

Chapter 7

Unravelling Microbial Nitrogen Pathway in Rhizosphere



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Abstract The rhizosphere is a biologically active zone of the soil around plant roots containing soil microbes including bacteria and fungi, where plant–microbe interactions taking place are often beneficial to the plant, the microbes or to neither of them. The developments in molecular biology methods of recent times are focusing on rhizospheric microbial diversity. The rhizosphere is a microenvironment which is very much different from non-rhizosphere soil. The activity of the microbes in the rhizosphere is high and leads to better cycling and availability of nutrients improving the chemical soil quality indicators. With the advances in second generation sequencing and omic technologies, several important mechanisms underlying plant–microbe interactions have been revealed. Metagenomics has revolutionized microbial ecology by facilitating the genomic characterization of microbial communities in various segments of the environment. This has provided a deep insight to the genes that are present in varying environments and constitutes powerful reference material for studying microbial gene expression. Another approach, metatranscriptomics, enables the characterization of community transcription patterns and is a significant advancement in understanding of plant and microbe gene expression. This chapter discusses about how metatranscriptomics could rapidly improve our understanding of plant–microbe interactions in the rhizospheric region.

Keywords Rhizosphere · Plant–microbe interactions · Metagenomics · Metatranscriptomics

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7.1 Introduction

The nitrogen cycle is an important collection of biogeochemical pathways responsible for transforming atmospheric nitrogen (not usable by plants) to organic and inorganic forms which can be used by plants in different ecosystem. Microbial communities are responsible for mediating these pathways. The process of nitrogen cycle is transforming one form of nitrogen to another with the help of different pathways that include nitrogen fixation, assimilation, ammonification, nitrification and some other oxidation-reduction reactions. Different microbes are responsible for providing plants with different sources of nitrogen (Cropnutrition.com).

Nitrogen is the most essential macronutrient required by plants to survive because it is an essential component of nucleotide and protein and because it is a major component of chlorophyll. Without proteins, plants would wither out and die, and without chlorophyll, they would not be able to carry out photosynthesis. Nitrogen is also a component of ATP (which is used for energy driven reactions), forms certain enzymes in plants and is also a component of DNA (stores genetic information). Without nitrogen there will be no plant life. Nitrogen in soil exists in two forms, i.e. organic form and inorganic form (which include ammonium and nitrate) (Jones et al. 2005). Although most of the nitrogen present in the soil is in organic form (90–95%), most of it is not available directly for plant use. Therefore NO_3^- (nitrate)- and NH_4^+ (ammonium) make up most of the plant available nitrogen. Unlike ammonium ions, nitrate ions are free flowing in soil and do not bind to negatively charged cations in soil. Thus, it is more readily available for plants (Courty et al. 2015). Despite most of the organic nitrogen sources being unavailable, some of them can be converted to available forms by microorganisms. Uptake of organic nitrogen is very energy efficient for plants because the assimilation step (conversion of inorganic nitrogen compounds into organic products) is bypassed (Chapin et al. 2002).

The relative proportion of different forms of nitrogen absorbed by plants can depend on various interacting factors (Fig. 7.3). Even though a lot is not known about these factors and how they interact, the relative amount of nitrogen present in soil is considered one of the major factors (Chapin et al. 2002; von Wirén et al. 1997). Other factors like pH of the soil, moisture, temperature, availability of a particular nitrogen form and amount of potassium available in the soil (alleviate ammonium toxicity) might be some of the soil properties determining N form uptake (Britto and Kronzucker 2013). Competition experienced by other plants and microorganisms might play their role in N-availability. Symbiotic relationships on the other hand can increase plant's ability to absorb certain N forms (Kuznyakov and Xu 2013; Li et al. 2012). The species of a plant also might determine the form of nitrogen uptake because the transporters of N vary in every plant species. The development stage of the plant can also be a factor (Britto and Kronzucker 2002). Rhizosphere is basically a micro-zone of soil biota surrounding the root and root hair of plants. This is the region which accounts for plant–microbe interactions. The size of rhizosphere however depends on the species of plant and microbes (Supriya 2018)

Rhizosphere can be classified into three zones, i.e. the inner zone (Closest to the root), Rhizoplane (Root system) and Outer zone (adjoining epidermis). Rhizosphere is favourable for microbial growth due to the presence of root exudates (the organic and in-organic compounds that ooze out of the root during unfavourable conditions). This helps in forming a link between plants and microbes (Krasilnikov 1961). Organic root exudates include compounds like proteins, vitamins, sugars, enzymes, etc. Inorganic root exudates include compounds like water, gases like oxygen, nitrogen, etc. Other than these, some miscellaneous compounds like auxins, glycosides, etc. are also released which might create a negative impact.

In the rhizosphere, deposition of root exudates serves as food and nutrition for microbes. These microbes convert the complex compounds into simpler compounds and these simple compounds can now be consumed by plants through roots (Supriya 2018). Recent studies have explored the role of rhizosphere microbes in providing plants with different forms of nitrogen. There are many omics approaches used for soil ecology (Fig. 7.1). Studies have also suggested that the nitrogen cycling abilities of these microbes change with changes in external conditions, i.e. soil structure, temperature, pH, etc. (Cobo-Díaz et al. 2015). Even after such major advances, in understanding the interactions between microbes and plants, we are still not very sure about how uptake of different nitrogen forms is modulated. In this chapter, we will be talking in detail about the microbial community present in rhizosphere, relation between plants and microorganisms to finally understand and metagenomically analyse the nitrogen cycling pathways carried out in rhizosphere microorganisms. This genomic study would help us understand the very basics of how nitrogen sources are mediated by plant–microbe interactions and why different microbes process different sources of nitrogen.

7.2 Microbial Communities in Rhizosphere

The existence of huge microbial diversity in the rhizosphere zone is due to the nutrition provided by root exudates and the favourable conditions like optimum pH, temperature, moisture, etc. These communities include bacteria, actinomycetes, fungi, algae and protozoa majorly (Fig. 7.2). These microbes can be either beneficial, deleterious or neutral in nature (Supriya 2018).

Beneficial ones include the group of microbes which are of benefit to the root system of plant, for example, mycorrhizal fungi, N₂ fixing bacteria, etc. They help in N₂ fixation, promoting plant growth, colonizing root, increasing absorption capacity of roots. Deleterious ones are those which cause harm to the root systems which includes nematodes, pathogens, etc. Neutral ones are those which show a neutral effect, i.e. neither harm nor benefit the plants (Table 7.1).

Certain organic growth factors like vitamins, auxins, some biotic elements are of great importance for microbial growth. It was observed that small doses of these substances were able to increase the growth of microbes and plants by promoting various biochemical processes. The organic compounds/soil humus is of greatest

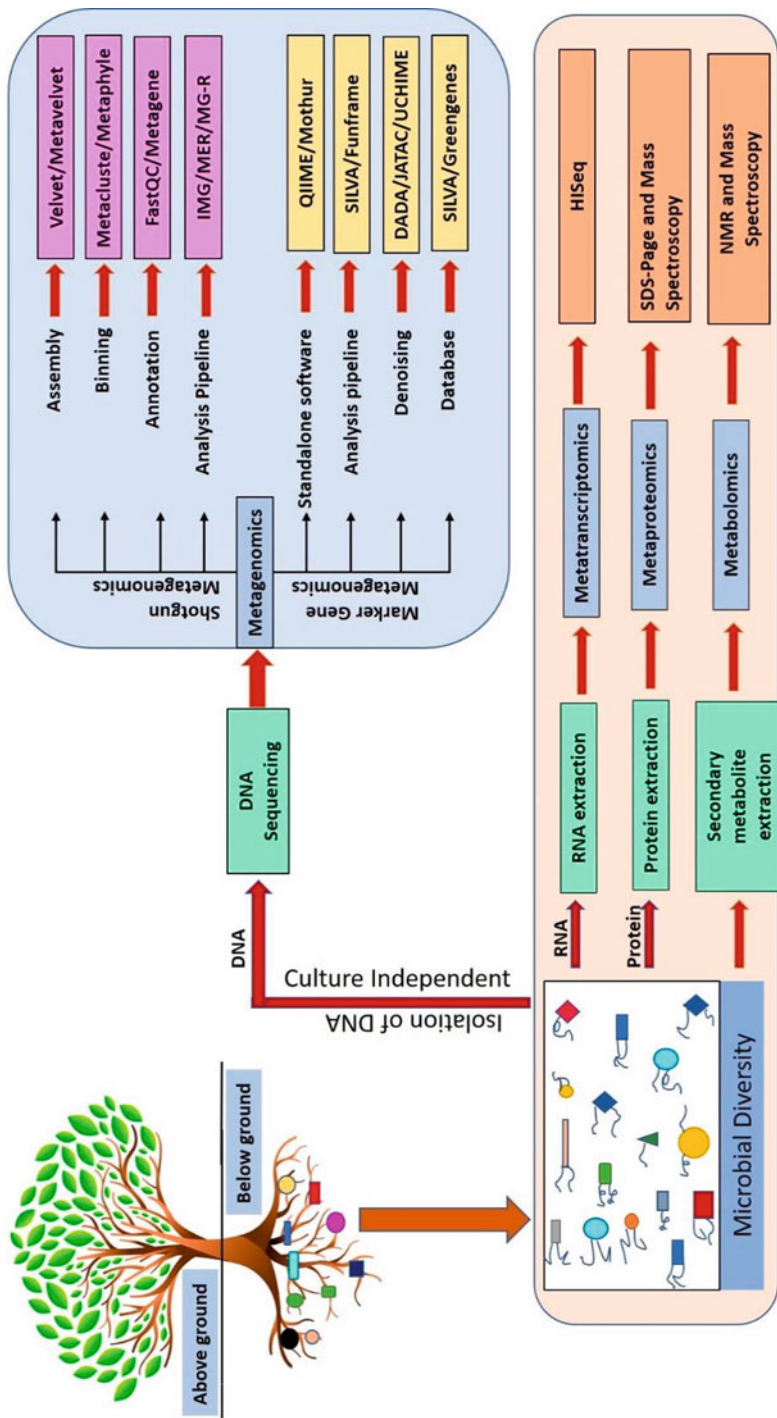


Fig. 7.1 Schematic representation of different omics approaches in soil ecology

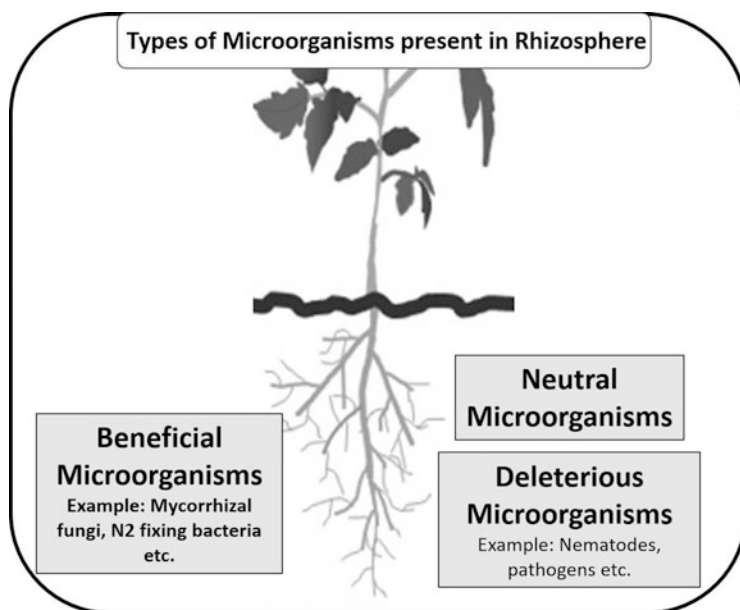


Fig. 7.2 Types of microorganisms present in rhizosphere

importance and thus its quantity and total number of microbial populations can be assumed to be directly proportional (Krasilnikov 1961).

7.3 Relation Between Plants and Microorganisms

Over the course of hundreds of years, plants have evolved various mutualistic associations with certain microbes which majorly include some fungi and bacteria. The most prominent example being that of Arbuscular mycorrhizal (AM) fungi which forms association with plant roots (Parniske 2008). 20% of the Carbon (C) is allocated to mycelia. Other than carbon it takes up nitrate, ammonium and provide Nitrogen to plants in return (Hodge and Storer 2015). This increases the nutrient absorption by plants and thus increases plant growth. Talking about bacteria, plants form mutualistic interactions with bacteria that can fix atmospheric N₂ to produce ammonia. This interaction takes place in the root nodules of plants where photosynthetically fixed carbon is provided to plant in return of fixed nitrogen (Moreau et al. 2019). Since, now we have an idea about the importance of plant and microorganisms in each other's life, we will discuss their effects in detail.

Table 7.1 Microbes found in rhizosphere with their features and functions

Microorganisms	Features	Functions	Examples	Reference
Bacteria	Small size, high in number, present in root hair, mostly gram negative	Fixing of nitrogen, solubilizing inorganic nitrogen	<i>Pseudomonas sp.</i> , <i>Bacillus sp.</i> , <i>Rhizobium sp.</i> , <i>Mycobacterium sp.</i>	Bashan et al. (2005)
Fungi	Second highest in number, work in association with roots	Symbiotic association, might be pathogenic, provides nutrition to roots, colonize in roots	<i>Aspergillus niger</i> , <i>Penicillium pinophilum</i>	Shaikh and Mokat (2018)
Actinomycetes	Forms a link between fungi and bacteria, gram +ve in nature	Compete with bacteria, recycle nutrients	<i>Allokutzneria sp.</i> , <i>Amycolatopsis sp.</i>	Poomthongdee et al. (2015)
Algae	Aerobic in nature, photoautotrophic	Symbiotic association with roots, maintain soil fertility	<i>Chlorella sp.</i> , <i>Chlamydomonas sp.</i>	Mendes et al. (2013)

7.3.1 Effect of Plants on Soil Microflora

Plants influence microorganism while alive and after their death. When they are alive, the root exudates and dying parts of roots provide nutrition for the microorganism's optimal growth in rhizosphere region. After their death, the leftovers of the plants nourish the soil and indirectly create favourable conditions for microbes to proliferate (Krasilnikov 1961).

7.3.1.1 Root Mass of Plants

Surface layer of soil constitutes the major root mass, i.e. the density of roots is highest in surface layer (Kachinskiĭ et al. 1950). This density keeps on decreasing as we move deeper into the soil. Climatic conditions, type of flora and fauna also affect root mass.

The significance of root system is dependent on its activity, i.e. ability to absorb nutrition from the surroundings. In return, products of metabolism are excreted, which are used by microbes to grow. Thus, higher root mass means more microbes (Krasilnikov 1961). This clearly shows that root mass has a direct and immense influence on the growth and development of microorganisms. It may be positive or negative.

7.3.1.2 Root Excretions

Roots are not only responsible for absorbing nutrients from the soil, but also excrete certain substances which play major role in determining fertility of soil. CO₂ is one of the major excretions by roots. The intensity of CO₂ formation by roots is directly influenced by the number of microorganisms in soil. Root excretions also include elements like Phosphorus, Potassium, Calcium, Sodium, etc. (Minina 1927; Sabinin 1940). Excretion from roots is achieved by the process of exosmosis. This happens in accordance with the concentration gradients across root membrane (Minina 1927; Sabinin 1940).

Plants were also found to excrete acetic, formic and oxalic acid through roots (Stoklasa et al. 1909). It was also observed that metabolic products can be transferred from one plant to another in an experiment where two plants were grown in a vessel, one was sprayed with methoxyphenyl acetic acid while the other was not. After some time of incubation, the substance was detected in the unsprayed plant, hence showing that the non-sprayed plant absorbed the substance from the sprayed plant via soil (Preston et al. 1954).

Roots of vegetative plants also secrete certain enzymes in soil. The nature and amount of enzyme secreted vary in different plant species (Virtanen et al. 1937). All these substances are in some way or the other, nutritional sources for microorganisms in soil and are responsible for increase in growth.

7.3.1.3 Root Residue

Root residue refers to dead root cells, hair, epidermis, etc. Different plants attract different types of microflora, which are responsible for decomposition of roots. Different products are formed as a result of this decomposition. These products are the basis of nutrition for some of the other species of microbes (Krasilnikov 1961). This shift of microbial population causes versatility in the final fauna.

7.3.2 *Effect of Soil Microorganisms on Plants*

The effect of soil microflora on life of plants has not been studied very vastly and only a little information is available. Microbes can either effect plant growth positively or negatively. We will talk about both the aspects.

7.3.2.1 Microbial Activators

These are the microorganisms which can produce biotic factors like vitamins, amino acids, etc. They are responsible for activating processes in soil (Zimmermann 1902).

Pure cultures of microbes including bacteria, fungi and actinomycetes affect the growth of plants positively. They increase percentage of germinating seeds and increase biochemical processes. Symbionts are responsible for nodule formation on roots of leguminous plants, they considerably improve growth and crop yield. Root nodule bacteria which act as microbial activators are thought to fix molecular nitrogen and supply it to plants (Krasilnikov 1961).

7.3.2.2 Microbial Inhibitors

These are microorganisms which during metabolism produce certain substrates that are responsible for suppression of growth and development of plant (Greig-Smith 1911). Some fungi and bacteria are known to produce toxins which act upon animals through food products, fodder and results is poisoning of those animals (Pidoplichko 1953). Talking about suppression of plant growth, these toxins are responsible for suppression of seed germination, sprout growth, sporulation process of lower plant, zygote formation in phycomyces (Krasilnikov 1961). Some common microbes responsible for toxin production are some Protozoa, bacteria belonging to genus *Pseudomonas*. Under certain conditions, the toxin produced might endow soil with its toxicity. Level of toxicity depends upon rate of toxin production by microorganisms.

7.4 Microbial Nitrogen Pathways

In terrestrial ecosystems, the availability of Nitrogen is limited, which results in competition in soil fauna and flora. To deal with this shortage of nitrogen, plants and microbes have developed ways to utilize other organic and mineral compounds as a source of Nitrogen (Merrick and Edwards 1995). Main sources of organic Nitrogen are usually plant and animal residue (Kögel-Knabner 2002). Proteins are the most important of them all, constituting 60% of organic nitrogen in soil (Cochrane 1958; Sinha 2004). A small proportion is also fulfilled by mineral sources like ammonium and nitrate (Schimel and Bennett 2004). Glutamate and glutamine are also used as nitrogen sources by various microorganisms (Wong et al. 2008). There are two broad pathways of utilizing nitrogen from soil:

1. *MIT route*: This stands for mineralization-immobilization turnover. This is the step where nitrogen is first mineralized to NH_4^+ before use by microbes and plants. When MIT route is dominant in soil, competition is experienced between plants and microbes (Manzoni and Porporato 2007).
2. *Direct route*: This involves uptake of organic molecules directly like amino acids (Barak et al. 1990; Hadas et al. 1992). In this case, microbes fulfil their requirement from organic resources while plants use mineral sources, hence no competition is experienced (Manzoni and Porporato 2007).

7.4.1 *Extracellular Depolymerase*

Organic polymerase released as residue from plants and microbes needs to be degraded to soluble forms before being utilized by microbe. This is done with the help of extracellular depolymerases. For hydrolysing/degrading nitrogen containing molecules, depolymerases like protease, chitinase and peptidoglycan hydrolases are most common. Protease can be produced by a wide variety of bacteria and fungi (Ahearn et al. 1968; Gupta et al. 2002) and has wide substrate specificities (Kalisz 1988). Extracellular protease is responsible for hydrolysing large protein molecules into smaller peptides and amino acids.

Chitinase is a polymerase secreted by wide variety of bacteria, fungi, plants but not archaea (Gooday 1990). It is found abundantly in nature. Chitinase produced by bacteria hydrolyses chitin. When produced by fungi, they also play role in cell wall development (Adams 2004; Bhattacharya et al. 2007).

7.4.1.1 Regulation

Production of extracellular hydrolases is regulated by the presence or absence of substrate. Here, substrate refers to the residues. For example, protease is activated by presence of protein in the medium (Kalisz 1988) so is chitinase activated by the presence of chitin (Felse and Panda 1999). Carbon sources in the medium might suppress production of these enzymes. For example, glucose might repress product of protease and chitinase (Duo-Chuan 2006). It was also found that inducing substrate in soil might affect production of enzymes by microbes. For example, protein induction increased the protease activity in Tundra soil (Zanuta and Bremner 1976). Similar results were obtained in case of chitinase (Rodriguez-Kabana et al. 1983).

Protease synthesis was repressed under limiting Nitrogen and Sulphur condition (Sims and Wander 2002) but in contrast it was observed that glucose addition to agar increases protease activity (Asmar et al. 1992). Due to different results in different conditions, it was believed that certain factors like soil age, fertility, type of microbes, etc. determine the result of an addition or reduction.

7.4.2 *Nitrogen Mineralization*

Now, that the organic molecules have been depolymerized by respective depolymerases, they are ready for mineralization. This is via the MIT route we talked about earlier. It includes mineralization of organic molecules to finally form ammonium (NH_4^+) (Geisseler et al. 2009). Mineralization of Nitrogen can be done with the help of enzymes like urease and amino-acid oxidase.

7.4.2.1 Urease

Urease is an enzyme which acts as a catalyst for hydrolysing urea to form NH_4^+ (Mobley et al. 1995). It has been extensively studied because of its presence in environment. It is released by mammals as an excretion product. It is also a product of degradation of uric acid extracted by birds (Cunin et al. 1986). More importantly urease can be produced by bacteria, algae, fungi and even plants (Mobley and Hausinger 1989; Follmer 2008). As in the case of protease and chitinase, urease synthesis too is repressed in presence of a more suitable nitrogen source and increased when urea is added (Mobley et al. 1995). It is estimated that most of the urease activity is extracellular, i.e. about 60% of total in soil (Pettit et al. 1976). Later it was believed that urease activity in soil was not a result of urea released by animals but because of urea produced by microbes and plants. This was believed on the basis of experiment carried out by (Zanuta and Bremner 1976). When tests were carried out in soil, it was found that addition of urea did not promote urease activity (Lloyd and Sheaffe 1973). Presence of protected extracellular urease could be one of the reasons or repression by NH_4^+ . Addition of Carbon increases urease activity, this is because of the increased nitrogen demand by microorganisms when carbon is added (Lloyd and Sheaffe 1973; Zanuta and Bremner 1976).

7.4.2.2 Amino-Acid Oxidase

Deamination of amino acids and uptake of ammonium are key reactions in nitrogen fixation, and these are carried out by amino-acid oxidase. This enzyme is mainly intracellular but may be produced by some bacteria and fungi extracellularly a well (on cell surface) (Böhmer et al. 1989; Braun et al. 1992; Davis et al. 2005). Some amino acids are very substrate specific; some have a broad spectrum (Braun et al. 1992). It has been found that production of amino-acid oxidases is repressed by NH_4^+ (Palenik and Morel 1990; Vallon et al. 1993), enhanced by Nitrogen starvation (Davis et al. 2005). Amino acid in the presence of Carbon induces amino-acid oxidases production (Vallon et al. 1993). Several fungal species like basidiomycetes and ascomycetes as well as some bacterial species can produce amino-acid oxidases (Braun et al. 1992; Davis et al. 2005) (Fig. 7.3).

7.5 Metagenomic Analysis

Metagenomics refers to study of genetic material recovered directly from environmental samples. This can also be referred to as environmental genomics.

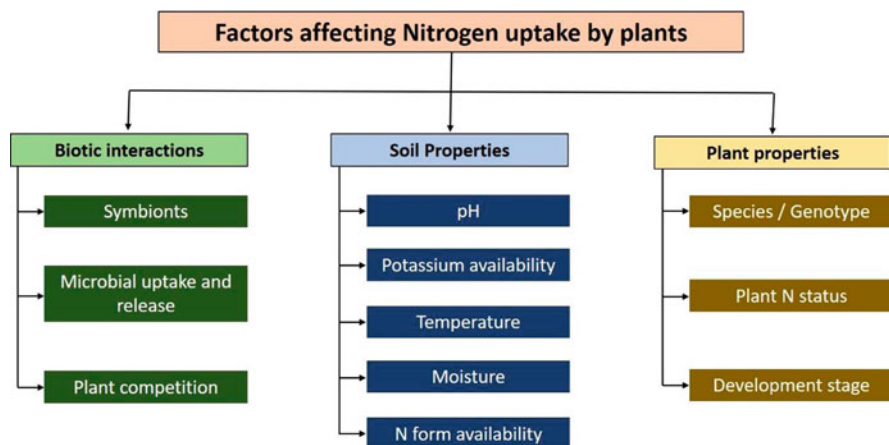


Fig. 7.3 factors affecting nitrogen uptake by plants

7.5.1 Metagenomic Analysis of Nitrogen Cycle

Nitrogen metabolism involves about 7–8 pathways including ammonia assimilation, nitrate and nitrite ammonification, nitric oxide synthase, allantoin utilization, cyanate hydrolysis, Dissimilatory nitrate reductase, denitrification, and Nitrogen fixation. To analyse nitrogen cycle pathway, metagenomics (Cobo-Díaz et al. 2015) was carried out in a study where soil sample was collected from Sierra Nevada Natural and National park and pyrosequencing was performed. About 520,430 reads were obtained which were trimmed to 412,302 sequences. These sequences obtained were studied and analysed with the help of genome library on the basis of functions a particular set of genome performs. At subsystem level (2) 52% of sequence were responsible for ammonia assimilation, 16 of sequence for Nitrate ammonification. In subsystem 3, Nitric oxide synthetase was 9.1%, nitrosative stress was 0.65%, Allantoinian utilization 5.18%, Dissimilatory nitrate reductase 4.19%, Denitrification 2.33%, Nitrogen fixation 1.33%. This analysis also helped in figuring bacterial community based on similarities found in proteins which are responsible for nitrogen cycle. The percentage abundance of microbes in soil is as follows: Bacteroidetes—42%, Proteobacteria—27%. Actinobacteria—14%, Firmicutes—2%, Acidobacteria—6%, and others.

7.5.2 Response of Nitrogen Cycle to CO₂

Earlier studies were focussed on gene families like *nifH* (important for Nitrogen fixation) (Collavino et al. 2014), *amoA* (Nitrification) (Bru et al. 2011; Leininger et al. 2006).

These studies suggested the importance of uncultured organisms in carrying out these processes. When CO₂ concentration is increases, it results in affecting several processes that affect N-cycling. Increase in photosynthesis and plant growth release more carbon into soil and in return demand more nitrogen. Therefore, microbial nitrogen fixation tends to increase under high concentration of CO₂ (Luo et al. 2006; Norby et al. 2010). Decrease in N-fixation was also observed in some studies (Hungate et al. 2004). Experiments (Tu et al. 2017) showed that long CO₂ exposure, produced a significant change in the number of gene families responsible for dissimilatory nitrate reduction, N-Metabolism, N₂ fixation while no such change was observed in case of nitrification, assimilatory Nitrate reduction and denitrification.

Overall, elevated CO₂ increased plant biomass both above ground and in root, increased concentration of ammonium, no change was observed in concentration of nitrate and an increase was observed in C/N ratio both above and below ground.

7.6 Conclusion

After unravelling the nitrogen cycling pathways followed in rhizosphere region with the help of metagenomics and metatranscriptomics approach, it can be concluded that the abundance or absence of certain nutrients in soil can be responsible for the nitrogen cycling pathways followed by microorganisms. These conditions also decide activity level of microbes and their ability to produce soluble nitrogen for plants to consume. The production of enzymes like urease, amino-acid oxidase also varies depending upon soil nutrient availability. This shows that microbes can follow a wide variety of N-cycling pathways ranging from MIT route to direct route as discussed above depending upon the requirements of plants and nutrient availability in soil.

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