Biofertilizer

Blue-green algae cultured in specific media. Blue-green algae can be helpful in agriculture as they have the capability to fix atmospheric nitrogen to soil. This nitrogen is helpful to the crops. Blue-green algae is used as a bio-fertilizer.

A **biofertilizer** (also *bio-fertilizer*) is a substance which contains living microorganisms which, when applied to seed, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant. Bio-fertilizers add nutrients through the natural processes of nitrogen fixation, solubilizing phosphorus, and stimulating plant growth through the synthesis of growth-promoting substances. Bio-fertilizers can be expected to reduce the use of chemical fertilizers and pesticides. The microorganisms in bio-fertilizers restore the soil's natural nutrient cycle and build soil organic matter. Through the use of bio-fertilizers, healthy plants can be grown, while enhancing the sustainability and the health of the soil. Since they play several roles, a preferred scientific term for such beneficial bacteria is "plant-growth promoting rhizobacteria" (PGPR). Therefore, they are extremely advantageous in enriching soil fertility and fulfilling plant nutrient requirements by supplying the organic nutrients through microorganism and their byproducts. Hence, bio-fertilizers do not contain any chemicals which are harmful to the living soil.

Bio-fertilizers eco friendly organic agro-input and more cost-effective than chemical fertilizers. Bio-fertilizers such as Rhizobium, Azotobacter, Azospirillum and blue green algae (BGA) have been in use a long time. Rhizobiuminoculant is used for leguminous crops. Azotobacter can be used with crops like wheat, maize, mustard, cotton, potato and other vegetable crops. Azospirillum inoculations are recommended mainly for sorghum, millets, maize, sugarcane and wheat. Blue green algae belonging to a general cyanobacteria genus, *Nostoc* or *Anabaena* or *Tolypothrix* or *Aulosira*, fix atmospheric nitrogen and are used as inoculations for paddy crop grown both under upland and low-land conditions. *Anabaena* in association with water fern Azolla contributes nitrogen up to 60 kg/ha/season and also enriches soils with organic matter.

Other types of bacteria, so-called phosphate-solubilizing bacteria, such as Pantoea agglomerans strain P5 or Pseudomonas putida strain P13, are able to solubilize the insoluble phosphate from organic and inorganic phosphate sources. In fact, due to immobilization of phosphate by mineral ions such as Fe, Al and Ca or organic acids, the rate of available phosphate (Pi) in soil is well below plant needs. In addition, chemical Pi fertilizers are also immobilized in the soil, immediately, so that less than 20 percent of added fertilizer is absorbed by plants. Therefore, reduction in Pi resources, on one hand, and environmental pollutions resulting from both production and applications of chemical Pi fertilizer, on the other hand, have already demanded the use of new generation of phosphate fertilizers globally known as phosphate-solubilizing bacteria or phosphate bio-fertilizers.

Benefits

A bio-fertilizer provides the following benefits:

1. Since a bio-fertilizer is technically living, it can symbiotically associate with plant roots. Involved microorganisms could readily and safely convert complex organic material in simple compounds, so that plants are easily taken up. Microorganism function is in long duration, causing improvement of the soil fertility. It maintains the natural habitat of the soil. It increases crop yield by 20-30%, replaces chemical nitrogen and phosphorus by 25%, and stimulates plant growth. It can also provide protection against drought and some soil-borne diseases.
2. Bio-fertilizers are cost-effective relative to chemical fertilizers. They have lower manufacturing costs, especially regarding nitrogen and phosphorus use.
3. It is environmentally friendly in that it not only prevents damaging the natural source but also helps to some extent cleanse the plant from precipitated chemical fertilizers.

List of Leading Countries in Biofetilizer

Cuba is now one of the world leaders in biofertilisers, with a highly impressive production of organic food.

1. Biofertilizers

Biofertilizers are defined as preparations containing living cells or latent cells of efficient strains of microorganisms that help crop plants' uptake of nutrients by their interactions in the rhizosphere when applied through seed or soil. They accelerate certain microbial processes in the soil which augment the extent of availability of nutrients in a form easily assimilated by plants.

Very often microorganisms are not as efficient in natural surroundings as one would expect them to be and therefore artificially multiplied cultures of efficient selected microorganisms play a vital role in accelerating the microbial processes in soil.

Use of biofertilizers is one of the important components of integrated nutrient management, as they are cost effective and renewable source of plant nutrients to supplement the chemical fertilizers for sustainable agriculture. Several microorganisms and their association with crop plants are being exploited in the production of biofertilizers. They can be grouped in different ways based on their nature and function.

S. No.	Groups	Examples
N2 fixing Biofertilizers		
1.	Free-living	*Azotobacter, Beijerinkia, Clostridium, Klebsiella, Anabaena, Nostoc,*
2.	Symbiotic	*Rhizobium, Frankia, Anabaena azollae*
3.	Associative Symbiotic	*Azospirillum*
P Solubilizing Biofertilizers		
1.	Bacteria	*Bacillus megaterium* var. *phosphaticum, Bacillus subtilis Bacillus circulans, Pseudomonas striata*
2.	Fungi	*Penicillium sp, Aspergillus awamori*
P Mobilizing Biofertilizers		
1.	Arbuscular mycorrhiza	*Glomus sp.,Gigaspora sp.,Acaulospora sp.*, *Scutellospora sp. & Sclerocystis sp.*
2.	Ectomycorrhiza	*Laccaria sp., Pisolithus sp.*, *Boletus sp.*, *Amanita sp.*
3.	Ericoid mycorrhizae	*Pezizella ericae*
4.	Orchid mycorrhiza	*Rhizoctonia solani*
Biofertilizers for Micro nutrients		
1.	Silicate and Zinc solubilizers	*Bacillus* sp.
Plant Growth Promoting Rhizobacteria		
1.	Pseudomonas	*Pseudomonas fluorescens*

2. Different types of biofertilizers

Rhizobium

Rhizobium is a soil habitat bacterium, which can able to colonize the legume roots and fixes the atmospheric nitrogen symbiotically. The morphology and physiology of Rhizobium will vary from free-living condition to the bacteroid of nodules. They are the most efficient biofertilizer as per the quantity of nitrogen fixed concerned. They have seven genera and highly specific to form nodule in legumes, referred as cross inoculation group. *Rhizobium* inoculant was first made in USA and commercialized by private enterprise in 1930s and the strange situation at that time has been chronicled by Fred (1932).

Initially, due to absence of efficient bradyrhizobial strains in soil, soybean inoculation at that time resulted in bumper crops but incessant inoculation during the last four decades by US farmers has resulted in the build up of a plethora of inefficient strains in soil whose replacement by efficient strains of bradyrhizobia has become an insurmountable problem.

Azotobacter

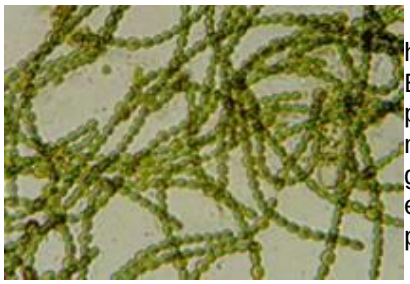
Of the several species of *Azotobacter*, *A. chroococcum* happens to be the dominant inhabitant in arable soils capable of fixing N2 (2-15 mg N2 fixed /g of carbon source) in culture media. The bacterium produces abundant slime which helps in soil aggregation. The numbers of *A. chroococcum* in Indian soils rarely exceeds 105/g soil due to lack of organic matter and the presence of antagonistic microorganisms in soil.

Azospirillum

Azospirillum lipoferum and *A. brasilense* (*Spirillum lipoferum* in earlier literature) are primary inhabitants of soil, the rhizosphere and intercellular spaces of root cortex of graminaceous plants. They perform the associative symbiotic relation with the graminaceous plants. The bacteria of Genus *Azospirillum* are N2 fixing organisms isolated from the root and above ground parts of a variety of crop plants. They are Gram negative, *Vibrio* or *Spirillum* having abundant accumulation of polybetahydroxybutyrate (70 %) in cytoplasm.

Five species of *Azospirillum* have been described to date *A. brasilense*, *A.lipoferum*, *A.amazonense*, *A.halopraeferens* and *A.irakense*. The organism proliferates under both anaerobic and aerobic conditions but it is preferentially micro-aerophilic in the presence or absence of combined nitrogen in the medium. Apart from nitrogen fixation, growth promoting substance production (IAA), disease resistance and drought tolerance are some of the additional benefits due to *Azospirillum* inoculation.

Cyanobacteria



Both free-living as well as symbiotic cyanobacteria (blue green algae) have been harnessed in rice cultivation in India. A composite culture of BGA having heterocystous *Nostoc*, *Anabaena*, *Aulosira etc*. is given as primary inoculum in trays, polythene lined pots and later mass multiplied in the field for application as soil based flakes to the rice growing field at the rate of 10 kg/ha. The final product is not free from extraneous contaminants and not very often monitored for checking the presence of desiredalgal flora.

Once so much publicized as a biofertilizer for the rice crop, it has not presently attracted the attention of rice growers all over India except pockets in the Southern States, notably Tamil Nadu. The benefits due to algalization could be to the extent of 20-30 kg N/ha under ideal conditions but the labour oriented methodology for the preparation of BGA biofertilizer is in itself a limitation. Quality control measures are not usually followed except perhaps for random checking for the presence of desired species qualitatively.

Azolla

Azolla is a free-floating water fern that floats in water and fixes atmospheric nitrogen in association with nitrogen fixing blue green alga *Anabaena azollae*. *Azolla* fronds consist of sporophyte with a floating rhizome and small overlapping bi-lobed leaves and roots. Rice growing areas in South East Asia and other third World countries have recently been evincing increased interest in the use of the symbiotic N2 fixing water fern *Azolla* either as an alternate nitrogen sources or as a supplement to

commercial nitrogen fertilizers. *Azolla* is used as biofertilizer for wetland rice and it is known to contribute 40-60 kg N/ha per rice crop.

Phosphate solubilizing microorganisms(PSM)

Several soil bacteria and fungi, notably species of *Pseudomonas, Bacillus, Penicillium, Aspergillus etc*. secrete organic acids and lower the pH in their vicinity to bring about dissolution of bound phosphates in soil. Increased yields of wheat and potato were demonstrated due to inoculation of peat based cultures of *Bacillus polymyxa* and *Pseudomonas striata*. Currently, phosphate solubilizers are manufactured by agricultural universities and some private enterprises and sold to farmers through governmental agencies. These appear to be no check on either the quality of the inoculants marketed in India or the establishment of the desired organisms in the rhizosphere.

AM fungi

The transfer of nutrients mainly phosphorus and also zinc and sulphur from the soil *milleu* to the cells of the root cortex is mediated by intracellular obligate fungal endosymbionts of the genera *Glomus, Gigaspora, Acaulospora, Sclerocysts* and *Endogone* which possess vesicles for storage of nutrients and arbuscles for funneling these nutrients into the root system. By far, the commonest genus appears to be *Glomus*, which has several species distributed in soil. Availability for pure cultures of AM (Arbuscular Mycorrhiza) fungi is an impediment in large scale production despite the fact that beneficial effects of AM fungal inoculation to plants have been repeatedly shown under experimental conditions in the laboratory especially in conjunction with other nitrogen fixers.

Silicate solubilizing bacteria (SSB)

Microorganisms are capable of degrading silicates and aluminum silicates. During the metabolism of microbes several organic acids are produced and these have a dual role in silicate weathering. They supply H+ ions to the medium and promote hydrolysis and the organic acids like citric, oxalic acid, Keto acids and hydroxy carbolic acids which from complexes with cations, promote their removal and retention in the medium in a dissolved state.

The studies conducted with a Bacillus sp. isolated from the soil of granite crusher yard showed that the bacterium is capable of dissolving several silicate minerals under *in vitro* condition. The examination of anthrpogenic materials like cement, agro inputs like super phosphate and rock phosphate exhibited silicate solubilizing bacteria to a varying degree. The bacterial isolates made from different locations had varying degree of silicate solubilizing potential. Soil inoculation studies with selected isolate with red soil, clay soil, sand and hilly soil showed that the organisms multiplied in all types of soil and released more of silica and the available silica increased in soil and water. Rice responded well to application of organic sliceous residue like rice straw, rice husk and black ash @ 5 t/ha. Combining SSB with these residues further resulted in increased plant growth and grain yield. This enhancement is due to increased dissolution of silica and nutrients from the soil.

Plant Growth Promoting Rhizobacteria (PGPR)

The group of bacteria that colonize roots or rhizosphere soil and beneficial to crops are referred to as plant growth promoting rhizobacteria (PGPR).

The PGPR inoculants currently commercialized that seem to promote growth through at least one mechanism; suppression of plant disease (termed Bioprotectants), improved nutrient acquisition (termed Biofertilizers), or phytohormone production (termed Biostimulants). Species of *Pseudomonas* and *Bacillus* can produce as yet not well characterized phytohormones or growth regulators that cause crops to have greater amounts of fine roots which have the effect of increasing the absorptive surface of plant roots for

uptake of water and nutrients. These PGPR are referred to as Biostimulants and the phytohormones they produce include indole-acetic acid, cytokinins, gibberellins and inhibitors of ethylene production.

Recent advances in molecular techniques also are encouraging in that tools are becoming available to determine the mechanism by which crop performance is improved using PGPR and track survival and activity of PGPR organisms in soil and roots. The science of PGPR is at the stage where genetically modified PGPR can be produced. PGPR with antibiotic, phytohormone and siderophore production can be made.

Despite of promising results, biofertilizers has not got widespread application in agriculture mainly because of the variable response of plant species or genotypes to inoculation depending on the bacterial strain used. Differential rhizosphere effect of crops in harbouring a target strain or even the modulation of the bacterial nitrogen fixing and phosphate solubilizing capacity by specific root exudates may account for the observed differences. On the other hand, good competitive ability and high saprophytic competence are the major factors determining the success of a bacterial strain as an inoculant.

Studies to know the synergistic activities and persistence of specific microbial populations in complex environments, such as the rhizosphere, should be addressed in order to obtain efficient inoculants. In this regards, research efforts are made at Agricultural College and Research Institute, Madurai to obtain appropriate formulations of microbial inoculants incorporating nitrogen fixing, phosphate- and silicate- solubilizing bacteria and plant growth promoting rhizobacteria which will help in promoting the use of such beneficial bacteria in sustainable agriculture.

Liquid Biofertilizers

Biofertilizers are such as *Rhizobium, Azospirillum* and Phosphobacteria provide nitrogen and phosphorous nutrients to crop plants through nitrogen fixation and phosphorous solubilization processes. These Biofertilizers could be effectively utilized for rice, pulses, millets, cotton, sugarcane, vegetable and other horticulture crops. Biofertilizers is one of the prime input in organic farming not only enhances the crop growth and yield but also improves the soil health and sustain soil fertility. At present, Biofertilizers are supplied to the farmers as carrier based inoculants. As an alternative, liquid formulation technology has been developed in the Department of Agricultural Microbiology, TNAU, Coimbatore which has more advantages than the carrier inoculants.

Benefits

The advantages of Liquid Bio-fertilizer over conventional carrier based Bio-fertilizers are listed below:

- Longer shelf life -12-24 months.
- No contamination.
- No loss of properties due to storage upto 45º c.
- Greater potentials to fight with native population.
- High populations can be maintained more than 109 cells/ml upto 12 months to 24 months.
- Easy identification by typical fermented smell.
- Cost saving on carrier material, pulverization, neutralization, sterilization, packing and transport.
- Quality control protocols are easy and quick.
- Better survival on seeds and soil.
- No need of running Bio-fertilizer production units through out the year.
- Very much easy to use by the farmer.
- Dosages is 10 time less than carrier based powder Bio-fertilizers.
- High commercial revenues.
- High export potential.
- Very high enzymatic activity since contamination is nil.

Characteritistics of different liquid Bio-fertilizers

Rhizobium

This belongs to bacterial group and the classical example is symbiotic nitrogen fixation. The bacteria infect the legume root and form root nodules within which they reduce molecular nitrogen to ammonia which is reality utilized by the plant to produce valuable proteins, vitamins and other nitrogen containing compounds. The site of symbiosis is within the root nodules. It has been estimated that 40-250 kg N / ha / year is fixed by different legume crops by the microbial activities of *Rhizobium*. The percentage of nodules occupied, nodules dry weight, plant dry weight and the grain yield per plant the multistrain inoculant was highly promising Table-2 shows the N fixation rates.

Quantity of biological N fixed by Liqiud *Rhizobium* in different crops

Host Group	*Rhizobium* Species	Crops	N fix kg/ha
Pea group	*Rhizobium leguminosarum*	Green pea, Lentil	62- 132
Soybean group	*R.japonicum*	Soybean	57- 105
Lupini Group	*R. lupine orinthopus*	Lupinus	70- 90
Alfafa grp.Group	*R.mellilotiMedicago Trigonella*	Melilotus	100- 150
Beans group	*R. phaseoli*	Phaseoli	80- 110
Clover group	*R. trifoli*	Trifolium	130
Cowpea group	*R. species*	Moong, Redgram, Cowpea, Groundnut	57- 105
Cicer group	*R. species*	Bengal gram	75- 117

Physical features of liquid Rhizobium

- Dull white in colour
- No bad smell
- No foam formation, pH 6.8-7.5

Azospirllium

It belongs to bacteria and is known to fix the considerable quantity of nitrogen in the range of 20-40 kg N/ha in the rhizosphere in non- non-leguminous plants such as cereals, millets, Oilseeds, cotton etc. The efficiency of *Azospirillium* as a Bio-Fertilizer has increased because of its ability of inducing abundant roots in several pants like rice, millets and oilseeds even in upland conditions. Considerable quantity of nitrogen fertilizer up to 25-30 % can be saved by the use of *Azospirillum* inoculant. The genus *Azospirillum* has three species viz., *A. lipoferum*, *A. brasilense* and *A. amazonense*. These species have been commercially exploited for the use as nitrogen supplying Bio-Fertilizers.

One of the characteristics of *Azospirillum* is its ability to reduce nitrate and denitrify. Both *A. lipoferum*,and *A. brasilense* may comprise of strains which can actively or weakly denitrify or reduce nitrate to nitrite and therefore, for inoculation preparation, it is necessary to select strains which do not possess these characteristics. *Azospirllium lipoferum* present in the roots of some of tropical forage grasses uch as Digitaria, Panicum, Brachiaria, Maize, Sorghum, Wheat and Rye.

Physical features of liquid *Azospirillum*

- The colour of the liquid may be blue or dull white.
- Bad odours confirms improper liquid formulation and may be concluded as mere broth.

- Production of yellow gummy colour materials comfirms the quality product.
- Acidic pH always confirms that there is no Azospirillum bacteria in the liquid.

N2 fixing capacity of Azospirillum in the roots of several plants and the amount of N2 fixed by them.

Plant	**Mg N2 fixed /g of substrate**
Oryza sativa (Paddy)	28
Sorghum bicolour (Sorghum)	20
Zea mays (Maize)	20
Panicum sp.	24
Cynodon dactylon	36
Setaria sp	12
Amaranthus spinosa	16

Production of growth hormones

Azospirillum cultures synthesize considerable amount of biologically active substances like vitamins, nicotinic acid, indole acetic acids giberllins. All these hormones/chemicals helps the plants in better germination, early emergence, better root development.

Role of Liquid *Azospirillum* under field conditions

- Stimulates growth and imparts green colour which is a characteristic of a healthy plant.
- Aids utilization of potash, phosphorous and other nutrients.
- Encourage plumpness and succulence of fruits and increase protein percentage.

Sign of non functioning of *Azospirillum* in the field

- No growth promotion activity
- Yellowish green colour of leaves, which indicates no fixation of Nitrogen

Azotobacter

It is the important and well known free living nitrogen fixing aerobic bacterium. It is used as a Bio-Fertilizer for all non leguminous plants especially rice, cotton, vegetables etc. *Azotobacter* cells are not present on the rhizosplane but are abundant in the rhizosphere region. The lack of organic matter in the soil is a limiting factor for the proliferation of *Azotobaceter* in the soil.

Field experiments were conducted in 1992, 1993 and 1994 during the pre-kharif wet seasons to find out the influence on rice grain yield by the combined use of N- fixing organisms and inorganic nitrogen fertilizer which recorded increase in was yield.

Physical features of liquid *Azotobacter*

The pigmentation that is produced by Azotobacter in aged culture is melanin which is due to oxidation of tyrosine by tyrosinase an enzyme which has copper. The colour can be noted in liquid forms. Some of the pigmentation are described below-

- *A. chroococcum*: Produces brown-black pigmentation in liquid inoculum.

- *A. beijerinchii*: Produces yellow- light brown pigementation in liquid inoculum
- *A. vinelandii*: Produces green fluorescent pigmentation in liquid inoculum.
- *A. paspali*: Produces green fluorescent pigmentation in liquid inoculum.
- *A. macrocytogenes*: Produces, pink pigmentation in liquid inoculum.
- *A. insignis*: Produces less, gum less, grayish-blue pigmentation in liquid inoculum.
- *A. agilies*: Produces green-fluorescent pigmentation in liquid inoculum.

Role of liquid *Azotobacter* in tissue culture

The study was conducted by Dr. Senthil et al (2004) on sugarcane variety CO 86032 in Tissue culture Laboratories of Rajashree Sugars and Chemicals Ltd, Varadaraj nagar, Theni, Tamilnadu. The liquid bioinoculants were provided by Dr. Krishnan Chandra, Regional Director, RCOF, Bangalore to evaluate their growth promoting effects on sugarcane micropropagation. He recorded Biometric observations like Plant height, leaf length, width, root length, no of roots. Chemical parameters –Protein, Carbohydrates, N, P,K total biomass and concluded as follows:

- The performance of *Azotobacter* liquid inoculant was c
- omparatively better than all the treatments in 10 % MS medium followed Azospirillum.
- The performance of *Azotobacter* liquid inoculant was comparatively better than all the treatments followed by Azosopirillum for the growth of the polybag sugarcane seedlings.

Role of liquid Azotobacter as a Bio-control agent

Azotobacter have been found to produce some antifungal substance which inhibits the growth of some soil fungi like *Aspergillus, Fusarium, Curvularia, Alternaria, Helminthosporium, Fusarium* etc.

Acetobaceter

This is a sacharophillic bacteria and associate with sugarcane, sweet potato and sweet sorghum plants and fixes 30 kgs/ N/ ha year. Mainly this bacterium is commercialized for sugarcane crop. It is known to increase yield by 10-20 t/ acre and sugar content by about 10-15 percent.

Effect of liquid *Acetobacter diazotrophicus* on sugarcane

In South India use of Azospirillum and Phospho-bacterium on the cash crop sugarcane is a regular practice for the past few years with a saving of nearly 20 % of chemical nitrogen and phosphate applications. Now, it has been reported that a bacteria *Acetobacter diazotrophicus* which is present in the sugarcane stem, leaves, soils have a capacity to fix up to 300 kgs of nitrogen. This bacteria first reported in brazil where the farmers cultivate sugarcane in very poor sub-soil fertilized with Phosphate, Potassium and micro elements alone, could produce yield for three consecutive harvests, without any nitrogen fertilizer. They have recorded yield 182- 244 tones per ha. This leads to the assumption that active nitrogen fixing bacteria has associated within the plant.

Do's and Don't for Entrepreneurs, Dealers and farmers

Do	Don't
Keep Bio-fertilizers bottles away from direct heat and sunlight. Store it in cool and dry place.	Don't store Bio-fertilizers bottles under heat and sunlight
Sell only Bio-fertilizers bottles which contain batch number, the name of the crop on which it has to be used, the date of manufacture and expiry period.	Don't sell Bio-fertilizers bottles after their expiry period is over.
If the expiry period is over, then discard it as it is not effective.	Don't prick holes into the bottles or puncture them to pour the content
Keep Bio-fertilizers bottles away from fertilizer or pesticide containers and they should not be mixed directly.	Do not mix the Bio-fertilizers with fungicides, insecticides, herbicides, herbicides and chemical fertilizers.

Liquid Bio-fertlizer application methodology
There are three ways of using Liquid Bio-fertilizers

1. Seed treatment
2. Root dipping
3. Soil application

Seed Treatment

Seed Treatment is a most common method adopted for all types of inoculants. The seed treatment is effective and economic. For small quantity of seeds (up to 5 kgs quantity) the coating can done in a plastic bag. For this purpose, a plastic bag having size (21" x 10") or big size can be used. The bag should be filled with 2 kg or more of seeds. The bag should be closed in such a way to trap the airs as much as possible. The bag should be squeezed for 2 minutes or more until all the seed are uniformly wetted. Then bag is opened, inflated again and shaked gently. Stop shaking after each seeds gets a uniform layer of culture coating. The bag is opened and the seed is dried under the shade for 20-30 minutes. For large amount of seeds coating can be done in a bucket and inoculant can be mixed directly with hand. Seed Treatment with *Rhizobium*, *Azotobacter, Azospirillum*, along with PSM can be done.

The seed treatment can be done with any of two or more bacteria. There is no side (antagonistic) effect. The important things that has to be kept in mind are that the seeds must be coated first with *Rhizobium, Azotobacter* or *Azospirillum*. When each seed get a layer of above bacteria then PSM inoculant has to be coated as outer layer. This method will provide maximum number of each bacteria required for better results. Treatments of seed with any two bacteria will not provide maximum number of bacteria on individual seed.

Root dipping

For application of *Azospirillum*/ /PSM on paddy transplating/ vegetable crops this method is used. The required quantity of *Azospirillum*/ /PSM has to be mixed with 5-10 litres of water at one corner of the field and the roots of seedlings has to be dipped for a minimum of half-an-hour before transplantation.

Soil application

Use 200ml of PSM per acre. Mix PSM with 400 to 600 kgs of Cow dung FYM along with ½ bag of rock phosphate if available. The mixture of PSM, cow dung and rock phosphate have to be kept under any tree or under shade for over night and maintain 50% moisture. Use the mixture as soil application in rows or during leveling of soil.

Dosage of liquid Bio-fertilizers in different crops

Recommended Liquid Bio-fertilizers and its application method, quantity to be used for different crops are as follows:

Crop	Recommended Bio-fertilizer	Application method	Quantity to be used
Field crops Pulses Chickpea, pea, Groundnut, soybean, beans, Lentil, lucern, Berseem, Green gram, Black gram, Cowpea and pigeon pea	*Rhizobium*	Seed treatment	200ml/acre
Cereals Wheat, oat, barley	*Azotobacter/Azospirillum*	Seed treatment	200ml/acre
Rice	*Azospirillum*	Seed treatment	200ml/acre
Oil seeds Mustard, seasum, Linseeds, Sunflower, castor	*Azotobacter*	Seed treatment	200ml/acre
Millets Pearl millets, Finger millets, kodo millet	*Azotobacter*	Seed treatment	200ml/acre
Maize and Sorghum	*Azospirillum*	Seed treatment	200ml/acre
Forage crops and Grasses Bermuda grass, Sudan grass, Napier Grass , ParaGrass, StarGrass etc.	*Azotobacter*	Seed treatment	200ml/acre
Other Misc. Plantation Crops Tobacco	*Azotobacter*	Seedling treatment	500ml/acre
Tea, Coffee	*Azotobacter*	Soil treatment	400ml/acre
Rubber, Coconuts	*Azotobacter*	Soil treatment	2-3 ml/plant
Agro-ForestRY/Fruit Plants All fruit/agro-forestry (herb,shrubs, annuals and perennial) plants for fuel wood fodder, fruits,gum,spice,leaves,flowers,nuts and seeds puppose	*Azotobacter*	Soil treatment	2-3 ml/plant at nursery
Leguminous plants/ trees	*Rhizobium*	Soil treatment	1-2 ml/plant

Note:
Doses recommended when count of inoculum is 1 x 108 cells/ml then doses will be ten times more besides above said Nitrogen fixers, Phosphate solubilizers and potash mobilizers at the rate of 200 ml/ acre could be applied for all crops.

Equipments required for Biofertilizer production

In biofertilizer production industry, equipments are the major infrastructure, which involves 70 percent of capital investment. Any compromise on the usage of the following mentioned equipments may finally decline in the quality of biofertilizer.After studying the principle behind the usage of all instruments, some of the instruments can be replaced with a culture room fitted with a U.V.Lamp. Autoclaves, Hot Air Oven, Incubators and sealing machines are indigenously made with proper technical specifications. The correct use of equipments will give uninterrupted introduction with quality inoculum.

Essential equipments

Autoclave

It is an apparatus in which materials are sterilized by air free saturated steam (under pressure) at a temperature above 100OC. If the steam pressure inside the autoclave is increased to 15 psi, the temperature will rise to 121°C. this is sufficient to destroy all vegetative cells. Normally all growth medium are sterilized in the autoclave.

Laminar air flow chamber

Laminar air flow chamber provides a uniform flow of filtered air. This continuous flow of air will prevent settling of particles in the work area.Air borne contamination is avoided in this chamber. Culture transfers and inoculation can be done here.

BOD incubators

Incubators providing controlled conditions (light, temperature, humidity, etc.) required for the growth and development of microorganisms. Multiplication of starter culture can be done in this instrument.

Rotary shaker

It is used for agitating culture flasks by circular motion under variable speed control. Shaking provides aeration for growth of cultures. Shakers holding upto 20-50 flasks are generally used. The capacity of the shaker may be increased if it is a double- decker type.

Hot air oven

Hot air oven is meant for sterilizing all glassware materials. Dry heat is used in this apparatus to sterilize the materials. Normally 180OC is used for two hours for sterilizing glasswares.

pH meter

An instrument for measuring pH of the solution using a 0-14 scale in which seven represents neutral points, less than seven is acidity (excess of H‘ over OH-) and more than seven is alkality (excess of OH- over H‘) useful in adjusting the pH of the growth medium.

Refrigerator

This equipment is used preserving all mother cultures used for biofertilizer production. The mother culture is periodically sub-cultured and stored in the refrigerator for long- term usage.

Fermentor

A fermentor is the equipment, which provides the proper environment for the growth of a desired organism. It is generally a large vessel in which, the organism may be kept at the required temperature, pH , dissolved oxygen concentration and substrate concentration. Different models of fermentors are available depending upon the necessity. A simple version model contains steam generator, sterilization process devices and agitator. A sophisticated fermentor contains pH regulator, oxygen level regulator, anti-foam device, temperature controller, etc.

3. Mass production of Bacterial Biofertilizer

Biofertilizers are carrier based preparations containing efficient strain of nitrogen fixing or phosphate solubilizing microorganisms. Biofertilizers are formulated usually as carrier based inoculants. The organic carrier materials are more effective for the preparation of bacterial inoculants. The solid inoculants carry more number of bacterial cells and support the survival of cells for longer periods of time.

- The mass production of carrier based bacterial biofertilizers involves three stages.
- Culturing of microorganisms
- Processing of carrier material
- Mixing the carrier and the broth culture and packing

Culturing of Microorganisms

Although many bacteria can be used beneficially as a biofertilizer the technique of mass production is standardizedfor *Rhizobium*, *Azospirillum*, *Azotobacter* and phosphobacteria. The media used for mass culturing are as follows:

Rhizobium : Yeast extract mannitol broth.

Growth on Congo red yeast extract mannitol agar medium

Mannitol	-	10.0 g
K2 HPO4	-	0.5 g
Mg So4 7H2 O	-	0.2 g
NaCl	-	0.1 g
Yeast extract	-	0.5 g
Agar		20.0 g
Distilled water		1000.0 ml

Add 10 ml of Congo red stock solution (dissolve 250 mg of Congo red in 100ml water) to 1 liter after adjusting the PH to 6.8 and before adding agar.

Rhizobium forms white, translucent, glistening, elevated and comparatively small colonies on this medium. Moreover, *Rhizobium* colonies do not take up the colour of congo red dye added in the medium. Those colonies which readily take up the congo red stain are not rhizobia but presumably *Agrobacterium*, a soil bacterium closely related to *Rhizobium.*

Azospirillum : Dobereiner's malic acid broth with NH4Cl (1g per liter)

Composition of the N-free semisolid malic acid medium

Malic acid	-	5.0g
Potassium hydroxide	-	4.0g
Dipotassium hydrogen orthophosphate	-	0.5g
Magnesium sulphate	-	0.2g
Sodium chloride	-	0.1g
Calcium chloride	-	0.2g
Fe-EDTA (1.64% w/v aqueous)	-	4.0 ml
Trace element solution	-	2.0 ml
BTB (0.5% alcoholic solution)	-	2.0 ml

Agar	-	1.75 g
Distilled water	-	1000 ml
pH	-	6.8
Trace element solution		
Sodium molybdate	-	200 mg
Manganous sulphate	-	235 mg
Boric acid	-	280 mg
Copper sulphate	-	8 mg
Zinc sulphate	-	24 mg
Distilled water	-	200 ml

Waksman medium No.77 (N-free Mannitol Agar Medium for *Azotobacter*)

Mannitol	:	10.0 g
Ca CO3	:	5.0 g
K2HPO4	:	0.5 g
Mg SO4.7H2O	:	0.2 g
NaCl	:	0.2 g
Ferric chloride	:	Trace
MnSO4.4H2O	:	Trace
N-free washed Agar	:	15.0 g
pH	:	7.0
Distilled Water	:	1000 ml

Phosphobacteria : Pikovskaya's Broth

Glucose	10.0 g
Ca3(PO4)2	5.0 g
(NH4)2SO4	0.5 g
KCl	0.2 g
MgSO4. 7H2O	0.1 g
MnSO4	Trace
FeSO4	Trace
Yeast Extract	0.5 g
Distilled Water	1000 ml

The broth is prepared in flasks and inoculum from mother culture is transferred to flasks. The culture is grown under shaking conditions at 30±2°C as submerged culture. The culture is incubated until maximum cell population of 1010 to 1011 cfu/ml is produced. Under optimum conditions this population level could be attained with in 4 to 5 days for *Rhizobium*; 5 to 7 days for *Azospirillum*; 2 to 3 days for phosphobacteria and 6-7 days for *Azotobacter*. The culture obtained in the flask is called **starter culture**. For large scale production of inoculant, inoculum from starter culture is transferred to large flasks/seed tank fermentor and grown until required level of cell count is reached.

Inoculum preparation

- Prepare appropriate media for specific to the bacterial inoculant in 250 ml, 500 ml, 3 litre and 5 litre conical flasks and sterilize.
- The media in 250 ml flask is inoculated with efficient bacterial strain under aseptic condition
- Keep the flask under room temperature in rotary shaker (200 rpm) for 5- 7 days.
- Observe the flask for growth of the culture and estimate the population, which serves as the starter culture.
- Using the starter culture (at log phase) inoculate the larger flasks (500 ml, 3 litre and 5 litre) containing the media, after obtaining growth in each flask.

- The above media is prepared in large quantities in fermentor, sterilized well, cooled and kept it ready.
- The media in the fermentor is inoculated with the log phase culture grown in 5 litre flask. Usually 1 -2 % inoculum is sufficient, however inoculation is done up to 5% depending on the growth of the culture in the larger flasks.
- The cells are grown in fermentor by providing aeration (passing sterile air through compressor and sterilizing agents like glass wool, cotton wool, acid etc.) and given continuous stirring.
- The broth is checked for the population of inoculated organism and contamination if any at the growth period.
- The cells are harvested with the population load of 109 cells ml-1 after incubation period.
- There should not be any fungal or any other bacterial contamination at 10-6 dilution level
- It is not advisable to store the broth after fermentation for periods longer than 24 hours. Even at 4o C number of viable cells begins to decrease.

Processing of carrier material

The use of ideal carrier material is necessary in the production of good quality biofertilizer. Peat soil, lignite, vermiculite, charcoal, press mud, farmyard manure and soil mixture can be used as carrier materials. The neutralized peat soil/lignite are found to be better carrier materials for biofertilizer production The following points are to be considered in the selection of ideal carrier material.

- Cheaper in cost
- Should be locally available
- High organic matter content
- No toxic chemicals
- Water holding capacity of more than 50%
- Easy to process, friability and vulnerability.

Preparation of carrier material

The carrier material (peat or lignite) is powdered to a fine powder so as to pass through 212 micron IS sieve.

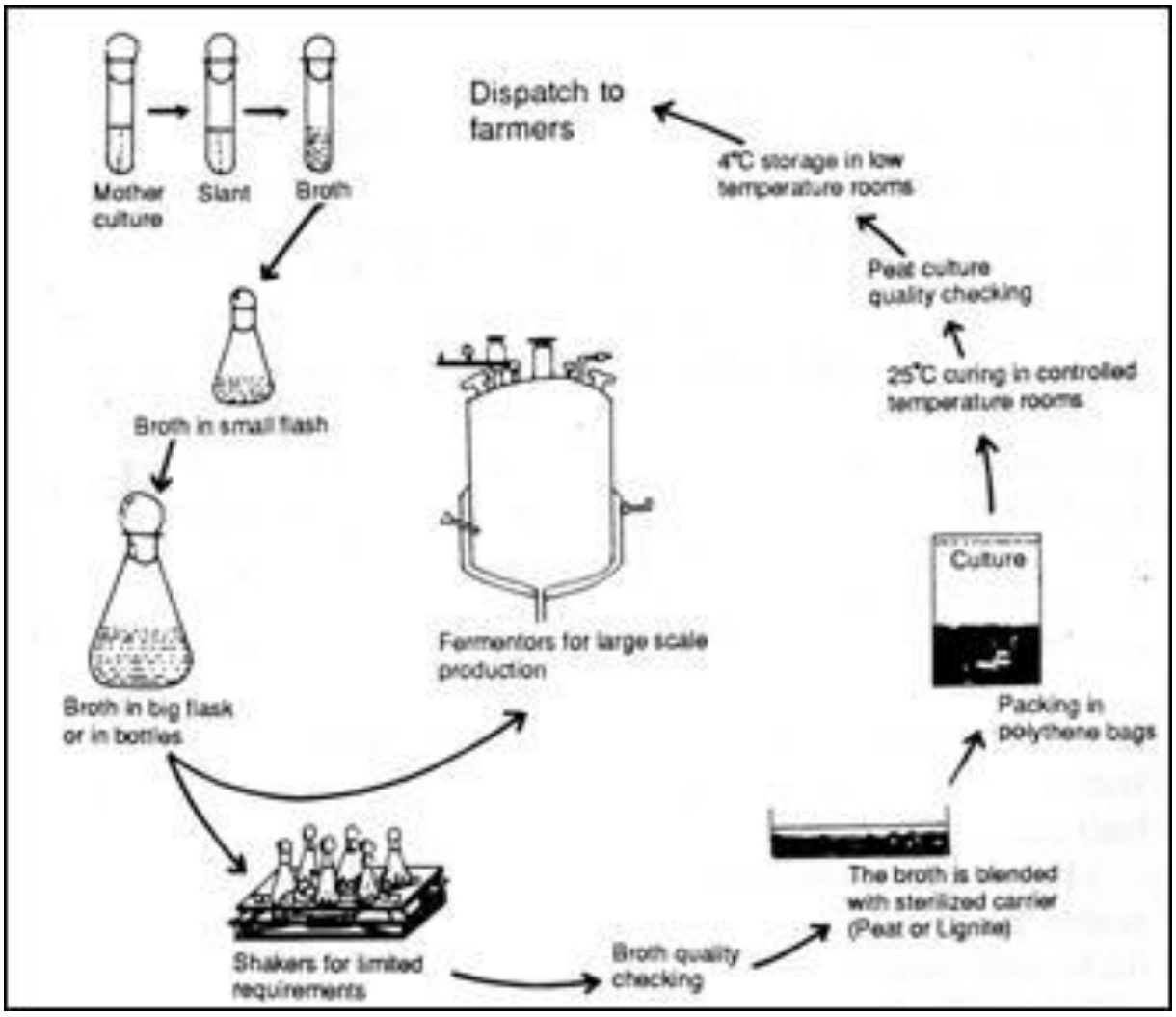
- The pH of the carrier material is neutralized with the help of calcium carbonate (1:10 ratio) , since the peat soil / lignite are acidic in nature (pH of 4 - 5)
- The neutralized carrier material is sterilized in an autoclave to eliminate the contaminants.

Mixing the carrier and the broth culture and packing

Inoculant packets are prepared by mixing the broth culture obtained from fermentor with sterile carrier material as described below:

Preparation of Inoculants packet

- The neutralized, sterilized carrier material is spread in a clean, dry, sterile metallic or plastic tray.
- The bacterial culture drawn from the fermentor is added to the sterilized carrier and mixed well by manual (by wearing sterile gloves) or by mechanical mixer. The culture suspension is to be added to a level of 40 – 50% water holding capacity depending upon the population.
- The inoculant packet of 200 g quantities in polythene bags, sealed with electric sealer and allowed for curing for 2 -3 days at room temperature (curing can be done by spreading the inoculant on a clean floor/polythene sheet/ by keeping in open shallow tubs/ trays with polythene covering for 2 -3 days at room temperature before packaging).



Schematic representation of mass production of bacterial biofertilizers

Specification of the polythene bags

- The polythene bags should be of low density grade.
- The thickness of the bag should be around 50 – 75 micron.
- Each packet should be marked with the name of the manufacturer, name of the product, strain number, the crop to which recommended, method of inoculation, date of manufacture, batch number, date of expiry, price, full address of the manufacturer and storage instructions etc.,

Storage of biofertilizerpacket

- The packet should be stored in a cool place away from the heat or direct sunlight.
- The packets may be stored at room temperature or in cold storage conditions in lots in plastic crates or polythene / gunny bags.
- The population of inoculant in the carrier inoculant packet may be determined at 15 days interval. There should be more than 109 cells / g of inoculant at the time of preparation and107 cells/ g on dry weight basis before expiry date.

Mass production of Mycorrhizal biofertilizer

The commercial utilization of mycorrhizal fungi has become difficult because of the obligate symbiotic nature and difficulty in culturing on laboratory media. Production of AM inoculum has evolved from the original use of infested field soils to the current practice of using pot culture inoculum derived

from the surface disinfected spores of single AM fungus on a host plant grown in sterilized culture medium. Several researches in different parts of the world resulted in different methods of production of AM fungal inoculum as soil based culture as well as carrier based inoculum. Root organ culture and nutrient film technique provide scope for the production of soil less culture.

As a carrier based inoculum, pot culture is widely adopted method for production. The AM inoculum was prepared by using sterilized soil and wide array of host crops were used as host. The sterilization process is a cumbersome one and scientists started using inert materials for production of AM fungi. The researchers tried use of perlite, montmorillonite clay etc., In TNAU vermiculite was tried as substrate for the replacement of soil sterilization, which resulted in the best method of inoculum production.

Method of production

- Tank for mass multiplication of *AM*
- Sprinkling of water in tank with vermiculite
- Making of furrows to sow maize seeds
- Sowing the seeds in furrows
- View of the maize sown AM pit

Vermiculite contained raised AM infected maize plants

- A trench (1m x 1m x 0.3m) is formed and lined with black polythene sheet to be used as a plant growth tub.
- Mixed 50 kg of vermiculite and 5 kg of sterilized soil and packed in the trench up to a height of 20 cm
- Spread 1 kg of AM inoculum (mother culture) 2-5 cm below the surface of vermiculite
- Maize seeds surface sterilized with 5% sodium hypochlorite for 2 minutes are sown
- Applied 2 g urea, 2 g super phosphate and 1 g muriate of potash for each trench at the time of sowing seeds. Further 10 g of urea is applied twice on 30 and 45 days after sowing for each trench
- Quality test on AM colonization in root samples is carried out on 30th and 45th day
- Stock plants are grown for 60 days (8 weeks). The inoculum is obtained by cutting all the roots of stock plants. The inoculum produced consists of a mixture of vermiculite, spores, pieces of hyphae and infected root pieces.
- Thus within 60 days 55 kg of AM inoculum could be produced from 1 sq meter area. This inoculum will be sufficient to treat 550 m2 nursery area having 11,000 seedlings.

AM fungi

Nursery application: 100 g bulk inoculum is sufficient for one metre square. The inoculum should be applied at 2-3 cm below the soil at the time of sowing. The seeds/cutting should be sown/planted above the VAM inoculum to cause infection.

For polythene bag raised crops: 5 to 10 g bulk inoculum is sufficient for each packet. Mix 10 kg of inoculum with 1000 kg of sand potting mixture and pack the potting mixture in polythene bag before sowing.

For out –planting: Twenty grams of VAM inoculum is required per seedling. Apply inoculum at the time of planting.
For existing trees: Two hundred gram of VAM inoculum is required for inoculating one tree. Apply inoculum near the root surface at the time of fertilizer application.

Mass production and field application of cyanobacteria

Blue green algal inoculation with composite cultures was found to be more effective than single culture inoculation. A technology for mass scale production of composite culture of blue green algae under rice field condition was developed at TNAU and the soil based BGA inoculum could survive for more than 2 years. At many sites where algal inoculation was used for three to four consecutive cropping seasons, the inoculated algae establish well and the effect persisted over subsequent rice crop. Technologies for utilizing nitrogen fixing organisms in low land rice were the beneficial role of blue green algal inoculation in rice soils of Tamil Nadu.

The blue green algal inoculum may be produced by several methods viz., in tubs, galvanized trays, small pits and also in field conditions. However the large-scale production is advisable under field condition which is easily adopted by farmers.

I. Multiplication in trays

- Big metallic trays (6'x 3'x 6"lbh) can be used for small scale production
- Take 10 kg of paddy field soil, dry powder well and spread
- Fill water to a height of 3"
- Add 250 g of dried algal flakes (soil based) as inoculum
- Add 150 g of super phosphate and 30 g of lime and mix well with the soil
- Sprinkle 25 g carbofuran to control the insects
- Maintain water level in trays
- After 10 to 15 days, the blooms of BGA will start floating on the water sources
- At this stage stop watering and drain. Let the soil to dry completely
- Collect the dry soil based inoculum as flakes
- Store in a dry place. By this method 5 to 7 kg of soil based inoculum can be obtained.

II. Multiplication under field condition

Materials

- Rice field
- Super phosphate
- Carbofuran
- Composite BGA starter culture

Procedure

Select an area of 40 m2 (20m x 2m) near a water source which is directly exposed to sunlight.
Make a bund all around the plot to a height of 15 cm and give it a coating with mud to prevent loss of water due to percolation.

- Plot is well prepared and levelled uniformly and water is allowed to a depth of 5-7.5 cm and left to settle for 12 hrs.
- Apply 2 kg of super phosphate and 200 g lime to each plot uniformly over the area.
- The soil based composite starter culture of BGA containing 8-10 species @ 5 kg / plot is powdered well and broadcasted.
- Carbofuran @ 200 g is also applied to control soil insects occurring in BGA.
- Water is let in at periodic intervals so that the height of water level is always maintained at 5 cm.
- After 15 days of inoculation, the plots are allowed to dry up in the sun and the algal flakes are collected and stored.

Observations

The floating algal flasks are green or blue green in colour. From each harvest, 30 to 40 kg of dry algal flakes are obtained from the plot.

Method of inoculation of BGA in rice field

Blue green algae may be applied as soil based inoculum to the rice field following the method described below.

- Powder the soil based algal flakes very well.
- Mix it with 10 kg soil or sand (10kg powdered algal flakes with 10 kg soil / sand).
- BGA is to be inoculated on 7-10 days after rice transplanting.
- Water level at 3-4" is to be maintained at the time of BGA inoculation and then for a month so as to have maximum BGA development.

Observation

A week after BGA inoculation, algal growth can be seen and algal mat will float on the water after 2-3 weeks. The algal mat colour will be green or brown or yellowish green.

Mass production and field application of *Azolla*

Azolla is a free-floating water fern that floats in water and fixes atmospheric nitrogen in association with nitrogen fixing blue green alga *Anabaena azollae*. *Azolla* fronds consist of sporophyte with a floating rhizome and small overlapping bi-lobed leaves and roots. Rice growing areas in South East Asia and other third World countries have recently been evincing increased interest in the use of the symbiotic N2 fixing water fern *Azolla* either as an alternate nitrogen sources or as a supplement to commercial nitrogen fertilizers. *Azolla* is used as biofertilizer for wetland rice and it is known to contribute 40-60 kg N ha-1 per rice crop. The agronomic potential of *Azolla* is quite significant particularly for rice crop and it is widely used as biofertilizer for increasing rice yields. Rice crop response studies with *Azolla* biofertilizer in the People's Republic in China and in Vietnam have provided good evidence that *Azolla* incorporation into the soil as a green manure crop is one of the most effective ways of providing nitrogen source for rice.

The utilization of *Azolla* as dual crop with wetland rice is gaining importance in Philippines, Thailand, Srilanka and India. The important factor in using *Azolla* as a biofertilizer for rice crop is its quick decomposition in soil and efficient availability of its nitrogen to rice. In tropical rice soils the applied *Azolla* mineralizes rapidly and its nitrogen is available to the rice crop in very short period. The common species of *Azolla* are *A. microphylla, A. filiculoides, A. pinnata, A. caroliniana, A. nilotica, A. rubra* and *A. mexicana*.

I. Mass multiplication of *Azolla* under field conditions

A simple *Azolla* nursery method for large scale multiplication of *Azolla* in the field has been evolved for easy adoption by the farmers.

Materials

- One cent (40 sq.m) area plot
- Cattle dung
- Super phosphate
- Furadan
- Fresh *Azolla* inoculum

Procedure

- Select a wetland field and prepare thoroughly and level uniformly.
- Mark the field into one cent plots (20 x 2m) by providing suitable bunds and irrigation channels.
- Maintain water level to a height of 10 cm.
- Mix 10 kg of cattle dung in 20 litres of water and sprinkle in the field.
- Apply 100 g super phosphate as basal dose.
- Inoculate fresh *Azolla* biomass @ 8 kg to each pot.
- Apply super phosphate @ 100 g as top dressing fertilizer on 4th and 8th day after *Azolla* inoculation.
- Apply carbofuran (furadan) granules @ 100 g/plot on 7th day after *Azolla* inoculation.
- Maintain the water level at 10 cm height throughout the growth period of two or three weeks.
- Observations
- Note the *Azolla* mat floating on the plot. Harvest the *Azolla*, drain the water and record the biomass.

II. Method of inoculation of *Azolla* to rice crop

The *Azolla* biofertilizer may be applied in two ways for the wetland paddy. In the first method, fresh *Azolla* biomass is inoculated in the paddy field before transplanting and incorporated as green manure. This method requires huge quantity of fresh *Azolla*. In the other method, *Azolla* may be inoculated after transplanting rice and grown as dual culture with rice and incorporated subsequently.

A. *Azolla* biomass incorporation as green manure for rice crop

- Collect the fresh *Azolla* biomass from the *Azolla* nursery plot.
- Prepare the wetland well and maintain water just enough for easy incorporation.
- Apply fresh *Azolla* biomass (15 t ha-1) to the main field and incorporate the *Azolla* by using implements or tractor.

***B. Azolla* inoculation as dual crop for rice**

- Select a transplanted rice field.
- Collect fresh *Azolla* inoculum from *Azolla* nursery.
- Broadcast the fresh *Azolla* in the transplanted rice field on 7th day after planting (500 kg / ha).
- Maintain water level at 5-7.5cm.
- Note the growth of *Azolla* mat four weeks after transplanting and incorporate the *Azolla* biomass by using implements or tranctor or during inter-cultivation practices.
- A second bloom of *Azolla* will develop 8 weeks after transplanting which may be incorporated again.
- By the two incorporations, 20-25 tonnes of *Azolla* can be incorporated in one hectare rice field.

4. Application of Biofertilizers

1. Seed treatment or seed inoculation
2. Seedling root dip
3. Main field application

Seed treatment

One packet of the inoculant is mixed with 200 ml of rice kanji to make a slurry. The seeds required for an acre are mixed in the slurry so as to have a uniform coating of the inoculant over the seeds and then shade dried for 30 minutes. The shade dried seeds should be sown within 24 hours. One packet of the inoculant (200 g) is sufficient to treat 10 kg of seeds.

Seedling root dip

This method is used for transplanted crops. Two packets of the inoculant is mixed in 40 litres of water. The root portion of the seedlings required for an acre is dipped in the mixture for 5 to 10 minutes and then transplanted.

Main field application

Four packets of the inoculant is mixed with 20 kgs of dried and powdered farm yard manure and then broadcasted in one acre of main field just before transplanting.

Rhizobium

For all legumes *Rhizobium* is applied as seed inoculant.

Azospirillum/Azotobacter

In the transplanted crops, *Azospirillum* is inoculated through seed, seedling root dip and soil application methods. For direct sown crops, *Azospirillum* is applied through seed treatment and soil application.

Phosphobacteria

Inoculated through seed, seedling root dip and soil application methods as in the case of *Azospirillum*. Combined application of bacterial biofertilizers.

Phosphobacteria can be mixed with *Azospirillum* and *Rhizobium*. The inoculants should be mixed in equal quantities and applied as mentioned above.

Points to remember

- Bacterial inoculants should not be mixed with insecticide, fungicide, herbicide and fertilizers.
- Seed treatment with bacterial inoculant is to be done at last when seeds are treated with fungicides.

Biofertilizers recommendation (one packet - 200 g)

S. No.	Crop	Seed	Nursery	Seedling dip	Main field	Total requirement of packets per ha
1.	Rice	5	10	5	10	30
2.	Sorghum	3	-	-	10	13
3.	Pearl millet	3	-	-	10	13
4.	Ragi	3	-	5	10	18
5.	Maize	3	-	-	10	13
6.	Cotton	3	-	-	10	13
7.	Sunflower	3	-	-	10	13
8.	Castor	3	-	-	10	13
9.	Sugarcane	10	-	-	36 (3 split)	46
10.	Turmeric	-	-	-	24 (2 split)	24
11.	Tobacco	1	3	-	10 g/pit	14
12.	Papaya	2	-	-	10	-
13.	Mandarin Orange	2	-	-	10 g/pit	-
14.	Tomato	1	-	-	10	14
15.	Banana	-	-	5	10 g/pit	-

Rhizobium (only seed application is recommended)

S. No.	Crop	Total requirement of packets per ha
1.	Soybean	5
2.	Groundnut	5
3.	Bengalgram	5
4.	Blackgram	3
5.	Greengram	3
6.	Redgram	3
7.	Cowpea	3

Phosphobacteria
The recommended dosage of *Azospirillum* is adopted for phosphobacteria inoculation; for combined inoculation, both biofertilizers as per recommendations are to be mixed uniformly before using.

5. Azolla – The best feed for cattle and poultry

Azolla is a free floating water fern that floats in water and fixes nitrogen in association with the nitrogen fixing blue green algae, *Anabaena azollae*. Azolla is considered to be a potential biofertilizer in terms of nitrogen contribution to rice. Long before its cultivation as a green manure, Azolla has been used as a fodder for domesticated animals such as pigs and ducks. In recent days, Azolla is very much used as a sustainable feed substitute for livestock especially dairy cattle, poultry, piggery and fish.

Azolla contains 25 – 35 per cent protein on dry weight basis and rich in essential amino acids, minerals, vitamins and carotenoids including the antioxidant b carotene. Cholorophyll a, chlorophyll b and carotenoids are also present in Azolla, while the cyanobiont *Anabaena azollae* contains cholorophyll a,

phycobiliproteins and carotenoids. The rare combination of high nutritive value and rapid biomass production make Azolla a potential and effective feed substitute for live stocks.

Inputs required

Azolla fronds, Polythene sheet, Super phosphate and Cow dung.

Methodology

The area selected for Azolla nursery should be partially shaded. The convenient size for Azolla is 10 feet length, 2 feet breadth and 1 feet depth. The nursery plot is spread with a polythene sheet at the bottom to prevent water loss. Soil is applied to a depth of 2 cm and a gram of super phosphate is applied along with 2 kg of vermicompost or cow dung in the nursery for quick growth. Azolla mother inoculum is introduced @ 5 kg/plot.

The contents in the plot are stirred daily so that the nutrients in the soil dissolve in water for easy uptake by Azolla. Azolla is harvested fifteen days after inoculation at the rate of 50-80 kg / plot. One third of Azolla should be left in the plot for further multiplication. Five kg cow dung slurry should be sprinkled in the Azolla nursery at ten days intervals. Neem oil can be sprayed over the Azolla at 0.5 5 level to avoid pest incidence.

Animal	Dosage / day
Adult cow , Buffalo, Bullock	1.5-2 kg
Layer, Broiler birds	20 – 30 grams
Goat	300 – 500 grams
Pig	1.5 – 2.0 kg
Rabbit	100 gram

Value of the technology

The egg yield is increased in layer birds due to Azolla feeding. The Azolla fed birds register an overall egg productivity of 89.0 per cent as against 83.7 per cent recorded by the birds fed with only concentrated feed. The average daily intake of concentrated feed is considerably low (106.0 g) for birds due to Azolla substitution as against 122.0 g in the control birds. More impotantly Azolla feeding shows considerable amount of savings in the consumption of concentrated feed (13.0 %) leading to reduced operational cost. By considering the average cost of the concentrated feed as Rs. 17/ Kg, a 13.0 % saving in the consumption ultimately leads to a feed cost savings of 10.0 paise /day/ bird and hence a layer unit maintaining 10,000 birds could cut down its expense towards feed to a tune of rs.1000/day.

Benefits

The Azolla feeding to layer birds increase egg weight, albumin, globulin and carotene contents. The total protein content of the eggs laid by the Azolla fed birds is high and the total carotene content of Azolla eggs(440 g 100 g-1 of edible portion)is also higher than the control. The rapid biomass production due to the high relative growth rate, increased protein and carotene contents and good digestability of the Azolla hybrid Rong ping favour its use as an effective feed supplement to poultry birds.

Effect of Azolla hybrid Rong Ping on the nutritional value of egg

Parameters	*Azolla* egg	Control	percentage increase over control
Egg weight (g)	61.20	57.40	6.62
Albumin (g /100 g of edible portion)	3.9	3.4	14.70
Globulin (g /100 g of edible portion)	10.1	9.5	6.31
Total protein (g/ 100 g of edible portion)	14.0	12.9	8.52
Carotenes (µg / 100 g of edible portion)	440	405	8.64

Application

In Indian conditions, agriculture is very much coupled with poultry farming. Azolla is an important low cost input, which plays a vital role in improving soil quantity in sustainable rice farming. The twin potentials as biofertilizer and animal feed make the water fern Azolla as an effective input to both the vital components of integrated farming, agricultural and animalo husbandry.

Limitation

Azolla is a water fern and requires a growth temperature of 35-38º C. The multiplication of Azolla is affected under elevated temperature. Hence adopting this technology in dry zones where the temperature exceeds 40ºc is difficult.

Achievements

Azolla hybrid Rong ping had been selected to supply to the tribal population. Azolla mother inoculum nursery was laid out in villages with the help of Krishi Vigyan Kendra, TNAU, Coimbatore and Krishi Vigyan Kendra, Karamadai, women entrepreneurs were selected and one day training was imparted to them on the cultivation of Azolla. Wet biomass (Starter inoculm) were supplied at free of cost @ 10 kg/women entrepreneur during the training so as to enable them to initiate commercial Azolla cultivation in their backyards.

Azolla multiplication plots had been laid out in Narasipuram. Azolla mass production training was conducted to the SHG in Narasipuram village with the help of Kalaimagal Arts and Science College, Narasipuram, Sappanimadai (tribal village) and Avinashilingam KVK, Karamadai. With the help of Avinashilingam KVK, Karamadai Azolla trainings were conducted to women volunteers and we have established Azolla village in Karamadai. The Avin milk producers union Coimbatore and the poultry owners association, Namakkal have been contacted and explained the importance of Azolla as feed supplement.

The Milk Producers Union also involved in the training and marketing of Azolla. They are purchasing Azolla fronds from the village level Azolla growers both under wet and dry conditions. Around 400 rural women and 370 tribal people have been trained on the cultivation of Azolla through this project. The Azolla laboratory and the Azolla germplasm center at AC& RI, TNAU, Coimbatore helped us in the maintenance of germplasm by providing the mother inoculum. The Animal Husbandry Unit at AC&RI, TNAU, Coimbatore helped us in standardizing the Azolla and concentrated feed mixing ratio.

6. List of Biofertilizer production units in Tamil Nadu

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T Stanes & Company Limited
Dr. S. Ramarethinam
8/23-24, Race Course Road,
COIMBATORE-641 018 (TN)
(0422-211514, 213515 Fax: 217432
e-mail: tstanes@vsnl.com

Esvin Advanced Technologies Limited
Mr. T. S. Venkataraman
"Esvin House" Perungudi,
CHENNAI-600 096 (TN)
(4961056,4960690 Fax: 4961002
e-mail: tsv@vsnl.com

Southern Petrochemical Industries Corporation Limited,
Mr. K. Raju
SPIC Ltd. Biotechnology Division, Chettiar Agaram Road, Gandhi Nagar, Porur,
CHENNAI-600 116 (TN)
(044-4768064 Tele-Fax: 044-4767347
e-mail: biotech.por@spic.co.in

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Mr. P. Mallikarjuna Reddy
Chief Manager –Bioproducts
Commercial Group, Madras Fertilizers Ltd., Manali,
CHENNAI-600 068 (TN)
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Biofertilizer Production Unit
Mr. S. Murugan
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SALEM-636 201 (TN)

Biofertilizer Production Unit,
Mr.Thiru P. Raman
Agricultural Chemist, Biofertilizer Production Unit,
KUDUMIAMALAI-622 104
Distt. Pudukkottai

Main Biocontrol Research Laboratory
(Unit of Tamilnadu Cooperative Sugar Federation)
2E/1, Rajeshwari Vedhachalam Street,
CHENGALPATTU-603 001 (TN)
(04114-431393

The SIMA Cotton Development and Research Association
Dr. M.A. Shanmugham
"Shanmukha Manram", Post Box No. 3871, Race Course,
COIMBATORE-641 018 (TN)
(0422-211391 Tele-Fax: 0422-216798

7. Constraints in Biofertilizer Technology

Though the biofertilizer technology is a low cost, ecofriendly technology, several constraints limit the application or implementation of the technology the constraints may be environmental, technological, infrastructural, financial, human resources, unawareness, quality, marketing, etc. The different constraints in one way or other affecting the technique at production, or marketing or usage.

Technological constraints

- Use of improper, less efficient strains for production.
- Lack of qualified technical personnel in production units.
- Unavailability of good quality carrier material or use of different carrier materials by different producers without knowing the quality of the materials.
- Production of poor quality inoculants without understanding the basic microbiological techniques
- Short shelf life of inoculants.

Infrastructural constraints

- Non-availability of suitable facilities for production
- Lack of essential equipments, power supply, etc.
- Space availability for laboratory, production, storage, etc.
- Lack of facility for cold storage of inoculant packets

Financial constraints

- Non-availability of sufficient funds and problems in getting bank loans
- Less return by sale of products in smaller production units.

Environmental constraints

- Seasonal demand for biofertilizers
- Simultaneous cropping operations and short span of sowing/planting in a particular locality
- Soil characteristics like salinity, acidity, drought, water logging, etc.

Human resources and quality constraints

- Lack of technically qualified staff in the production units.
- Lack of suitable training on the production techniques.
- Ignorance on the quality of the product by the manufacturer
- Non-availability of quality specifications and quick quality control methods
- No regulation or act on the quality of the products
- Awareness on the technology
- Unawareness on the benefits of the technology

- Problem in the adoption of the technology by the farmers due to different methods of inoculation.
- No visual difference in the crop growth immediately as that of inorganic fertilizers.

Awareness on the technology

- Unawareness on the benefits of the technology.
- Problem in the adoption of the technology by the farmers due to different methods of inoculation.
- No visual difference in the crop growth immediately as that of inorganic fertilizers.
- Unawareness on the damages caused on the ecosystem by continuous application of inorganic fertilizer.

Marketing constraints

- Non availability of right inoculant at the right place in right time.
- Lack of retain outlets or the market network for the producers.

8. Biofertilizer strains developed from TNAU

Azospirillum	**Strains**
Normal soils	Az. 204
Acid soils	Az Y2
Dry lands	Azt. 11
Rhizobium	
Groundnut	TNAU 14
Soybean	Cos 1
Cowpea	Coc 10
Redgram	Cc 1
Greengram &	COG 15
Blackgram	GMBS 1
Bengalgram	CoBe 13
Phosphobacteria	
All crops	PB 1

9. Economics

The increasing demand for the biofertilizers and the awareness among farmers and planters in the use of biofertilizers have paved way for the fertilizer manufactures and new entrepreneurs to get into biofertilizer production. A number of biofertilizer production units have been started recently particularly in the southern states of our country.

Nationalized banks have started their Hi-Tech agricultural programme providing loan and motivated the entrepreneurs to start their own production units. The Government of India is also encouraging this low cost technology by providing a subsidy upto Rs.20 lakhs to start a production unit with the capacity of 150 metric tonnes per annum. However, we are all aware that the success of the project entirely depends on the economic viability. With the objective of giving an overall economics of the biofertilizer production and sales, an approximate estimate is prepared.

Total estimate for starting a biofertilizer production unit with the capacity of 150 metric tonnes/annum.

S. No.	Particulars		Amount (Rs.in lakhs)
I.	Expenditure*		
A.	Capital Investment (Fixed cost)		
i.	Building including cost of site (App. 1200 sq. ft.)	:	12.00
ii.	Equipment and apparatus	:	41.00
B.	**Operational cost (variable cost)**		
i.	Working capital (Raw materials)	:	10.00
ii.	Staff salary	:	2.04
iii.	Labour	:	2.50
iv.	Electricity	:	0.50
v.	Travelling expenses	:	0.50
vi.	Administrative expenses	:	0.50
vii.	Interest on loan and depreciation	:	0.70
viii.	Miscellaneous expenses	:	0.26
	Total (variable cost)	**:**	**17.00**
	Total investment	**:**	**70.00**
	Actual initial investment	**:**	**50.00**

* The expenditures does not include the marketing expenses

Expenditure details (Rupees in lakhs)

S.No.	Equipment and apparatus	Qty (Nos.)	Amount (Rs.in lakhs)
1.	Fermentor (200 lit. capacity)	4	26.00
2.	Shaker	2	1.50
3.	Laminar air flow chamber	1	0.60
4.	Autoclave	2	0.30
5.	Hot air oven	1	0.10
6.	Incubator	1	0.10
7.	Refrigerator	1	0.30
8.	Microscope	1	0.75
9.	pH meter	1	0.15
10.	Physical balance	1	0.10
11.	Electronic balance	1	0.75
12.	Counter-poise balance	5	0.25
13.	Sealing machine	5	0.25
14.	Work benches	4	0.30
15.	Plastic trays	50	0.25
16.	Trays (Zinc/Aluminium)	10	0.20
17.	Trolley	1	0.10
18.	Automatic packing machine (optional)	1	9.00
	Total		**41.00**

Working capital

1.	cost of mother culture	**:**	0.05
2.	Glasswares	**:**	0.70
3.	Chemicals	**:**	2.50
4.	Polythene bags	**:**	3.50
5.	Carrier materials	**:**	3.00
6.	Miscellaneous items	**:**	0.25
	Total	**:**	**10.00**

Staff salary

Technical staff (1 No.)	:	9000 x 12	1,08,000
Laboratory staff (2 Nos.)	:	4000 x 2 x 12	96,000
Total			**2,04,000**

II. Production

60% capacity	:	90 MT per year
75% capacity	:	112.5 MT per year
100% capacity	:	150 MT per year

III. Receipts

Cost of 1 kg of biofertilizer (present Govt./University rate)	:	Rs.25/-
Cost of 90 MT (60% capacity)	:	22.500 lakh rupees
112.5 MT (75% capacity)	:	28.125 lakh rupees
135 MT (90% capacity)	:	33.750 lakh rupees
150 MT (100% capacity)		37.500 lakh rupees

IV. Profitability

Year	**Production**	**Receipt (Lakh Rs.)**	**Expenditure (Lakh Rs.)**	**Gain (Lakh Rs.)**
I	60%	22.500	50.000	-27.500
II	75%	28.125	18.700*	9.425
III	90%	33.750	20.570*	13.180
IV	100%	37.500	22.630*	14.870
	Profit anticipated after 4 years			**9.975**

**Every year 10% increase in the expenditure is calculated to balance the price escalation*

Economics of AM biofertilizer – Mass production

I	Capital cost (for construction of pits size of 4x 3x1.5 ft including construction material sand labour cost)	Rs.3,000/-
2	Inoculum cost (from TNAU) 20 KG @ Rs.20/- per kg	Rs.400/-
3	Vermiculite cost (including transport charges) 500kg@ Rs.6.50	Rs.3,250/-
4	Labour cost-Since it is a single pit, family members can look after	NA
5	Seed materials and mesh for covering for pits	Rs.100+100
6	Quality control charges at TNAU (This will be done after 1 year and before selling the product & need not be carried out after each harvest)	Rs.1,000/-
7	Bag- cost of packing the materials-30 @ Rs.10 each Labour cost of harvesting and packing	Rs.300/- Rs.200/-
	Total	Rs.8,350/-
8	Benefit expected by the sale of produced inoculum 500kg @ Rs.20/- per kg (In TNAU) Rs.35/- per kg (In Private)	Rs.10,000/- Rs.17,500/-
9	Net Income (First harvest) Rs.10,000-8,350(Sl.No.8 – Sl.No 1 to 7) Rs.17,500-8,350	Rs.1,197/- Rs.9,150/-
10	For the II harvest the cost will be	Rs.4,950/-
11	From the second harvest benefit will be of Rs.10,000/ - Rs.4,950/ Rs.17,500/ - Rs.4,950/	Rs. 5,050/- Rs.12,550/-
12	The Net Income for one year will be Rs.50,000/ -Rs.24,750/ Rs.87,500/ -Rs.24,750/	Rs.25,250/- Rs.62,750/-

10. Cost and availability of Biofertilizers

Name of Biofertilizers	Cost of Biofertilizers	Availabilty
Azospirillum	**Rs.40/Kg**	**Professor and Head Department of Agricultural Microbiology Tamil Nadu Agricultural University Coimbatore - 641 003 Phone: 91-422-6611294 Fax: 91-422-2431672 Email: microbiology@tnau.ac.in**
Phosphobacteria	**Rs.40/Kg**	
Rhizobium	**Rs.40/Kg**	
Azotobacter	**Rs.40/Kg**	
VAM	**Rs.30/Kg**	

Ashbys Mannitol Agar M706 Ashbys Mannitol Agar is used for cultivation of Azotobacter species that can use mannitol and atmospheric nitrogen as source of carbon and nitrogen respectively.

Composition**

Ingredients Gms / Litre

Mannitol	20.000
Dipotassium phosphate	0.200
Magnesium sulphate	0.200
Sodium chloride	0.200
Potassium sulphate	0.100
Calcium carbonate	5.000
Agar	15.000
Final pH (at 25°C)	7.4±0.2

**Formula adjusted, standardized to suit performance parameters

Directions Suspend 40.7 grams in 1000 ml distilled water. Heat just to boiling . Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well and pour into sterile Petri plates.

Principle And Interpretation

Azotobacter is a genus of free-living diazotrophic bacteria which have the highest metabolic rate compared to any other microorganisms. Azotobacters are chemoorganotrophic, using sugars, alcohols and salts of organic acids for growth. Azotobacters can non-symbiotically fix atmospheric nitrogen aerobically due to their unique mode of metabolism. Besides the ability to fix atmospheric nitrogen, Azotobacter also synthesize biologically active substances, which attributes to improving seed germination, plant growth etc.

Ashbys Agar Media are formulated as described by Subba Rao (1). It is used for isolation of Azotobacter , a non-symbiotic nitrogen fixing bacteria which uses mannitol as a carbon source and atmospheric nitrogen as nitrogen source. Dipotassium phosphate provides buffering to the medium. Various essential ions required for promoting growth of Azotobacter are also available in this medium

Quality Control Appearance White to cream homogeneous free flowing powder Gelling Firm, comparable with 1.5% Agar gel Colour and Clarity of prepared medium Whitish opalescent gel forms in Petri plates Reaction

Reaction of 4.07% w/v aqueous solution at 25°C.

pH : 7.4±0.2 pH 7.20-7.60

Cultural Response M706: Cultural characteristics observed after an incubation at 35-37°C for upto 5 days. Organism Growth Azotobacter nigricans ATCC 35009 good-lu

Dobereiner's medium

Composition	**g/l**
Malic acid	5 g
KOH	4 g
Yeast Extract	5 g
Mn SO_4H_2O (1%)	1 ml
$MgSO_47H_2O$ (10%)	1 ml
NaCl (10%)	2 ml
K_2HPO_4 (10%)	4 ml
$NaMoO_4$ (0.1%)	0.2 ml
$CaCl_2$ (10%)	1 ml
$FeSO_4.7H_2O$ (5%)	1 ml
1 m NH_4Cl	5 ml
Bromthymol Blue	3 ml

Preparation of Bromthymol blue
- 0.5 g in 53 ml of 95% ETOH and add 47 ml of distilled water.

Preparation of 5M NH_4Cl
- Weigh 24 g ammonium chloride
- Dissolve in 100 ml distilled water

Preparation for 5M KOH
- Weigh 35.05 g potassium hydroxide
- Dissolve in 125 ml distilled water

Preparation of Dobereiners medium
1. Prepare stock solution of the 8 chemical reagents and label each bottle.
2. Pipette the aliquot (volume) of the stock and complete volume to 1000 ml.
3. Add 3 ml Bromthymol Blue to the liquid medium,
4. Adjust pH using 1.0 N KOH until its greenest color is attained (pH 6.5-6.8).
5. Dispense medium into desired bottles properly covered and Sterilize at 15 psi for 15 minutes.

Yeast Mannitol Agar w/ 1.5% Agar

Yeast Mannitol Agar w/ 1.5% Agar is used for cultivation, isolation and enumeration of soil microorganisms like Rhizobium species.

Composition**	Ingredients Gms / Litre
Yeast extract	1.000
Mannitol	10.000
Dipotassium phosphate	0.500
Magnesium sulphate	0.200
Sodium chloride	0.100

Calcium carbonate	1.000
Agar	15.000
Final pH (at 25°C)	6.8±0.2

**Formula adjusted, standardized to suit performance parameters

Directions Suspend 27.8 grams in 1000 ml distilled water. Heat just to boiling. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well and pour into sterile Petri plates. Note : Due to presence of calcium carbonate, the prepared medium forms opalescent solution with white precipitate.

Principle And Interpretation

Beijerinck was first to isolate and cultivate an aerobic gram negative rod-shaped microorganism from the nodules of legume. He named it Bacillus radicicola , which was subsequently placed under the genus Rhizobium . Bacteria belonging to the genus Rhizobium live freely in soil and in the root region of both leguminous and non-leguminous plants. However they can enter into symbiosis only with leguminous plants by infecting their roots and forming nodules on them. Rhizobium present in these root nodules fixes atmospheric nitrogen i.e. gaseous nitrogen from air to organic nitrogen compounds, which is absorbed by plants. Thus role of Rhizobium is noteworthy for their major contributions to soil fertility. Yeast Mannitol Agar is used for the cultivation of symbiotic nitrogen fixing organisms viz. Rhizobium species (1) Yeast extract serves as a good source of readily available amino acids, vitamin B complex and accessory growth factors for Rhizobia. It also poises oxidation - reduction potential of medium in the range favorable for Rhizobia and serves as hydrogen donor in respiratory process (2). Mannitol is the fermentable sugar alcohol source. Calcium and magnesium provide cations essential for the growth of Rhizobia.

pH 6.60-7.00

Cultural Response

M715: Cultural characteristics observed after an incubation at 25-30°C for upto 5 days.

Organism	Growth
Rhizobium leguminosarum ATCC 10004 luxuriant	
Rhizobium meliloti ATCC 9930	

luxuriant Storage and Shelf Life Store below 30°C in tightly closed container and the prepared medium at 2- 8°C. Use before expiry date on the label

a. Fogg's medium with or without N2 source (Fogg, 1949)for Azolla
   Preparation for 1 litre medium:

Potassium di-hydrogen phosphate (K2HPO4)	0.2 g
Magnesium sulphate (MgSO4. 7H2O)	0.2 g
Calcium chloride (CaCl2. 2H2O)	0.1 g
Fe-EDTA stock solution	1.0 ml
A5 micronutrient solution	1.0 ml
Distilled water	1000 ml
p H	6.5- 7.0

Add 1.5 g of NaNO3 per litre to the above composition of Fogg's medium with N2 sources for non-nitrogen fixing blue green algae growth.

Ashbys Mannitol Agar M706

Ashbys Mannitol Agar is used for cultivation of *Azotobacter* species that can use mannitol and atmospheric nitrogen as source of carbon and nitrogen respectively.

Composition**

Ingredients	Gms / Litre
Mannitol	20.000
Dipotassium phosphate	0.200
Magnesium sulphate	0.200
Sodium chloride	0.200
Potassium sulphate	0.100
Calcium carbonate	5.000
Agar	15.000

Final pH (at 25°C) 7.4±0.2

**Formula adjusted, standardized to suit performance parameters

Directions

Suspend 40.7 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at
15 lbs pressure (121°C) for 15 minutes. Mix well and pour into sterile Petri plates.
Note: Due to presence of calcium carbonate, the prepared medium forms opalescent solution with white precipitate .

Principle And Interpretation

Azotobacter is a genus of free-living diazotrophic bacteria which have the highest metabolic rate compared to any other microorganisms. *Azotobacters* are chemoorganotrophic, using sugars, alcohols and salts of organic acids for growth. *Azotobacters* can non-symbiotically fix atmospheric nitrogen aerobically due to their unique mode of metabolism. Besides the
ability to fix atmospheric nitrogen, *Azotobacter* also synthesize biologically active substances, which attributes to improving
seed germination, plant growth etc.
Ashbys Agar Media are formulated as described by Subba Rao (1). It is used for isolation of *Azotobacter* , a non-symbiotic nitrogen fixing bacteria which uses mannitol as a carbon source and atmospheric nitrogen as nitrogen source. Dipotassium phosphate provides buffering to the medium. Various essential ions required for promoting growth of *Azotobacter* are also available in this medium

Quality Control

Appearance
White to cream homogeneous free flowing powder
Gelling
Firm, comparable with 1.5% Agar gel
Colour and Clarity of prepared medium
Whitish opalescent gel forms in Petri plates
Reaction
Reaction of 4.07% w/v aqueous solution at 25°C. pH : 7.4±0.2
pH
7.20-7.60

Cultural Response

M706: Cultural characteristics observed after an incubation at 35-37°C for upto 5 days.

Organism Growth

Azotobacter nigricans ATCC 35009

Bio fertilizer

Bio fertilizers are not fertilizers. **"Bio fertilizer"** is a substance which **contains living microorganisms** which, when applied to seed, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and **promotes growth** by increasing the supply or availability of primary nutrients to the host plant.

Why bio fertilizers?

It's a microbial green revolution. Bio fertilizers are having it sown advantages over chemical fertilizers and it is economically and environmental friendly too. With the increasing demand in agriculture it has become important for us to increase the Productivity by using various fertilizers insecticides Pesticides .But with the tremendous use of these products the soil has been affected badly because of the depletion in the essential minerals of the soil. So to overcome this problem it has become important for all of us to use a different remedy for the production of various bio fertilizers. They are the best at economic value.

Types of bio fertilizers:

- Nitrogen bio fertilizers
- Compost bio fertilizers
- Phosphorous bio fertilizers

Nitrogen fixing fertilizers:

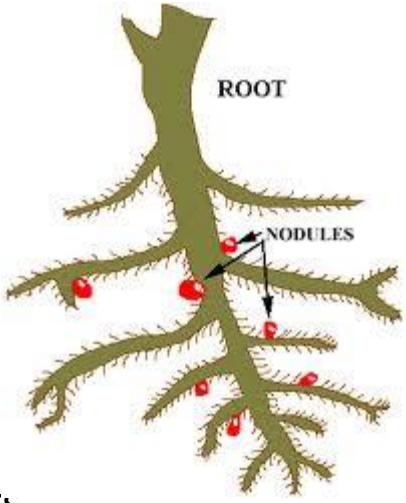
Symbiotic: Rhizobium

It belongs to rhizobiaceae family, the rhizobium bacteria present in the nodules of these crops are not always efficient. Therefore, the competitive, efficient bacteria are isolated, screened, selected and produced as carrier based inoculants

Morphology:

1) Unicellular, cell size less than 2µ wide. Short to medium rod, pleomorphic
2) Motile with peritricus flagella
3) Gram negative
4) Accumulate poly β-hydroxyl butyrate granules.

Physiology:

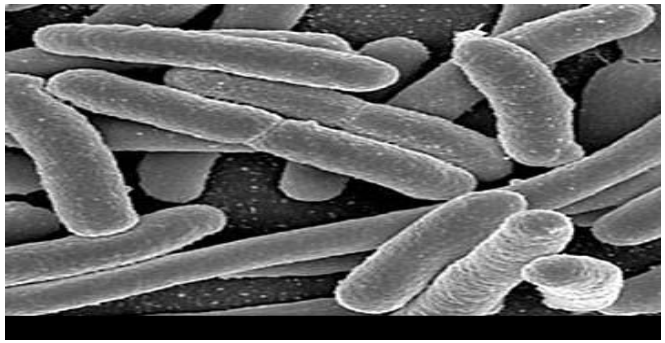


1) Nature : chemo heterotrophic, symbiotic with legume
2) C source: supplied by legume through photosynthesis, mono disaccharide.
3) N source: fixed from atmosphere.
4) Respiration: aerobic.
5) Growth: fast (rhizobium), slow (Brady rhizobium)
6) Doubling time: fast grower- 2-4 hours slow grower 6-12 hours.
7) Growth media : YEMA

Recommended for :

Pulses: chickpea, pea, lentil, black gram, green gram, cowpea, pigeon pea.
Oil seeds: soybean, groundnut.

Non symbiotic: Azospirillum, Azotobacter.



Azotobacter

It belongs to azotobacteriaceae .It produces growth promoting substances which improve seed germination and growth of extended root system. It produces polysaccharides which improve soil aggregation. Azotobacter suppresses the growth of saprophytic and pathogenic micro-organism near the root system of crop plants

Morphology:

1) Cell size: Large ovoid cells, size ranging from 2.0-7.0×1.0-2.5µ.
2) Cell character: polymorphic
3) Accumulate poly β-hydroxyl butyrate granules.
4) Gram reaction: negative

Physiology:

1) Nature: chemo heterotrophic, free living
2) C source: a variety of carbon source (mono, di and certain polysaccharide) organic acids.
3) N sources: Nitrogen through fixation, amino acid, NH_4, NO_3
4) Respiration: aerobic
5) Growth media: Ashby Jensen's medium
6) Doubling time: 3 hours

Contribution :

1) 20-40 mg BNF/g of C source in laboratory condition equivalent to 20-40 kg N/ha.
2) Production of growth promoting substance like vitamins of B groups, indoleacetic acid and gibberellin acid.
3) Biological control of plant disease by suppressing Aspergillus, Fusarium.

Recommended for:

Rice, wheat, millets, other cereals, cotton, vegetable, sunflower, mustard, flowers.

Increase in yield: 20 to 30%

Azospirillium

It belongs to family spirillaceae. The bacteria have been found to live within the root of sorghum, bajra and rage plants. They are chemoheterotrophic and association in nature secrete growth regulatory substance

The use of azospirillium inoculants help in increasing yield of millets. It significantly increase the growth, chlorophyll content and mycorrhyzal infection in root. Increased growth and nutrient uptake by barley plants were observed when seed were co-inoculated with A. baselines and Glomus vermiform.

Morphology:

1) Cell size: curved rod, 1mm in diameter, size and shape vary.
2) Accumulate: poly β-hydroxyl butyric acid.
3) Gram reaction: negative
4) Development of white pellicles 2-4mm below the surface of NFB medium.

Physiology:

1) Nature: chemoheterotrophic, associative.
2) C source: organic acid, L-arabinose, D-gluconate, D-fructose, D-glucose, sucrose, pectin.
3) N sources: nitrogen through fixation, amino acids, NH_4, NO_3
4) Respiration: aerobic, micro aerobic.
5) Growth media: N free bromothymol blue (NBF)
6) Doubling time: 1hr in ammonia containing medium, 5.5 to 7hr. on malate containing semi-solid medium

Contribution:

1) 20-40 mg N/g malate under laboratory condition equivalent to 20-40 kg N/ha.
2) Results in increase mineral and water uptake, root development, vegetative growth and crop yield.

Recommended for:

Rice, millets maize, wheat, sorghum, sugarcane and co-inoculants for legumes.

Response: Average increase in yield 15-30%.

Phosphate solubilizing bio fertilizer

Phosphorus is one of the most important plant nutrients and may be critical nutrient for the optimum growth of plants. Most of our soils are in available forms of phosphorus required phosphate application.
In the rhizosphere of crops will render insoluble soil phosphate available to plants due to production and secretion of organic acid by them. The use of this bio fertilizer will also increase the availability of phosphate from rock phosphate applied directly even to neutral to alkaline soil or when used for preparation of phosphor-compost. Phosphate solubilizing micro-organism include efficient strain of bacteria, fungi, yeast and actinomycetes in that order

Bacteria Morphology:

1) Cell size: rod shape, 1.1 to 2.2µm in diameter.
2) Gram reaction: for Bacillus positive and for Pseudomonas negative
3) Transparent zones of clearing around microbial colonies indicate extent of Phosphate solubilization.

Physiology

1) Nature: chemoheterotrophic.
2) C source: Glucose is the main C source but they can utilize other carbon sources.
3) Respiration: aerobic, micro aerobic.
Growth media: Pikovskaya's media

Advantages of using bio fertilizers:

1)They help to get high yield of crops by making the soil rich with nutrients and useful microorganisms necessary for the growth of the plants.

2) Bio fertilizers have replaced the chemical fertilizers as chemical fertilizers are not beneficial for the plants. They decrease the growth of the plants and make the environment polluted by releasing harmful chemicals.

3) Plant growth can be increased if bio fertilizers are used, because they contain natural components which do not harm the plants but do the vice versa.

4) If the soil will be free of chemicals, it will retain its fertility which will be beneficial for the plants as well as the environment, because plants will be protected from getting any diseases and environment will be free of pollutants.

5) Bio fertilizers destroy those harmful components from the soil which cause diseases in the plants. Plants can also be protected against drought and other strict conditions by using bio fertilizers.

6) Bio fertilizers are not costly and even poor farmers can make use of them.

7) They are environment friendly and protect the environment against pollutants.

Disadvantages

- Much lower nutrient density -- requires large amounts to get enough for most crops.
- Requires a different type of machine to apply than chemical fertilizers.
- Sometimes hard to locate in certain areas odor.

Why do we need bio fertilizers……?????????????

An estimate shows 100million tons of fixed N2 is required for global food production. Chemical fertilizers is the most common practice to increase crop yeilds Besides the cost factor the use of chemical fertilizers is associated with environmental pollution.

Process of making bio fertilizer:

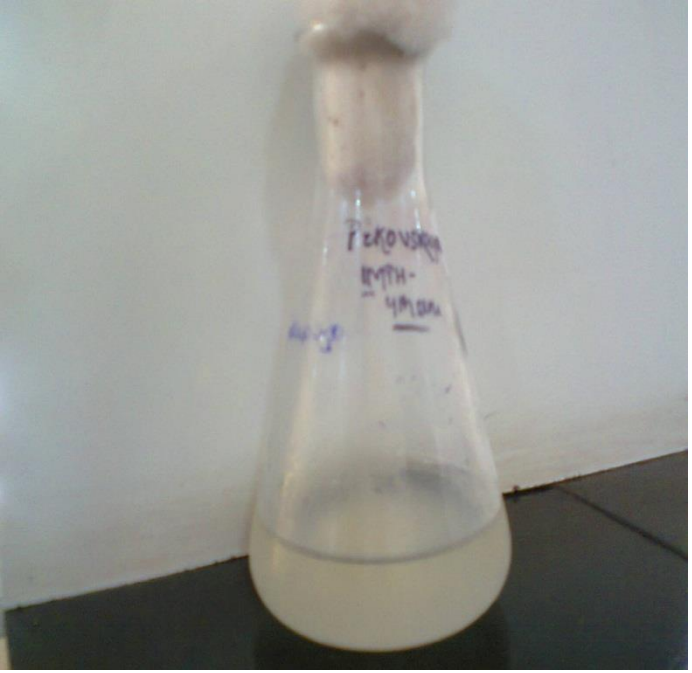
- Bio fertilizers are usually prepared as carrier-based inoculants containing effective microorganism.
- Incorporation of microorganisms in carrier material enables easy-handling, long-term storage and high effectiveness of bio fertilizers.
- Among various types of bio fertilizers, bacterial inoculant is one major group which includes rhizobia, nitrogen-fixing rhizobacteria, plant growth promoting rhizobacteria, phosphate-solubilizing bacteria.
- Basically, the carrier-based inoculant of these bacteria can be prepared by a common procedure.

The most common way of inoculation

Seed inoculation

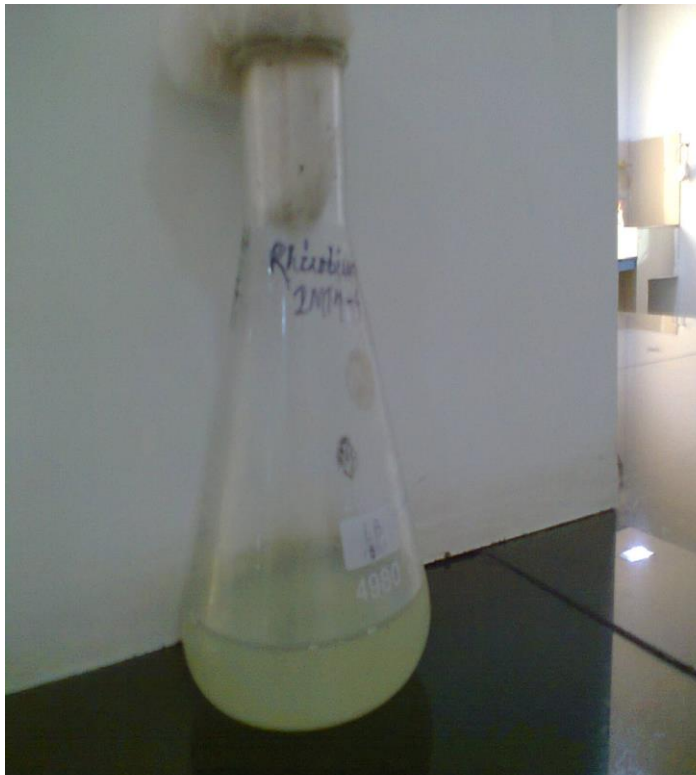
The inoculant (bacteria-carrier mixture) is mixed with water to make slurry-form, and then mixed with seeds. In this case, the carrier must be a form of fine powder. To achieve the tight coating of inoculant on seed surface, use of adhesive, such as gum arabic,methylethylcellulose, sucrose solutions, and vegetable oils, is recommended. Seed inoculation may not always be successful, i.e. the inoculation resulted in low nodule occupancy of the inoculated rhizobial strain, or low establishment of the inoculated rhizobacterial strain. This might be due to low population and/or low survival of the inoculated bacterial strain on the seed surface and in the soil.

“soil inoculation” will be adopted, whereby a large population of a bacterial strain can be introduced into the soil. For soil inoculation in general, granular inoculant is placed into the furrow under or alongside the seed. This enhances the chance for the inoculated strain to be in contact with plant roots.



Phosphate solubilizing culture

Rhizobium culture



Azospirillum culture

Azotobacter and Rhizobium

Carrier material

Various types of material are used as carrier for seed or soil inoculation. For preparation of seed inoculant, the carrier material is milled to fine powder with particle size of 10 -40 µm.

The properties of a good carrier material for seed inoculation are:

(1) Non-toxic to inoculant bacterial strain.
(2) Good moisture absorption capacity.
(3) Easy to process and free of lump-forming materials.
(4) Easy to sterilize by autoclaving or gamma-irradiation.
(5) Available in adequate amounts
(6) Inexpensive.
(7) Good adhesion to seeds, and
(8) Good pH buffering capacity.
(9) Need less non-toxic to plant, is another important property.

Essential criteria for carrier selection relating to survival of the inoculant bacteria should be considered :

(1) Survival of the inoculant bacteria on seed. Seeds are not always sown immediately after seed coating with the inoculant bacteria. The bacteria have to survive on seed surface against drying condition until placed into soil.
(2) Survival of the inoculant bacteria during the storage period.
(3) Survival of the inoculant bacteria in soil.

After being introduced into the soil, the inoculant bacteria have to compete with native soil microorganisms for the nutrient and habitable niche, and have to survive against grazing protozoa. Such carrier materials that offer the available nutrient and/or habitable micro-pore to the inoculant bacteria will be desirable. In this sense, materials with micro-porous structure, such as soil aggregate and charcoal, will be good carrier for soil inoculant.

Sterilization:

Carrier sterilization is autoclaving. Carrier material is packed in partially opened, thin-walled polypropylene bags and autoclaved for 60 min at 121 ºC. It should be noted that during autoclaving, some materials changes their properties and produce toxic substance to some bacterial strains.

Soils

Dr.R.Stephan
Government Arts College
Ariyalur

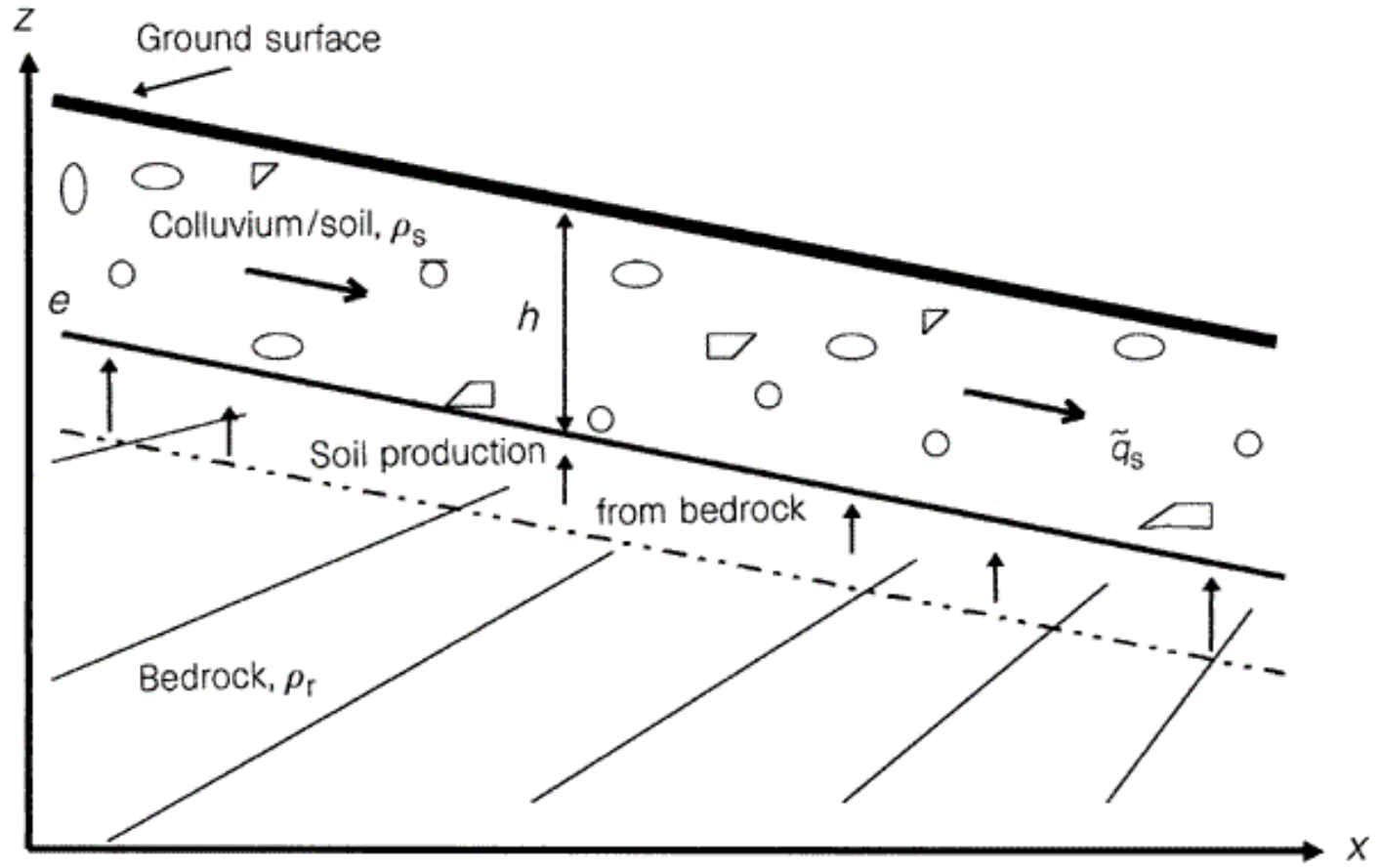
Soils

Soil: Definition

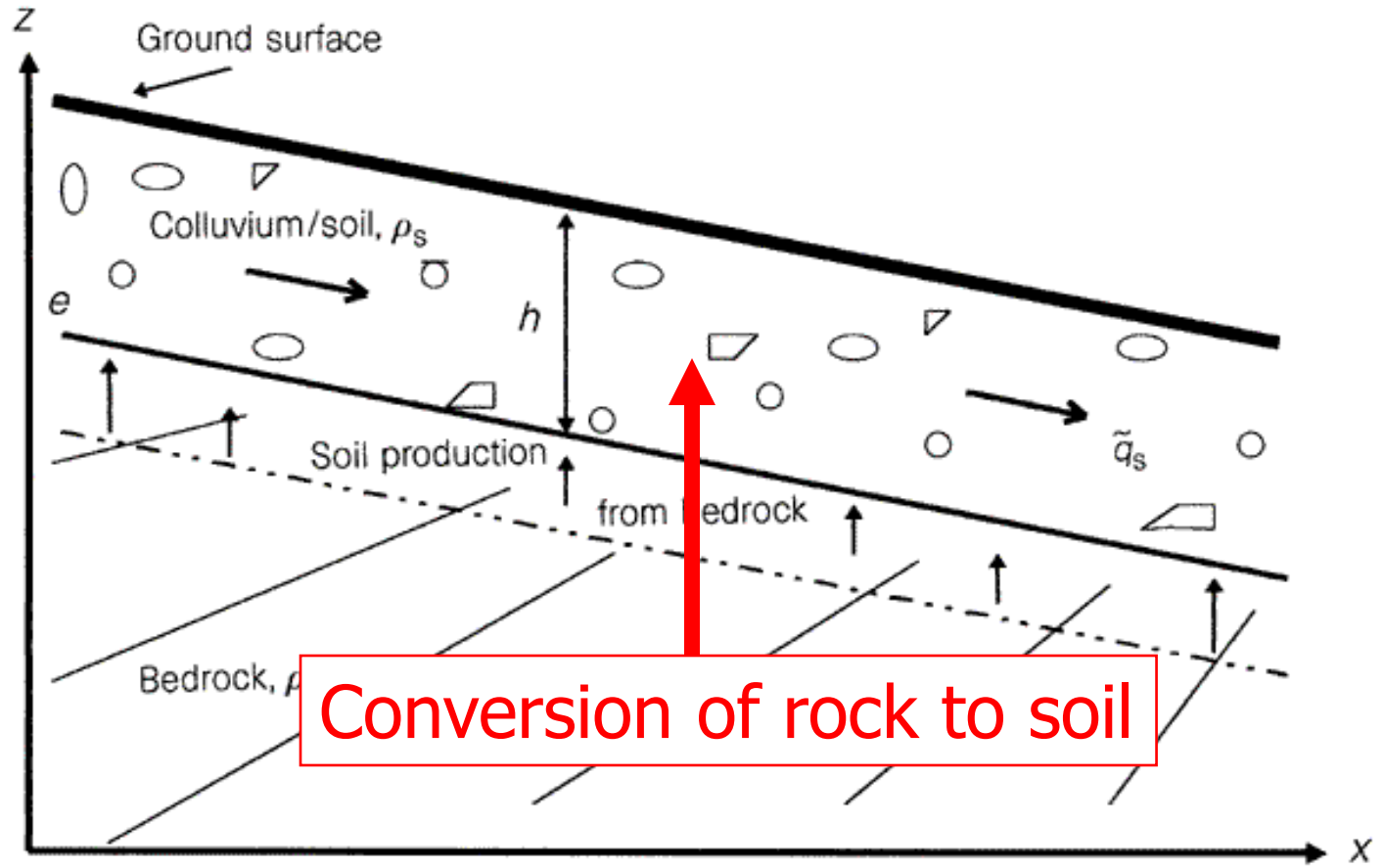
Solid earth material that has been altered by physical, chemical and organic processes so that it can support rooted plant life.

Engineering definition: Anything that can be removed without blasting

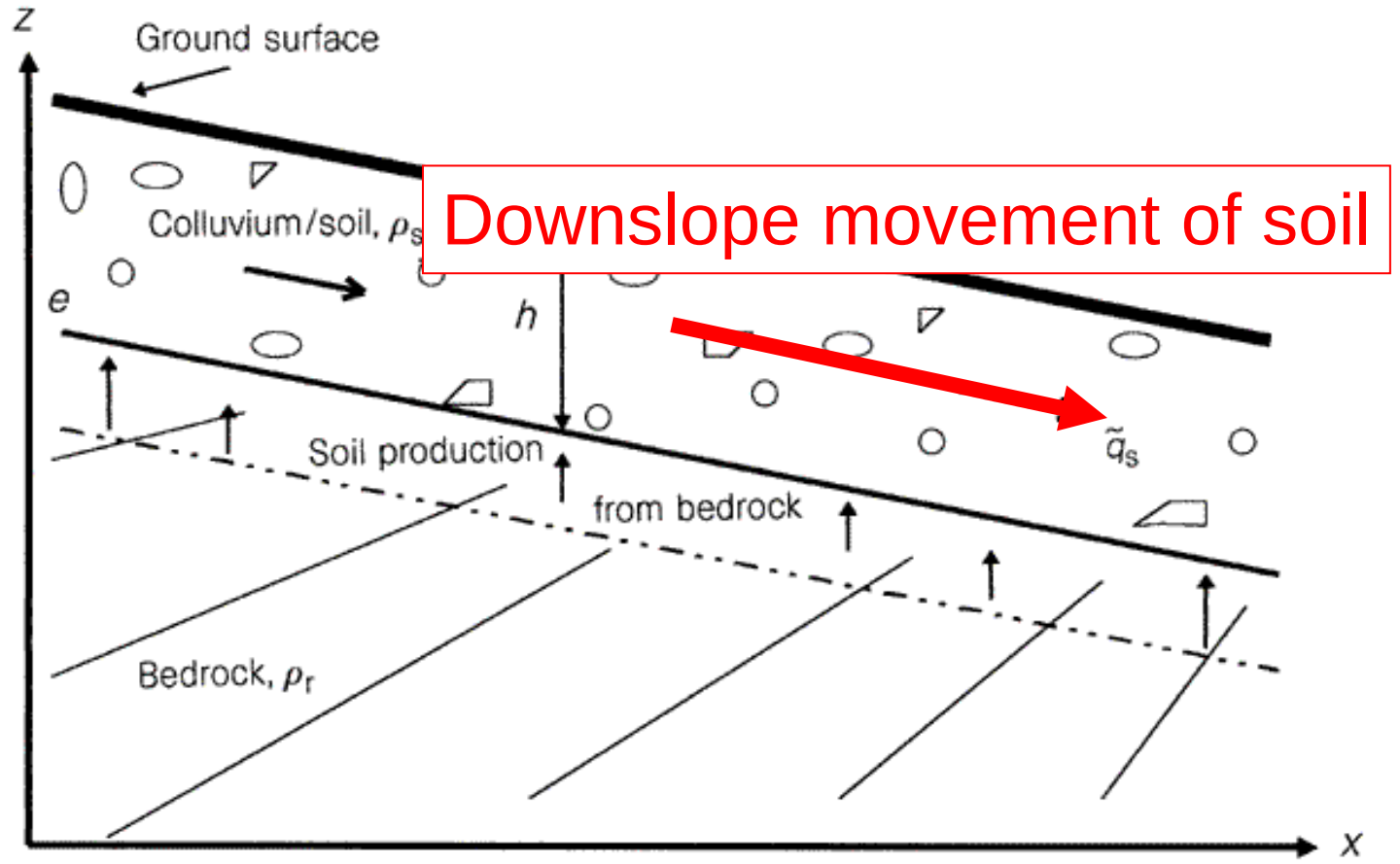
Soil Production



Soil Production: Inputs



Soil Production: Outputs



Soil Thickness: Storage

input ± output = soil thickness

or:

rock weathering ± soil transport = thickness

Soil thickness reflects the *balance* between rates of soil production and rates of downslope soil movement.

Factors of Soil Formation

- Climate
- Organisms
- Parental Material
- Topography
- Time

Factors of Soil Formation

Climate

- Temperature and precipitation
- Indirect controls (e.g., types of plants)
- Weathering rates

The greater the rainfall, the faster the rates of erosion and leaching.

Factors of Soil Formation

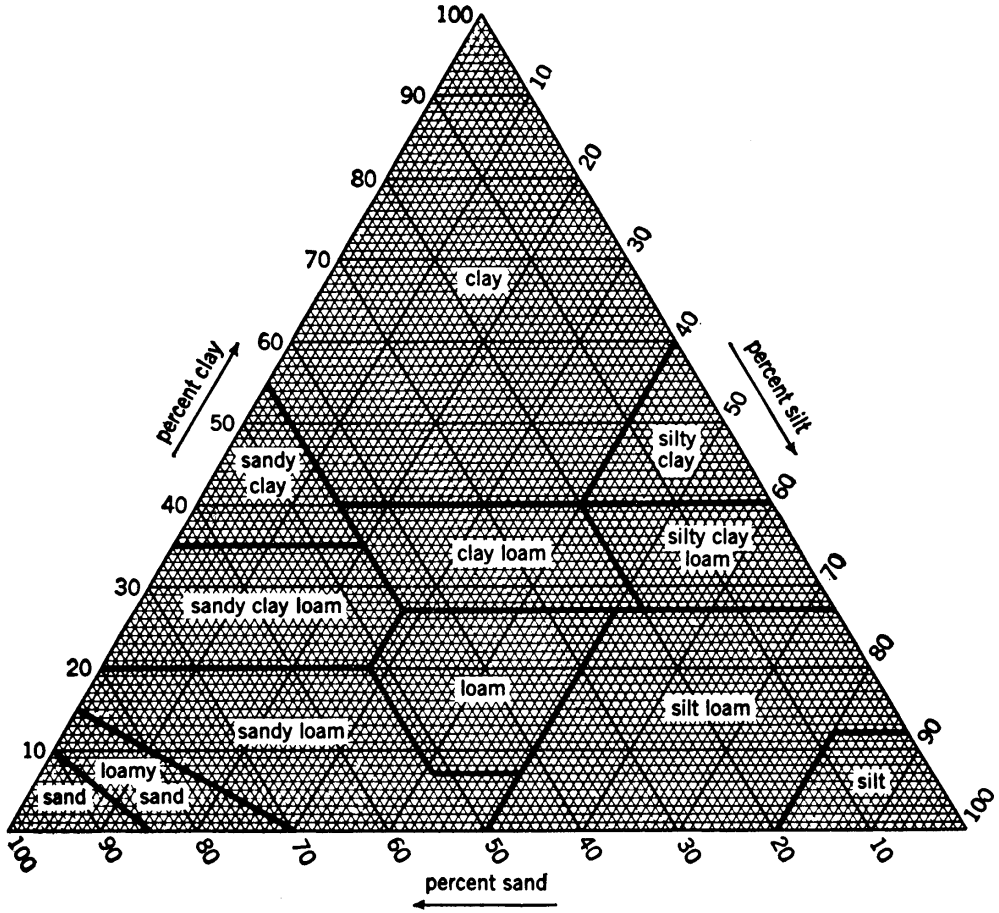
Organisms

- Types of native vegetation
- Weathering is dependent of plant growth
- Plant and animal activity produces humic acids that are powerful weathering agents.
- Plants can physically erode as well as chemically erode.
- Plants stabilize soil profiles, Animals (including humans) tend to increase erosion.

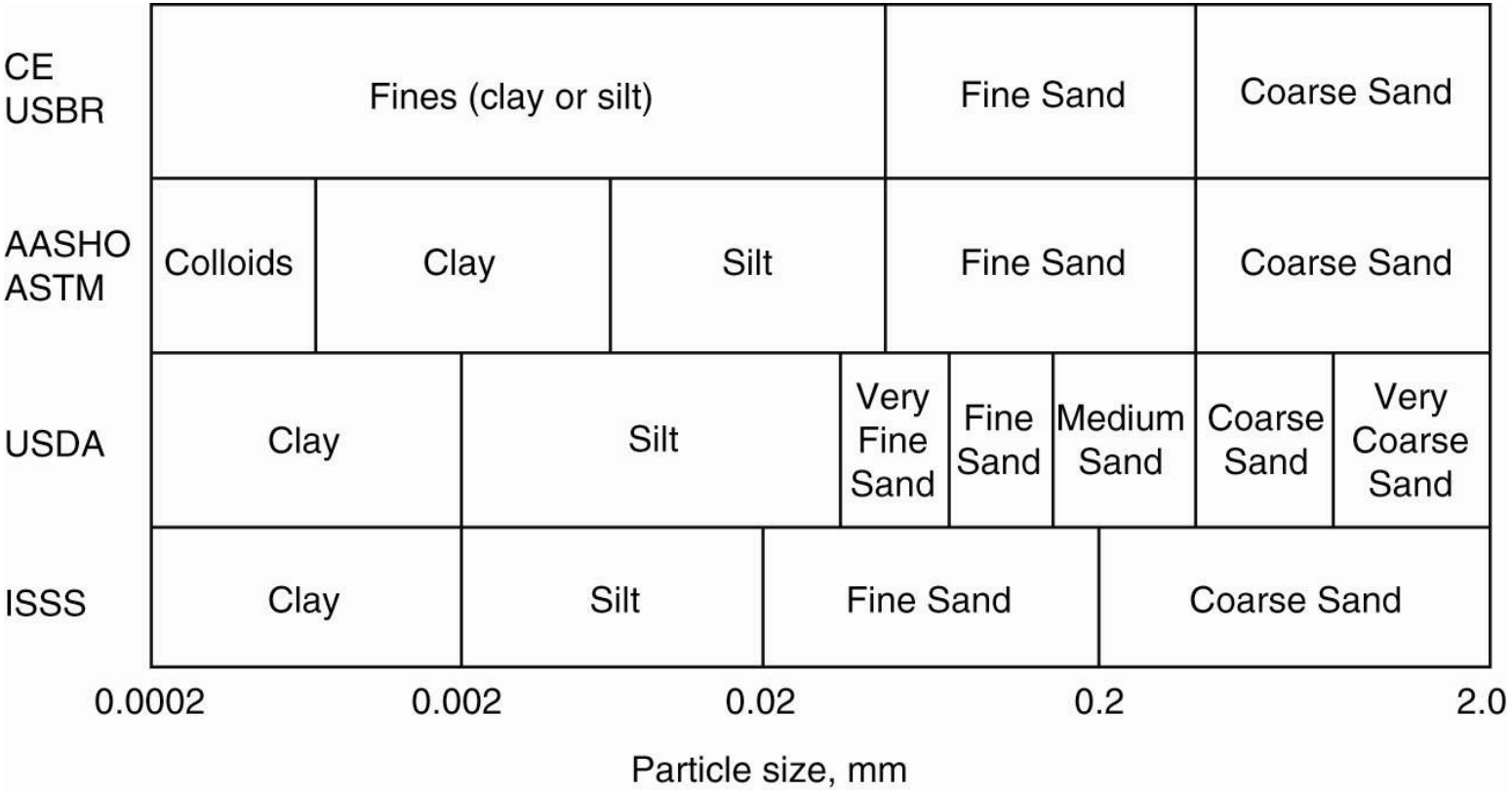
Factors of Soil Formation

Parental Material:

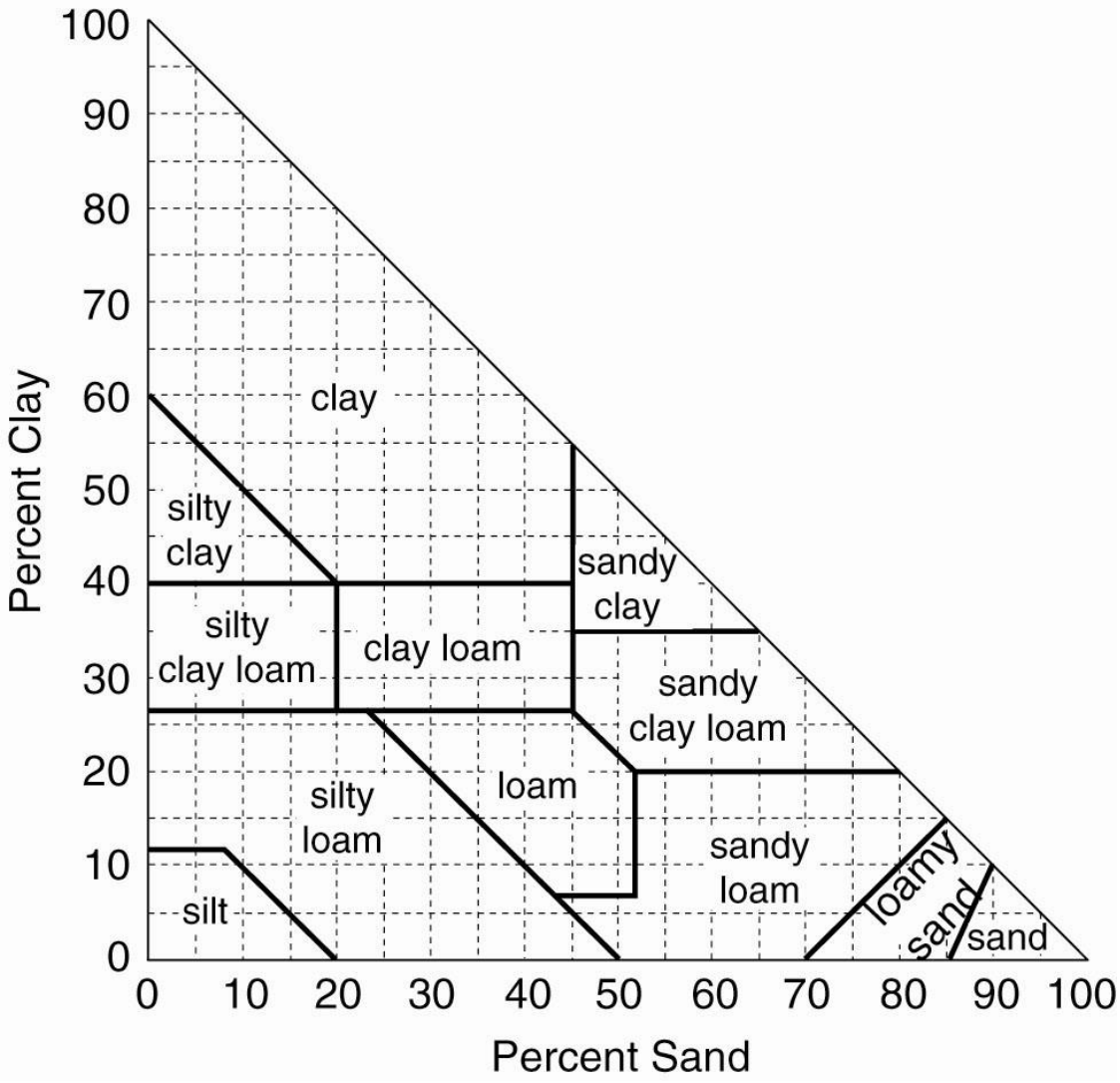
- Chemistry
- Mineralogy
- Grain size



Soil Texture



Soil Texture



Factors of Soil Formation

Topography:

- Ground slope
- Elevation
- Aspect (e.g., north facing vs. south facing slopes)

Factors of Soil Formation

Downslope transport of soil is a function of slope:

$$\text{Erosion rate} = f(S)$$

The steeper the surface slope, the more likely any eroded material is to be transported out of the system.

Factors of Soil Formation

Soils on hillslopes reach an equilibrium thickness, often about 1 m.

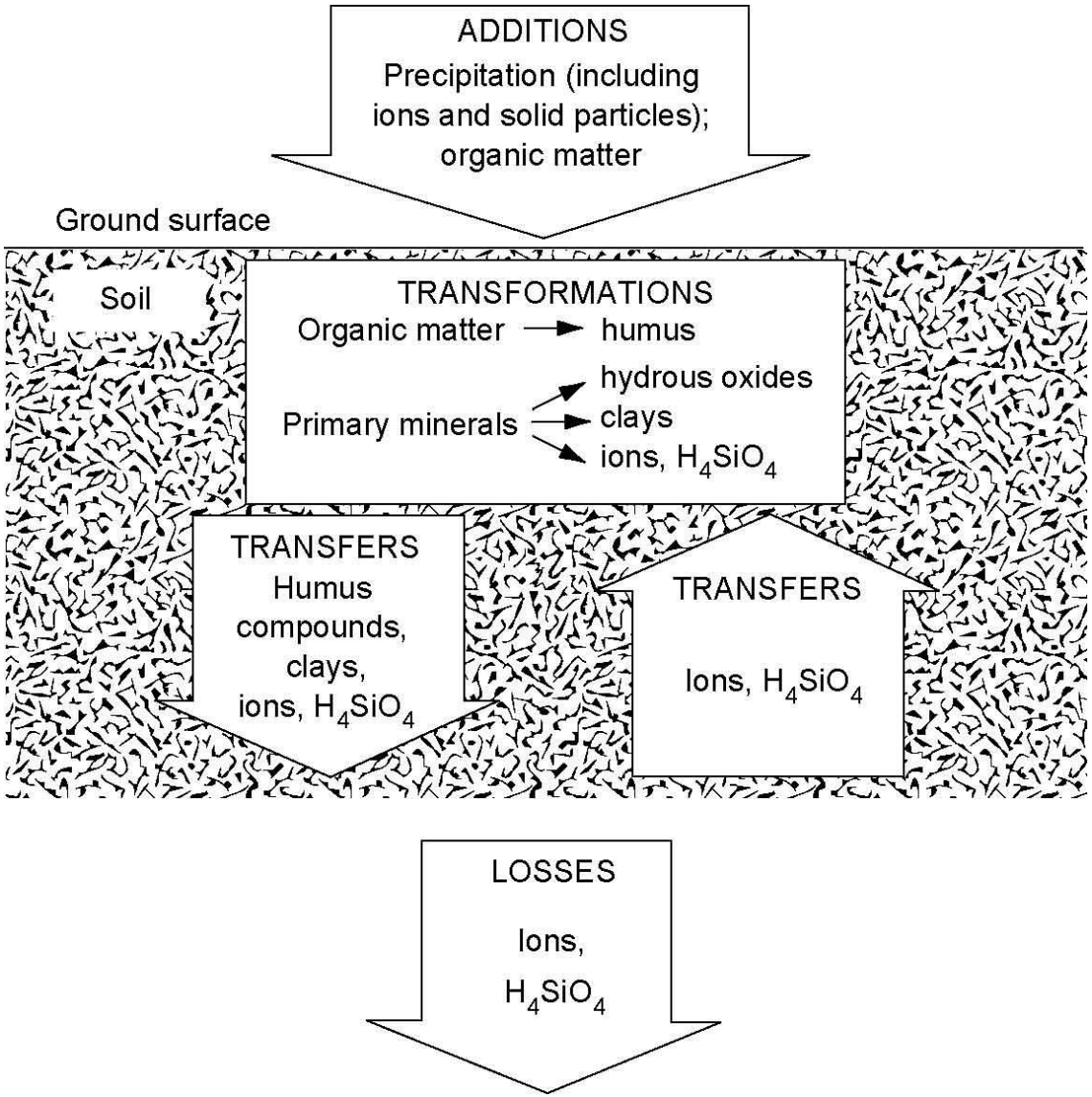
Soils on flat surfaces, such as floodplains or plateaus, tend to thicken through time due to weathering rates being greater than sediment transport rates.

Factors of Soil Formation

Time

- Development and destruction of soil horizons
- Reaction rates are slow, the longer a rock unit has been exposed, the more likely it is to be weathered.

Soil Development



Additions to Soils

Inputs from outside ecosystem

- Atmospheric inputs
  - Precipitation, dust, deposition
- Horizontal inputs
  - Floods, tidal exchange, erosion, land-water movement

Inputs from within ecosystem

- Litterfall and root turnover

Transformations

- *Decomposition* of organic matter
- *Humification* to form complex organic matter
- Weathering of rocks to produce more stable forms
  - Physical weathering
    - Fragmentation of rock
      - Freeze-thaw; drying-wetting; fire
    - Physical abrasion
      - Abrasion by glaciers
  - Chemical weathering
    - Dissolves primary minerals
    - Forms secondary minerals

Decomposition

- Breakdown of soil organic matter to form soluble compounds that can be absorbed or leached
- Depends on
  - Quantity of input
  - Location of input (roots vs. leaves)
  - Environment
    - Temperature
    - moisture

Soil Horizons and Profiles

Soil Horizons

- Layers in Soil
- Not Deposited, but zones of chemical action

Soil Profile

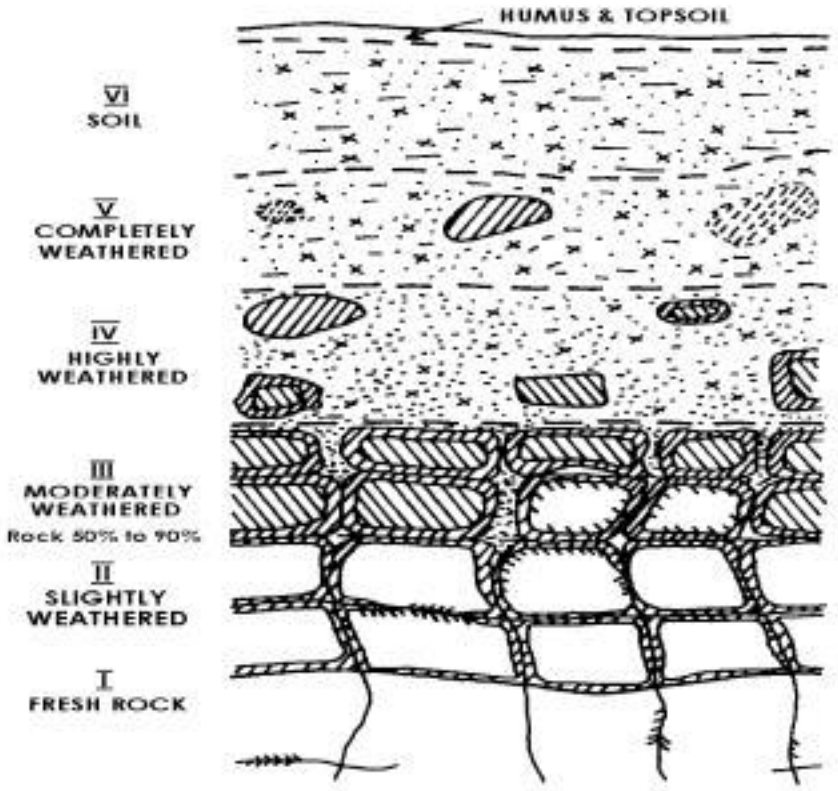
- Suite of horizons at a given locality

Soil Horizons and Profiles

Soil Horizons

- Over time, soil layers differentiate into distinct ‘horizons’ defined by zones of chemical action.
- Chemical reactions and formation of secondary minerals (clays).
- Leaching by infiltrating water (*elluviation*).
- Deposition and accumulation of material leached from higher levels in the soil (*illuviation*).

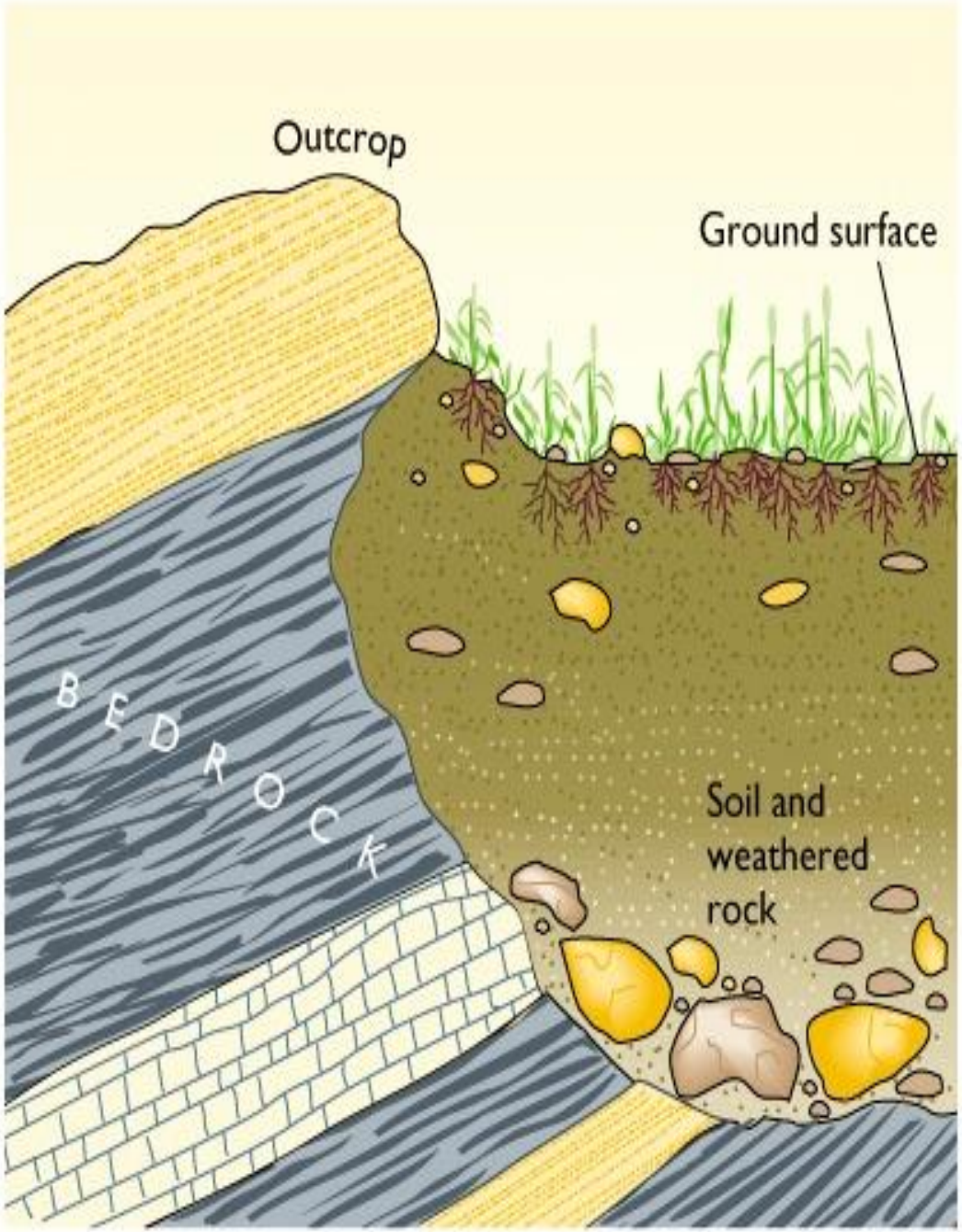
Saprolite is weathered rock that retains remnant rock structure.



Soil

Saprolite

Bedrock

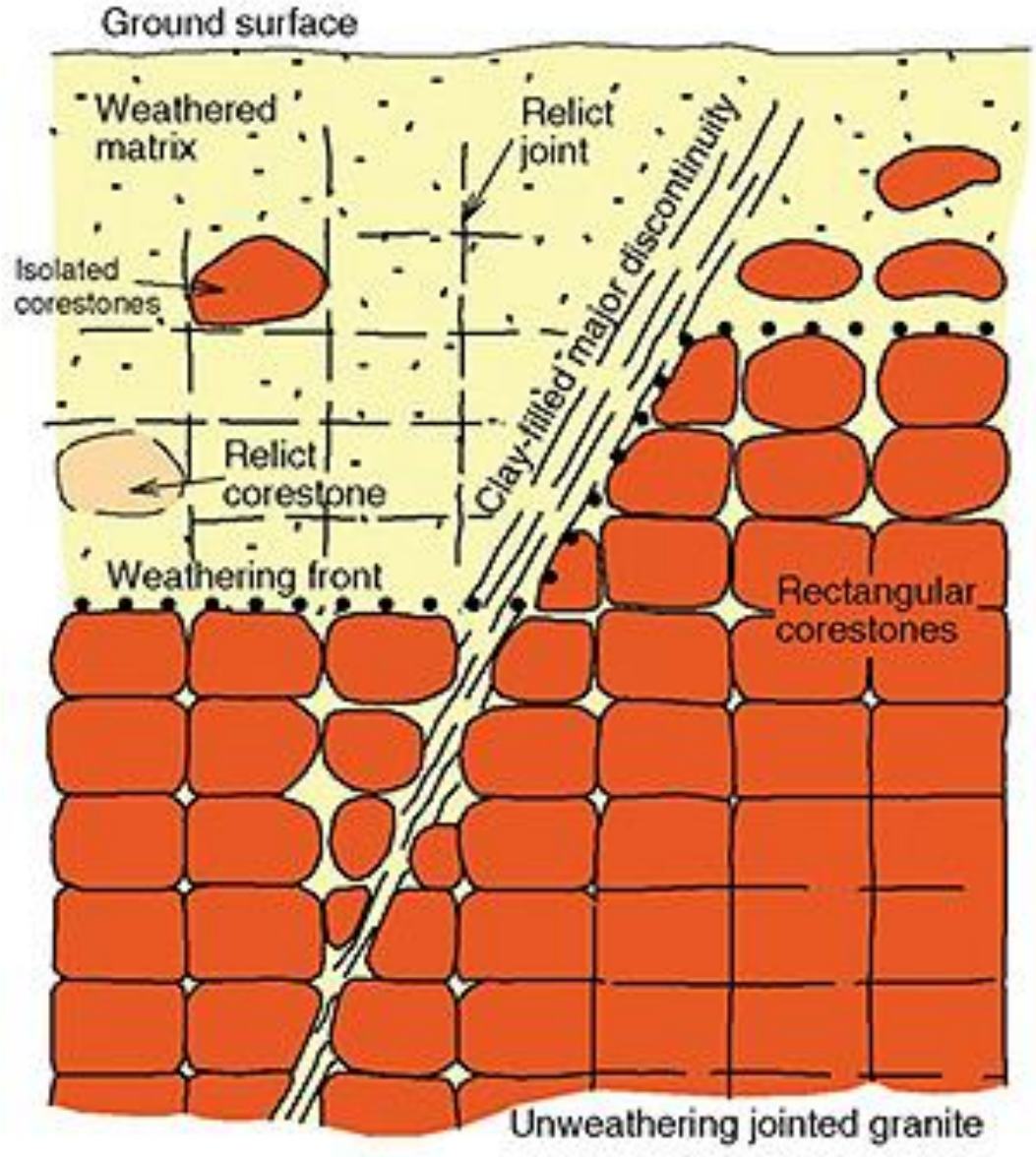


Weathering Products:

Product of weathering is regolith or soil

Regolith or soil that is transported is called sediment

Weathering often proceeds along fractures and joints, resulting in progressive development of corestones.

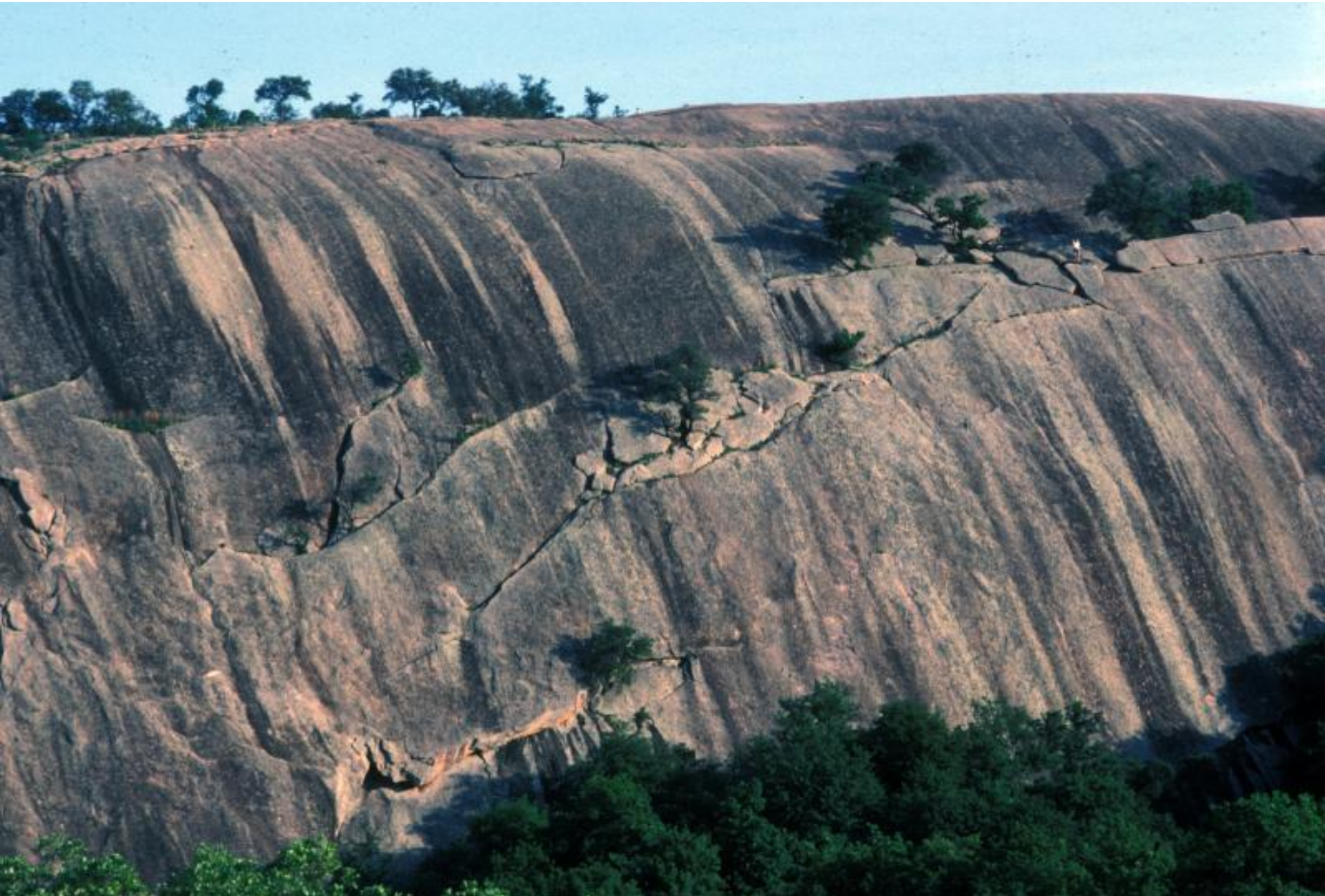


Weathering often proceeds along fractures and joints, resulting in progressive development of corestones that when exposed at the surface can form **tors** and larger **bornhardts** (also known as **inselbergs** or rock islands).

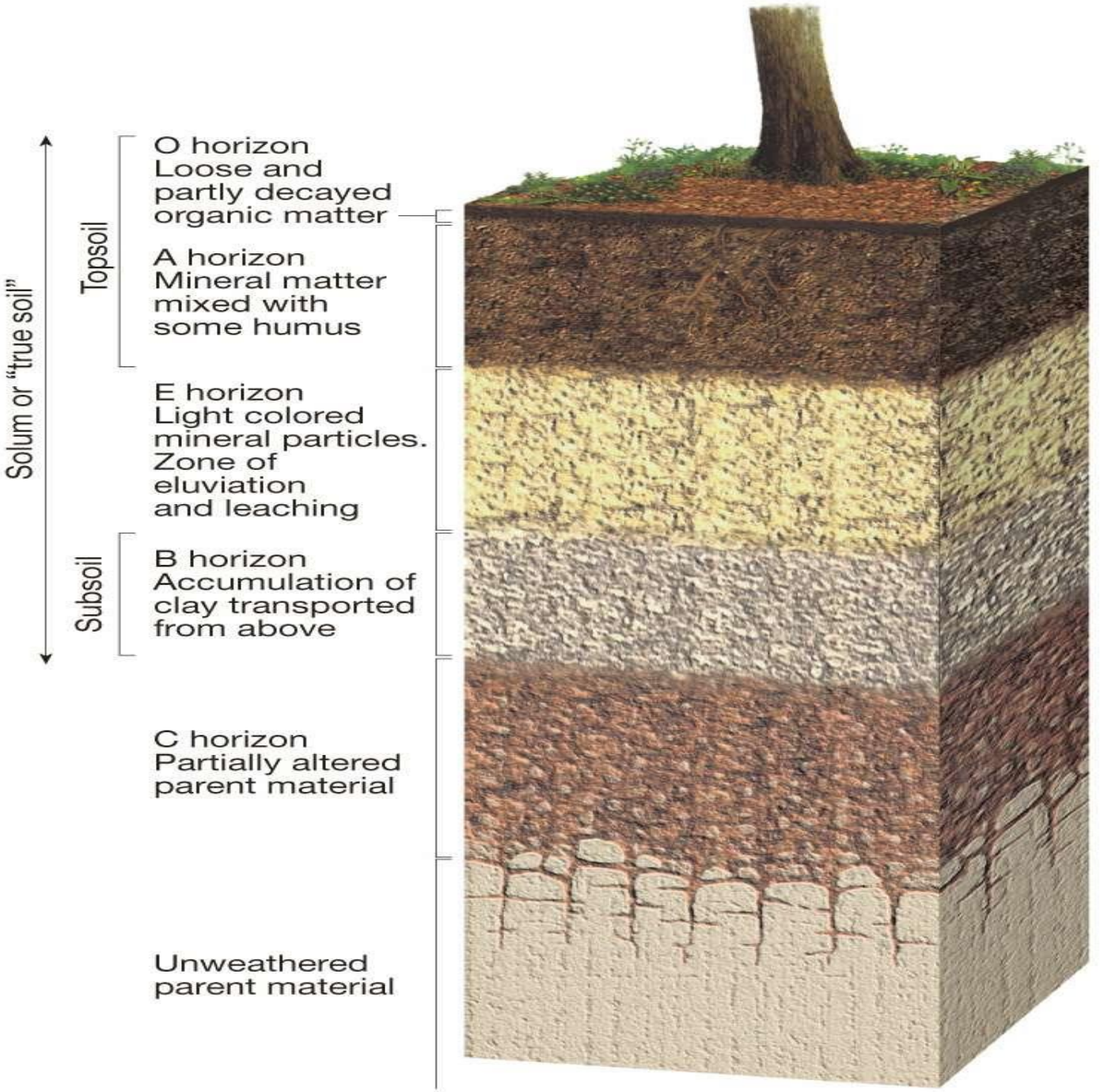
Inselberg (bornhardt), Mali

Inselberg (bornhardt), Ayers Rock, Australia

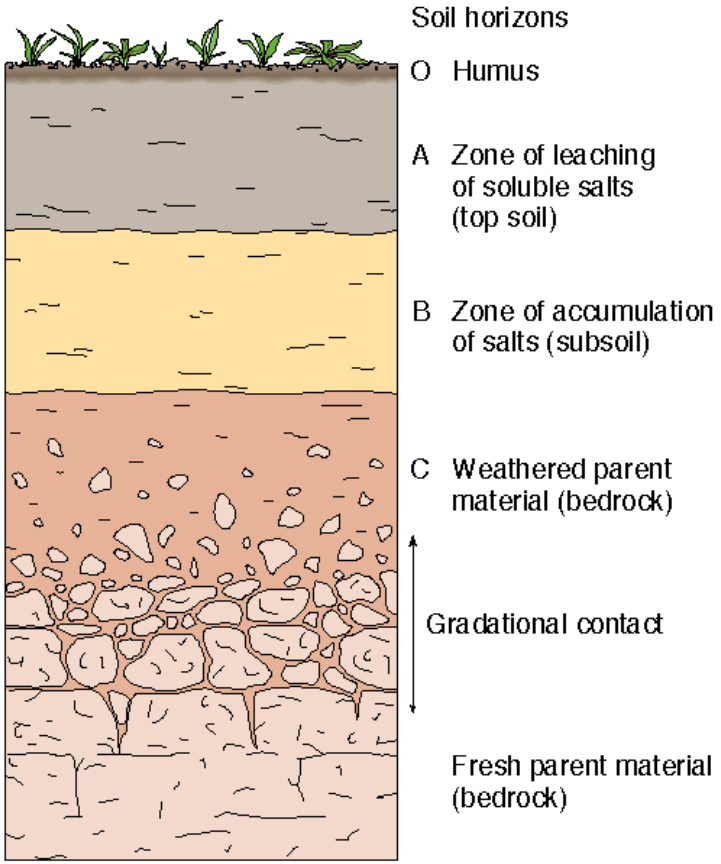
Inselberg (bornhardt), precambrian granite, central Texas

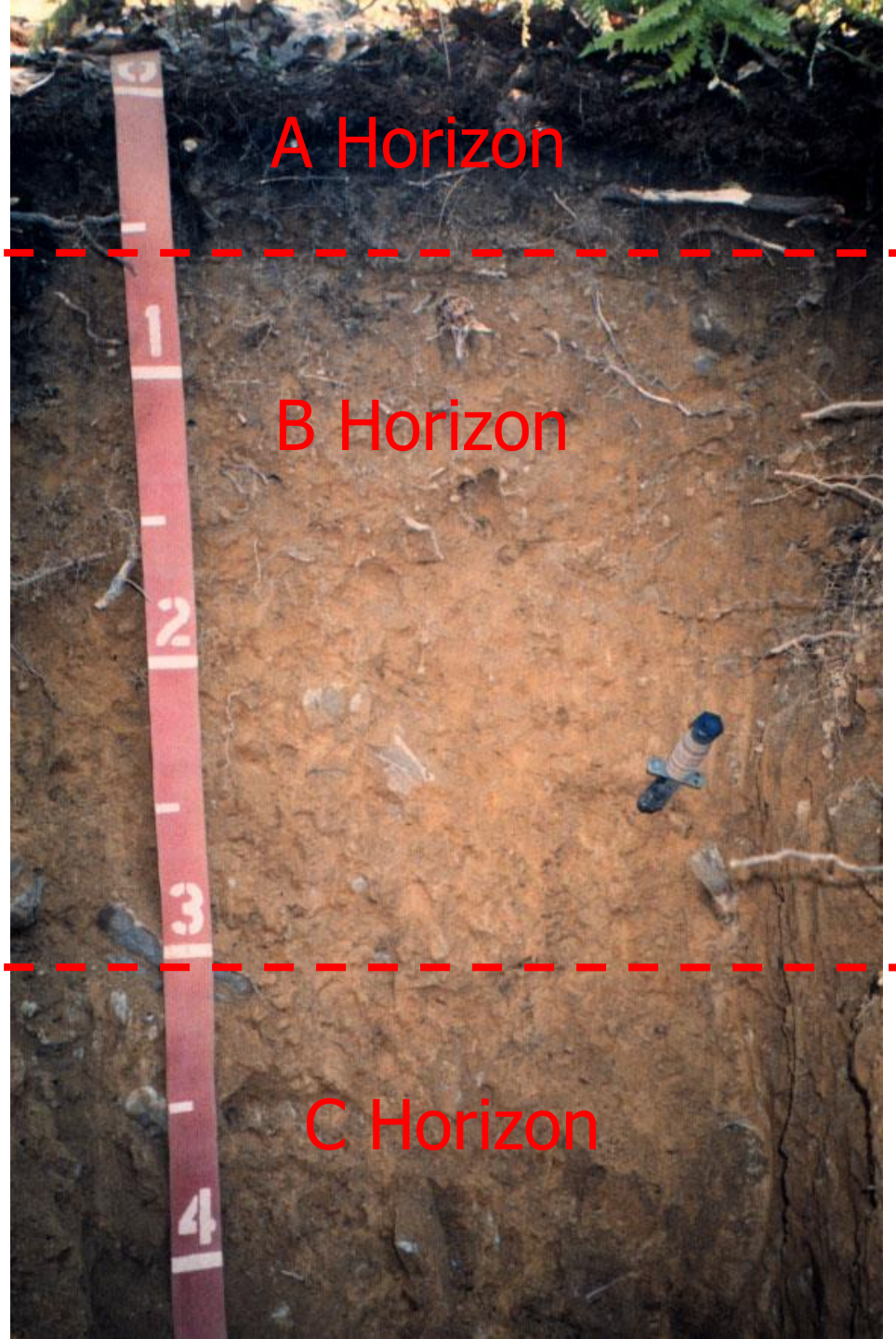


Typical soil profile



Cookport soil, Pennsylvania





Soil classification = Soil orders

- Aridisols = arid zone soils (calcic horizons)
- Mollisols = grassland soils (thick A horizon)
- Alfisols, Ultisols, and
- Spodosols = forest soils (thick B horizon)
- Oxisols = tropical soils (quite oxidized)
- Histosols = wetland soils
- Gelisols = polar soils
- Andosols = volcanic parent material
- Vertisols = swelling clays
- Entisols = weak A over C horizon
- Inceptisols = weak B horizon

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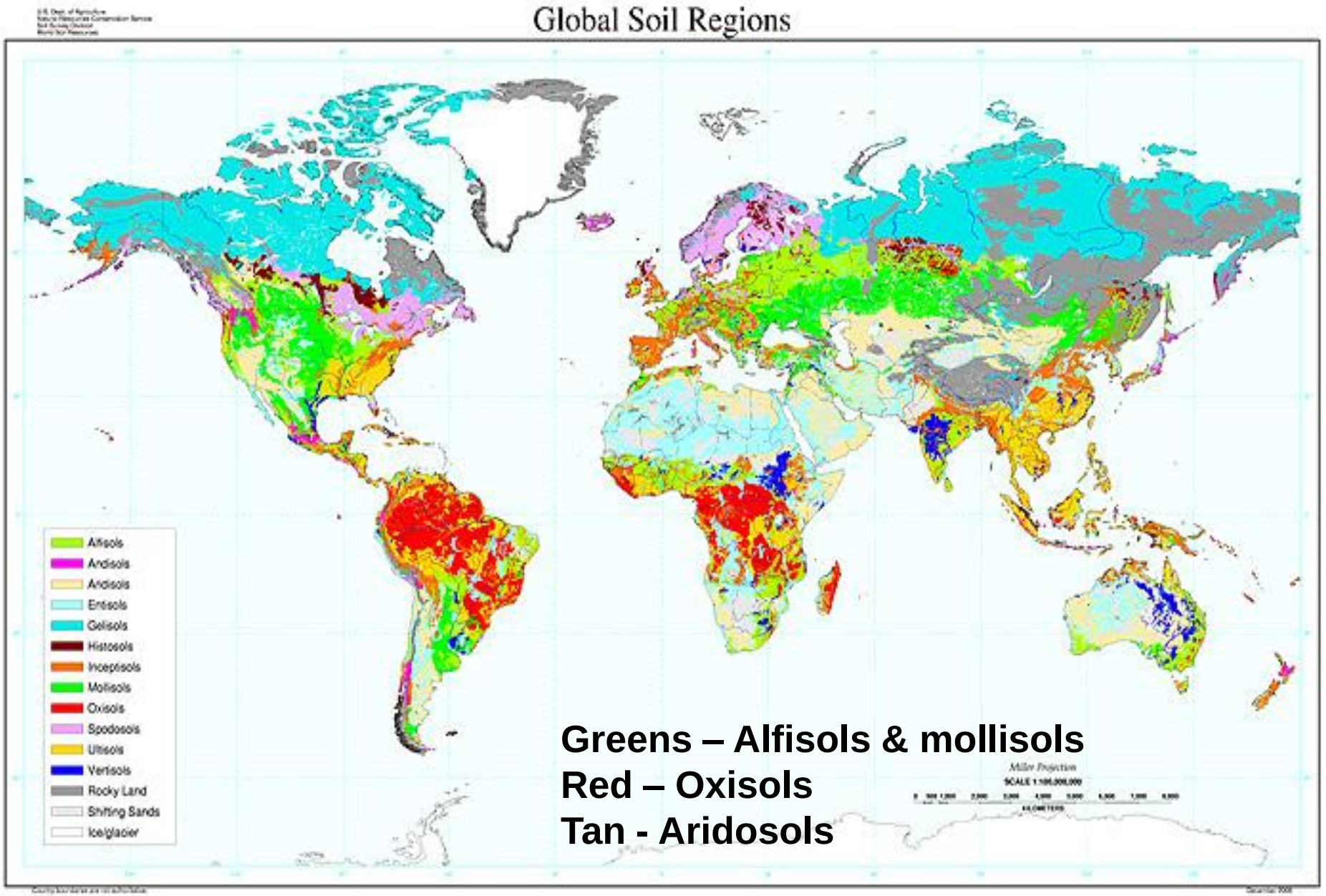
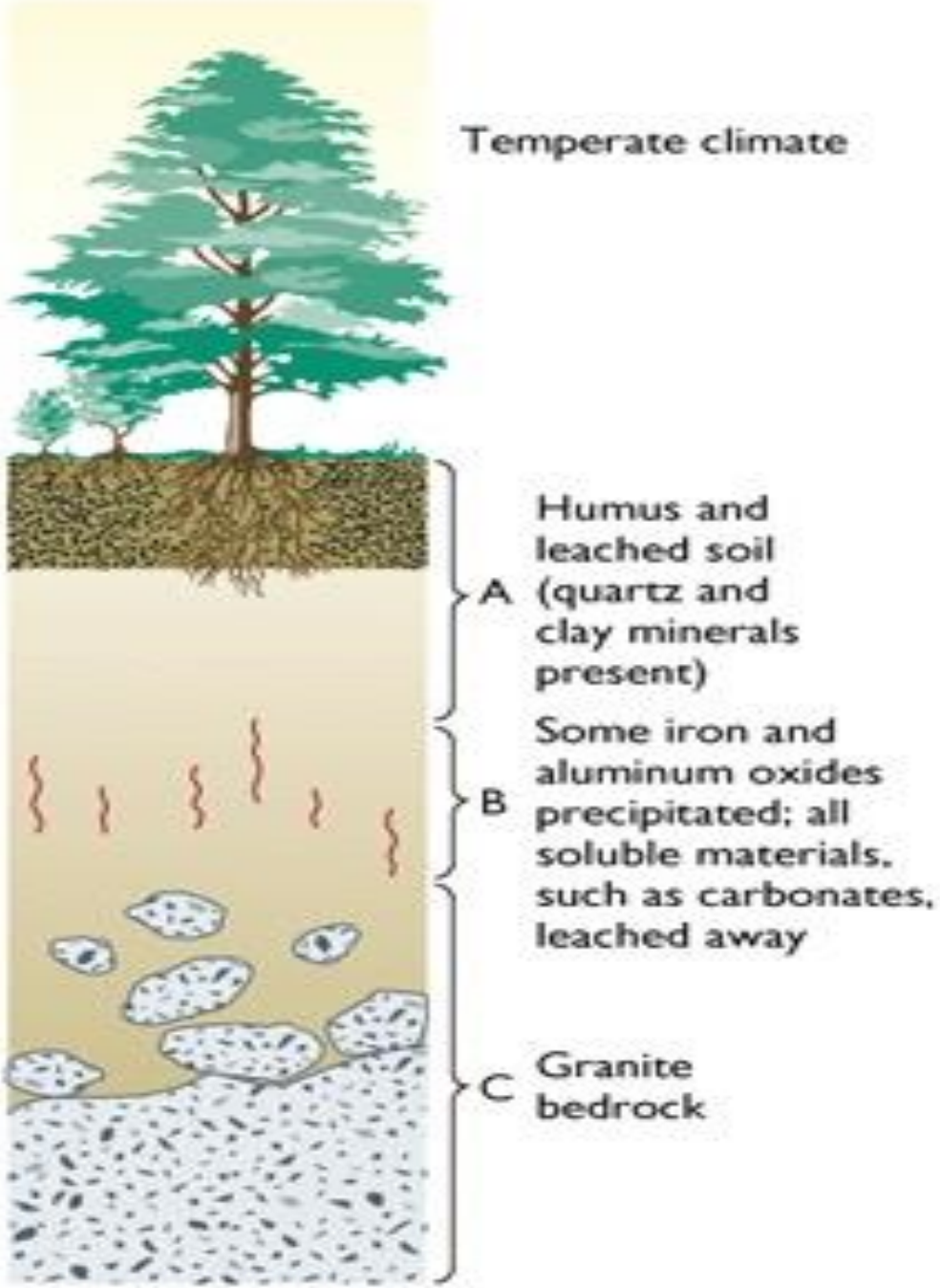
Soil map of world



Soil types: mollisols

Decomposing organic material from plants and animals mixes with accumulated soil minerals.



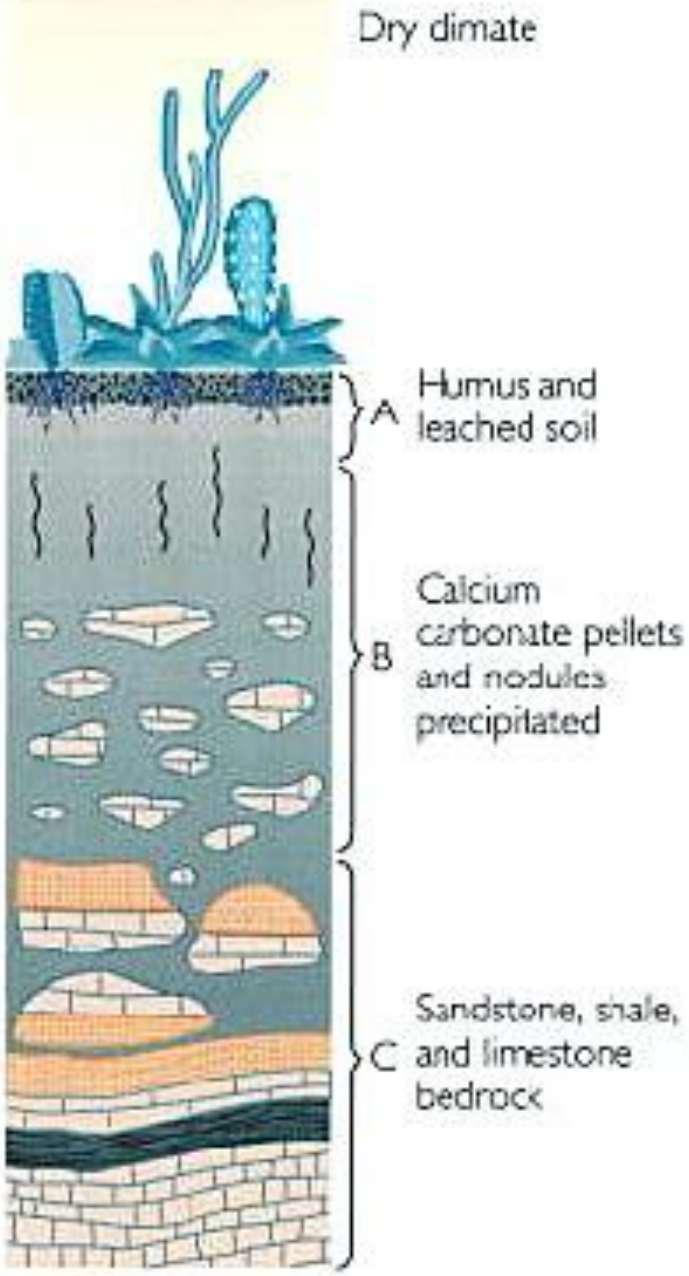
Alfisols and mollisols:
Form in warm or cool, temperate climates.
Soil is clay-rich and fertile.

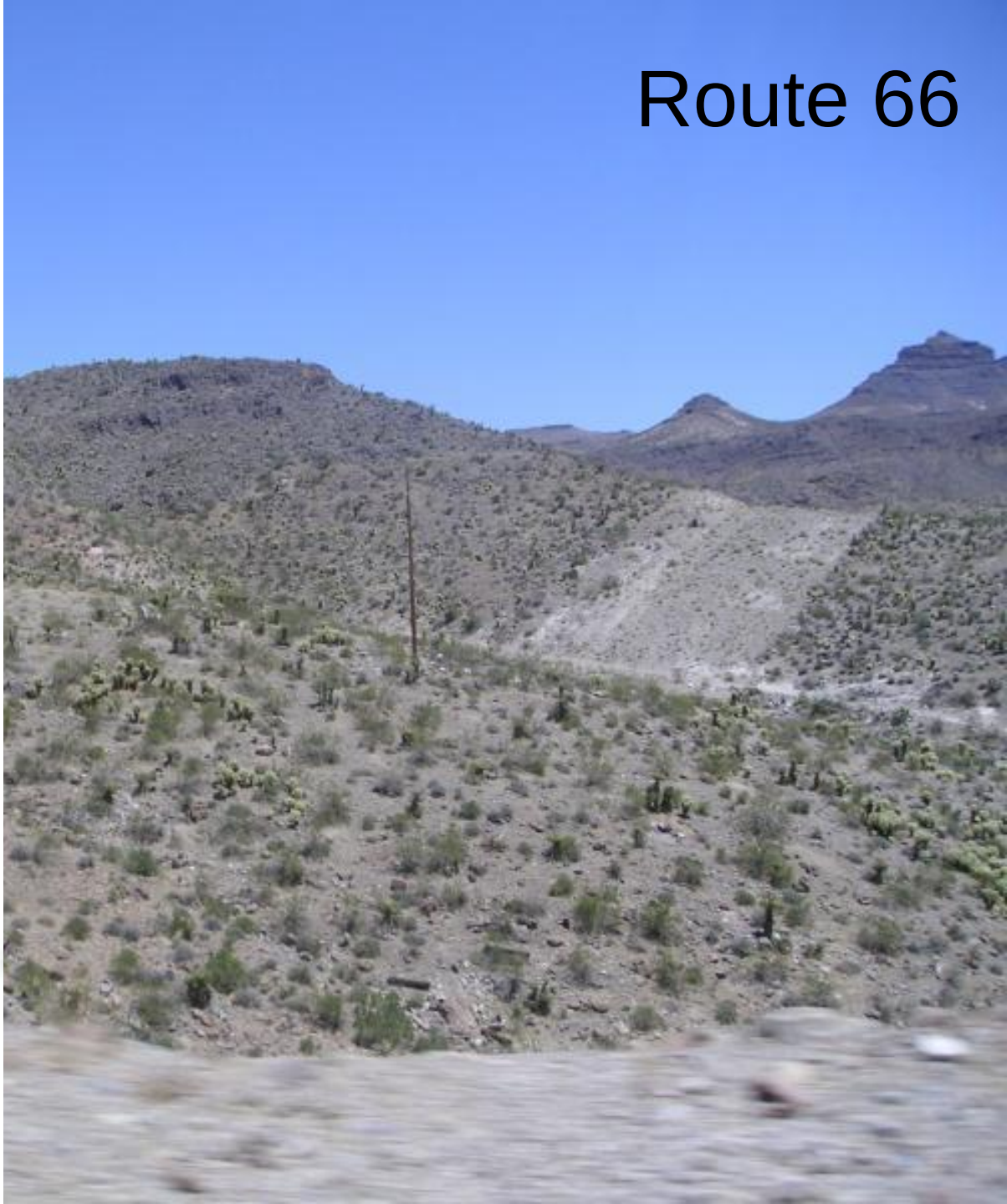
Alfisols are forest soils
Mollisols are grassland soils

Soil types: aridisols

Physical weathering breaks rocks into small mineral particles.

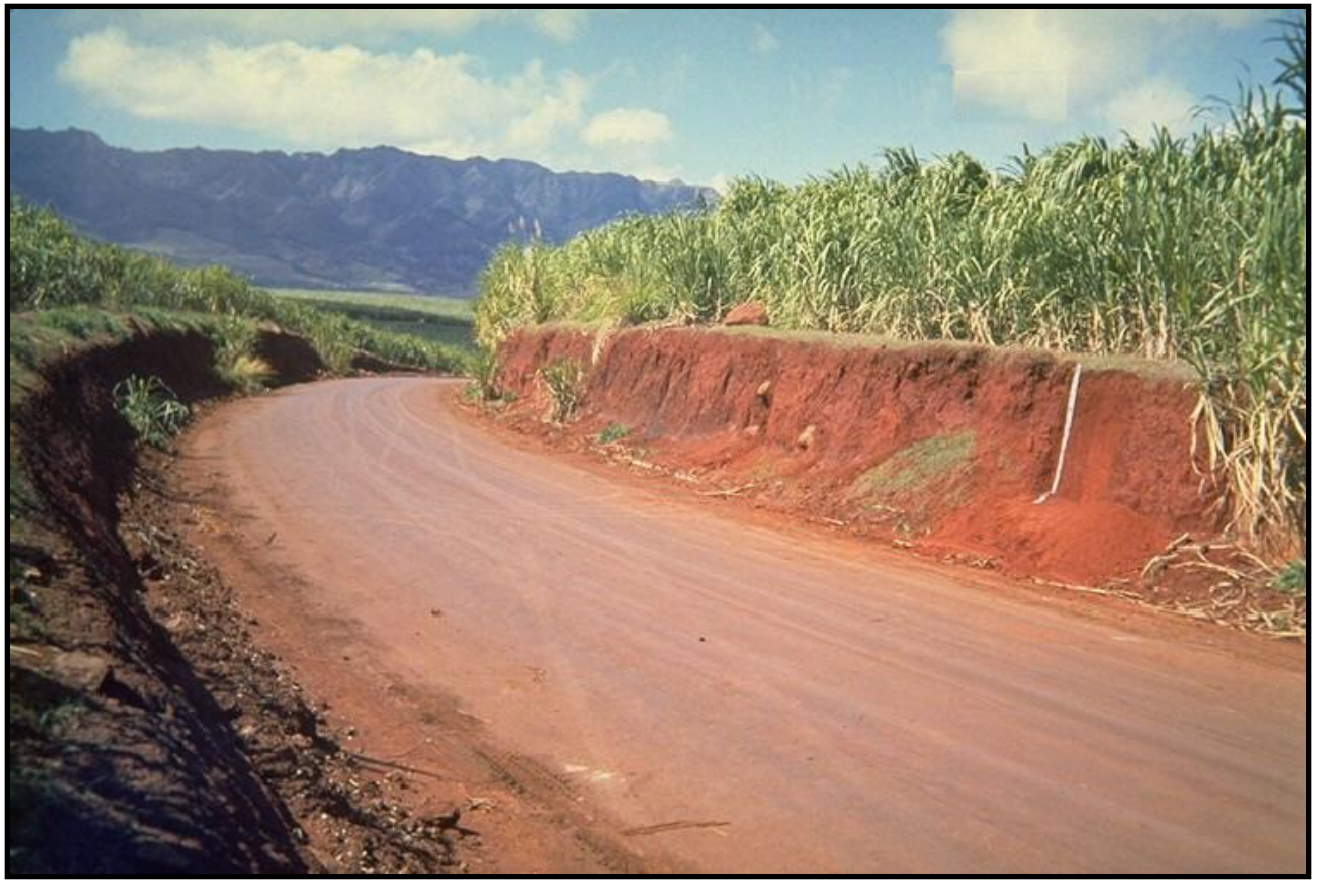
Aridisols


Dry dimate
A
Humus and leached soil
B
Calcium carbonate pellets and nodules precipitated
C
Sandstone, shale, and limestone bedrock


Route 66

Soil types: oxisols

Intensive leaching and oxidation combine to create iron-rich residual soils.



Soil types: oxisols

Laterite = highly developed oxisol.

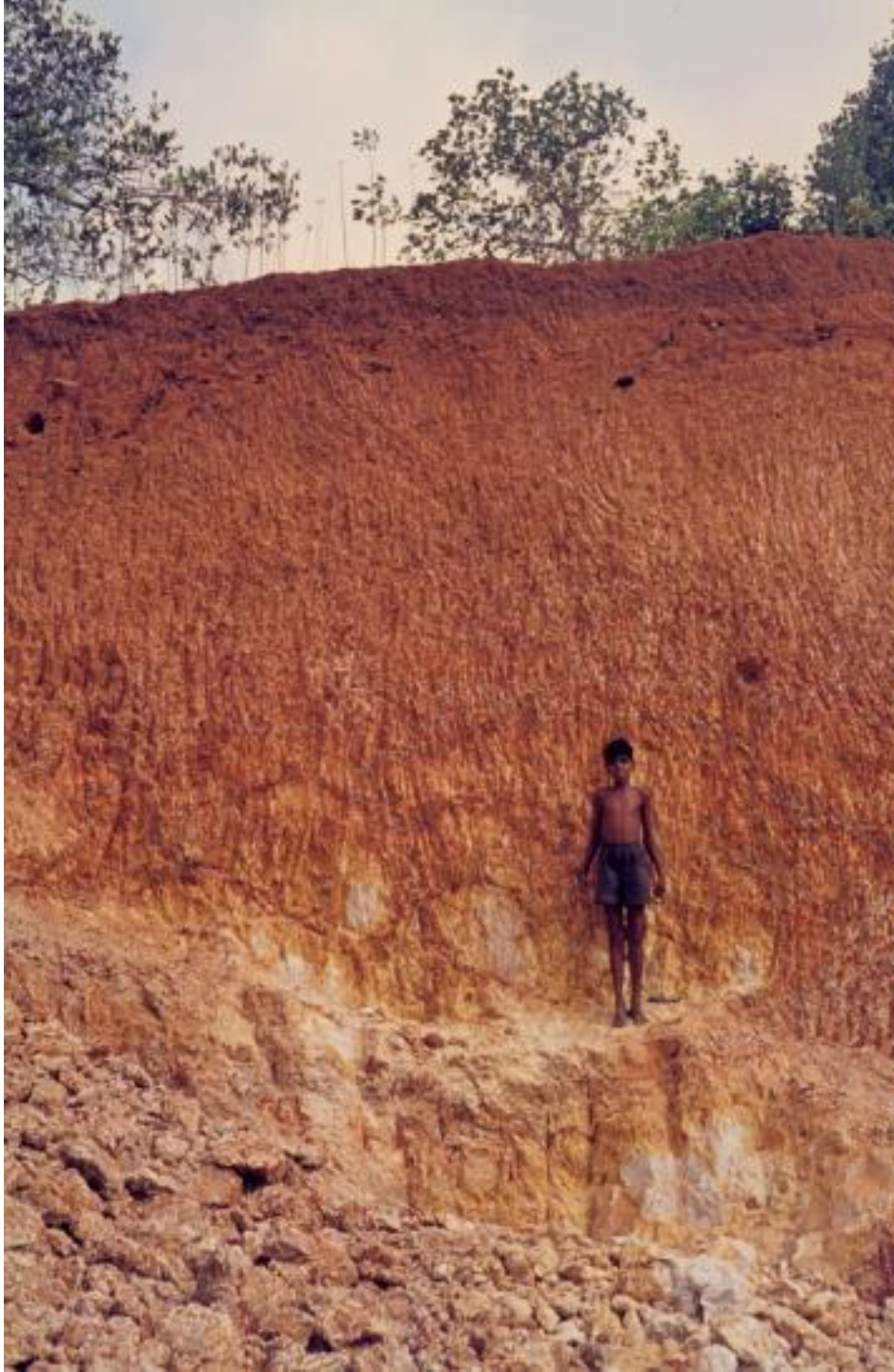
Forms in a hot, humid climate. Soil is deep red, hard and infertile.

Plants recycle nutrients in a thin A and O horizon.

Laterite formation on gneiss, Kerala, India

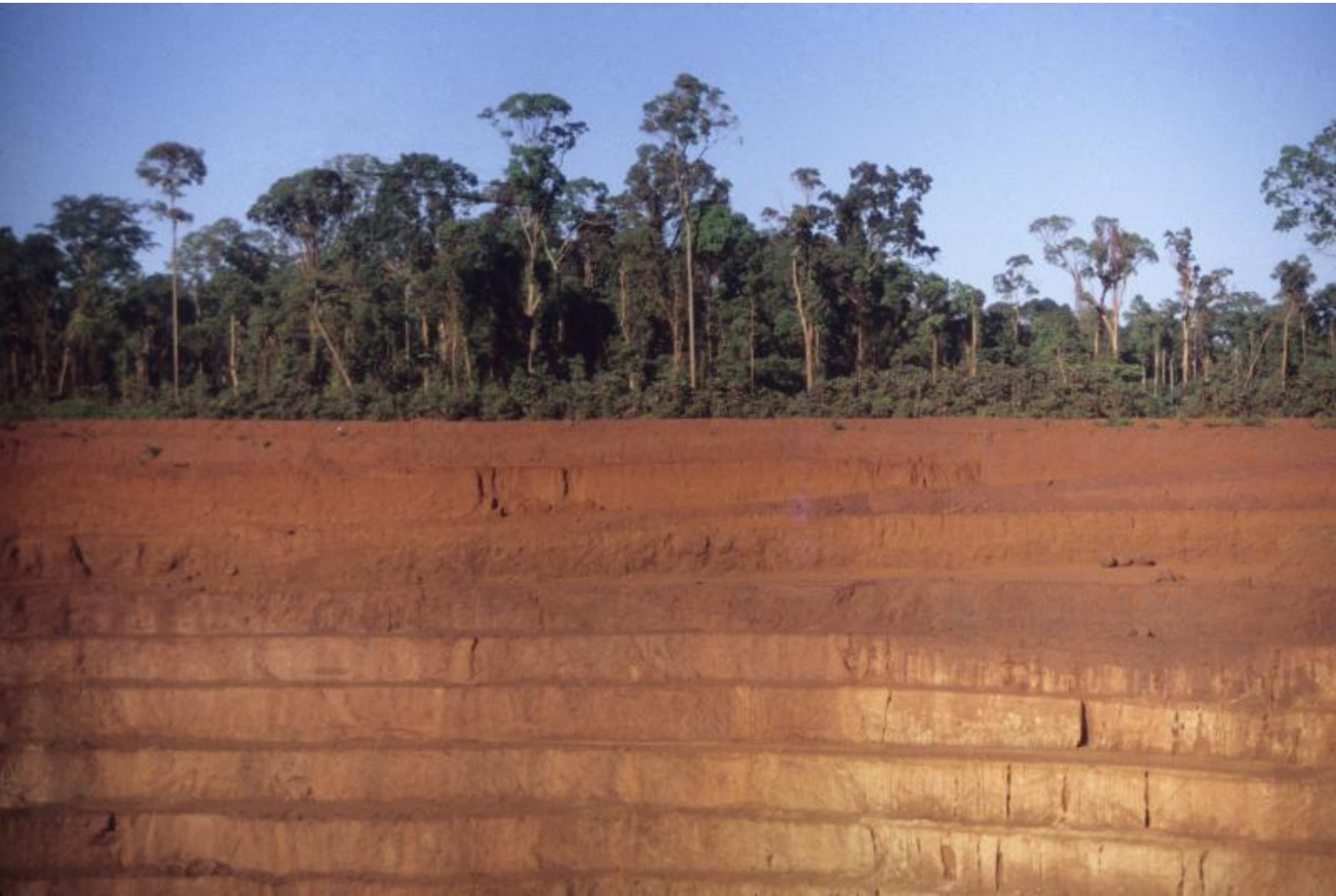
Laterites


Wet climate
Thin or absent humus
Thick masses of insoluble iron and aluminum oxides; occasional quartz
Iron-rich clays and aluminum hydroxides
Thin leached zone
Mafic igneous bedrock



Deforestation removes the fertile organic layer. The underlying soil is infertile, dries to brick-like hardness when it dries out, and is difficult to cultivate. Aluminum (from bauxite) and iron (from limonite) can be mined from these soils.

Carajas, Brazil



Limits of Soil Development

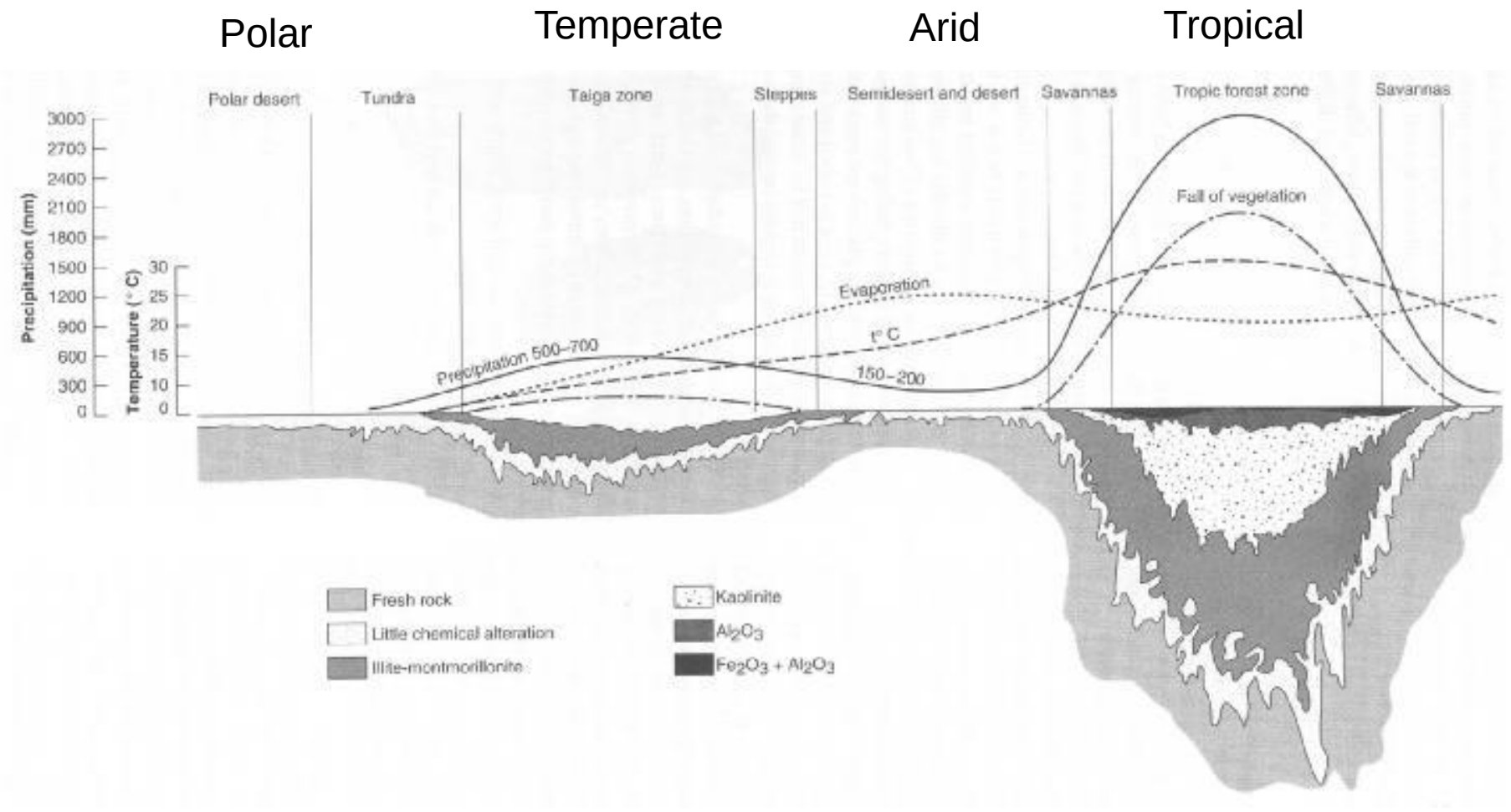
Balance Between:

- Downward Lowering of Surface
- Downward Migration of Horizons

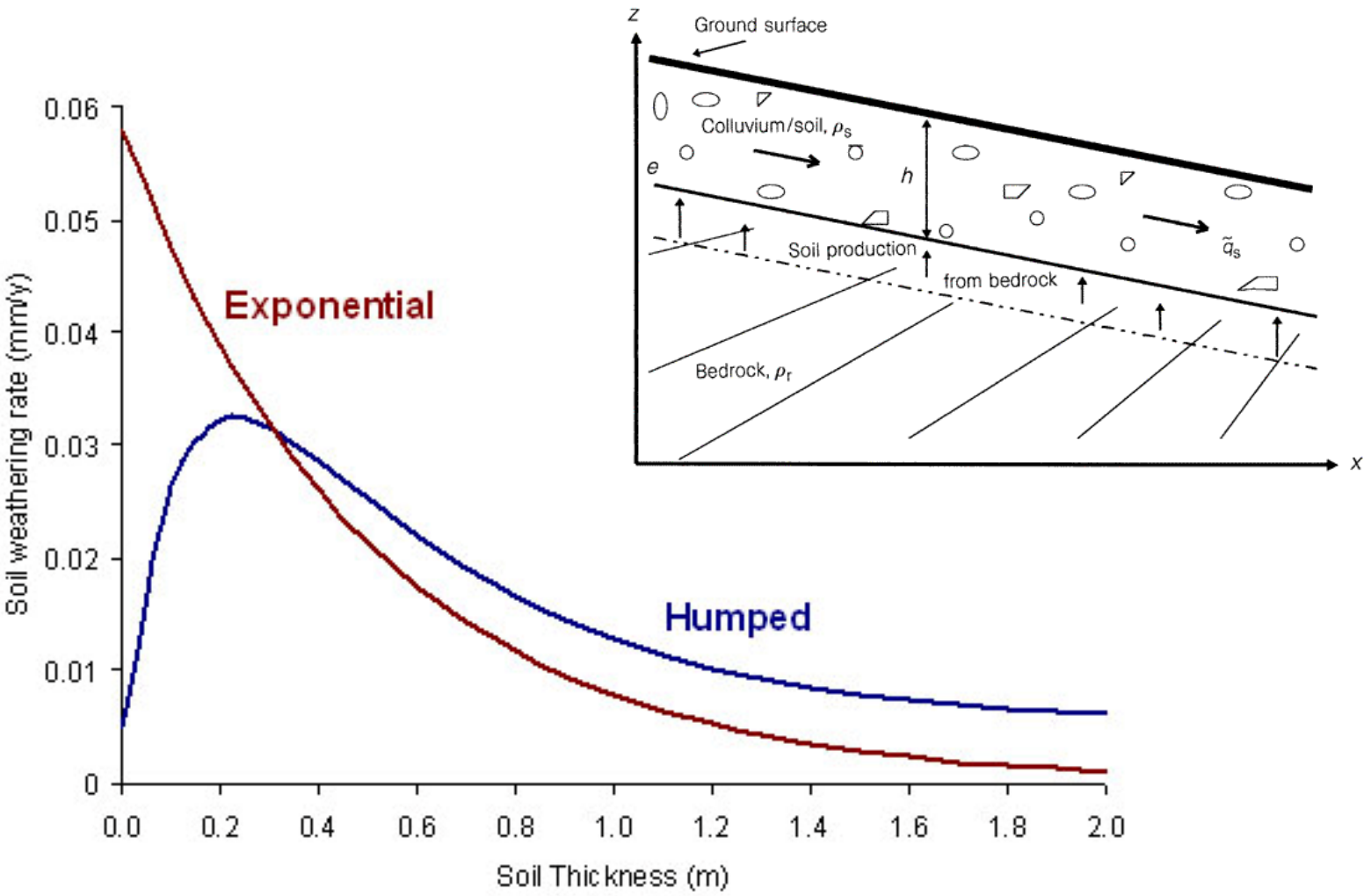
If erosion rapid or soil evolution slow, soils may never mature beyond a certain point.

Extremely ancient soils may have lost everything movable

Weathering with Climate



Soil Production Function

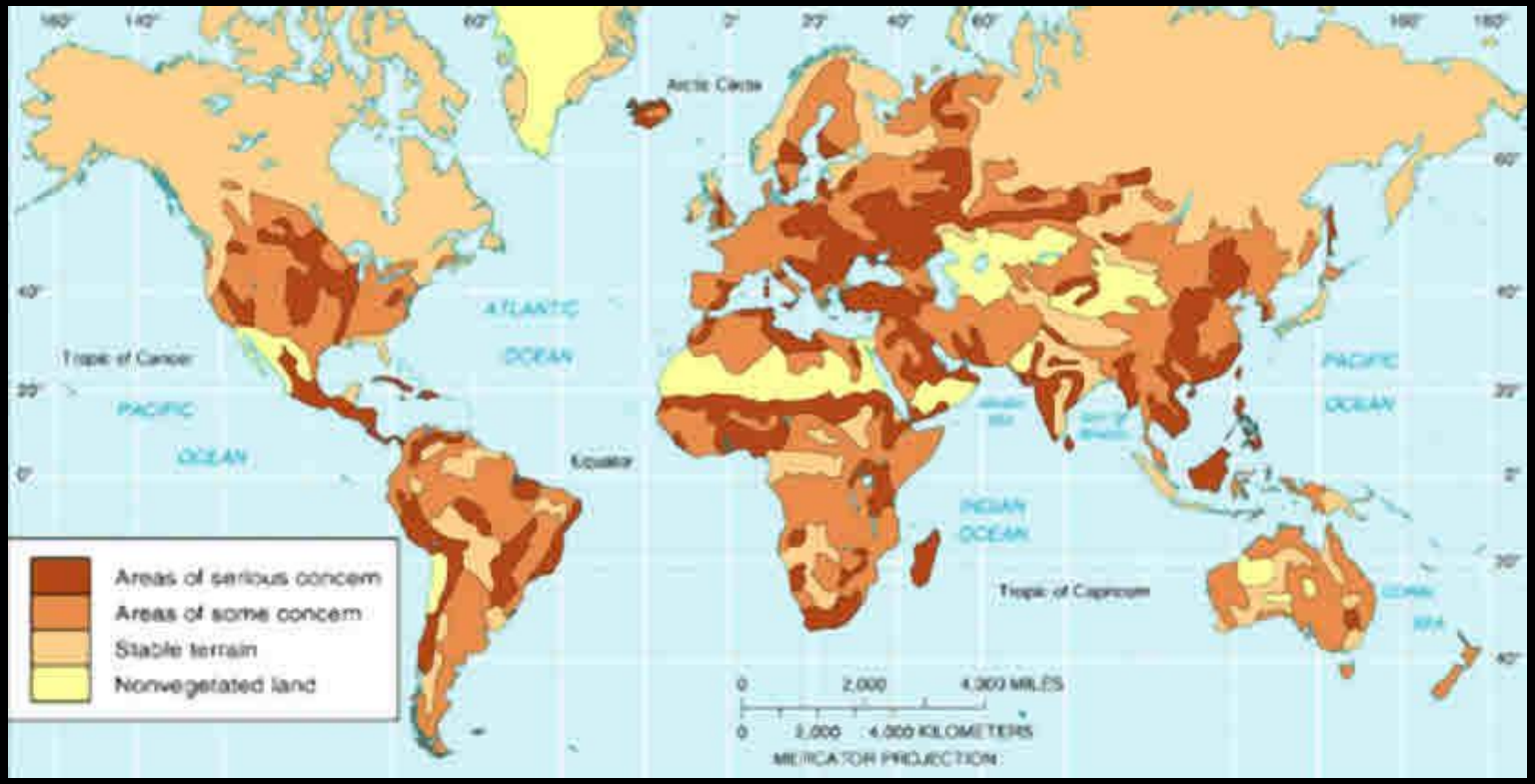


Sugar Loaf, Rio De Janiero, Brazil

Convex, soil-mantled hillslopes, Northern California

Agricultural Soil Loss

Erosion of unprotected soils during periods where they remain fallow and without vegetative cover leads to increased erosion



We know more about the movement of celestial bodies than about the soil underfoot.

- Leonardo da Vinci

Biological Nitrogen Fixation

Conversion of dinitrogen gas (N_2) to ammonia (NH_3)

Availability of fixed N often factor most limiting to plant growth

N-fixation ability limited to few bacteria, either as free-living organisms or in symbiosis with higher plants

First attempt to increase forest growth through N-fixation in Lithuania, 1894 (lupines in Scots pine)

Biological nitrogen fixation:

$$N_2 + 8\ \text{flavodoxin}^- + 8H^+ + 16\ MgATP^{2-} + 18\ H_2O \xrightarrow{\text{nitrogenase}}$$

$$2NH_4^+ + 2OH^- + 8\ \text{flavodoxin} + 16\ MgADP^- + 16H_2PO_4^- + H_2$$

1. Rare, extremely energy consuming conversion because of stability of triply bonded N_2
2. Produces fixed N which can be directly assimilated into N containing biomolecules

Ecology of nitrogen-fixing bacteria

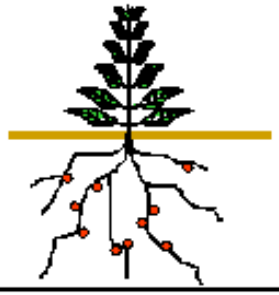
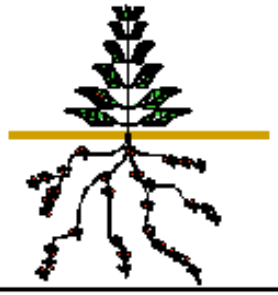
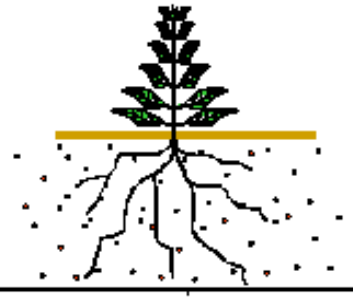
System of N_2 fixation (and microbes involved) ($N_2 \rightarrow NH_3$)	SYMBIOSIS (e.g. *Rhizobium*)	ASSOCIATION (e.g. *Azospirillum*)	FREE-LIVING (e.g. *Rhodospiorillum*)	
				
Energy source (Organic C)	Sucrose from the host plant	Root exudates from the host plant	Heterotroph (plant residues)	Autotroph (photo-synthesis)
Estimates of fixation rate (kg N/ha/y)	50-400	10-200	1-2	10-80

Tableau I. **Une sélection de quelques bactéries fixatrices d'azote.**

Groupes phylogéniques, nombre de fixateurs caractérisés, exemples	Métabolisme énergétique, tension d'oxygène compatible avec la fixation de l'azote, interaction avec les plantes
Bactéries vertes sulfureuses	
4 genres, 6 espèces	
Chlorobium limicola	PAT Anaérobiose
Firmibactéries (Gram+)	
3 genres, 22 espèces	
Bacillus polymixa	CHT Microaérobiose
Clostridium acetobutylicum	CHT Anaérobiose
Clostridium pasteurianum	CHT Anaérobiose
Thallobactéries (Gram+)	
4 genres, x espèces	
Arthrobacter sp	CHT Microaérobiose
Frankia	CHT Microaérobiose. Symbiote actinorhizien (pe aulne, casuarina)
Héliobactéries	
3 genres, 3 espèces	
Heliobacterium chlorum	PHT Anaérobiose
Heliospirillum gestii	PHT Anaérobiose
Cyanobactéries	
14 genres, x espèces	
Anabaena 7120	PAT Aérobiose
Anabaena azollae	PAT Aérobiose. Symbiote de la fougère *Azolla*
Nostoc 73102	PAT Aérobiose
Gloeothece 6501	PAT Microaérobiose
Campylobactéries	
1 genre, 1 espèce	
Protéobactéries α	
20 genres, 54 espèces	
Acetobacter diazotrophicus	CHT Microaérobiose. Endophyte de la canne à sucre
Azorhizobium caulinodans	CHT Microaérobiose. Symbiote de *Sesbania rostrata*
Azospirillum brasilense	CHT Microaérobiose. Associé aux racines des Graminées
Bradyrhizobium japonicum	CHT Microaérobiose. Symbiote du soja
Rhizobium leguminosarum	CHT Microaérobiose. Symbiote du pois
Rhizobium meliloti	CHT Microaérobiose. Symbiote de la luzerne
Rhodobacter capsulatus	PHT Anaérobiose
Rhodospirillum rubrum	PHT Anaérobiose
Protéobactéries β	
7 genres, 11 espèces	
Alcaligenes faecalis	CHT Microaérobiose. Associé aux racines du riz
Azoarcus spp	CHT Microaérobiose. Endophyte de l'herbe de Kallar (*Leptochloa fusca*)
Derxia gummosa	CHT Microaérobiose
Herbaspirillum seropedicae	CHT Microaérobiose. Endophyte de la canne à sucre
Thiobacillus ferrooxidans	CAT Microaérobiose
Protéobactéries γ	
18 genres, 44 espèces	
Azotobacter vinelandii	CHT Aérobiose
Beggiatoa alba	CAT Microaérobiose
Enterobacter agglomerans	CHT Anaérobiose
Klebsiella pneumoniae	CHT Anaérobiose
Pseudomonas stutzeri	CHT Microaérobiose
Protéobactéries δ	
2 genres, 10 espèces	
Desulfovibrio gigas	CHT Anaérobiose
Archaebactéries	
4 genres, 7 espèces	
Methanobacterium ivanovii	CAT Anaérobiose
Methanococcus thermolithotrophicus	CAT Anaérobiose

CAT : chimioautotrophe ; CHT : chimiohétérotrophe ; PAT : photoautotrophe ; PHT : photohétérotrophe.

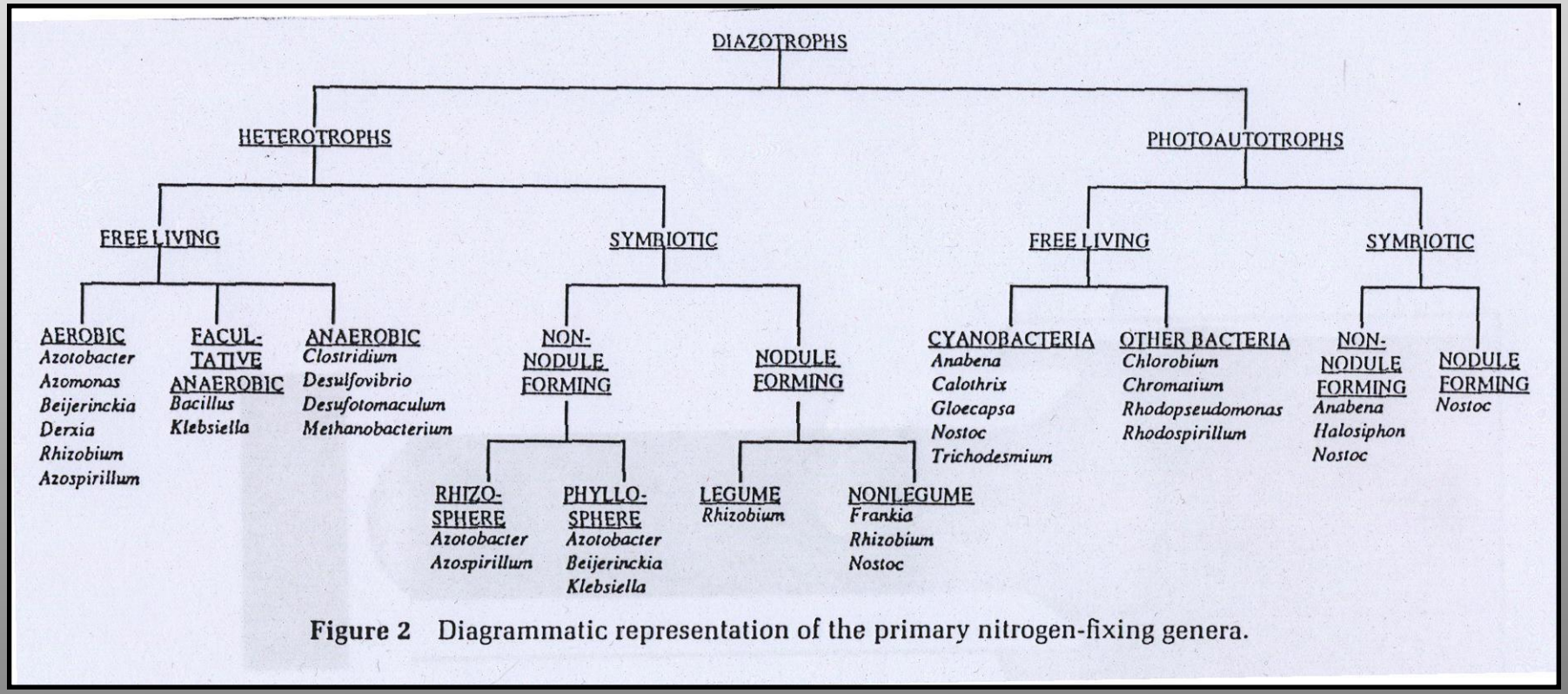


Figure 2 Diagrammatic representation of the primary nitrogen-fixing genera.

N-fixation requires energy input:

- Reduction reaction, e^- must be added (sensitive to O_2)
- Requires ~35 kJ of energy per mol of N fixed (theoretically)
- Actual cost: ~15-30g CH per g of NH_3 produced
- Assimilation of NH_3 into organic form takes 3.1-3.6 g CH

Enzymology of N fixation

Only occurs in certain prokaryotes

- **Rhizobia fix nitrogen in symbiotic association with leguminous plants**
- **Rhizobia fix N for the plant and plant provides Rhizobia with carbon substrates**
- **All nitrogen fixing systems appear to be identical**
- **They require nitrogenase, a reductant (reduced ferredoxin), ATP, O-free conditions and regulatory controls (ADP inhibits and NH_4^+ inhibits expression of nif genes**

Biological nitrogen fixation is the reduction of atmospheric nitrogen gas (N_2) to ammonium ions (NH_4^+) by the oxygen-sensitive enzyme, **nitrogenase**.
Reducing power is provided by NAPH/ferredoxin, via an Fe/Mocentre.

Plant genomes lack any genes encoding this enzyme, which occurs only in prokaryotes (bacteria).

Even within the bacteria, only certain free-living bacteria (Klebsiella, Azospirillum, Azotobacter), blue-green bacteria (Anabaena) and a few symbiotic Rhizobial species are known nitrogen-fixers.

Another nitrogen-fixing association exists between an Actinomycete (Frankia spp.) and alder (Alnus spp.)

The enzyme **nitrogenase** catalyses the conversion of atmospheric, gaseous dinitrogen (N_2) and dihydrogen (H_2) to ammonia (NH_3), as shown in the chemical equation below:

$$\mathbf{N_2 + 3\ H_2 \Rightarrow\ 2\ NH_3}$$

The above reaction seems simple enough and the atmosphere is 78% N_2, so why is this enzyme so important?

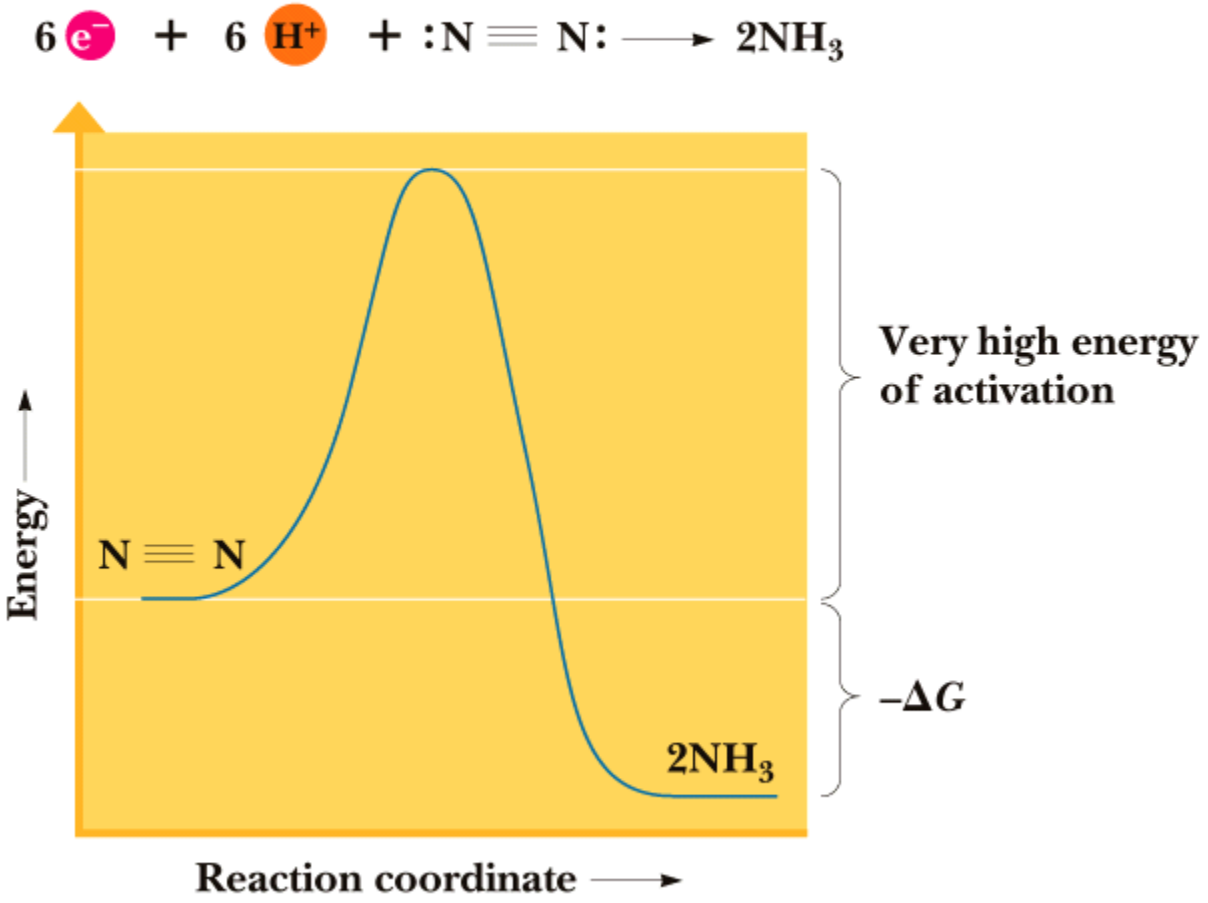
The incredibly strong (triple) bond in N_2 makes this reaction very difficult to carry out efficiently.
In fact, nitrogenase consumes ~16 moles of ATP for every molecule of N_2 it reduces to NH_3, which makes it one of the most energy-expensive processes known in Nature.

Nitrogenase Complex

Two protein components: nitrogenase reductase and nitrogenase

- Nitrogenase reductase is a 60 kD homodimer with a single 4Fe-4S cluster
- Very oxygen-sensitive
- Binds MgATP
- 4ATP required per pair of electrons transferred
- Reduction of N_2 to $2NH_3 + H_2$ requires 4 pairs of electrons, so **16 ATP are consumed per N_2**

Garrett & Grisham: Biochemistry, 2/e
Figure 26.4



Saunders College Publishing

Why should nitrogenase need ATP???

- N_2 reduction to ammonia is thermodynamically favorable
- However, the activation barrier for breaking the N-N triple bond is enormous
- **16 ATP** provide the needed activation energy

Nitrogenase

A 220 kD heterotetramer

- Each molecule of enzyme contains 2 Mo, 32 Fe, 30 equivalents of acid-labile sulfide (FeS clusters, etc)
- Four 4Fe-4S clusters plus two FeMoCo, an iron-molybdenum cofactor
- Nitrogenase is **slow - 12 e^- pairs per second, i.e., only three molecules of N_2 per second**

Genetic Clusters

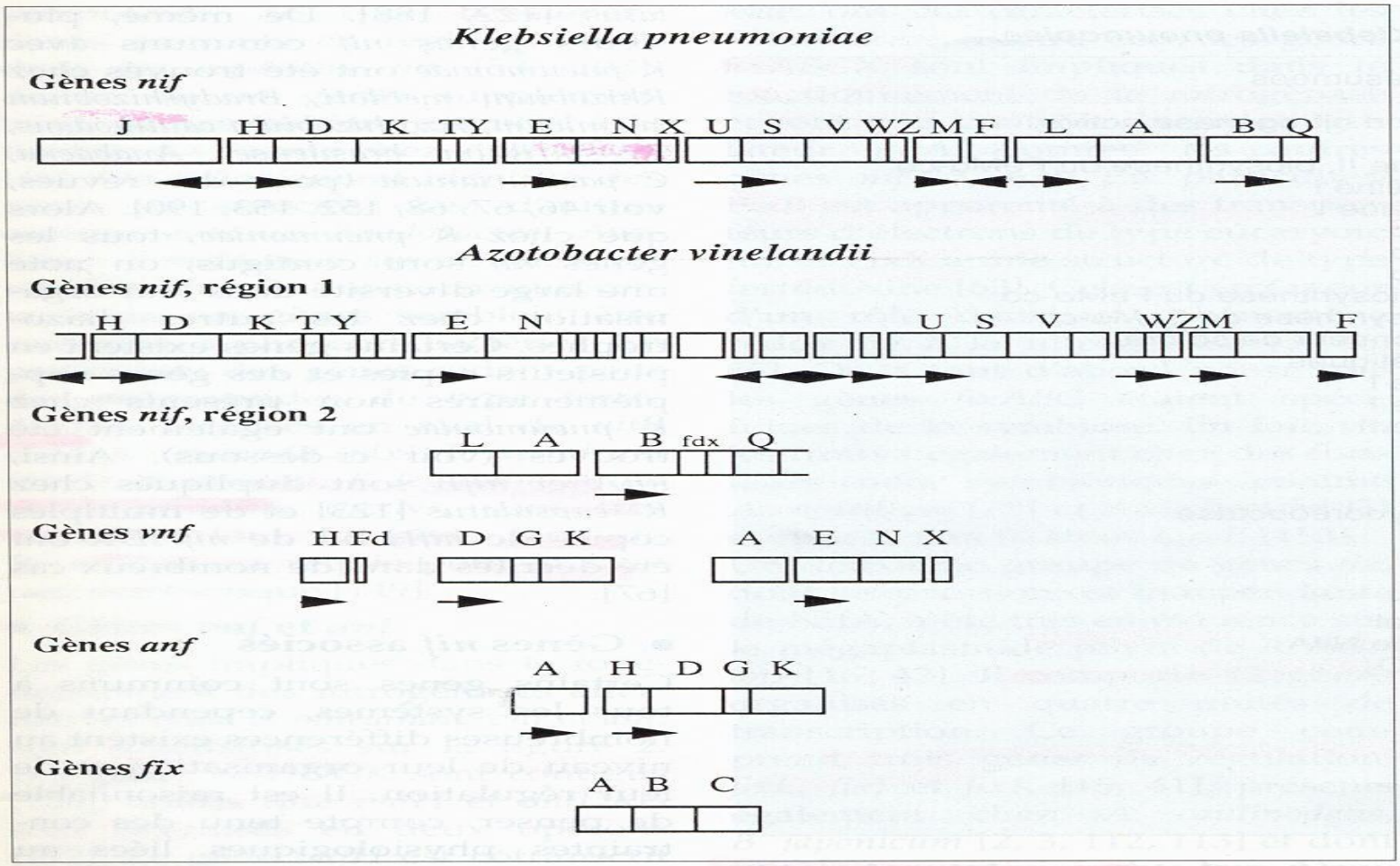
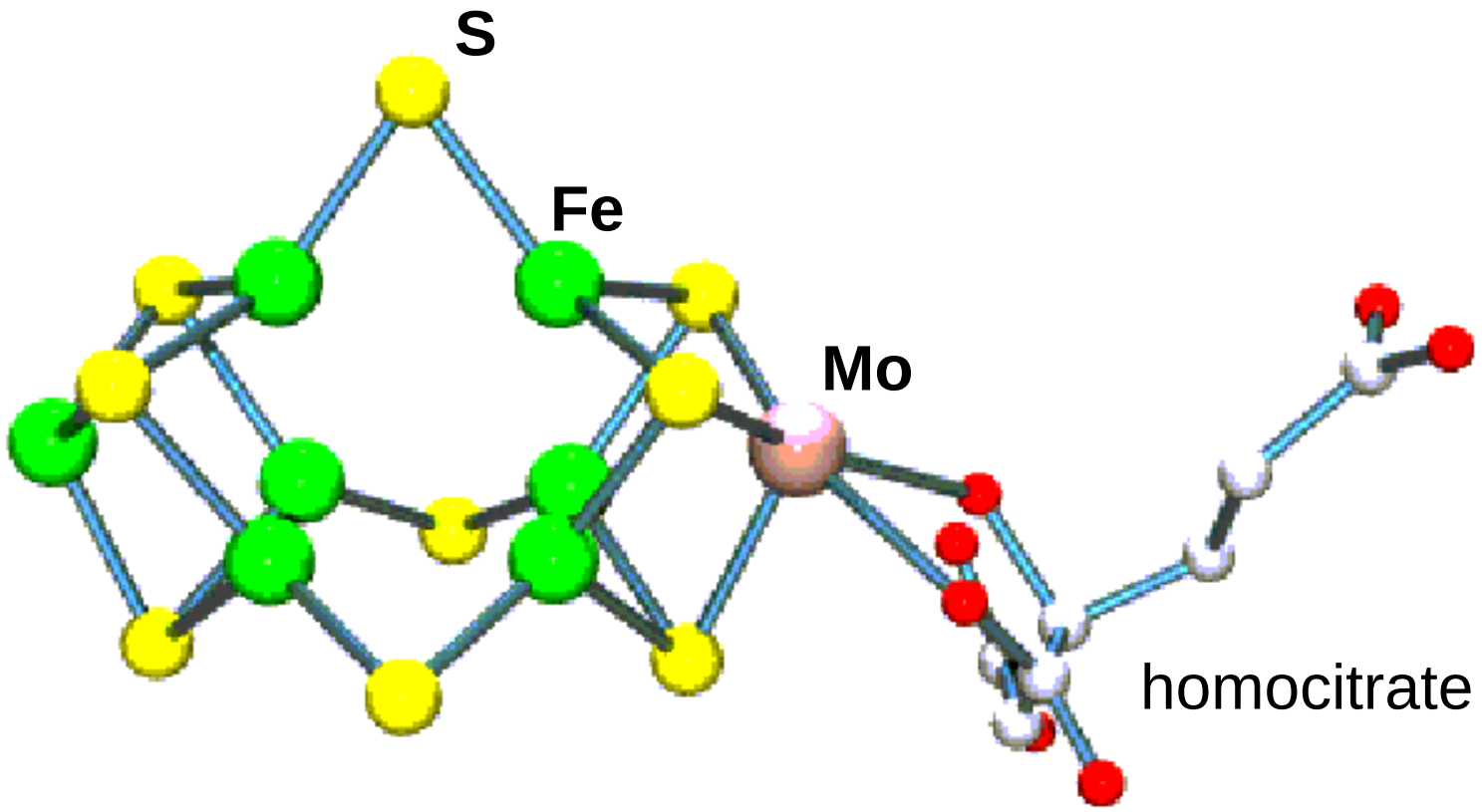


Fig 2. Organisation des gènes de la fixation de l'azote de Klebsiella pneumoniae *et d'*Azotobacter vinelandii. *Les gènes contigus correspondent à des opérons polycistroniques. Les flèches indiquent le sens de transcription à partir de promoteurs dépendant du facteur* σ^{54}.

The genes and products

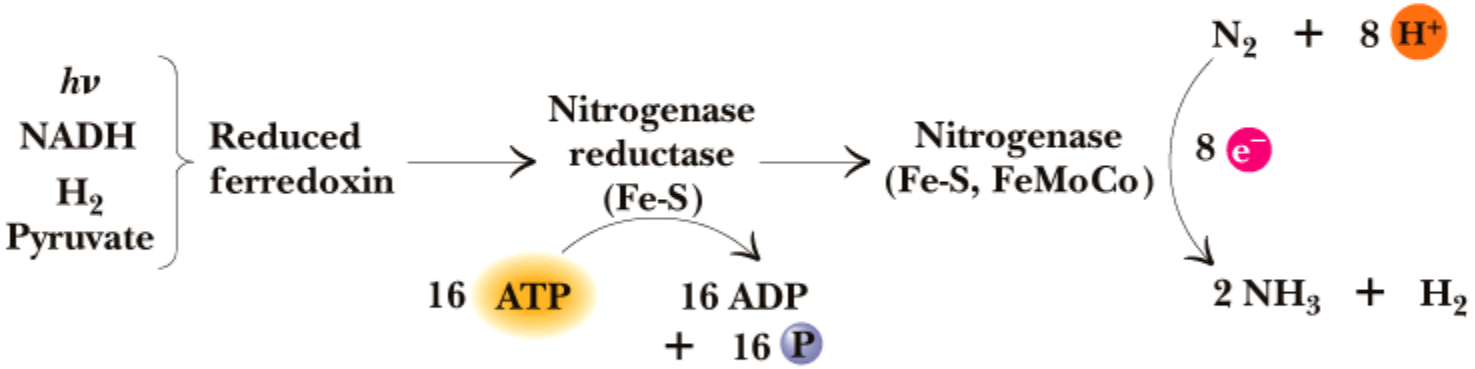
Tableau II. **Fonction des gènes *nif* de *Klebsiella pneumoniae*.**

Gènes	Fonctions établies ou présumées
1. Gènes impliqués dans la synthèse d'une nitrogénase active	
nifH	Polypeptide de la protéine II, biosynthèse du FeMo-co
nifD	Polypeptide α de la protéine I
nifK	Polypeptide β de la protéine I
nifE	Biosynthèse du FeMo-co
nifN	Biosynthèse du FeMo-co
nifB	Biosynthèse du FeMo-co
nifV	Homocitrate synthase, biosynthèse du FeMo-co
nifQ	Métabolisme du Mo, biosynthèse du FeMo-co
nifS	Cystéine désulfurase, donneur de soufre pour les groupes prosthétiques
nifW	Maturation de la protéine I
nifZ	Maturation de la protéine I
nifM	Maturation de la protéine II
2. Transport des électrons	
nifJ	Pyruvate-flavodoxine oxydoréductase
nifF	Flavodoxine
3. Régulation	
nifA	Activateur transcriptionnel
nifL	Modulateur de l'activité de NifA en présence de NH_3 ou O_2
4. Gènes non essentiels	
nifT	Inconnu
nifY	Inconnu
nifX	Inconnu
nifU	inconnu



Fe - S - Mo electron transfer cofactor in nitrogenase

Garrett & Grisham: Biochemistry, 2/e
Figure 26.6



Three Types of N-fixers Important in Forest Soils

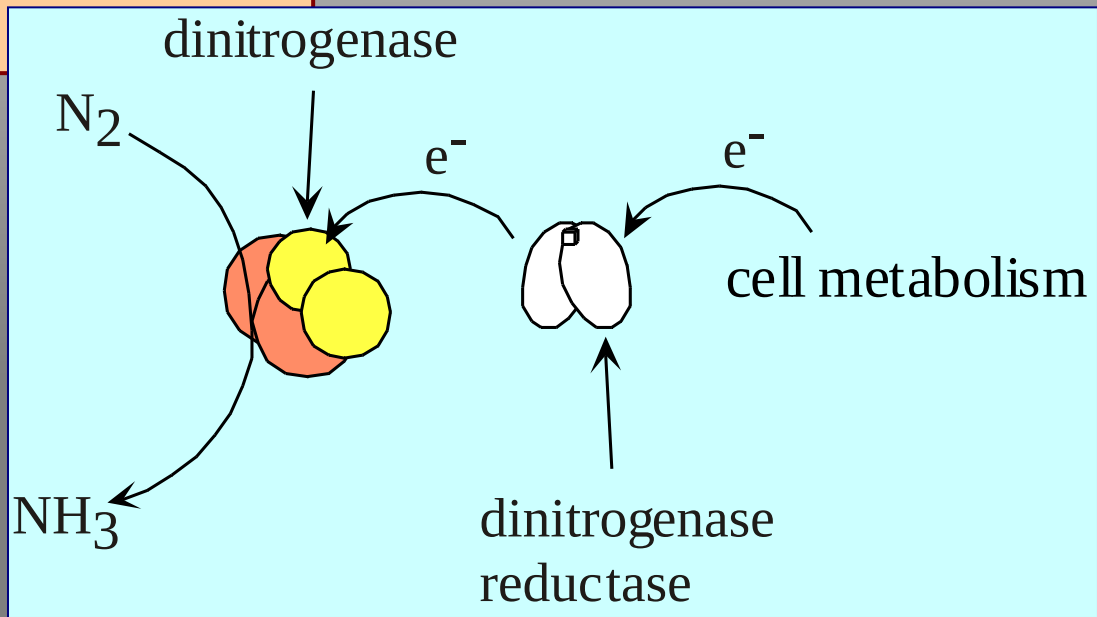
Cyanobacteria: Autotrophic N-fixers, protect nitrogenase with specialized *heterocyst* cells.

Heterotorophic bacteria: Free-living or associative with rhizosphere. Use energy from decomposing organic matter to fix N, protect nitrogenase by rapidly converting O_2 to CO_2 through respiration.

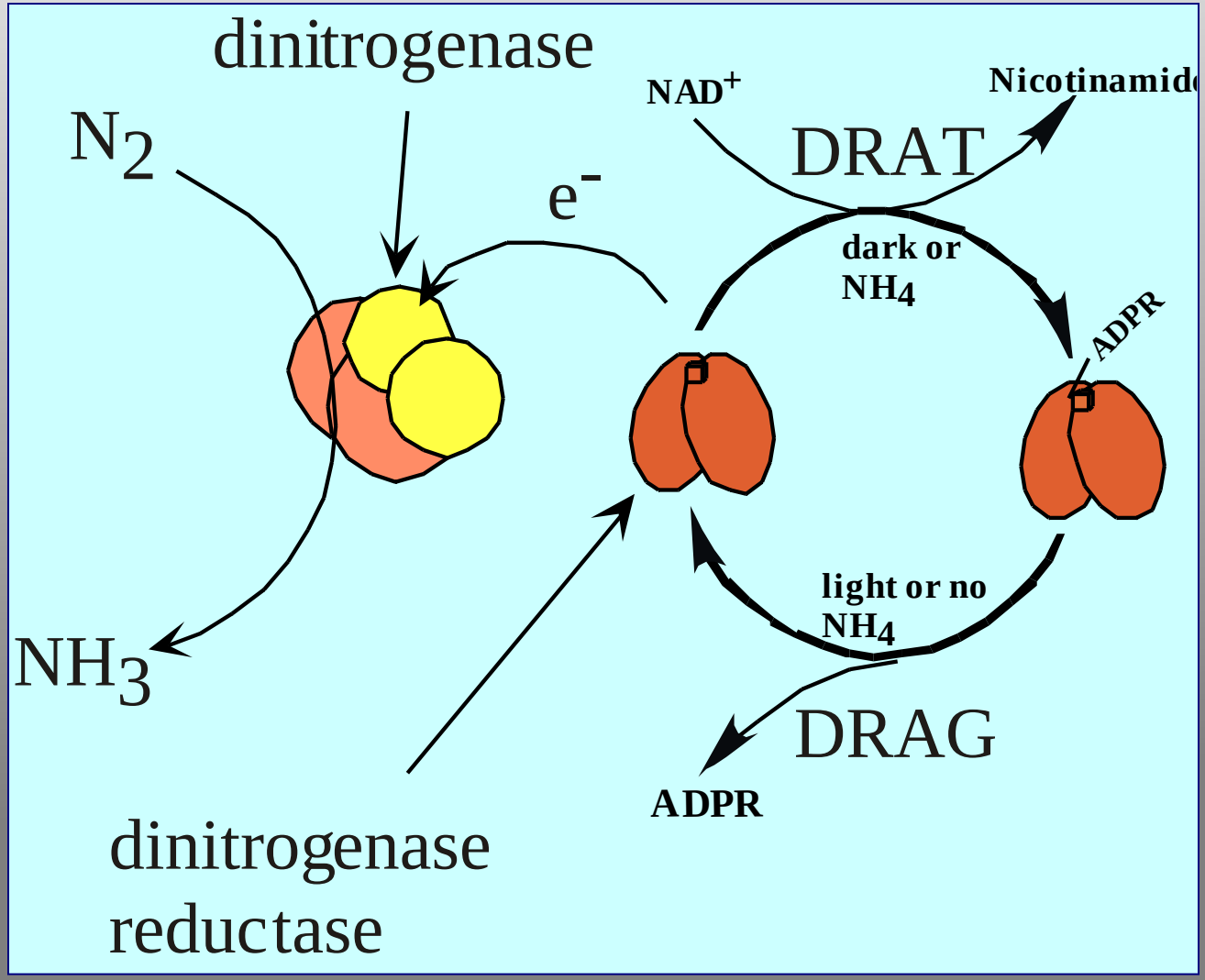
Symbiotic bacteria: Plants form nodules to house bacteria and provide C as energy source (*Rhizobium*/*Bradyrhizobium* for legumes, *Frankia* for non-legumes). Nodules contain a form of hemoglobin which binds O_2, protecting nitrogenase enzyme.

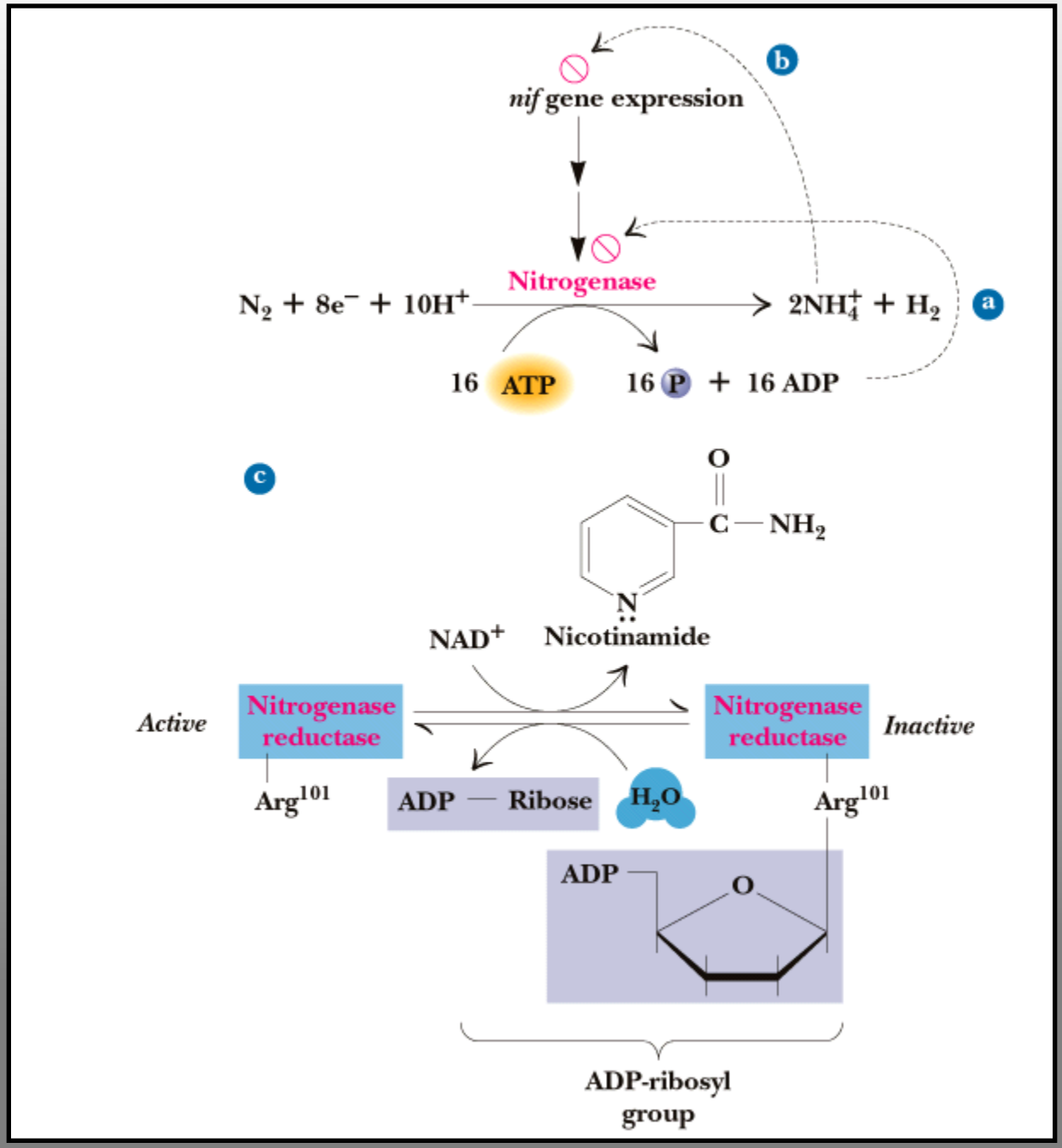
Nitrogen fixation in Klebsiella

- **Nif system is turned on when**
  - **No fixed nitrogen**
  - **Anaerobic**
  - **Temperature below 30° C**
- **Nitrogenase is made**
  - **Converts N_2 to NH_3**



ADP ribosylation of dinitrogenase reductase




b
nif gene expression
Nitrogenase
$N_2 + 8e^- + 10H^+$
$2NH_4^+ + H_2$
a
16 ATP
16 P + 16 ADP
c
O
C — NH_2
N
NAD+
Nicotinamide
Active
Nitrogenase reductase
Arg101
ADP — Ribose
H_2O
Nitrogenase reductase
Inactive
Arg101
ADP
O
ADP-ribosyl group

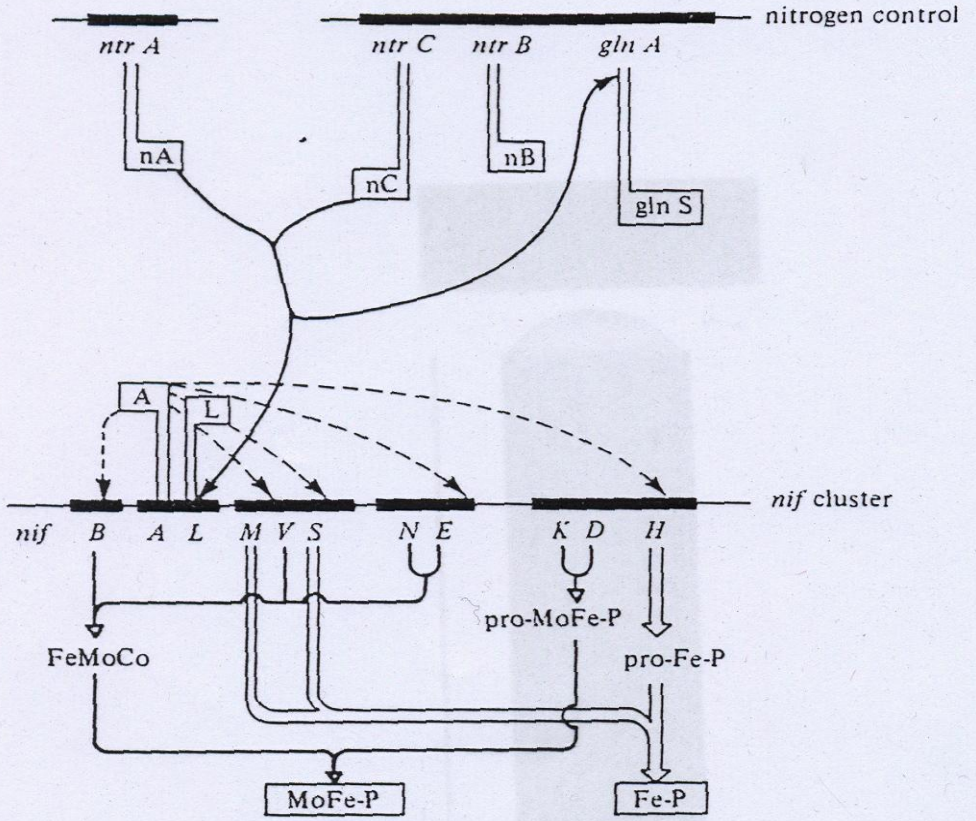


Figure 10.5. Organization and control of the *nif* cluster in *K. pneumoniae*. The situation under the condition of derepression is shown. The products of *ntrA* + *ntrC*, designated as nA and nC, together activate the promotor of *glnA* and of *nifA-nifL*. The *nifA* product (A) then activates the remaining promotors of the *nif* cluster. pro-Fe-P, Polypeptide of azoferredoxin; incorporation of the iron-sulfur centers is controlled by *nifM* and *nifS* products; pro-MoFe-P, precursor of molybdoferredoxin; the iron-molybdenum cofactor results from the products of genes *nifB*, *nifN*, *nifV*, and *nifE*. In the presence of ammonia product nB prevents activation at the nitrogen control level and product L at the *nif* cluster level. Solid arrows indicate the promotors at which transcription is derepressed by the gene products nA + nC. Dotted arrows indicate the promotors at which transcription is derepressed by the gene product A.

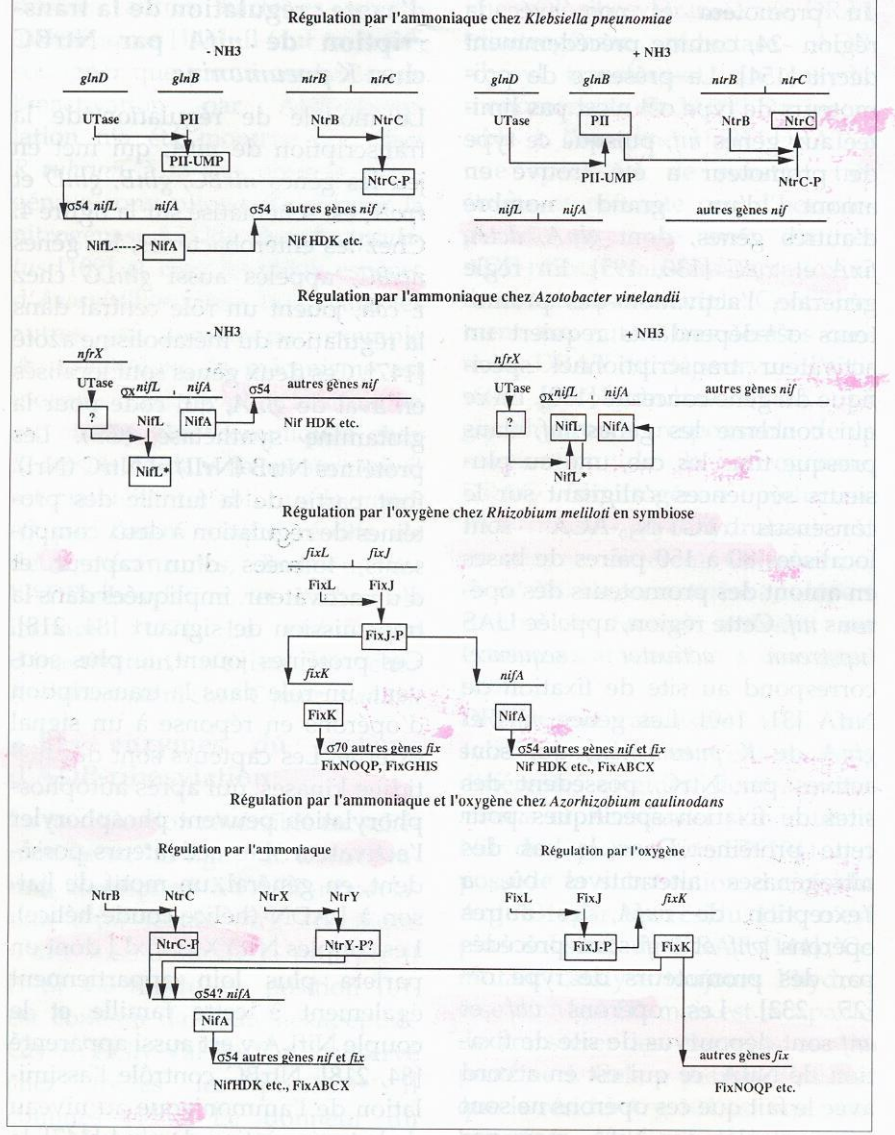


Fig 4. Quelques modèles de régulation chez différentes bactéries fixatrices d'azote. K pneumoniae *fixe l'azote en anaérobiose et en absence d'ammoniaque,* A vinelandii *dans l'air et en absence d'ammoniaque,* R meliloti *seulement en symbiose,* A caulinodans *en symbiose et à l'état libre en microaérobiose en absence d'ammoniaque. Le modèle présenté pour* A caulinodans *correspond aux conditions de fixation à l'état libre. Les principales protéines régulatrices sont encadrées.*

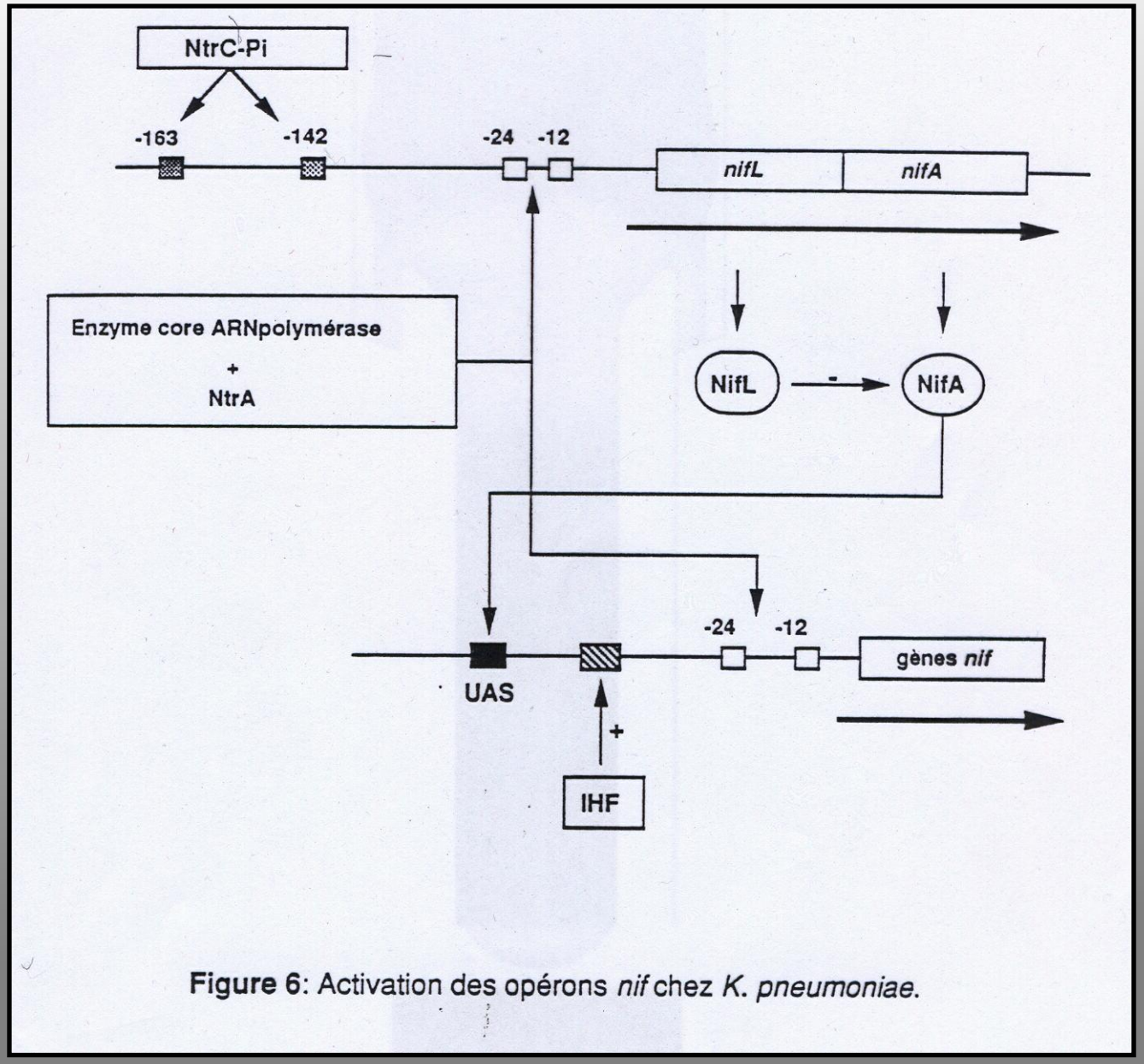
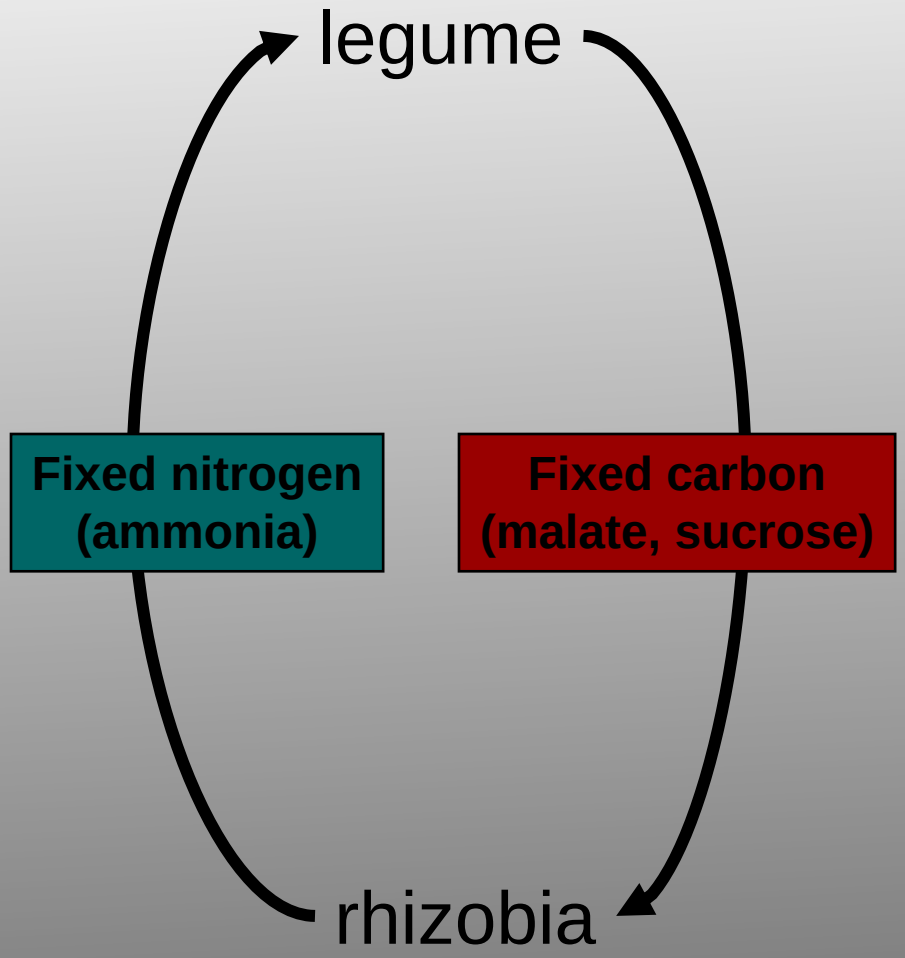
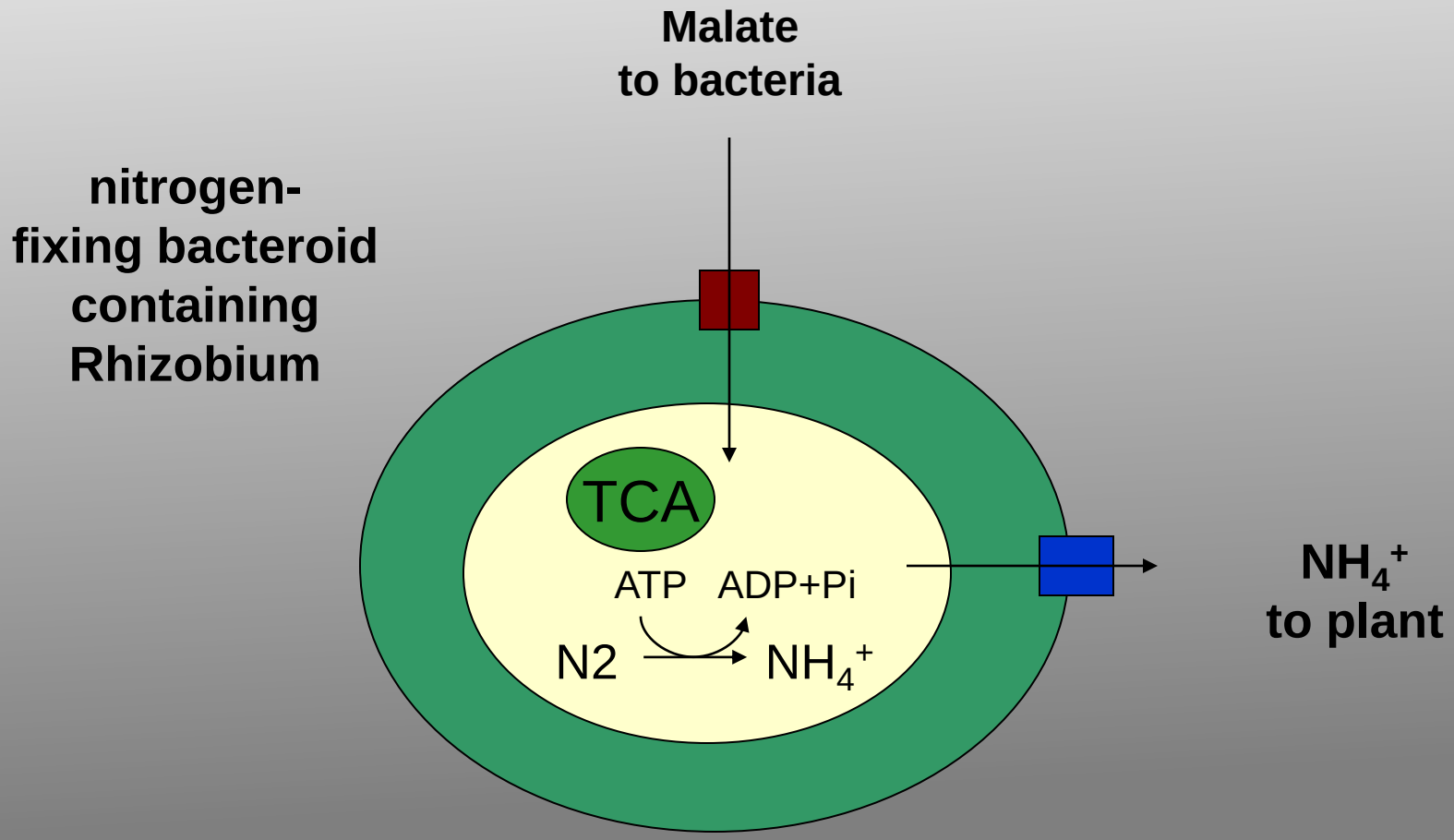


Figure 6: Activation des opérons *nif* chez *K. pneumoniae*.


legume
Fixed nitrogen
(ammonia)
Fixed carbon
(malate, sucrose)
rhizobia

Exchange of nutrients during Rhizobium-legume symbiosis

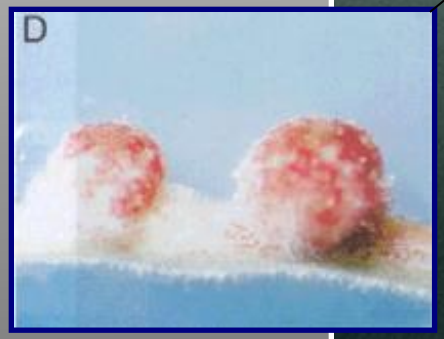


Symbiotic Nitrogen Fixation

The *Rhizobium*-legume association

Bacterial associations with certain plant families, primarily **legume** species, make the largest single contribution to biological nitrogen fixation in the biosphere

Pea Plant



R. leguminosarum nodules

Pink color is leghaemoglobin a protein that carries oxygen to the bacteroids

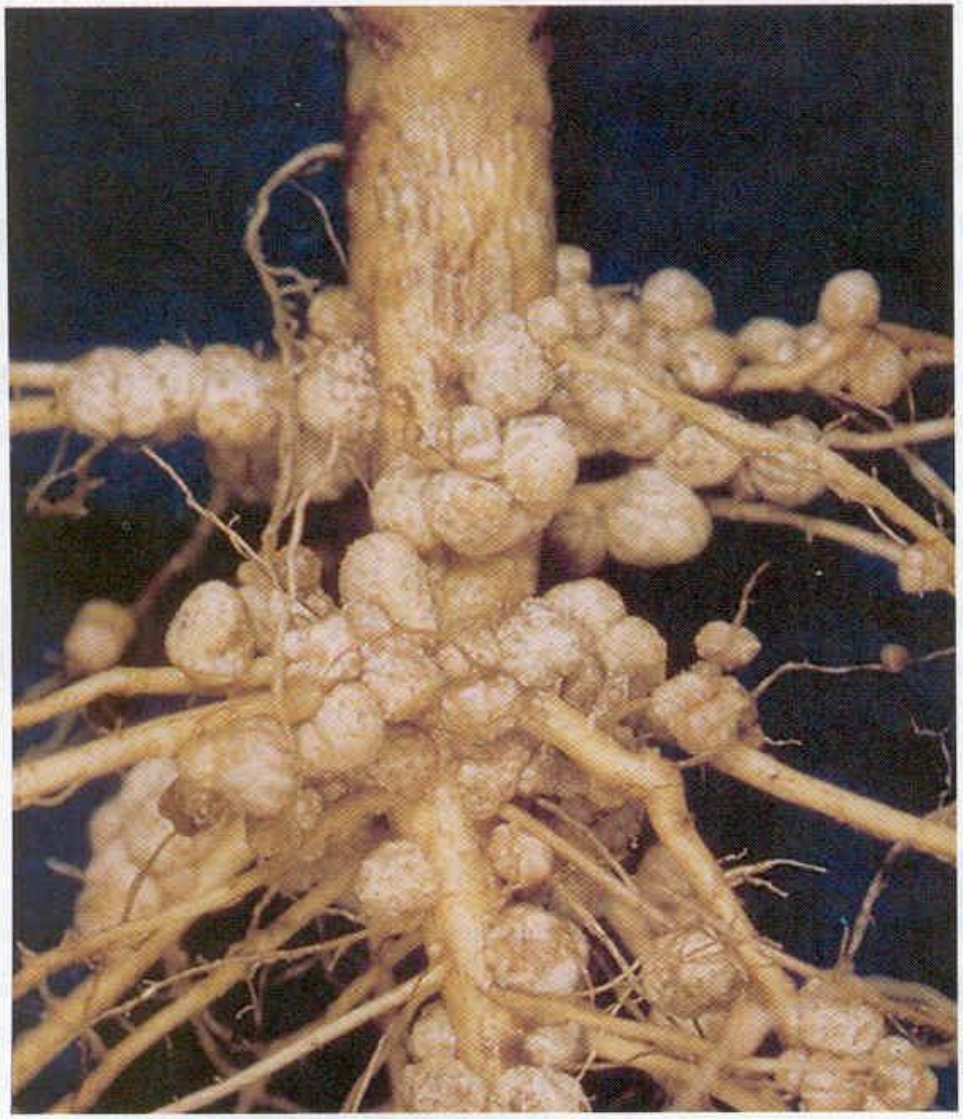
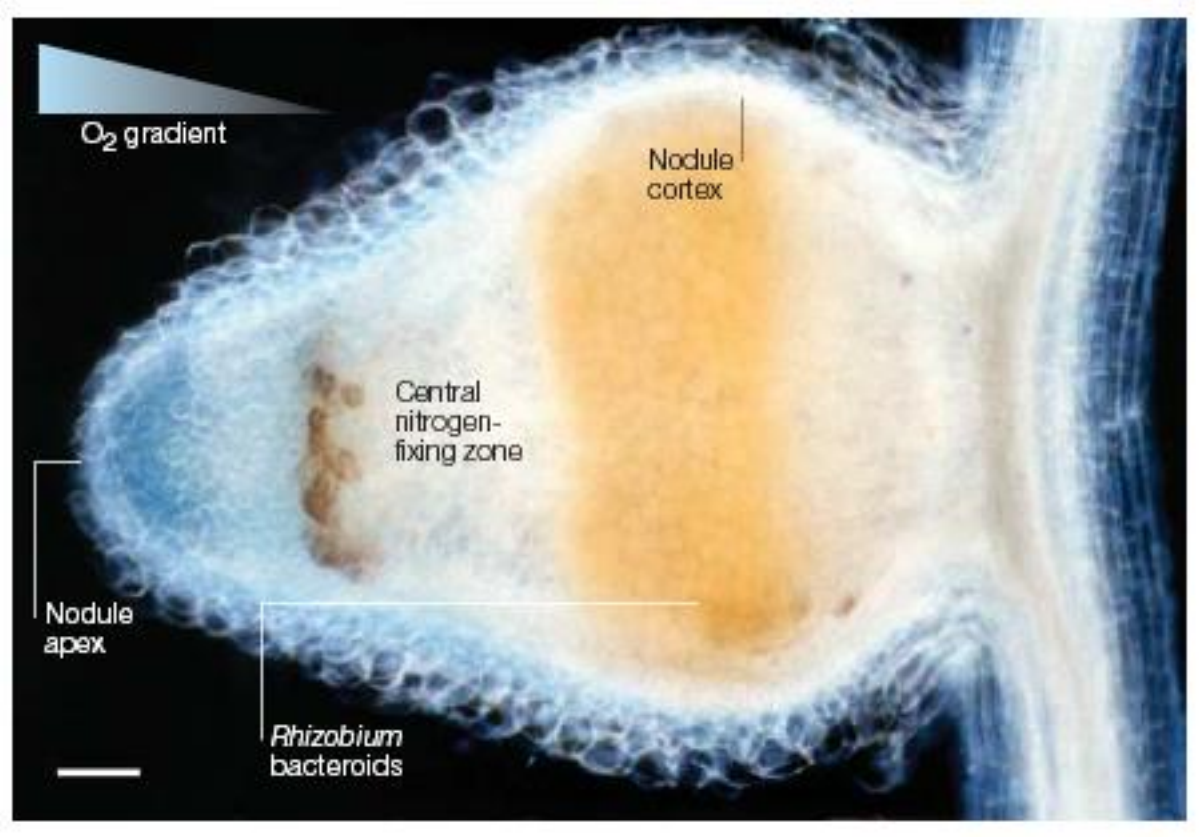
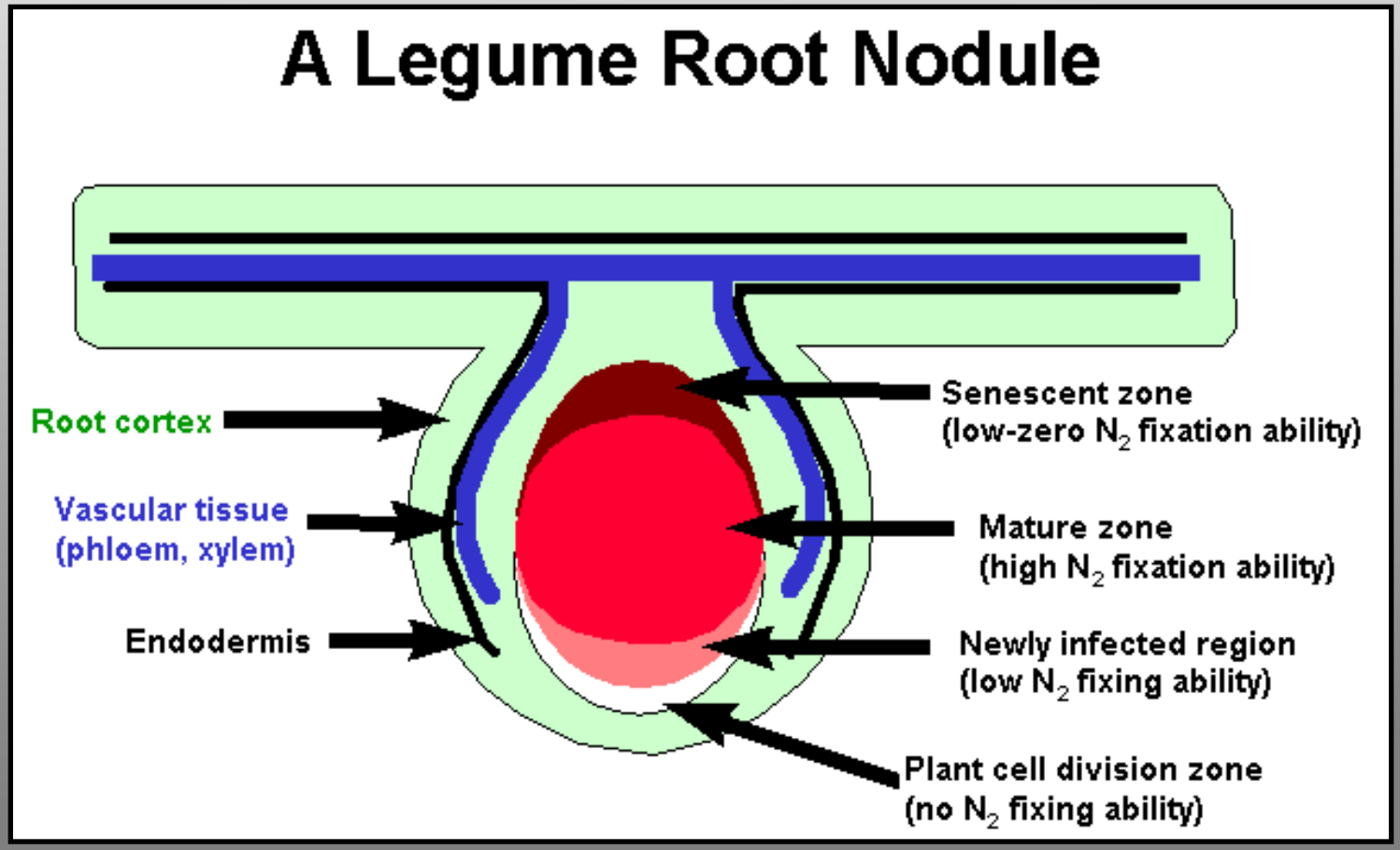
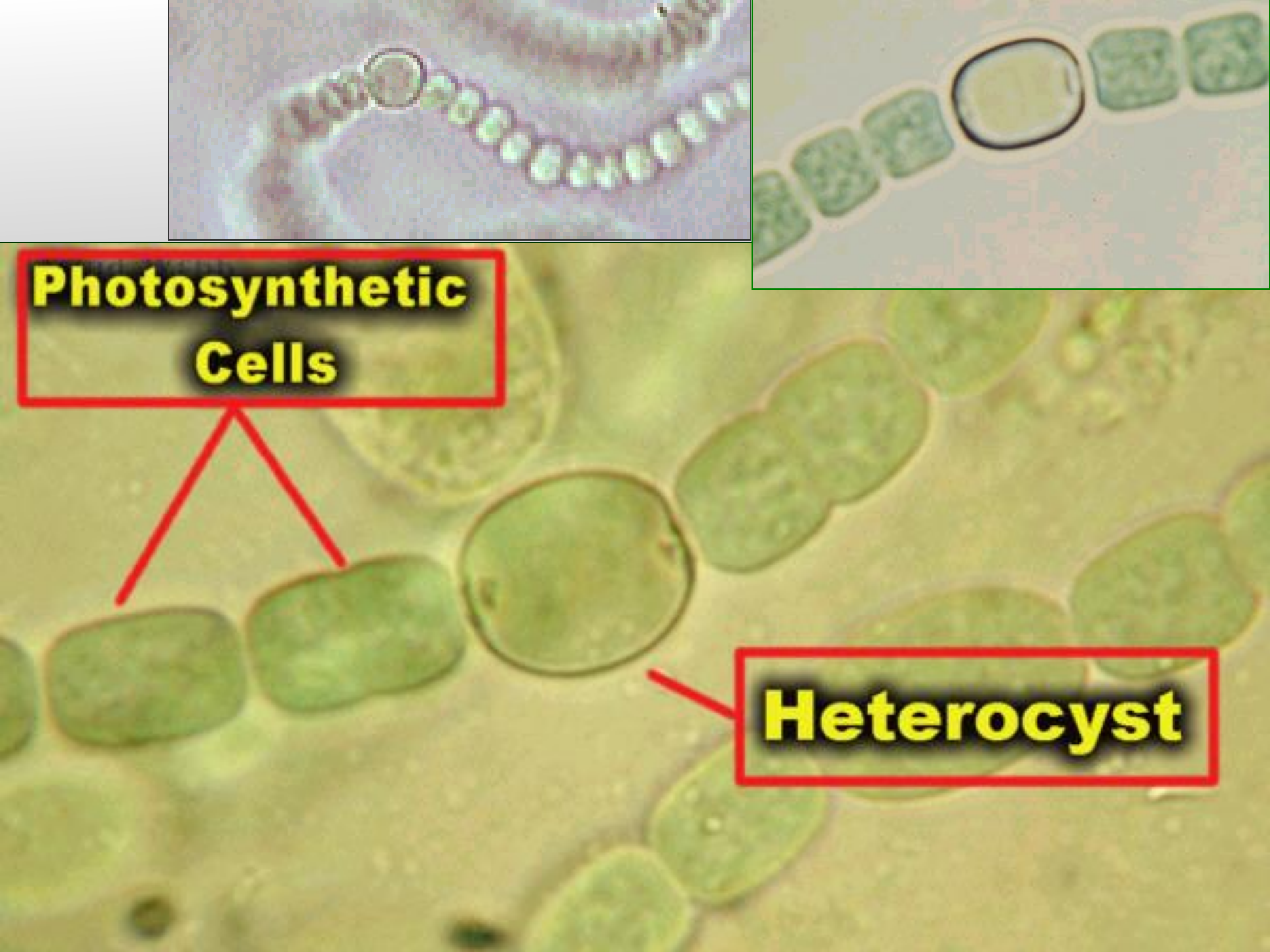


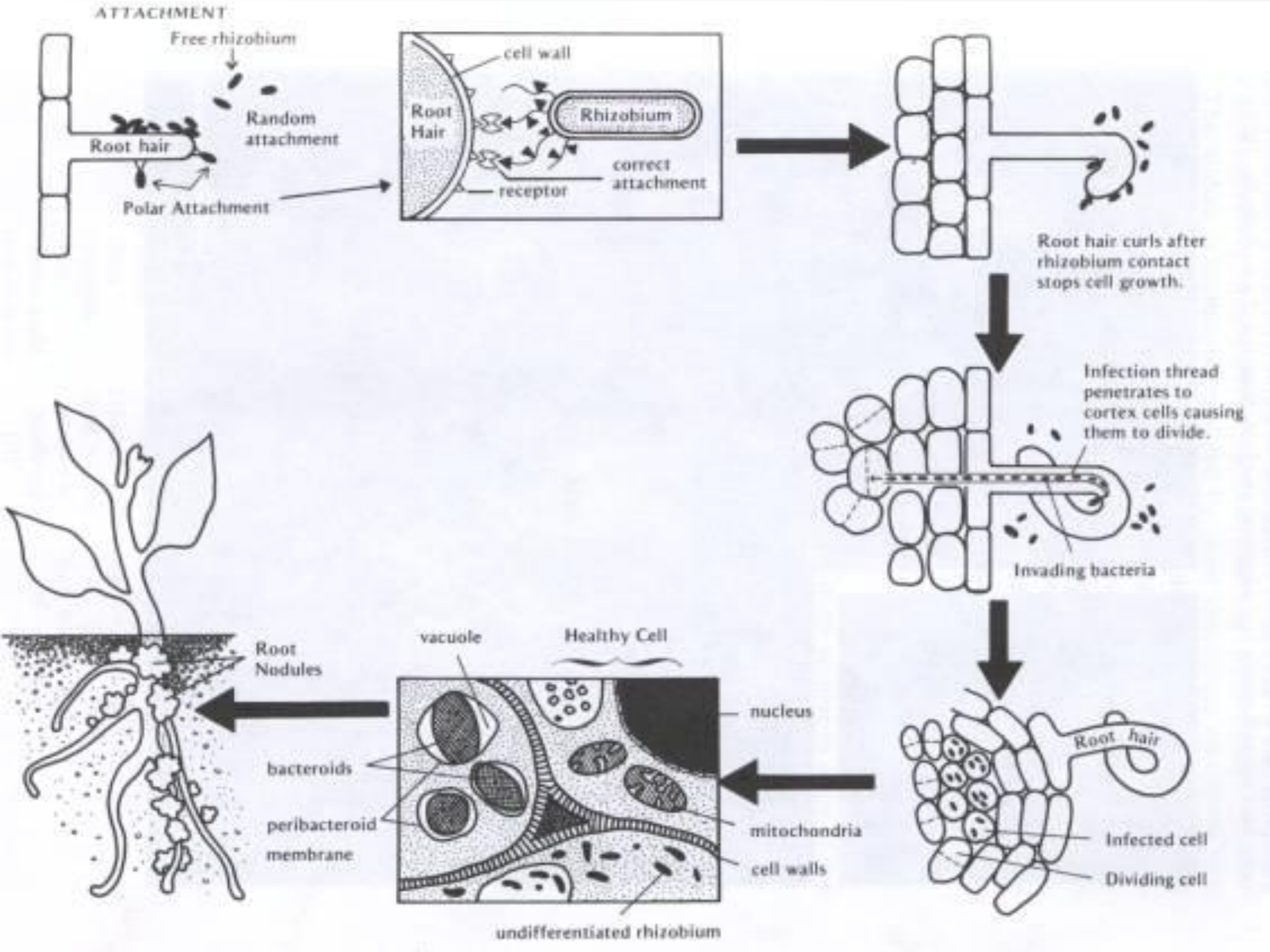
FIGURE 16.73 Soybean root nodules. The nodules develop by infection with *Bradyrhizobium japonicum*.


O_2 gradient
Nodule cortex
Central nitrogen-fixing zone
Nodule apex
Rhizobium bacteroids

Physiology of a legume nodule




Photosynthetic Cells
Heterocyst


ATTACHMENT
Free rhizobium
Random attachment
Root hair
Polar Attachment
cell wall
Root Hair
Rhizobium
correct attachment
receptor
Root hair curls after rhizobium contact stops cell growth.
Infection thread penetrates to cortex cells causing them to divide.
Invading bacteria
Root hair
Infected cell
Dividing cell
nucleus
mitochondria
cell walls
vacuole
Healthy Cell
bacteroids
peribacteroid membrane
undifferentiated rhizobium
Root Nodules

The nodulation process

1. Chemical recognition of root and *Rhizobium*
2. Root hairs curl
3. Formation of infection threads
4. Invasion of the roots by *Rhizobia*
5. Nodule tissue forms
6. Bacteria convert to bacteriods and begin to form nitrogenase enzyme
7. Legume provides *Rhizobia* with carbon. *Rhizobia* provide the legume with fixed N

The Nodulation Process

- Chemical recognition of roots and Rhizobium
- Root hair curling
- Formation of infection thread
- Invasion of roots by Rhizobia
- Cortical cell divisions and formation of nodule tissue
- Bacteria fix nitrogen which is transferred to plant cells in exchange for fixed carbon

Biological NH_3 creation (nitrogen fixation) accounts for an estimated 170×10^9 kg of ammonia every year. Human industrial production amounts to some 80×10^9 kg of ammonia yearly.

The industrial process (Haber-Bosh process) uses an Fe catalyst to dissociate molecules of N_2 to atomic nitrogen on the catalyst surface, followed by reaction with H_2 to form ammonia. This reaction typically runs at ~450º C and 500 atmospheres pressure.

These extreme reaction conditions consume a huge amount of energy each year, considering the scale at which NH_3 is produced industrially.

The Dreams…..

If a way could be found to **mimic nitrogenase catalysis**
(a reaction conducted at 0.78 atmospheres N_2 pressure
and ambient temperatures), huge amounts of energy
(and money) could be saved in industrial ammonia production.

If a way could be found to
transfer the capacity to form N-fixing symbioses
from a typical legume host
to an important non-host crop species such as corn or wheat,
far less fertilizer
would be needed to be produced and applied
in order to sustain crop yields

Because of its current and potential **economic importance**, the interaction between Rhizobia and leguminous plants has been intensively studied.

Our understanding of the process by which these two symbionts establish a functional association is still not complete, but it has provided a **paradigm** for many aspects of cell-to-cell communication between microbes and plants (e.g. during pathogen attack), and even between cells within plants (e.g. developmental signals; fertilization by pollen).

Symbiotic Rhizobia are classified in two groups:

Fast-growing ***Rhizobium*** spp. whose nodulation functions (**nif, fix**) are encoded on their symbiotic megaplasmids (**pSym**)

Slow-growing ***Bradyrhizobium*** spp. whose N-fixation and nodulation functions are encoded on their chromosome.

There are also two types of nodule that can be formed:

determinate

and

indeterminate

This outcome is controlled by the plant host

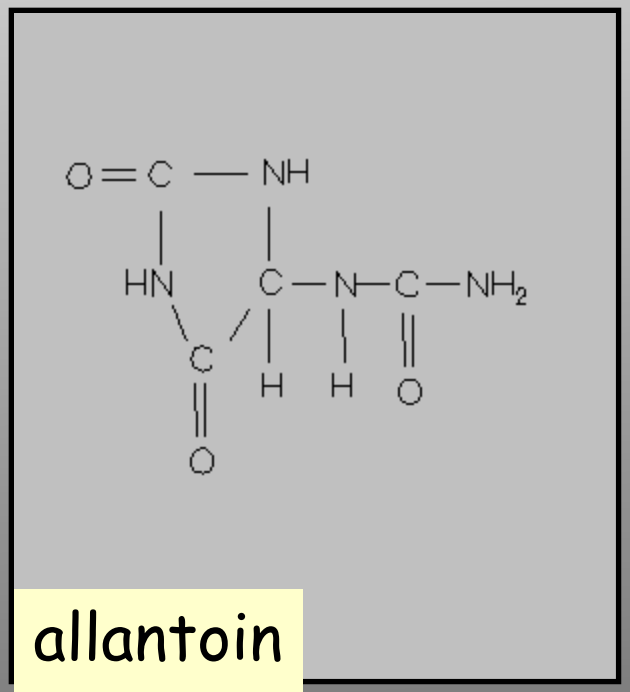
Determinate nodules

Formed on **tropical legumes** by *Rhizobium* and *Bradyrhizobium*

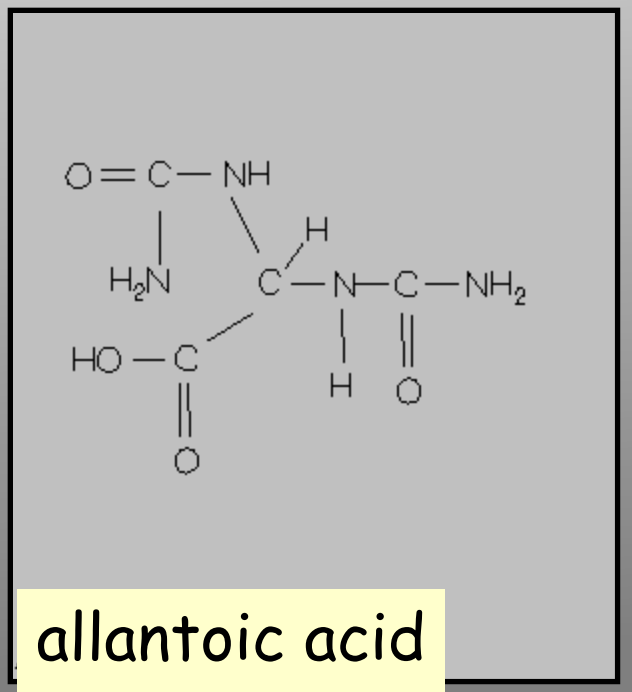
Meristematic activity not persistent - present only during early stage of nodule formation;
after that, cells simply expand rather than divide, to form **globose nodules.**

Nodules arise just below epidermis; largely internal vascular system

Uninfected cells dispersed throughout nodule; equipped to assimilate NH_4^+ as **ureides** (allantoin and allantoic acid)



allantoin



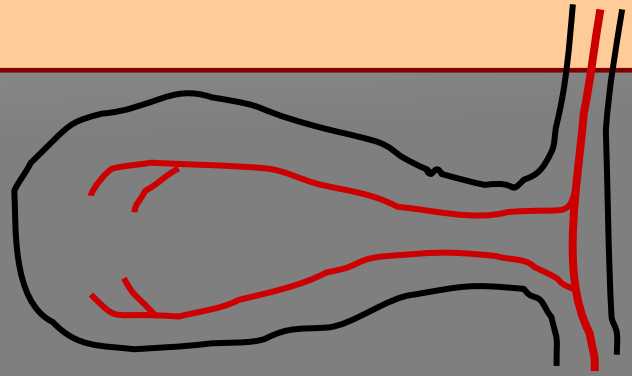
allantoic acid

Indeterminate nodules

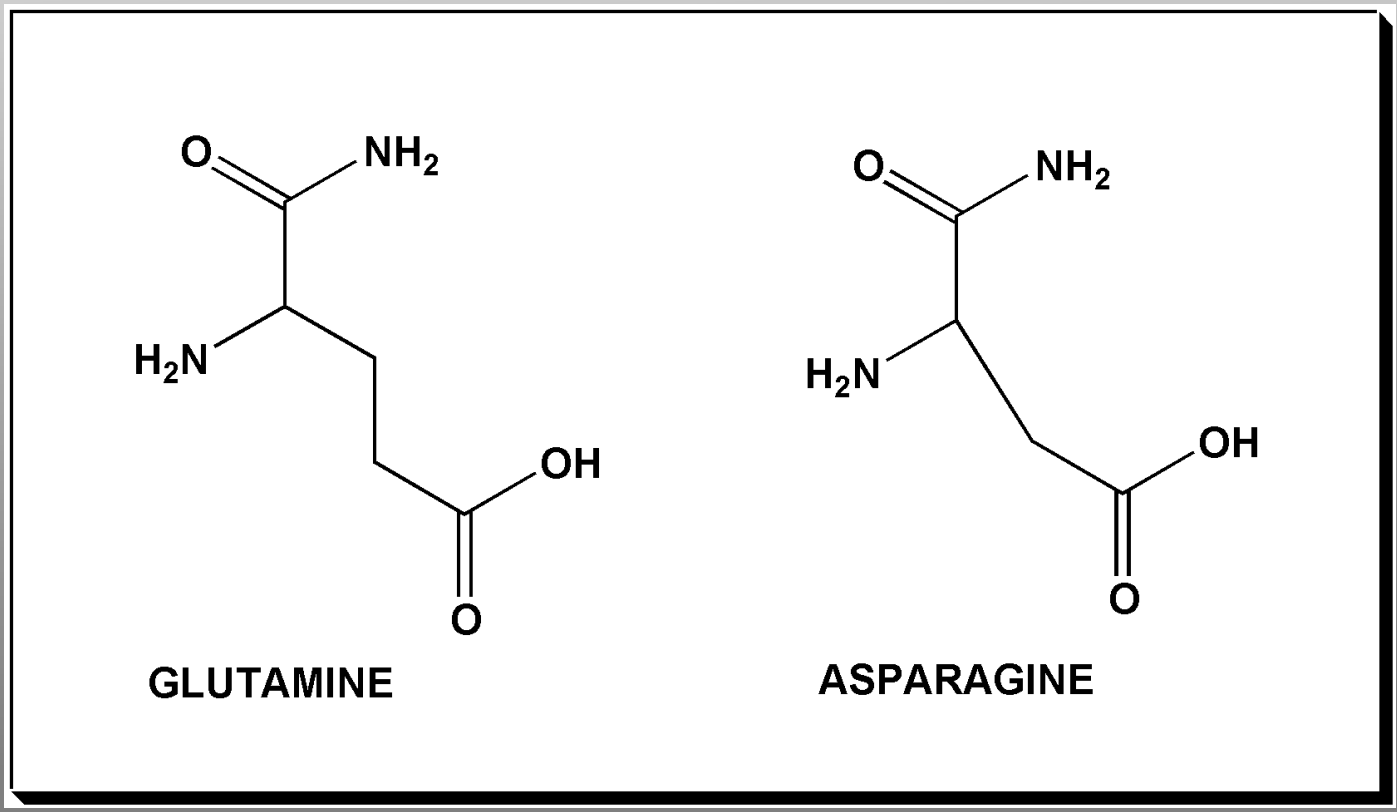
Formed on **temperate** legumes
(pea, clover, alfalfa);
typically by *Rhizobium* spp.

Cylindrical nodules with a **persistent meristem**;
nodule growth creates zones of different developmental stages

Nodule arises near endodermis, and nodule vasculature clearly connected with root vascular system



Uninfected cells of indeterminate nodules assimilate NH_4^+ as **amides** (asparagine, glutamine)



Rhizobium

- establish highly specific symbiotic associations with legumes
  - form **root nodules**
  - fix nitrogen within root nodules
  - nodulation genes are present on large plasmid

Rhizobium-legume symbioses

Host plant	**Bacterial symbiont**
Alfalfa	*Rhizobium meliloti*
Clover	*Rhizobium trifolii*
Soybean	*Bradyrhizobium japonicum*
Beans	*Rhizobium phaseoli*
Pea	*Rhizobium leguminosarum*
Sesbania	*Azorhizobium caulinodans*

Complete listing can be found at at:
http://cmgm.stanford.edu/~mbarnett/rhiz.htm

Both plant and bacterial factors determine specificity

TABLE 16.8 Major cross-inoculation groups of leguminous plants

Host plant	Nodulated by
Pea	*Rhizobium leguminosarum* biovar *viciae*[a]
Bean	*Rhizobium leguminosarum* biovar *phaseoli*[a]
Bean	*Rhizobium tropici*
Lotus	*Mesorhizobium loti*
Clover	*Rhizobium leguminosarum* biovar *trifolii*[a]
Alfalfa	*Sinorhizobium meliloti*
Soybean	*Bradyrhizobium japonicum*
Soybean	*Bradyrhizobium elkanii*
Soybean	*Rhizobium fredii*
Sesbania rostrata (a tropical legume)	*Azorhizobium caulinodans*

[a] Several varieties (biovars) of *Rhizobium leguminosarum* exist, each capable of nodulating a different legume.

Typical Associations (cross-inoculation groups)

R.l.* biovar *viciae
colonizes **pea** (*Pisum* spp.) and vetch
(temperate; indeterminate nodules)

R.l.* biovar *trifolii
colonizes **clover** (*Trifolium* spp.)
(temperate; indeterminate nodules)

Rhizobium leguminosarum* biovar *phaseoli
colonizes **bean** (*Phaseolus* spp.)
(tropical; determinate nodules)

Rhizobium meliloti
colonizes **alfalfa** (*Medicago sativa*)
temperate; indeterminate nodules

Rhizobium fredii
colonizes **soybean** (*Glycine max*)
tropical; determinate nodules

Bradyrhizobium japonicum
colonizes **soybean**
tropical; determinate nodules

Rhizobium NGR 234
colonizes ***Parasponia*** and tropicals;
very broad host range

Very early events in the Rhizobium-legume symbiosis

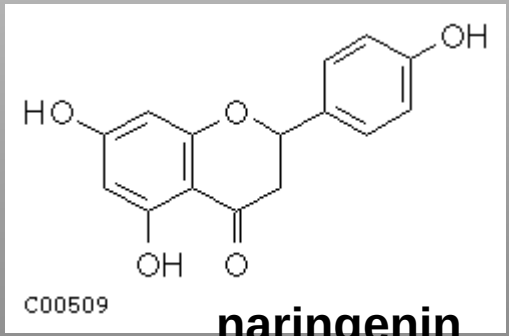
Flavonoids nod-gene inducers

rhizosphere

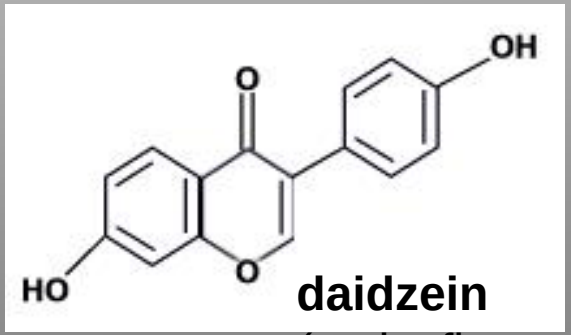
Nod-factor

Nodule development process

1. Bacteria encounter root;
they are chemotactically attracted toward specific plant chemicals (**flavonoids**) exuding from root tissue, especially in response to nitrogen limitation

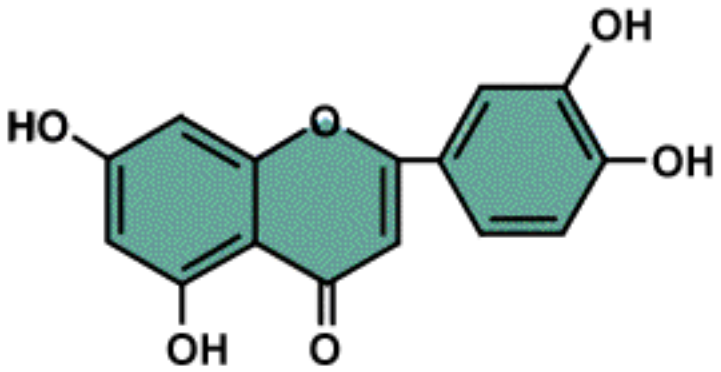


naringenin
(a flavanone)



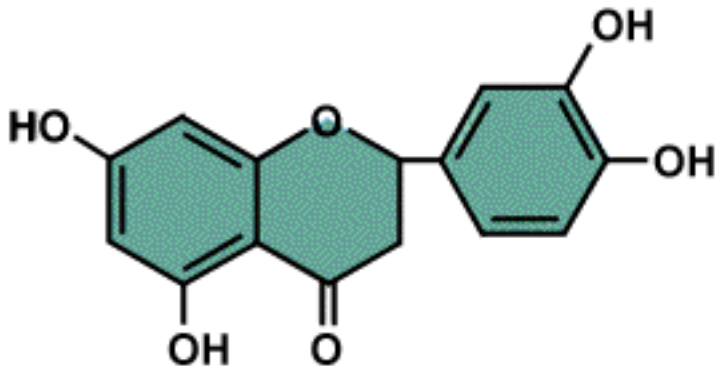
daidzein
(an isoflavone)

Inducers of nodulation in *Rhizobium leguminosarum bv viciae*



5, 7, 3', 4'-Tetrahydroxyflavone

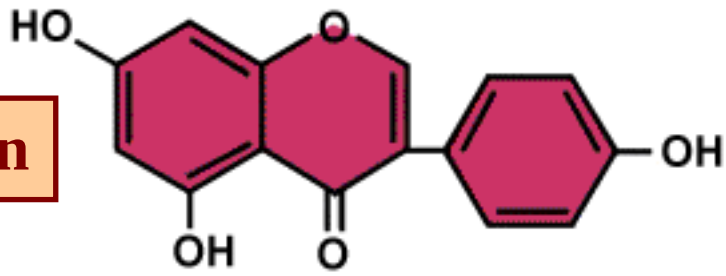
luteolin



5, 7, 3', 4'-Tetrahydroxyflavone

eriodictyol

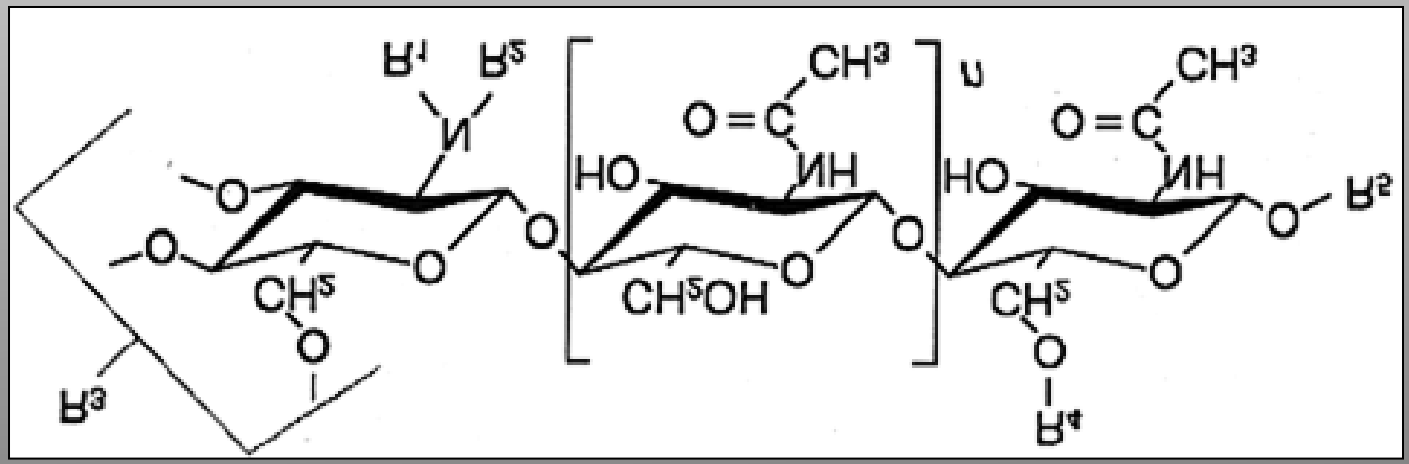
Inhibitor of nodulation



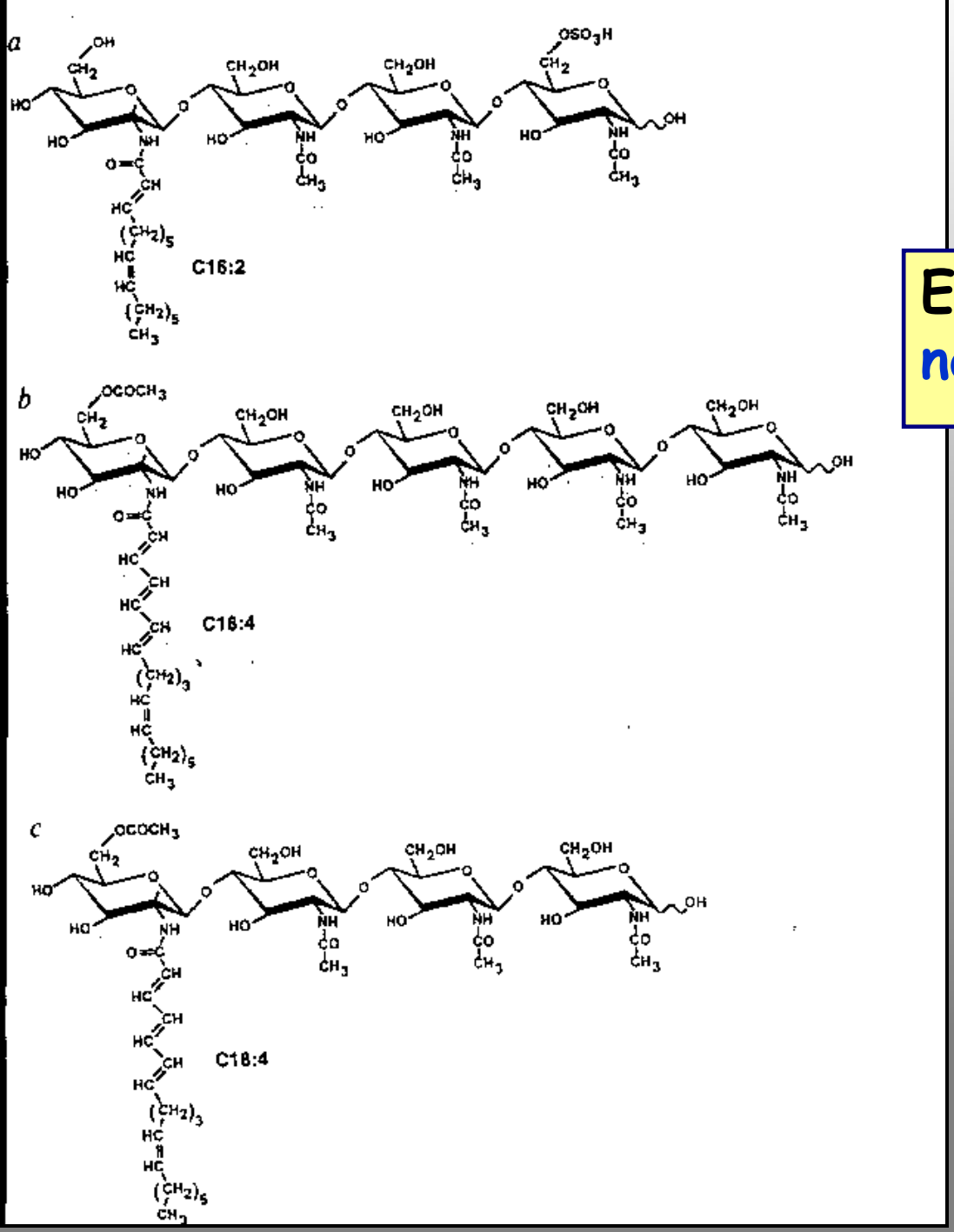
5, 7, 4'-Trihydroxyisolflavone

genistein

2. Bacteria attracted to the root attach themselves to the root hair surface and secrete specific **oligosaccharide** signal molecules (**nod factors**).



nod factor

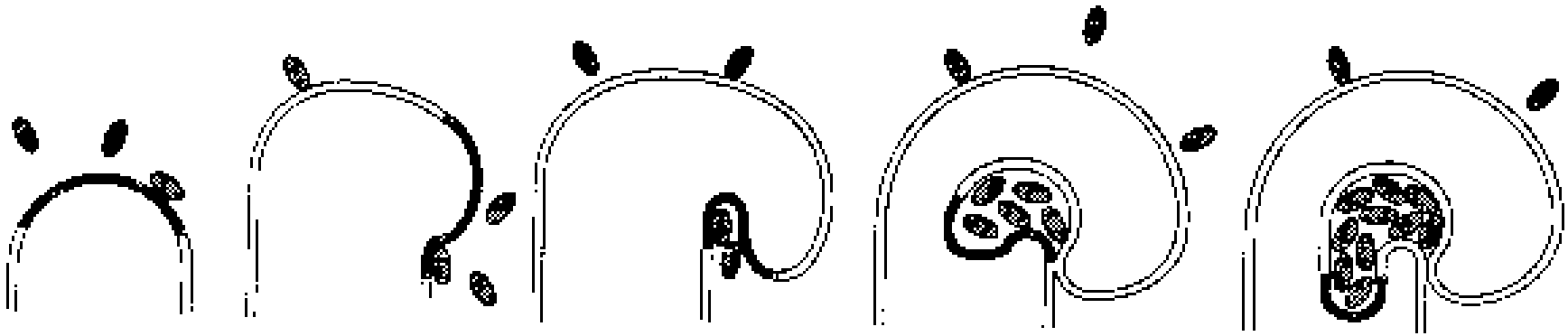


Examples of different nod factors

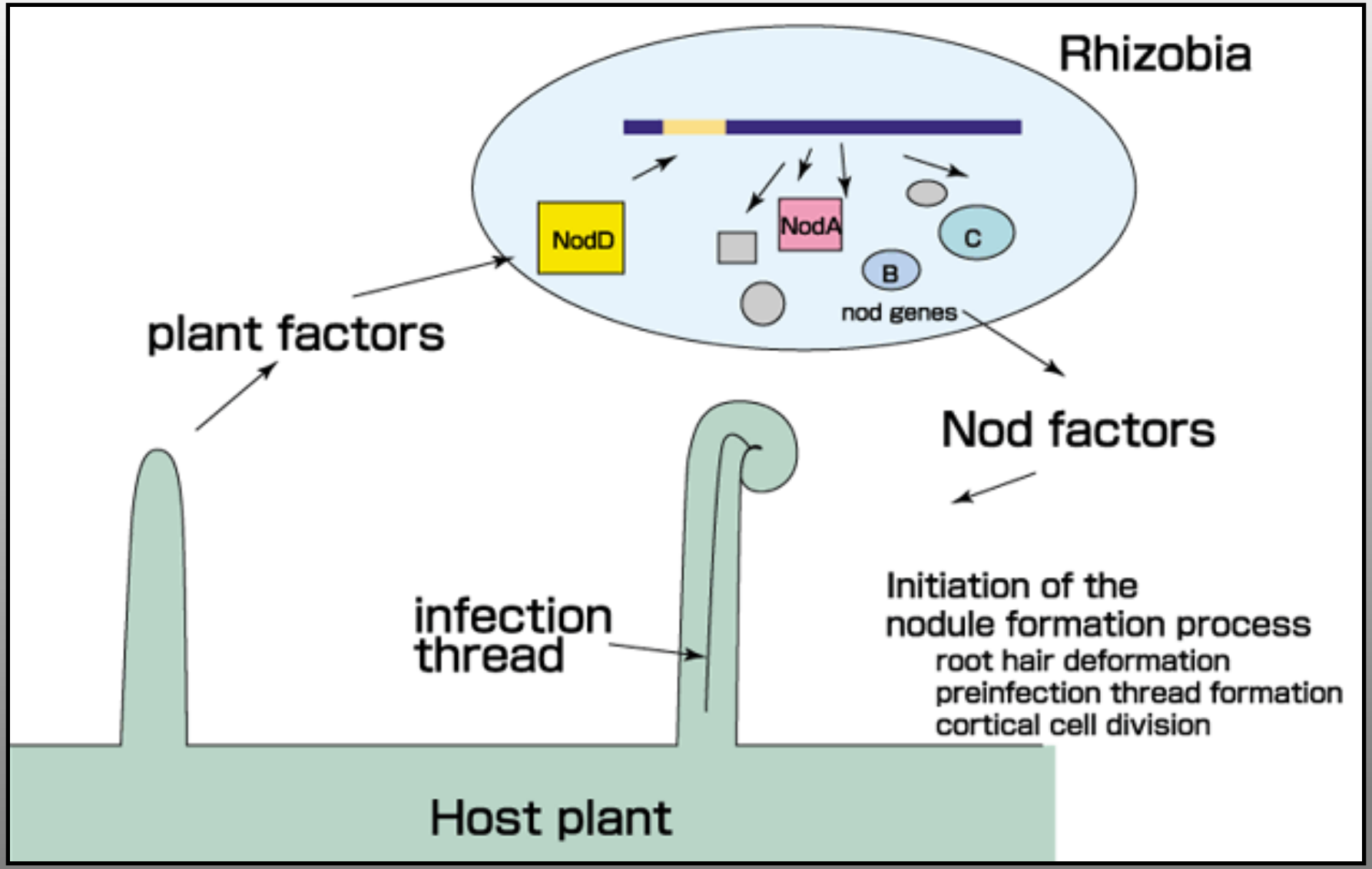
3. In response to oligosaccharide signals, the root hair becomes deformed and **curls** at the tip; bacteria become enclosed in small pocket.

Cortical **cell division** is induced within the root.

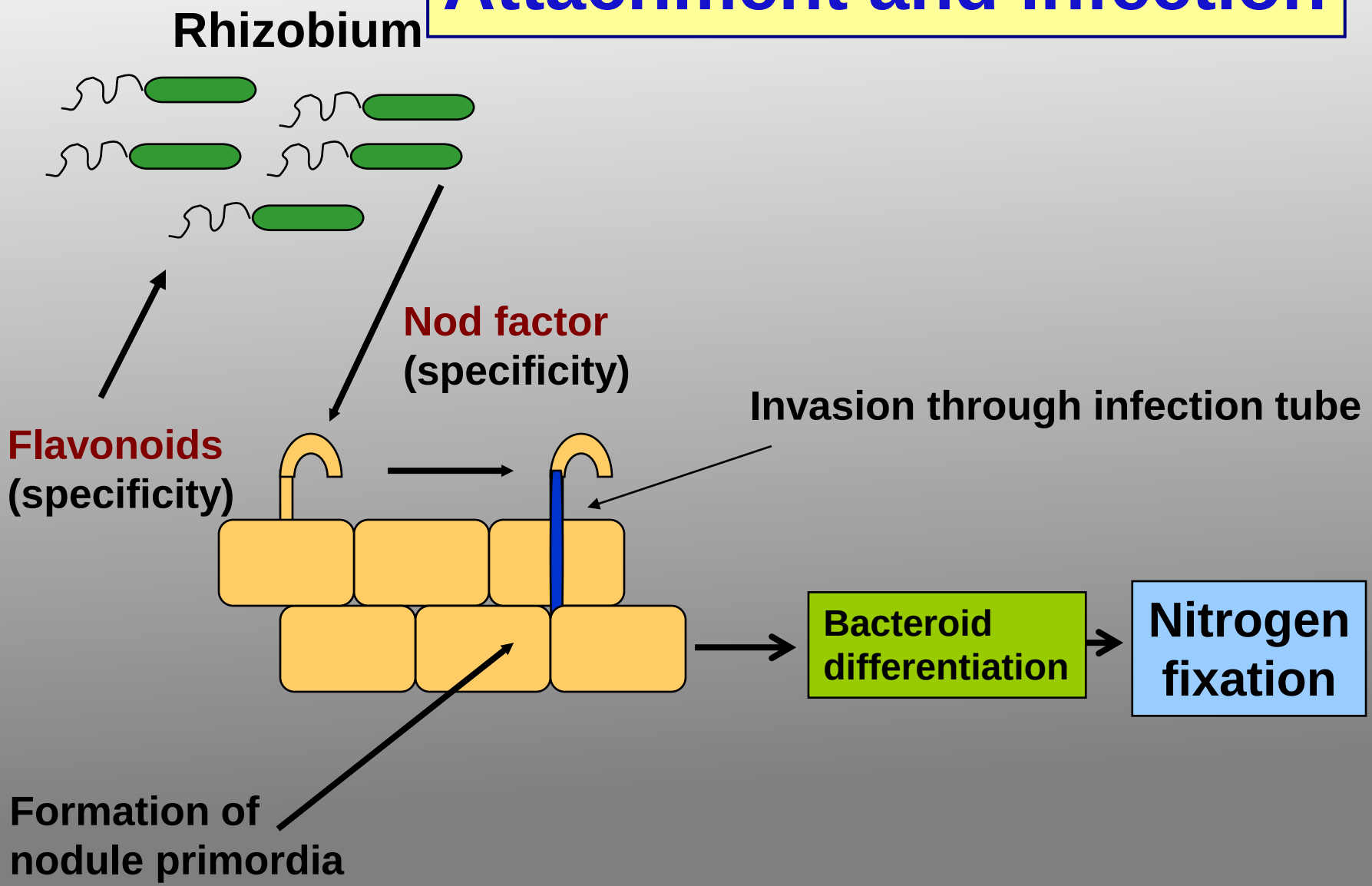
Root hair attachment and curling

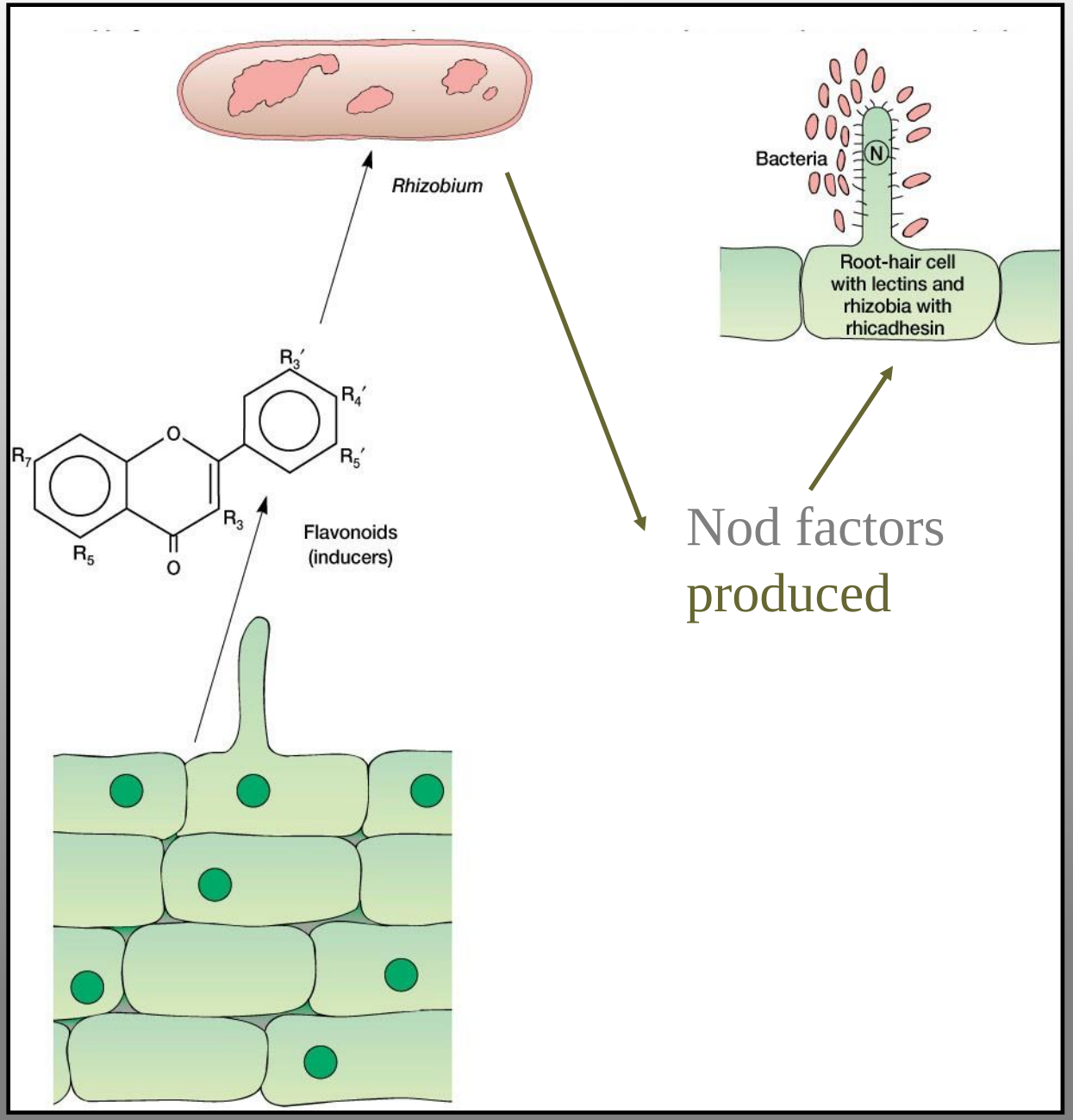


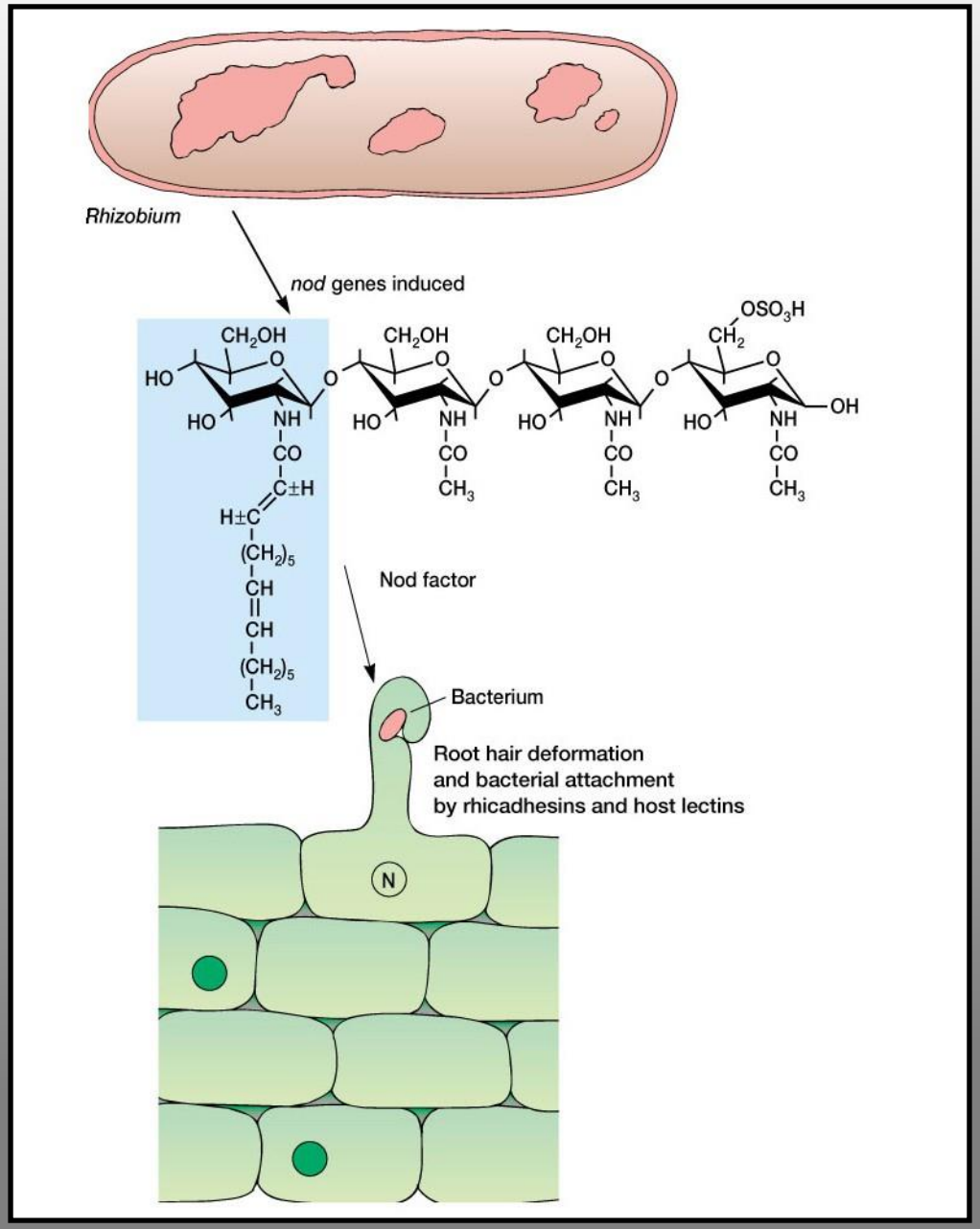
root hair beginning to curl


Rhizobia
NodD
NodA
C
B
nod genes
plant factors
Nod factors
infection thread
Initiation of the nodule formation process
root hair deformation
preinfection thread formation
cortical cell division
Host plant

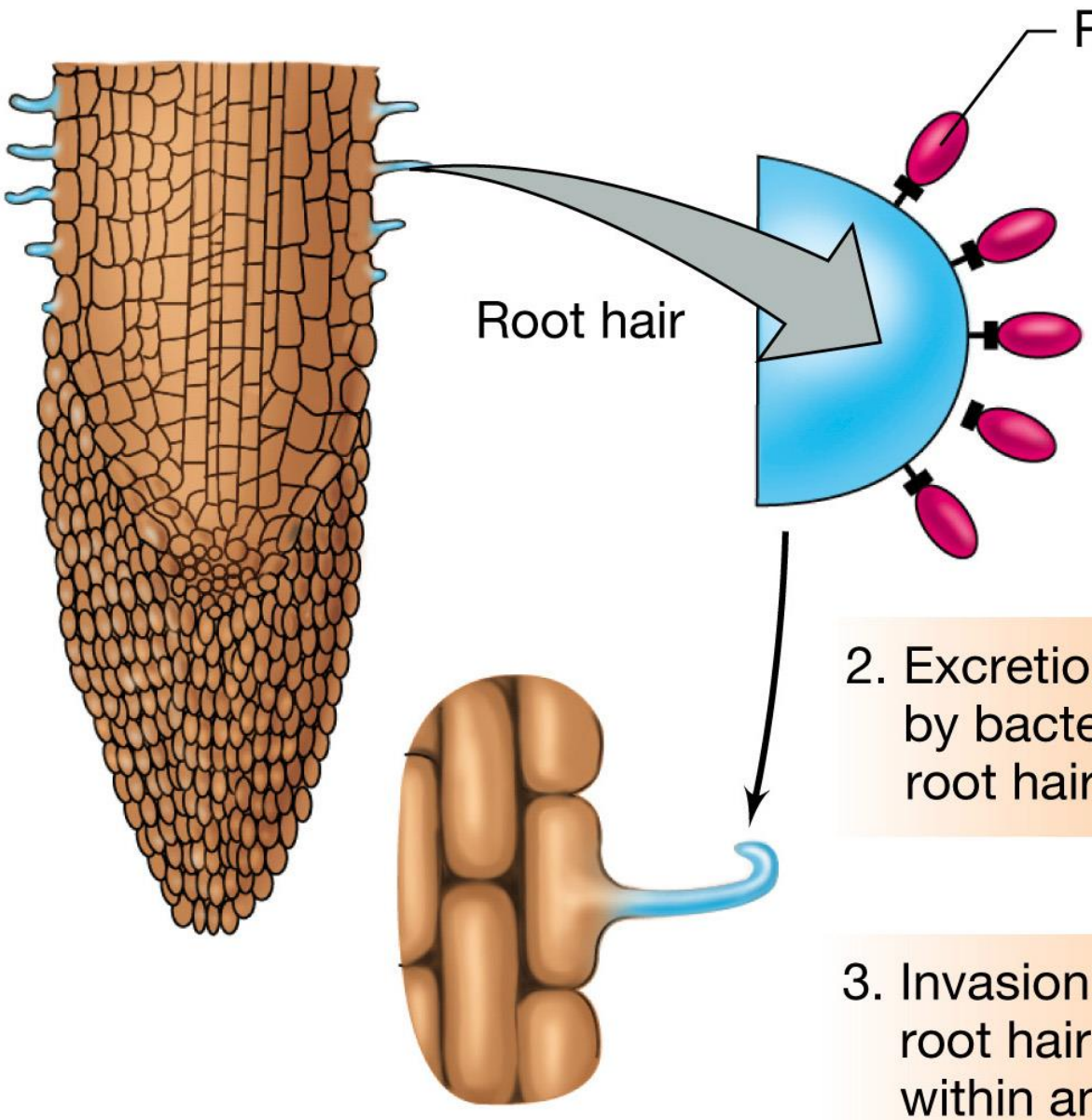
Attachment and infection




Rhizobium
Bacteria
N
Root-hair cell with lectins and rhizobia with rhicadhesin
Flavonoids (inducers)
Nod factors produced

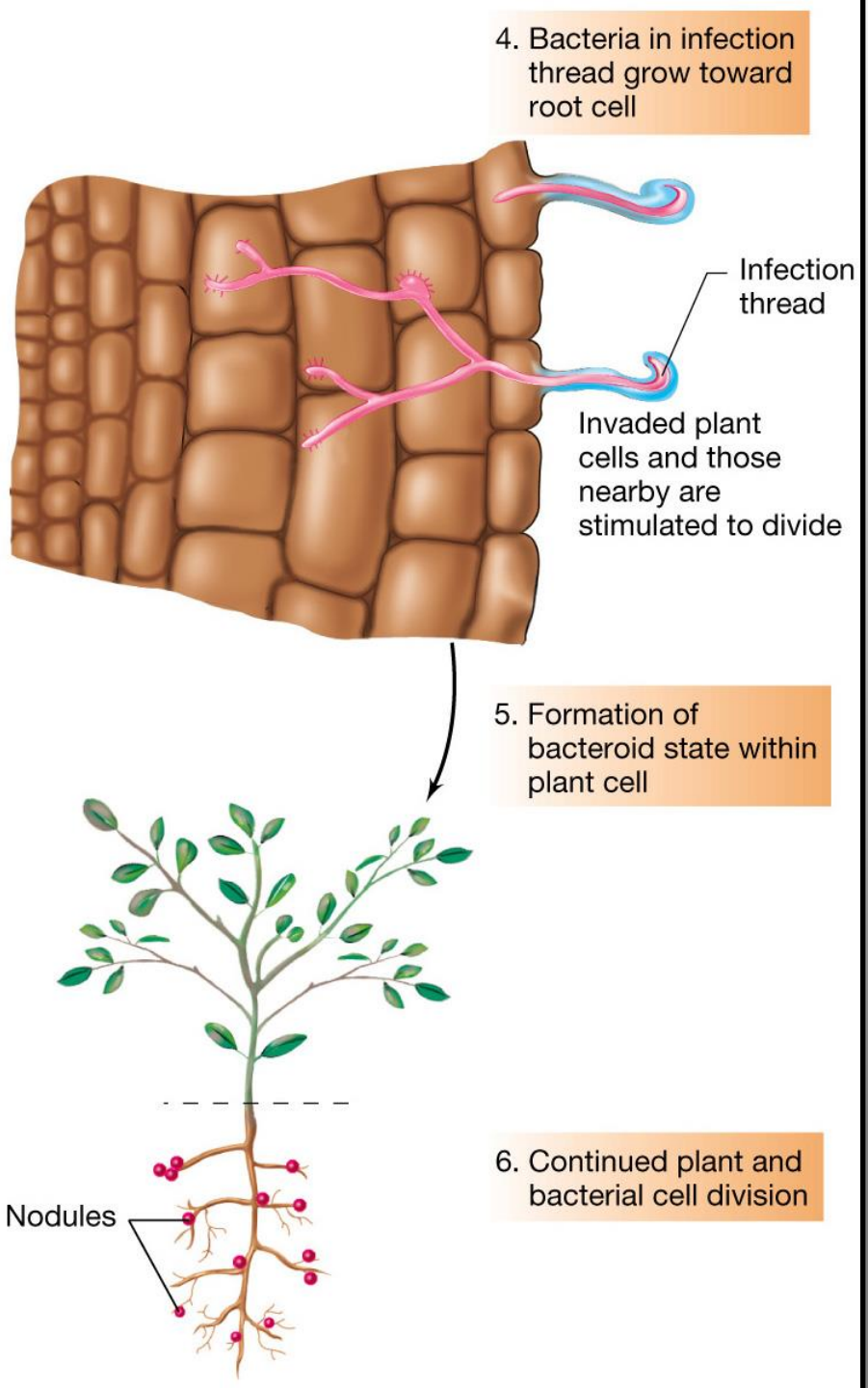

Rhizobium
nod genes induced
Nod factor
Bacterium
Root hair deformation
and bacterial attachment
by rhicadhesins and host lectins
N

1. Recognition and attachment (rhicadhesin-mediated)

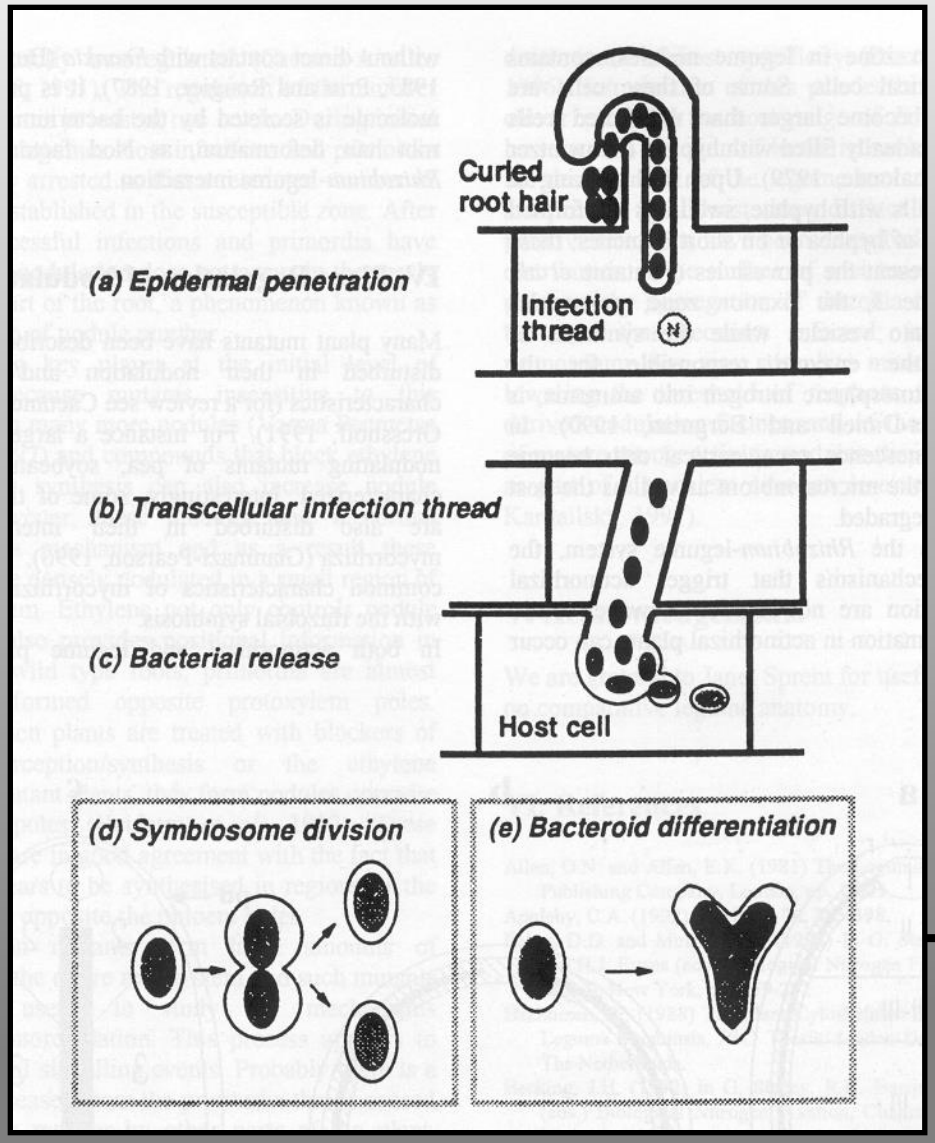


2. Excretion of nod factors by bacterium causing root hair curling

3. Invasion. Rhizobia penetrate root hair and multiply within an "infection thread"


4. Bacteria in infection thread grow toward root cell
Infection thread
Invaded plant cells and those nearby are stimulated to divide
5. Formation of bacteroid state within plant cell
6. Continued plant and bacterial cell division
Nodules

Nodule development



Enlargement of the nodule, nitrogen fixation and exchange of nutrients

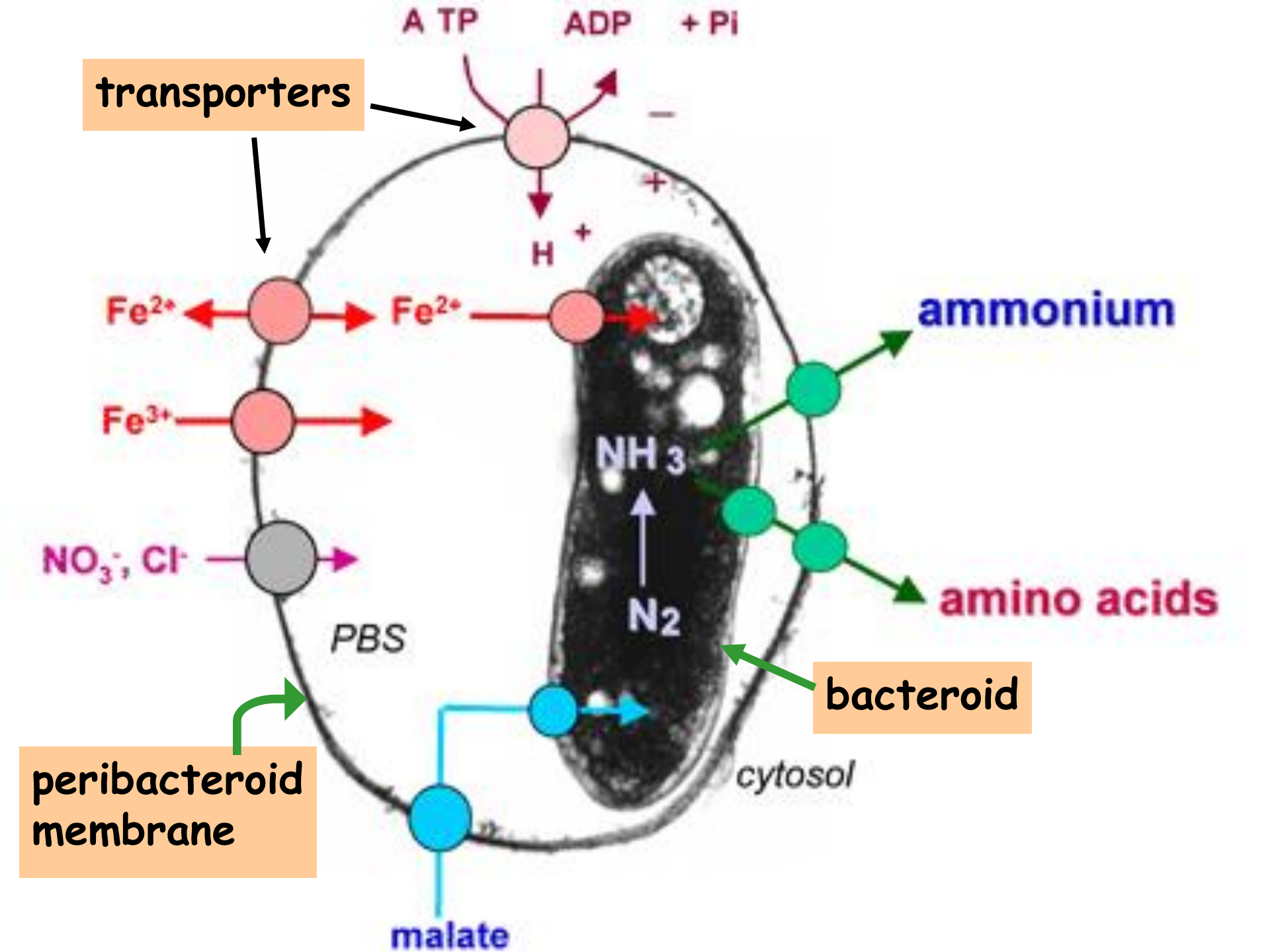
5. Infection thread penetrates through several layers of cortical cells and then ramifies within the cortex. Cells in advance of the thread divide and organize themselves into a nodule primordium.

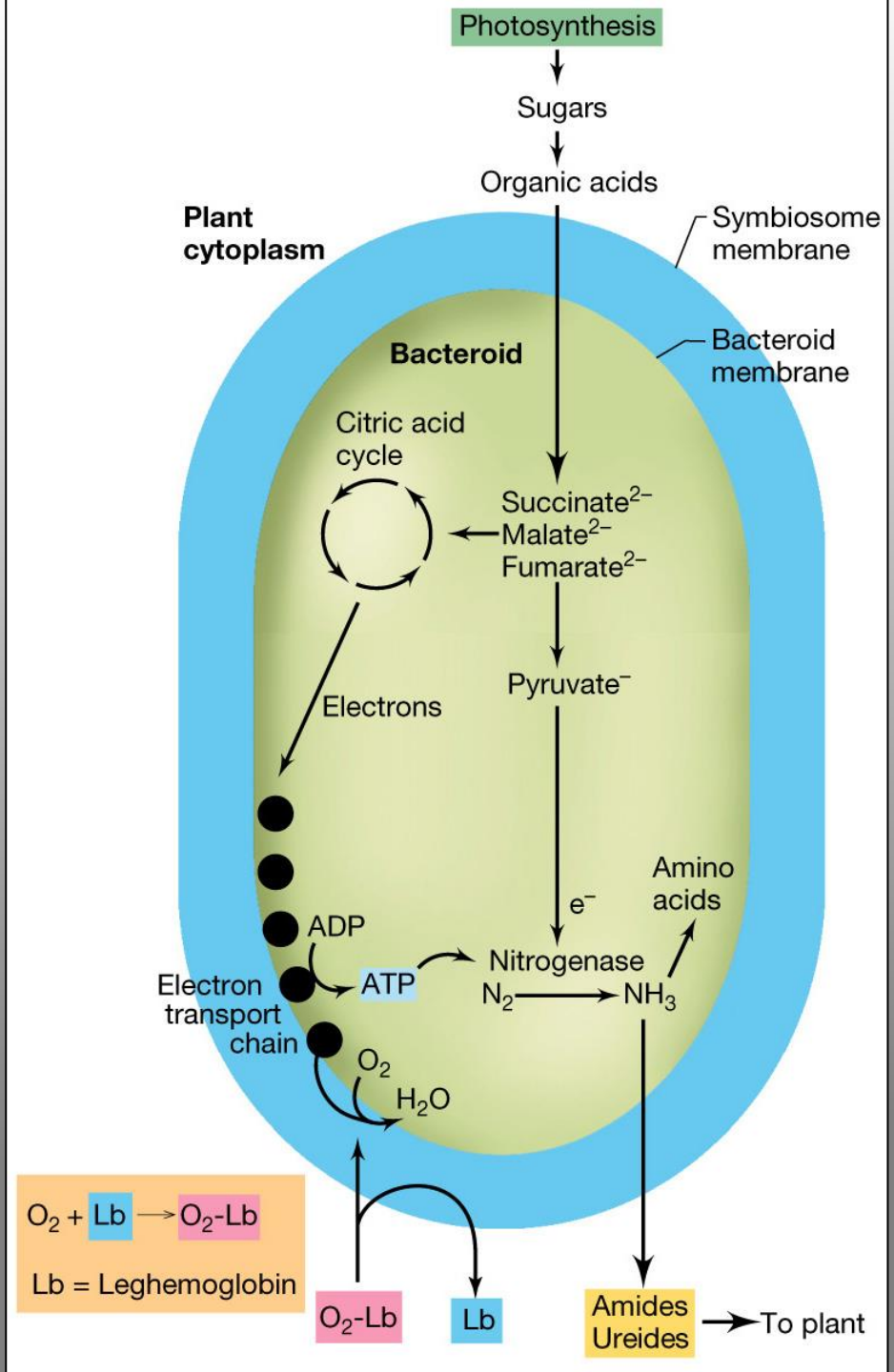
6. The branched infection thread enters the nodule primordium zone and penetrates individual primordium cells.

7. Bacteria are released from the infection thread into the cytoplasm of the host cells, but remain surrounded by the **peribacteroid membrane**. Failure to form the PBM results in the activation of host defenses and/or the formation of ineffective nodules.

8. Infected root cells swell and cease dividing. Bacteria within the swollen cells change form to become endosymbiotic **bacteroids,** which begin to fix nitrogen.

The nodule provides an **oxygen-controlled** environment (leghemoglobin = pink nodule interior) structured to facilitate transport of reduced nitrogen metabolites from the bacteroids to the plant vascular system, and of photosynthate from the host plant to the bacteroids.


A TP
ADP
+ Pi
transporters
H +
Fe2+
Fe2+
Fe3+
NO3-, Cl-
NH 3
N2
ammonium
amino acids
PBS
bacteroid
peribacteroid membrane
cytosol
malate


Photosynthesis
Sugars
Organic acids
Plant cytoplasm
Symbiosome membrane
Bacteroid
Bacteroid membrane
Citric acid cycle
Succinate2–
Malate2–
Fumarate2–
Pyruvate–
Electrons
e–
Amino acids
ADP
ATP
Nitrogenase
N2
NH3
Electron transport chain
O2
H2O
O2 + Lb → O2-Lb
Lb = Leghemoglobin
O2-Lb
Lb
Amides
Ureides
To plant

Types of bacterial functions involved in nodulation and nitrogen fixation

nod (nodulation) and nol (nod locus) genes

mutations in these genes block nodule formation or alter host range

most have been identified by transposon mutagenesis, DNA sequencing and protein analysis, in *R. meliloti*, *R. leguminosarum* bv *viciae* and *trifolii*

fall into four classes:

- nodD
- nodA, B and C (common nodgenes)
- hsn (host-specific nod genes)
- other nod genes

Gene clusters on *R. meliloti* pSym plasmid

(nol) (nod) (nif) (fix)

F G H I N D_1 A B C I J Q P G E F H D_3 E K D H A B C

N M L R E F D A B C I J T C B A H D K E N

Gene clusters on *R. leguminosarum* bv *trifolii* pSym plasmid

--- D_2 D_1 Y A B C S U I J ---

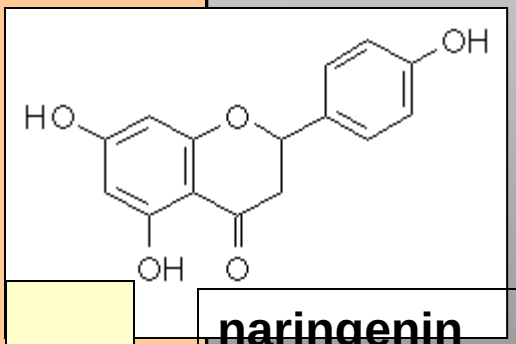
Gene cluster on *Bradyrhizobium japonicum* chromosome

Nod D (the sensor)

the **nod D** gene product recognizes molecules (phenylpropanoid-derived **flavonoids**) produced by plant roots and becomes activated as a result of that binding

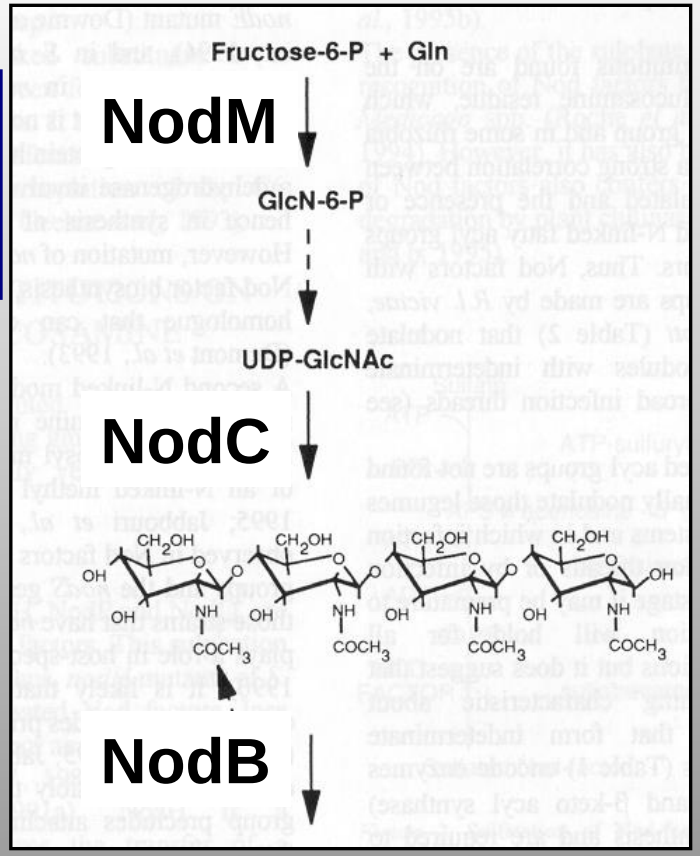
activated nodD protein positively controls the expression of the other genes in the nod gene "regulon" (signal transduction)

different nodD alleles recognize **various flavonoid** structures with different affinities, and respond with differential patterns of nod gene activation

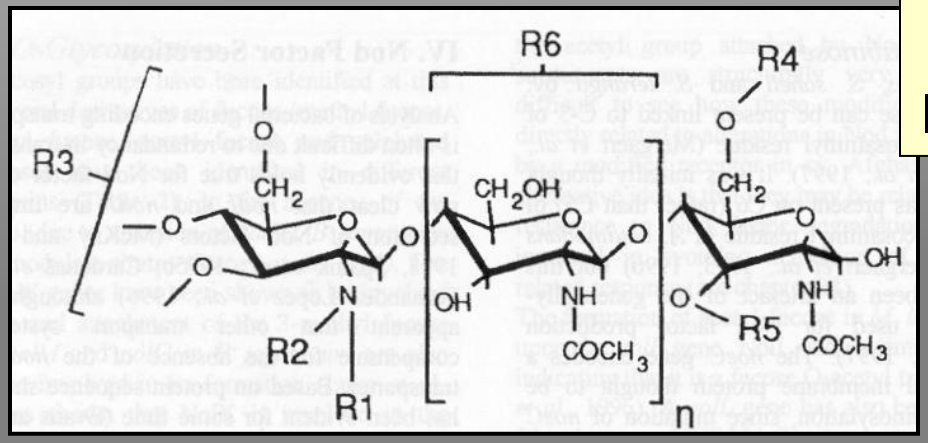


naringenin
(**a flavanone**)

Nod factor biosynthesis



Nod factor R-group "decorations" determine host specificity



Nod Factor: a lipooligosaccharide

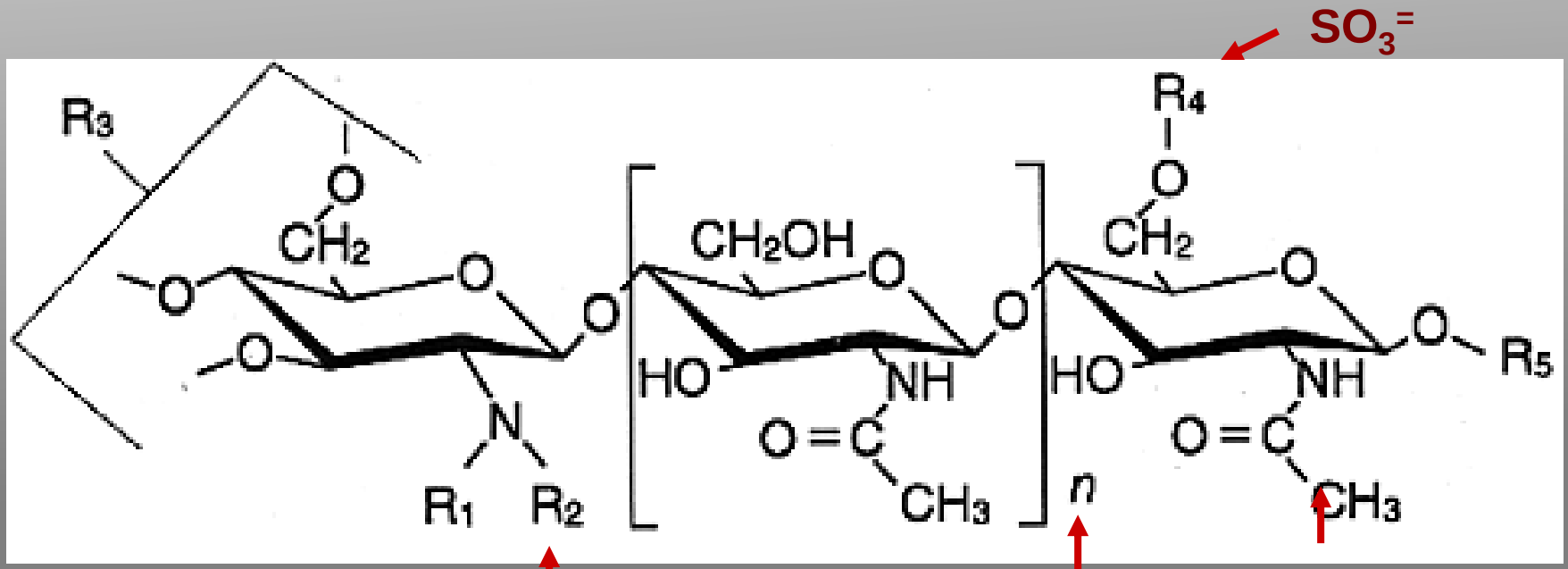
Common nod genes - nod ABC

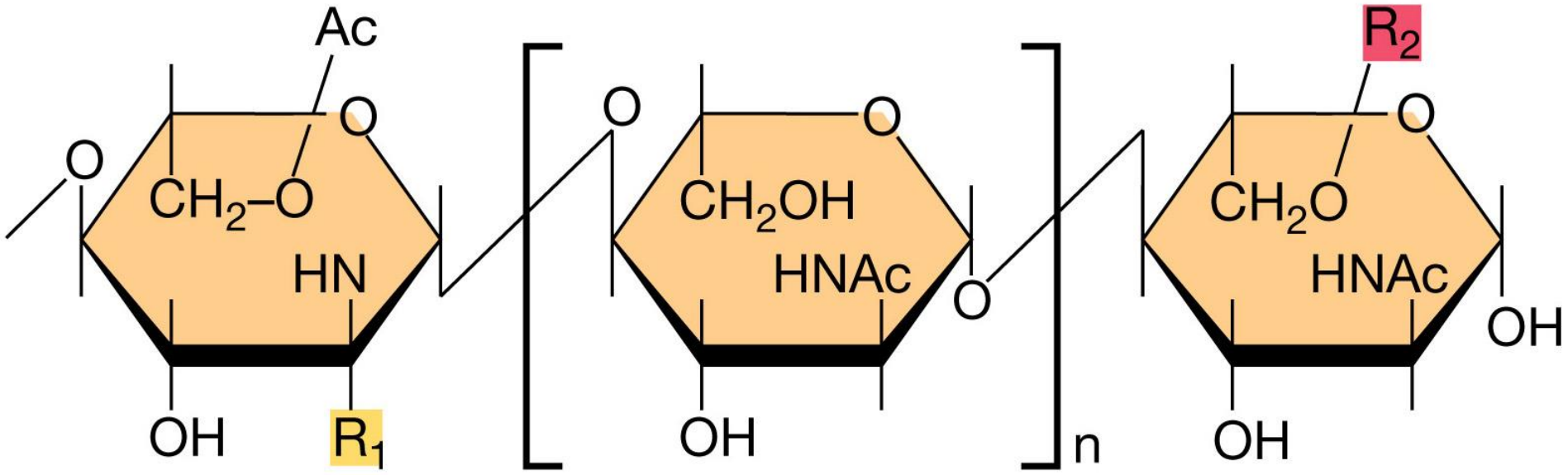
mutations in nodA,B or C completely abolish the ability of the bacteria to nodulate the host plant; they are found as part of the nod gene "regulon" in all Rhizobia (∴ common)

products of these genes **are required** for bacterial induction of root cell hair deformation and root cortical cell division

The **nod ABC** gene products are enzymes responsible for synthesis of diffusible nod factors, whcih are sulfated and acylated *beta*-1,4-oligosaccharides of glucosamine

(other gene products, e.g. NodH, may also be needed for special modifications)





(a)

Species	R_1	R_2
Sinorhizobium meliloti	C16 : 2 or C16 : 3	SO_4^{2-}
Rhizobium leguminosarum biovar *viciae*	C18 : 1 or C18 : 4	H or Ac

(b)

nod factors are active on host plants at very low concentrations (10^{-8} to 10^{-11} M) but have no effect on non-host species

Host-specific nod genes

mutations in these genes elicit abnormal root reactions on their usual hosts, and sometimes elicit root hair deformation reactions on plants that are not usually hosts

Example:
loss of nodH function in *R. meliloti* results in synthesis of a nod factor that is no longer effective on alfalfa but has gained activity on vetch

The ΔnodH nod factor is now more hydrophobic than the normal factor - no sulfate group on the oligosaccharide.

The role of the nodH gene product is therefore to add a specific sulfate group, and thereby change host specificity

Other nod genes

May be involved in the attachment of the bacteria to the plant surface, or in export of signal molecules, or proteins needed for a successful symbiotic relationship

exo (exopolysaccharide) genes

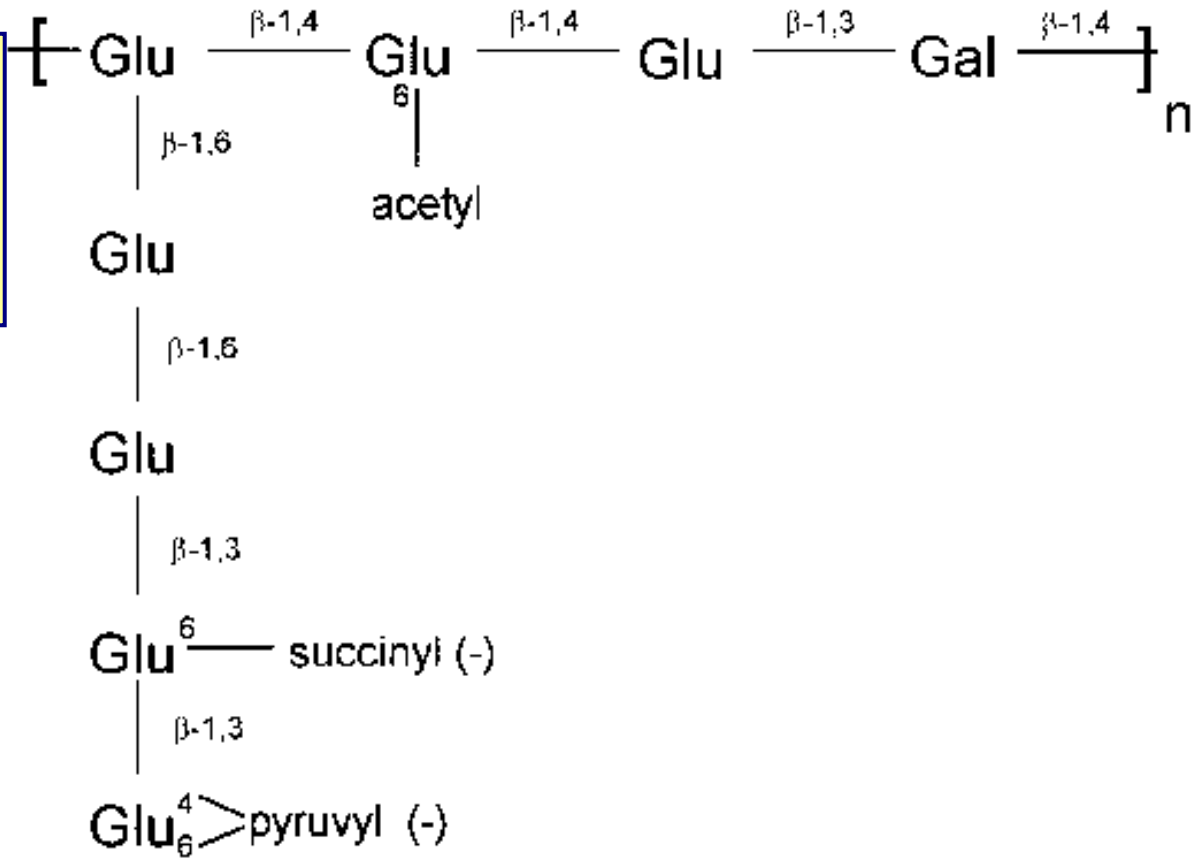
Encode proteins needed for exopolysaccharide synthesis and secretion

In *Rhizobium*-legume interactions that lead to indeterminate nodules, exo mutants cannot invade the plant properly. However, they do provoke the typical plant cell division pattern and root deformation, and can even lead to nodule formation, although these are often empty (no bacteroids).
In interactions that usually produce **determinate** nodules, exo mutations tend to have no effect on the process.

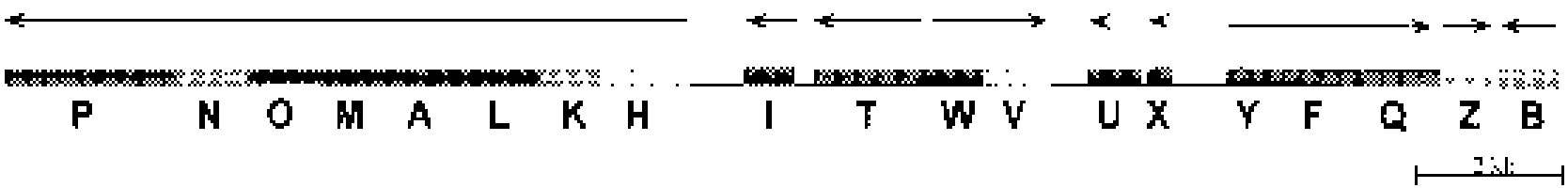
Exopolysaccharides may provide substrate for signal production, osmotic matrix needed during invasion, and/or a recognition or masking function during invasion

example of Rhizobial exopolysaccharide

Succinoglycan



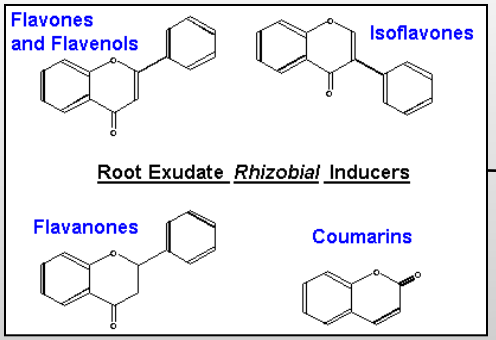
Map of the *exo* Gene Cluster



Functions of the *exo* gene products

- addition of first galactose to lipid carrier
- glucosyltransferase
- octamer modification
- nucleotide sugar biosynthesis
- polymerization or transport
- glycanase
- putative regulatory protein
- function unknown

Sinorhizobium meliloti



nod-gene inducers from alfalfa roots (specificity)

NodD

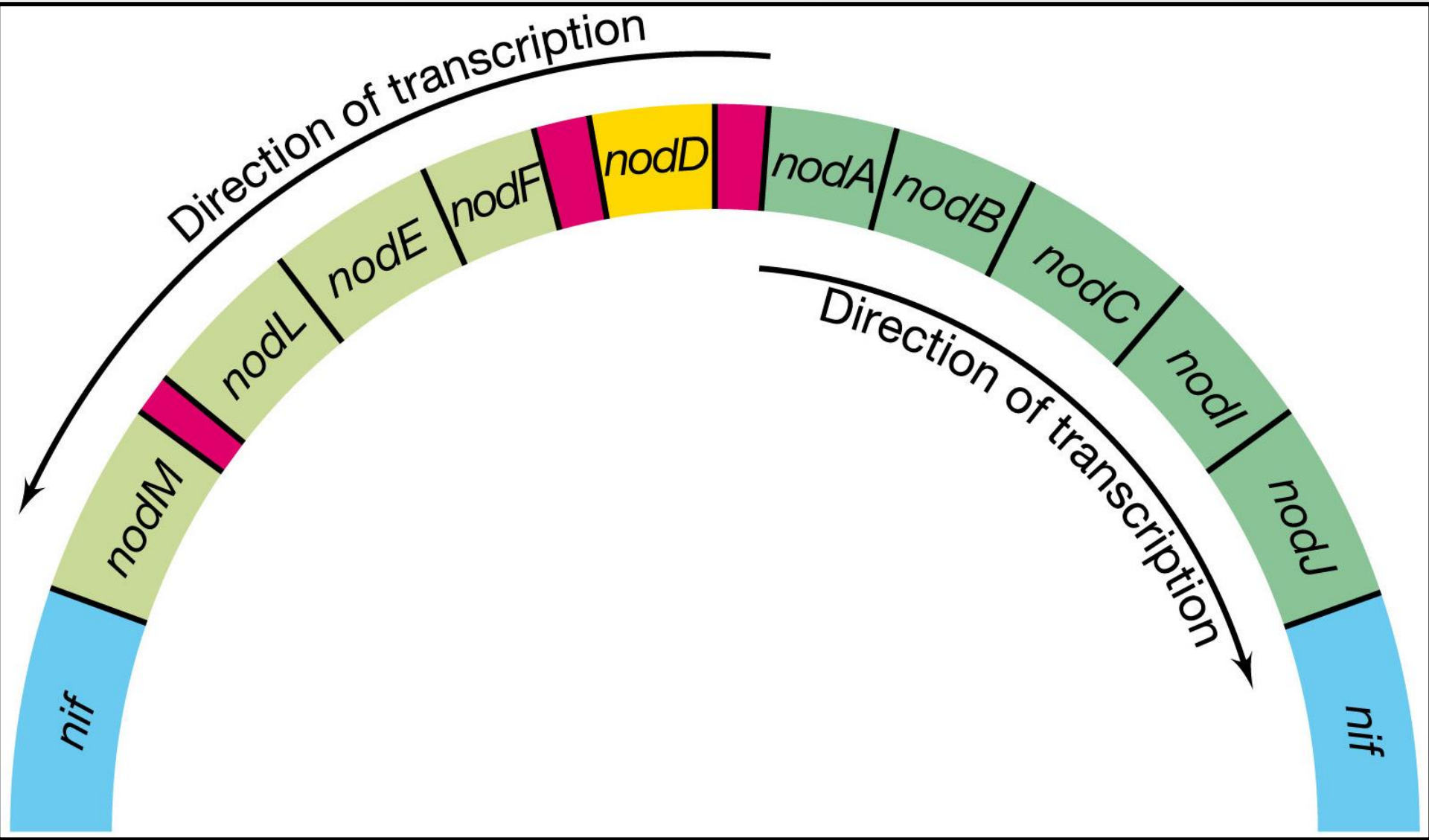
chromosome

plasmid

pSym

activated NodD positively regulates nod genes

nod genes


Direction of transcription
nodM
nodL
nodE
nodF
nodD
nodA
nodB
nodC
nodI
nodJ
nif
nif
Direction of transcription

nif (nitrogen fixation) genes

Gene products are required for symbiotic nitrogen fixation, and for nitrogen fixation in free-living N-fixing species

Example: subunits of nitrogenase

Table 1. The *nif-gene* Products and Their Role (Known or Proposed) in Nitrogen Fixation

nif-GENE	IDENTITY/ROLE
nifH	Dinitrogenase reductase. Obligate electron donor to dinitrogenase during nitrogenase turnover. Also is required for FeMo-co biosynthesis and apodinitrogenase maturation
nifD	α subunit of dinitrogenase. Forms an $a_2ß_2$ tetramer with ß subunit. FeMo-co, the site of substrate reduction, is present buried within the α subunit of dinitrogenase
nifK	ß subunit of dinitrogenase. P-clusters are present at the ß subunit-interface
nifT	Unknown
nifY	In *K. pneumoniae,* aids in the insertion of FeMo-co into apodinitrogenase
nifE	Forms $a_2ß_2$ tetramer with NifN. Required for FeMo-co synthesis. Proposed to function as a scaffold on which FeMo-co is synthesized
nifN	Required for FeMo-co synthesis
nifX	Involved in FeMo-co synthesis. Specific role is not known
nifU	Involved in mobilization of Fe for Fe-S cluster synthesis and repair
nifS	Involved in mobilization of S for Fe-S cluster synthesis and repair
nifV	Homocitrate synthase, involved in FeMo-co synthesis
nifW	Involved in stability of dinitrogenase. Proposed to protect dinitrogenase from 0_2 inactivation
nifZ	Unknown
nifM	Required for the maturation of NifH
nifF	Flavodoxin. Physiologic electron donor to NifH
nifL	Negative regulatory element
nifA	Positive regulatory element
nifB	Required for FeMo-co synthesis. Metabolic product, NifB-co is the specific Fe and S donor to FeMo-co
fdxN	Ferredoxin. In *R. capsulatus,* serves as electron donor to nitrogenase
nifQ	Involved in FeMo-co synthesis. Proposed to function in early MoO_4^{2-} processing
nifJ	Pyruvate:flavodoxin (ferredoxin) oxidoreductase. Involved in electron transport to nitrogenase

fix (fixation) genes

Gene products required to successfully establish a functional N-fixing nodule.
No **fix** homologues have been identified in free-living N-fixing bacteria.

Example: regulatory proteins that monitor and control oxygen levels within the bacteroids

FixL senses the oxygen level; at low oxygen tensions, it acts as a **kinase** on **FixJ**, which regulates expression of two more transcriptional regulators:

NifA, the upstream activator of nif and some fix genes;

FixK, the regulator of *fixN* (another oxgen sensor?)

This key transducing protein, FixL, is a novel hemoprotein kinase with a complex structure. It has an N-terminal membrane-anchoring domain, followed by the heme binding section, and a C-terminal kinase catalytic domain.

Result?

Low oxygen tension activates nif gene transcription and permits the oxygen-sensitive nitrogenase to function.

Metabolic genes and transporters

Dicarboxylic acid (malate) transport and metabolism

Genes for other functions yet to be identified….

⇒ DNA microarray analysis of gene expression patterns

⇒ Proteomic analysis of bacteroids and peribacteroid membrane preparations

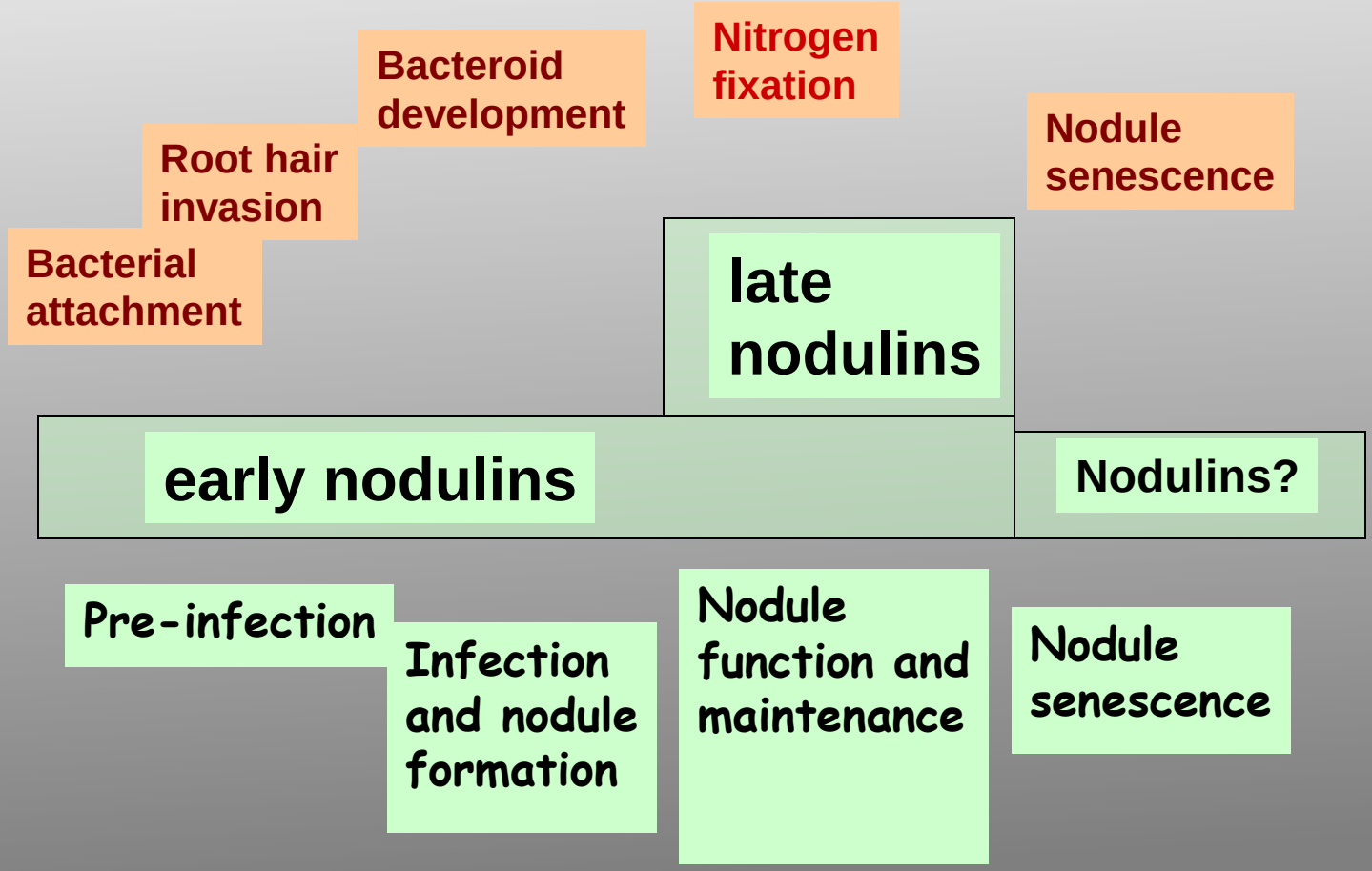
Host plant role in nodulation

1. Production and release of nod gene inducers
   - **flavonoids**

2. Activation of plant genes specifically required for successful nodule formation - **nodulins**

3. Suppression of genes normally involved in repelling microbial invaders - **host defense genes**

Nodulins


Bacterial attachment
Root hair invasion
Bacteroid development
Nitrogen fixation
Nodule senescence
late nodulins
early nodulins
Nodulins?
Pre-infection
Infection and nodule formation
Nodule function and maintenance
Nodule senescence

Early nodulins

At least 20 nodule-specific or nodule-enhanced genes are expressed in plant roots during nodule formation; most of these appear after the initiation of the visible nodule.

Five different nodulins are expressed only in cells containing growing infection threads.
These may encode proteins that are part of the plasmalemma surrounding the infection thread, or enzymes needed to make or modify other molecules

Twelve nodulins are expressed in root hairs and in cortical cells that contain growing infection threads. They are also expressed in host cells a few layers ahead of the growing infection thread.

Late nodulins

The best studied and most abundant late nodulin is the protein component of **leghemoglobin**.
The **heme** component of leghemoglobin appears to be synthesized by the bacteroids.

Other **late nodulins** are enzymes or subunits of enzymes that function in nitrogen metabolism (glutamine synthetase; uricase) or carbon metabolism (sucrose synthase). Others are associated with the peribacteroid membrane, and probably are involved in transport functions.

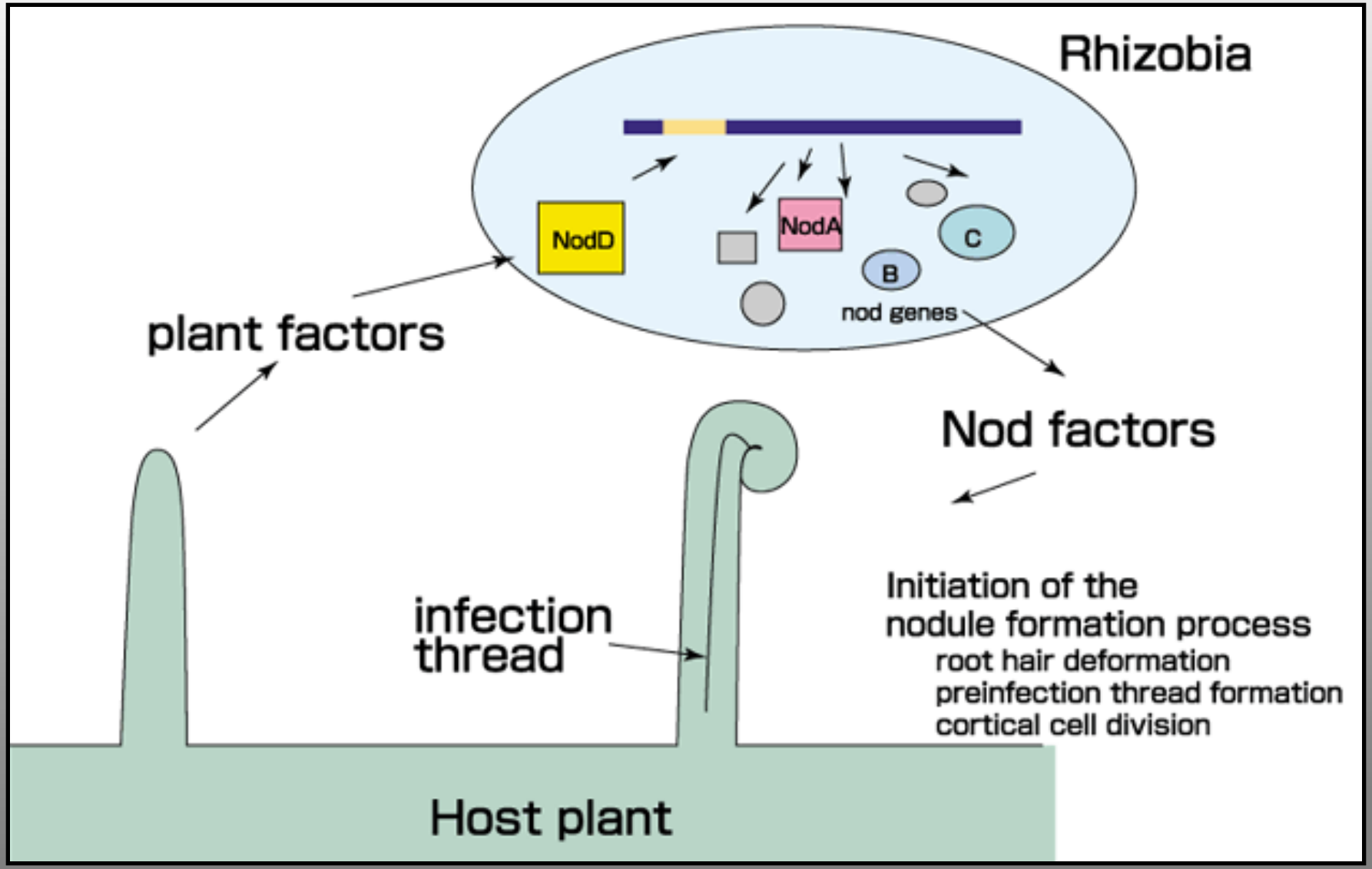
These late nodulin gene products are usually not unique to nodule function, but are found in other parts of the plant as well. This is consistent with the hypothesis that nodule formation evolved as a specialized form of root differentiation.

There must be **many other host gene functions** that are needed for successful nodule formation.

Example: what is the **receptor** for the nod factor?

These are being sought through genomic and proteomic analyses, and through generation of plant mutants that fail to nodulate properly

The full genome sequencing of *Medicago truncatula* and *Lotus japonicus* , both currently underway, will greatly speed up this discovery process.


Rhizobia
NodD
NodA
C
B
nod genes
plant factors
Nod factors
infection thread
Initiation of the nodule formation process
root hair deformation
preinfection thread formation
cortical cell division
Host plant

A plant receptor-like kinase required for both bacterial and fungal symbiosis

S. Stracke *et al* Nature 417:959 (2002)

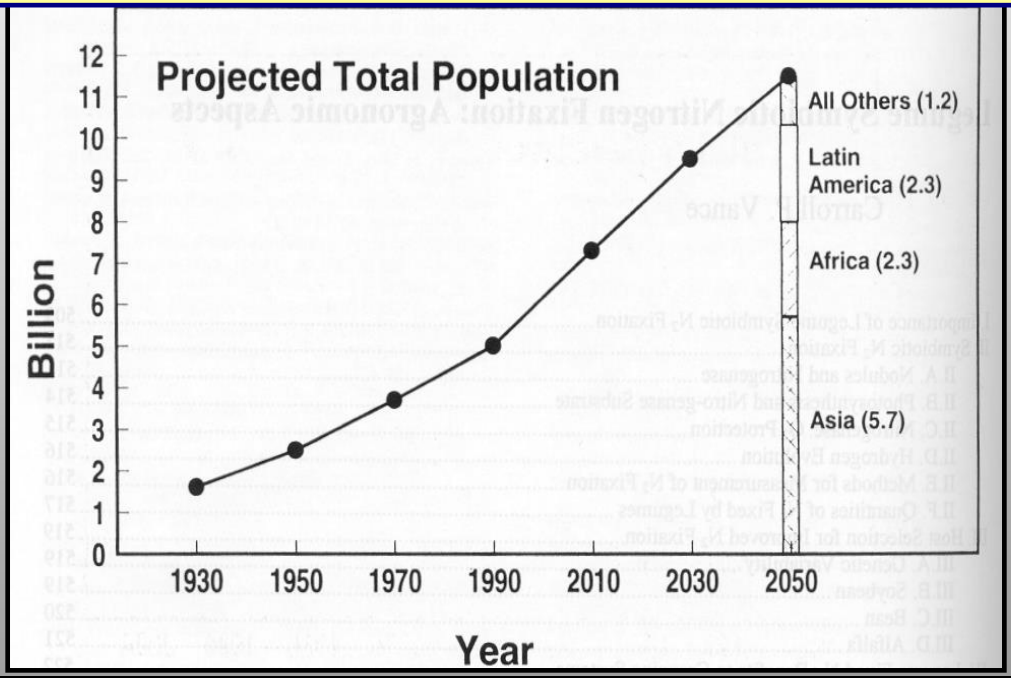
Screened mutagenized populations of the legume *Lotus japonicus* for mutants that showed an inability to be colonized by VAM

Mutants found to also be affected in their ability to be colonized by nitrogen-fixing bacteria ("symbiotic mutants")

Stem-nodulating bacteria

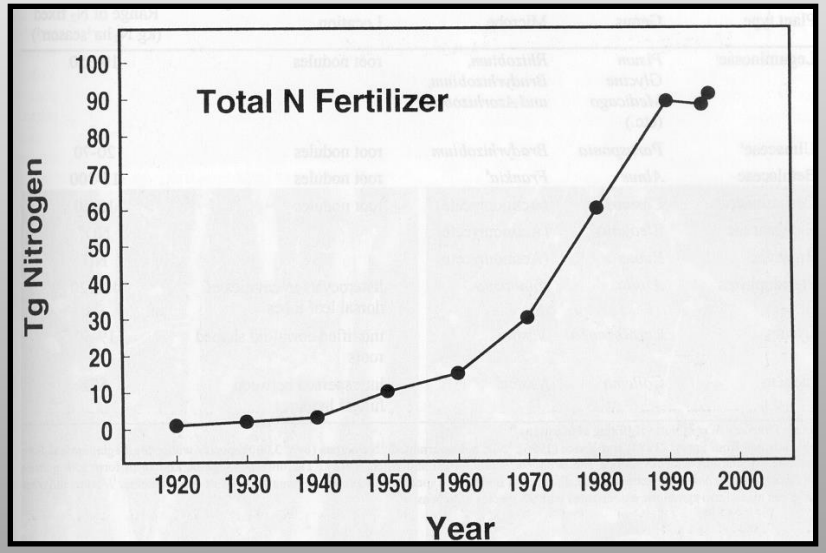
- observed primarily with tropical legumes

A growing population must eat!



- **Combined nitrogen is the most common limiting nutrient in agriculture**
- **Estimated that 90% of population will live in tropical and subtropical areas where (protein-rich) plant sources contribute 80% of total caloric intake.**
- **In 1910 humans consumed 10% of total carbon fixed by photosynthesis, by 2030 it is predicted that 80% will be used by humans.**

Why chemical fertilizers aren't the answer



The Haber-Bosch process

$N_2 + H_2 \longrightarrow 2NH_3$

300 to 1000 bar pressure

400 to 600 C

Catalyst

Consumes 1.4% of total fossil fuels annually

Electrical discharge

$N_2 + O_2 + \text{lightning} \longrightarrow NO_x$

$NO_x + H_2O \longrightarrow HNO_2 \text{ or } HNO_3$

- **Production of nitrogenous fertilizers has "plateaued" in recent years because of high costs and pollution**

- **Estimated 90% of applied fertilizers never reach roots and contaminate groundwater**

Current approaches to improving biological nitrogen fixation

1. **Enhancing survival of nodule forming bacterium by improving competitiveness of inoculant strains**

2. **Extend host range of crops, which can benefit from biological nitrogen fixation**

3. **Engineer microbes with high nitrogen fixing capacity**

What experiments would you propose if you were to follow each of these approaches?

Biological Nitrogen Fixation

Conversion of dinitrogen gas (N_2) to ammonia (NH_3)

Availability of fixed N often factor most limiting to plant growth

N-fixation ability limited to few bacteria, either as free-living organisms or in symbiosis with higher plants

First attempt to increase forest growth through N-fixation in Lithuania, 1894 (lupines in Scots pine)

Biological nitrogen fixation:

$$N_2 + 8\ \text{flavodoxin}^- + 8H^+ + 16\ MgATP^{2-} + 18\ H_2O \xrightarrow{\text{nitrogenase}}$$

$$2NH_4^+ + 2OH^- + 8\ \text{flavodoxin} + 16\ MgADP^- + 16H_2PO_4^- + H_2$$

1. Rare, extremely energy consuming conversion because of stability of triply bonded N_2
2. Produces fixed N which can be directly assimilated into N containing biomolecules

Ecology of nitrogen-fixing bacteria

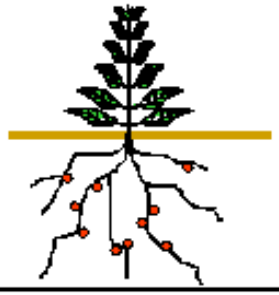
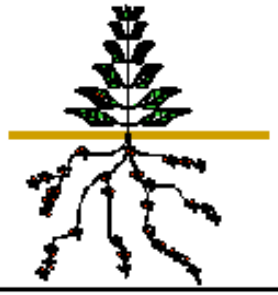
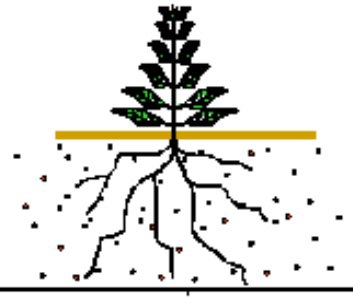
System of N_2 fixation (and microbes involved) ($N_2 \rightarrow NH_3$)	SYMBIOSIS (e.g. *Rhizobium*)	ASSOCIATION (e.g. *Azospirillum*)	FREE-LIVING (e.g. *Rhodospiorillum*)	
				
Energy source (Organic C)	Sucrose from the host plant	Root exudates from the host plant	Heterotroph (plant residues)	Autotroph (photo-synthesis)
Estimates of fixation rate (kg N/ha/y)	50-400	10-200	1-2	10-80

Tableau I. **Une sélection de quelques bactéries fixatrices d'azote.**

Groupes phylogéniques, nombre de fixateurs caractérisés, exemples	Métabolisme énergétique, tension d'oxygène compatible avec la fixation de l'azote, interaction avec les plantes
Bactéries vertes sulfureuses	
4 genres, 6 espèces	
Chlorobium limicola	PAT Anaérobiose
Firmibactéries (Gram+)	
3 genres, 22 espèces	
Bacillus polymixa	CHT Microaérobiose
Clostridium acetobutylicum	CHT Anaérobiose
Clostridium pasteurianum	CHT Anaérobiose
Thallobactéries (Gram+)	
4 genres, x espèces	
Arthrobacter sp	CHT Microaérobiose
Frankia	CHT Microaérobiose. Symbiote actinorhizien (pe aulne, casuarina)
Héliobactéries	
3 genres, 3 espèces	
Heliobacterium chlorum	PHT Anaérobiose
Heliospirillum gestii	PHT Anaérobiose
Cyanobactéries	
14 genres, x espèces	
Anabaena 7120	PAT Aérobiose
Anabaena azollae	PAT Aérobiose. Symbiote de la fougère *Azolla*
Nostoc 73102	PAT Aérobiose
Gloeothece 6501	PAT Microaérobiose
Campylobactéries	
1 genre, 1 espèce	
Protéobactéries α	
20 genres, 54 espèces	
Acetobacter diazotrophicus	CHT Microaérobiose. Endophyte de la canne à sucre
Azorhizobium caulinodans	CHT Microaérobiose. Symbiote de *Sesbania rostrata*
Azospirillum brasilense	CHT Microaérobiose. Associé aux racines des Graminées
Bradyrhizobium japonicum	CHT Microaérobiose. Symbiote du soja
Rhizobium leguminosarum	CHT Microaérobiose. Symbiote du pois
Rhizobium meliloti	CHT Microaérobiose. Symbiote de la luzerne
Rhodobacter capsulatus	PHT Anaérobiose
Rhodospirillum rubrum	PHT Anaérobiose
Protéobactéries β	
7 genres, 11 espèces	
Alcaligenes faecalis	CHT Microaérobiose. Associé aux racines du riz
Azoarcus spp	CHT Microaérobiose. Endophyte de l'herbe de Kallar (*Leptochloa fusca*)
Derxia gummosa	CHT Microaérobiose
Herbaspirillum seropedicae	CHT Microaérobiose. Endophyte de la canne à sucre
Thiobacillus ferrooxidans	CAT Microaérobiose
Protéobactéries γ	
18 genres, 44 espèces	
Azotobacter vinelandii	CHT Aérobiose
Beggiatoa alba	CAT Microaérobiose
Enterobacter agglomerans	CHT Anaérobiose
Klebsiella pneumoniae	CHT Anaérobiose
Pseudomonas stutzeri	CHT Microaérobiose
Protéobactéries δ	
2 genres, 10 espèces	
Desulfovibrio gigas	CHT Anaérobiose
Archaebactéries	
4 genres, 7 espèces	
Methanobacterium ivanovii	CAT Anaérobiose
Methanococcus thermolithotrophicus	CAT Anaérobiose

CAT : chimioautotrophe ; CHT : chimiohétérotrophe ; PAT : photoautotrophe ; PHT : photohétérotrophe.

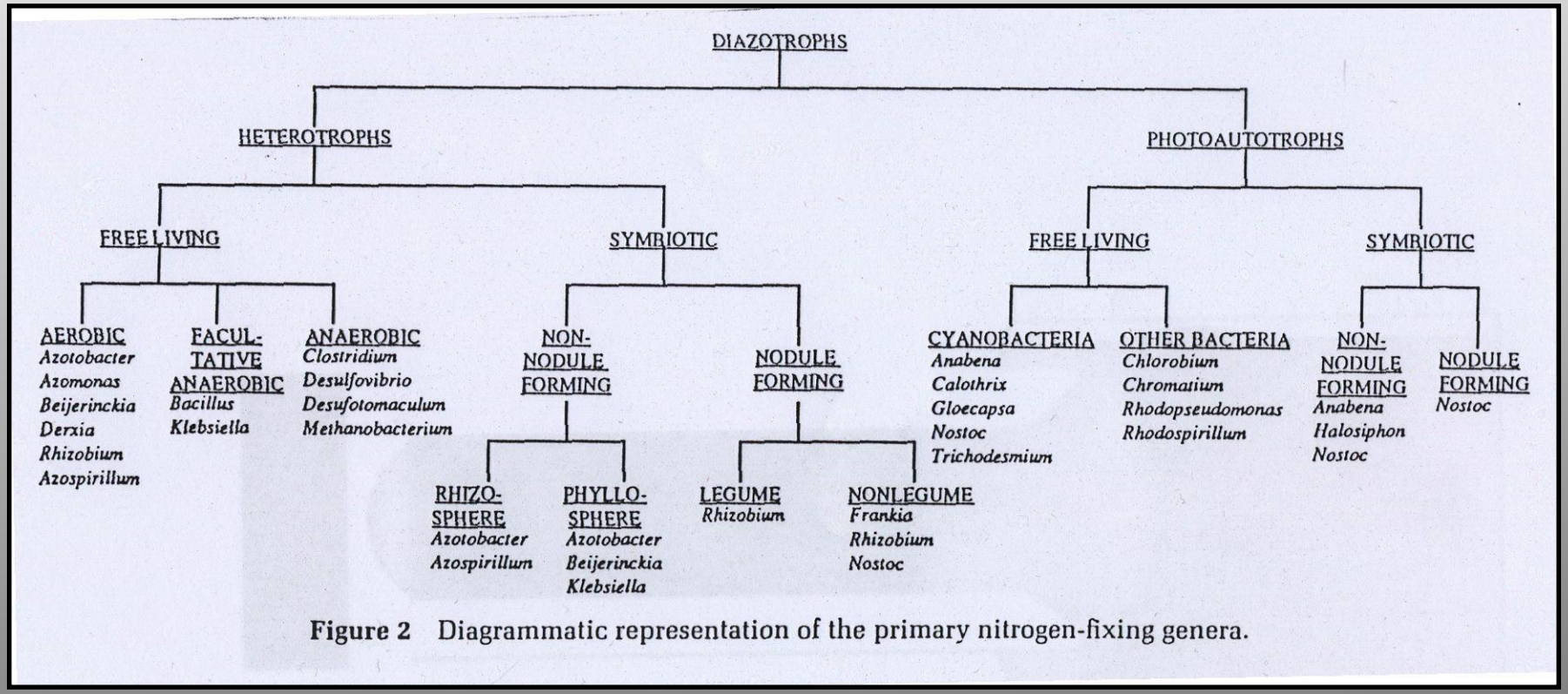


Figure 2 Diagrammatic representation of the primary nitrogen-fixing genera.

N-fixation requires energy input:

- Reduction reaction, e^- must be added (sensitive to O_2)
- Requires ~35 kJ of energy per mol of N fixed (theoretically)
- Actual cost: ~15-30g CH per g of NH_3 produced
- Assimilation of NH_3 into organic form takes 3.1-3.6 g CH

Enzymology of N fixation

Only occurs in certain prokaryotes

- **Rhizobia fix nitrogen in symbiotic association with leguminous plants**
- **Rhizobia fix N for the plant and plant provides Rhizobia with carbon substrates**
- **All nitrogen fixing systems appear to be identical**
- **They require nitrogenase, a reductant (reduced ferredoxin), ATP, O-free conditions and regulatory controls (ADP inhibits and NH_4^+ inhibits expression of nif genes**

Biological nitrogen fixation is the reduction of atmospheric nitrogen gas (N_2) to ammonium ions (NH_4^+) by the oxygen-sensitive enzyme, **nitrogenase**.
Reducing power is provided by NAPH/ferredoxin, via an Fe/Mocentre.

Plant genomes lack any genes encoding this enzyme, which occurs only in prokaryotes (bacteria).

Even within the bacteria, only certain free-living bacteria (Klebsiella, Azospirillum, Azotobacter), blue-green bacteria (Anabaena) and a few symbiotic Rhizobial species are known nitrogen-fixers.

Another nitrogen-fixing association exists between an Actinomycete (Frankia spp.) and alder (Alnus spp.)

The enzyme **nitrogenase** catalyses the conversion of atmospheric, gaseous dinitrogen (N_2) and dihydrogen (H_2) to ammonia (NH_3), as shown in the chemical equation below:

$$\mathbf{N_2 + 3\ H_2 \Rightarrow\ 2\ NH_3}$$

The above reaction seems simple enough and the atmosphere is 78% N_2, so why is this enzyme so important?

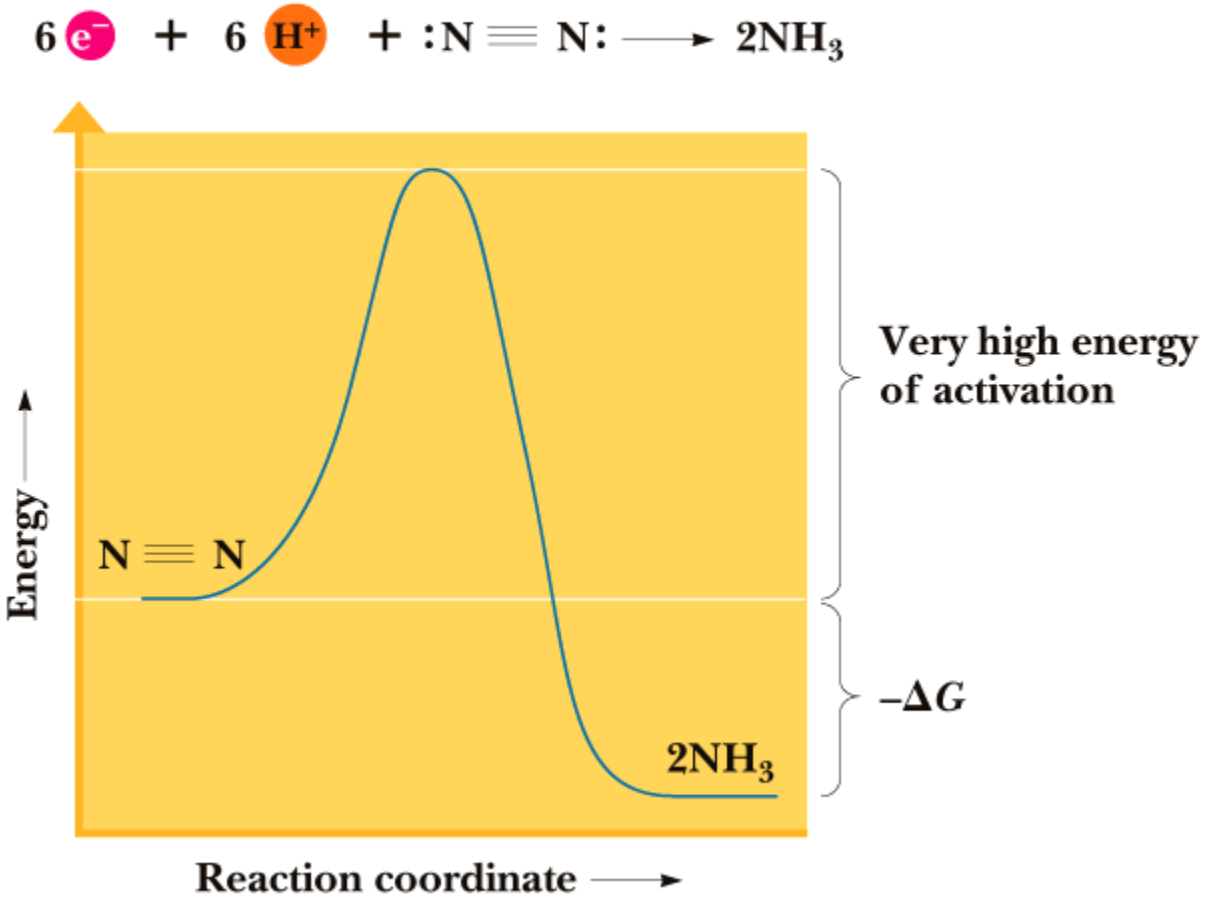
The incredibly strong (triple) bond in N_2 makes this reaction very difficult to carry out efficiently.
In fact, nitrogenase consumes ~16 moles of ATP for every molecule of N_2 it reduces to NH_3, which makes it one of the most energy-expensive processes known in Nature.

Nitrogenase Complex

Two protein components: nitrogenase reductase and nitrogenase

- Nitrogenase reductase is a 60 kD homodimer with a single 4Fe-4S cluster
- Very oxygen-sensitive
- Binds MgATP
- 4ATP required per pair of electrons transferred
- Reduction of N_2 to $2NH_3 + H_2$ requires 4 pairs of electrons, so **16 ATP are consumed per N_2**

Garrett & Grisham: Biochemistry, 2/e
Figure 26.4



Saunders College Publishing

Why should nitrogenase need ATP???

- N_2 reduction to ammonia is thermodynamically favorable
- However, the activation barrier for breaking the N-N triple bond is enormous
- **16 ATP** provide the needed activation energy

Nitrogenase

A 220 kD heterotetramer

- Each molecule of enzyme contains 2 Mo, 32 Fe, 30 equivalents of acid-labile sulfide (FeS clusters, etc)
- Four 4Fe-4S clusters plus two FeMoCo, an iron-molybdenum cofactor
- Nitrogenase is **slow - 12 e^- pairs per second, i.e., only three molecules of N_2 per second**

Genetic Clusters

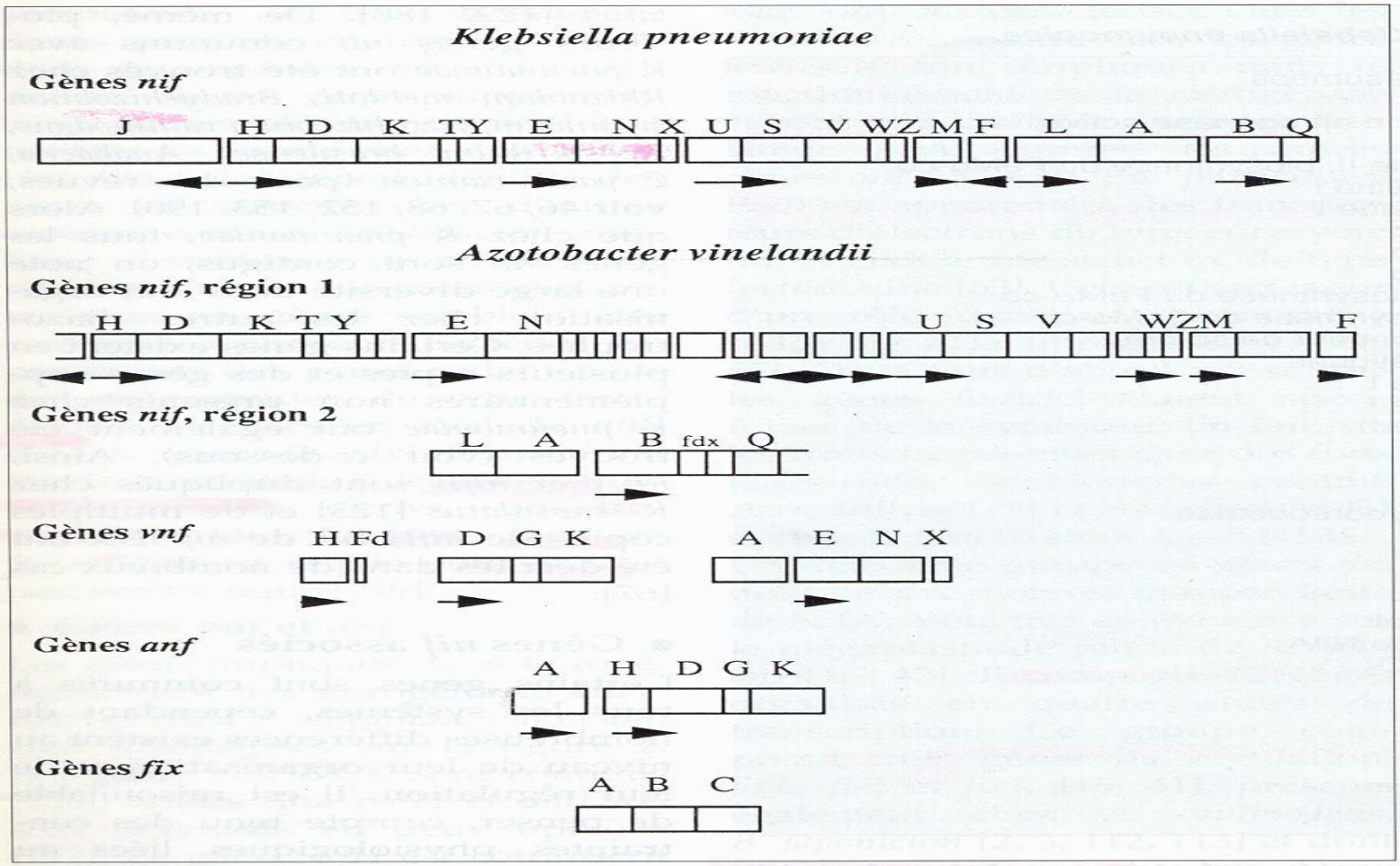
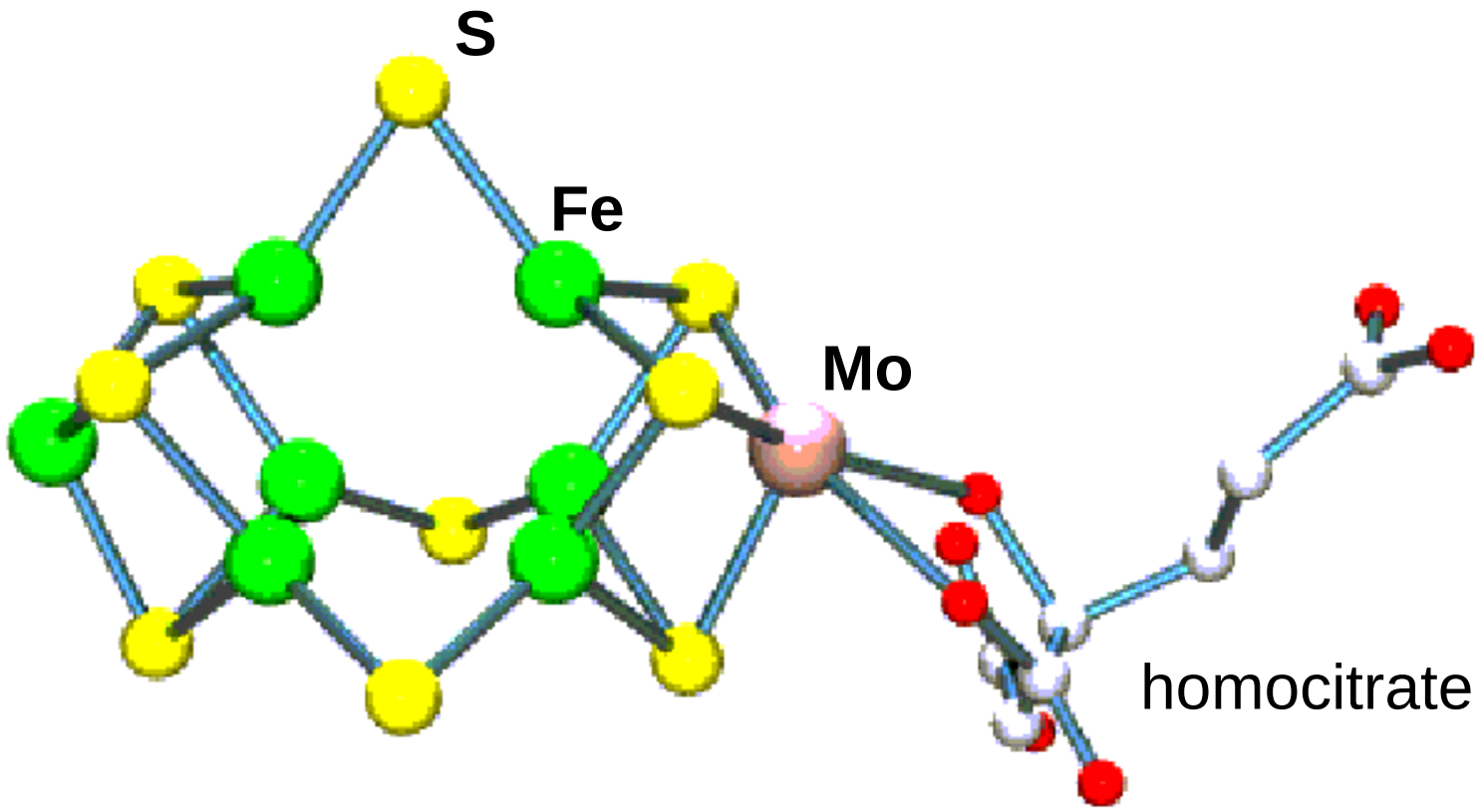


Fig 2. Organisation des gènes de la fixation de l'azote de Klebsiella pneumoniae *et d'*Azotobacter vinelandii. *Les gènes contigus correspondent à des opérons polycistroniques. Les flèches indiquent le sens de transcription à partir de promoteurs dépendant du facteur* σ^{54}.

The genes and products

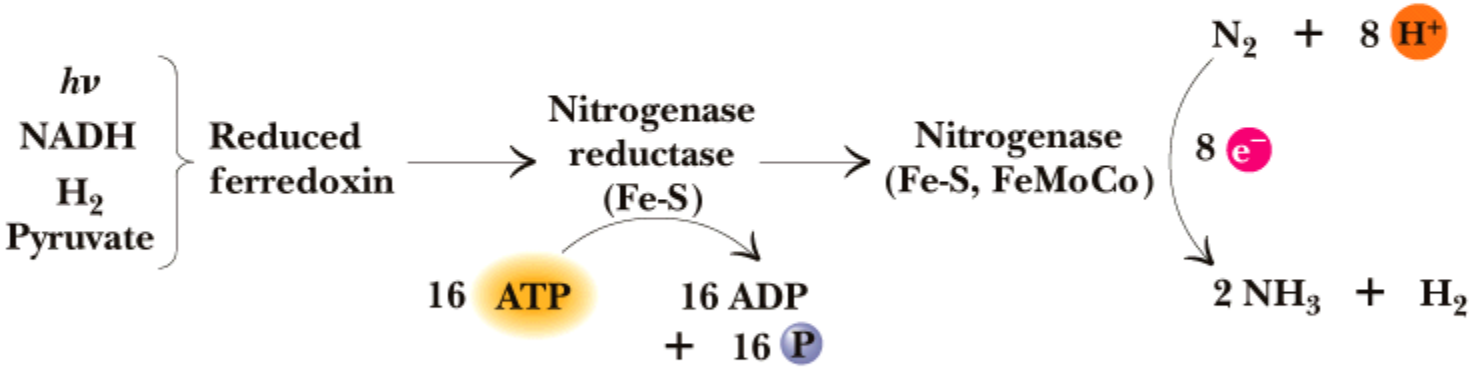
Tableau II. **Fonction des gènes *nif* de *Klebsiella pneumoniae*.**

Gènes	Fonctions établies ou présumées
1. Gènes impliqués dans la synthèse d'une nitrogénase active	
nifH	Polypeptide de la protéine II, biosynthèse du FeMo-co
nifD	Polypeptide α de la protéine I
nifK	Polypeptide β de la protéine I
nifE	Biosynthèse du FeMo-co
nifN	Biosynthèse du FeMo-co
nifB	Biosynthèse du FeMo-co
nifV	Homocitrate synthase, biosynthèse du FeMo-co
nifQ	Métabolisme du Mo, biosynthèse du FeMo-co
nifS	Cystéine désulfurase, donneur de soufre pour les groupes prosthétiques
nifW	Maturation de la protéine I
nifZ	Maturation de la protéine I
nifM	Maturation de la protéine II
2. Transport des électrons	
nifJ	Pyruvate-flavodoxine oxydoréductase
nifF	Flavodoxine
3. Régulation	
nifA	Activateur transcriptionnel
nifL	Modulateur de l'activité de NifA en présence de NH_3 ou O_2
4. Gènes non essentiels	
nifT	Inconnu
nifY	Inconnu
nifX	Inconnu
nifU	inconnu



Fe - S - Mo electron transfer cofactor in nitrogenase

Garrett & Grisham: Biochemistry, 2/e
Figure 26.6



Three Types of N-fixers Important in Forest Soils

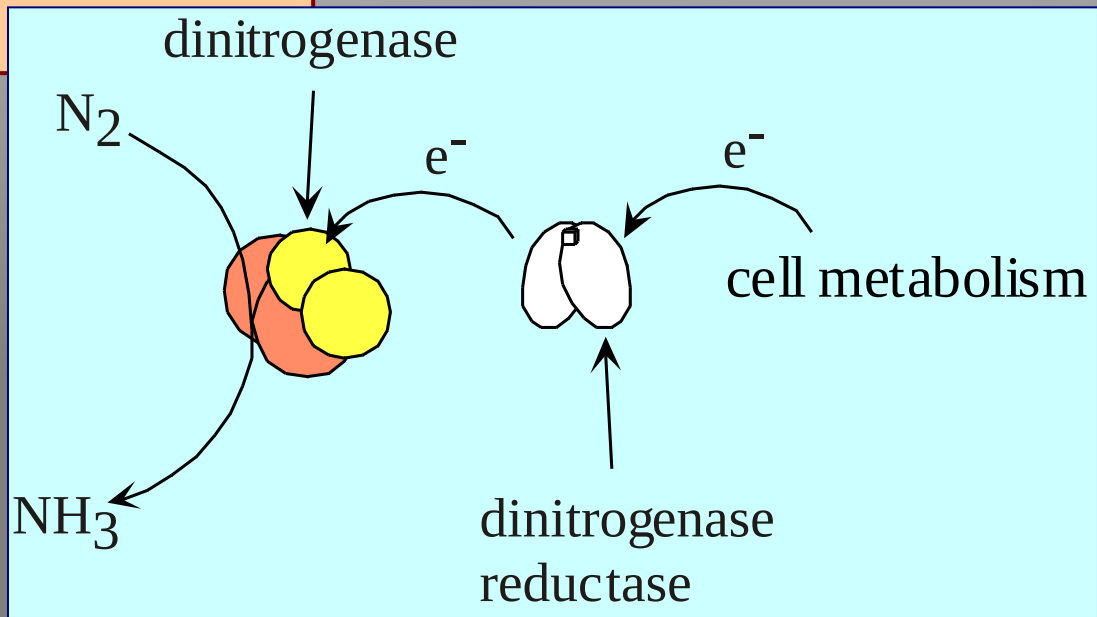
Cyanobacteria: Autotrophic N-fixers, protect nitrogenase with specialized *heterocyst* cells.

Heterotorophic bacteria: Free-living or associative with rhizosphere. Use energy from decomposing organic matter to fix N, protect nitrogenase by rapidly converting O_2 to CO_2 through respiration.

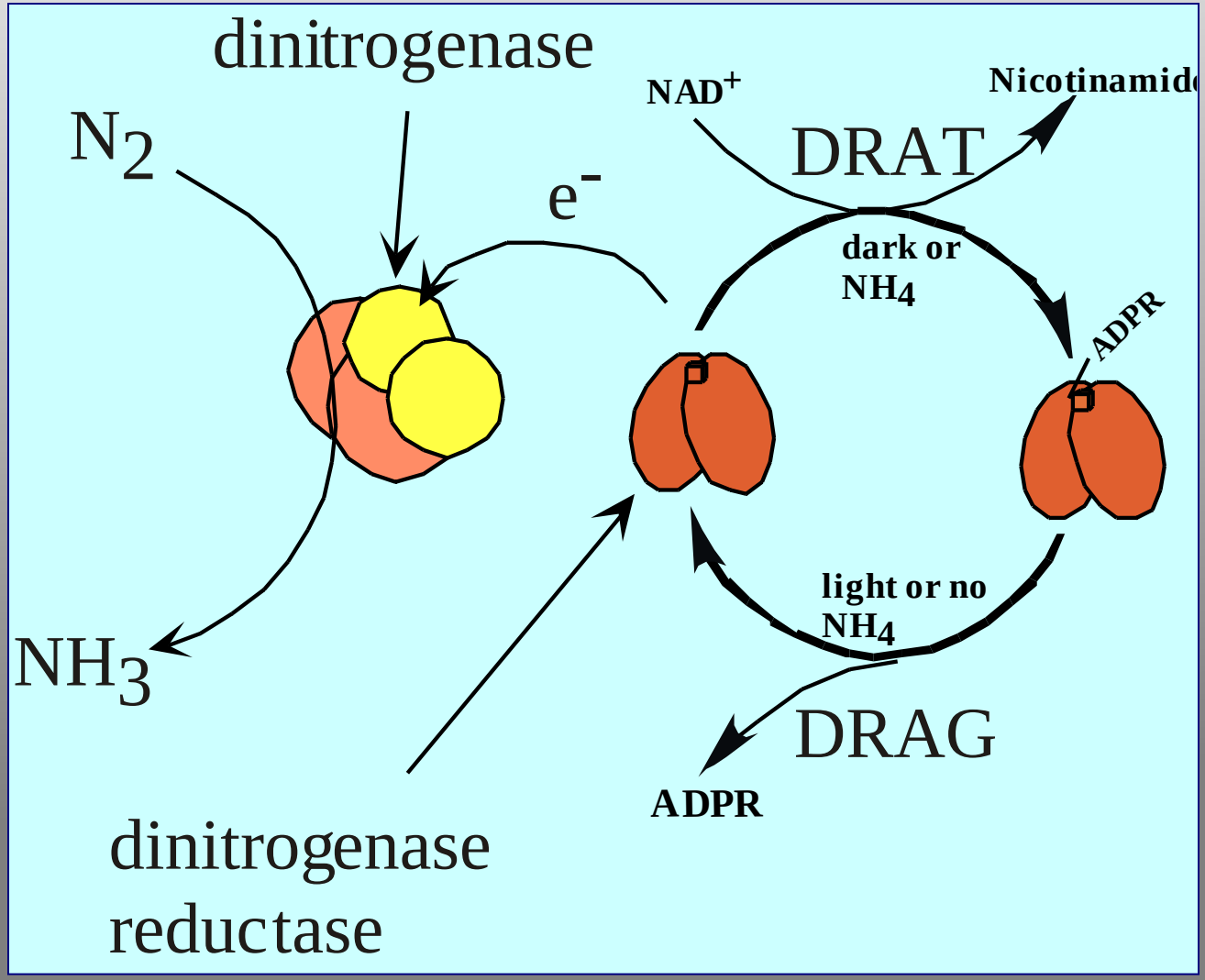
Symbiotic bacteria: Plants form nodules to house bacteria and provide C as energy source (*Rhizobium*/*Bradyrhizobium* for legumes, *Frankia* for non-legumes). Nodules contain a form of hemoglobin which binds O_2, protecting nitrogenase enzyme.

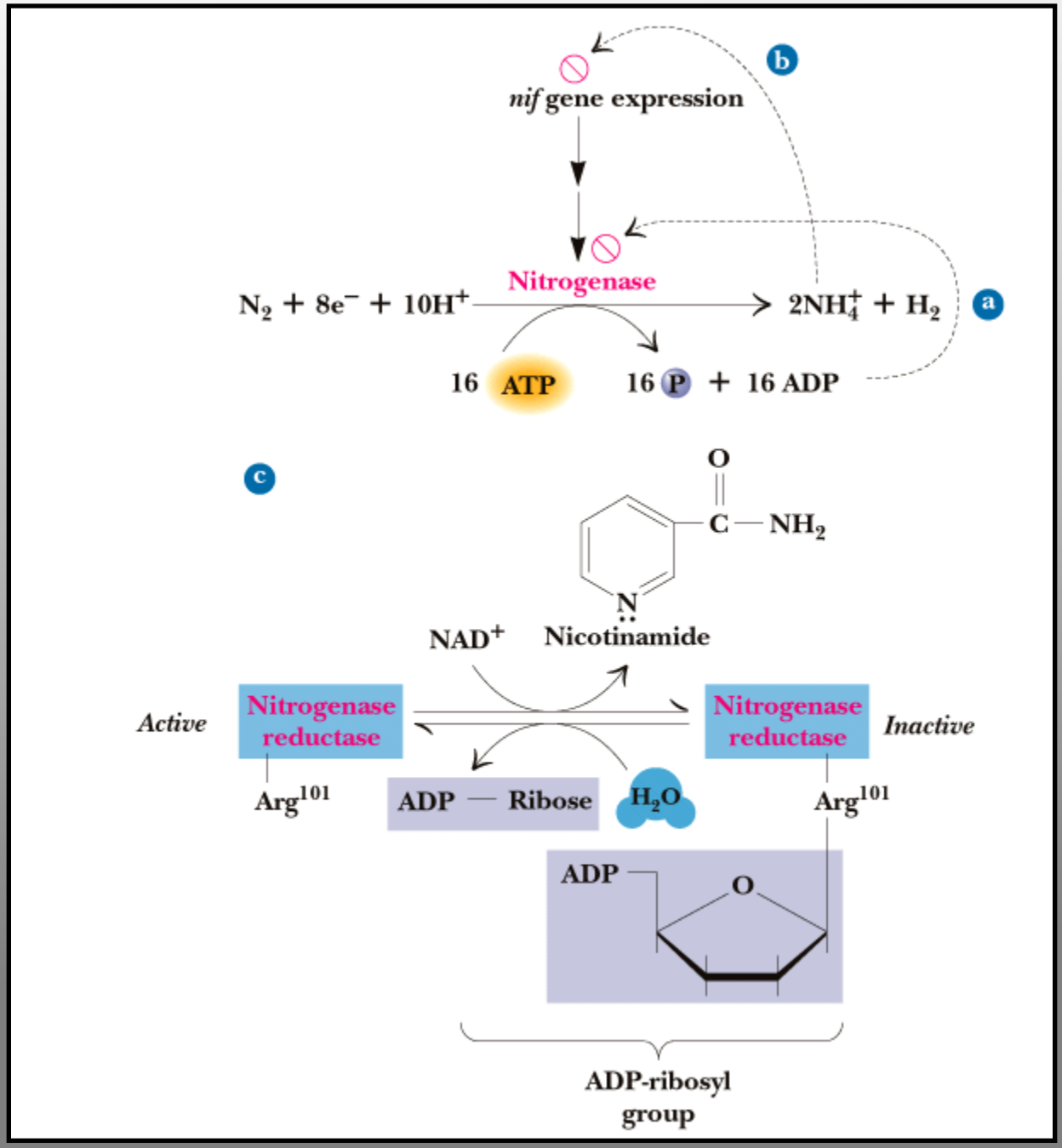
Nitrogen fixation in Klebsiella

- **Nif system is turned on when**
  - **No fixed nitrogen**
  - **Anaerobic**
  - **Temperature below 30° C**
- **Nitrogenase is made**
  - **Converts N_2 to NH_3**



ADP ribosylation of dinitrogenase reductase




b
nif gene expression
Nitrogenase
$N_2 + 8e^- + 10H^+$
$2NH_4^+ + H_2$
a
16 ATP
16 P + 16 ADP
c
O
C — NH_2
N
NAD+
Nicotinamide
Active
Nitrogenase reductase
Arg101
ADP — Ribose
H_2O
Nitrogenase reductase
Inactive
Arg101
ADP
O
ADP-ribosyl group

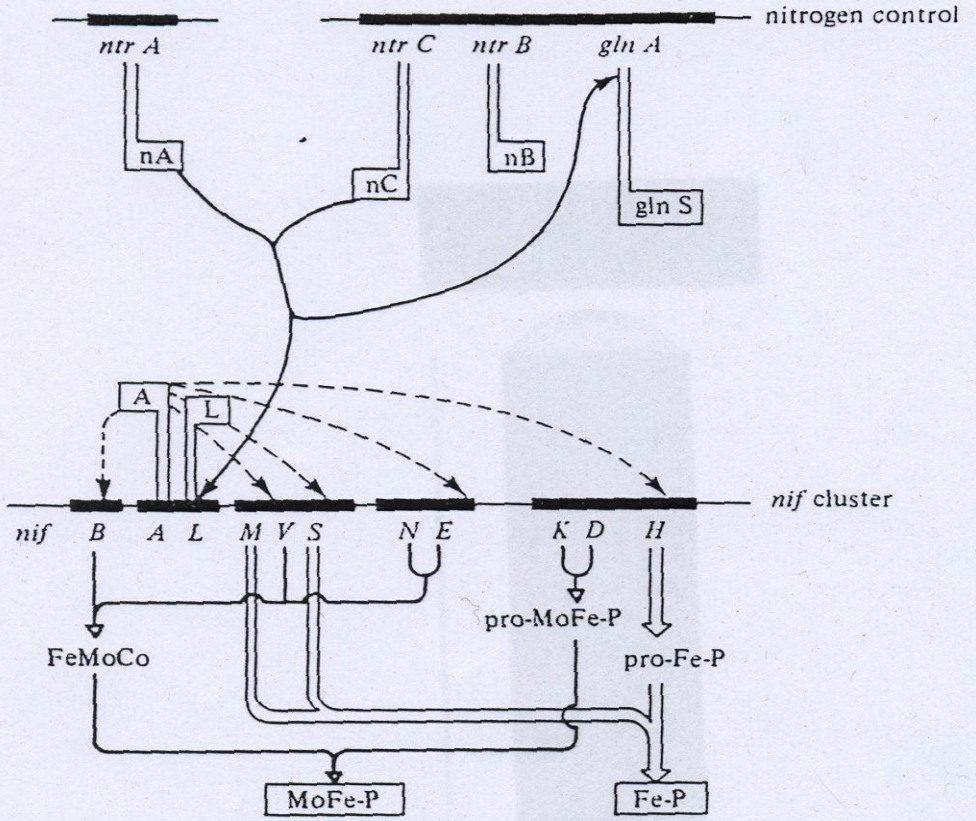


Figure 10.5. Organization and control of the *nif* cluster in *K. pneumoniae*. The situation under the condition of derepression is shown. The products of *ntrA* + *ntrC*, designated as nA and nC, together activate the promotor of *glnA* and of *nifA-nifL*. The *nifA* product (A) then activates the remaining promotors of the *nif* cluster. pro-Fe-P, Polypeptide of azoferredoxin; incorporation of the iron-sulfur centers is controlled by *nifM* and *nifS* products; pro-MoFe-P, precursor of molybdoferredoxin; the iron-molybdenum cofactor results from the products of genes *nifB*, *nifN*, *nifV*, and *nifE*. In the presence of ammonia product nB prevents activation at the nitrogen control level and product L at the *nif* cluster level. Solid arrows indicate the promotors at which transcription is derepressed by the gene products nA + nC. Dotted arrows indicate the promotors at which transcription is derepressed by the gene product A.

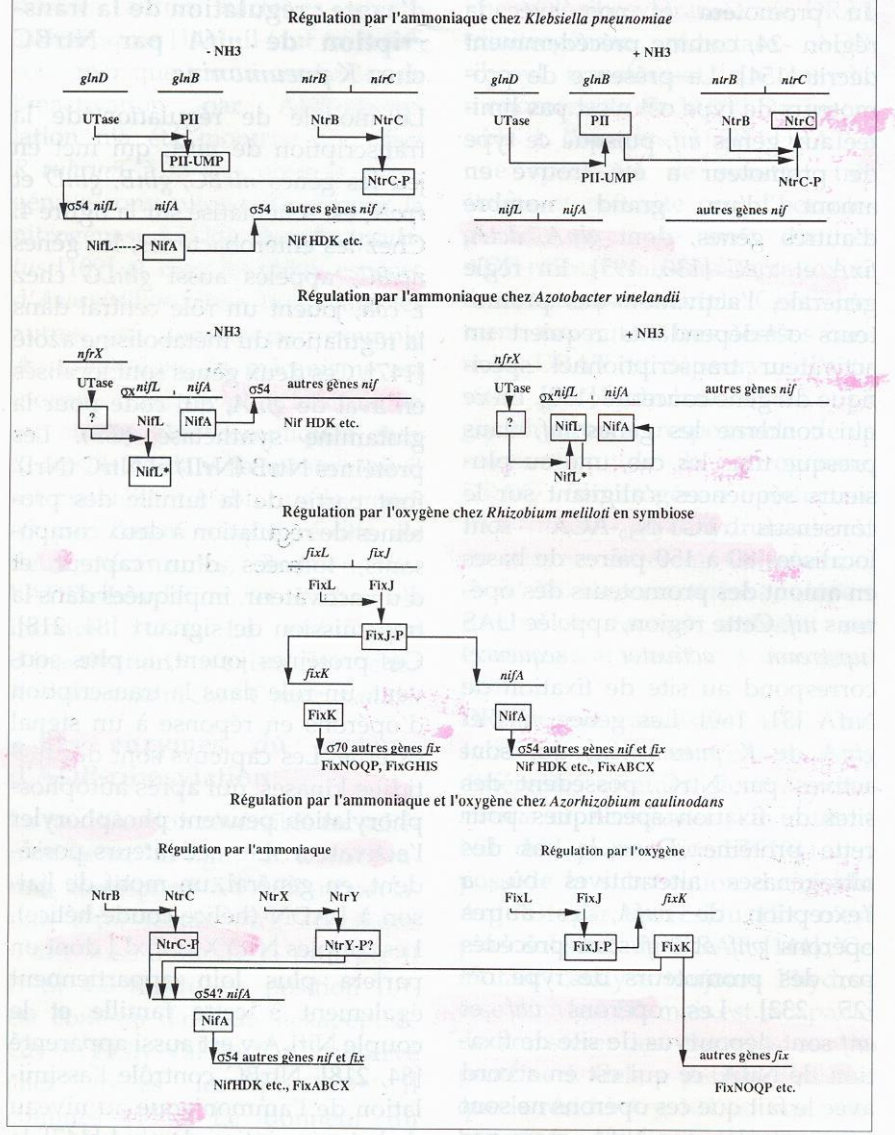


Fig 4. Quelques modèles de régulation chez différentes bactéries fixatrices d'azote. K pneumoniae *fixe l'azote en anaérobiose et en absence d'ammoniaque,* A vinelandii *dans l'air et en absence d'ammoniaque,* R meliloti *seulement en symbiose,* A caulinodans *en symbiose et à l'état libre en microaérobiose en absence d'ammoniaque. Le modèle présenté pour* A caulinodans *correspond aux conditions de fixation à l'état libre. Les principales protéines régulatrices sont encadrées.*

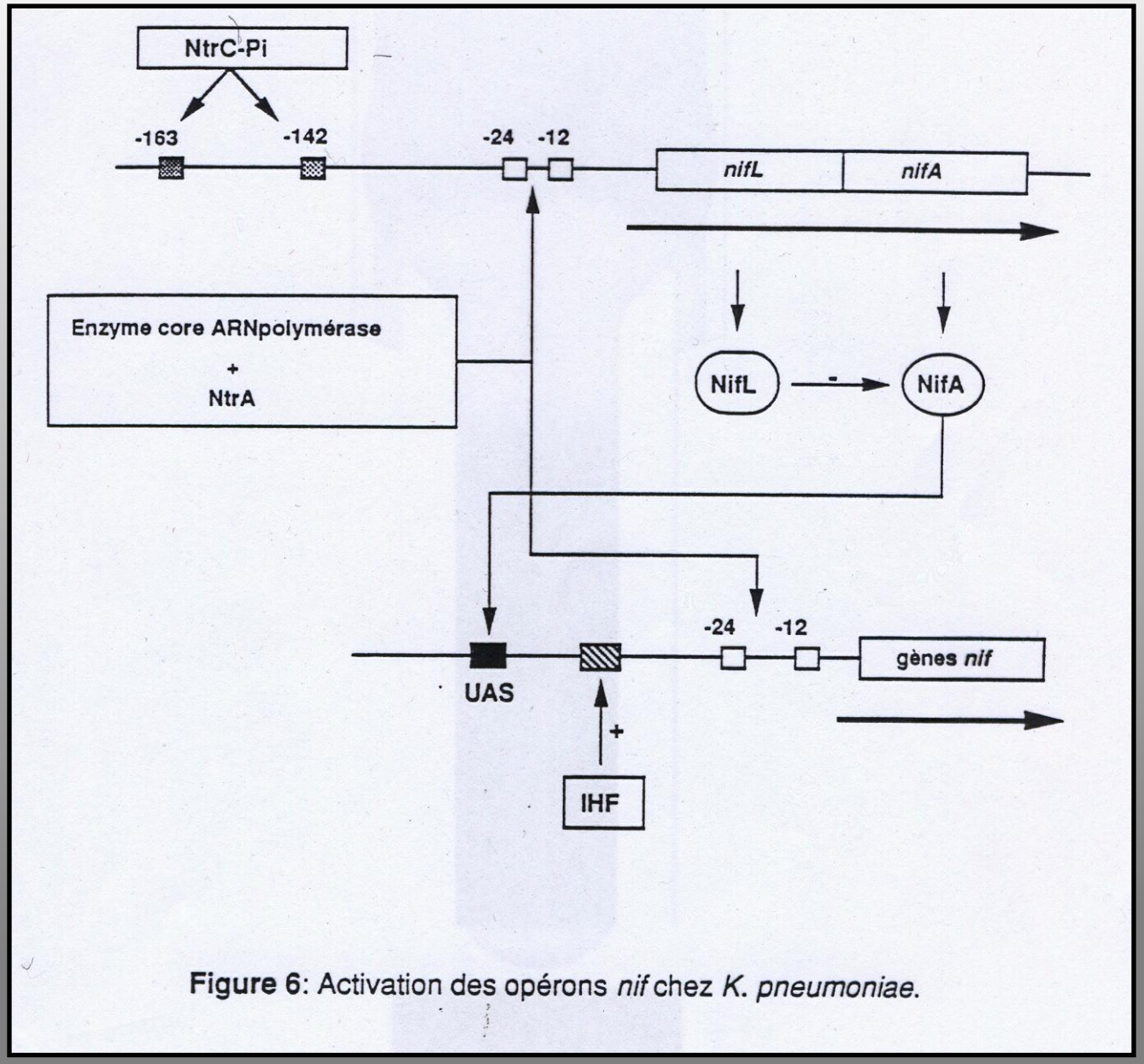
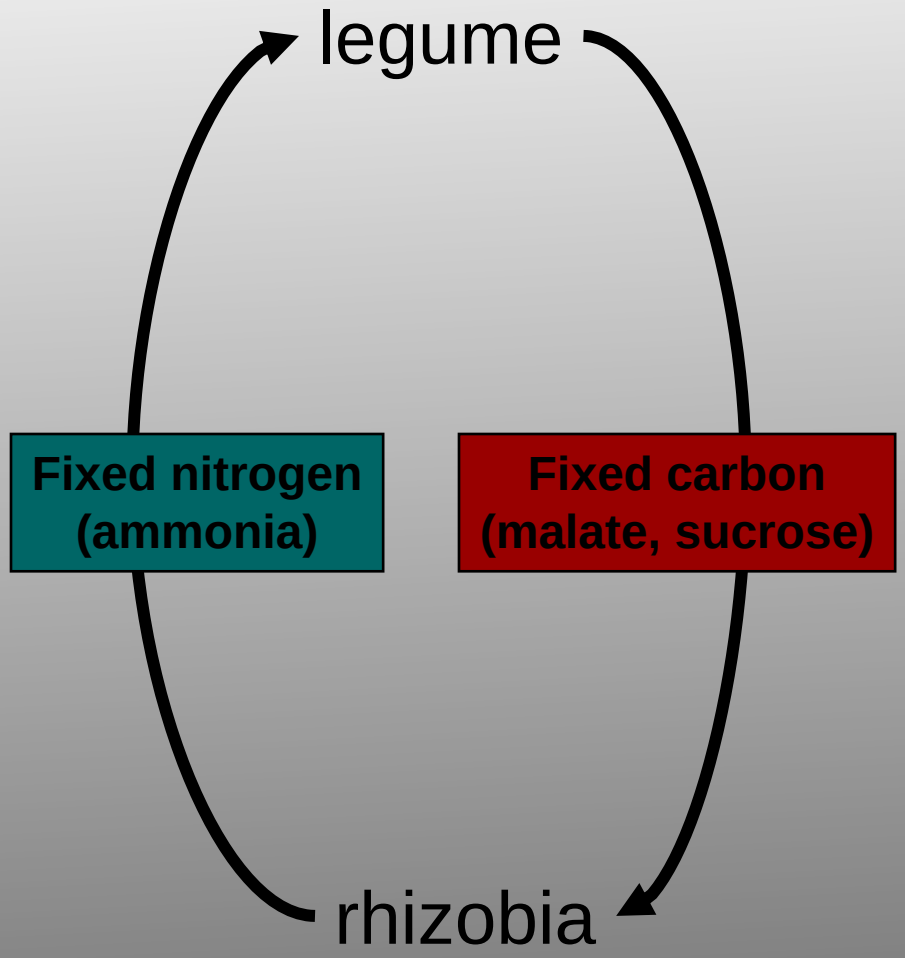
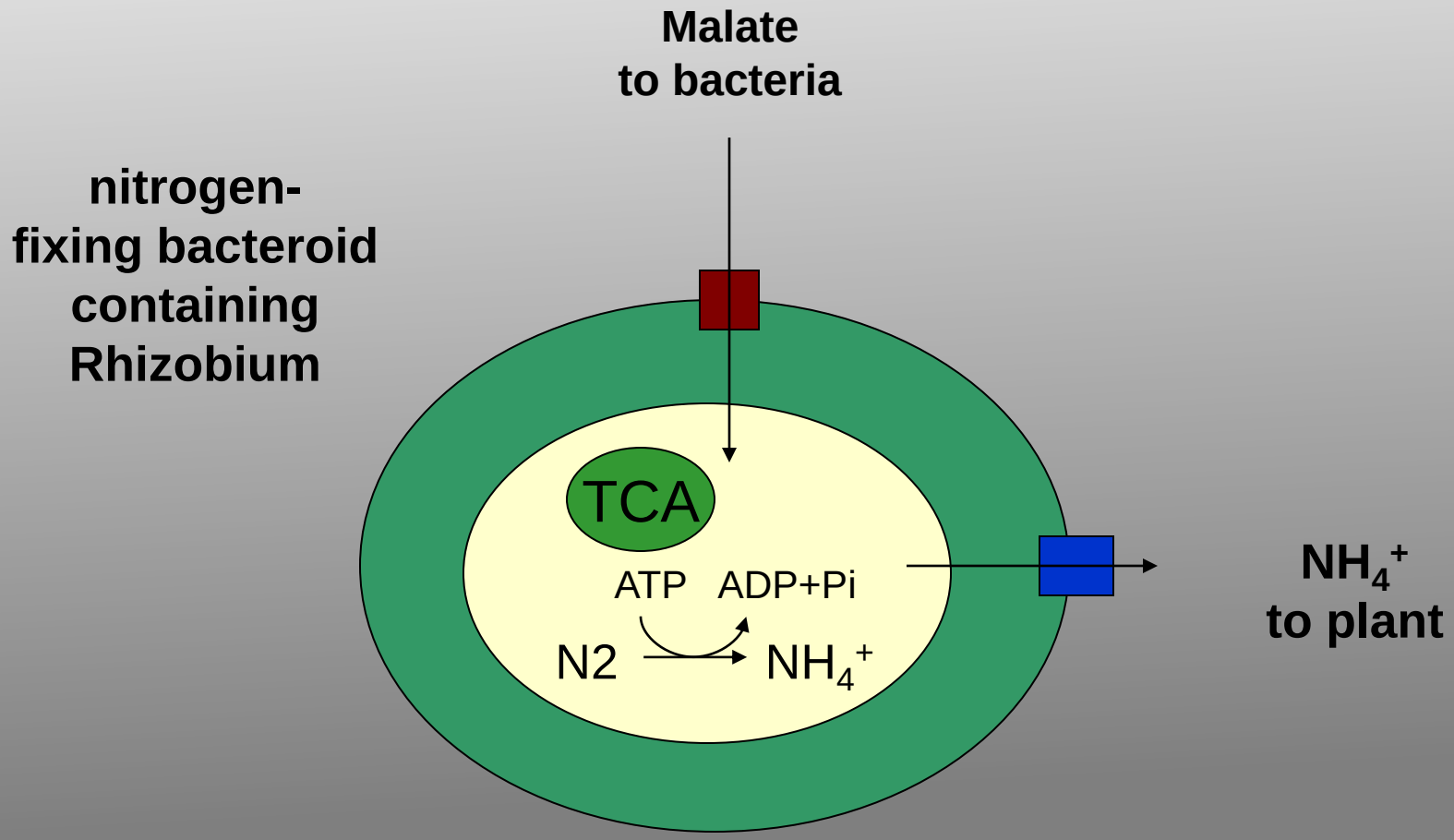


Figure 6: Activation des opérons *nif* chez *K. pneumoniae*.


legume
Fixed nitrogen
(ammonia)
Fixed carbon
(malate, sucrose)
rhizobia

Exchange of nutrients during Rhizobium-legume symbiosis

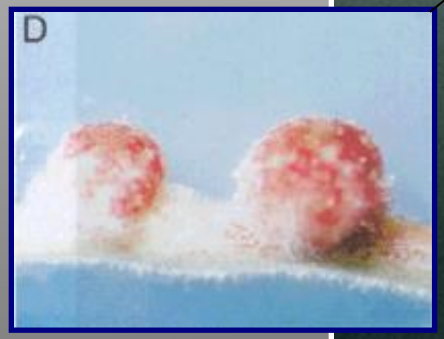


Symbiotic Nitrogen Fixation

The *Rhizobium*-legume association

Bacterial associations with certain plant families, primarily **legume** species, make the largest single contribution to biological nitrogen fixation in the biosphere

Pea Plant



R. leguminosarum nodules

Pink color is leghaemoglobin a protein that carries oxygen to the bacteroids

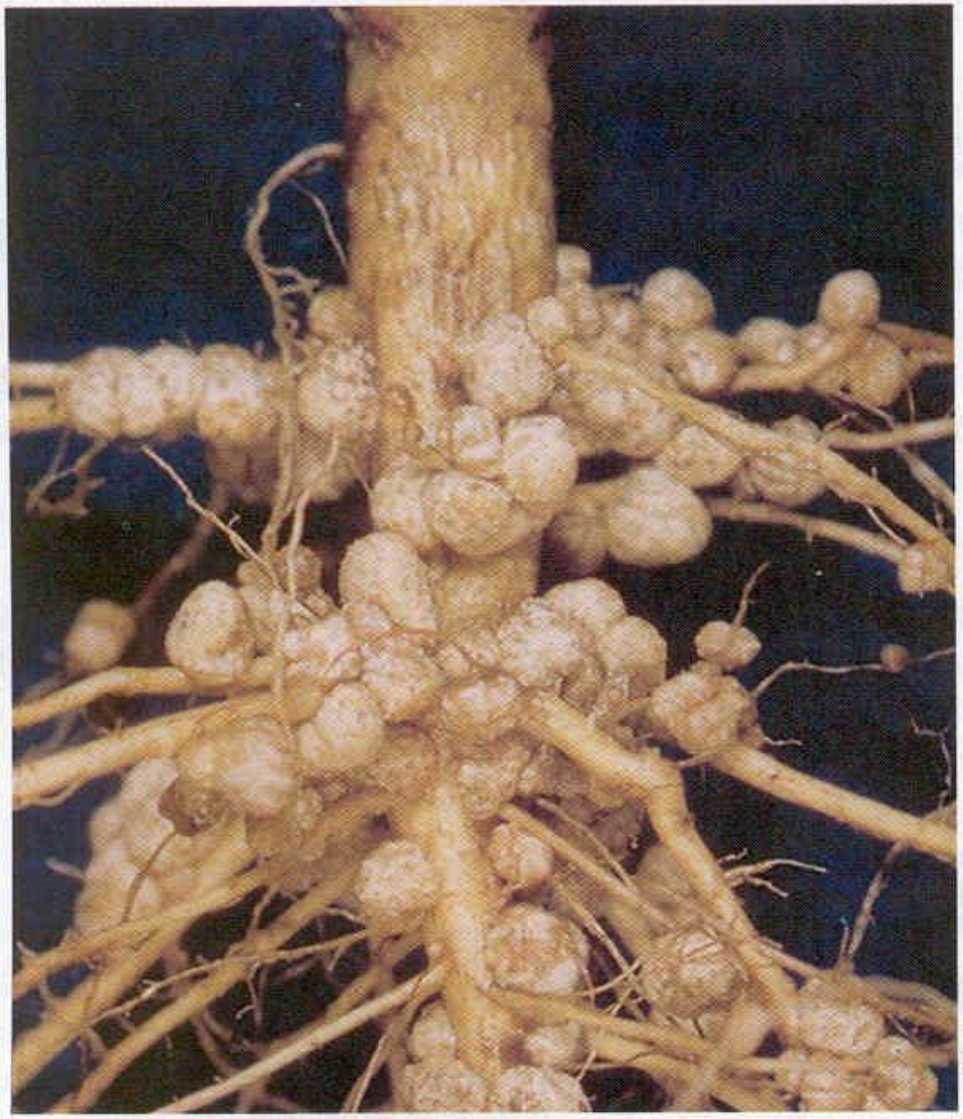
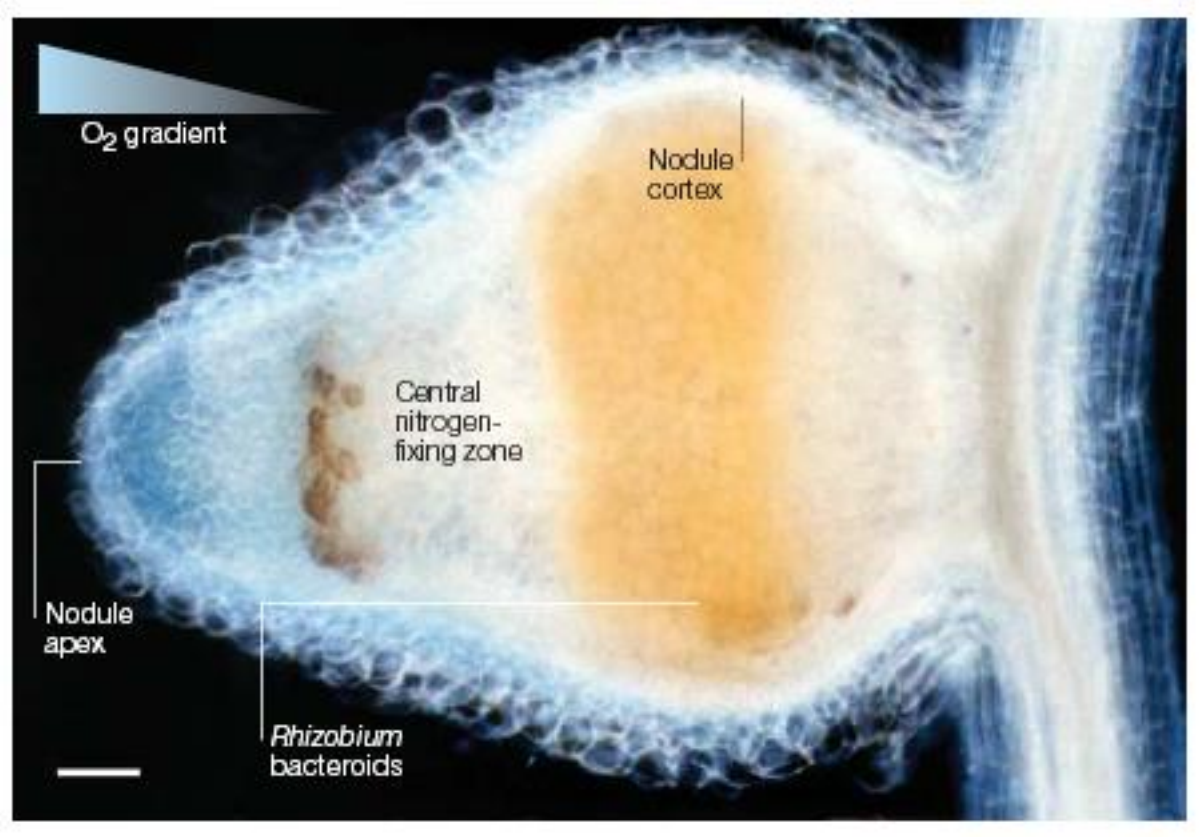
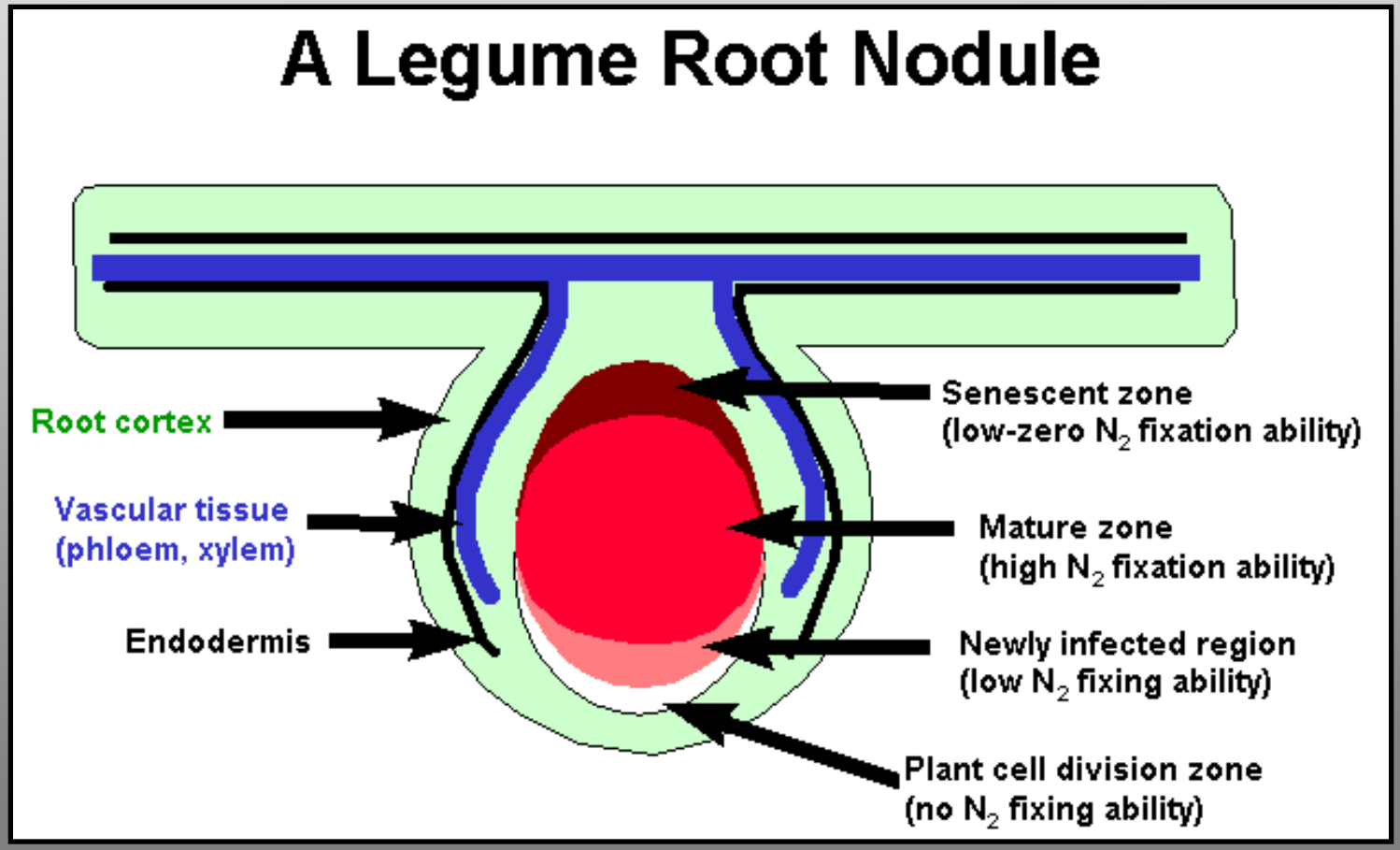
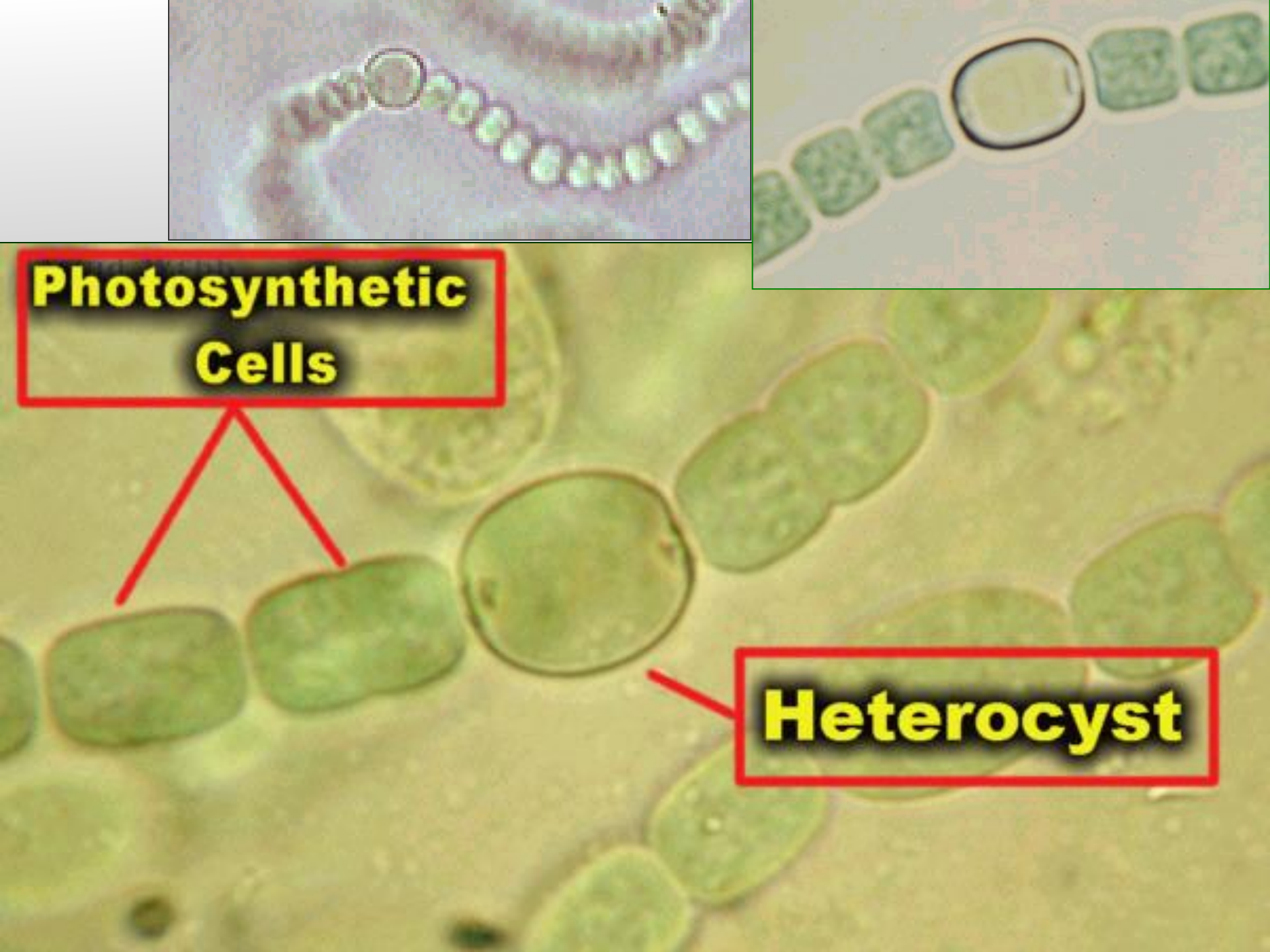


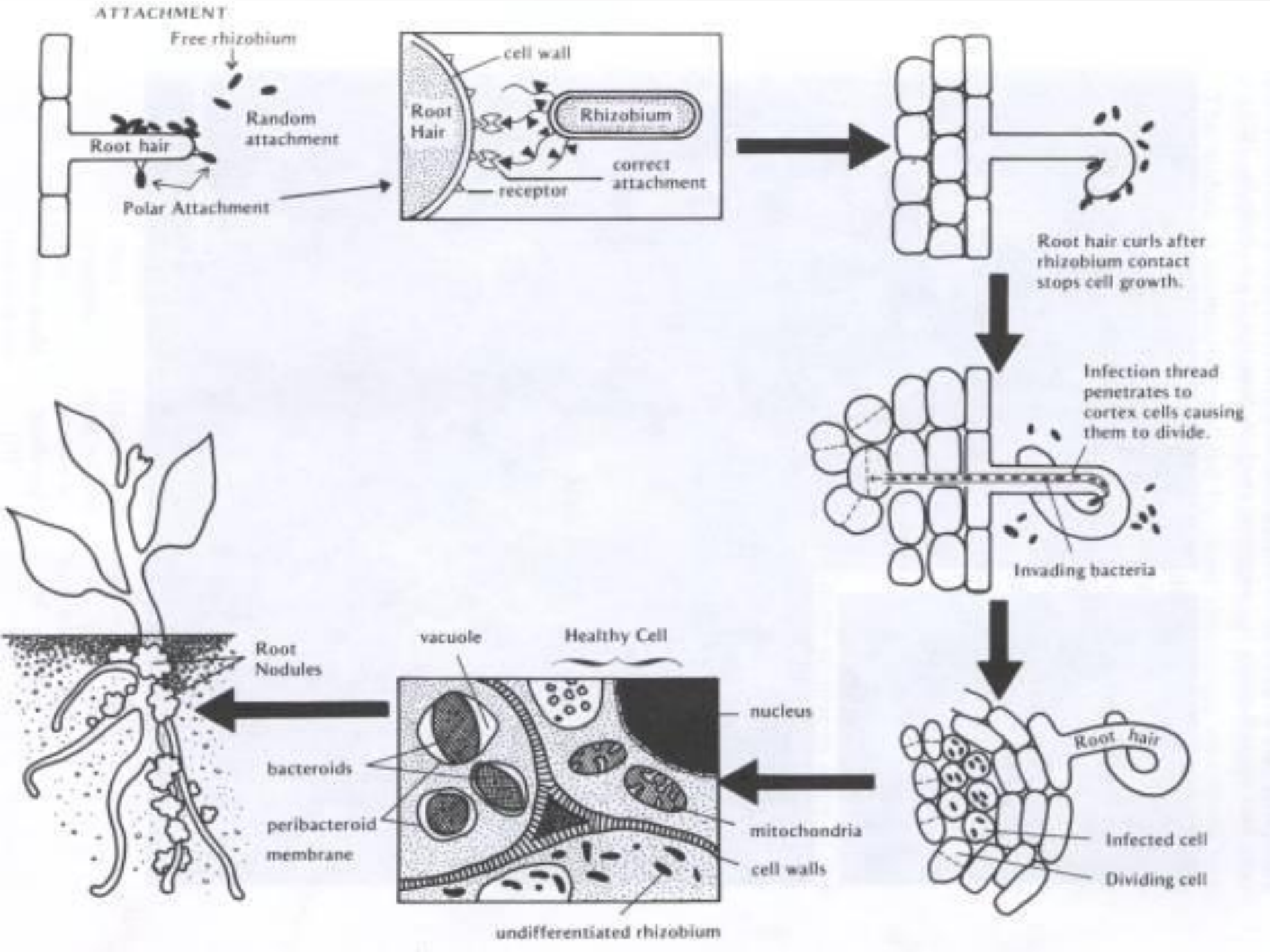
FIGURE 16.73 Soybean root nodules. The nodules develop by infection with *Bradyrhizobium japonicum*.


O_2 gradient
Nodule cortex
Central nitrogen-fixing zone
Nodule apex
Rhizobium bacteroids

Physiology of a legume nodule




Photosynthetic Cells
Heterocyst


ATTACHMENT
Free rhizobium
Random attachment
Root hair
Polar Attachment
cell wall
Root Hair
Rhizobium
correct attachment
receptor
Root hair curls after rhizobium contact stops cell growth.
Infection thread penetrates to cortex cells causing them to divide.
Invading bacteria
Root hair
Infected cell
Dividing cell
nucleus
mitochondria
cell walls
vacuole
Healthy Cell
bacteroids
peribacteroid membrane
undifferentiated rhizobium
Root Nodules

The nodulation process

1. Chemical recognition of root and *Rhizobium*
2. Root hairs curl
3. Formation of infection threads
4. Invasion of the roots by *Rhizobia*
5. Nodule tissue forms
6. Bacteria convert to bacteriods and begin to form nitrogenase enzyme
7. Legume provides *Rhizobia* with carbon. *Rhizobia* provide the legume with fixed N

The Nodulation Process

- Chemical recognition of roots and Rhizobium
- Root hair curling
- Formation of infection thread
- Invasion of roots by Rhizobia
- Cortical cell divisions and formation of nodule tissue
- Bacteria fix nitrogen which is transferred to plant cells in exchange for fixed carbon

Biological NH_3 creation (nitrogen fixation) accounts for an estimated 170×10^9 kg of ammonia every year. Human industrial production amounts to some 80×10^9 kg of ammonia yearly.

The industrial process (Haber-Bosh process) uses an Fe catalyst to dissociate molecules of N_2 to atomic nitrogen on the catalyst surface, followed by reaction with H_2 to form ammonia. This reaction typically runs at ~450º C and 500 atmospheres pressure.

These extreme reaction conditions consume a huge amount of energy each year, considering the scale at which NH_3 is produced industrially.

The Dreams…..

If a way could be found to **mimic nitrogenase catalysis**
(a reaction conducted at 0.78 atmospheres N_2 pressure
and ambient temperatures), huge amounts of energy
(and money) could be saved in industrial ammonia production.

If a way could be found to
transfer the capacity to form N-fixing symbioses
from a typical legume host
to an important non-host crop species such as corn or wheat,
far less fertilizer
would be needed to be produced and applied
in order to sustain crop yields

Because of its current and potential **economic importance**, the interaction between Rhizobia and leguminous plants has been intensively studied.

Our understanding of the process by which these two symbionts establish a functional association is still not complete, but it has provided a **paradigm** for many aspects of cell-to-cell communication between microbes and plants (e.g. during pathogen attack), and even between cells within plants (e.g. developmental signals; fertilization by pollen).

Symbiotic Rhizobia are classified in two groups:

Fast-growing ***Rhizobium*** spp. whose nodulation functions (**nif, fix**) are encoded on their symbiotic megaplasmids (**pSym**)

Slow-growing ***Bradyrhizobium*** spp. whose N-fixation and nodulation functions are encoded on their chromosome.

There are also two types of nodule that can be formed:

determinate

and

indeterminate

This outcome is controlled by the plant host

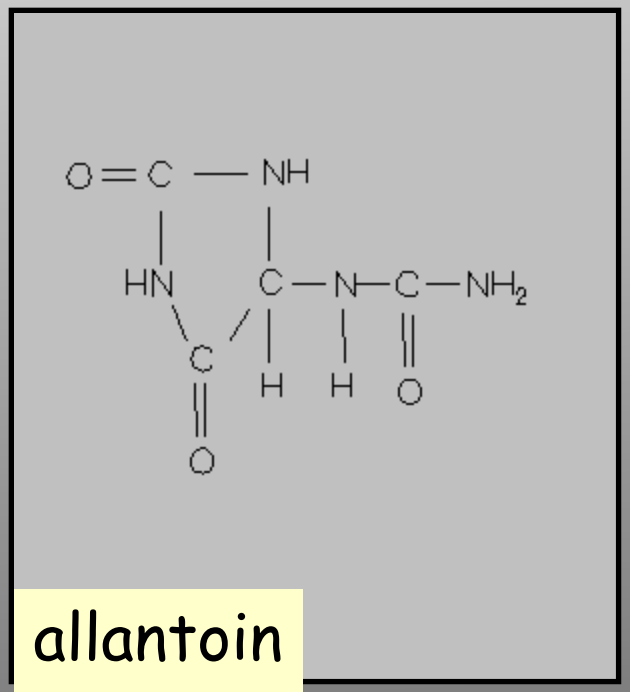
Determinate nodules

Formed on **tropical legumes** by *Rhizobium* and *Bradyrhizobium*

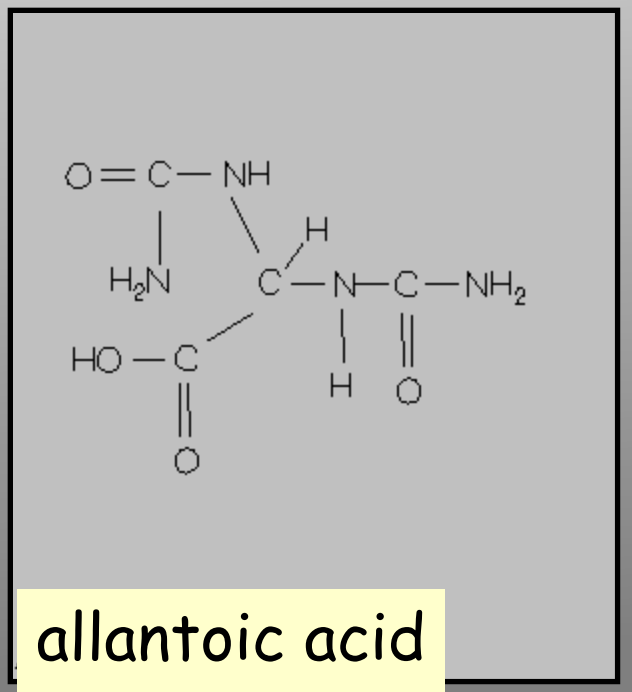
Meristematic activity not persistent - present only during early stage of nodule formation;
after that, cells simply expand rather than divide, to form **globose nodules.**

Nodules arise just below epidermis; largely internal vascular system

Uninfected cells dispersed throughout nodule; equipped to assimilate NH_4^+ as **ureides** (allantoin and allantoic acid)



allantoin



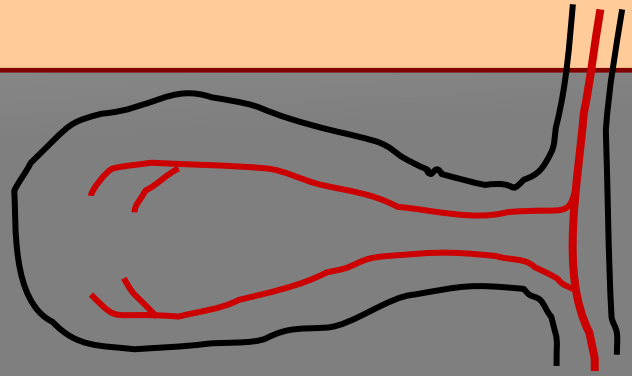
allantoic acid

Indeterminate nodules

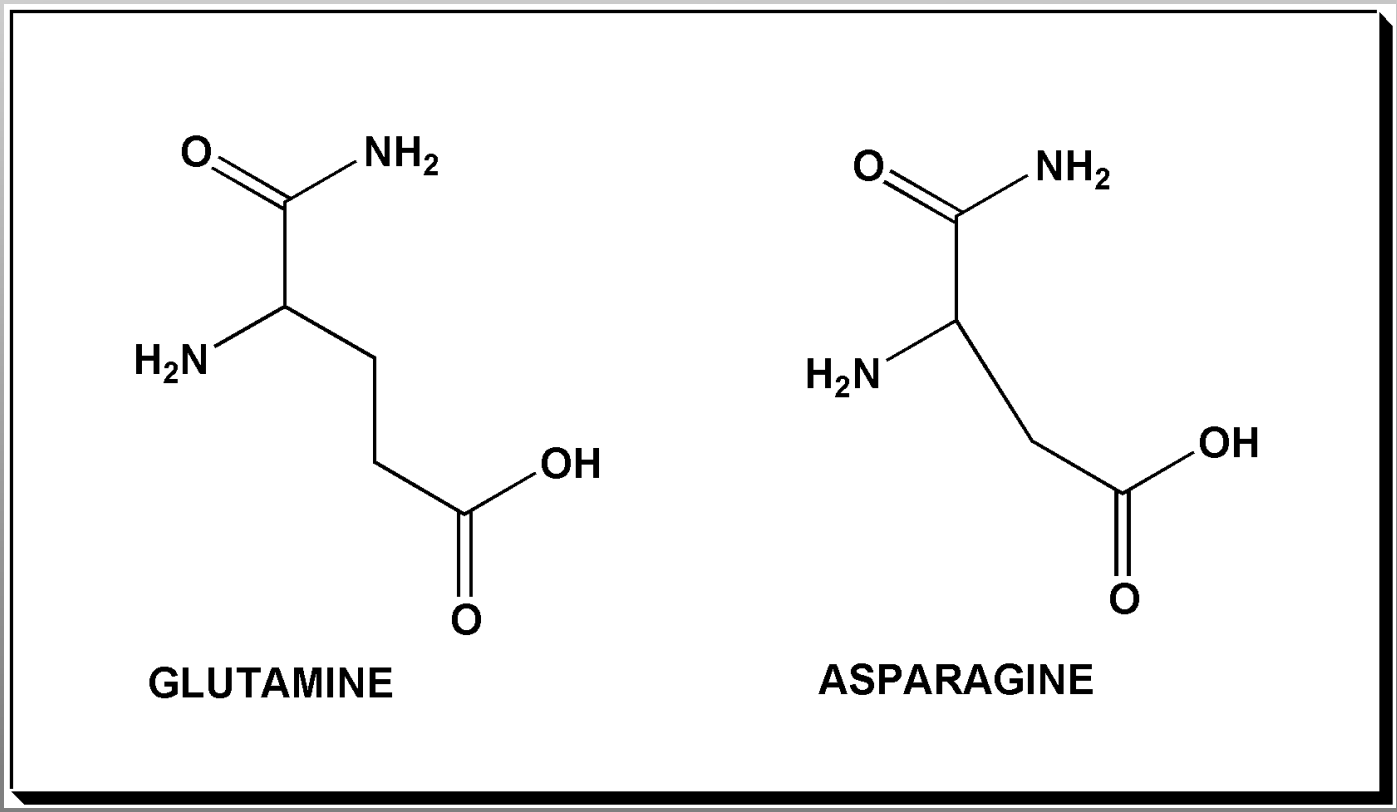
Formed on **temperate** legumes
(pea, clover, alfalfa);
typically by *Rhizobium* spp.

Cylindrical nodules with a **persistent meristem**;
nodule growth creates zones of different developmental stages

Nodule arises near endodermis, and nodule vasculature clearly connected with root vascular system



Uninfected cells of indeterminate nodules assimilate NH_4^+ as **amides** (asparagine, glutamine)



Rhizobium

- establish highly specific symbiotic associations with legumes
  - form **root nodules**
  - fix nitrogen within root nodules
  - nodulation genes are present on large plasmid

Rhizobium-legume symbioses

Host plant	**Bacterial symbiont**
Alfalfa	*Rhizobium meliloti*
Clover	*Rhizobium trifolii*
Soybean	*Bradyrhizobium japonicum*
Beans	*Rhizobium phaseoli*
Pea	*Rhizobium leguminosarum*
Sesbania	*Azorhizobium caulinodans*

Complete listing can be found at at:
http://cmgm.stanford.edu/~mbarnett/rhiz.htm

Both plant and bacterial factors determine specificity

TABLE 16.8 Major cross-inoculation groups of leguminous plants

Host plant	Nodulated by
Pea	*Rhizobium leguminosarum* biovar *viciae*[a]
Bean	*Rhizobium leguminosarum* biovar *phaseoli*[a]
Bean	*Rhizobium tropici*
Lotus	*Mesorhizobium loti*
Clover	*Rhizobium leguminosarum* biovar *trifolii*[a]
Alfalfa	*Sinorhizobium meliloti*
Soybean	*Bradyrhizobium japonicum*
Soybean	*Bradyrhizobium elkanii*
Soybean	*Rhizobium fredii*
Sesbania rostrata (a tropical legume)	*Azorhizobium caulinodans*

[a] Several varieties (biovars) of *Rhizobium leguminosarum* exist, each capable of nodulating a different legume.

Typical Associations (cross-inoculation groups)

R.l.* biovar *viciae
colonizes **pea** (*Pisum* spp.) and vetch
(temperate; indeterminate nodules)

R.l.* biovar *trifolii
colonizes **clover** (*Trifolium* spp.)
(temperate; indeterminate nodules)

Rhizobium leguminosarum* biovar *phaseoli
colonizes **bean** (*Phaseolus* spp.)
(tropical; determinate nodules)

Rhizobium meliloti
colonizes **alfalfa** (*Medicago sativa*)
temperate; indeterminate nodules

Rhizobium fredii
colonizes **soybean** (*Glycine max*)
tropical; determinate nodules

Bradyrhizobium japonicum
colonizes **soybean**
tropical; determinate nodules

Rhizobium NGR 234
colonizes ***Parasponia*** and tropicals;
very broad host range

Very early events in the Rhizobium-legume symbiosis

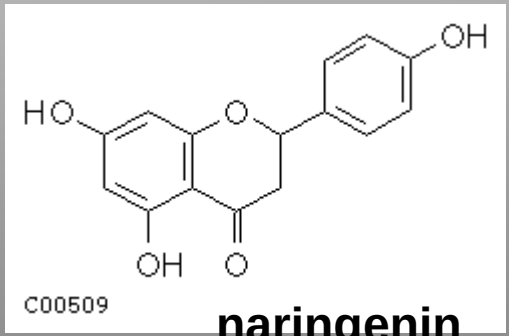
Flavonoids nod-gene inducers

rhizosphere

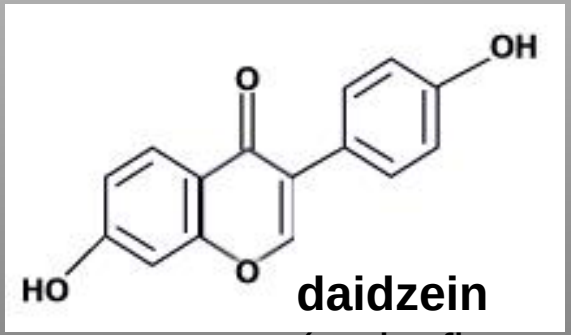
Nod-factor

Nodule development process

1. Bacteria encounter root;
they are chemotactically attracted toward specific plant chemicals (**flavonoids**) exuding from root tissue, especially in response to nitrogen limitation

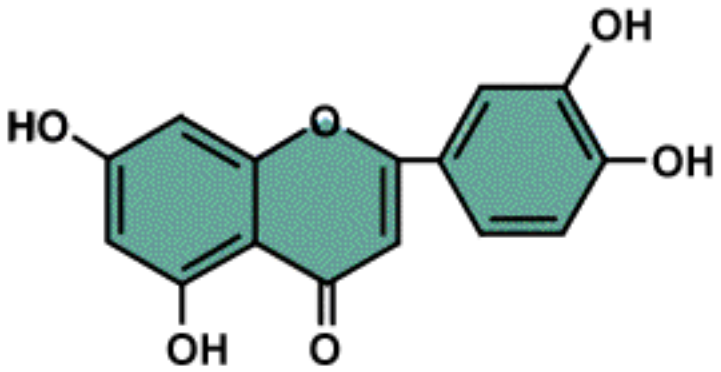


naringenin
(a flavanone)



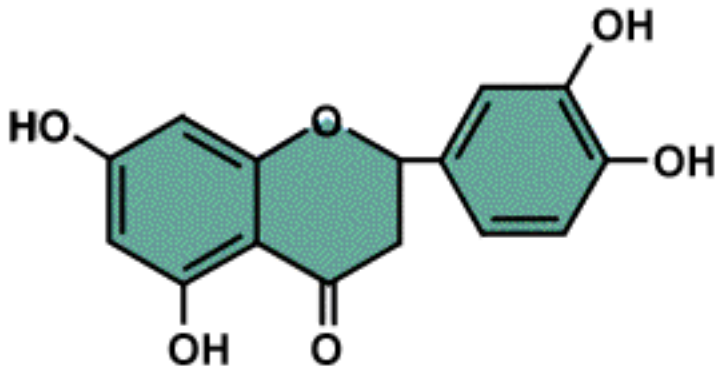
daidzein
(an isoflavone)

Inducers of nodulation in *Rhizobium leguminosarum bv viciae*



5, 7, 3', 4'-Tetrahydroxyflavone

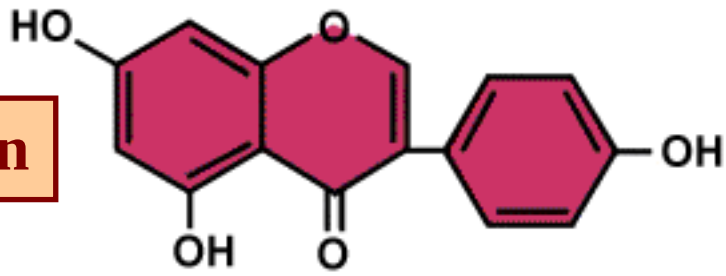
luteolin



5, 7, 3', 4'-Tetrahydroxyflavone

eriodictyol

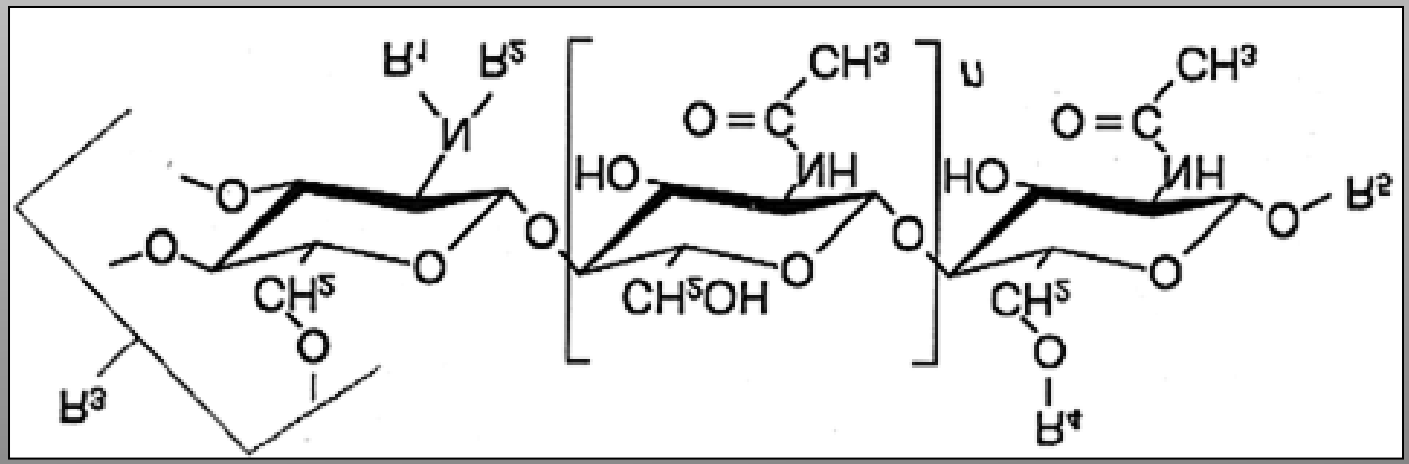
Inhibitor of nodulation



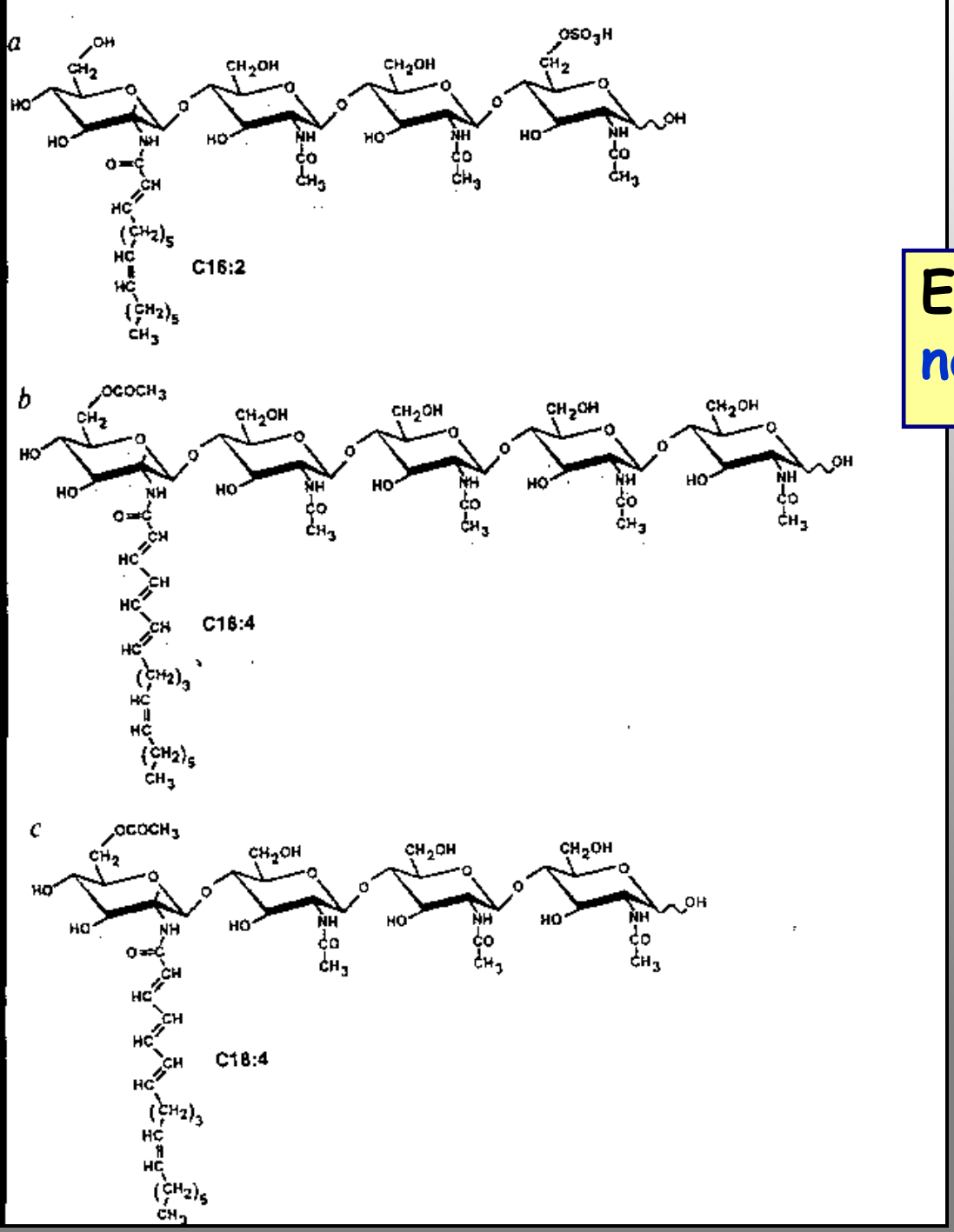
5, 7, 4'-Trihydroxyisolflavone

genistein

2. Bacteria attracted to the root attach themselves to the root hair surface and secrete specific **oligosaccharide** signal molecules (**nod factors**).



nod factor

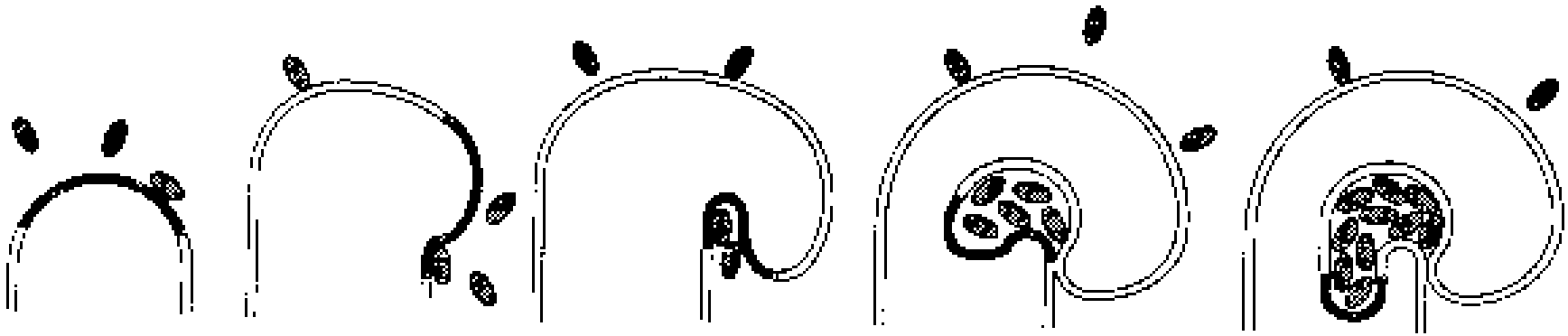


Examples of different nod factors

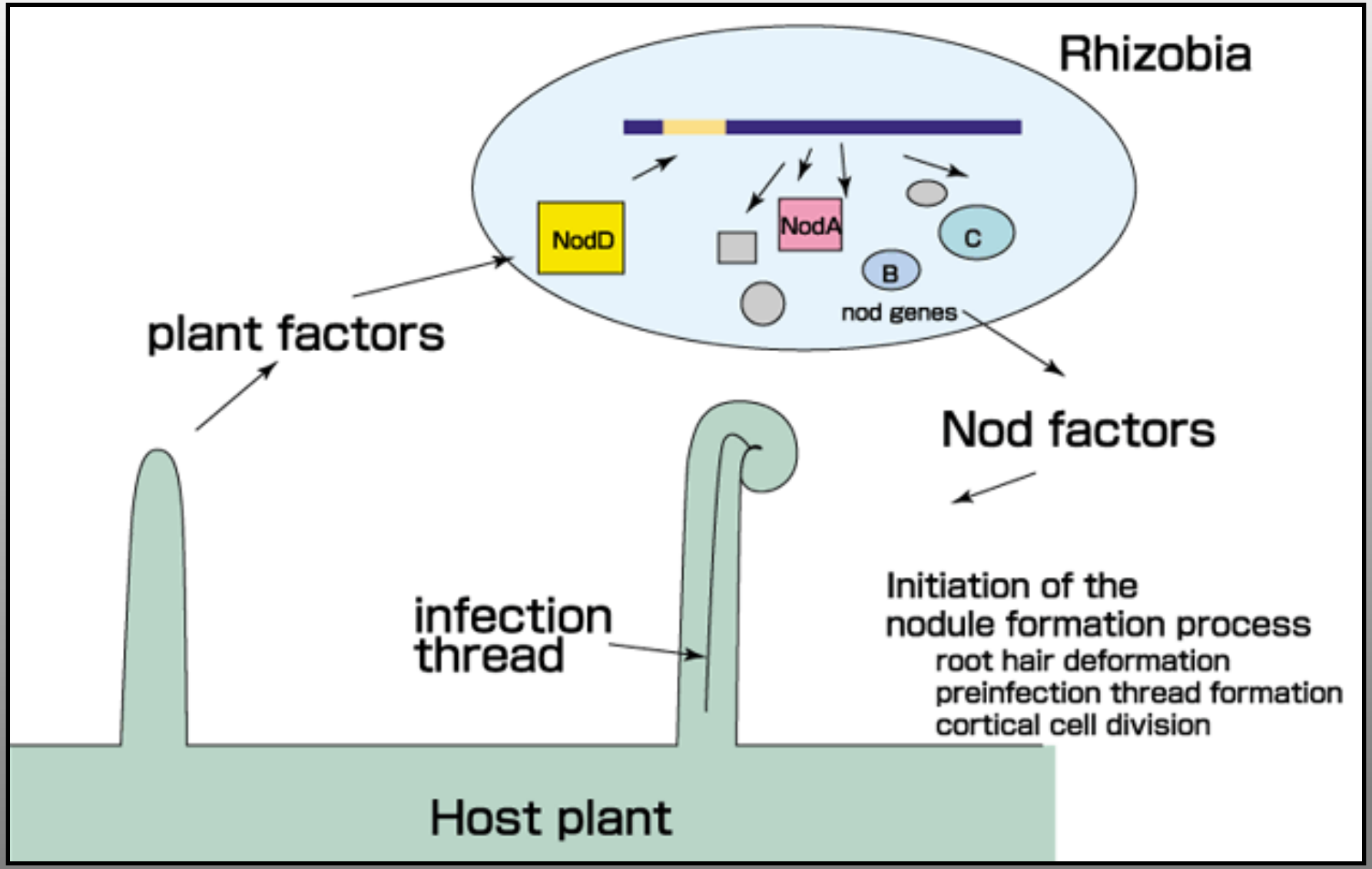
3. In response to oligosaccharide signals, the root hair becomes deformed and **curls** at the tip; bacteria become enclosed in small pocket.

Cortical **cell division** is induced within the root.

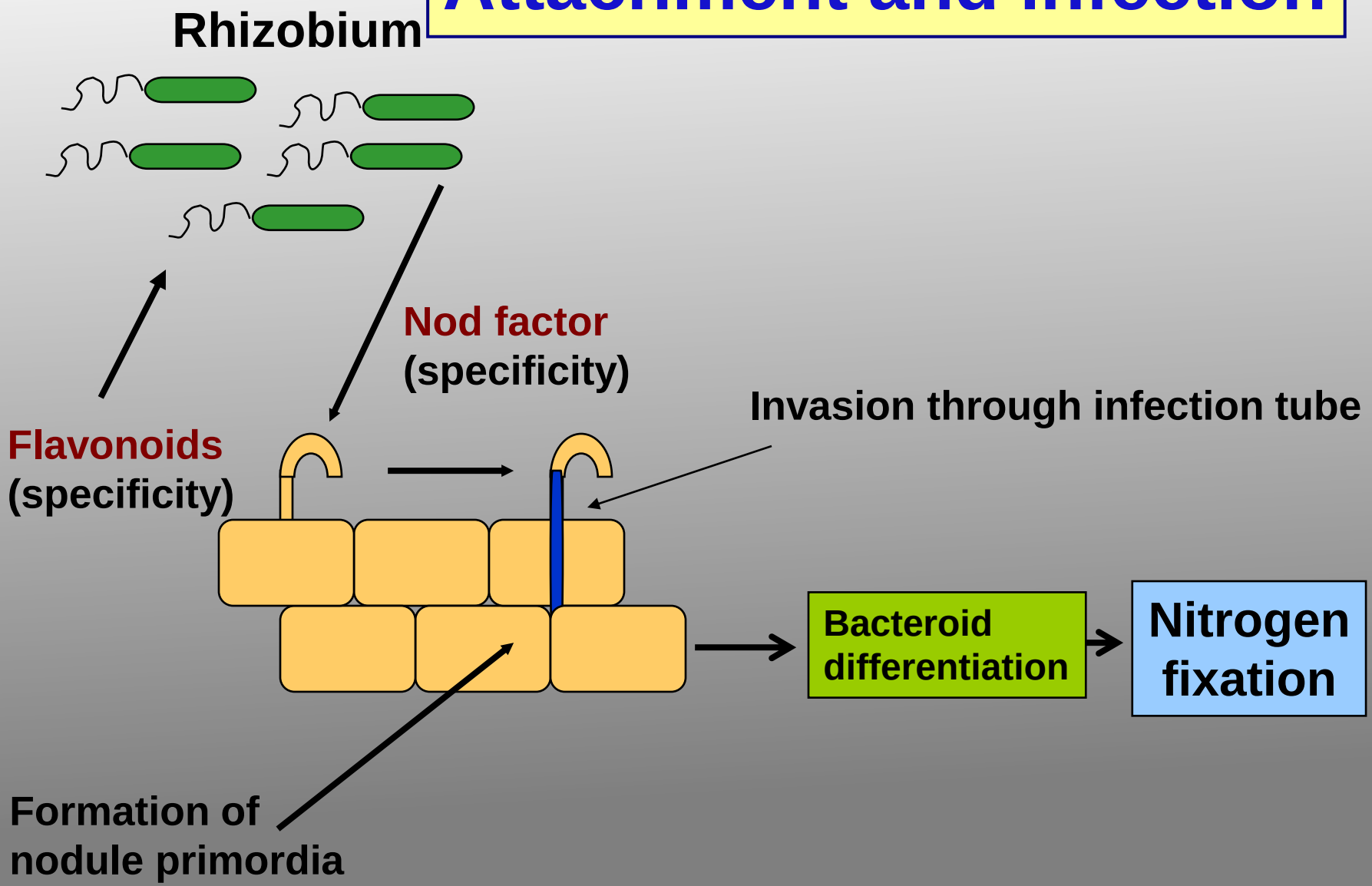
Root hair attachment and curling

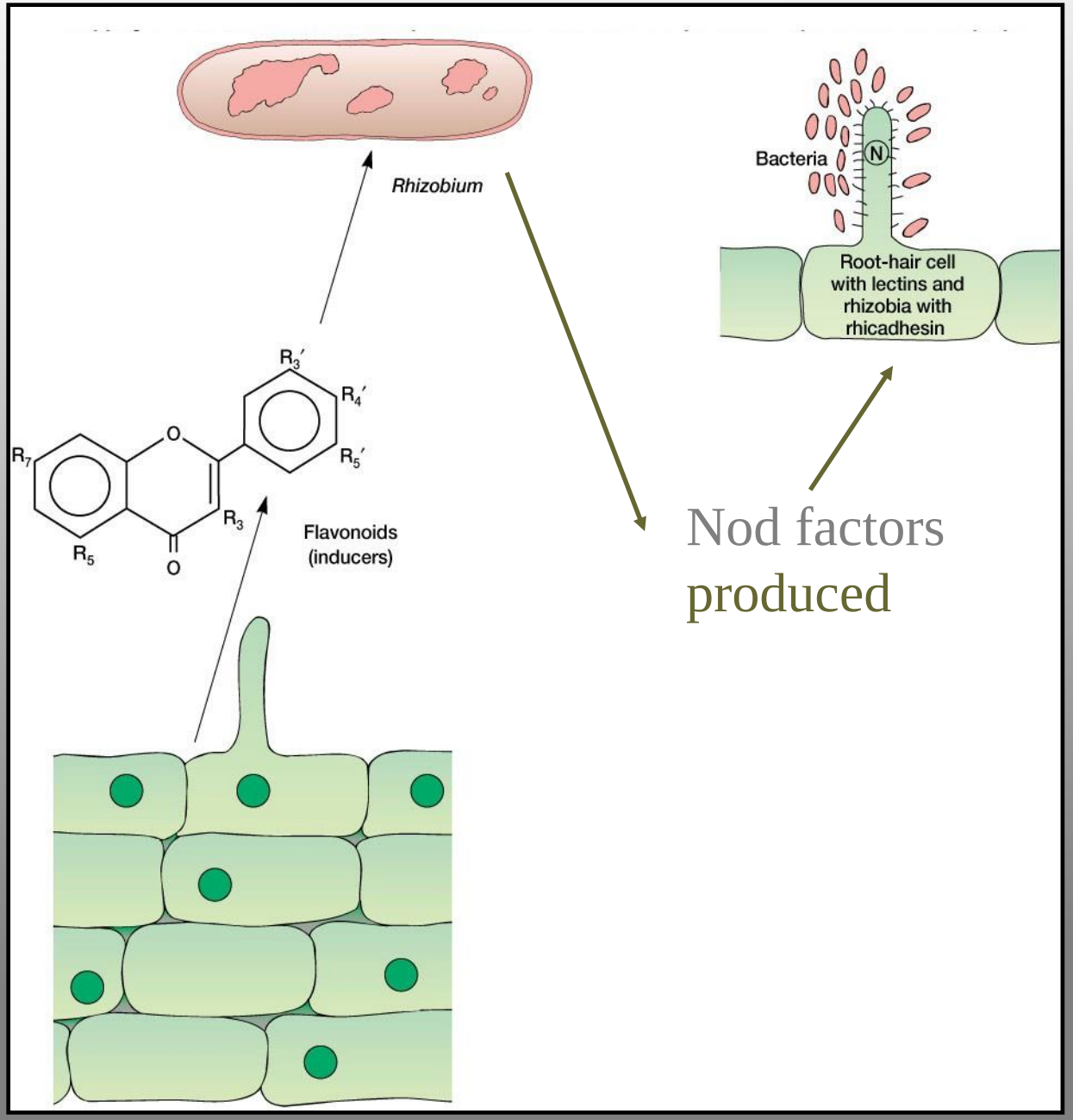


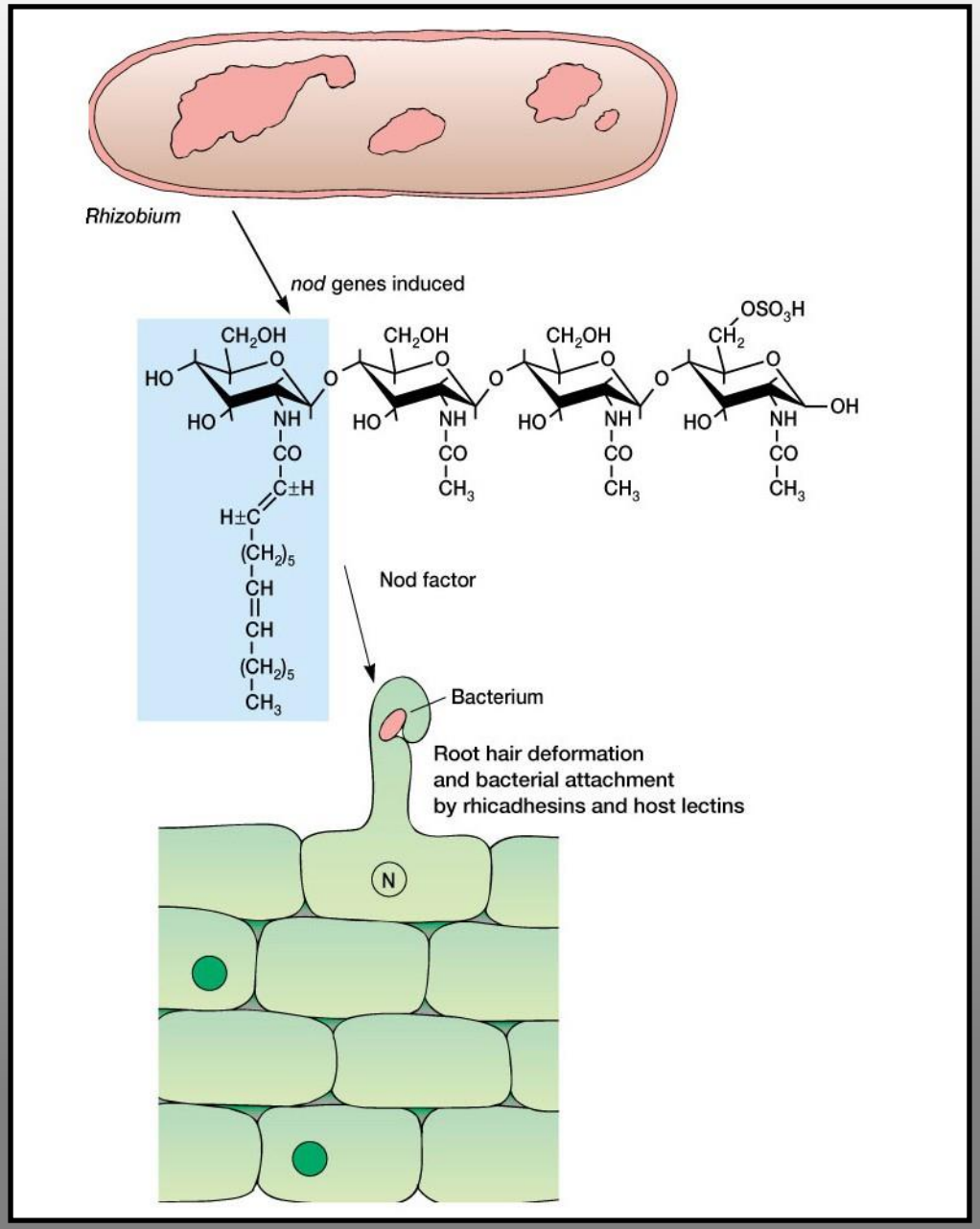
root hair beginning to curl


Rhizobia
NodD
NodA
C
B
nod genes
plant factors
Nod factors
infection thread
Initiation of the nodule formation process
root hair deformation
preinfection thread formation
cortical cell division
Host plant

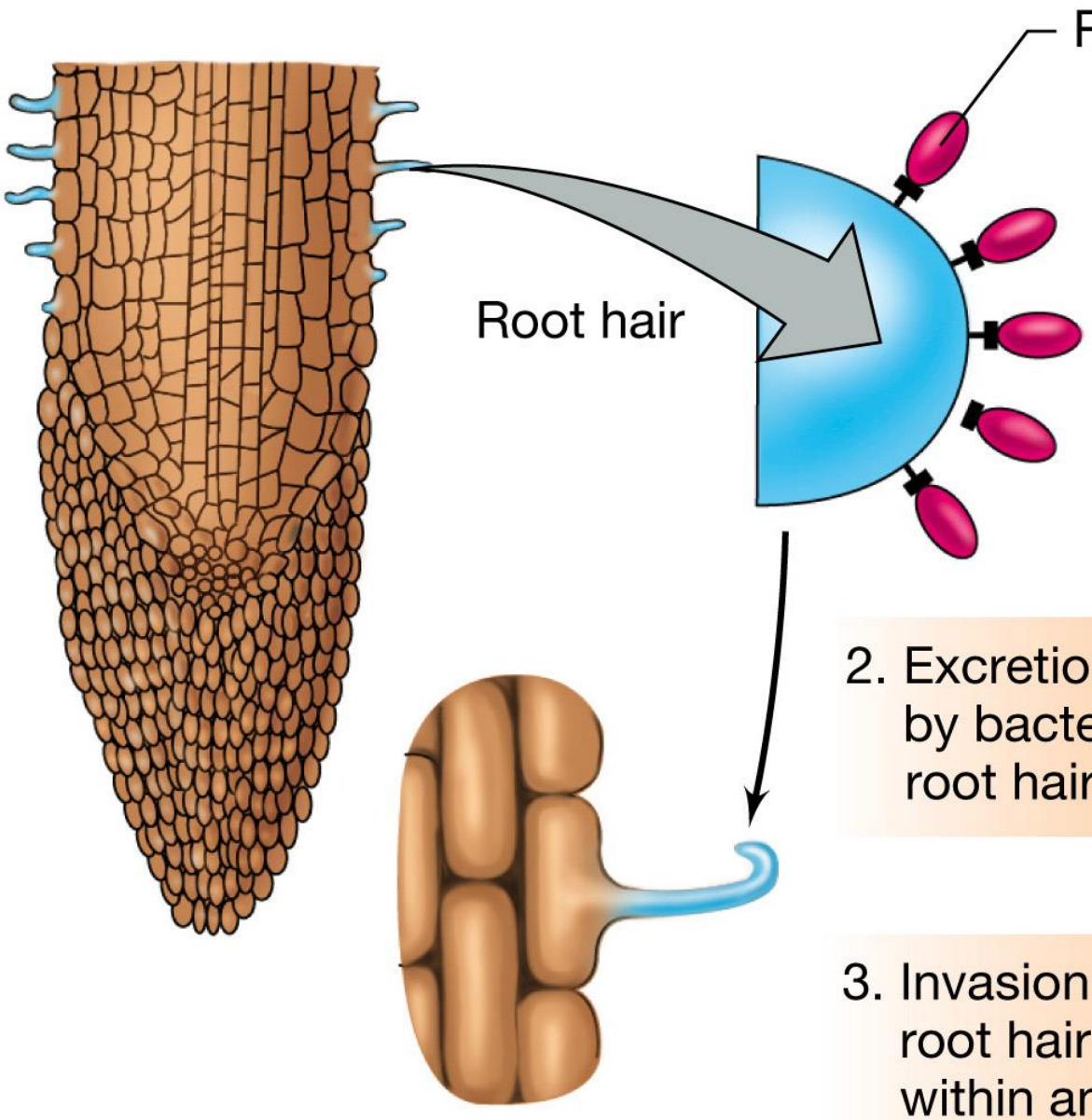
Attachment and infection




Rhizobium
Bacteria
N
Root-hair cell with lectins and rhizobia with rhicadhesin
Flavonoids (inducers)
Nod factors produced

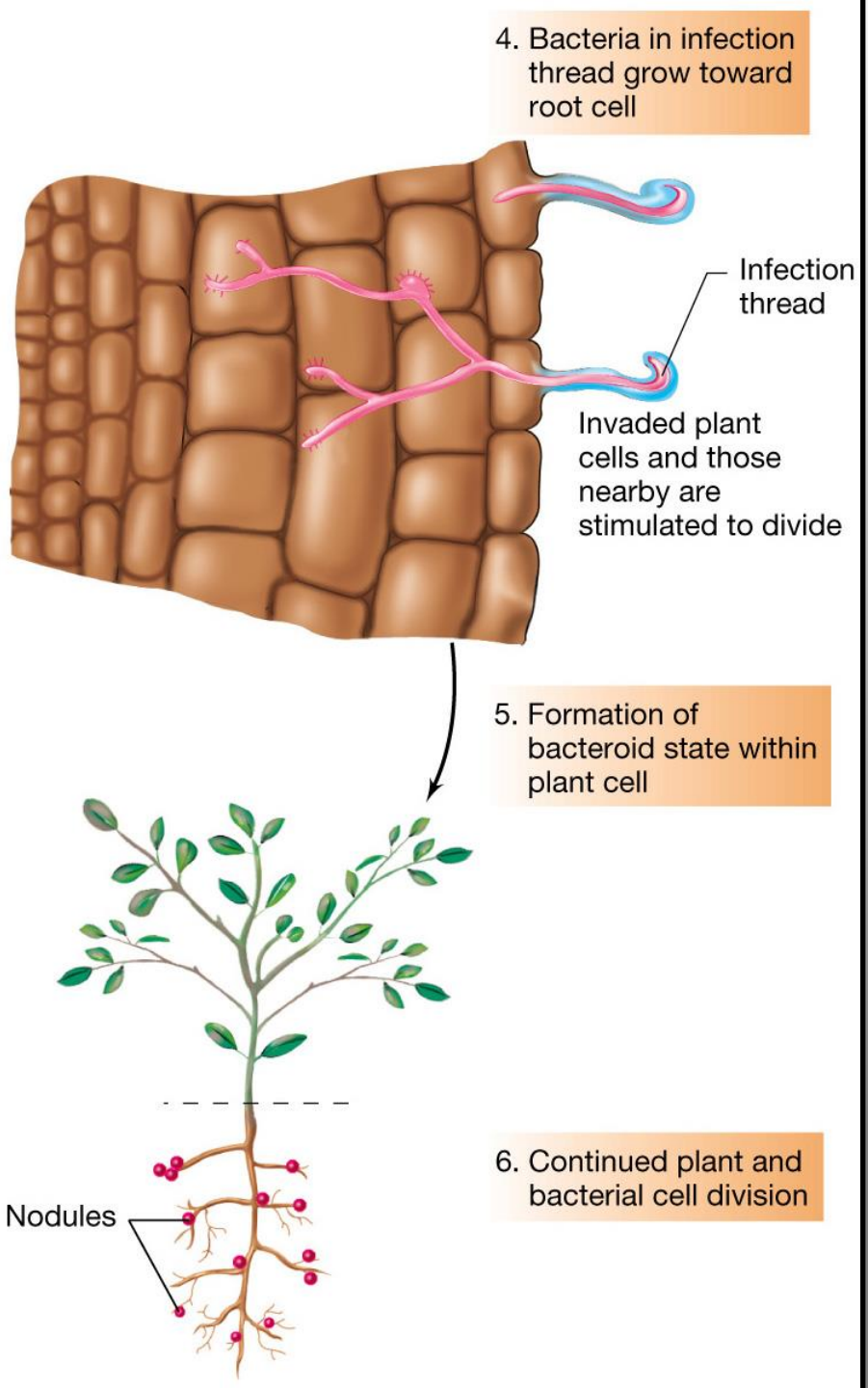

Rhizobium
nod genes induced
Nod factor
Bacterium
Root hair deformation
and bacterial attachment
by rhicadhesins and host lectins
N

1. Recognition and attachment (rhicadhesin-mediated)

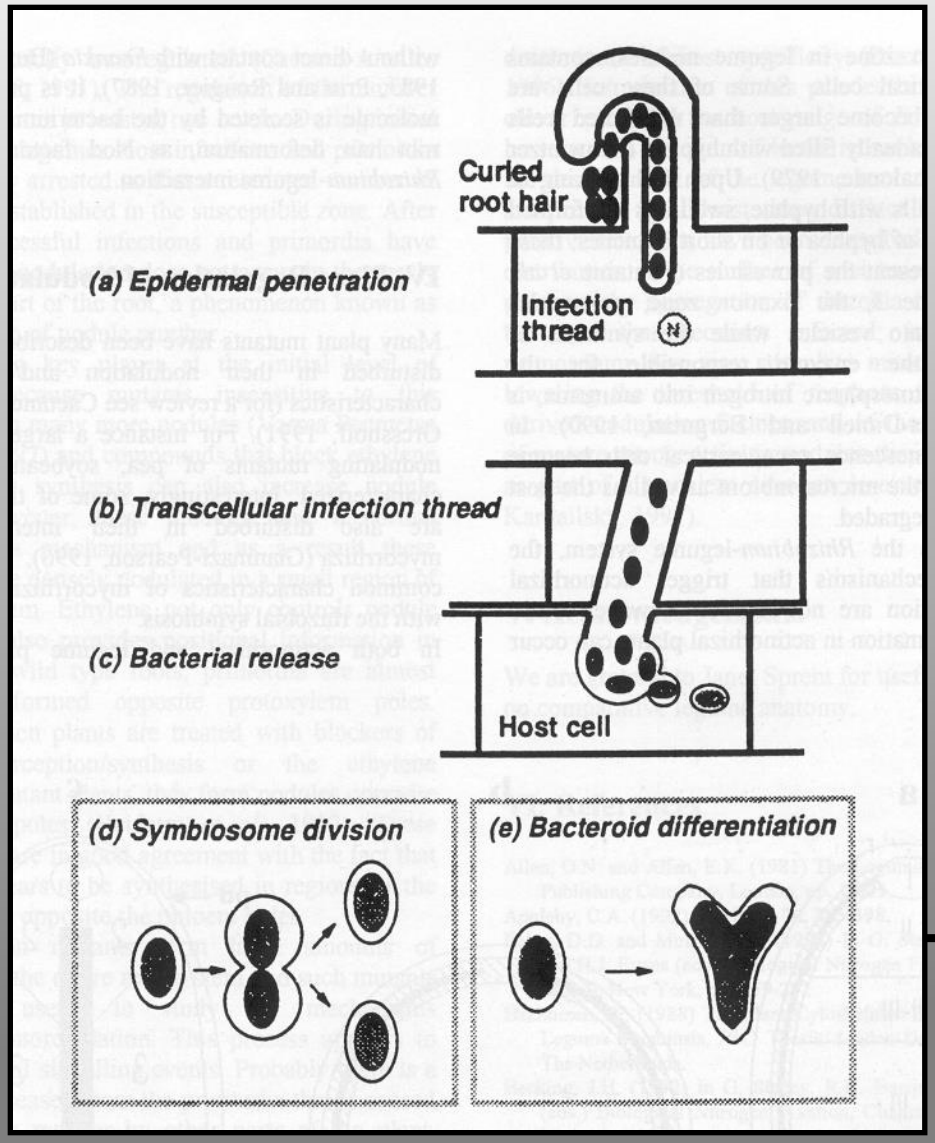


2. Excretion of nod factors by bacterium causing root hair curling

3. Invasion. Rhizobia penetrate root hair and multiply within an "infection thread"


4. Bacteria in infection thread grow toward root cell
Infection thread
Invaded plant cells and those nearby are stimulated to divide
5. Formation of bacteroid state within plant cell
6. Continued plant and bacterial cell division
Nodules

Nodule development



Enlargement of the nodule, nitrogen fixation and exchange of nutrients

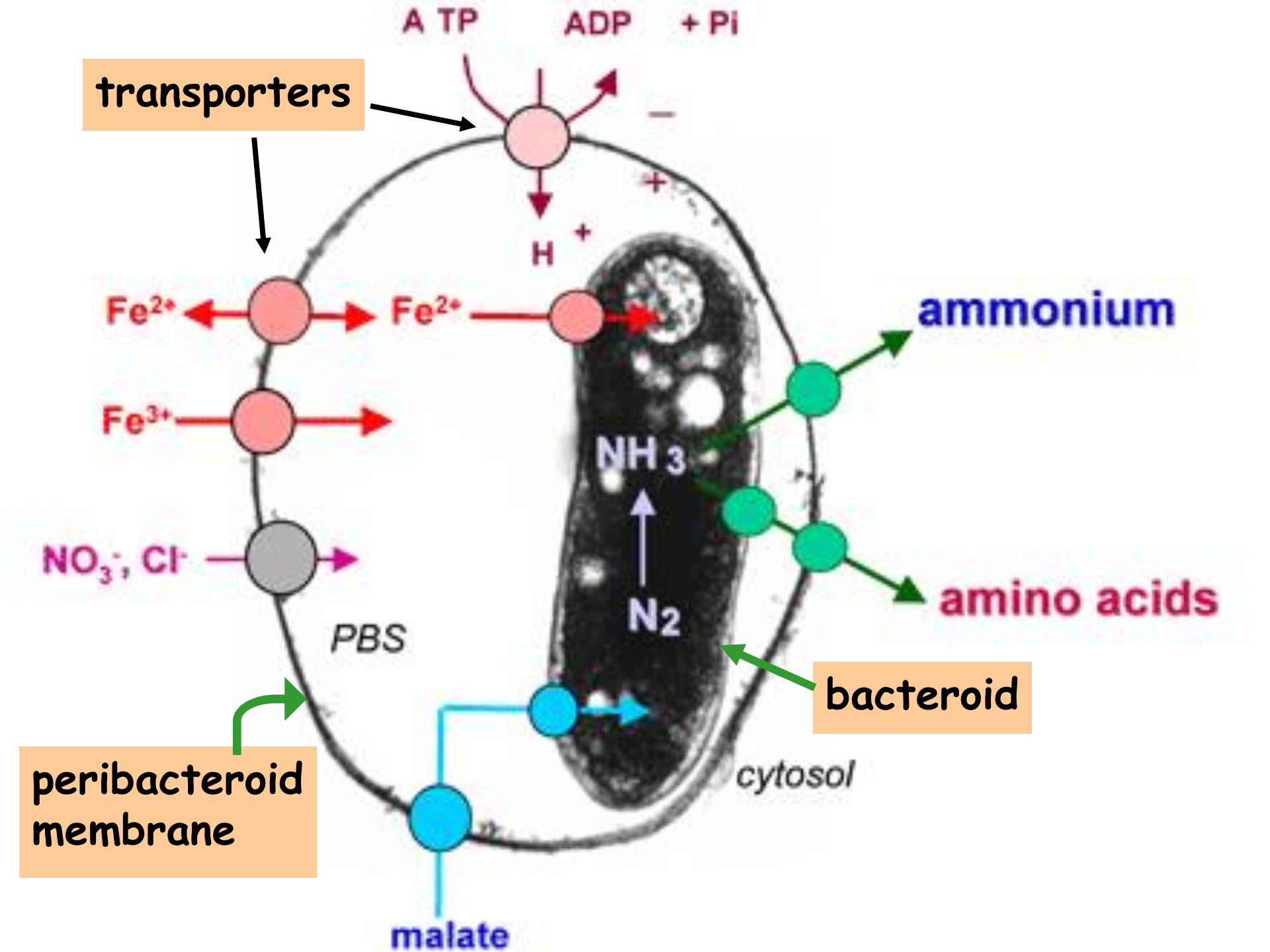
5. Infection thread penetrates through several layers of cortical cells and then ramifies within the cortex. Cells in advance of the thread divide and organize themselves into a nodule primordium.

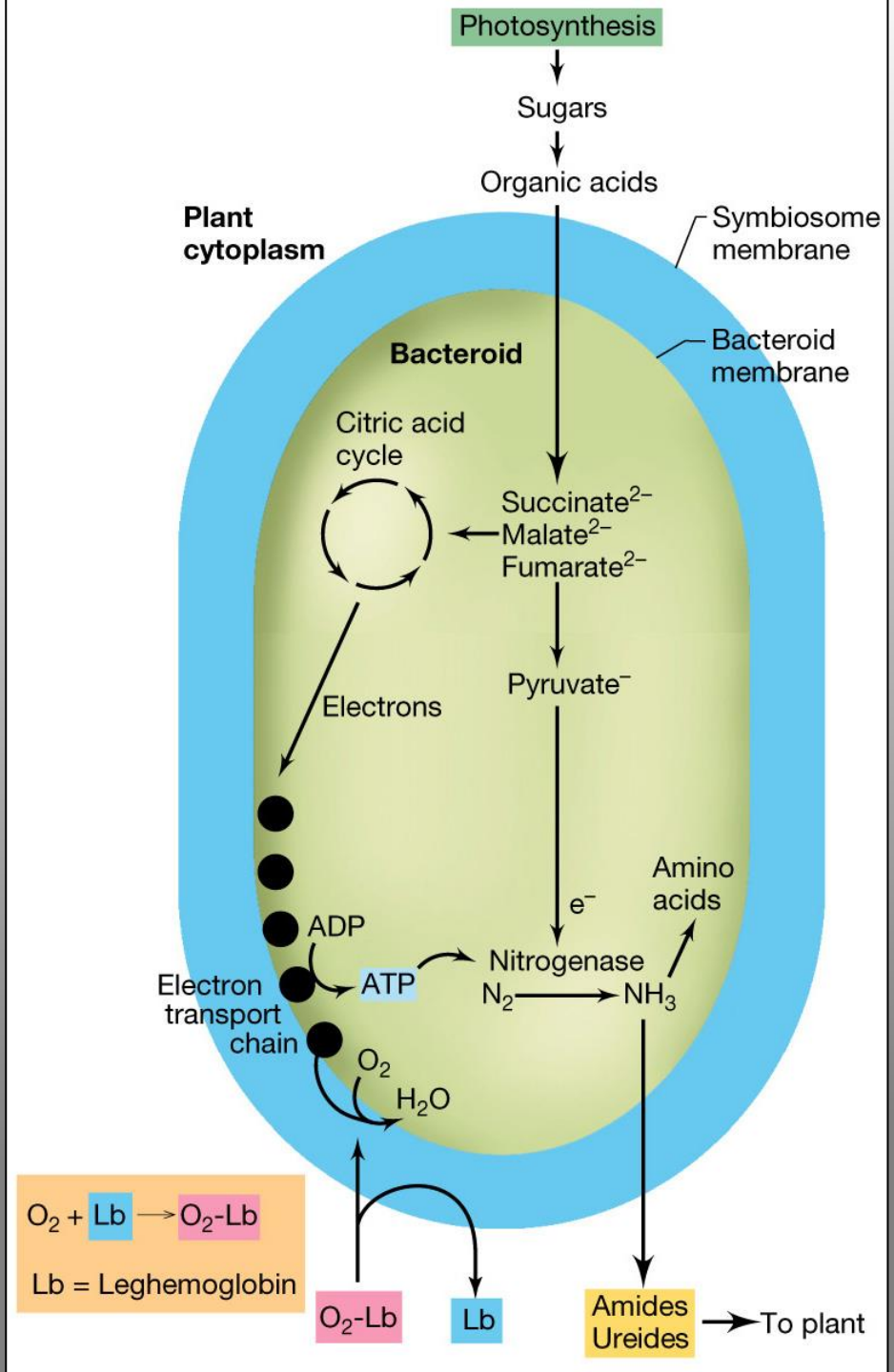
6. The branched infection thread enters the nodule primordium zone and penetrates individual primordium cells.

7. Bacteria are released from the infection thread into the cytoplasm of the host cells, but remain surrounded by the **peribacteroid membrane**. Failure to form the PBM results in the activation of host defenses and/or the formation of ineffective nodules.

8. Infected root cells swell and cease dividing. Bacteria within the swollen cells change form to become endosymbiotic **bacteroids,** which begin to fix nitrogen.

The nodule provides an **oxygen-controlled** environment (leghemoglobin = pink nodule interior) structured to facilitate transport of reduced nitrogen metabolites from the bacteroids to the plant vascular system, and of photosynthate from the host plant to the bacteroids.


A TP
ADP
+ Pi
transporters
H +
Fe2+
Fe2+
Fe3+
NO3-, Cl-
NH 3
N2
ammonium
amino acids
PBS
bacteroid
peribacteroid membrane
cytosol
malate


Photosynthesis
Sugars
Organic acids
Plant cytoplasm
Symbiosome membrane
Bacteroid
Bacteroid membrane
Citric acid cycle
Succinate2–
Malate2–
Fumarate2–
Pyruvate–
Electrons
e–
Amino acids
ADP
ATP
Nitrogenase
N2
NH3
Electron transport chain
O2
H2O
O2 + Lb → O2-Lb
Lb = Leghemoglobin
O2-Lb
Lb
Amides
Ureides
To plant

Types of bacterial functions involved in nodulation and nitrogen fixation

nod (nodulation) and nol (nod locus) genes

mutations in these genes block nodule formation or alter host range

most have been identified by transposon mutagenesis, DNA sequencing and protein analysis, in *R. meliloti*, *R. leguminosarum* bv *viciae* and *trifolii*

fall into four classes:

- nodD
- nodA, B and C (common nodgenes)
- hsn (host-specific nod genes)
- other nod genes

Gene clusters on *R. meliloti* pSym plasmid

(nol) (nod) (nif) (fix)

F G H I N D_1 A B C I J Q P G E F H D_3 E K D H A B C

N M L R E F D A B C I J T C B A H D K E N

Gene clusters on *R. leguminosarum* bv *trifolii* pSym plasmid

--- D_2 D_1 Y A B C S U I J ---

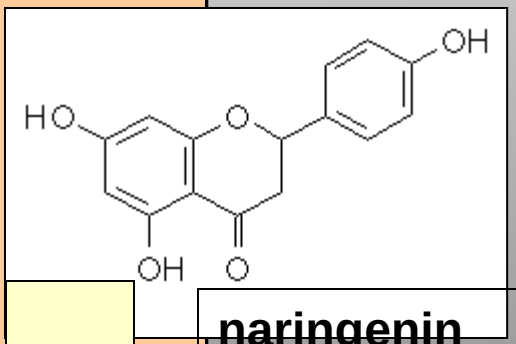
Gene cluster on *Bradyrhizobium japonicum* chromosome

Nod D (the sensor)

the **nod D** gene product recognizes molecules (phenylpropanoid-derived **flavonoids**) produced by plant roots and becomes activated as a result of that binding

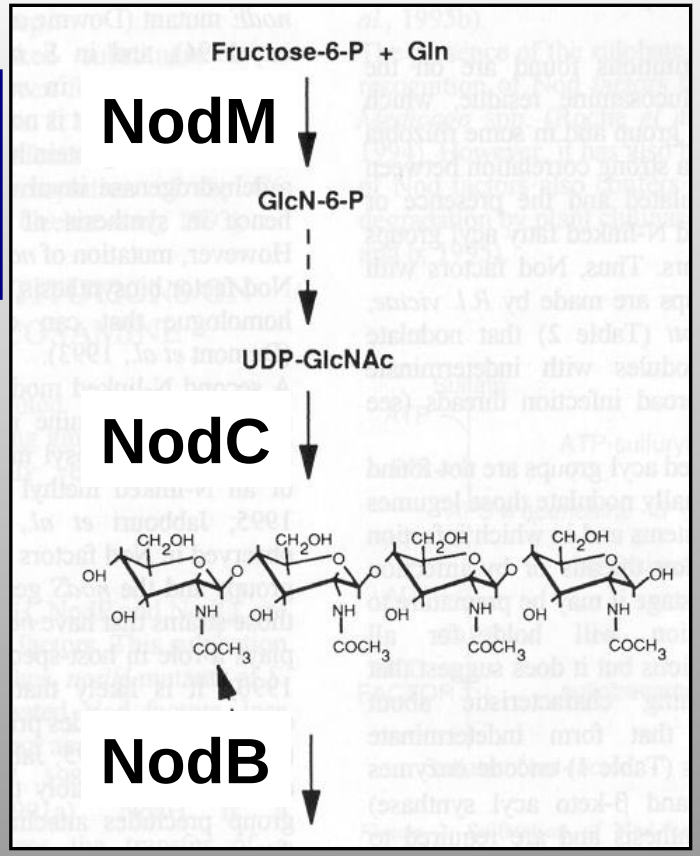
activated nodD protein positively controls the expression of the other genes in the nod gene "regulon" (signal transduction)

different nodD alleles recognize **various flavonoid** structures with different affinities, and respond with differential patterns of nod gene activation

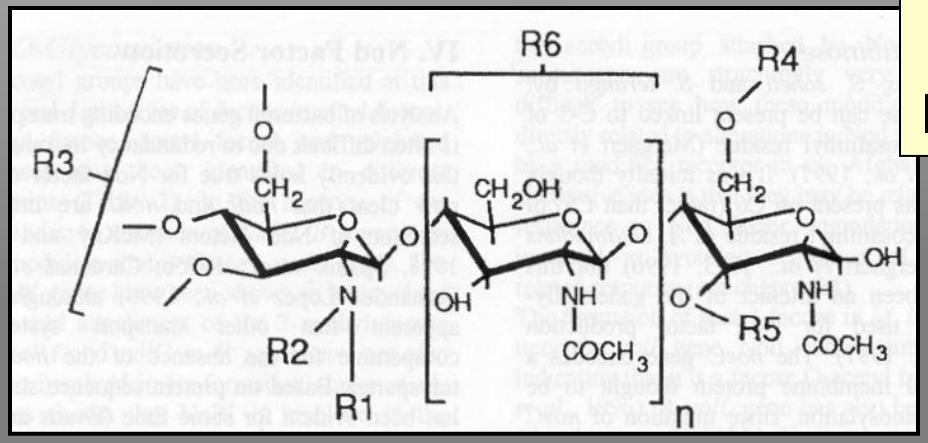


naringenin
(**a flavanone**)

Nod factor biosynthesis



Nod factor R-group "decorations" determine host specificity



Nod Factor: a lipooligosaccharide

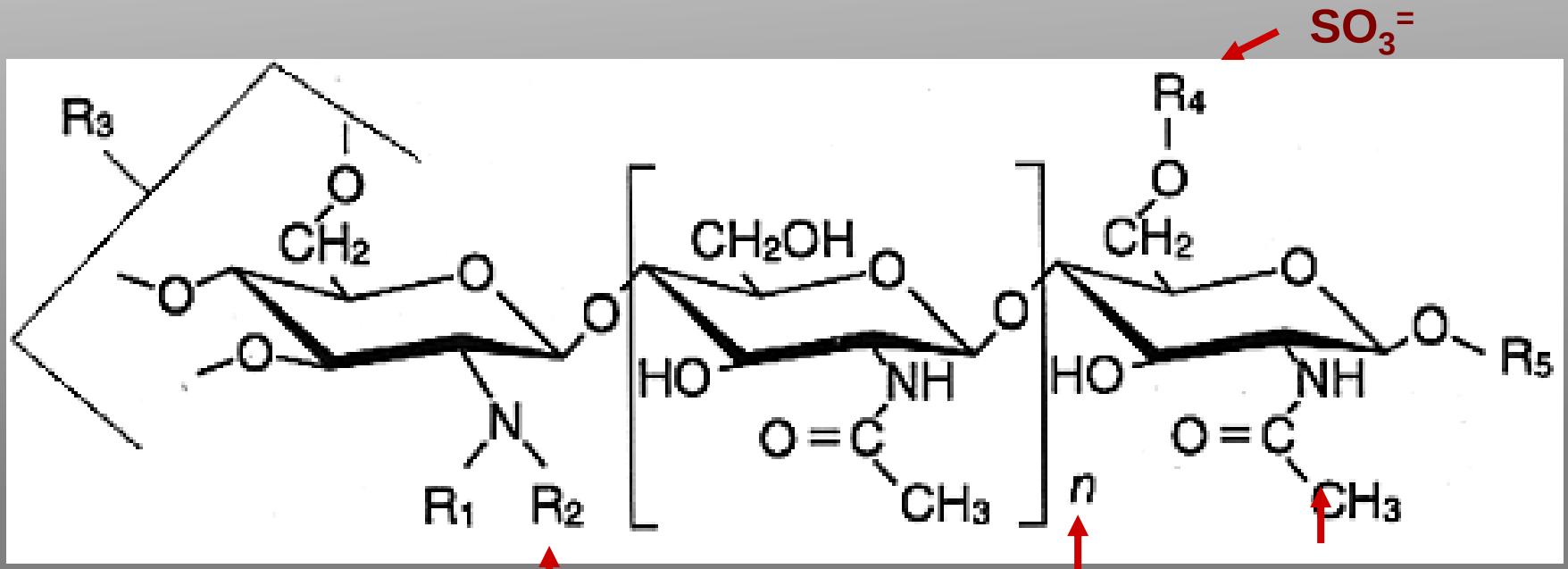
Common nod genes - nod ABC

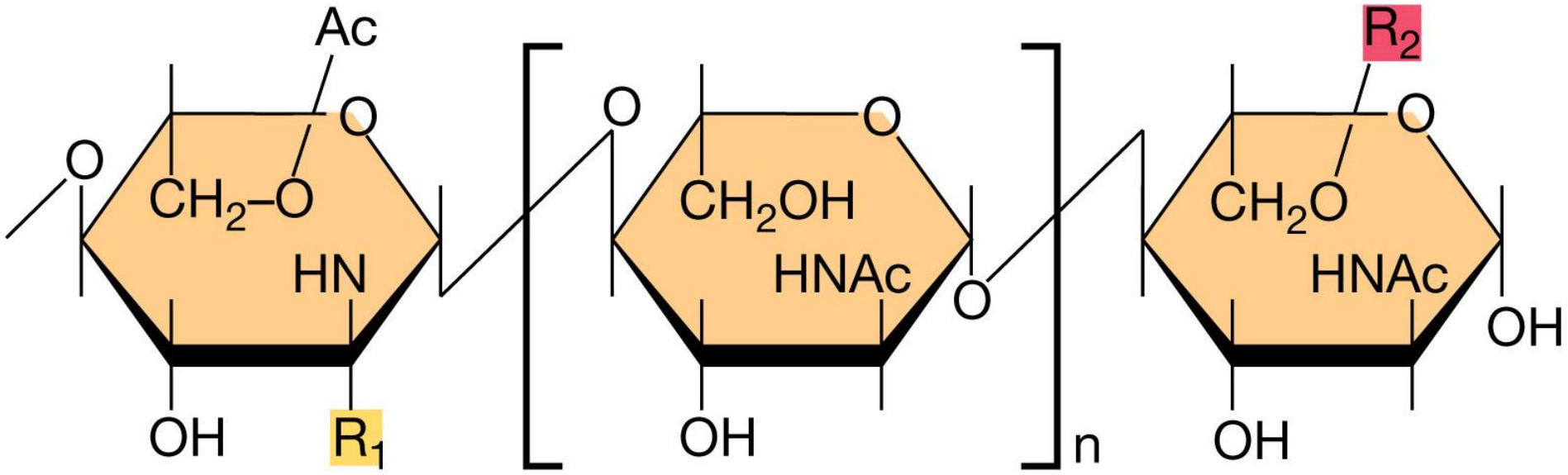
mutations in nodA,B or C completely abolish the ability of the bacteria to nodulate the host plant; they are found as part of the nod gene "regulon" in all Rhizobia (∴ common)

products of these genes **are required** for bacterial induction of root cell hair deformation and root cortical cell division

The **nod ABC** gene products are enzymes responsible for synthesis of diffusible nod factors, whcih are sulfated and acylated *beta*-1,4-oligosaccharides of glucosamine

(other gene products, e.g. NodH, may also be needed for special modifications)





(a)

Species	R_1	R_2
Sinorhizobium meliloti	C16 : 2 or C16 : 3	SO_4^{2-}
Rhizobium leguminosarum biovar *viciae*	C18 : 1 or C18 : 4	H or Ac

(b)

nod factors are active on host plants at very low concentrations (10^{-8} to 10^{-11} M) but have no effect on non-host species

Host-specific nod genes

mutations in these genes elicit abnormal root reactions on their usual hosts, and sometimes elicit root hair deformation reactions on plants that are not usually hosts

Example:
loss of nodH function in *R. meliloti* results in synthesis of a nod factor that is no longer effective on alfalfa but has gained activity on vetch

The ΔnodH nod factor is now more hydrophobic than the normal factor - no sulfate group on the oligosaccharide.

The role of the nodH gene product is therefore to add a specific sulfate group, and thereby change host specificity

Other nod genes

May be involved in the attachment of the bacteria to the plant surface, or in export of signal molecules, or proteins needed for a successful symbiotic relationship

exo (exopolysaccharide) genes

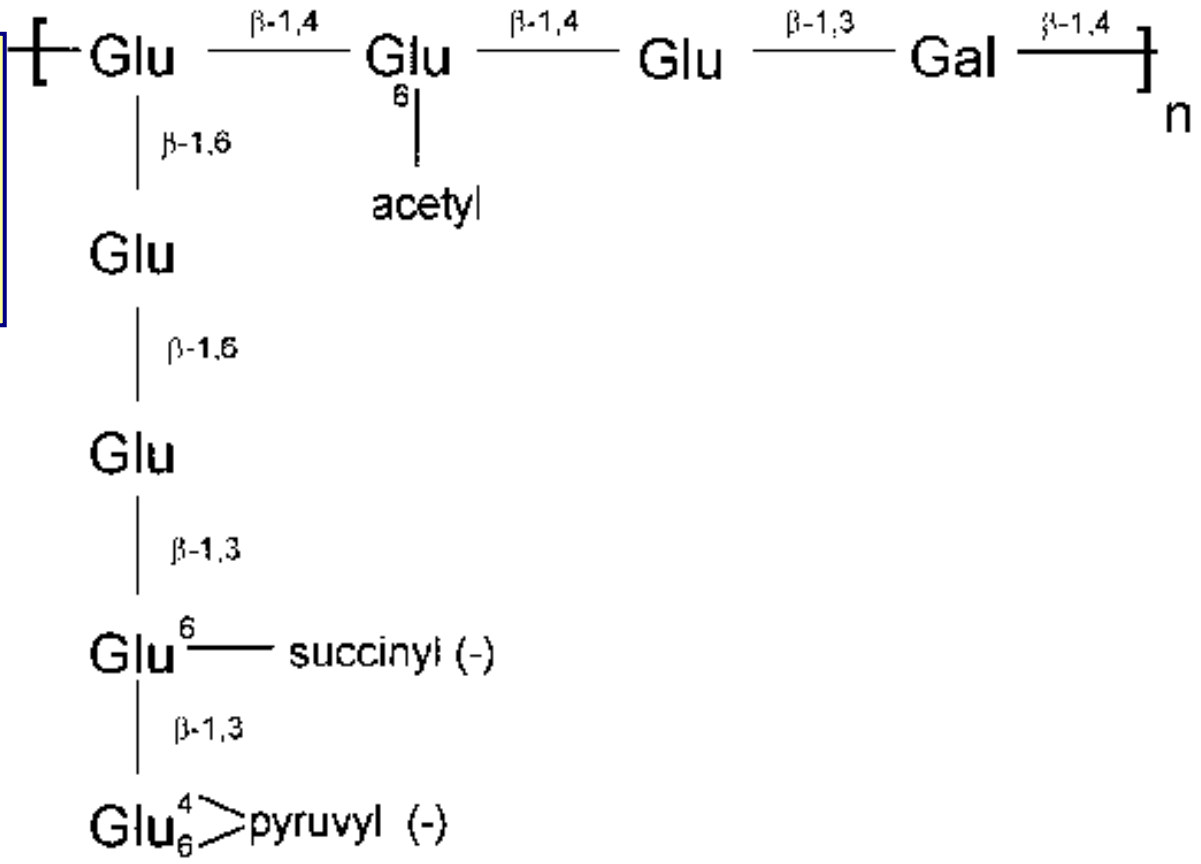
Encode proteins needed for exopolysaccharide synthesis and secretion

In *Rhizobium*-legume interactions that lead to indeterminate nodules, exo mutants cannot invade the plant properly. However, they do provoke the typical plant cell division pattern and root deformation, and can even lead to nodule formation, although these are often empty (no bacteroids).
In interactions that usually produce **determinate** nodules, exo mutations tend to have no effect on the process.

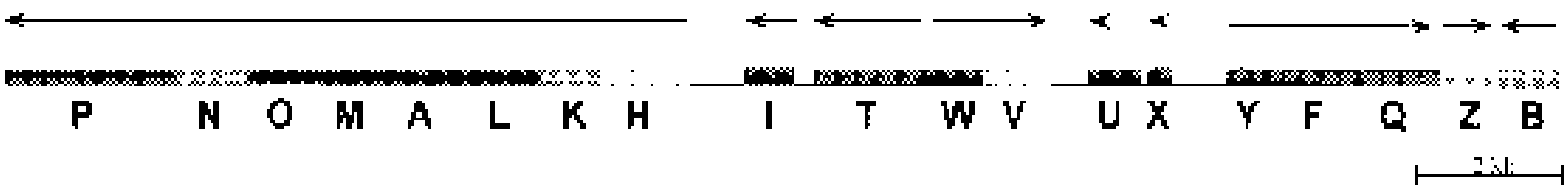
Exopolysaccharides may provide substrate for signal production, osmotic matrix needed during invasion, and/or a recognition or masking function during invasion

example of Rhizobial exopolysaccharide

Succinoglycan



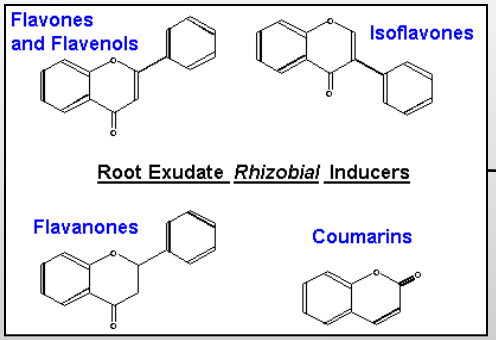
Map of the *exo* Gene Cluster



Functions of the *exo* gene products

- addition of first galactose to lipid carrier
- glucosyltransferase
- octamer modification
- nucleotide sugar biosynthesis
- polymerization or transport
- glycanase
- putative regulatory protein
- function unknown

Sinorhizobium meliloti



nod-gene inducers from alfalfa roots (specificity)

NodD

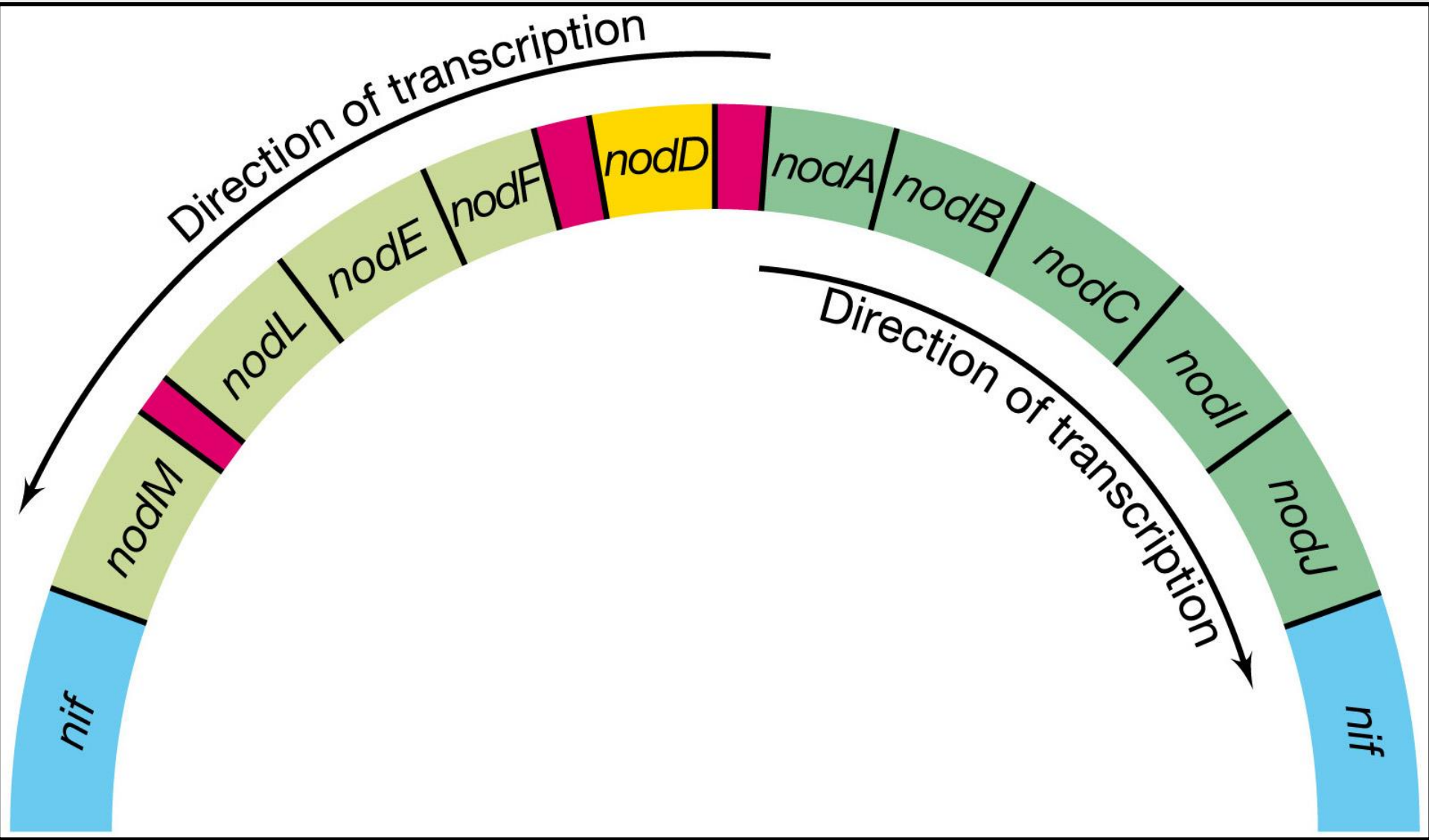
chromosome

plasmid

pSym

activated NodD positively regulates nod genes

nod genes


Direction of transcription
nodM
nodL
nodE
nodF
nodD
nodA
nodB
nodC
nodI
nodJ
nif
nif
Direction of transcription

nif (nitrogen fixation) genes

Gene products are required for symbiotic nitrogen fixation, and for nitrogen fixation in free-living N-fixing species

Example: subunits of nitrogenase

Table 1. The *nif-gene* Products and Their Role (Known or Proposed) in Nitrogen Fixation

nif-GENE	IDENTITY/ROLE
nifH	Dinitrogenase reductase. Obligate electron donor to dinitrogenase during nitrogenase turnover. Also is required for FeMo-co biosynthesis and apodinitrogenase maturation
nifD	α subunit of dinitrogenase. Forms an $a_2ß_2$ tetramer with ß subunit. FeMo-co, the site of substrate reduction, is present buried within the α subunit of dinitrogenase
nifK	ß subunit of dinitrogenase. P-clusters are present at the ß subunit-interface
nifT	Unknown
nifY	In *K. pneumoniae,* aids in the insertion of FeMo-co into apodinitrogenase
nifE	Forms $a_2ß_2$ tetramer with NifN. Required for FeMo-co synthesis. Proposed to function as a scaffold on which FeMo-co is synthesized
nifN	Required for FeMo-co synthesis
nifX	Involved in FeMo-co synthesis. Specific role is not known
nifU	Involved in mobilization of Fe for Fe-S cluster synthesis and repair
nifS	Involved in mobilization of S for Fe-S cluster synthesis and repair
nifV	Homocitrate synthase, involved in FeMo-co synthesis
nifW	Involved in stability of dinitrogenase. Proposed to protect dinitrogenase from 0_2 inactivation
nifZ	Unknown
nifM	Required for the maturation of NifH
nifF	Flavodoxin. Physiologic electron donor to NifH
nifL	Negative regulatory element
nifA	Positive regulatory element
nifB	Required for FeMo-co synthesis. Metabolic product, NifB-co is the specific Fe and S donor to FeMo-co
fdxN	Ferredoxin. In *R. capsulatus,* serves as electron donor to nitrogenase
nifQ	Involved in FeMo-co synthesis. Proposed to function in early MoO_4^{2-} processing
nifJ	Pyruvate:flavodoxin (ferredoxin) oxidoreductase. Involved in electron transport to nitrogenase

fix (fixation) genes

Gene products required to successfully establish a functional N-fixing nodule.
No **fix** homologues have been identified in free-living N-fixing bacteria.

Example: regulatory proteins that monitor and control oxygen levels within the bacteroids

FixL senses the oxygen level; at low oxygen tensions, it acts as a **kinase** on **FixJ**, which regulates expression of two more transcriptional regulators:

NifA, the upstream activator of nif and some fix genes;

FixK, the regulator of *fixN* (another oxgen sensor?)

This key transducing protein, FixL, is a novel hemoprotein kinase with a complex structure. It has an N-terminal membrane-anchoring domain, followed by the heme binding section, and a C-terminal kinase catalytic domain.

Result?

Low oxygen tension activates nif gene transcription and permits the oxygen-sensitive nitrogenase to function.

Metabolic genes and transporters

Dicarboxylic acid (malate) transport and metabolism

Genes for other functions yet to be identified….

⇒ DNA microarray analysis of gene expression patterns

⇒ Proteomic analysis of bacteroids and peribacteroid membrane preparations

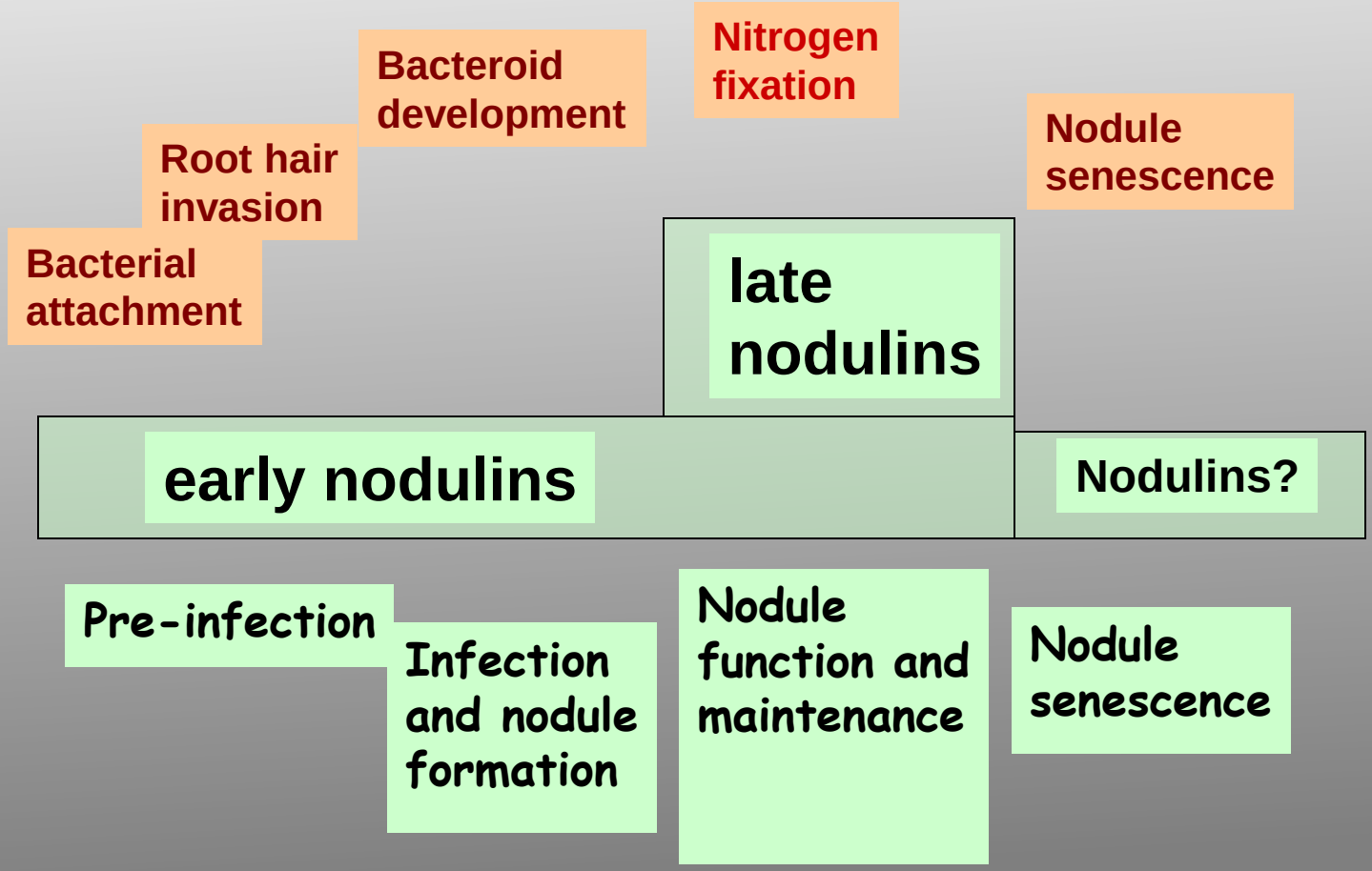
Host plant role in nodulation

1. Production and release of nod gene inducers
   - **flavonoids**

2. Activation of plant genes specifically required for successful nodule formation - **nodulins**

3. Suppression of genes normally involved in repelling microbial invaders - **host defense genes**

Nodulins


Bacterial attachment
Root hair invasion
Bacteroid development
Nitrogen fixation
Nodule senescence
late nodulins
early nodulins
Nodulins?
Pre-infection
Infection and nodule formation
Nodule function and maintenance
Nodule senescence

Early nodulins

At least 20 nodule-specific or nodule-enhanced genes are expressed in plant roots during nodule formation; most of these appear after the initiation of the visible nodule.

Five different nodulins are expressed only in cells containing growing infection threads.
These may encode proteins that are part of the plasmalemma surrounding the infection thread, or enzymes needed to make or modify other molecules

Twelve nodulins are expressed in root hairs and in cortical cells that contain growing infection threads. They are also expressed in host cells a few layers ahead of the growing infection thread.

Late nodulins

The best studied and most abundant late nodulin is the protein component of **leghemoglobin**.
The **heme** component of leghemoglobin appears to be synthesized by the bacteroids.

Other **late nodulins** are enzymes or subunits of enzymes that function in nitrogen metabolism (glutamine synthetase; uricase) or carbon metabolism (sucrose synthase). Others are associated with the peribacteroid membrane, and probably are involved in transport functions.

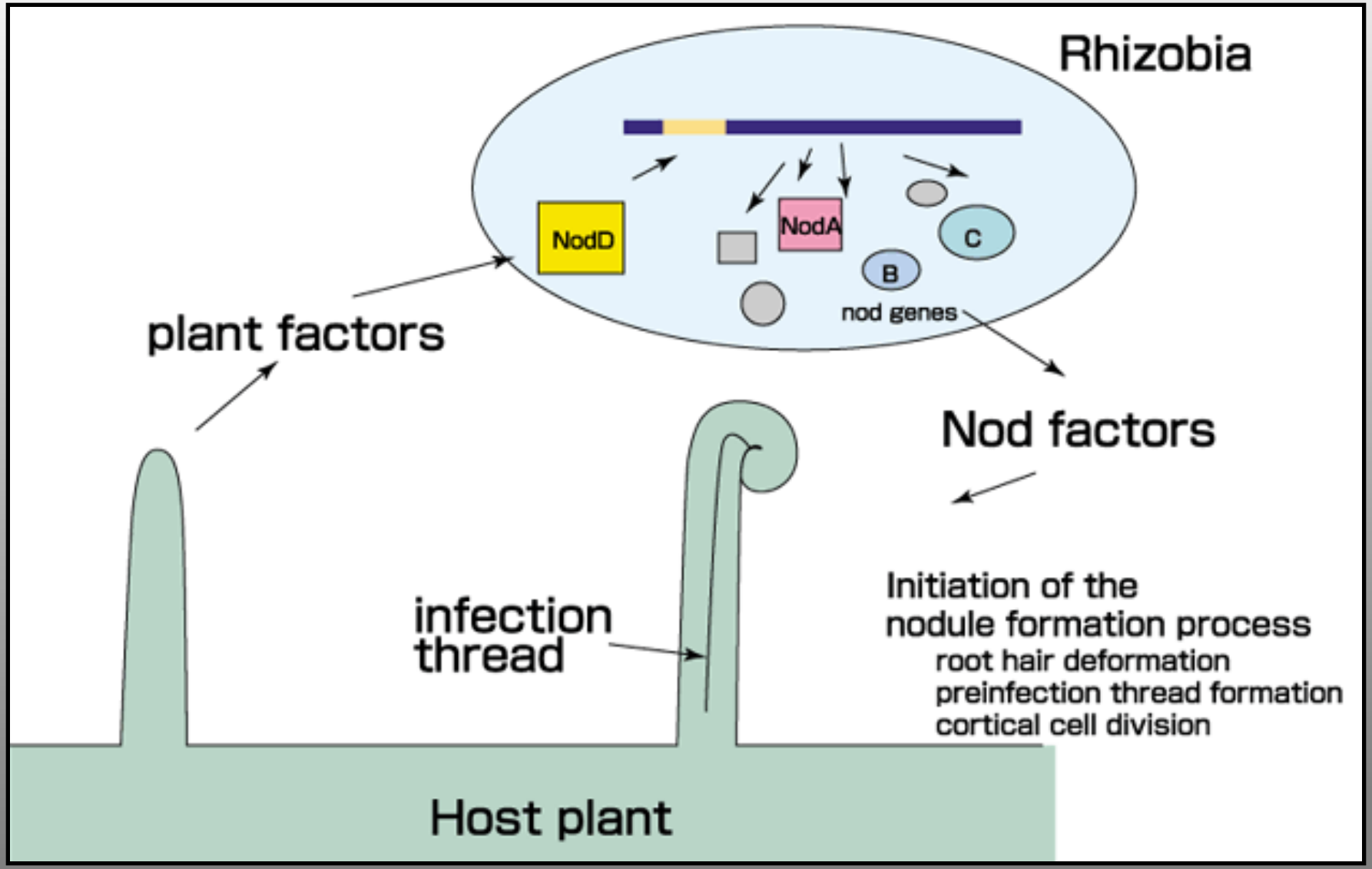
These late nodulin gene products are usually not unique to nodule function, but are found in other parts of the plant as well. This is consistent with the hypothesis that nodule formation evolved as a specialized form of root differentiation.

There must be **many other host gene functions** that are needed for successful nodule formation.

Example: what is the **receptor** for the nod factor?

These are being sought through genomic and proteomic analyses, and through generation of plant mutants that fail to nodulate properly

The full genome sequencing of *Medicago truncatula* and *Lotus japonicus* , both currently underway, will greatly speed up this discovery process.


Rhizobia
NodD
NodA
C
B
nod genes
plant factors
Nod factors
infection thread
Initiation of the nodule formation process
root hair deformation
preinfection thread formation
cortical cell division
Host plant

A plant receptor-like kinase required for both bacterial and fungal symbiosis

S. Stracke *et al* Nature 417:959 (2002)

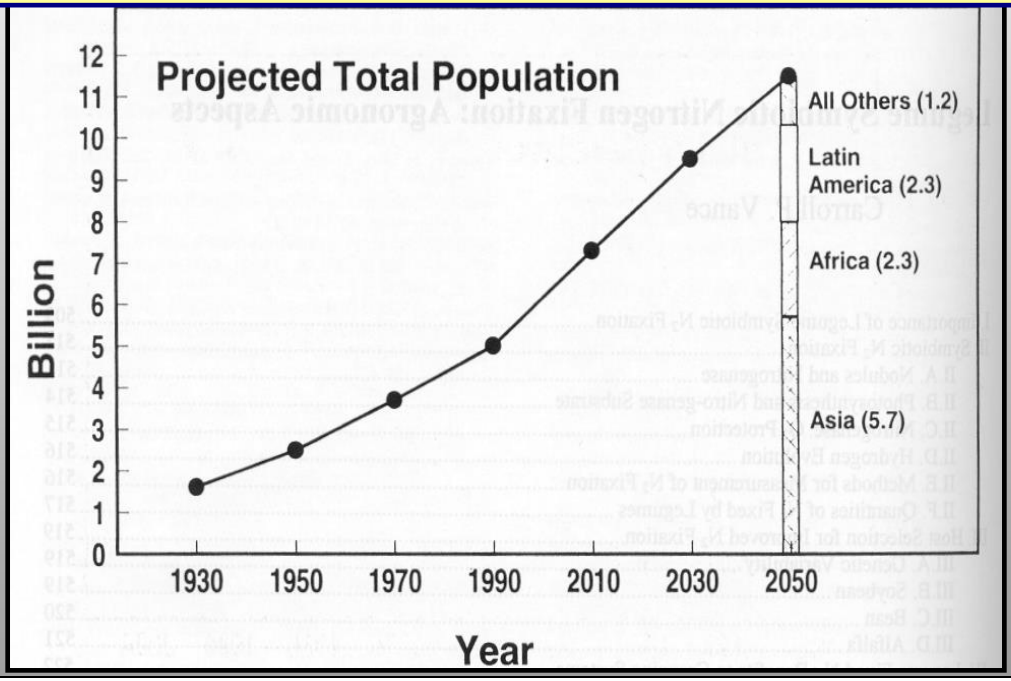
Screened mutagenized populations of the legume *Lotus japonicus* for mutants that showed an inability to be colonized by VAM

Mutants found to also be affected in their ability to be colonized by nitrogen-fixing bacteria ("symbiotic mutants")

Stem-nodulating bacteria

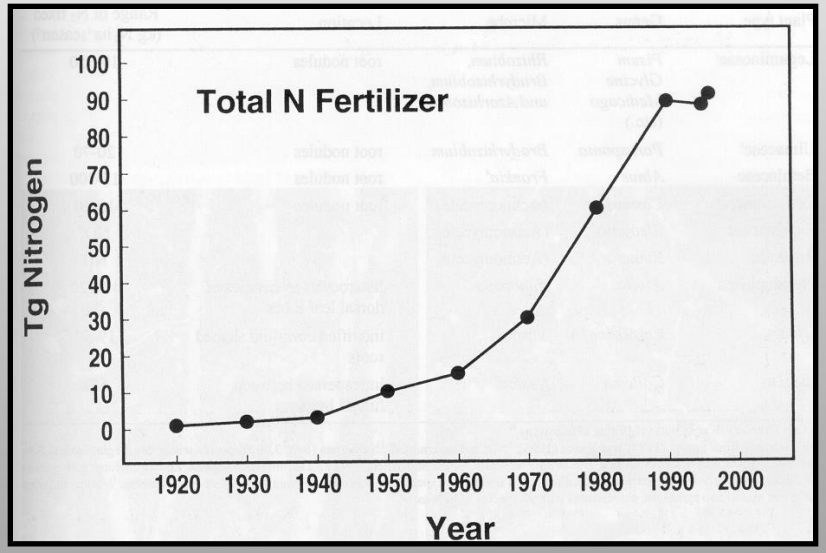
- observed primarily with tropical legumes

A growing population must eat!



- **Combined nitrogen is the most common limiting nutrient in agriculture**
- **Estimated that 90% of population will live in tropical and subtropical areas where (protein-rich) plant sources contribute 80% of total caloric intake.**
- **In 1910 humans consumed 10% of total carbon fixed by photosynthesis, by 2030 it is predicted that 80% will be used by humans.**

Why chemical fertilizers aren't the answer



The Haber-Bosch process

$N_2 + H_2 \longrightarrow 2NH_3$

300 to 1000 bar pressure

400 to 600 C

Catalyst

Consumes 1.4% of total fossil fuels annually

Electrical discharge

$N_2 + O_2 + \text{lightning} \longrightarrow NO_x$

$NO_x + H_2O \longrightarrow HNO_2 \text{ or } HNO_3$

- **Production of nitrogenous fertilizers has "plateaued" in recent years because of high costs and pollution**

- **Estimated 90% of applied fertilizers never reach roots and contaminate groundwater**

Current approaches to improving biological nitrogen fixation

1. **Enhancing survival of nodule forming bacterium by improving competitiveness of inoculant strains**

2. **Extend host range of crops, which can benefit from biological nitrogen fixation**

3. **Engineer microbes with high nitrogen fixing capacity**

What experiments would you propose if you were to follow each of these approaches?