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Review: Nitrogen Fixing Microorganisms

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Abstract: Microorganisms employed to enhance the availability of nutrients, viz., nitrogen (by fixing atmosphere N_2), to the crops are called biofertilizers. In recent years, biofertilizers have emerged as an important component for biological nitrogen fixation. It offers an economically attractive and ecologically sound route for augmenting nutrient supply. Plant growth promoting species are commonly used to improve crop yield. In addition to their agricultural usefulness, there are potential benefits in environmental applications. Thus various species of *Rhizobium* for legumes, blue - green algae (BGA) or *cynaobacteria* and *Azolla* (a fern containing symbiotic N_2 - fixing BGA, i.e., *Azolla Anabaena Azollae*) for wet land rice and *Azotobacter / Azospirillum* for several crops can play significant role in agriculture.

Key words: Nitrogen · Nitrogenase · Nitrogen Fixation · Symbiosis · Environmental Problems

INTRODUCTION

Nitrogen (N_2) is an element essential for the support of all forms of life. It is found in amino acids and proteins and many other organic compounds are derived from the nitrogen fixation process [1]. N_2 is the most abundant gas in Earth's atmosphere, it is extremely unreactive [2]. Biological nitrogen fixation is an important part of the microbial processes [3]. Biological nitrogen fixation is carried out only by prokaryotes, which may be symbiotic or free living in nature. It is well documented that biological nitrogen fixation mediated by nitrogenase enzymes is a process important to the biological activity of soil. Nitrogenase activity in soil depends on ecological conditions in association with the specific nitrogen fixation capabilities of certain microorganisms and plant genotypes under various climatic conditions. However, the degree of nitrogenase activity is plant specific. The nitrogen fixing activity of free-living, non-photosynthetic aerobic bacteria is strongly dependent on favorable moisture conditions, oxygen concentration and a supply of organic C substrates [4].

Nitrogen-fixing organisms are generally active in plant root zone soil. Plants that are capable of releasing exudates exhibit higher nitrogen fixation activity in soil [1]. Before it can be incorporated into biological molecules, N_2 must be chemically reduced to the equivalent of ammonia. The biological reduction of nitrogen is catalyzed by a multimeric enzyme complex, nitrogenase [2]. Nitrogenase consists of two conserved proteins: an iron (Fe) containing dinitrogenase reductase (or Fe protein), encoded by the nifH gene and a molybdenum iron (MoFe) dinitrogenase (or MoFe protein) that is encoded by the nifDK genes [4]. This enzyme is irreversibly inhibited by molecular oxygen and reactive oxygen species. Oxygen stress on diazotrophic (nitrogen-fixing) organisms triggers a wide range of protective responses aimed at deterring the inhibitory effects of oxygen on nitrogenase. The level of resistance to oxygen stress and the mechanisms involved vary among diazotrophs and influence niche selection. The evolutionary trajectory of adaptive mechanisms that protect nitrogenase from molecular oxygen and reactive oxygen species can be discerned in physioecological patterns in microbial morphology, biochemistry, physiology and community structure along a gradient from anaerobic to fully aerobic environments [2]. Nitrogen fixing free living microorganisms have frequently been reported as plant growth promoters [5].

Plants are predominantly made up of carbon, oxygen and hydrogen which are supplied by air and water. Beyond these three elements, nitrogen is required in the greatest quantity [6]. Nitrogen is an element whose content is minimal as compared to that of other mineral nutrients; it determines the intensity of the organic matter accumulation [7]. The source of soil nitrogen is the atmosphere where nitrogen gas occupies about 79% of the total atmospheric gases. Living organisms that are present in the soil have profound effect on

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transformation, which provides food and fiber for an expanding world population. Although nitrogen is very abundant in nature, it often limits plant productivity because atmospheric nitrogen is only available to a very lesser range of organisms symbiotically associate with higher plants and non-symbiotically. About 386 x 1016 kg nitrogen exists in the Earth's atmosphere. It is stated that nitrogen returned to the earth every year, microbiologically is of the order of 139 x 109 kg of which about 65% (89 x 109 kg) is contributed by nodulated legumes [8].

Biological fixation of the atmospheric nitrogen can be estimated at about 175 million metric tons per year or about 70% of all nitrogen fixed on the Earth per year, the remaining is by some micro-organisms, autotrophs or heterotrophs 'free' fixers [9]. The transformation, or 'fixation' of nitrogen from the unavailable gaseous form in the atmosphere to forms that plants and other organisms can use (either NH_4^+ orNO_3^-) is mediated by (i) bacteria in symbiotic relation- ships with vascular plants, (ii) symbioses between *cyanobacteria* and fungi (lichens) or plants, (iii) free living heterotrophic or autotrophic bacteria that are typically associated with soil or detritus and (iv) abiotic reactions occur without microbes in the atmosphere associated with lightening [6].

In this study the Nitrogen fixing free living microorganisms have frequently been reported as plant growth promoters. Plant growth-promoting bacteria (PGPB) were defined as free-living soil, rhizosphere, rhizoplane and phylosphere bacteria that, under some conditions, are beneficial for plants. These bacteria are capable of fixing atmospheric nitrogen, solubilize phosphorus and iron and enhance production of plant hormones.

A Hierarchy of Explanation for Patterns of Nitrogen Fixation: Mechanistic explanation of nitrogen fixation can be sought at cellular/molecular, physiological (whole organism) and sub-ecosystem levels (i.e., ecological controls). At the lowest level are controls at the sub-organismal level, including genetic control, enzyme synthesis and other mechanisms. At the whole organism level, nitrogen fixers are subjected to physiological controls that determine nitrogen fixation can occur; for example, oxygen concentrations or the ability to acquire molybdenum. In addition, the ability of nitrogen-fixing organisms to colonize or persist in a given environment is a function of competitive interactions, predation pressure and availability of limiting nutrients. The third hierarchical level comprises this suite of ecological controls. At the ecosystem level, the patterns and balance of nitrogen inputs and outputs set constraints on the rates of nitrogen fixation, while at the final and highest level regional and global patterns of nitrogen fixation are controlled by patterns of land cover and use, biome distribution, global climatic patterns and patterns of N_2 deposition [9].

Biological Nitrogen Fixation and Symbiosis: Biological nitrogen fixation can be an important source of nitrogen for supporting aquatic primary productivity [10]. Nitrogen is one of the most essential elements for all forms of life; a basic material for synthesizing proteins, nucleic acids and other organic nitrogenous compounds. Unfortunately no plant species is able to reduce atmospheric dinitrogen into ammonia and use it directly for its growth. It appears that only a number of prokaryotic microorganisms including bacteria and *cyanobacteria* have been shown to posses the ability to fix dinitrogen [11]. Bacteria known collectively as the ''*Rhizobia*'' are famous for their ability to induce nodules on the roots (and occasionally, stems) of legume plants. Within these nodules, the differentiated, ''bacteroid'' forms fix atmospheric nitrogen and the resultant ammonia being used as a source of fixed nitrogen. This symbiosis provides the bacteria with an exclusive niche and, in return, the plants obtain a

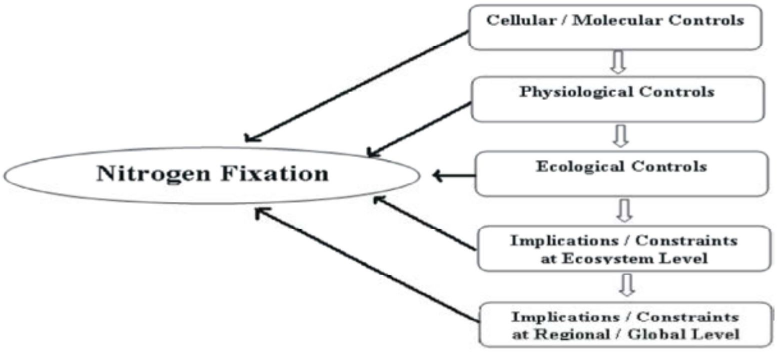

Fig. 1: Patterns of Nitrogen Fixation

personalized nitrogen source [12]. They occur in the so - called free - living forms e.g. aerobic azotobacter, anaerobic Clostridia or in symbiosis with certain higher plants e.g. *Rhizobia* with legumes or *Azolla Anabaena Azollae* with *Azolla*. The potential for biological nitrogen fixation is increased greatly by the fact that there is a close relationship between plants and nitrogen Prokaryotes. Nitrogen fixing prokaryotes are able to make completely useful associations with plants: from loose associations to intercellular symbioses. There exist associative symbioses in which nitrogen fixing prokaryotes (e.g. *Azospirillum, Azotobacter, Enterobacter species*) have been found to occur in rhizosphere of different plants such as sugarcane, maize, wheat, rice, grasses and others [10].

Both plant and bacterium can live separately but the association is vary beneficial for them. It was reported that in plants, up to 25% of total nitrogen came from nitrogen fixation. Activity of nitrogen fixing microorganisms depends greatly upon excessive amount of carbon compounds and adequately low level of combined nitrogen [12]. Carbohydrates come directly from photosynthesis (in the case of *cynobacteria*) or from decay of organic wastes in soils. Recently it was shown that roots of plants release substances into soil, which - in certain measure support colonization and nitrogen fixing activity of bacteria in rhizosphere of plants [11]. It is known that besides ammonia fixed biologically, many microorganisms are able to produce hormones and these substances can influence plant growth effectively [12]. In the case of *Azolla* -Anabaena symbiosis, *Azolla Anabaena Azollae Cynobacteria* live and reproduce in cavities of leaves, but the association remains extracellular. A special form of symbiosis is intracellular symbiosis or endocytobiosis in which the endosymbionts or endocytobionts live in special cells of their hosts such as Rhizoba in rot nodules of legumes or frankia actinomycetes in non-leguminous plants. The *Rhizobium* -legume symbiosis is one of the most efficient fixing systems which is able to fix approximately from 100 up to more than 300 kg nitrogen per hectare per year [11].

Rhizobia are found in most soils and usually present in large number in such soils where the appropriate host plants are grown. However, plants do not reproduce bacteria and the infection always occurs during the life of every individual plant in soil. *Rhizobia* infect their host plants through root hairs. First they are contained within a so - called infection thread and then are released from the infection thread into the cytoplasm of cells in the cortex. Then bacteria are differentiated into endosymbionts namely bacteroid forms which can reduce dinitrogen into ammonia and this can be assimilated directly by host plants [11]. The plant canopy hosts a wide array of microorganisms having beneficial, harmful and neutralistic effects. It is well-established that many soil- and plant-associated bacterial groups are able to synthesize phytohormones. The genera under this list are steadily growing and presently include Gram-negative and Gram-positive, symbiotic and nitrogen-fixing bacteria [13]. N_2 Fixing bacteria such as *Azotobacter, Azospirillum*, *Rhizobium*, Meso*Rhizobium* and Sino*Rhizobium* are well known for their ability to improve plant development [14,15]. Many of these bacteria can produce and excrete in their cultures more than one hormone type: *Rhizobium* isolates synthesize *gibberellins* (GA) and *auxin; Azotobacter spp.,* GA, *auxin* and *cytokininsand Acetobacter* and *Herbaspirillum* isolates, indole-3-acetic acid (IAA) and GA [13].

Azospirillum: *Azospirillum* species belong to the facultative endophytic diazotrophs groups which colonize the surface and the interior of roots and this kind of association is considered as the starting point of most ongoing BNF (Biological Nitrogen Fixation) programs with non-legume plants world wide. Nitrogen fixing organisms such as *Azospirillum*, directly benefits plants improving shoot and root development and increasing the rate of water and mineral uptake by roots [14].

Azotobacter: *Azotobacter* is an obligate aerobe, although it can grow under low O_2 concentration. The ecological distribution of this bacterium is a complicated subject and is related with diverse factors which determine the presence or absence of this organism in a specific soil [14]. Bacteria of the genus *Azospirillum* are a well-known example of so-called associative nitrogen fixers, which are widespread in the soils of tropical, subtropical and temperate regions. These bacteria develop in close relationships with the roots of various wild and agricultural plants [16, 17].

Azolla Spp: *Azolla* spp. comprises several floating water fern species found in tropical and temperate ecosystems. It has the ability to fix atmospheric nitrogen through symbiosis with blue green algae (Nostoc anabaena). Therefore, it is considered an important potential source of nitrogen for wetland rice. The contribution of nitrogen from *Azolla* spp., to wetland rice plants has been found to be maximum when incorporated into the soil as green manure [18].

Cyanobacteria: *Cyanobacteria* are important for global nitrogen cycle [19]. N_2-fixing *cyanobacteria* are among the most widespread and important N_2 fixers on Earth [9]. *Cyanobacteria* or blue green algae are a diverse group of prokaryotes that often form complex associations with bacteria and green algae in structures known as *cyanobacteria*l mats [19]. They are the major N_2 fixers in freshwater and marine systems [9]. In large areas of the world's oceans, the activities of nitrogen -fixing microorganisms (diazotrophs) provide an important source of nitrogen to the marine ecosystem [4]. They also grow and fix nitrogen in many terrestrial environments, from rainforests to deserts [9]. Much of our understanding of the ecology and biogeochemistry of oceanic diazotrophy has been derived from studies on the filamentous *cyanobacterium Trichodesmium spp.,* a cosmopolitan *cyanobacterium* in tropical and subtropical marine systems, including oligotrophic regions throughout the Atlantic and Pacific Oceans [4]. *Cyanobacteria* are able to survive in extreme environments because of unique adaptations such as their capability of fixing nitrogen and their resistance to desiccation. Because of the ability to fix atmospheric nitrogen, *cyanobacteria*l mats have been used as biofertilizer in modern agriculture [19].

Gluconacetobacter Diazotrophicus: Gluconacetobacter diazotrophicus is a nitrogen-fixing, acetic acid bacterium first isolated from sugarcane plants. It belongs to *phylum Proteobacteria* (comprising Gram negative bacteria) in section a-Proteobacteria, order *Rhodospirillales* and family *Acetobacteraceae*. Currently, this family contains three nitrogen-fixing genera, comprising of seven species, namely *Acetobacter nitrogenifigens, Gluconacetobacter kombuchae, Gluconacetobacter johannae, Gluconacetobacter azotocaptans, Gluconacetobacter diazotrophicus, Swaminathania salitolerans* and *Acetobacter peroxydans* [20].

Rhizobia: *Rhizobia* are known for their ability to establish symbiotic interactions with leguminous plants by the formation and colonization of root nodules, where bacteria fix nitrogen to ammonia and make it available for the plant. The bacteria are mostly rhizospheric microorganisms, despite its ability to live in the soil for long period of time [14].

Beneficial Interaction Between Microorganisms and Plants: Agricultural enhancers are defined as biological or non biological agents which reduce the time of growth, increase the production and/or quality of the agricultural products as well as the time of the flowering and fruition, the fruit size, etc. The participation of microorganisms as enhancers is related to their enzymatic activity. For example, potential for nitrogen fixing is a property of some prokaryotes. It is known, that during the growth, the plant interacts intimately with microorganisms of soils. The interaction between the microorganisms and the plants can be beneficial, neutral or detrimental. Three classes of microorganisms beneficial for the plants may be defined as: i) Microorganisms that can increase the supplement of mineral nutrients essential for growth such as nitrogen and phosphorus; ii) Microorganisms that stimulate the growth of the plants at an indirect form by means of the repression of pathogenic organisms (for example *Bacillus thuringensis* produces a toxin lethal for the phytopathogenic insects). These microorganisms are utilized for biocontrol and are called such to differentiate it from the use of chemical insecticide. iii) Microorganisms that direct the biological growth of the plants, for example by phytohormones [21].

Environmental Problems: Human activity is causing large imbalances in the nitrogen and phosphorus cycles of coastal marine waters as a consequence of increased fertilizer use [22]. Intensive application of chemical fertilizers in agriculture has caused damage to the ecological state of the agricultural systems [21]. Nitrogen (N_2) is the major essential nutrient in rice production and therefore, N_2-supplying capacity in paddy soils has a great influence on rice yields. Recently, it has been known that an excessive use of chemical fertilizer induces environmental pollution such as nitrate leaching and nitrous oxide emission [23]. On the other hand, nitrogen is becoming one of the major concerns as a pollutant in terrestrial ecosystems. Nitrogen Fertilizer not utilized by rice plants may contribute to environmental pollution. Some portions of nitrogen fertilizer are easily lost through various processes, such as leaching and denitrification. Furthermore, the increased use of nitrogen fertilizer may substantially increase leaching NO_3^-, which potentially pollutes groundwater. Increased amounts of NO_3^- may enhance direct and indirect N_2O emission to the atmosphere. Thus, attention should be focused on nitrogen losses in paddy fields because this may cause serious environmental problems [24-26]. Therefore, concerns on biological nitrogenfixation, a natural N_2-supplying source, are increasing [23]. Soil salinity is a major factor limiting plant productivity, affecting about 95 million hectares worldwide. The UNEP (United Nations

Environment Program) estimates that 20% of the agricultural land and 50% of the cropland in the world is salt-stressed. Salinity imposes serious environmental problems that affect grassland cover and the availability of animal feed in arid and semi-arid regions, some crops are moderately tolerant of saline conditions; many crops are negatively affected by even low levels of salt. Salt stress unfavorably affected plant growth and productivity during all developmental stages [27].

Management of soil-plant microbial interactions has been suggested as an appropriate strategy to improve the capture and cycling of nutrients, thus leading to a more rational use of land resources by reducing the use-abuse of chemical fertilizers [5]. The use of biofertilizers is an alternative to improve the conditions of Mexican fields and world-wide. Biological fertilizers do not contaminate in the soil and atmosphere and help to produce healthy foods [21].

Ecosystem Effects and Ecological Controls: Both the ecological and evolutionary changes that accompany biological invasions can dramatically alter ecosystems and their functioning by altering resource availability, food web structure, community interactions, chemical composition and the physical structure of the ecosystem itself. Classical examples are the introduction of the fire weed Myrica faya into native ecosystems in Hawaii which until then lacked plant species with nitrogen fixing symbionts. The invading shrub enabled vegetation growth on nitrogen-poor soil and had a direct impact on the biogeochemical cycling within the invaded ecosystem [28]. By ecological controls, we mean controls over the rate of nitrogen fixation that are or can be influenced by interactions between the nitrogen fixer and other organisms (excluding symbiotic partners, if any) and/or the nitrogen fixer and its environment. N_2 fixers, like all other organisms, are subject to a very wide variety of biotic and abiotic controls; it can be too hot or too cold, too dry or too wet, too acid or too alkaline; there can be too many competitors for crucial resources, or too many grazers that restrict N_2 fixers' distribution or abundance. We are particularly interested in such controls where they influence N_2 fixers (or their activity) to a greater extent than they affect non-fixing organisms, because only in those circumstances N_2 fixers will be constrained relative to other organisms. Differential suppression of nitrogen fixers can occur where nitrogen fixers require a resource that other organisms do not need, or where they require more of a resource or less of another environmental factor than do non-fixers. It can also occur when N_2 fixers experience systematically higher mortality than nonfixers, or when environmental conditions are outside the limits of adaptation for all N_2 fixers [9]. While requiring a resource that other organisms need much less of (e.g., molybdenum) it could be considered as physiological rather than an ecological control and conditions outside the bounds of all organisms might be considered an environmental constraint, we will treat these together. Also, the ecological distribution of N_2-fixing organisms may be wider than that of their ability to fix nitrogen and we focus on the ability to fix nitrogen. What are the general features of the N_2-fixation process that could lead to differential suppression of nitrogen fixers in some environments?.

- Nitrogen fixation is relatively energy-intensive.
- Nitrogenase enzymes are inactivated by O_2. Organisms maintain a delicate balance between the efficiency of using O_2 as an electron acceptor and the inactivation of nitrogenase and free-living photosynthetic N_2 fixers must segregate the O_2 they produce from their nitrogenase system.
- Most nitrogenases require molybdenum in order to function; many nonfixers require much less molybdenum. As discussed below, N_2 fixers may also need more P, Fe and/or other nutrients than other organisms.
- In most N_2-fixing organisms, the synthesis and/or activity of nitrogenase is inhibited by high levels of combined nitrogen.
- Many N_2-fixing organisms are rich in nitrogen compared to non-fixers and so may be grazed preferentially.

How can these overall differences between nitrogen fixers and other organisms translate into ecological controls of nitrogen fixation? In this analysis, we will consider three major groups of N_2 fixers - free-living *cyanobacteria*, bacteria and *cyanobacteria* in symbiotic associations with plants and heterotrophic bacteria. There are many other nitrogen fixers, including lichens with *cyanobacteria*l phycobionts, bacteria in animal digestive systems and many minerotrophic bacteria. These fixers are important to the metabolism of particular organisms and to the nitrogen budgets of particular ecosystems; lichens especially have been evaluated in a number of ecosystems. However, symbiotic N_2 fixers, free-living *cyanobacteria* and heterotrophs are by far the most important contributors of fixed N_2 in most ecosystems and if we can understand and model what controls their rates of fixation, that will contribute substantially to explaining the interactions between nitrogen limitation and nitrogen fixation in most ecosystems globally [9].

CONCLUSION

The relevance of biofertilizers is increasing rapidly since chemical fertilizers (nitrogenous fertilizers) damage the environment. In contrast, biofertilizers lead to soil enrichment and are compatible with long-term sustainability. Further they are ecofriendly and pose no danger to the environment.

Future Work: Choice of the strain is vary critical task because an efficient strain of the nitrogen- fixing microorganisms is as important in biofertilizer production as seed is in crop cultivation. To find out alternative is to use the strains from reliable source along with all technical specifications for large scale production of nitrogen-fixing microorganisms.

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