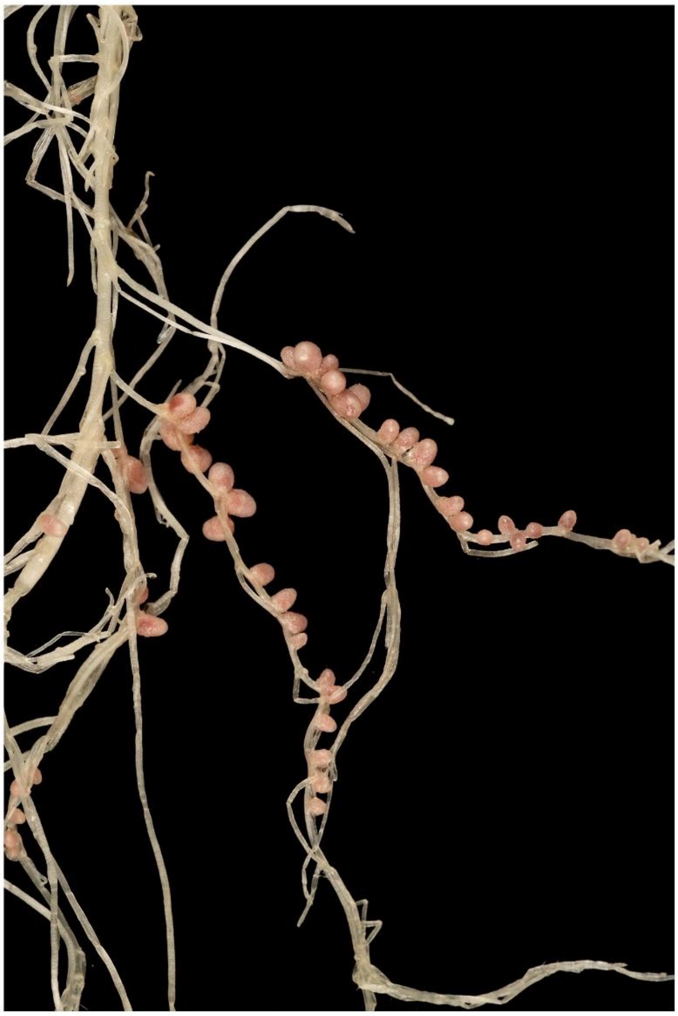
Microbial nitrogen Fixation

Pam: What are those strange pink bumps on the roots of our pea plants? Do all plants have them?



Nitrogen-fixing nodules on the root of a pea plant

Allan Downie

Department of Molecular Microbiology, John Innes Centre, Norwich, U.K.

Nitrogen Fixation

Storyline

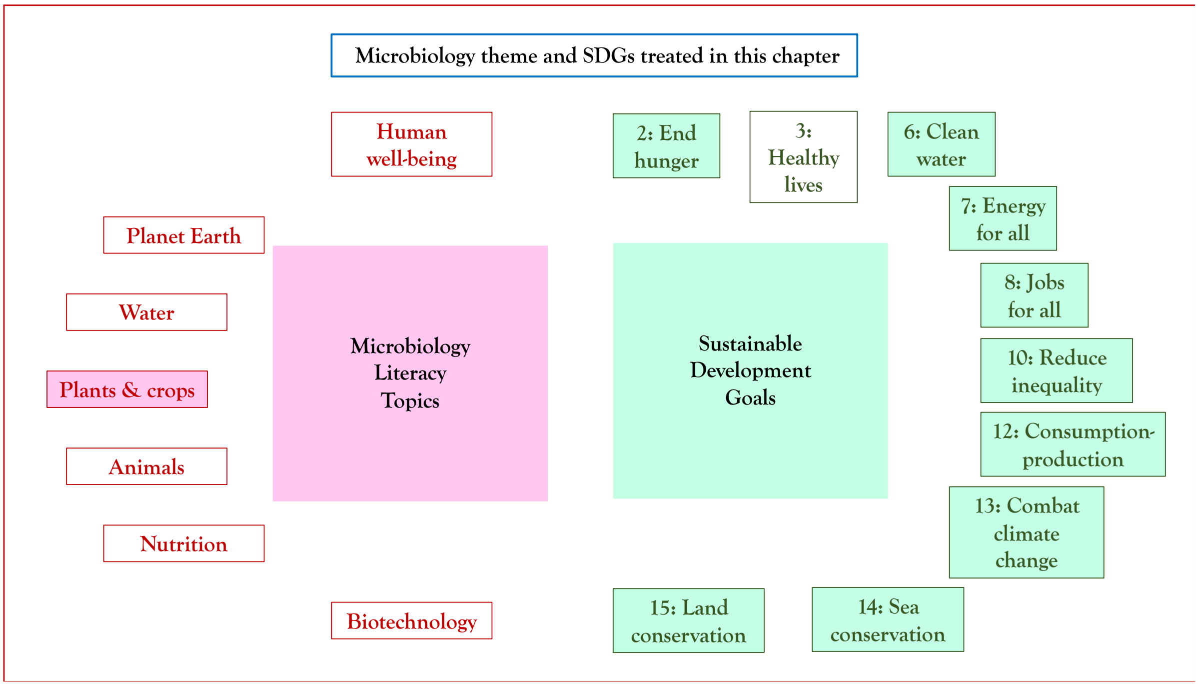
Nitrogen is an essential element for all life forms because it is required to produce proteins, nucleic acids and chlorophyll, the most important pigment needed for photosynthesis. Although nitrogen gas (N_2) makes up about 78% of our atmosphere, it cannot be used in this form by any organisms except some specialist bacteria. These 'nitrogen fixing' bacteria can break the triple bond linking the two nitrogen atoms in N_2 by reduction (adding hydrogen) to produce two molecules of ammonia (NH_3), which then gets incorporated into amino acids to form proteins and ultimately nucleic acids, **chlorophyll** and other N-containing compounds.

A sub-group of these nitrogen-fixing bacteria called **rhizobia** can form a **symbiosis** with certain plants, called **legumes**, in which the nitrogen-fixing rhizobia provide ammonia to the plant in exchange for a carbon and energy source. In legumes like peas and beans, rhizobia infect the plant roots and then grow in specialised plant-made structures called **nodules**, that form as bumps on the roots when the rhizobia invade the roots. The plants ensure the environment within the nodules is ideal for the bacteria to reduce nitrogen to **ammonia**, a process that is both difficult and requires a lot of energy. The bacterial growth and energy requirements are met by the plant supplying carbon from photosynthesis; in return, the rhizobia supply the plant with nitrogen fertiliser in the form of **ammonium** which is formed when ammonia is in water. The net result is a symbiosis in which the legumes benefit because they grow better and the numbers of rhizobia in the soil increase after the plants die off (or are harvested). Additional benefits are:

- *legume grains such as peas, lentils and soybean are high in protein, so excellent as food for humans and farm animals*
- *residual N in legume roots, leaves and stems becomes incorporated into the soil*
- *nitrogen leaching from the soil into the wider environment is minimised*
- *the enhanced growth of legume roots and exudate from them stimulates the growth of diverse microorganisms around legume roots increasing soil fertility.*

In addition to the symbiosis between legumes and rhizobia, there are some other symbiotic interactions in which other nitrogen-fixing bacteria transfer ammonia to plants and photosynthetic microorganisms in exchange for carbon compounds that ultimately come from photosynthesis. Some nitrogen-fixing bacteria are even able to carry out their own photosynthesis to produce carbon compounds that provide energy for nitrogen fixation and growth.

The microbiology: bacterial infection of roots leads to enhanced plant growth associated with nitrogen fixation. The rhizobial-legume symbiosis along with other nitrogen-fixing symbioses and nitrogen fixation by free-living bacteria, all play a key role in the **nitrogen cycle**, by taking nitrogen gas from the atmosphere converting it to ammonia which can be used for plant growth.
Sustainability issues: **biological nitrogen fixation** enhances crop yields and minimises the release into the wider environment of reactive nitrogen in the form of ammonia or **oxides of nitrogen** that can contribute to global warming, **eutrophication** of oceans and lakes or production of algal blooms, all of which can be caused by overuse of chemical nitrogen fertilisers. Biological nitrogen fixation also helps produce high protein grains such as peas, beans, soybeans, lentils and chickpeas, that are particularly important as foods in impoverished regions.



Nitrogen Fixation: The Microbiology

1. ***Legumes and the nitrogen-fixing bacteria that infect their roots have shaped agronomic practises for thousands of years.*** It has been known for over two thousand years that if crop plants are grown in land previously used to grow legumes, the harvest yield of the following crop is significantly improved. This was discovered independently in Asia, where rice was grown after soybeans, in the middle east, where wheat or barley was grown after peas or lentils, and in the Americas, where maize was grown after (or with) blackbeans. It was also well known that most legumes produced small bumps on their roots and it was recognised in the late 1800s that these small bumps were full of bacteria that were named *Rhizobium* species (generically referred to as '**rhizobia**'). It was shown that these bacteria take nitrogen gas (N_2) from the atmosphere and convert it into a form (**ammonia**, NH_3) that reacts with water to make ammonium, which can be used by the plant. This process is called '**symbiotic nitrogen fixation**' and plays an important part in the global **nitrogen cycle**; it promotes the growth of both the legumes and of subsequent crops planted in the fields where legumes had been grown, thereby helping to establish intensive agriculture.

2. ***Specific bacterial strains are required for different legumes: the start of an industry that produces rhizobia to be added to fields where legumes are grown.*** The advantages of legumes were so great that legumes were moved between continents to increase farm productivity. However, initially crops like soybeans did not grow as well in America, Australia or Europe as in their native habitat in Asia. Similarly, clover or alfalfa taken from Europe to Australia did not grow well. The problem was that, although the different regions of the world had their own legumes with their own native rhizobia in the soils, these native rhizobia were not able to carry out efficient nitrogen fixation on the introduced legumes. This issue was resolved when rhizobia isolated from the nodules of legumes grown in their native regions were cultured and then grown in large amounts and inoculated into the soil. It quickly became evident that whereas one type of rhizobial bacterium could be used to form nitrogen-fixing nodules on some groups of legumes, a different rhizobial bacterium would be required for other legumes. For example, rhizobia isolated from pea nodules will not form a symbiosis with soybean plants. So an **inoculum** industry was established to identify those rhizobial strains that induced optimal nitrogen fixation on different legumes. These rhizobia are now cultured and stored in ways that enable their use as soil inoculants, resulting in increased legume crop yields in soils that lacked or had few of the appropriate rhizobia.

The pea plant on the left was grown without a rhizobial inoculant; its roots lack nodules and its leaves have grown less well and show signs of N-limitation. The pea on the right was inoculated and has formed about 100 nitrogen-fixing nodules on the roots; (two clumps of nodules are highlighted in yellow boxes) and the leaves have grown more and look healthier.

3. ***Nitrogen fixation by rhizobia not only benefits the plant.*** The grain seeds (e.g. from soybeans, lentils, beans and peas) and leaves of fodder crops (such as alfalfa and clover) have high protein content. For example, soybean seeds contain about 40% protein compared with 10-15% protein in maize or wheat seeds, and this protein is important in human and animal nutrition. An additional advantage is that crops grown in **crop rotations** after legumes have increased

growth yields, mostly due to nitrogen that was released from legume roots and **crop residues** as they slowly degraded in the soil.

4. ***Legume nodules provide an ideal environment for nitrogen fixation.*** Microscopy revealed that legume nodules were full of rhizobia and that most of these bacteria were actually within plant cells. These nitrogen-fixing rhizobia have **differentiated**, increasing their size and are referred to as **bacteroids**. *In some regards, these rhizobial bacteroids behave a bit like **organelles** such as **mitochondria** or **chloroplasts**, that evolved from incorporated bacteria into organelles millions of years ago. However instead of working with the gases oxygen or carbon dioxide from the atmosphere (as used by mitochondria or chloroplasts, respectively), the rhizobial bacteroids work with nitrogen gas.* N_2 is relatively inert and only one enzyme has been identified that can react with it. This enzyme is called **nitrogenase,** and it sequentially adds protons and electrons to each molecule of N_2, finally producing two molecules of NH_3 (ammonia) that are then released into the plant cell.

Nitrogenase is an enzyme complex that is very sensitive to oxygen, which inactivates it. The legume accommodates this limitation by restricting the flow of oxygen into nodules, and by producing in the rhizobially-infected plant cells, large amounts of a protein called **leghaemoglobin.** This is similar to **haemoglobin** in blood and you can see a characteristic pink-red colour if you cut a nitrogen-fixing nodule in half. The leghaemoglobin in nodules mops up **free oxygen** and then releases it in such a way as to keep the free oxygen levels low enough not to inactivate the nitrogenase (in essence the leghaemoglobin acts as an oxygen buffer). Another limitation of nitrogenase is that it requires a large amount of energy and the plants provide a steady supply of carbon compounds that the bacteria can use to produce energy in the form of **ATP**. To do this the bacteria use a special **respiratory system** with a **cytochrome oxidase** that can work with the very low levels of oxygen released in nodule cells by the leghaemoglobin.

A pea nodule cut in half showing the red colour of the plant-made leghaemoglobin that is required for symbiotic nitrogen fixation. The root is at the left and the growing tip of the nodule is at the right.

5. **Both legumes and rhizobia benefit from the symbiosis**. Each nodule formed on a root is usually filled with many rhizobial cells that all result from an infection initiated by a single bacterial cell (i.e. the bacteria within a nodule are usually **clonal**). A single nodule can contain 10^8 (100 million) rhizobial cells all derived from the one bacterium that infected the nodule. Even if only a very low proportion of these cells escape back to the soil, then the bacterial numbers in soil increase. For example, if only 0.001% (one in a hundred thousand) of the bacteria in such a nodule get released into the soil, then that is equivalent to the one bacterial cell that clonally infected the nodule, producing one thousand progeny that get released into the soil in one growing season. These rhizobia that escape from nodules enter the soil population and so have the potential to infect and nodulate the growing roots of young legumes.

The bacterial growth in the nodules is supported by the plant, which supplies carbon mostly in the form of the organic acid **malate**, that is generated in the root nodules from sucrose that came from photosynthesis in the leaves. This supply of carbon from the plant depends on the rhizobia providing ammonia; if the rhizobia in the nodule do not supply ammonia to the legume, then the legume shuts down the nodule, inducing lysis that kills most of the bacteria.

This is important in nature because the nodule provides such a good potential growth niche, that other bacteria try to benefit from it. These might be rhizobia that do not fix nitrogen on that host plant, but also might be other (non-nitrogen-fixing) bacteria that sometimes co-infect nodules alongside a nitrogen-fixing rhizobial strain. Such bacteria that try to freeload on the back of the nitrogen-fixing rhizobia are referred to as 'cheaters' – they try to get a free lunch, and the plant has many sanctions to try to prevent nodules being infected by cheaters.

6. **Rhizobia and legumes have several levels of mutual recognition systems that enable recognition between rhizobia and their appropriate host legume**. Rhizobia bacteria grow around roots and are very well adapted to growing in the soil immediately adjacent to plant roots (the **rhizosphere**). They are attracted by, and can grow on, **root exudates** –food products of photosynthesis released by the roots - and can preferentially attach to legume roots via specific surface interactions between proteins on the plant cell wall and the **rhizobial polysaccharide surface** (different rhizobia have different surface polysaccharides). There they can detect **signalling compounds** secreted from legume roots. These plant-made chemicals induce the rhizobia to make signals called **Nodulation (or Nod) factors**. These Nod factors are specifically recognised by membrane receptors on the surface of root cells on potential partner legumes. The recognition is based on the various different chemical groups that decorate the Nod factors (a bit like a lock and key mechanism). These Nod factors can determine which legumes can be nodulated by a given rhizobial strain. Thus, the Nod factors made by a pea-nodulating strain are subtly different from those made by soybean-nodulating strains. In most legumes the Nod factors activate pathways that lead to development of nodules and of specialised plant-made structures that help the bacteria infect the roots and enter nodule cells. Proteins secreted by the rhizobia can assist in the recognition process and, in a few cases, can even bypass the need for Nod factors. The Nod-factor signals used by the rhizobia are very similar to the signals made by some **endomycorrhizal fungi** when they enter into a symbiosis with land plants. This latter symbiosis is very ancient and the more recent evolution of the symbiosis between legumes and rhizobia repurposed some of the signalling components from the endomycorrhizal symbiosis.

7. **Other nitrogen-fixing bacterial symbioses.**

Picture of a nodule induced by a Frankia bacterium on an alder root. The insert shows a group of five nodules on a pea root for size comparison

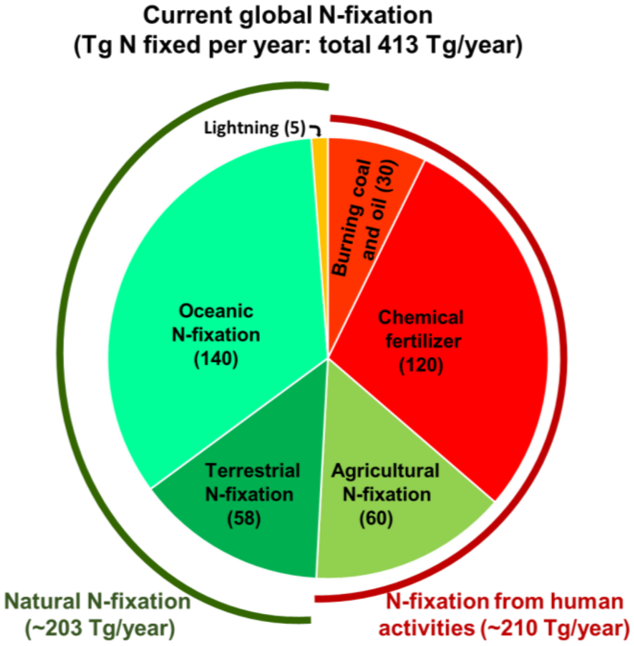
Nitrogen-fixing nodules also occur as a result of a symbiosis involving **filamentous** bacteria called **Frankia** and non-legume plants such as alder, casuarina, eleagnus and datisca. The plants involved are mostly trees or woody shrubs and these symbioses play an important global role in nitrogen fixation, particularly in forests. There are close similarities with the rhizobial legume symbioses, but the *Frankia*-induced nodules can last for several years and some become much larger than legume nodules.

Other symbioses occur with photosynthetic nitrogen-fixing bacteria called **cyanobacteria** that can form symbiotic nitrogen-fixing symbioses. The only known intracellular symbiosis between cyanobacteria and a flowering plant occurs in specialised cells in the stem glands of

Gunnera. However, cyanobacteria can form nitrogen-fixing nodules on **cycads**, and form a nitrogen fixing symbiosis with some **lichens**. Another example of a cyanobacterial symbiosis is that which occurs with the water fern ***Azolla***, that is grown in many rice paddy fields; the fixed nitrogen that is captured during this symbiosis becomes incorporated into the soil, increasing rice yields by up to 50%. Other cyanobacterial nitrogen-fixing symbioses occur widely in oceanic phytoplankton (including dinoflagellates and diatoms) influencing the global ocean biogeochemical cycle of N.

In addition to these nitrogen-fixing symbioses, there are many species of bacteria that can fix nitrogen without being in a symbiosis. For example, cyanobacteria that live in water can use photosynthesis to provide the energy required for nitrogen fixation to produce ammonia for their own growth. In addition, there are diverse nitrogen-fixing soil bacteria that rely on plant-made carbon root exudates to energise nitrogen fixation, but this is much less effective at stimulating plant growth than symbiotic nitrogen fixation.

8. **Role of microbial nitrogen fixation in the global nitrogen cycle.** Before the onset of industrialisation, it is estimated that globally, bacterial nitrogen fixation captured about 200 million tons of nitrogen per annum, about three quarters of that coming from bacteria in oceans and the rest coming from land-based nitrogen-fixing bacteria. At that time the only other major source of generating reactive N from N_2 was lightning, the high energy of which produced about another 5 million tons of reactive N (as oxides of N).

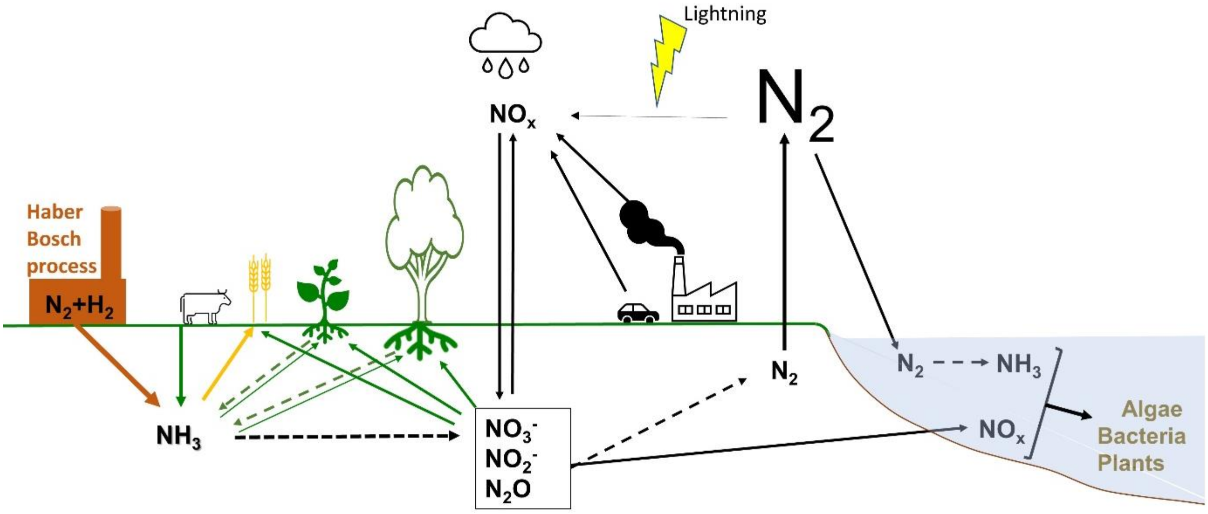


By the early 21st century, human activity had doubled the amount of nitrogen fixation and hence '**reactive nitrogen**' (forms of N other than N_2) released into the biosphere in the form of oxides of nitrogen and ammonia. This occurred mostly because of the invention of the Haber-Bosch industrial production of ammonia (invented in 1910); nowadays this streamlined process uses catalysts acting at a high temperature (about 500 °C) and pressure (about 200 times atmospheric pressure). Currently it produces about 120 million tons of fixed nitrogen per annum, most of which is used as plant fertilisers. Additional reactive N arising from human activity comes from biological nitrogen fixation in agriculture (about 60 million tons per annum, mostly from cultivated legumes). Combustion of fossil fuels (coal and oil contain nitrogen from the organisms that produced them) adds about 30 million tons of **reactive nitrogen** to the atmosphere each year. This doubling of the production of reactive nitrogen by humans has increased crop yields enabling the human population to increase to its current level near 8 billion people. Without industrial production of nitrogen fertiliser, it is estimated that the maximum human population that could be supported would be in the region of 4 billion people. The increases in the production of reactive nitrogen puts enormous strain on the global nitrogen cycle. Consequences of this are:

- *increased levels of reactive nitrogen in the atmosphere, including the greenhouse gas* N_2O *which remains in the atmosphere for about 100 years*

- *reactive nitrogen compounds in the atmosphere can affect levels of ozone, increase atmospheric particulate material and degrade air quality*
- *increased levels of reactive N in soils can cause decreases in terrestrial biodiversity*
- *leaching of reactive nitrogen into water tables affect the quality of drinking water*
- *run off of reactive nitrogen into rivers and oceans can cause profound changes to their ecosystems contributing to decreased biodiversity and even* ***eutrophication****, and the production of low oxygen zones in water bodies resulting in the death of animals like fish.*

The reactive forms of nitrogen in terrestrial, freshwater and marine environments are used by bacteria which, can interconvert different forms of reactive nitrogen and can even produce N_2 gas that can return to the atmosphere. However, this equilibrium is currently out of balance, primarily because of the large amounts of ammonium fertilisers being produced and added to soils.



The Nitrogen Cycle. The atmosphere is mostly nitrogen gas (N_2 makes up 78% of the gases), which cannot be used in that form by any animals, plants or fungi. Nitrogen-fixing bacteria can convert N_2 into ammonia NH_3 which can enter the nitrogen cycle. These ammonia-producing bacteria can grow in nitrogen fixing symbioses with plants (e.g. legumes) and fungi (e.g. lichens) or by bacteria in soil. In lakes and oceans, photosynthetic cyanobacteria can also fix nitrogen converting N_2 to NH_3, which can then be used by phytoplankton, algae, water plants and other bacteria. Animal excretions are rich in nitrogen which can be converted to ammonia by bacteria. Furthermore, when animals, plants or microorganisms die, the nitrogen they contain can be converted to ammonia by soil bacteria. All the ammonia produced can be converted to nitrite and nitrate by other bacteria. Most of the nitrate and ammonium can then be used again by plants, but some of the oxides of nitrogen can be converted to N_2 gas by bacteria and this N_2 can return to the atmosphere completing the nitrogen cycle. Reactive nitrogen can also be released by combustion of fossil fuels and by lightning adding to the nitrogen cycle. Broken arrows represent microbial interconversions of different forms of nitrogen.

9. What are the advantages and disadvantages to growing legumes in agriculture? Why are more legumes not grown in cultivation? One of the advantages of legumes is that farmers do not have to pay for **nitrogenous fertiliser** and astonishingly (when compared with industrial nitrogen fixation), the bacteria in their nodules are able to fix nitrogen at soil temperatures.

Because of the high energy costs in industrial production of nitrogen fertiliser, its cost is usually linked to the cost of oil. This cost factor is particularly important in some economically disadvantaged regions (e.g. parts of Asia and Africa), especially where high distribution costs can also add to the price of applying nitrogen fertilizer. An environmental advantage of symbiotic nitrogen fixation is that during legume growth and senescence, reactive N is released slowly to the soil, and so nitrogen-associated pollution is lower. Nevertheless, when the legumes are eaten by humans or animals, most of the nitrogen is excreted in urine or faeces, and this reactive nitrogen then enters the eco-system. Legume grains are usually low in the sulphur containing amino-acids methionine and cysteine essential for human nutrition, but this can be complemented by the availability of these amino-acids in cereal grains, eggs and meat.

Given their high protein content and environmental advantages, why are legumes not grown more widely? The EU average is that about 3.3% of arable land is used to grow legumes; this compares with over 20% of arable land being used for legumes in some other areas. One issue is that legume yields per unit area are usually less than half of that obtained with cereals. Furthermore, legumes are more susceptible to diseases, are more sensitive to temperature extremes and are more sensitive to drought. These factors mean they require skilful management and make it harder to obtain reproducible high yields of legumes. Part of the problem comes down to less effort having been put into the breeding of legumes. However, soybeans have been highly bred and are used widely because they produce relatively high yields, and their seeds are rich in both protein and oil. They require a relatively warm growing season, and they are a good cash crop because of the high value of both soya oil and the protein-rich soybean residue that is used widely to produce animal feed. Unfortunately, in several parts of the world, the high cash value of soybeans has resulted in tropical forest land being cleared to plant the soybeans that are used to produce food for farm animals for human consumption. The environmental impact would be much lower if humans ate more legumes and less meat.

Relevance for Sustainable Development goals and Grand Challenges

The symbioses between plants and nitrogen-fixing bacteria relate to several SDGs (microbial aspects *in italics) including:*

- **Goal 2. End hunger, achieve food security and improved nutrition and promote sustainable agriculture** *(end hunger and malnutrition, increase agricultural productivity) (access to nutritious food, end hunger and malnutrition, increase productivity of small-scale farmers, sustainable food production, resilient agriculture, genetic diversity in food production).* Using legumes (with appropriate rhizobial inoculants where needed) in agricultural practices reduces the need for added nitrogen fertilizer, provides high protein foods and can stimulate the growth of other crops grown together with or after legumes. This, together with other nitrogen-fixing symbioses such as growing rice along with the Azolla-fern symbiosis and the use of other nitrogen fixing symbioses, can contribute toward sustainable and resilient agriculture with genetically diverse crops.
- **Goal 6. Ensure availability and sustainable management of water and sanitation for all** *(improve water quality, reduce pollution, protect water-related ecosystems).* Increased use of symbiotic nitrogen fixation in agriculture reduces pollution of ground water with nitrogen fertilisers that are converted by soil bacteria into nitrates and nitrite that decrease water quality.
- **Goal 7. Ensure access to affordable, reliable, sustainable and modern energy for all** (*ensure access to clean, renewable and sustainable energy*). Sustainability requires the increasing use of renewables and growing nitrogen fixing crops can contribute to that by increasing soil fertility for subsequent growth of biomass/bioenergy crops.

- **Goal 8. Promote sustained, inclusive and sustainable economic growth, full and productive employment and decent work for all** *(promote economic growth, productivity and innovation, enterprise and employment creation).* Growing nitrogen-fixing legumes and inoculating appropriate rhizobia has been done by smallholder farmers and in poor regions and can provide cash crops such as beans. This promotes local economic growth and productivity. It is often enterprising women and smallholders who drive these innovations stimulating employment.
- **Goal 10. Reduce income inequality within and among countries** (r*educe income inequalities and promote economic inclusion).* The local sale of legumes such as beans, chickpeas, cowpeas, peanuts, pigeon pea and soybeans grown by smallholder farmers can help integrate these farmers into the cash economy in poor regions. Using appropriate rhizobial inoculants as needed can increase yields and profits.
- **Goal 12. Ensure sustainable consumption and production patterns** *(achieve sustainable production and use/consumption practices, reduce waste production/pollutant release into the environment, attain zero waste lifecycles, inform people about sustainable development practices).* The growth of nitrogen-fixing crops with appropriate rhizobial inoculants reduces the use of nitrogenous fertiliser and is critical for sustainable agricultural production with minimal release of reactive nitrogen into the environment and hence reduced pollution. In addition to legume grains providing good nutritional value, the crop residues are a valuable source of fertiliser when incorporated into the soil. There are active programmes to inform farmers how to implement such sustainable development practices, but these need to be expanded at the local level
- **Goal 13. Take urgent action to combat climate change and its impacts** *(reduce greenhouse gas emissions, mitigate consequences of global warming, develop early warning systems for global warming consequences, improve education about greenhouse gas production and global warming).* Ammonia contributes to the formation of particulate matter in the atmosphere causing decreased visibility and subsequent deposition of nitrogen to sensitive ecosystems. Microbes in soil and water readily react with ammonia to produce the oxides of nitrogen (nitric oxide, NO; nitrous acid, HONO; nitrous oxide N_2O; nitrite, NO_2; and nitrate, NO_3). Of these, N_2O is a powerful greenhouse gas (about 260-300 fold stronger than CO_2), that remains in the atmosphere for about 100 years. Lowering the amount of ammonia fertiliser used on crops decreases the production of the oxides of nitrogen. Another source of these is from excretion of reactive N by animals, and replacing meat and dairy foods with legume proteins would decrease the release of reactive N and hence levels of atmospheric NO_2.
- **Goal 14. Conserve and sustainably use the oceans, seas and marine resources for sustainable development** *(reduce pollution of marine systems by agricultural nutrients/wastes, develop mitigation measures for acidification, enhance sustainable use of oceans and their resources).* Reactive N from fertilizers makes its way into marine systems, substantially changing their ecology, and use of legumes can reduce this.
- **Goal 15. Protect, restore and promote sustainable use of terrestrial ecosystems, sustainably manage forests, combat desertification, and halt and reverse land degradation and halt biodiversity loss.** *(Conserve and restore terrestrial and freshwater ecosystems, restore degraded forests, restore degraded land, protect biodiversity and natural habitats).* Plants such as legumes nodulated by rhizobia and plants nodulated by nitrogen-fixing actinobacteria are often primary colonisers of nutrient poor land because of their ability to fix atmospheric nitrogen. Therefore, they can be used to recolonise and fertilize degraded land. They play critical roles in the nitrogen cycles of forests contributing to maintenance of biodiversity. The growth of nitrogen fixing legumes helps preserve freshwater ecosystems by reducing pollution caused by addition of nitrogen fertilisers.

Potential Implications for Decisions

1. *Individual*

a. Assess whether legumes such as peas, beans, chickpeas, lentils, soybeans, lupins and peanuts can play a larger role in diets, potentially replacing some of the animal-sourced proteins currently in diets.

b. Consider using processed legumes such as tofu, soya milk, peanut butter and legume-based vegetable protein products in diets.

c. Think about cultivating legumes in vegetable gardens or allotments and the potential advantages of co-cultivating nitrogen-fixing legumes with other vegetables (such as maize with beans, cauliflower with peas, broadbeans with potatoes).

d. Increasing consumption of vegetables including legumes in the diet is an important food source for beneficial bacteria in the human gut and will increase the diversity and health of the human gut microbiota.

e. Non-microbial benefits: increased variety in diet, potential increase in roughage that helps the digestive system.

2. *Community policies*

a. Promote restoration of wildflower meadowlands, which are rich in native legumes.

b. Promote increased availability of allotments for growth of fruit and vegetables and associated legumes

c. Non-microbial parameters: Engagement of schools in replanting. Awareness of the benefits of integration of diverse planting and restoration of natural ecosystems and the knock-on benefits on insect, bird and animal diversity.

3. *National policies related to growth of plants with nitrogen fixing symbioses*

a. Farming subsidies related to restoration of native meadows and diverse planting in field margins.

b. Support for restoration of woodland containing trees such as Black Locust, Mimosa, Alder, Redbud, Autumn Olive, Acacia and Mesquite that enter into nitrogen-fixing symbioses with bacteria.

c. Decreased environmental pollution related to decreased use of nitrogenous fertilisers.

d. Decreased release of ammonia and nitrous oxide (N_2O) that contribute to atmospheric pollution.

e. Promotion of breeding of legumes with improved agronomic potential such as ability to stand tall in fields, improved harvesting and enhanced nitrogen fixation.

f. Non-microbial parameters: improved wildlife diversity. Consider whether genetic modification of legumes could enhance their usefulness.

Pupil Participation

1. ***Class discussion on nitrogen-fixing symbioses***: what legumes do they know of and what do they get used for? Can you distinguish grain legumes and forage legumes?

2. Pupil stakeholder awareness

a. What legumes do you eat regularly? What other legumes that you do not currently eat would be interesting to include in your diet? Can you think of foods from other parts of the world that include legumes that you eat only seldom or not at all?

b. Can you suggest different menus that include legumes as a major source of dietary protein?

c. What food legumes are grown in your local environment?

d. What grain legumes are grown to be fed to animals? Does it make environmental sense to grow large areas of legumes specifically for animal foods?

e. What forage legumes are routinely fed to animals and in what form (pasture, hay, silage)?

f. What non-food legumes grow in your local environment?

g. What bushes and trees do you know that form symbioses with nitrogen fixing bacteria in your local area? In what kind of environments are they found?

3. Exercises

a. Most legumes have small nodules on their roots. These nodules can be up to the size of a match head and are usually smaller on roots of small legumes. Can you dig up some legumes and examine the nodules? Try cutting a nodule in half and see if you can see the pink colour of the plant-made leghaemoglobin inside.

b. How would you recognise a legume? What is special about the flower shape? Having looked at this shape, are there any other plants you did not recognise as legumes?

c. Try planting some legume seeds in a garden and watch them grow, while also looking at legumes that are already growing. What are the problems with growing them? You may find that the slugs and snails love them. Some legumes grow to be a bit straggly and need help, because many use their tendrils to climb.

d. What are the challenges and the opportunities of growing crops with nitrogen-fixing symbioses in relation to sustainable living?

The Evidence Base, Further Reading and Teaching Aids

Wagner, S. C.. (2011) Biological Nitrogen Fixation. Nature Education Knowledge 3(10):15 https://www.nature.com/scitable/knowledge/library/biological-nitrogen-fixation-23570419/

Nitrogen Fixation. Wikipedia https://en.wikipedia.org/wiki/Nitrogen_fixation

Aczel. M (2019) What Is the Nitrogen Cycle and Why Is It Key to Life? Front. Young Minds. 7:41 https://kids.frontiersin.org/articles/10.3389/frym.2019.00041

Zaleski A (2021). The nitrogen emergency: How to fix our forgotten environmental crisis. New Scientist 15 May 2021 pp 41-45 https://www.newscientist.com/article/mg25033340-800-the-nitrogen-emergency-how-to-fix-our-forgotten-environmental-crisis/

Cartoon animations explaining nitrogen fixation and the Nitrogen Cycle
https://www.youtube.com/watch?v=ZaFVfHftzpI
https://www.youtube.com/watch?v=HOpRT8BRGtk
https://www.youtube.com/watch?v=tCrgTV20BD4

https://www.youtube.com/watch?v=tKOXZKcpCaQ

Glossary

Ammonia: the reduced form of nitrogen (NH_3) that interconverts with ammonium (NH_4^+) in water.

ATP: adenosine triphosphate, a high energy compound that can be generated by the oxidation of compounds via the respiratory chain. It is present in all life forms and provides the energy to drive many processes in living cellscurrency

***Azolla*:** a rapidly growing small water fern that forms a symbiosis with nitrogen-fixing cyanobacteria. It is co-cultivated with rice because the mat it forms prevents weed growth and when it dies off it releases nitrogen that promotes rice growth.

Bacteroids: differentiated forms of rhizobia that can fix nitrogen within plant cells.

Biological nitrogen fixation: the enzymic process of reducing one molecule of N_2 to two molecules of ammonia (NH_3).

Chlorophyll: a pigment that is required for photosynthesis and so is very abundant worldwide. Each chlorophyll molecule contains four N atoms.

Chloroplast: a membrane enclosed structure within plant cells that carries out photosynthesis. They initially evolved millions of years ago by the incorporation of a photosynthetic bacterium into a cell.

Clonal: a population derived from a single individual.

Crop residues: those parts of plants that remain after harvest. Often they are incorporated back into the soil to return nutrients back to the soil.

Crop rotations: the sequential growth of different crops in agriculture. It is done to avoid depletion of soil nutrients and accumulation of pathogenic organisms

Cyanobacteria: a phylum of bacteria that obtain their energy from photosynthesis. Some can fix nitrogen.

Cycads: ancient group of vascular plants that is separate from flowering plants

Cytochrome oxidase (in rhizobia): the last enzyme in the respiratory system donating electrons to oxygen. Nitrogen-fixing bacteroids have a special cytochrome oxidase that has a very high affinity for oxygen.

Differentiated: occurs when an organism changes from one type into another form

Endomycorrhizal fungi: fungi that grow in a symbiosis with many (over 80%) plant species, invading cells in plant roots and transferring nutrients such as phosphate and nitrate from the soil into plant cells.

Eutrophication: enrichment of a body of water with minerals and nutrients; it can lead to enhanced algal growth that can then deplete the oxygen levels in the water.

Filamentous: describes types of microorganisms in which the cells grow attached end to end resulting in long strings of microbial cells.

***Frankia*:** a genus of bacteria that form nitrogen-fixing symbioses with some non-legume plants. Unlike many bacteria, they grow as continuous filaments, and these grow from the soil into root cells

Free oxygen: oxygen molecules that are not bound (e.g.to leghaemoglobin) and so are free to react.

Haber Bosch process: an industrial scale chemical process that uses high pressures and temperatures to catalyse the reduction of N_2 by hydrogen to form ammonia (NH_3).

Haemoglobin; a protein in blood cells containing a red pigment called haem that binds oxygen. This carries oxygen around the body allowing mitochondria to produce energy from respiration.

Inoculum/inoculant: microorganisms such as rhizobia added to plants to improve plant growth

Legumes: plants in the family *Fabaceae*, many of which interact with rhizobia to facilitate biological nitrogen fixation.

Leghaemoglobin: a red oxygen-binding haem protein found in abundance in nitrogen-fixing legume nodules; it promotes nitrogen fixation by acting as an oxygen buffer by binding free oxygen in nodules cells and releasing it at a low enough concentration to prevent denaturation of nitrogenase.

Lichens: composite organism made up of a photosynthestic green alga or cyanobacterium in s symbiotic relationship with a fungus.

Malate: a biochemical intermediate in the degradation of sugars.

Mitochondria: membrane enclosed structures within which oxygen is reduced to water resulting in the production of energy. They originally evolved by the incorporation of a bacterium into a cell.

Nodule (root nodule): a plant-made structure containing nitrogen-fixing bacteria, usually initiated on specific plant roots by infection with either rhizobia or Frankia bacteria.

Nitrogenase: the enzyme complex that reduces N_2 to ammonia.

Nitrogen cycle: The conversion of N_2 gas into reactive nitrogen (usually as ammonium or nitrate) that can be taken up by plants or microorganisms where it is assimilated into proteins and other organic compounds that can be used by animals and humans. After excretion or death, these compounds are degraded by microorganisms in soil or water, producing either reactive nitrogen that can be taken up by plants and microbes, or producing N_2 gas that escapes to the atmosphere completing the cycle.

Nitrogen fixation: The process of breaking the triple bond of N_2 gas forming reactive forms of nitrogen that can then enter the nitrogen cycle. This can be done biologically by nitrogen-fixing bacteria, or it can be done chemically by the Haber Bosch process or it can be done by lightning.

Nitrogenous fertiliser: Fertilisers that are rich in nitrogen; they can include inorganic compounds such as ammonium, nitrate and urea. Organic nitrogen-rich fertilisers include farmyard manure and other materials such as bone, hoof and horn which contain nitrogen that is slowly released to plants by microbial degradation.

Nod (nodulation) factors: Signalling molecules produced by rhizobia in response to chemicals secreted by legume roots. Nod factors play key roles in rhizobial infection of legume roots and in initiating nodule development.

Organelle: subcellular structures bounded by membranes. These include mitochondria and plastids that evolved millions of years ago from bacteria taken up into larger cells.

Oxides of nitrogen: oxidised forms of nitrogen including N_2O (nitrous oxide), NO (nitric oxide), NO_2^- (nitrite) and NO_3^- (nitrate).

Phytoplankton: the self-feeding photosynthetic component of the marine plankton.

Reactive nitrogen. Forms of nitrogen in its reduced form ammonia (NH_3) or in its various oxides such as N_2O, NO, NO2, NO3 or HONO. These are all much more reactive than nitrogen gas (N_2).

Respiratory system (in rhizobia): a linked series of membrane bound redox reactions that transfer electrons down an electrochemical gradient to oxygen and producing energy (in the form of ATP) that can be used in many reactions.

Rhizosphere: the region of the soil in very close proximity to plant roots.

Rhizobia: soil bacteria that can infect legume plants and form nitrogen-fixing nodules. There are several genera including *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Ensifer (Sinorhizobium)* and *Paraburkholderia,* all containing different species that preferentially nodulate specific legumes.

Rhizobial polysaccharide surface: the outer layer of the cell wall surrounding rhizobia.

Root exudates: organic molecules such as polysaccharides, proteins and metabolites released by plant roots or by the lysis of plant root cells during normal root growth.

Signalling compounds: biochemical compounds released by one cell type and recognised by another cell, inducing a response

Symbiosis: a very close interaction between two different organisms that grow together.