt & are extremely sensitive to O2. This property is the reason why facultative anaerobes can fix N2 under anoxic conditions, cyanobacteria only in heterocysts & Rhizobium can fix N2 only in presence of leghemoglobin.

- This reaction is performed exclusively by prokaryotes (the bacteria and related organisms), using an O_2 sensitive enzyme complex termed nitrogenase.
- This enzyme consists of 2 proteins - an iron protein and a molybdenum-iron protein.

The bacteria penetrate the root cortex within the infection thread. Plant cells start dividing and vesicles containing the bacteria, bacteriods, bud into the cells from the branching infection thread

Growth continues in the affected regions of the cortex and pericycle and these fuse to form the nodule



In symbiotic nitrogen-fixing organisms such as *Rhizobium*, root nodules can contain *oxygen-scavenging molecules* such as ***leghaemoglobin***, which shows as a pink colour when the active nitrogen-fixing nodules of legume roots are cut open. Leghaemoglobin may regulate the supply of oxygen to the nodule tissues in the same way as haemoglobin regulates the supply of oxygen to mammalian tissues

- These plants thus protects the nitrogenase of bacteria from O_2. It does this by surrounding the bacteria with a red *O_2-binding pigment* k/a **Leghemoglobin** that resembles Hb of animal blood in many aspects..

Clover root nodules.

Leghaemoglobin is found only in the nodules and is not produced by either the bacterium or the plant when grown alone.

N2 fixation can proceed only at very low oxygen concentrations

- Nitrogenase is extremely sensitive to oxygen. Thus N2 fixation can proceed only at very low oxygen concentrations. The nodules form an anaerobic compartment. Since N2 fixation depends on the uptake of nitrogen from the air, the question arises how is the enzyme protected against the oxygen present in air? The answer is that oxygen, which has diffused together with nitrogen into the nodules, is consumed by the **respiratory chain** contained in the bacteroid membrane. Due to a very high affinity of the bacteroid cytochrome-*a*/*a*3 complex, respiration is still possible with an O2 concentration of only 10^{-9} mol/L.
- As described previously, at least a total of 16 molecules of ATP are required for the fixation of one molecule of N2. Upon oxidation of one molecule of NADH, about 2.5 molecules of ATP are generated by the mitochondrial respiratory chain.
- In bacterial respiratory chain, which normally has a lower degree of coupling than that of mitochondria, only ~ 2 molecules of ATP may be formed per molecule of NADH oxidized. Thus ~ 4 molecules of O2 have to be consumed for formation of 16 molecules of ATP.
- If the bacteroids possess a hydrogenase, due to the oxidation of H_2 formed during N2 fixation, oxygen consumption is further increased by half an O_2 molecule.
- Thus during N_2 fixation, for each N_2 molecule at least four O_2 molecules are consumed by bacterial respiration ($O_2/N_2 \geq 4$). In contrast, the O_2/N_2 ratio in air is about 0.25. This comparison shows that air required for N_2 fixation contains in relation to nitrogen far too little oxygen.

- The outer layer of the nodules is a considerable **diffusion barrier** for the entry of air. The diffusive resistance is so high that bacteroid respiration is limited by the uptake of oxygen. This leads to the astonishing situation that N2 fixation is limited by the influx of O2 for formation of the required ATP.
- Fraser Bergersen have shown in soybean nodules that a doubling of the O2 content in air (with a corresponding decrease of the N2 content) resulted in doubling of rate of N2 fixation. But, because of the O2 sensitivity of nitrogenase, a further increase in O2 resulted in a steep decline in N2 fixation.
- Since the bacterial respiratory chain is located in the membrane and nitrogenase in the interior of the bacteroids, O2 is kept at a safe distance from nitrogenase. The high diffusive resistance for O2, which, as shown in the experiment, can limit N2 fixation, ensures that even at low temperatures, at which N2 fixation and the bacterial respiration are slowed down, oxygen is kept away from the nitrogenase complex.
- The cells infected by rhizobia form **leghemoglobin**, which is very similar to the myoglobin of animals, but has a 10-fold higher affinity for oxygen.
- The oxygen concentration required for half saturation of leghemoglobin amounts to only 10–20 \ 10-9 mol/L. Leghemoglobin is located in the cytosol of the host cell—outside the peribacteroid membrane—and present there in unusually high concentrations (3 \ 10^{-3} mol/L in soybeans). Leghemoglobin can amount to 25% of the total soluble protein of the nodules and gives them a pink color. It has been proposed that leghemoglobin plays a role in the transport of oxygen within the nodules. However, it is more likely that it serves as an **oxygen buffer** to ensure continuous electron transport in the bacteroids at the very low prevailing O2 concentration in the nodules.

The energy costs for utilizing N_2 as a nitrogen source are much higher than for the utilization of NO_3^-

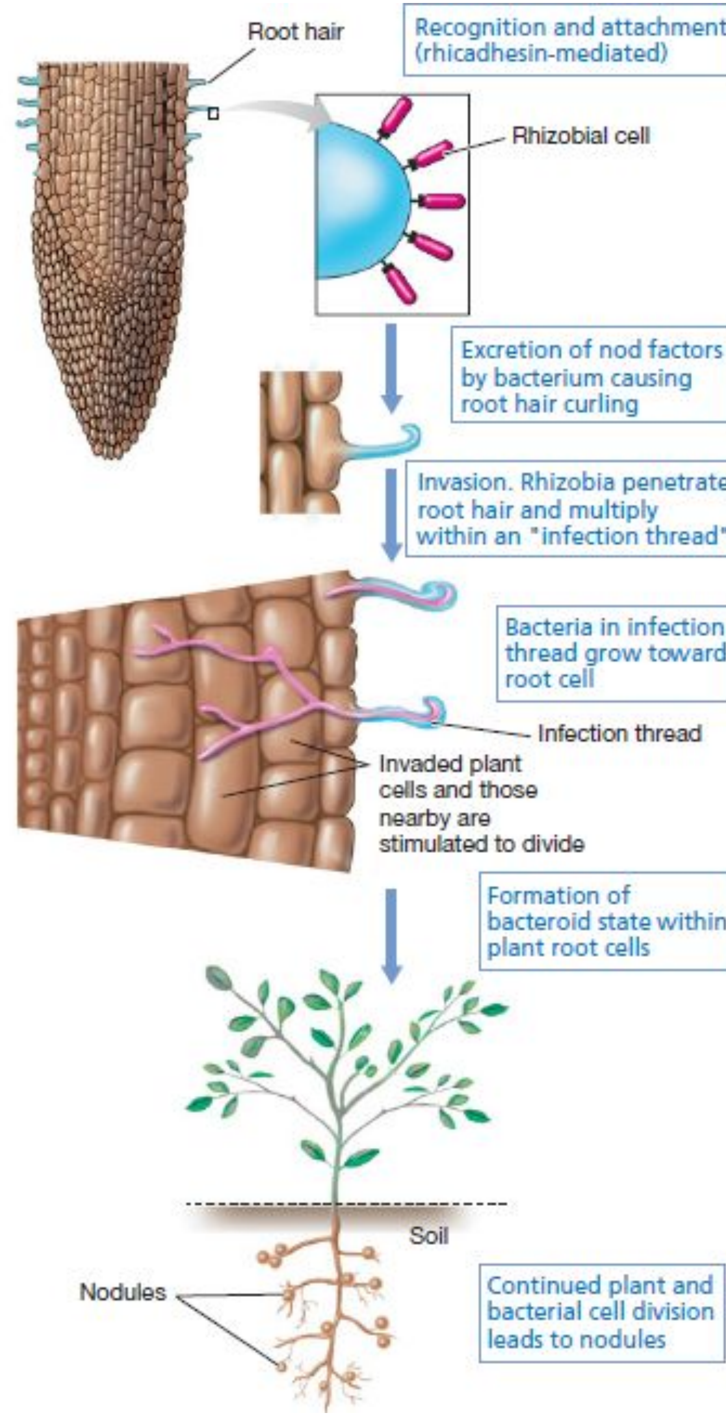
At least six molecules of NADH are consumed in the formation of one molecule of NH_4^+ from molecular nitrogen. Assimilation of nitrate, in contrast, requires only four NAD(P)H equivalents for the formation of NH_4^+. In addition, it costs the plant much metabolic energy to form the nodules. Therefore it is much more economical for plants, which have the potential to fix N_2 with the help of their symbionts, to satisfy their nitrogen demand by nitrate assimilation. This is why the formation of nodules is regulated. Nodules are formed only when the soil is nitrate-deficient. The advantage of this symbiosis is that legumes and actinorhizal plants can grow in soils with very low nitrogen content, where other plants have no chance.

Steps in Root Nodule Formation in a legume infected by Rhizobium

The steps are as follows:

1. Recognition of the correct partner by both plant and bacterium and attachment of the bacterium to the root hairs
2. Secretion of oligosaccharide signaling molecules (NOD FACTORS) by the bacterium
3. Bacterial invasion of the root hair
4. Movement of bacteria to main root by way of infection thread
5. Formation of modified bacterial cells (*bacteroids*) within the plant cells and development of the N_2-fixing state
6. Continued plant and bacterial cell division, forming the mature root nodule

Another mechanism of nodule formation that does not require nod factors is used by some species of phototrophic rhizobia. This mechanism has yet to be elucidated, but appears to require bacterial production of cytokinins. Cytokinins are plant hormones, derived from adenine or phenylurea, necessary for cell growth and differentiation.



Attachment and Infection

- A RHIZOBIAL cell penetrates into the root hair, which curls in response to substances (Nod factors) secreted by the bacterium

o The bacterium then induces formation by the plant of a cellulosic tube, called the infection thread, which spreads down the root hair

Bacteroids

- The rhizobia multiply rapidly within the plant cells and become transformed into swollen, misshapen, and branched cells called **bacteroids.**
- A microcolony of bacteroids becomes surrounded by portions of the plant cytoplasmic membrane to form a structure called the symbiosome.

ONLY AFTER the sybiosomes for does N2 fixation begins

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Nodule Formation:

Rhizobial genes that direct the steps in nodulation of a legume are called **nod genes**. It is thought that the ability to form nodules has independently emerged multiple times through the horizontal transfer of such genes as nod and nif that are located on plasmids or transferable regions of chromosomal DNA.

In *Rhizobium leguminosarum* biovar viciae, which nodulates peas, ten nod genes have been identified. The nodABC genes encode proteins that produce oligosaccharides called **nod factors**; these induce root hair curling and trigger cell division in the pea plant, eventually leading to formation of the nodule

Nodule Formation
- **Nod Factors** – proteins that produce oligosaccharides; these induce root hair curling and trigger cell division in the pea plant, eventually leading to formation of the nodule
- NodD inducers are plant **flavonoids**, organic molecules widely secreted by plants
o Some **flavonoids** that are structurally very closely related to nod inducers in R. leguminosarum biovar viciae INHIBIT nod gene expression in other rhizobial species

Leghomoglobin - In the absence of its bacterial symbiont, a legume cannot fix N_2
o RHIZOBIA can fix N_2 when grown in pure culture under MICROAEROPHILIC conditions (due to nitrogenase inactivation from high levels of O_2)
o In nodule, O_2 levels are precisely controlled by the O_2-binding protein leghemoglobin

 Leghemoglobin (iron-containing protein) production is INDUCED through the interaction of the plant and bacterial partners
 Functions as an "OXYGEN BUFFER," ***cycling between the oxidized (Fe3+) and reduced (Fe2+) forms of iron to supply sufficient O_2 for BACTERIAL RESPIRATION while keeping unbound O_2 within the nodule low***

Summary: Legume-root nodule symbiosis

- ***Rhizobium, Bradyrhizobium, Sinorhizobium, Mesorhizobium, Azorhizobium and Photorhizobium*** are genera of gm –ve Alphaproteobacteria that can grow in soil or infect leguminous plants and establish a symbiotic relationship. (collectively k/a **rhizobia**)
- Infection of roots of a legume by these bacteria leads to formation of **root nodules** that fix N_2. (of enormous agricultural importance as increases combined N2 in soil). As unfertilized bare soils are N2 deficient, nodulated legumes grow well in areas where other plants grow poorly.
- Rhizobia= microaerophiles., but their nitrogenases – inactivated by O_2.
- In nodule, precise O2 levels are controlled by O_2-binding protein **leghemoglobin.**
- This Fe- containing protein present in healthy N_2 fixing nodules and is induced by interation of plant host and bacterial symbiont.
- Acts as O_2 buffer, cycling between Fe^{3+} and Fe^{2+} forms to keep unbound O_2 in low amounts. Ratio of leghemoglobin-bound O_2 to free O_2 is 10,000:1.

Soil Microorganism Associations with Vascular Plants

- Plants are covered by microbes, on both the above- and below ground surfaces. A wide variety of microbes are found on and in aerial surfaces of plants, called the **phyllosphere.** The genera present on plant leaves and stems include *Sphingomonas,* which is especially equipped to survive with the high levels of UV irradiation occurring on these plant surfaces (protective effect)

- **Rhizosphere and Rhizoplane Microorganisms**
- Plant roots release a wide variety of materials to their surrounding soil, -various *alcohols, ethylene, sugars, amino and organic acids, vitamins, nucleotides, polysaccharides, and enzymes***.** These materials create unique environments for the soil microorganisms.
- These environments include the **rhizosphere,** first described by Lorenz Hiltner in 1904, **the volume of soil around the root influenced by the materials released from the root.**
- The plant root surface, termed the **rhizoplane,** also provides a unique environment for microbes, as these gaseous, soluble, and particulate materials move from the plant to the soil.
- A wide range of **bacteria in the rhizosphere can promote plant growth**, orchestrated by rhizosphere bacteria that **communicate with the plant** using complex chemical signals. These chemical signal compounds, including auxins, gibberellins, glycolipids, and cytokinins, are only now beginning to be fully appreciated in terms of their biotechnological potential.
- **Plant growth promoting rhizobacteria** (**PGPR**) include the genera *Pseudomonas* and *Achromobacter.* These can be added to the plant, even in the seed stage, if the bacteria have the required attachment surface proteins. The genes that control the expression of these attachment proteins are still being identified.

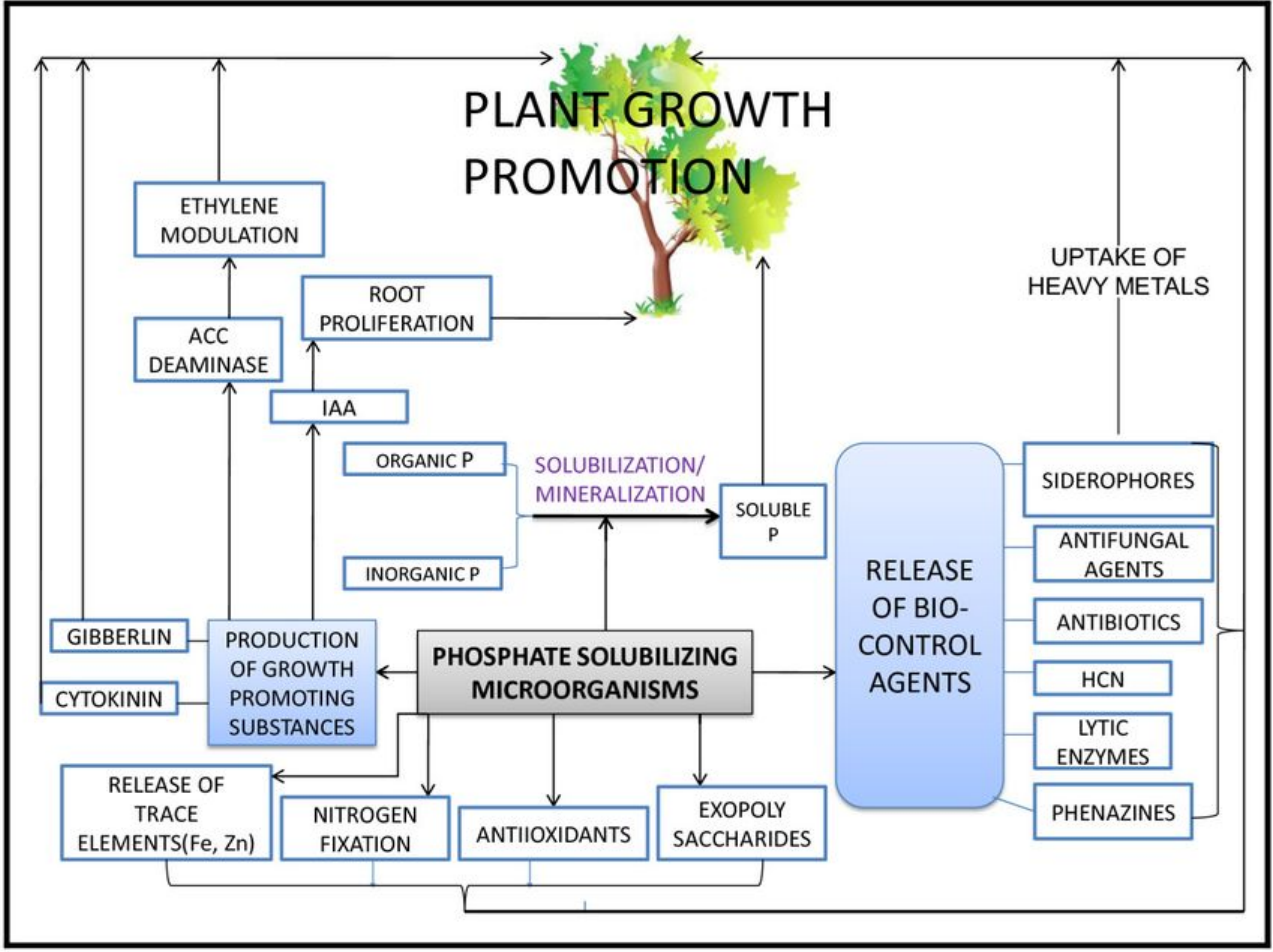
Bio-fertilizers are micro-organisms which bring about nutrient enrichment of soil by enhancing the availability of nutrients to crops. The micro-organisms which act as bio-fertilizers are bacteria, cyanobacteria (blue green algae) and mycorrhizal fungi. Bacteria and cynobacteria have the property of nitrogen fixation while mycorrhizal fungi preferentially withdraw minerals from organic matter for the plant with which they are associated.

(B) Phosphatic/Microphos Biofertilizers:
They release phosphate from bound and insoluble states

1)Bacterial: Bacillus *polymyxa*, *Pseudomonas st*riata,
2)Fungal*: (*I) As Aspergillus species. **(ii) Mycorrhiza**

- A critical process that occurs on the surface of the plant, and particularly in the root zone, is **associative nitrogen fixation,** in which the nitrogen-fixing microbes are on the surface of the plant root, the rhizoplane, as well as in the rhizosphere.
- This process is carried out by *Azotobacter, Azospirillum* and *Acetobacter.*
- contribute to nitrogen accumulation by tropical grasses.

- Major contribution may not be in nitrogen fixation but in production of **growth promoting hormones** that **increase root hair development** and thus greater **ability of the plant to take up nutrients**.
- area of research- particularly important in tropical agricultural areas.



PLANT GROWTH PROMOTION
ETHYLENE MODULATION
ROOT PROLIFERATION
ACC DEAMINASE
IAA
UPTAKE OF HEAVY METALS
ORGANIC P
SOLUBILIZATION/ MINERALIZATION
SOLUBLE P
INORGANIC P
RELEASE OF BIO-CONTROL AGENTS
SIDEROPHORES
ANTIFUNGAL AGENTS
ANTIBIOTICS
HCN
LYTIC ENZYMES
PHENAZINES
GIBBERLIN
CYTOKININ
PRODUCTION OF GROWTH PROMOTING SUBSTANCES
PHOSPHATE SOLUBILIZING MICROORGANISMS
RELEASE OF TRACE ELEMENTS(Fe, Zn)
NITROGEN FIXATION
ANTIIOXIDANTS
EXOPOLY SACCHARIDES

Plants improve their nutrition by symbiosis with fungi

- Frequently plant growth is limited by the supply of nutrients other than nitrate (e.g., phosphate). Because of its low solubility, the extraction of phosphate by the roots from the soil requires very efficient uptake systems. For this reason, plant roots possess very **high affinity transporters**, with a half saturation of 1 to 5 mM phosphate, where the phosphate transport is driven by proton symport, similar to the transport of nitrate. In order to increase the uptake of phosphate, but also of other mineral nutrients (e.g., nitrate and potassium), most plants enter a symbiosis with fungi.
- Fungi are able to form a mycelium with hyphae, which have a much lower diameter than root hairs and which are therefore well suited to penetrate soil particles and to mobilize their nutrients. The symbiotic fungi (microsymbionts) deliver these nutrients to the plant root (macrosymbiont) and are in turn supplied by the plant with substrates for maintaining their metabolism.
- The supply of the symbiotic fungi by the roots demands a high amount of assimilates. For this reason, many plants make the **establishment of the mycorrhiza dependent on the phosphate availability in the soil**. In the case of a high phosphate concentration in the soil, when the plant can do without, it treats the fungus as a pathogen and activates its defense system against fungal infections

Mycorrhizae

Mycorrhiza (pl-Mycorrhizae Frank,. 1885): It is a mutually beneficial or symbiotic association of a fungus with the root of a higher plant, in which nutrients are transferred in both directions. The fungus provides nutrients such as phosphorus from the soil to the plant, and the plant in turn transfers carbohydrates to the fungus.

From fungal spores produced in culture or from root scrapings of infected plants, soil inoculants are produced that enhance plant growth.

- Mycorrhizae are **fungus-root associations**, The term "mycorrhizae" comes from the Greek words meaning fungus and roots. These microorganisms contribute to plant functioning in natural environments, agriculture, and reclamation. Roots of ~ 95% of all vascular plants are normally involved in symbiotic associations with mycorrhizae.

Two Kinds of Mycorrhizae:

(a) Endomycorrhiza : the fungus penetrates the plant cells where it forms characteristic structures, including arbuscules and coils. Vesicles are not consistently observed.

(b) Ectomycorrhiza (= Ectotrophic Mycorrhiza): The fungus forms a mantle extensive sheath on the surface of root with only a slight penetration into the root tissue itself.

Mycorrhiza advantages : i) Absorption of water,

(ii) Solubilisation of organic matter of soil humus, release of inorganic nutrients, absorption and their transfer to root,

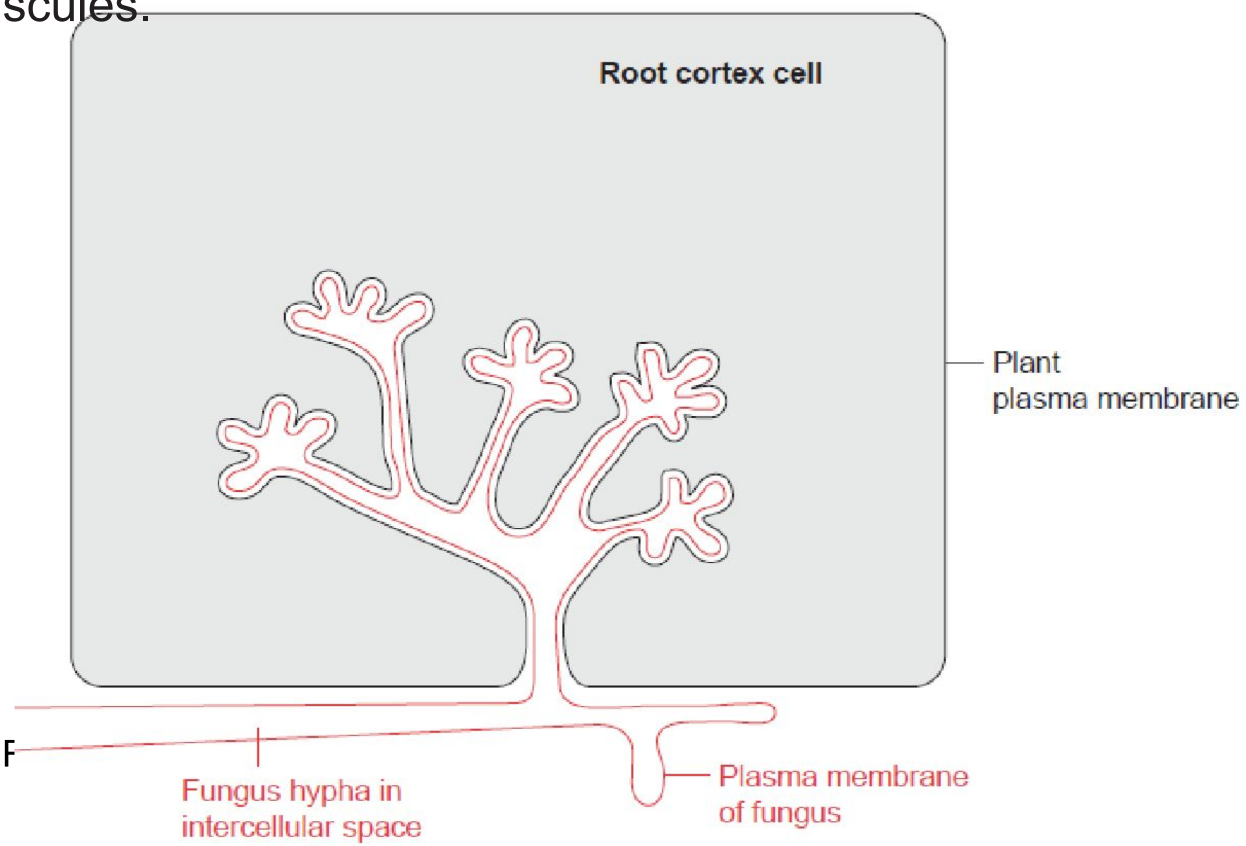
(iii) Direct absorption of minerals from the soil over a large area and handing over the same to the root. Plants with ectomycorrhiza are known to absorb 2-3 times more of nitrogen, phosphorus, potassium and calcium,

(iv) The fungus secretes antimicrobial substances which protect the young roots from attack of pathogens. Ectomycorrhiza occurs in the trees like Eucalyptus, Oak (Quercus), Peach, Pine, etc. The fungus partner is generally specific. It belongs to basidiomycetes.

The arbuscular mycorrhiza is widespread

Arbuscular mycorrhiza has been detected in >80% of all plant species. In this symbiosis the fungus penetrates the cortex of plant roots and forms there a network of hyphae, which protrude into cortical cells and form there treelike invaginations, termed **arbuscules** or form **hyphal coils**. The arbuscules form a large surface, enabling an efficient exchange of substances between the fungus and the host. The fungus delivers phosphate, nitrate, K+-ions, and water, and the host delivers carbohydrates. The arbuscules have a lifetime of only max. 2 weeks, but the subsequent degeneration does not damage the corresponding host cell. Thus, the maintenance of symbiosis requires a constant formation of new arbuscules.

Schematic representation of an arbuscule. The hypha of a symbiotic fungus traverses the rhizodermis cells and spreads in the intercellular space of the root cortex. From there treelike invaginations into the inner layer of the cortex are formed. The large boundary surface between the host and the microsymbiont enables an effective exchange of substances.



Ectomycorrhiza supplies trees with nutrients: Many trees in temperate and cool climates form a symbiosis with fungi termed **Ectomycorrhiza**. In this the hyphae of the fungi do not penetrate the cortex cells, but colonize only the surface and the intercellular space of the cortex with a network of hyphae, termed **Hartig net**, which is connected with a very extensive mycel in the soil. Microsymbionts are *Asco*- and *Basidiomycetae* from more than 60 genera, including several mushrooms. The plant root tips colonized by the fungi are thickened and do not form any root hairs. The uptake of nutrients and water is delegated to the microsymbiont, which in turn is served by plant with substrates to maintain its metabolism, Exchange of substances occurs, as in arbuscular mycorrhiza, via closely neighbored fungal and plant plasma membranes. The ectomycorrhiza also enables a transfer of assimilates between adjacent plants.

Ectomycorrhiza is of great importance for the growth of trees, such as beech, oak, and pine, as it increases the uptake of phosphate by a factor of three to five.

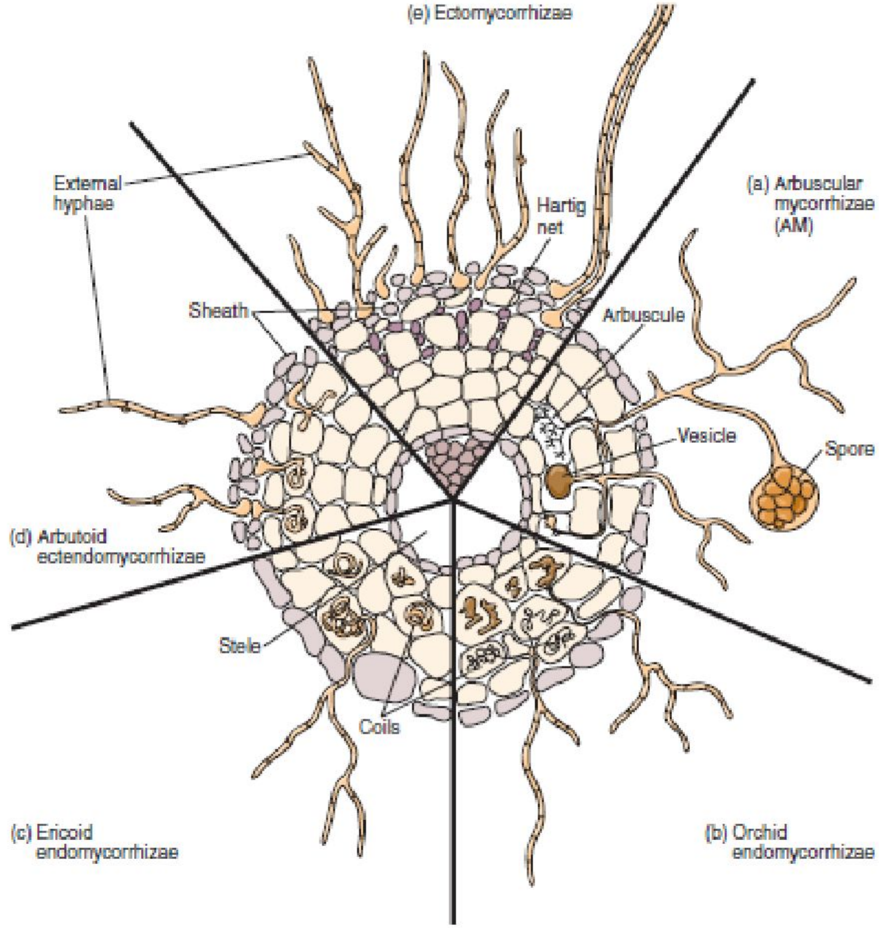


Figure 30.11 **Mycorrhizae.** Fungi can establish mutually beneficial relationships with plant roots, called mycorrhizae. Root cross sections illustrate different mycorrhizal relationships. See text for details.

Arbuscular mycorrhizae (endomycorrizae)
Arbuscules in root. Endotrophic (orchids, non pathogenic penetration) vs AM (mos common, intracellular penetration, cannot grow on culture, high SA for nutrient exchange - covered by plant periarbuscular membrane (seperates cell from fungus, therefore, non pathogenic) Root secrete strigolactomes sensed by germinating AM fungal spore -> release lipochito-oligosaccharides and COs -> molecular trigger -> cyoplasmic Ca -> activate AM fungal induced gene exp-> pre penetration -> allow for abuscular growth)

Root nodule symbioses may have evolved from a preexisting pathway for the formation of arbuscular mycorrhiza:

In both arbuscular mycorrhiza and root nodule symbiosis cases, receptor-like kinases are involved, linked to **signal cascades**, which induce the synthesis of the proteins required for the controlled infection. These signal cascades probably involve G-proteins, MAP-kinases, and Ca++ ions as messenger. For several legume species, mutants are known that have lost the ability to establish both root nodule symbiosis and arbuscular mycorrhiza. One of the genes that cause such a defect in different legume species has been identified to encode an RLK, indicating that this RLK has an essential function in the formation of both arbuscular mycorrhiza and root nodule symbiosis.