

BIOLOGICAL NITROGEN FIXATION: MECHANISM, MICROORGANISMS AND ITS ROLE IN SUSTAINABLE AGRICULTURE

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ABSTRACT

Nitrogen is one of the most essential elements required for the growth, development and survival of living organisms. It plays an important role in the formation of amino acids, proteins, nucleic acids, chlorophyll, enzymes and several other biologically important compounds. Although atmospheric nitrogen is abundant in the form of molecular nitrogen (N_2), most plants are unable to use it directly because the two nitrogen atoms are strongly linked by a stable triple covalent bond. Nitrogen -fixation is therefore an important natural and biological process that converts atmospheric nitrogen into forms that can enter biological systems and support plant growth. Free-living organisms such as Azotobacter, Rhizobium and Clostridium etc. can contribute to nitrogen availability without forming a direct symbiotic relationship with plants. Associative nitrogen fixers occur in close proximity to plant roots and benefit from compounds released by the plant. The interaction between Rhizobium and legumes involves several stages, including recognition of plant signals, production of Nod factors, root-hair responses, formation of infection threads, development of root nodules and differentiation of bacteria into nitrogen-fixing bacteroid. This highly coordinated interaction enables the exchange of nutrients between the plant and microorganism, in which the plant supplies carbon compounds and the bacterial partner contributes fixed nitrogen. Overall, biological nitrogen fixation represents a significant connection between atmospheric nitrogen, microorganisms, plants and soil fertility. A better understanding of nitrogen-fixing organisms, their mechanisms, isolation methods, culture conditions and agricultural applications can support the development of sustainable strategies for improving plant nutrition and maintaining soil health.

KEYWORDS: Nitrogen fixation, Nitrogen cycle, Soil fertility, Symbiotic and non-symbiotic, Free living nitrogen fixers.

1. INTRODUCTION

Nitrogen is very important for living things. It is needed to make proteins, DNA, RNA, Chlorophyll and energy-related substances. However, atmospheric nitrogen exists predominantly as nitrogen gas (N_2). Plants cannot directly use this nitrogen because the two nitrogen atoms are strongly bonded together. So, biological Nitrogen fixation is very important. In BNF, some special microorganisms called diazotrophs convert the nitrogen in the air into a form that plants can use. These microorganisms can live freely in soil, live near plant roots, or live together with plants. For example, Rhizobium bacteria live in the root nodules of legume plants like pea and groundnut. They fix nitrogen inside these nodules. The enzyme responsible for nitrogen fixation is called nitrogenase, and it is sensitive to oxygen. BNF is also important in biotechnology because

excessive use of chemical fertilizers can cause fertilizer loss and environmental pollution. Therefore, scientists are trying to improve BNF so that plants can get nitrogen more efficiently and the use of chemical fertilizers can be reduced.^[1] The field has now entered an engineering phase. Synthetic biology is being used to tune nitrogenase expression, improve oxygen tolerance, alter nitrogen regulation and explore heterologous expression in non-diazotrophic organisms. Reviews by Mus et al. and later engineering studies show both the promise and the complexity of transferring nitrogen fixation beyond its natural hosts.^[2,3]

1.1 Nitrogen fixation

Sustainability is measured as a ratio of input and output by taking into account stock depletion by the economist. In agricultural stocks means soil, water, renewable

energy sources and also environmental quality. Modern agriculture is based upon the principle of maximum output in the short term, with inadequate concern for input efficiency [or] stock maintenance [Odum, 1989].^[4] From 1.6 billion in 1900, the world population has grown to more than 7 billion today and will reach 9 billion by 2050.^[5]

Nitrogen-fixing organism can be classified into three categories they are: Free living nitrogen fixer That is free-living nitrogen fixers are microorganisms that live freely in soil [or] water and convert atmospheric nitrogen into ammonia without forming a symbiotic association with plants. Importance of the Free-living nitrogen fixers are Increase soil fertility naturally, improve plant growth by adding usable nitrogen to the soil, support sustainable agriculture. Example: Azotobacter [aerobic], Clostridium [anaerobic], Nostoc.

Associative nitrogen fixers: These are nitrogen fixing bacteria that live closely associated with plant roots, especially around the root surface, but they do not form true root nodules like *Rhizobium*. They take nitrogen from the atmosphere. They live near the roots and get food/carbon compounds from the plant. In return, the plant gets some fixed nitrogen. Example; Azo-spirillum, Azotobacter (often discussed as free-living rather than strictly associative).

Symbiotic nitrogen fixers: Symbiotic nitrogen fixers are microorganisms, mainly bacteria, that live in close association with plants and convert atmospheric nitrogen (N_2) into a usable form of nitrogen for the plant. Example: *Rhizobium* lives inside the root nodules of leguminous plants like pea, gram, groundnut and soybean. The last two groups can be found in the rhizosphere of legumes and nonlegume plant.^[6,7] Nitrogen fixation is the process by which atmospheric nitrogen (N_2) is converted into chemically usable nitrogen compounds, mainly ammonia (NH_3). Although nitrogen gas is abundant in the atmosphere, most plants

and animals cannot use atmospheric N_2 directly because of its strong and stable triple bond.^[8] Nitrogen fixation is an essential component of the global nitrogen cycle because it introduces atmospheric nitrogen into biologically available forms. The fixed nitrogen can subsequently enter different biochemical pathways and become part of important cellular compounds such as amino acids, proteins and nucleic acids.^[9] Nitrogen fixation can occur through biological, industrial and natural physicochemical processes. Biological nitrogen fixation is carried out by specialized microorganisms called diazotrophs, whereas industrial fixation is mainly performed through the Haber–Bosch process. Lightning and other atmospheric processes can also convert a small amount of atmospheric nitrogen into reactive nitrogen compounds.^[10] In biological nitrogen fixation, atmospheric N_2 is reduced to ammonia by the enzyme complex nitrogenase. Nitrogenase is found in certain microorganisms, including free-living and symbiotic nitrogen-fixing bacteria and archaea.^[11] The nitrogenase enzyme requires a continuous supply of electrons and energy in the form of ATP to drive the reduction of the highly stable N_2 molecule. The commonly studied molybdenum-dependent nitrogenase contains two major protein components: the Fe protein and the Mo protein.^[12] The ammonia produced during nitrogen fixation does not usually remain as free ammonia. It is rapidly incorporated into cellular nitrogen metabolism and converted into nitrogen-containing organic compounds. In plant–microbe symbioses, the fixed nitrogen can be transferred to the host plant and used for growth and development.^[13] Overall, nitrogen fixation can be considered a key biological bridge between atmospheric nitrogen and living organisms. By converting relatively inaccessible N_2 into ammonia and subsequently into biologically useful nitrogen compounds, nitrogen-fixing microorganisms contribute significantly to plant nutrition, soil fertility, agricultural productivity and the functioning of the global nitrogen cycle.^[14]

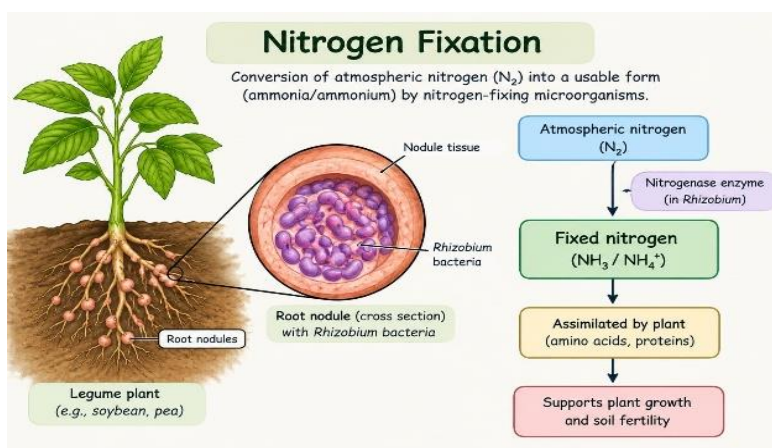


Fig. 1: nitrogen fixation.

1.2 Role of nitrogen fixation

The main function of *Rhizobium* in this symbiotic relationship is the conversion of atmospheric nitrogen

into ammonia through the nitrogenase enzyme complex. Nitrogen fixation requires energy from the plant and suitable low-oxygen conditions inside the root nodule. The fixed nitrogen is incorporated into nitrogen-containing compounds that support plant growth, protein synthesis, and other essential biological processes. Rhizobium–legume symbiosis is therefore an important natural source of nitrogen for sustainable agriculture and reduces dependence on chemical nitrogen fixers.^[15,16]

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Fig. 2: Root nodules of leguminous plant.

1.2 Nitrogen cycle

Nitrogen is an essential nutrient required for the growth and development of all living organisms. It is a major component of proteins, nucleic acids, enzymes and other important biological molecules. Although the atmosphere contains approximately 78% nitrogen gas (N_2), most plants cannot use atmospheric nitrogen directly. The conversion of nitrogen into usable forms and its movement through the environment are controlled by the nitrogen cycle.^[17,18]

The nitrogen cycle is a natural process in which nitrogen circulates between the atmosphere, soil, plants, animals and microorganisms. This cycle involves several important processes, including nitrogen fixation, nitrification, assimilation, ammonification and denitrification. Soil microorganisms play a central role in

these processes by converting nitrogen from one form to another, thereby maintaining soil fertility and supporting plant growth.^[19] Among these processes, nitrogen fixation is particularly important because it converts atmospheric nitrogen gas into ammonia or related biologically available nitrogen compounds. Biological nitrogen fixation is mainly carried out by specialized microorganisms known as diazotrophs.

These include free-living bacteria, associative bacteria and symbiotic bacteria such as Rhizobium, which forms a beneficial association with leguminous plants. The enzyme nitrogenase is responsible for the conversion of atmospheric nitrogen into ammonia utilized by plants for the synthesis of essential nitrogen-containing compounds.^[20,21]

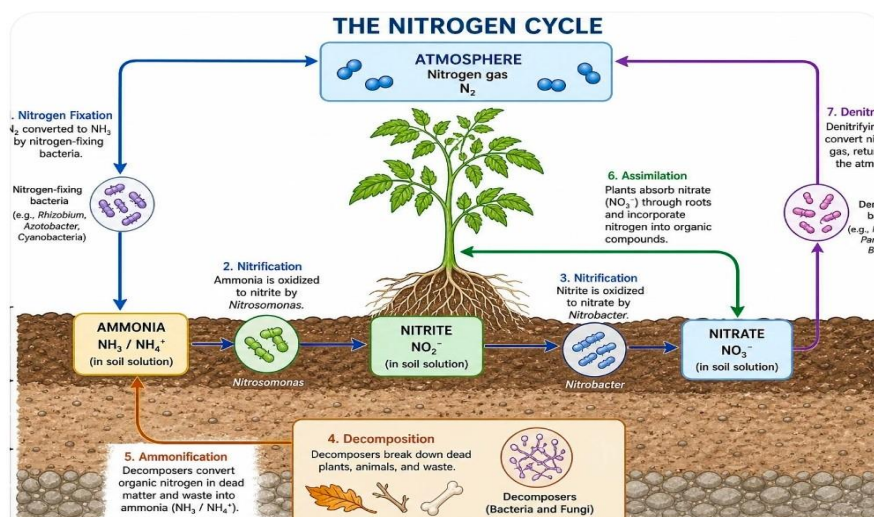


Fig. 3: Nitrogen Cycle.

1.3 Importance of nitrogen in living organisms

Nitrogen is an important nutrient required by all living organisms for their growth and development. It is present in proteins, amino acids, nucleic acids and other essential biological compounds. Plants need nitrogen for the formation of chlorophyll, which helps them carry out photosynthesis and produce food. It is also involved in the formation of enzymes and other substances necessary for normal plant growth. Animals obtain nitrogen mainly through their food, especially from plant and animal proteins. Because of its importance in the formation of cellular components, nitrogen availability has a direct effect on the growth and productivity of living organisms. The availability of nitrogen in a usable form is therefore essential for maintaining life and supporting the functioning of ecosystems.^[22] The nitrogen cycle is a natural process through which nitrogen moves between the atmosphere, soil, plants, animals and microorganisms. Atmospheric nitrogen is present mainly in the form of nitrogen gas (N_2), which cannot be directly utilized by most plants. Through different biological and chemical processes, nitrogen is converted into forms that can be taken up by plants and used for their growth. After plants and animals die, the nitrogen present in their bodies is returned to the soil through decomposition. Microorganisms then convert it into different nitrogen compounds, and some of the nitrogen is eventually released back into the atmosphere. In this way, nitrogen continuously circulates through the environment and maintains the balance of this important nutrient in nature.^[23]

1.4 Atmospheric nitrogen and its availability

The atmosphere contains approximately 78% nitrogen gas, making it the largest reservoir of nitrogen on Earth.

However, the nitrogen present in the atmosphere is relatively stable because the two nitrogen atoms are joined by a strong triple bond. Most plants and animals cannot break this bond and use atmospheric nitrogen directly. For this reason, atmospheric nitrogen must first be converted into biologically available forms such as ammonia, ammonium or nitrate. This conversion is carried out through processes such as biological nitrogen fixation, lightning and industrial nitrogen fixation. Among these, biological nitrogen fixation is particularly important in natural ecosystems and agriculture because it provides a pathway through which atmospheric nitrogen enters the biological system.^[24]

1.5 Environmental importance of nitrogen cycle

The nitrogen cycle is closely associated with the health and stability of natural ecosystems. Nitrogen is required for plant growth, and its availability influences the productivity of grasslands, forests, agricultural fields and aquatic environments. Natural nitrogen cycling helps maintain nutrient balance between the soil, plants and atmosphere. However, human activities such as excessive use of nitrogen fertilizers, industrial emissions and changes in land use have altered the natural nitrogen cycle. Increased nitrogen inputs may contribute to nutrient pollution, eutrophication and other environmental problems. Understanding these changes is important for maintaining ecosystem health and developing agricultural practices that use nitrogen more efficiently.^[25]

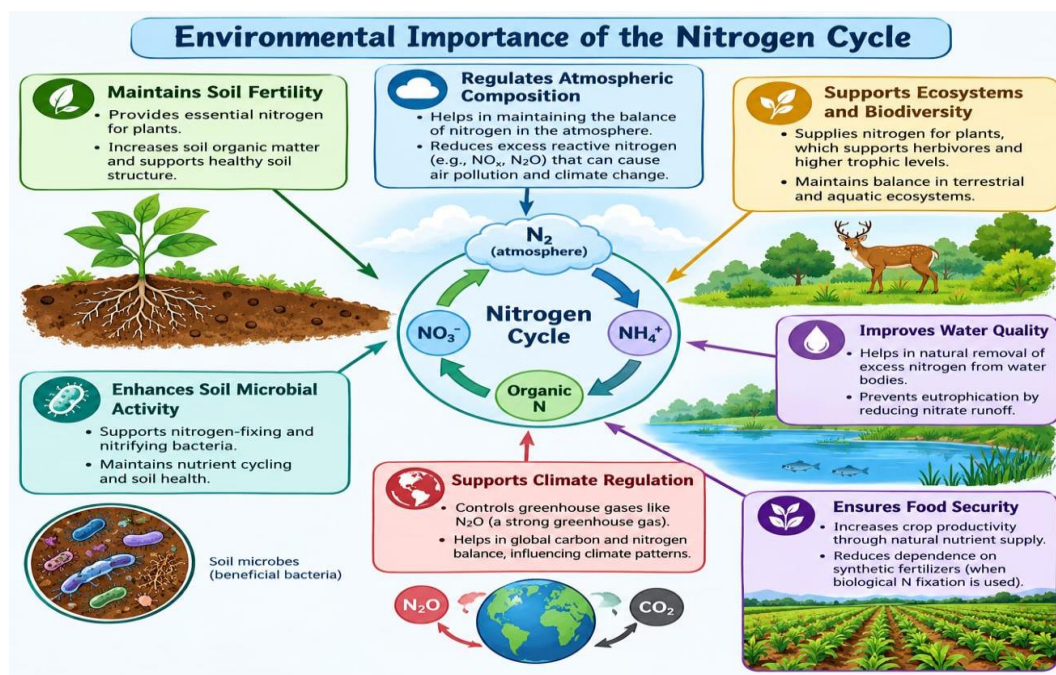


Fig. 4: environmental importance of nitrogen cycle.**1.6 Plant available nitrogen**

Nitrogen in soil occurs predominantly in two major forms: **organic nitrogen (approximately 98%)** and **inorganic or mineral nitrogen (approximately 2%)**. Inorganic nitrogen mainly occurs as ammonia (NH_3), ammonium (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-). Organic nitrogen is present in both living and non-living components of soil organic matter, including soil microorganisms, fresh plant and animal residues, humified organic matter, and non-humified organic compounds. Among the inorganic forms, **ammonium ($\text{NH}_4^+\text{-N}$) and nitrate ($\text{NO}_3^-\text{-N}$)** are the principal forms of nitrogen available for plant uptake. In contrast, most organic nitrogen is not directly available to plants. It must first undergo microbial decomposition and **mineralization**, during which organic nitrogen is converted into inorganic forms, primarily ammonium, which may subsequently be transformed into nitrate through nitrification. Thus, the transformation of organic nitrogen into mineral forms is an important process governing nitrogen availability and plant nutrition in soil.^[26] Once nitrogen becomes available in the soil, it is subject to competition between plants and soil microorganisms for uptake. At the same time, soil nitrogen is continuously depleted through several pathways, including soil erosion, denitrification, leaching, ammonia volatilization, and the removal of nitrogen-rich crop residues from agricultural fields. These processes can substantially reduce the amount of nitrogen retained in soil and influence its availability for subsequent plant growth. Therefore, efficient management of soil nitrogen is essential for maintaining soil fertility and ensuring adequate nitrogen supply to crops.^[27,28] Nitrogen deficiency is a common constraint in many agricultural soils and can adversely affect crop growth and productivity. To overcome inadequate nitrogen availability, synthetic nitrogen fertilizers have been widely used to supplement the nitrogen requirements of crops. Industrial ammonia production is primarily based on the **Haber–Bosch process**, which was developed in the early twentieth century. This process enables the synthesis of ammonia (NH_3) by reacting atmospheric nitrogen (N_2) with hydrogen (H_2) under controlled industrial conditions. The ammonia produced through this process serves as an important raw material for manufacturing a wide range of nitrogen-containing fertilizers and compounds, including ammonium-based fertilizers, nitrate fertilizers and urea. Consequently, the Haber–Bosch process has played a major role in increasing the availability of reactive nitrogen for modern agriculture.^[29] The widespread use of industrially produced nitrogen fertilizers has increased the input of reactive nitrogen into the global nitrogen cycle, which is regulated through interconnected processes such as nitrogen fixation, assimilation, mineralization, nitrification and denitrification.^[30–32] In addition, the manufacture of nitrogen fertilizers is highly dependent on fossil-fuel resources and accounts for

approximately 2% of global energy consumption.^[33] A considerable proportion of fertilizer-derived nitrogen is not taken up by crops and may consequently be lost from agricultural soils. It is estimated that nearly 25% of applied fertilizer nitrogen can be lost through leaching and other nitrogen-loss processes occurring during agricultural production.^[34] Such losses reduce nitrogen-use efficiency and can increase the release of reactive nitrogen into surrounding ecosystems. Consequently, excessive nitrogen inputs and associated losses may contribute to environmental pollution and negatively influence soil quality and overall ecosystem health.^[35] These challenges emphasize the importance of improving nitrogen-use efficiency and developing more sustainable approaches to nitrogen management in agriculture.

3. METHODS USED FOR ESTIMATION NITROGEN FIXATION

These methods are used to determine the nitrogen-fixing activity or estimate the amount of nitrogen fixed by microorganisms and plants.

3.1 Acetylene reduction assay (ARA)

The acetylene reduction assay is an indirect method for measuring nitrogenase activity. Nitrogenase reduces acetylene (C_2H_2) to ethylene (C_2H_4). The ethylene produced is measured using gas chromatography. This method is useful for studying nitrogenase activity in root nodules, bacteria and soil samples.^[36]

3.2 Nitrogen balance method

The nitrogen balance approach estimates biological nitrogen fixation by assessing the difference between nitrogen inputs and outputs, together with changes in the nitrogen content of the soil–plant system. This method provides an indirect estimate of nitrogen fixation based on the overall nitrogen balance within the system. It is useful for studying nitrogen accumulation in agricultural fields and ecosystems. However, accurate estimation requires consideration of all other potential sources of nitrogen entering the system as well as the different pathways through which nitrogen may be lost.^[37]

3.3 Nitrogen difference method

The nitrogen difference method compares the total nitrogen content of a nitrogen-fixing crop with that of a suitable non-fixing reference crop. The difference in nitrogen content provides an estimate of nitrogen supplied through biological fixation.^[38]

3.4 Xylem sap ureide method

The xylem sap ureide method is used mainly in certain leguminous plants. Fixed nitrogen is transported from root nodules to the shoots in the form of ureides, such as allantoin and allantoic acid. Measuring ureide concentration in xylem sap helps estimate the contribution of symbiotic nitrogen fixation.^[39]

3.5 Molecular methods

Molecular approaches provide valuable tools for the identification and characterization of nitrogen-fixing microorganisms and enable the detection and analysis of genes associated with biological nitrogen fixation. The nitrogen fixation protein H gene is an important marker associated with nitrogenase. PCR, quantitative PCR, sequencing and other molecular techniques can be used to study nitrogen-fixing microbial communities.^[40]

4. RHIZOBIUM BACTERIA

Rhizobium is a group of beneficial soil bacteria that plays an important role in biological nitrogen fixation. These microorganisms establish mutually beneficial symbiotic relationships with the root systems of leguminous crops, including pea, chickpea, soybean, and common bean. During this association, Rhizobium bacteria enter the root tissues and stimulate the formation of specialized structures called root nodules. Inside these nodules, atmospheric nitrogen (N_2) is converted into ammonia by the enzyme nitrogenase. This process makes nitrogen available to plants for their growth and development. Rhizobium bacteria are generally Gram-negative, rod-shaped bacteria belonging mainly to the class Alpha-proteobacteria. They can live freely in soil, but under suitable conditions they establish a symbiotic relationship with leguminous plants. The bacteria recognize compatible plant roots through chemical signals and initiate the formation of root nodules. Within these nodules, the bacteria differentiate into nitrogen-fixing forms called bacteroid. The plant supplies carbohydrates and energy to the bacteria, while the bacteria provide fixed nitrogen to the plant. This mutual relationship with nutrition and contributes to soil fertility.^[41,42] Rhizobium is a group of Gram-negative, soil-dwelling bacteria that forms a beneficial association mainly with leguminous plants. These bacteria are important members of the soil microbial community and can contribute to plant nutrition through biological nitrogen fixation.^[43] Rhizobia bacteria are attracted towards the roots of suitable host plants by chemical compounds released from plant roots. This early interaction allows the bacterium to recognize a compatible host and initiate the process of symbiosis.^[44] Legume roots release flavonoids and other signalling molecules into the surrounding soil. These compounds activate specific bacterial genes involved in establishing the rhizobium-legume association.^[45] Following recognition of plant signals, rhizobia synthesize Nod factors, which are important signalling molecules required for the initiation of nodule development. The plant recognizes these molecules through specific receptors present on root cells.^[46] Recognition of Nod

factors produces several changes in the root, particularly root-hair deformation and curling. This response helps the bacteria become closely associated with the developing infection site.^[47] Rhizobia cells enter the root through a tubular structure called the infection thread. The infection thread provides a controlled route through which bacterial cells move towards the internal tissues of the developing root nodule.^[48] During infection, the plant undergoes controlled cellular division and differentiation, resulting in the formation of a specialized structure known as a root nodule. The nodule provides a suitable environment for the nitrogen-fixing association.^[49] Inside the developing nodule, rhizobia cells differentiate into specialized forms called bacteroid. These differentiated bacterial cells are responsible for carrying out biological nitrogen fixation within the symbiotic tissue.^[50] The interior of an active root nodule maintains a carefully regulated oxygen concentration. Leg haemoglobin, an oxygen-binding molecule associated with nodules, helps maintain conditions that support respiration while protecting the oxygen-sensitive nitrogenase enzyme.^[51] Nitrogen fixation in rhizobia bacteroid is performed by the nitrogenase enzyme complex. This enzyme enables the conversion of atmospheric nitrogen (N_2) into ammonia (NH_3), which can subsequently be incorporated into biologically useful nitrogen compounds.^[52] Nitrogenase requires substantial amounts of ATP and reducing power to convert molecular nitrogen into ammonia. The energy required for this process is ultimately supported by carbon compounds supplied by the host plant.^[53] The symbiotic relationship involves an exchange of nutrients between the two partners. The plant provides photosynthetically derived carbon compounds to the bacteria, whereas the rhizobia symbiont supplies fixed nitrogen to the plant.^[54] Rhizobium-legume associations are generally host-specific or host-selective, meaning that a particular rhizobia strain may establish an effective symbiosis only with certain plant species. This specificity is controlled by molecular recognition between the plant and bacterium.^[55] The efficiency of biological nitrogen fixation is influenced by environmental conditions such as soil pH, temperature, moisture, salinity, nutrient availability and soil nitrogen concentration. Unfavourable conditions can reduce bacterial survival, nodulation and nitrogen-fixing activity.^[56] Rhizobia have considerable agricultural importance because their association with legumes can introduce biologically fixed nitrogen into cropping systems. Effective rhizobial inoculation can therefore contribute to plant nitrogen nutrition and may reduce the requirement for synthetic nitrogen fertilizers.^[57]

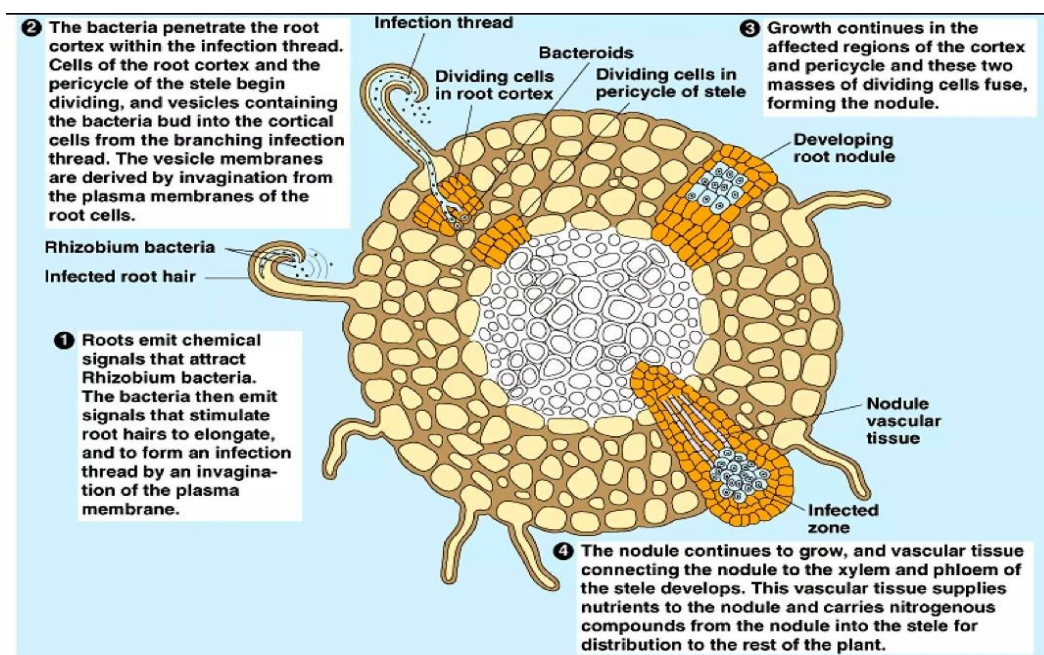


Fig. 5: Rhizobium Bacteria.

5. ISOLATION OF NITROGEN FIXING-BACTERIA

5.1 Introduction to isolation

Isolation of nitrogen-fixing bacteria is an important first step in studying biological nitrogen fixation. These microorganisms are present in different environments, including agricultural soil, the rhizosphere, root nodules, and plant tissues. Depending on their relationship with plants, nitrogen-fixing bacteria may be classified as free-living, associative, endophytic, or symbiotic microorganisms. The isolation method depends on the type of bacterium being studied and the source from which it is collected. In the case of leguminous plants, bacteria belonging to the rhizobia group are commonly obtained from root nodules. On the other hand, free-living and associative nitrogen-fixing bacteria can be isolated from soil and the root surface of plants. The main purpose of isolation is to obtain individual bacterial strains in pure culture so that their growth characteristics, identity, and nitrogen-fixing ability can be examined.^[58]

5.2 Selection and collection of samples

The first stage of isolation is the selection of a suitable source of nitrogen-fixing bacteria. Soil from agricultural fields, the rhizosphere of healthy plants, and root nodules of leguminous crops are commonly selected for this purpose. The choice of sample depends on whether the study is aimed at isolating free-living nitrogen fixers or bacteria that form a symbiotic association with plants. For isolation of rhizobium, healthy legume plants are preferred because their root nodules may contain bacteria involved in nitrogen fixation. Crops such as soybean, chickpea, pea, bean, and other legumes are useful sources. Root nodules are collected carefully along with a small portion of the root to reduce damage during removal. Soil and root samples should be handled carefully and transferred to the laboratory under suitable

conditions so that the microorganisms remain viable. Sampling from diverse plant species and geographical locations can increase the likelihood of recovering a broader diversity of bacterial isolates.

Environmental conditions, plant species, soil properties, and the age of the plant may influence the microorganisms present in the sample.^[59]

5.3 preparation of soil and root samples

The collected soil or root samples are prepared before bacterial isolation. For soil samples, a measured quantity of soil is mixed with sterile distilled water or sterile saline solution to release the microorganisms into suspension. Root nodules are washed to remove adhering soil particles and may be surface sterilized when bacteria present inside the nodules are being studied.

The prepared material is then crushed or homogenized under aseptic conditions to obtain a bacterial suspension. Surface sterilization is particularly important for endophytic bacterial isolation because it helps remove microorganisms present on the outer surface of plant tissues. The treated tissue can then be processed to recover bacteria from inside the plant.^[60]

5.4 Serial dilution of the bacterial suspension

Serial dilution is carried out to reduce the number of microorganisms present in the original sample. A portion of the soil or root suspension is transferred into sterile diluent, and further tenfold dilutions are prepared. Dilutions such as 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5} may be used according to the sample and bacterial population. This technique helps obtain well-separated bacterial colonies on culture plates. Appropriate dilutions are selected for plating so that individual colonies can be observed and picked for further study.^[61]

5.5 Inoculation on nitrogen-free selective medium

The diluted bacterial suspension is inoculated onto nitrogen-free culture media to encourage the growth of bacteria capable of obtaining nitrogen from atmospheric nitrogen or other available sources. Media such as Ashby's nitrogen-free medium, Jensen's medium, and other suitable nitrogen-free media are used in the isolation of diazotrophs. The culture medium provides essential nutrients for bacterial growth while lacking an easily accessible source of combined nitrogen. Bacteria growing on such media are considered potential nitrogen-fixing microorganisms. However, growth on nitrogen-free medium alone is not sufficient to prove nitrogen fixation, and further confirmation is required.^[62]

5.6 Incubation of inoculated culture plates

After inoculation, the culture plates are incubated under suitable conditions to allow bacterial growth. The incubation temperature and duration depend on the bacterial group and culture medium. For many agricultural nitrogen-fixing bacteria, incubation around 28–30°C is commonly used. During incubation, bacterial colonies may develop on the nitrogen-free medium. Some nitrogen-fixing bacteria require oxygen-limited conditions, so semisolid media or controlled oxygen conditions may be used when isolating particular free-living diazotrophs.^[63]

5.7 Selection of distinct bacterial colonies

After incubation, the culture plates are examined for bacterial growth. Individual colonies are selected based on their visible characteristics, such as colony size, shape, colour, margin, elevation, and texture. In some cases, colonies with mucoid or creamy appearances may be observed among the growing bacteria. The selected colonies are transferred carefully to fresh culture media for purification. Colony morphology is useful for preliminary selection, but it cannot by itself establish the identity or nitrogen-fixing ability of a bacterium.^[64]

5.8 Purification of bacterial isolates

Purification is an important step in obtaining a pure bacterial culture. The selected colonies are repeatedly streaked on fresh agar medium using aseptic techniques. Repeated streaking helps separate individual bacterial cells and obtain colonies derived from a single bacterial type.

The purified cultures are examined for uniform colony characteristics and checked for contamination. Pure

cultures are maintained on suitable media for subsequent identification, nitrogen-fixation testing, and other laboratory studies.^[65]

5.9 Confirmation of nitrogen-fixing ability

The purified isolates are screened to determine whether they possess nitrogen-fixing activity. Growth on nitrogen-free medium may be used as a preliminary screening method. Additional tests, such as the acetylene reduction assay, can be used to assess nitrogenase activity.

Molecular methods, including detection of the nitrogen fixation gene, can provide further evidence related to nitrogen fixation. These tests are important because not every bacterium that grows on nitrogen-free medium is necessarily a confirmed nitrogen fixer. A combination of physiological and molecular evidence gives a stronger basis for identifying diazotrophic bacteria.^[66]

5.10 Characterization and identification of isolated bacteria

The isolated bacteria are characterized using morphological, biochemical, and molecular methods. Microscopic examination provides information about cell shape and other visible characteristics. Biochemical tests help study the physiological properties of the isolates.

Molecular identification, particularly 16S rRNA gene sequencing, is widely used to determine the taxonomic identity of bacterial isolates. In the case of root nodule bacteria, additional studies involving symbiotic genes and host-plant relationships may be useful for understanding their nitrogen-fixing association.^[67]

5.11 Preservation of pure nitrogen-fixing bacterial cultures

After purification and characterization, the selected bacterial isolates are preserved for further research. Cultures may be maintained on agar slants or stored using suitable cryopreservation methods. Proper preservation helps maintain the bacterial strain for future experiments, including nitrogenase activity studies, plant-growth promotion experiments, and molecular characterization.

The final isolates can be used for further investigations into their role in biological nitrogen fixation and their possible applications in sustainable agriculture.^[68]

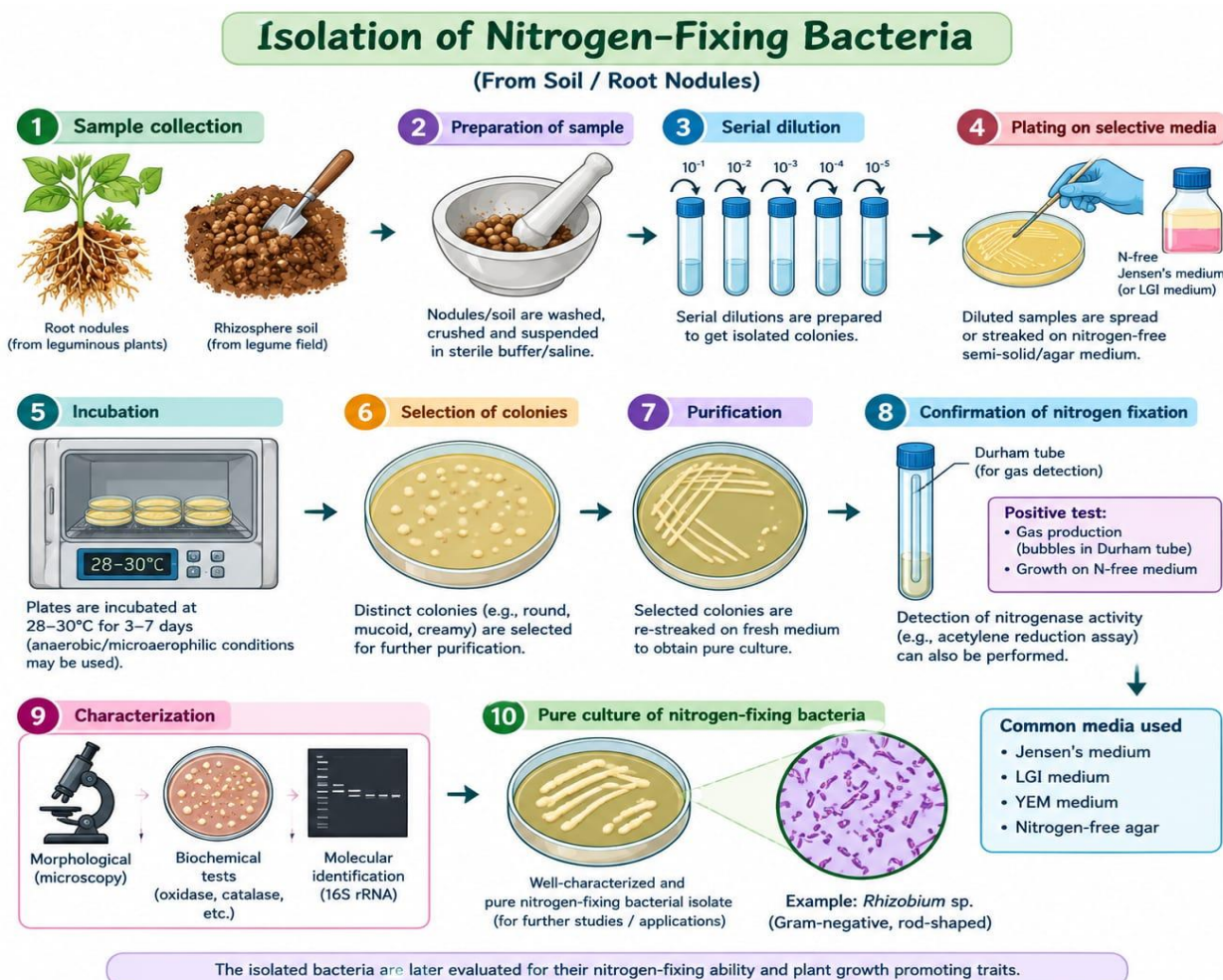


Fig. 6: Isolation of nitrogen fixing bacteria.

6. CULTURE MEDIA USED FOR NITROGEN FIXING BACTERIA

6.1 Jensen's Nitrogen-Free Medium

Jensen's nitrogen-free medium is used for the cultivation and preliminary isolation of free-living nitrogen-fixing bacteria, particularly members of the genus *Azotobacter*. The medium supplies mineral nutrients and a carbon source while excluding a readily available combined nitrogen source. This arrangement helps in screening microorganisms that may utilize atmospheric nitrogen for their growth. Jensen's medium has been used in studies involving the isolation and assessment of nitrogen-fixing bacteria from soil. However, bacterial growth on this medium should be followed by additional tests to confirm nitrogen-fixation activity.^[69]

6.2 Nutrient Agar

Nutrient agar is a general-purpose solid culture medium used for the growth and maintenance of many non-fastidious bacteria. It contains nutrients such as peptone, beef extract, sodium chloride, and agar, which support ordinary bacterial growth. In nitrogen-fixation studies, nutrient agar may be useful for maintaining purified bacterial isolates and obtaining sufficient biomass for characterization. It is not a nitrogen-free selective

medium, so growth on nutrient agar alone cannot be considered evidence of nitrogen fixation.^[70]

6.3 Ashby's Mannitol Agar

Ashby's mannitol agar is a nitrogen-free medium commonly associated with the isolation of free-living nitrogen-fixing bacteria such as *Azotobacter*. Mannitol acts as a carbon source, while the mineral components support microbial growth.

The absence of combined nitrogen creates a screening condition in which organisms capable of obtaining nitrogen from the atmosphere may develop. *Azotobacter* isolates can produce characteristic colonies on Ashby-based media, although colony appearance must be supported by further identification and nitrogenase activity testing.^[71]

6.4 Yeast Extract Mannitol Agar (YEMA)

Yeast extract mannitol agar is widely used for the cultivation and maintenance of *Rhizobia* bacteria associated with legume root nodules. Mannitol provides a carbon source, while yeast extract supplies growth-supporting nutrients.

YEMA is highly effective for promoting the growth of well-defined rhizobial colonies and for evaluating their cultural characteristics. Congo red may be incorporated into some rhizobial media as a differential aid. Since YEMA contains organic nitrogen from yeast extract, it should not be described as a nitrogen-free selective medium for proving nitrogen fixation.^[72]

6.5 Nitrogen-Free or Reduced-Nitrogen Broth

Nitrogen-free and reduced-nitrogen broth media are used in the enrichment and screening of potential nitrogen-fixing bacteria. Such media are designed to provide

suitable carbon and mineral nutrients while limiting the availability of combined nitrogen.

In enrichment procedures, environmental samples may be introduced into nitrogen-free semisolid media to encourage the development of diazotrophic populations. Growth in these media can then be followed by transfer to fresh medium, isolation on solid agar, and further characterization. The precise composition and oxygen conditions depend on the bacterial group being investigated.^[73]

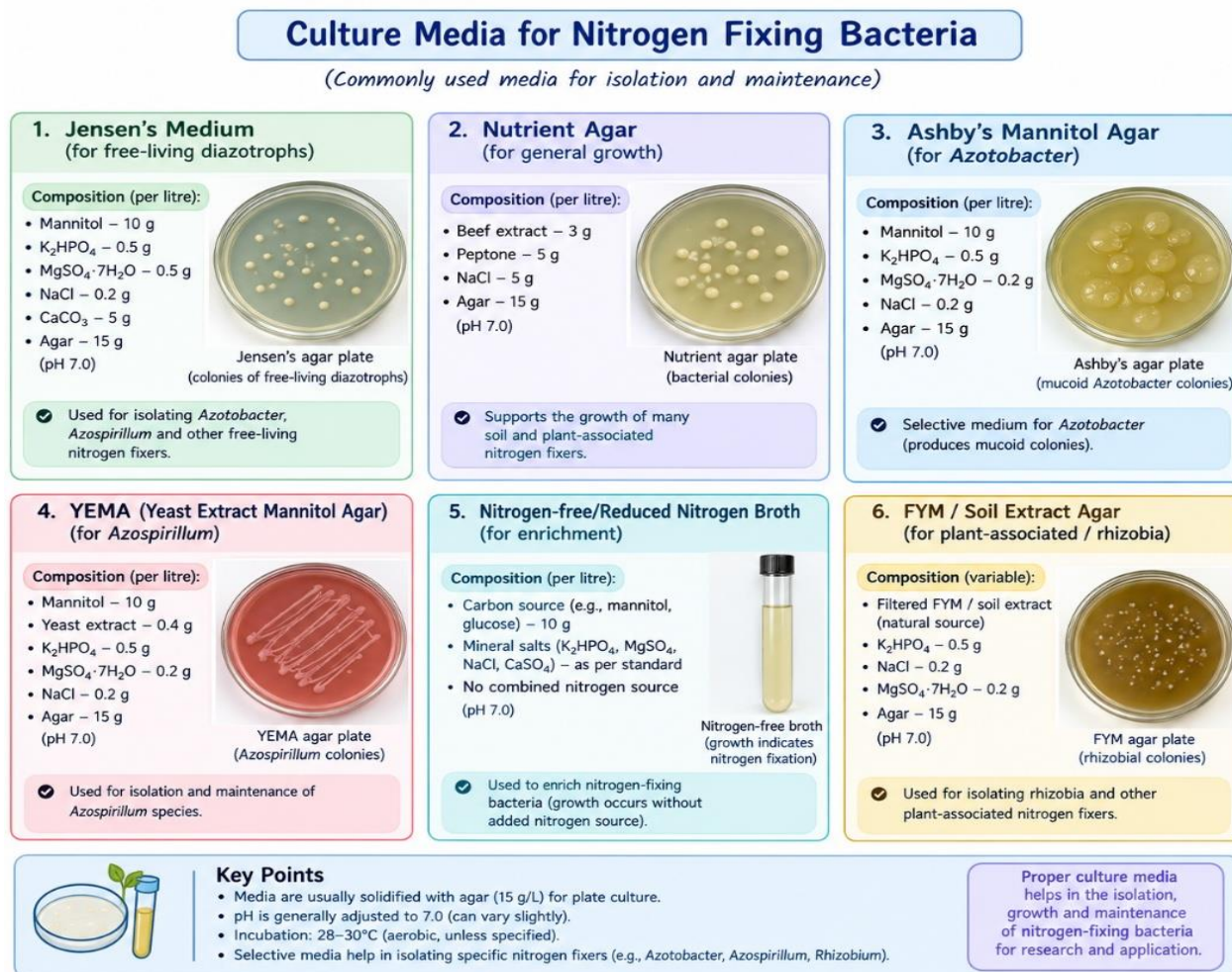


Fig. 7: Culture media of nitrogen fixing bacteria.

7. Introduction of nitrogen fixing bacteria into soil after isolation and culture media

After isolation, purification, identification, and multiplication of an effective nitrogen-fixing bacterial strain, the selected culture can be introduced into agricultural soil through different inoculation methods.

The main aim is to bring viable bacteria close to the developing roots so that they can survive, colonize the rhizosphere, and establish an effective association with the plant.

7.1 Selection of an effective strain

A suitable bacterial strain is selected based on its nitrogen-fixing ability, plant compatibility, survival under soil conditions, and ability to colonize the root environment.

For symbiotic bacteria such as *Rhizobium*, the strain should preferably be compatible with the target legume crop.^[74]

7.2 Preparation of bacterial inoculum

The selected strain is multiplied in a suitable culture medium to obtain a sufficient population of active bacterial cells.

The resulting culture is then prepared as an inoculum suitable for agricultural application.^[75]

7.3 Formulation with a carrier material

The bacterial culture may be mixed with an appropriate carrier material to protect the cells and maintain their viability during storage and application.

Peat-based and other organic carrier materials have been used for microbial inoculant formulations.^[76]

7.4 Seed inoculation

One of the common methods is to coat seeds with the bacterial inoculant before sowing.

This allows the bacteria to reach the developing root system soon after germination and increases the possibility of early root colonization.^[77]

7.5 Direct soil application

The inoculant can also be applied directly to the soil, particularly near the seed or root zone.

This method places the bacterial cells closer to the region where roots develop and where plant-microbe interactions occur.^[78]

7.6 Application with organic manure or carrier material

In some agricultural practices, the bacterial inoculant can be mixed with suitable organic materials and then applied to the soil.

This approach can provide a favourable environment for bacterial survival and facilitate their distribution in the root zone.^[79]

7.7 Establishment in the rhizosphere

After application, the bacteria need to survive and establish themselves in the rhizosphere. Root exudates provide various organic compounds that can support microbial growth and help beneficial bacteria maintain their association with the plant.^[80]

7.8 Root association and nitrogen fixation

In compatible legume-Rhizobium associations, the bacteria colonize the roots and participate in nodule formation. Inside the nodules, the bacteria fix atmospheric nitrogen into forms that can be utilized by the plant.^[81]

7.9 Evaluation of inoculation efficiency

The effectiveness of inoculation can be assessed by observing bacterial establishment, root colonization, nodule formation, plant growth, and nitrogen status.

These observations help determine whether the introduced strain has successfully established and contributed to biological nitrogen fixation.^[82]

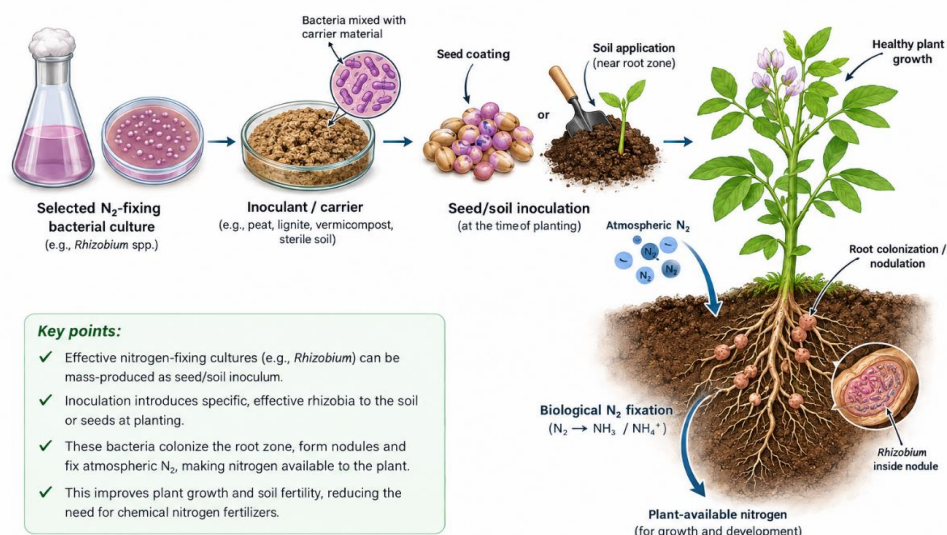


Fig. 7: Isolation of nitrogen fixing bacteria.

CONCLUSION

Nitrogen fixation is a fundamental biological process that makes atmospheric nitrogen available to living systems. Although molecular nitrogen is abundant in the atmosphere, its stable chemical structure prevents most

plants from using it directly. Nitrogen-fixing microorganisms overcome this limitation by converting atmospheric N_2 into biologically useful nitrogen compounds through the activity of nitrogenase. In this way, biological nitrogen fixation provides an important

connection between atmospheric nitrogen and plant nutrition and contributes to the continuous functioning of the nitrogen cycle.

The association between *Rhizobium* and leguminous plants is one of the most important examples of biological nitrogen fixation. The formation of root nodules provides a suitable environment for bacterial nitrogen fixation, while the plant supplies carbon and energy to the bacterial partner. The effectiveness of this association depends on factors such as plant–bacterial compatibility, soil pH, temperature, moisture, salinity, nutrient availability and the existing nitrogen level in soil. Understanding these interactions is important for improving the performance of beneficial nitrogen-fixing microorganisms in agricultural systems.

The isolation and characterization of nitrogen-fixing bacteria provide an important foundation for studying their biological properties and agricultural potential. Nitrogen-free media can be used for preliminary screening, but confirmation requires additional physiological or molecular evidence. Techniques such as the acetylene reduction assay, nitrogen estimation methods and molecular detection of nitrogen-fixation-related genes can provide stronger evidence of nitrogen-fixing activity. Proper characterization and preservation of promising isolates also make them available for future research and application.

From an agricultural perspective, the use of effective nitrogen-fixing microorganisms has considerable potential for improving soil fertility and plant nitrogen nutrition. Microbial inoculants can be introduced through seed treatment, direct soil application or suitable carrier-based formulations. When compatible microorganisms successfully establish themselves around plant roots, they may contribute to nitrogen availability and support plant growth. This biological approach can complement conventional nutrient management and may help reduce excessive dependence on synthetic nitrogen fertilizers.

In conclusion, nitrogen fixation is not only an important biochemical process but also a valuable component of sustainable agriculture and environmental management. Future research combining microbiology, plant science, molecular biology, biotechnology and synthetic biology may improve the efficiency and reliability of biological nitrogen fixation. Better selection of microbial strains, improved inoculant formulations and a deeper understanding of plant–microbe interactions can help develop practical strategies for maintaining soil fertility, improving crop productivity and reducing the environmental problems associated with excessive nitrogen fertilizer use. Thus, effective utilization of biological nitrogen fixation can contribute to a more balanced nitrogen cycle and support long-term sustainable agricultural production.

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