Chapter 5
Diversity and Evolution of Nitrogen Fixing Bacteria

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Abstract Nitrogen is a major element for plant life, yet environmental nitrogen is poorly available to plant, and thus classified as a 'limiting element'. As a consequence, most plants, except the insectivorous florae, rely upon microbial partners to maintain the nitrogen supply. Nitrogen-fixing prokaryotes are widely distributed and account for the fixation of nearly 50–200 megatonnes of nitrogen per year. Nitrogen-fixing microorganisms are potent agents for applications in agricultural fields. Reduction of gaseous dinitrogen to bioavailable nitrogen is mainly done by the molybdenum-dependent nitrogenase in archaea and eubacteria. In plants, the process of nodulation has evolved from 100 million years ago, confering the nodulation capability to about 70% of leguminous plant species. The genes necessary for the nitrogen fixation evolved only after the divergence of bacteria and archaea. Furthermore, the nitrogen-fixing endosymbionts are supposed to have evolved many times in the higher plants, especially in angiosperms. This chapter reviews the diversity and evolution of nitrogen-fixing bacteria.

Keywords Nitrogen · Nitrogen fixation · Endosymbiosis · Rhizobiaceae · Nitrogenase · Symbiotic nitrogen fixation · Associative nitrogen fixation · *Nostoc* · Evolution · Evolutionary analysis

5.1 Introduction

Nitrogen, an unambiguously regulating element meant for plant growth, is primarily present in the atmosphere in the form of dinitrogen gas (N_2), which is an incongruous source for plants. As an alternative, plants depend on reduced forms of

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nitrogen, such as nitrate (NO_3^-) or ammonium (NH_4^+), which can be easily absorbed from the soil. In agroecosystems, nitrogen supply is maintained by introducing plant-available forms in the form of fertilizers fashioned either through various chemical methods, e.g., the Haber–Bosch process that transforms the atmospheric nitrogen into ammonia by expending a large quantity of energy, or, sporadically, from mining mineral deposits, e.g., rocks encompassing potassium or sodium nitrate. Conversely, in most of the unobstructed non-agricultural systems (along with a few agricultural ones), the soluble form of nitrogen (ammonium) is made accessible to the plants through a process commonly acknowledged as biological nitrogen fixation (James 2017). The process of biological nitrogen fixation, which is defined as the capability of converting atmospheric nitrogen (N_2) to its reduced form, is certainly the earliest innovation utterly accomplished by various members of bacteria and archaea and is also one among the ecological services of supreme significance that are offered by microbes to different eukaryotes.

A diverse array of organisms, comprising plants, animals, protists, and fungi form symbiotic relations with nitrogen-fixing bacteria, but such associations appear to be unfastened and only a little indication of any kind of evolutionary amendment is found to be prevailing in either of the partners (Masson-Boivin and Sachs 2018). However, some plants have evolved the ability to make some specific root structures called nodules where diazotrophic bacteria are hosted intracellularly (van Velzen et al. 2018). A diverse community of leguminous and non-leguminous plants hosts a polyphyletic crowd of diazotrophs belonging to α and β proteobacteria which are together known as rhizobia. The additional group of nodulating species of plants is said to be 'actinorhizal' as a result of their passion for hosting diazotrophic filamentous Actinobacteria belonging to the genus *Frankia*.

The nitrogen fixation in leguminous plants is well documented; however, it differs from that of nitrogen fixation in actinorhizal plants. The actinorhizal plants develop an endosymbiosis with the nitrogen-fixing soil actinomycete *Frankia*. The establishment of the symbiotic process results in the formation of root nodules in which *Frankia* provides fixed nitrogen to the host plant in exchange for reduced carbon. As in the case of rhizobium–legume symbioses, the compatible interaction between *Frankia* and actinorhizal plants due to fine-tuned exchange of signals between the two partners leads to the development of nitrogen-fixing nodules. On the plant side, although the involvement of flavonoids in symbiosis is poorly understood, several studies indicate that they may play a significant role in the early stage of the interaction. The flavonols present in the root exudates enhance the level nodulation. Several genes of the isoflavonoid biosynthesis pathway are also known to be up-regulated during the early steps of plant-microbe interactions. The *Frankia* root hair deforming factors are known to play a similar role as played by Nod factors in legume-rhizobium symbiosis.

Although, their chemical properties differ from that of Nod factors but these are also known to be structurally similar to *Rhizobium* nod factors owing to the presence of *N*-acetyl-glucosamine, the backbone of *Rhizobium* Nod factors. After the successful completion of plant-microbe interactions, the actinobacterial auxin possibly plays a role in plant cell expansion, cell-wall remodeling, induction of adventitious roots, and in increasing the level of auxin in nodule primordial. The root infection may proceed either intracellularly or intercellularly depending upon the plant species involved. Unlike legume nodules, actinorhizal nodules are modified lateral roots (Santi et al. 2013). However, legume nodule primordia are formed in the root cortex and develop into stem-like organs with a peripheral vascular system and infected cells in the central tissue (Pawlowski and Sprent 2007).

Inside these specialized structures, these microsymbionts are provided with physiological circumstances favoring the catalysis of the conversion of atmospheric N_2 to NH_4^+ by the bacterial enzyme complex nitrogenase which is a complex and energy-intensive enzyme system, demanding 16 moles of adenosine triphosphate for a single mole of nitrogen fixed (Hoffman et al. 2014). Universally, the process of biological nitrogen fixation is solely accountable for the fixation of around 50–200 megatonnes of nitrogen per year added to earthly ecosystems, which makes up about 80% of the total fixed nitrogen. While it is anticipated that terrestrial contributions of biological nitrogen fixation are principally owing to the legume-rhizobial symbiosis, however, it is not easy to estimate biological nitrogen fixation by legumes in the field conditions (James 2017).

The evolution of the process of nodulation in plants is believed to have happened firstly nearby 100 million years ago leading to the possession of nodulation capability in around 70% of the leguminous plant species, and also in several plants, predominantly called as actinorhizal plants, which find their distributionwide three Angiosperm orders (Werner et al. 2014; Van Velzen et al. 2017). In parallel, the capacityof nitrogen fixation within legumes has found its way to hundreds of species in both α-Proteobacteria as well as β –Proteobacteria (Remigi et al. 2016), referred to as rhizobia, while the ability to nodulate actinorhizal plants has been restricted to the *Frankia* genus in Actinobacteria. Incongruously, most of the essential food crops like wheat, rice, corn, and tuber and root crops have not evolved with the nodule-based symbiotic association thereby lacking their nitrogen-fixing partners. However, another kind of, somewhat fewer formal relations, entitled as associative symbioses, is well known among several kinds of grass and bacteria, for instance, the tropical grasses, *Digitaria* and *Paspalum* often subordinate with the bacteria, *Azospirillum brasilense*, and *Azotobacter paspali*, respectively (Döbereiner et al. 1972).

A vast array of nitrogen-fixing microbes, however, is not associated with either animals or plants. They are freely living inhabitants of the soil which fix nitrogen for their own use. These free-living nitrogen-fixing bacteria also encompass some bacteria of agricultural importance such as cyanobacteria. The most comprehensively studied members of free-living nitrogen-fixing bacteria are represented by the members such as *Azotobacter vinelandii* (an obligate aerobe), *Clostridium pasteurianum* (an obligate anaerobe), *Klebsiella pneumoniae* (a facultative anaerobe), *Rhodobacter capsulatus* (a photosynthetic bacterium), and numerous *Nostoc* and *Anabaena* species. The death of such free-living nitrogen-fixing microbes marks the availability of nitrogen fixed by these organisms to the neighboring ecosystems (Fisher and Newton 2002). The present chapter targets the diversity of different nitrogen-fixing

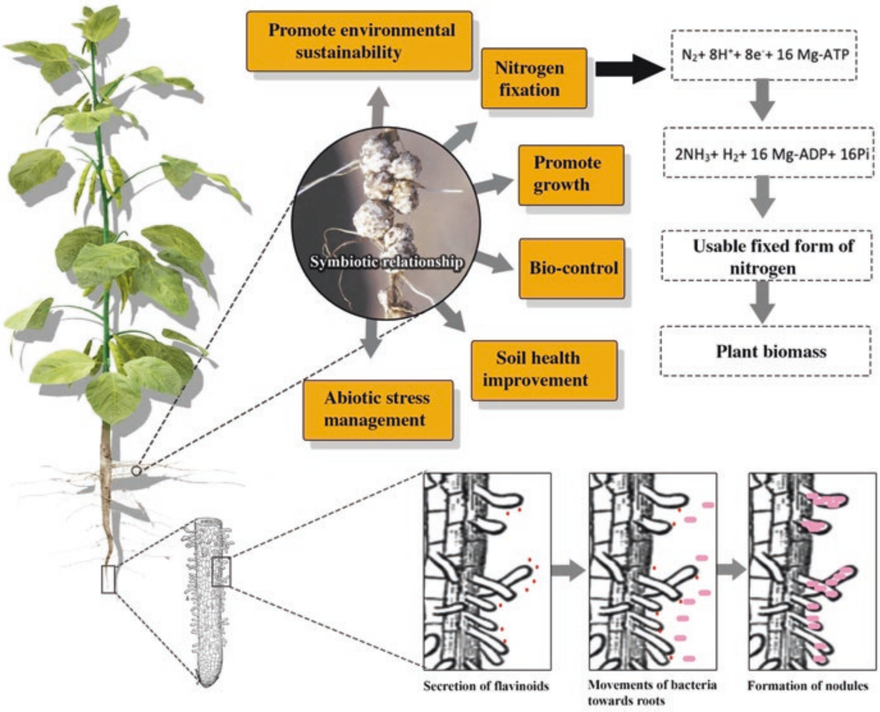

Fig. 5.1 Effects of nitrogen fixing microorganisms on plant growth and soil health

bacteria along with their importance for different ecosystems. The evolution of nitrogen-fixing bacteria is also guided further along with the evolution of the nitrogen-fixingsymbiotic relationship. Figure 5.1 enumerates the overall effects of nitrogen fixation by N_2-fixing microbes.

5.2 Diversity of Nitrogen-Fixing Bacteria

Although nitrogen is present as the most copious compound on the crust of the earth, it is however inaccessible for the majority of microorganisms. Nonetheless, nitrogen is the central part of almost all the proteinaceous compounds as well as nucleic acids; it is a fundamental need of all active living organisms. The conversion of nitrogen into ammonia via a natural process catalyzed by an enzyme complex known as nitrogenase requires an excessive amount of energy at its end (Canfield et al. 2010). This enzyme complex is usually present in some selective members of bacteria as well as archaeal taxa (Young 1992).

5.2.1 Diversity of Free-Living Nitrogen-Fixing Bacteria

The cycling of nitrogen in natural systems, including the agricultural sector, depends upon the fixation of nitrogen by biological means mainly by diazotrophic bacteria which are remarkably diversified and broadly dispersed athwart Bacteria and Archaea phyla. The preeminently acknowledged nitrogen-fixing bacteria make symbiotic relationships with their host plant and contribute to fixing a large amount of nitrogen (about 80%) by biological means (Peoples et al. 1995). However, these nitrogen fixers are restricted to only selective plants factions *e.g.,* leguminous plants, alders as well as cycad plants, whereas a very wide variety of plants is not able to make a symbiotic association with these nitrogen fixers. Free-living nitrogen fixers have got lesser consideration because of their lesser nitrogen-fixing efficiency as well as lower population size as compared to symbiotic fixers in the clement cultivation sector. These free-living nitrogen-fixing bacteria are usually heterotrophs residing in soil and contribute to fixing a noticeable amount of nitrogen devoid of any straight relationship with the plants. The advantageous characteristic of free-living nitrogen fixers over symbiotic nitrogen fixers is that the benefits of free-living nitrogen fixers are accessible to all the plants and not restricted to a particular plant group, thereby playing a good task in nitrogen delivery to the ecological system (Belnap 2002; Yeager et al. 2007).

Nitrogen-fixing bacteria free-living in soil exemplify a diverse group of bacteria counting saprophytes residing on plants litters and bacteria residing in close vicinity of plants roots with intimate relationships. For instance, bacterial species belonging to genera *Azotobacter, Bacillus, Clostridium Pseudomonas*, *Azospirillum, Klebsiella,* and cyanobacteria are examples of different types of nitrogen-fixing bacteria living freely in soil (Table 5.1). These saprophytic, freely living bacteria make their peculiar way to arrange energy supply either by oxidation of natural compounds discharged by other life forms or by the decay of residual plant materials. Some freely residing nitrogen fixers are chemolithotrophic in nature; therefore, having the potential to utilize inorganic molecules to accomplish their energy needs. In a particular set of conditions, freely residing nitrogen fixers might be able to fix a considerable amount of nitrogen (0–60 kg N ha^{-1} $year^{-1}$) (Kahindi et al. 1997; Burgmann et al. 2004). In aquatic organizations, cyanobacteria can fix up to 300 kg N ha^{-1} $year^{-1}$ in relationship with *Azolla* sp. whereas, in earthly organizations, these can fix approximately 30 kg N ha^{-1} $year^{-1}$ in close interactions with grasslands and approximately 3–5 kg N ha^{-1} $year^{-1}$ in different cultivationsystem (Paul and Clark 1989). *Azotobacter* sp. has been found to fix approximately 1 kg N ha^{-1} $year^{-1}$ while residing freely and approximately 1–20 kg N ha^{-1} $year^{-1}$ when present in baggy relationships with grasses (Paul and Clark 1989). These fixations of nitrogen occur under peculiar conditions where the readily accessible nitrogen to these bacteria is usually limited (Rosen and Allan 2007). These conditions can be created by amendments of farming lands with such organic compounds which are having lesser accessible nitrogen contents.

Table 5.1 Grouping of nitrogen-fixing bacteria

S. no.	Family	Bacteria
	Associative N_2-fixers	
1.	Rhodospirillaceae	*Azospirillum lipoferum, A. brasilense, A. amazonense, A. halopraeferens, A. irakense, A. oryzae, A. rugosum, A. formosense, A. palatum, A. canadense, A. doebereinerae, A. thiophilum, A. picis, A. zeae, A. humicireducens, A. fermentarium, A. agricola.*
	Free-living N_2-fixers	
2.	Azotobacteraceae	*Azotobacter vinelandii, A. chroococcum, A. paspali, A. salinestris, A. tropicalis, A. nigricans, A. beijernckii, A. armeniacus*
3.	Clostridiaceae	*Clostridium pasteurianum*
4.	Pseudomonadaceae	*Pseudomonas stutzeri*
	Symbiotic N_2-fixers	
5.	Rhizobiaceae	*Rhizobium hainanense, R. indigoferae, R. leguminosarum, R. lupine, R. lusitanum, R. miluonense, R. tropici, R. cellulosilyticum, R. daejeonense, R. etli, R. gallicum, R. giardinii, Sinorhizobium meliloti, S. americanum, S. fredii, S. medicae, Allorhizobium borbori, A. vitis, A. oryzae, A pseudooryzae*
6.	Bradyrhizobiaceae	*Bradyrhizobium japonicum, B. elkanii, B. yuanmingense, B. liaoningense, B. betae, B. cytisi*

Free-living nitrogen-fixing bacteria perform nitrogen fixation under such conditions where the amount of oxygen is either limited or absent due to the sensitivity of the nitrogenase enzyme towards oxygen which is the main enzyme responsible for nitrogen fixation. The abundance of free-living nitrogen-fixing bacteria in soil is generally low due to the limited amount of suitable carbon and energy resources. Furthermore, the conditions to access the suitable carbon and energy resources also happen to be least favorable (Smercina et al. 2019). However, the prevalence of favorable conditions and availability of suitable energy sources can encourage these bacteria to fix a considerable amount of nitrogen, accessibility of which is not limited to specific plant taxa but it is widespread.

5.2.2 *Diversity of Symbiotic Nitrogen-Fixing Bacteria*

The highly proficient form of nitrogen fixation is established with a symbiotic relationship with higher plants where the energy for fixing nitrogen, usually, the oxygen defense organization is provided by the plant counterpart. The two major groups of the symbiotic association are represented by the prokaryotic companions which are mainly soil bacteria, rhizobia in case of symbiotic associations with legumes, and *Frankia* in case of actinorhizal symbiotic relationships. However, in the case of a symbiotic association of *Gunnera*, the microbial counterpart responsible for fixing nitrogen is the cyanobacterium, *Nostoc* sp. Although, the habitats where the symbionts are located in differs from the companion microbe, for instance, in *Gunnera* the cyanobacterial partner reside in already present stem glands, while in the case of leguminous and actinorhizal plants, specialized organs called as the root nodules, are fashioned by the plant when it comes in contact with symbiont followed by the infection process.

All the above-mentioned systems harbor prokaryotes for fixing nitrogen inside the host cells; however, they are also disjointed from the plant cytoplasm utilizing the membranes which find their origin from the plant plasmalemma (Mylona et al. 1995). The most studied symbiotic relationship involves the leguminous plant and bacteria which are cooperatively regarded as rhizobia. Rhizobial partners collaborate in this symbiotic relationship of major biological significance occurring on all the landforms and account for one-fourth of total biological nitrogen fixed yearly on earth. This relationship involves around 18,000 legume species organized with a mounting assortment of alpha and beta-Proteobacteria. The term “rhizobia” is used here as the collective term that designs bacteria to be able to form a nitrogen-fixing symbiosis with legumes (Masson-Boivin et al. 2009). Along with a greater deal of phylogenetic variety, rhizobia also display a great piece of metabolic possessions (Prell and Poole 2006).

However, only one member, *Azorhizobium caulinodans*, seems to be a candid diazotroph, which can propagate *ex planta* that too at the expenditure of fixed nitrogen (Dreyfus et al. 1988) at a relatively high oxygen concentration. Along with the presence of diazotrophy, the other amazing metabolic features are, methylotrophy in *Methylobacterium nodulans* and the ability to photosynthesize in *Bradyrhizobium* which brings nodulation in the legumes belonging to the genus *Aeschynomene.* Both of these metabolic sorts are supposed to subsidize the symbiotic relationship (Masson-Boivin et al. 2009).

5.2.3 Diversity of Associative Nitrogen Fixing Bacteria

Symbiotic nitrogen fixation is well acknowledged in different actinorhizal and leguminous plants along with its occurrence in non-nodulating species. In grasses, nitrogen fixation takes place via loose associations with different rhizospheric and endophytic bacteria. Such a type of nitrogen fixation is recognized as associative nitrogen fixation. It can solely justify for about 60% of sugarcane’s yearly nitrogen supplies. Associative nitrogen fixation has been principally premeditated in tropical species; however, it also ensues in various moderate grasses where mesocosm reports have interpreted that such kind of nitrogen fixation process contributes up to 50 kg N ha^{-1} yr.$^{-1}$ (Roley et al. 2019).

It is quite indistinct that which microbial taxa are involved when the process of fixation takes place along with uncertainty in the place of fixation in a plant that whether it is rhizosphere or any other part supporting the fixation. In the case of sugarcane, both endophytes, as well as other present rhizosphere soil, account for nitrogen fixation (Dobereiner 1961; Boddey 1987; James 2000). The site for associative nitrogen fixation is largely presumed to happen in rhizomes and roots, coupled with the subsequent transfer of fixed nitrogen to the above ground tissues. However, the presence of diazotrophs throughout the plant's structures has been highly acknowledged which clearly indicates the occurrence of nitrogen fixation in above ground plant tissues (Roley et al. 2019). The most encouraging aspirants for nitrogen fixation in grasses are *Acetobacter diazotrophicus, Azoarcus* spp. in the case of kallar grass and *Herbaspirillum* spp. in case of sugar cane, and several other species of *Pseudomonas*, *Bacillus*, *Enterobacter*, *Alcaligenes*, *Azospirillum*, *Herbaspirillum*, *Klebsiella*, and *Rhizobium*, in case of rice and maize (James 2000). Several associative, non-endophytic, and diazotrophs, nitrogen fixers find their habitat on the root surfaces, predominantly in the elongation zones and root hair, or inside dislocated epidermises.

Conversely, the endophytic diazotrophs, for instance, *Azoarcus spp.*, *Acetobacter diazotrophicus*, *Herbaspirillum spp.*, and a few strains of *Azospirillum brasilense*, incline on colonizing the root cortex, and also pierce the endodermis layer to occupy the stele, so that there can be a subsequent and perpetual translocationtowards the aerial plant parts. Another endophytic bacterium, *H. seropedicae* enters through cracks shaped at root junctions which act as another apparent site for entry in rice plants.

5.3 Importance of Nitrogen Fixing Microbes

Extensive utilization of chemical fertilizers and pesticides has directed to severe upshots counting worsening of soil as well as ecological attributes. Maintenance and reinstatement of soil health attribute to buttress the proper development of plants are necessary to sustain agricultural production. The effect of soil deterioration on gross domestic production can be remunerated using superior management practices (Meena et al. 2016). It is vitally important to increase productivity not merely to meet increased food requirements but to improve soil fruitfulness as well. The nitrogen-fixing microorganisms utilizing their communication with other microbes contribute considerably to improve soil health and environmental sustainability.

5.3.1 Maintenance of Soil Health

The constancy and production efficiency of bio-network mainly depend upon the health of the soil. Thus, the management of agricultural systems should necessarily contemplate the health of the soil and its sustainability. The properties of soil are elementary not merely for the production of good food or to sustain functional bio-network but for prevention of soil attrition and minimizing ecological influences as well. The functions of soil microorganisms in elevated input agricultural systems have received lesser interest for the reason that organically mediated practices such as regulation of soil edifice; nutrient supply and management of ailments have been majorly substituted with manmade inputs including chemical fertilizers and pesticides utilization which eventually depends upon the use of non-renewable energy reservoirs (Barrios 2007). Substantial information are mounting up about maintaining various living microorganisms and their functioning in soil reflecting elemental tasks to soil health (Barea et al. 2005).

Positive communications between plants and microorganisms in soil are supposed to be fundamental determinants of the healthiness of plants as well as soil. Nutrient cycling in the soil is a very crucial ecological task that is vital to life on this planet. Degradation and elemental conversion have recognized greater interest as well due to their necessary position in the natural cycling of nutrients (Coleman et al. 2004). The affirmative effects of soil microorganisms present in symbiotic associations on crop productivity enhance because of the increase in the number of accessible nutrients by plants, particularly nitrogen which is fixed using N_2-fixing microorganisms. The fixation of N_2 by biological means plays a significant part in increasing crop production by various modes including, (1) assimilation of fixed nitrogen straightly in crop products, (2) assimilation of fixed nitrogen in silage, (3) addition to perpetuation and reinstatement of soil health (Giller 2001).

The edifice of soil is a result of the organization of sand, silt, and clay particles in addition to soil organic matter into accumulates of various sizes using living and non-living agents. The proper dimension, amount, and constancy of soil accumulates indicates a proper equilibrium among accumulates materializing things (such as the addition of organic materials, various microbes dwelling in soil, and different vegetations) and the factors responsible for their disruption such as bioturbation and agriculture (Six et al. 2002).

The importance of soil-dwelling microorganisms in soil edifice alteration had been identified earlier by planters but the effects of soil microorganisms on accumulated construction were first hypothesized in the shape of the hierarchical model of soil aggregation just merely 25 years back (Tisdal and Oades 1982). It was additionally projected as the ‘aggregate dynamic model’ which connects aggregate construction and degradation in a straight line in soils to the turnover of particulate organic matter which are arbitrated by activities of microorganisms as well as macro-fauna (Six et al. 1998). This model suggests that various natural practices *viz.*, various fungal and bacterial activities, roots of plants, and macro-fauna in soil result in the construction of ‘biological macro-aggregates, and steadiness of these aggregates accounts to soil edifice.

Being a major part of the plant rhizosphere, nitrogen-fixing microbes play a very crucial role in the construction and maintenance of these aggregates signifying their role in soil health maintenance. A beneficial soil community has a very distinct food network that prevents various phytopathogens and diseases in control by antagonism, predation, and parasitic interaction (Susilo et al. 2004). Therefore, there is a sturdy connection between soil-dwelling microorganisms, soil health, and plant healthiness. For instance, the cultivation of crops in impecunious soil is feebler due to deprived nutrients making the cropincreasingly vulnerable to the attack of phytopathogens and diseases assail (Altieri and Nichols 2003). The restraint of various diseases is mainly due to enriched nutrients in the soil leading to healthier plants which in turn are more capable to combat phytopathogens as well as various diseases. Thus, diverse nitrogen-fixing microorganisms help to enrich the soil with different nutrients via several means, thereby, rendering the plants healthier. It is a harmony that diversity of soil communities not merely assist the prevention of losses because of various pathogens and ailments but stimulate various central natural processes in the soil as well (Wall and Virginia 2000).

5.3.2 Environmental Sustainability

Although non-conventional agricultural practices can produce high crop yield yet it brings about considerable amounts of adverse consequences by significant utilization of inorganic fertilizers and pesticides as well as utilization of non-renewable energy sources (Sharma et al. 2020). Nitrogen-fixing microbes involved in nitrogen dynamics, as well as numerous aspects of sustaining ecology, are thus well-thought-out as a vital means to improve soil healthiness (Fig. 5.2). The present explorations draw attention to the magnitude of scheduling agricultural systems through environmental standards and bio-network facilities for augmentation of ecological as well as agricultural sustainability and productivity, reducing the amount of chemicals utilization and utilization of nonrenewable energy sources. The long-standing sustainability of the agriculture system should majorly depend on utilization and effectual dealing of natural resources (Rani et al. 2019; Sharma et al. 2019; Singh et al. 2019).

Fixing of nitrogen using microorganisms presents an inexpensively appealing and economically sensible means of decreasing exterior inputs as well as enhancing the qualities and quantities of natural sources. Biologically fixed nitrogen is a chief supplier of nitrogen by the use of symbiotically associated microorganisms. A diverse variety of nitrogen-fixing microbes present in soil plays a vital role in handling the performance as well as the sustainability of microbes in the soil, thereby maintaining the ecological constancy. The substantial alteration in types of soil or amateurish agricultural approaches including inappropriate utilization of agrochemicals has overwhelming effects on the quality of soil that in turn is destructive to soil-dwelling microbes resulting in severe damaging effects on the ecosystem (Huang et al. 2013). Numerous bacteria including nitrogen fixers present in the rhizosphere have received noticeable interest due to their handy interactions with plants in the rhizosphere and their capabilities to impart advantages to the plants by enhancing crop production, resistance to stressful conditions, and improving defense systems in plants.

Additionally, these microorganisms alleviate ecological apprehensions by imparting constancy amid plants and surroundings as well (Ahkami et al. 2017). The presence of endophytic microorganisms either bacteria or fungi in associations with plants make a multifaceted, lively as well as enthralling cross-talk environment towards prevalent applications for attaining environmental sustainability (Kusari

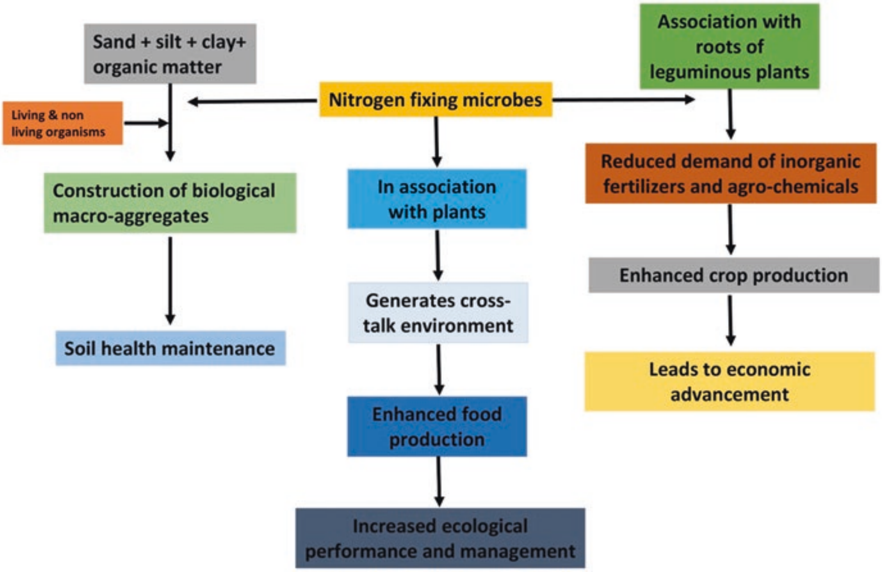

Fig. 5.2 Benefits of dinitrogen (N_2) fixing microbes

et al. 2014). Also, with the biologically fixed nitrogen, worldwide food production scaffolds can be additionally reliable in comparison to the utilization of nitrogen fertilizers. The complicated interchange among numerous microorganisms at various crossing points is imperative to feature the ecosystem and evolution for enhanced food production, ecological performance, and ecological management. The comprehension of these associations among plants and microorganisms will offer novel possibilities for sustaining the agriculture system, maintenance of vital microorganisms as well as plants, and protection of soil qualities and fruitfulness.

5.3.3 *Economic Importance*

According to the estimates of various studies, more than 925 million people worldwide are living in famine conditions (Hunger Statistic World Food Programme 2020). Furthermore, more than 2 billion people are anticipated to be added by 2050 (Eldakak et al. 2013). To overcome the doubts of food certainty and productivity in these circumstances, the traditional agriculture system is majorly reliant on the utilization of inorganic agrochemicals (Stehfest and Bouwman 2006). In certain mounting countries, farmers utilize inorganic fertilizers in a very intense amount in their agricultural terrains to obtain enhanced crop production because of being short of consciousness and appropriate guidance concerning modernization of agricultural approaches and their effects on ecological systems. The enlarged food products can be allocated to enhance the utilization of inorganic nitrogen fertilizers

(Rahman and Zhang 2018) and by viewing current agriculture approaches, it is anticipated to increase additionally in nearby future (Vance 2001). Because of the massive utilization of inorganic fertilizers across the world, the escalation in expenses of inorganic N_2 fertilizers has come about 104 million tonnes in 2006 from 11.6 million tonnes in 1961 (Mulvaney et al. 2009) that is anticipated to enhance additionally to a very larger extent.

Rhizobacteria present in the rhizosphere make associations with the roots of legume plants through a thread-like structure and make polymorphological structures on the roots denoting the main site of nitrogen fixation. The biologically fixed nitrogen is conferred to the plants present in symbiotic associations and makes the plants propagate in the scarcity of external nitrogen supply (Bardos et al. 2011). Furthermore, if the portion of this fixed nitrogen becomes available to the subsequently grown crop, nitrogen-fixing microorganisms play a key task in diminishing the demand for inorganic fertilizers (Crozat and Fustec 2006). The biologically fixed nitrogen symbolizes a crucial reservoir of nitrogen in an ecological system that is estimated to be about 122 million tonnes per year (Hirel et al. 2011). As a result, nitrogen-fixing microorganisms assist the decrease in the amount of chemical N-fertilizers inputs, therefore advancing the economy of the agricultural system.

5.4 Evolution of Nitrogen-Fixing Bacteria

Different life forms necessitate fixed bases of nitrogen and its accessibility frequently restricts efficiency in different natural systems. A major proportion of nitrogen on Earth is represented by dinitrogen (N_2), and its non-bio-availability is an unhidden and unquestionable phenomenon. On initial Earth, some abiotic developments, for instance, electrical oxidation of dinitrogen, were solely responsible for secure nitrogen sources (Boyd and Peters 2013).

The principal enzyme, responsible for reducing the dinitrogen to the bio-available form (ammonia), is a molybdenum-dependent nitrogenase. However, there are other phylogenetically related forms of nitrogenase divergent in the arrangement of metals at the active site, to contribute to ammonia in environments that face limitations of molybdenum (Joerger and Bishop 1988; Kessler et al. 1997). The nitrogenase enzyme is solely accountable for the fabrication of more than half of the total fixed nitrogen on Earth (Falkowski 1997). This progression of nitrogen fixation utilities to discharge limitation of fixed nitrogen in the natural environments (Zehr et al. 2003) and seems to have an unequal upshot on the working of any bio-network, comparative to involvements from any further inhabitants. Accordingly, the description of organisms capable of nitrogen fixation in the natural communities makes them keystone species (Hamilton et al. 2011).

Nature has strongly constrained the dispersal of enzyme nitrogenase to archaea and bacteria only, with no reported example of genes coding for a similar course taking place within the members of eukaryotes. The enzyme nitrogenase finds only a restricted circulation among Archea with only methanogens as the sole representatives distributed among the orders Methanococcales, Methanosarcinales, and Methanobacteriales. Any further evidence, which supports the occurrence of nitrogenase enzyme in the members of the remaining groups of Archea, is, however, has not been reported so far. Similarly, the nitrogenase enzyme also displays a narrow distribution among the bacterial communities.

For instance, the occurrence of *nif* has been advocated in numerous aerobic soil bacteria and has been identified in various cyanobacterial genomes, accounting for the inhabitants of different terrestrial as well as marine atmospheres. Furthermore, the persistence of *nif* gene clusters is frequently perceived in the genomes of Chlorobi, Chloroflexi, Firmicutes, and Bacteroidetes and numerous lines of Proteobacteria and Actinobacteria (Boyd and Peters 2013). The microorganisms exhibiting the ability of nitrogen fixation exhibit an extensive variability of physiologies ranging from strict anaerobes to obligate aerobes (Raymond et al. 2004; Boyd et al. 2011a; Dos Santos et al. 2012).

The extreme sensitivity of nitrogenase to oxygen exposure has allowed a diverse community of nitrogen-fixing microbes to evolve several strategies to perform the functioning of nitrogenase enzyme effectively under toxic environment (Gallon 1981; Berman-Frank et al. 2003). The evolution of symbiotic nitrogen fixation provides the microbial partner with an environment that can maintain low oxygen concentration. Leghemoglobin, a protein synthesized by the host plant, plays a unique role in lowering oxygen tension due to its advanced empathy for binding oxygen (Ott et al. 2005).

The evolution of another stratagem is confiscating oxygen which permits the symbiont to uphold respiration aerobically while catalyzing nitrogen fixation. Additionally, the process of nitrogen fixation takes place in the absence of oxygen in obligate anaerobes, and in the case of facultative anaerobes, it takes place merely in the course of anaerobic progression. Another member of nitrogen-fixing microbes, cyanobacteria, is the solitary diazotrophic descent which releases molecular oxygen as its metabolic product, have established numerous tools for nitrogen fixation (Fay 1992; Berman-Frank et al. 2003). For instance, the cyanobacteria which are non-filamentous in nature lean towards a diurnal cycle where the fixation of nitrogen is promoted during the night time when the concentration of oxygen has plunged significantly as a result of simultaneous reductions in the oxygen production coupled with the enlarged consumption of oxygen by co-dwelling heterotrophic inhabitants.

On the other hand, there is joint existence of oxygen production and nitrogen fixation in filamentous cyanobacteria which are made probable by the segregation of enzymes in some specialized structures called as ‘heterocysts’ where the photoreduction of oxygen to water upsurges the shield of the nitrogenase complex (Milligan et al. 2007), and this process is also said to be the Mehler reaction (Mehler 1957). Conversely, in the case of strict aerobes, the machinery of nitrogen fixation is sheltered by the cytochrome-reliant respiratory defense machinery whereby the oxygen consumption is ensured by a higher respiration rate thus contributing towards the maintenance of low intracellular oxygen (Poole and Hill 1997).

However, such mechanisms developed far ahead in the evolutionary history attributable to the augmented complication of *nif* gene groups accompanying microorganisms evolved with the ability of nitrogen fixation in an oxygen-rich environment. Table 5.2 enumerates the reported *nif* genes which play a role in nitrogen fixation. The modest congregations of genes specifically accompanying nitrogen fixation ensue in strict anaerobes. Nonetheless, sketching the evolutionary path of the entire process and ascertaining the earliest nitrogen fixers in existing biology have always been a challenge (Boyd and Peters 2013). Figure 5.3 predicts the 16S rRNA-based phylogeny tree depicting the evolution of nitrogen-fixing bacteria inferred by using the Maximum Likelihood method and Tamura-Nei model. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. All positions with less than 95% site coverage were eliminated, *i.e.*, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 1098 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

The process of biological nitrogen fixation is supposed to be the earliest and possibly even primordial process (Falkowski 1997; Fani et al. 2000). This prevalent

Table 5.2 List of *nif* genes reported for nitrogen fixation

S. no.	Gene name	Enzyme/enzyme unit encoded
1	*Nif H*	Nitrogenasereductase
2	*Nif D*	Nitrogenase molybdenum-iron protein subunit alpha
3	*Nif K*	Nitrogenase molybdenum-iron protein subunit beta
4	*Nif E*	Nitrogenase molybdenum-cofactor biosynthesis protein
5	*Nif N*	Nitrogenase molybdenum-cofactor biosynthesis protein
6	*Nif X*	Iron-molybdenum cofactor processing protein
7	*Nif A*	Nif-specific regulatory protein
8	*Nif B*	Fe-Mo cofactor biosynthesis protein
9	*Nif Q*	Molybdenum ion binding protein
10	*Nif S*	Cysteine desulfurase
11	*Nif T*	Nitrogen fixation protein
12	*Nif U*	Fe-S cluster assembly protein
13	*Nif V*	Homocitrate synthase
14	*Nif W*	Nitrogenase stabilizing/protective protein
15	*Nif Z*	Iron-sulfur cofactor synthesis protein
16	*Nif J*	Pyruvate-flavodoxinoxidoreductase
17	*Nif L*	Nitrogen fixation negative regulator
18	*Nif P*	Serine acetyltransferase
19	*Nif Y*	Facilitate insertion of Fe-Mo cofactor into apodinitrogenase
20	*Nif M*	Nif H maturation
21	*Nif F*	Electron donor to Nif H
22	*Nif N*	Electron donor toNitrogenase
23	*Nif R*	No protein encoded (repressor binding site only)

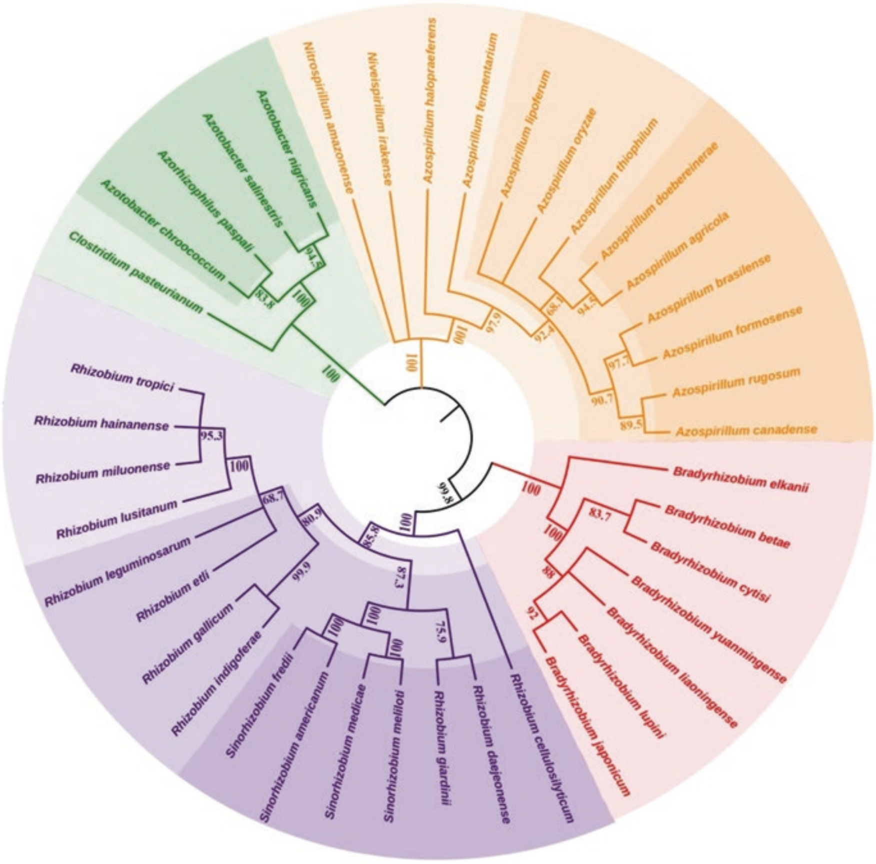

Fig. 5.3 16S rRNA-based phylogenetic tree depicting the evolution of nitrogen-fixing bacteria. The phylogeny is based on the Maximum Likelihood method and Tamura-Nei model

opinion is grounded on simulations of distinctive chemistry of Archaean community that oppose that decreasing concentration of carbon dioxide and a parallel drop in abiotic oxidation of dinitrogen to nitric oxide which headed towards nitrogen crunches at ~3.5 Giga-annum (Kasting and Siefert 2001). The non-biological bases of nitrogen fashioned through several tools such as lightning emancipation or mineral-based catalysis are believed to have become restrictive to an intensifying comprehensive biome. Meanwhile, existent nitrogenase plays an important role to relieve nitrogen restraint in environments (Zehr et al. 2003; Rubio and Ludden 2008), the disparity governing the stock as well as a claim for fixed forms of nitrogen is supposed to have epitomized a robust selective pressure that would have triggered the development of nitrogen fixation (Boyd and Peters 2013). However, if the accessibility to ammonia or other reduced forms of nitrogen is taken into consideration over the progression of geological time, there is the existence of ample pieces of evidence which have suggested enough supply of ammonia that is sufficient enough to upkeep the existence of nitrifying populations in the late Archean >2.5 Giga-annum (Garvin et al. 2009; Godfrey and Falkowski 2009).

The geological records are not that potent till now that they can conclusively resolve when the fixed nitrogen sources started becoming limited, it can be a question of general consideration that whether the distribution and evolutionary history of nitrogenase enzyme along with its accompanying functionalities in existing biology are unswerving with a prehistoric process or an important possession of the last universal common ancestor. Besides, unlike other progressions and functionalities that we attribute towards the possessions of last universal common ancestor, the enzyme nitrogenase is not usually allied with intensely entrenched lines identified by 16S ribosomal RNA evolutionary routes (Boyd and Peters 2013).

The presence of nitrogenase gene clusters has also been acknowledged in the Aquificales genomes (*i.e.*, *Thermocrinis albus* and *Hydrogenobacter thermophilus*) (Wirth et al. 2010; Zeytun et al. 2011). The documentation of the existence of *nif* gene groups in the genetic materials of Aquificales has been considered by sundry as the utmost deeply rooted bacterial line (Reysenbach et al. 2005). The very clear suggestion of this analysis is that profoundly rooted bacteria possess the restricted dispersal of *nif* which is subject to widespread gene loss or was not in the possession of the last universal common ancestor. However, the phylogenetic analyses of a concatenation of the building proteins obligatory for fixing nitrogen (homologs of H, D, and K) clearly specify the paraphyletic attitude of archaea as compared to that of bacteria (Boyd et al. 2011a, b), proposing the emergence of Nif next to the separation of bacteria and archaea.

5.5 Evolution of Nitrogen-Fixing Endosymbiosis

Plants have established several strategies to overcome the deficiency of different nutrients. The paucity of fixed nitrogen is partially fulfilled by living in close connections with the diazotrophic bacteria. These kinds of potent interdependent connections happen as a result of different heights of intimate relationships which range from temporary and adaptable loose links to the enduring and everlasting intracellular lodging of bacteria (Reinhold-Hurek et al. 2015; Santi et al. 2013).

The rhizospheric and phyllosphere portions of plants are inhabited by a diverse array of nitrogen-fixing microbes, thereby fueling opportunities for inaugurating various kinds of symbiotic relations. The endosymbiosis specifically targeting nitrogen fixation is considered to be most lucrative and is supposed to have evolved several times in the higher plants especially angiosperms. Thus, the evolutionary pathway towards endosymbiosis is not a multifaceted one (Geurts et al. 2016). The nitrogen-fixing symbioses are generally represented by two major sections: plant cyanobacterial symbioses and root nodule symbioses.

The symbiotic association between plant and cyanobacteria find a wide distribution through land plants whereas the root nodule symbiosis is constrained, taking

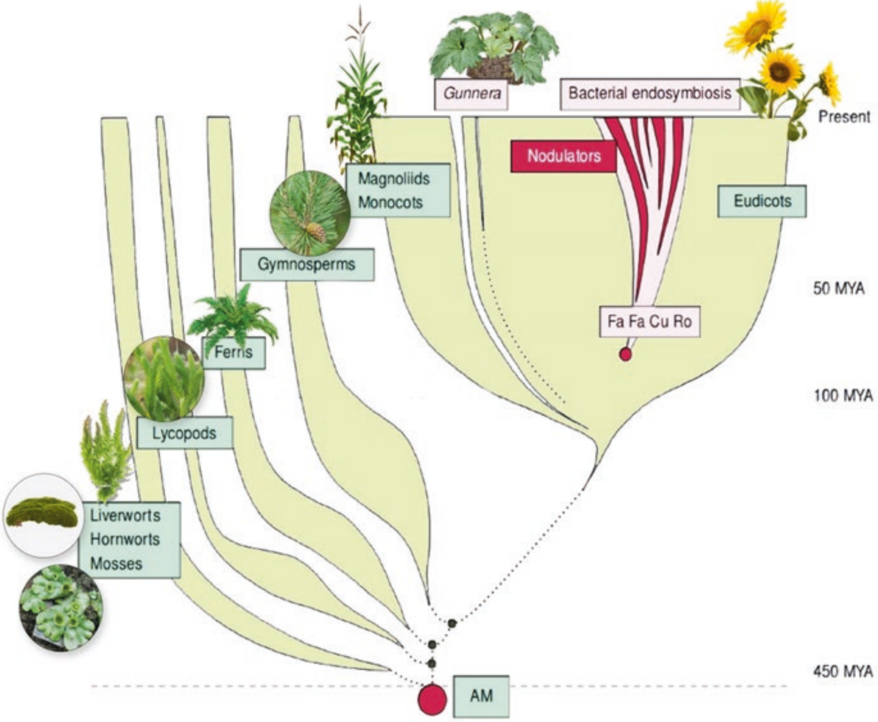

Fig. 5.4 Evolution of the plant root endosymbiosis. FaFa Cu Ro stands for Fabales, Fagales, Cucurbitales, and Rosales members of Eurosid I (Rosids)

place in numerous plant species fitting to only some related orders of flowering plants (Adams 2000; Delaux et al. 2015). The Angiosperms hosts several kinds of symbiotic partners which can be easily differentiated based on the involvement of microbial companions, such as, the cyanobacteria belonging to the genus *Nostoc* is a heterocystous bacteria, which can infect *Gunnera* species (Osborne and Bergman 2009) (Fig. 5.4). The filamentous actinomycetes belonging to the genus *Frankia* also form root nodules in plants called 'actinorhizal' plants (a polyphyletic crowd encompassing 25 genera demonstrating eight taxonomic families) (Li et al. 2015).

There is an appearance of several advances in the plants which range from macroscopic attributes to biochemical alterations. The appearance of such innovations is totally sovereign in different nitrogen-fixing lines. There is a continuous release of signals from the host plant for attracting the symbiont, followed by its enclosure in a freshly advanced assembly, which is later nourished with carbon and an appropriate atmosphere for nitrogen fixation. The development of such multifaceted plant attributes has started with initial adjustments and the later signs of progress are followed through manytransitionalphases (Christin et al. 2015; Delaux et al. 2015).

Despite alterations in the target tissues, the nitrogen-fixing endosymbioses share a collective fact that accommodation of the microbial companion is headed by the stimulation of cell division (Uheda and Silvester 2001; Pawlowski and Demchenko 2012). Cells accommodating the housing of nitrogen-fixing bacteria usually instigate from the mitotic cell divisions which are induced by a signal from the microbial partner.

The cells appearing permissive to infection are usually small in size and have got comparatively thin cell walls. There is a modification in the structure of the cell wall at the point of interactions which makes it less rigid, and also permits invaginations in the cell membrane thereby facilitating the uptake of the microbial partner. Subsequently, the symbiont microbes are not in direct contact with the cytoplasm as they persist walled in some specialized membrane sections (Geurts et al. 2016). The signaling encouraged by the symbiotic bacteria is found to be interfering with the auxin homeostasis in the plants (Kefford et al. 1960). Therefore, the accretion of auxin in the objective cells is prompted indirectly as an outcome of the microbe-induced signaling.

It has been established that the symbiotic signaling grid springs from the cell membrane-localized LysM-type and LRR-type receptor kinases down to a nuclear-localized transcriptional network. Another hallmark in the symbiotic signaling pathways is the generation of consistent oscillatory waves in the calcium concentration of the nuclear region. This calcium−/calmodulin-dependent kinase is often employed by the plant systems for decoding the signal, which sequentially triggers a network of transcription factors crucial for the development of root nodules. Figure 5.5 represents the graphical representation of the signaling cascade involved in root nodule formation. The expression of the *nodule inception* (NIN) gene is much important and adequate for the root nodule establishment (Marsh et al. 2007; Soyano et al. 2013; Vernié et al. 2015). Additionally, it has also been shown that the interdependent signaling elicits Ca^{2+} undulations in *Parasponia andersonii* as well as the actinorhizal plants *Alnus glutinosa* and *C. glauca* (Vernié et al. 2015; Granqvist et al. 2015).

Most of the symbiotic relationships, including the association of rhizobia, instigate the common signaling pathways by secretion of some special compounds called as lipochitooligosaccharides (D'Haeze and Holsters 2002; Persson et al. 2015). Therefore, such kind of symbiotic relationship signifies convergent evolutionary proceedings. The evolution of endosymbiosis of plants with different soil microbes, resulting in the facilitation of nutrient procurement, happened some 450 million years ago and now ensues in more than 80% of the higher plants. The other symbiotic relationships appeared during the preceding 60 million years, comprising the mutually valuable relationship of plants with nitrogen-fixing soil bacteria, harbored in nodules (Granqvist et al. 2015). Additionally, such kind of endosymbiotic relations has evolved several times in the plant kingdom: while rhizobia facing two to five times evolutionary activities whereas it was found to be almost nine times in the case of *Frankia* (Soltis et al. 1995; Doyle 2011; Werner et al. 2014; Li et al. 2015).

Since all the nodulating species of plants mainly find their position in the monophyletic group encompassing the orders Rosales, Fagales, Fabales, and Cucurbitales, and this group is often denoted as the nitrogen-fixing clade (Soltis et al. 1995). However, some of the lineages of this clade have not evolved with the ability to

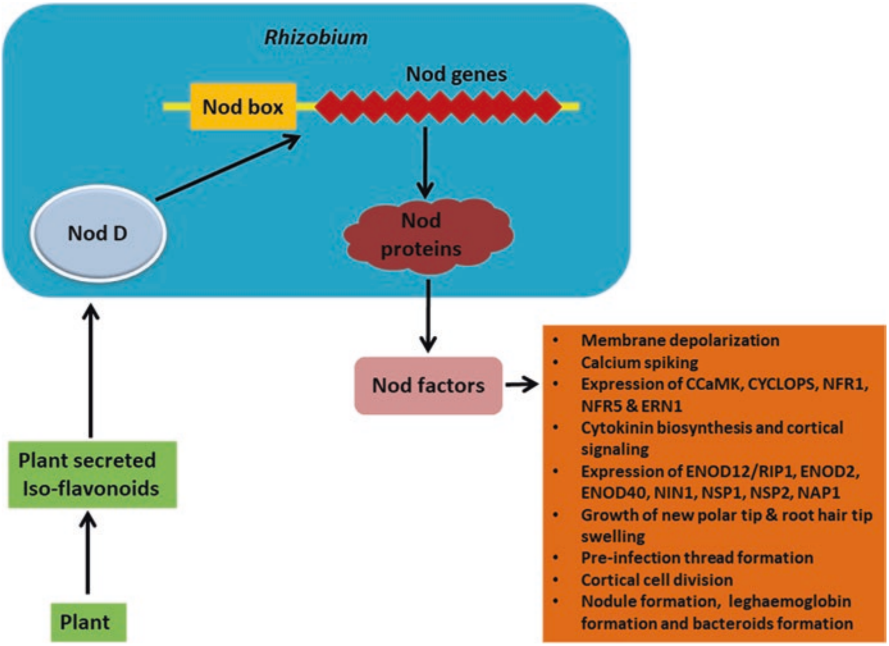

Fig. 5.5 *Rhizobium* bacteria recognize the plant-secreted iso-flavonoids with the help of the transcription factor nod D. nod D upon activation leads to the translation of nod proteins from various nod genes (both common and host-specific. Nod proteins are further involved in the production of Nod factors. These nod factors, in turn, induce several responses in the epidermis and cortex

make symbiotic relationships, two different theories have been proposed to account for such type of phylogenetic relationship: (i) the solitary achievement of nodulation in some antecedent of this clade which was further followed by an enormous and corresponding loss of the same attribute in most of the progenies, or (ii) a parallel progression of nodulation in some progenies along with fewer damages. The former assumption has not been able to get much support thus has received a universal dismissal, although the latter has got extensive acceptance (van Velzen et al. 2018).

Though the latter hypothesis has also got several constraints and one among those is confinement of nodulating species to the nitrogen-fixing clade only. To guide this kind of ostensible clash, one common assumption is that a genetic predisposition happening directed towards an originator state for the process of nodulation developed in some mutual antecedent of this clade about 110 million years ago. This kind of precursor state simplified the equivalent origin of the nodulation process in diverse inheritor roots (Soltis et al. 1995; Werner et al. 2014). However, even after the long-term devotional research in this field, the existence of this proposed precursor state has endured vague and also lacks any pragmatic sustenance. The consideration of the phylogenetic perspective of this nitrogen-fixing clade, for the hypothetical single gain of nodulation to be true, there is a requirement of minimum

7, 5, 17, and 36 losses in Fagales, Cucurbitales, Rosales, and Fabales, respectively. The precise refurbishment of all the evolutionary happenings that ensued 110 million years ago is a fallacious job (vanVelzen et al. 2019).

5.6 Conclusion

The evolution of the process of nitrogen fixation is one of several perfect examples of nature's success stories. Nitrogen is a vital element along with being part of the nucleic acid composition, therefore, often limits the growth and acts as a limiting agent. A vast array of microbehas evolved with the ability to fix atmospheric nitrogen, which otherwise is unavailable, to usable forms. Such microbes are harbored by plants and they form various kinds of associations thus increasing the availability of nitrogen to the plants and thereby decreasing the input demand of chemical fertilizers which is a major economic concern.

The process of evolution of nitrogen fixation seems to be a multifaceted one and appears to have evolved many times. Although, linking the events in a correct sequence that happened millions of years ago seems to be impossible but the understanding of 16S rRNA, which is a conserved sequence often used for tracing phylogeny, gives an idea of the evolutionary history of different nitrogen-fixing microbes. Since the evolution of the ability to harbor nitrogen-fixing microbes is restricted to some genera of the higher plants, a major proportion of crops rely heavily on the use of chemical fertilizers for meeting nitrogen demands. Therefore, a clear understanding of the evolutionary history of nitrogen fixation and evolution of different endosymbiotic relationships could pave a way for various genetic engineering experiments which could enable us to engineer the trait of nodulation in various crops like cereals and thereby would help in saving a vast amount of energy spent for the production of chemical fertilizers along with the promotion and safeguarding environmental sustainability.

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