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REVIEW ARTICLE

Role of Nitrogen Fixers as Biofertilizers in Future Perspective: A Review

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ABSTRACT:
Diazotrophs are nitrogen-fixing microorganisms that are capable for biotransformation of nitrates and nitrites into ammonia, which are directly used by plants. Nitrogenase enzyme is useful in Nitrogen fixation and they are used as potential biofertilizers to provide the growth and nutrition supply for plants. Various microorganisms are involved in nitrogen fixation and responsible genes of various diazotrophs were analyzed. The results reveal the most efficient gene sequences for nitrogen fixation and provide a homologous similarity between those microorganisms. The study relates the potential Nif gene transfer in various microorganisms. More research is needed to focus on the more effective alternatives for biofertilizers and development of genetically modified diazotrophs to enhance the future demands.

KEYWORDS: Nitrogen fixation, Diazotrophs, Nitrogenase, Nif gene, GM Diazotrophs.

INTRODUCTION:
Nitrogen is one of major elements useful for plant growth. Our atmosphere contains about 80% gaseous nitrogen but green plants are unable to use it directly from the atmosphere. The reduction of atmospheric nitrogen into ammonia by soil-borne microorganisms is called as Biological nitrogen fixation. The global nitrogen fixation is 175 x 10^6 tons/year. Biological nitrogen fixation is an important part of the microbial processes. Biological nitrogen fixation is carried out only by prokaryotes, which may be symbiotic or free-living in nature and catalysed by nitrogenase enzyme. Biological Nitrogen fixation is catalyzed by the enzyme nitrogenase. Some soil bacteria and blue-green algae are capable of reducing the atmospheric nitrogen into ammonia in their cells. The process of nitrogen reduction is called diazotrophy or nitrogen fixation.

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Microbes which reduce the atmospheric nitrogen are called nitrogen fixers or diazotrophs. Ammonia produced during nitrogen fixation is readily available to plants directly.

Biofertilizers are introduced as an alternative or substitute of chemical fertilizer. Now due to the substantial benefits by biofertilizers over chemical fertilizers, the development and usage of biofertilizers are gradually increasing globally. Biofertilizers able to increase soil fertility, crop growth and effective nutritive suppliers to plants, whereas chemical fertilizers pollute soil fertility, inorganic nutritive supply and also increases toxicity to plants and soil. Biofertilizers provide a pollution-free stimulant for plants[1].

Soil salinity is a major factor limiting plant productivity, affecting about 95 million hectares worldwide. The UNEP (United Nations Environmental Program) estimated that 20% of agricultural land and 50% of cropland in the world is salt stressed. Due to high cost and reduced availability of biofertilizers, farmers are still not able to benefit the maximum from their agricultural farms. By rapid urbanization and usage of

chemical fertilizers in agricultural fields may disrupt the natural available nutrition source for plants and thereby affects overall ecosystem globally. This leads to food scarcity ultimately thereby humans' predominant natural resource would be withdrawn, it enlightens world's dangerous global threat in future and may arise in present too. If the situation prevails for another 20 years, the future generation wholesomely depends on capsules and tablets for their diet. Development of more effective biofertilizers using diazotrophs only can resolve this global threat. More research needs to be focused in the improvement of biofertilizers by availability and at reduced cost[2].

Diazotrophs as bio-fertilizers:

The potential microorganisms can reduce the atmospheric nitrogen into ammonia called nitrogen fixers or diazotrophs. Green plants use ammonia from the nitrogen fixers to synthesize nitrogen-containing compounds such as arginine, asparagine, allantoin and allantoic acid. These nitrogens containing compounds synthesized directly from ammonia are known as urides. Urides are utilized in the metabolism of nucleic acids and proteins.

The concept of nitrogen fixation by legumes was proposed by J.B. Boussingault as early as 1834, but the process of symbiotic nitrogen fixation was discovered by Hellriegel and Wilfrath. The nitrogen-fixing bacterium *Rhizobium leguminosarum* was first isolated from legumes by Beijerinck in 1888. Thereafter, Beijerinck discovered free-living nitrogen-fixing bacterium *Azotobacter*. Now, many bacteria and blue-green algae do nitrogen fixation. Soon after discovery of nitrogen-fixing capability of *Rhizobium*, *Nobbe* and *Hitner* in the USA, mass cultured *Rhizobium* inoculant in the trade name 'Nitrogen' to fertilize leguminous crops. Since then several production units have been initiated for mass production of different symbiotic and free-living nitrogen-fixing bacteria in countries all over the world.

Bioactive ligands called Myc factors and Nod factors secreted by mycorrhiza and Rhizobium were perceived by host roots to trigger the signal transduction pathway, which initiates further signal transduction pathway through unknown receptors (SYMRK and NORK) which trigger release of Ca2+ in the cytosol. The whole pathway involves receptor-like kinases or other kinase related proteins like DMI and SYM71 to phosphorylate their substrates. Nuclear pore complex (NPC) and some of its proteins (NUP) play role in calcium spiking. DM1 proteins play role in maintaining periodic oscillation of calcium ions inside and outside the nucleus. Several channels proteins (Ca2+channel proteins) also facilitate this process with the help of various transporters. CCaMK is a calcium-calmodulin-dependent protein kinase, which phosphorylate the product of CYCLOPS protein thus initiating activation of various genes involving formation of structures like nodule and (PPA) pre-penetration apparatus[3,4,5].

Role of Micro-organisms as Bio-fertilizers:

Mechanistic explanation of nitrogen fixation can be sought at cellular/molecular, physiological (whole organism) and sub-ecosystem levels (i.e. ecological controls). 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.

Diazotrophs are broadly divided into two groups, namely symbiotic nitrogen fixers and non-symbiotic nitrogen fixers. Based on the association of microorganisms, they are classified[6,7].

Symbiotic Nitrogen fixers:

Nitrogen reduction by symbiotic microbes is called Symbiotic Nitrogen fixation. Some micro-organisms establish symbiotic association with plant roots and they do nitrogen fixations which are called as symbiotic nitrogen fixers or symbiotic diazotrophs. The diazotrophs derive nourishment from the plant roots and provide nitrogen to it. During symbiotic association, some diazotrophs induce nodule development on the plant roots are called as rhizocoensis. Eg. *Rhizobium, Frankia*, etc.

Rhizobium establish symbiotic association with roots of legumes and form root nodules. Rarely, they induce nodulation in the rots of non-legumes such as *Trema canabena*. *Frankia* induces root nodulation in *Alnus, Casuarina, Myrica, Discaria,* etc. Some symbiotic diazotrophs do not form root nodules in host plants. Eg. *Azospirillum, Beijerinckia, Azotobacter paspali,* etc. Symbiotic diazotrophs cannot fix the atmospheric nitrogen when they are living alone in the soil. But certain cyanobacteria are fixing nitrogen both when they

are residing in the host and when they are alive free in the soil. Eg. *Nostoc, Anabena,* etc[8,9,10].

Non-Symbiotic Nitrogen Fixation:

Some micro-organisms live independently in the soil and do nitrogen fixation. Such microbes are called non-symbiotic nitrogen fixers or non-symbiotic diazotrophs. Nitrogen fixation by these microorganisms is known as non-symbiotic nitrogen fixation. Of the 175 x 10^6 tons of total global nitrogen fixation, 190 x 10^5 tons is contributed by non-symbiotic nitrogen fixers. Non-symbiotic nitrogen fixers are divided into two groups based on their nutrition mode as follows:

i. Free-living autotrophic diazotrophs
ii. Free-living heterotrophic diazotrophs

The free-living autotrophic diazotrophs synthesize their own food either by photosynthesis using the sunlight or by chemicals. Among these diazotrophs, some are aerobes. Eg. *Oscillatoria, Cylindrospermum, Plectonema, Tolypotrix* etc. Some others are anaerobes. Eg. *Chlorobium vibriforme, Chlorobium limicola, Chromatium minus, Thiocystis formosa, Rhodopseudomonas viridis* etc. The free-living heterotrophic diazotrophs use dead organic matter as food and do nitrogen fixation. Eg. *Azotobacter, Derixa, Mycobacterium* (aerobes), *Beijerinckia, Clostridium, Klebsiella pneumoniae,* (anaerobes) etc. Blue-green algae are photo auto-trophic diazotrophs living on moist soils, they are also called as Cyanobacteria[4].

Nitrogenase:

Biological nitrogen fixation is catalyzed by the nitrogenase enzyme which consists of one larger sub-unit and one smaller sub-unit. The larger sub-unit is called molybdenum ferrous protein (Mo-Fe protein) or nitrogenase reductase and smaller sub-unit is called ferrous protein or dinitrogenase reductase. Nitrogenase reductase is a cold tolerant protein but it is inactive at 0^0 C. The ferrous protein carries Mg-ATP which supplies energy and donate electrons for nitrogen reduction. These two subunits are held together by two S-S linkages between the peptides. The Fe protein act as redox site for nitrogen reduction[11,12].

Characteristics of nitrogen complex:

Nitrogenase can reduce a wide variety of substrates such as N_2, N_3, N_2O, HCN, C_2H_2, $2H^+$, acetylene, cyclopropane, etc. Among them, N_2 forms a natural and abundant substrate. A few reduction reactions catalyzed by nitrogenase are given below:

The nitrogenase complex carries out the fixation of nitrogen by reducing molecular dinitrogen (N_2) to ammonium (NH_4^+) and also reduces acetylene to ethylene. The two-component proteins are dinitrogenase and dinitrogenase reductase. Although different metals can form the metal clusters contained in nitrogenase proteins, all nitrogenases have similar properties.

$$N_2 + 3H_2 \xrightarrow{Nitrogenase} 2NH_3$$

$$N_2O + H_2 \xrightarrow{Nitrogenase} N_2 + H_2O$$

$$CH_3NC + 6H_2 \xrightarrow{Nitrogenase} CH_3NH_2 + CH_4$$

$$2H^+ \xrightarrow{Nitrogenase} CH_3NH_2 + CH_4$$

All diazotrophs contain the enzyme nitrogenase which catalyzes the reduction of N_2 into NH_3. Nitrogenase is sensitive to oxygen, so it prefers anaerobic conditions for nitrogen fixation. But in microbes, the oxygen level is usually high which leads to oxidation of nitrogenase and hence that enzyme becomes inactive[13].

MECHANISM OF NITROGEN FIXATION:

Basic Requirements of Nitrogen Fixation:

Nitrogenase requires an energy source and electron donors for nitrogen reduction. ATPs released during the metabolism of carbohydrates, proteins and lipids react with Mg^{++} ions to form Mg-ATPs. The Mg^{++} of Mg-ATP binds with a Fe protein to form an active complex. This Mg-ATP is hydrolyzed into Mg-ADP and inorganic phosphate (ip) to supply energy. About 12-15 Mg-ATPs are required to reduce one molecule of N_2 into NH_3.

$$\text{Mg-ATP} + H_2O \longrightarrow \text{Mg-ADP - ip;}$$

$$\Delta G^0 = -7.3 \text{ k.cal/mole}$$

$$N_2 + 6H^+ + 6e^- \xrightarrow{12\ \text{Mg - ATP}} 2NH_3$$

$$12\ \text{Mg-ADP} + 12\text{ip}$$

Bacteroids synthesize ATPs, proton (H^+), electron donors such as $NADPH_2$ and ferredoxin by oxidizing the sugars. From reduced ferredoxin electrons flow to Mo-Fe protein. The enzyme nitrogenase complex receives energy from Mg-ATPs by hydrolysis. The Mo-Fe protein reduces N_2 into NH_3 by using the electrons. At least six electrons are required to reduce one molecule of N_2 into two molecules of NH_3. The Mo-Fe protein and Fe protein then separate from each other. If free H^+ ions are available in large amounts, the nitrogenase reduces

two H^+ ions into one H_2 molecule while passing the electrons from Fe protein to Mo-Fe protein.

Assimilation of Ammonia:
Ammonia produced during nitrogen fixation is unstable at the physiological pHs, so it is readily converted into ammonia (NH_4). The ammonium is involved in the biosynthesis of amino acids.

$$NH_3 + H \longrightarrow NH_4$$

Anammox:
Anammox (anaerobic ammonium oxidation) is anaerobic state of conversion of NO_2^- and NH_4 to NO_2. Anammox may be an important pathway in global N_2 cycling since it can account as much as 67% of benthic Nitrogen production. In anoxic water columns, it tends to account 35% of the nitrogen removal. It is estimated that anammox in sediments axonic water bodies may amount upto 35% to 50% of global marine nitrogen removal[14].

Genetics of Bio-fertilizers:
Nitrogen fixation in diazotrophs is genetically controlled by a cluster of genes called nitrogen-fixing genes or Nif genes. The Nif genes codes for the enzyme nitrogenase that reduces N_2 into NH_3. Nif genes in free-living diazotrophs occur in chromosomal DNA, but in most symbiotic diazotrophs, they are seen in extrachromosomal DNA called megaplasmid. The structure and regulation of Nif gene cluster vary from species to species.

1. Nif-genes of Klebsiella pneumonia:
Klebsiella pneumonia is a free-living diazotrophic bacterium. Nif genes are present in its chromosomal DNA. The Nif gene cluster is 24 x 10^3 basepairs in length and 18 x 10^6 daltons in the molecular weight. It lies between the genes coding for histidine (his) and uptake of shikimic acid (shi) and consists of 17 Nif genes located in 7 operons. They are Nif-QB, Nif-AL, Nif-F, Nif-MVSU, Nif-XNE, Nif-YKDH and Nif-J.

The chromosomal genes of *Klebsiella pneumoniae* other than Nif genes also control the expression of Nif genes. When there is a scarcity for NH_4 and glutamate, product of ntrC gene binds with Nif-A and activates that Nif gene cluster. If their concentration is high in the cell, product of ntrB gene binds with the product of ntrC on Nif-A and represses transcription. The product of Nif-L, when O_2 or NH_4 level is high, binds with the product of Nif-A and stops transcription.

2. Nif-genes of Azotobacter:
Nif genes of Azotobacter is a free-living diazotroph which are located in a 35 Kb segment segment of the chromosomal DNA. 12 Nif genes among the 17 of *Klebsiella pneumoniae* have been discovered in one cluster. They are in 4 operons –F, MVSUX, NE and YKDH, Nif-Q, Nif-B, Nif-A, Nif-L and Nif-J have not been noticed in Azotobacter. Nif-F and Nif-M are located 9 Kb segment segment apart from one another. Nif X and Nif N are separated by 9 Kb segment.

Nif genes of Azotobacter are regulated by ntrA, ntrB, ntrC and gln A genes which are located elsewhere in the chromosomal DNA. The product of ntr A binds with promoter of Nif gene cluster and induces transcription. The regulatory functions of ntr B and ntr C are still to be explored[15].

3. Nif-genes of Anabena:
Anabena is a photoautotrophic diazotroph. In Anabena 7210, Nif genes are located in the chromosomal DNA. The Nif gene cluster consists of four Nif genes namely, Nif-K, Nif-D, Nif-H and Nif-S. In vegetative cells, Nif K is 11 Kb segment apart from Nif D. Nif-D and Nif-S are near Nif-H. However, in heterocyst, Nif K is 4.8 Kb segment segment apart from Nif D. The length of Nif gene cluster in heterocyst is 6 Kb segment segment. The Nif H, Nif D and Nif K are in a single operon and Nif S is in a separate operon.

Genetics of Legume – Rhizobium Nitrogen Fixation:
Rhizobium is a symbiotic, nodule-forming diazotroph. It fixes the atmospheric nitrogen in root nodules of legumes. The nitrogen fixation is genetically controlled by some Rhizobial genes and certain legume genes. Genes for nodule development and nitrogenase are in a megaplasmid in the Rhizobium. The genes for leghaemoglobin (LHb) and some plant nodules are seen in the genome of the leguminous plant. A co-ordinate regulation of all these genes are required for the nitrogen fixation.

1. Rhizobial Genes:
In Rhizobium, nitrogen-fixing genes (Nif genes) and nodule forming genes (nod genes) are found close to each other in a megaplasmid pRL 1 JI. The nod genes and Nif genes are contained in a 135 Kb segment fragment of that plasmid. However, the genes occupy only about 50 Kb segment. There are about 19 genes in the gene cluster.

a. Nod Genes:
Nod genes lie in a cluster in between Nif L and Nif B genes. Nod A, nod B, nod c and nod D are common to all species of Rhizobium. They are found close together in an 8.5 Kb segment Eco RI segment and called common nod genes. The other nod genes vary between the species known as HSN genes.

b. Nif Genes:
Nif genes E, K, D, H, M, L, A and B are identified in the gene cluster. They are homologous to those genes present in *Klebsiella pneumoniae.* These genes codes for the synthesis of nitrogenase enzyme that reduces N_2 into NH_3. In addition to these Nif genes, there are three genes in a cluster and are designated as fix-A, fix-B and fix-C. Products of the fix genes allow the transfer of electrons through them for nitrogen reduction. But the exact function of fix genes in nitrogen fixation have hardly been known.

c. Hup Genes:
Some species of Rhizobium uptake Hydrogen produced during nitrogen fixation and use it to make water known as Hup^+ species. They contain Hup genes or uptake hydrogenase gene that codes for enzyme uptake hydrogenase. The Hup gene may be found elsewhere away from the Nif genes in the megaplasmid and it occupies about 15 Kb segment in the plasmid which are located in two or three operons. Hup genes of *Rhizobium leguminosarum* and *Rhizobium japonicum* are identical in their base sequence and arrangement. The hydrogenase encoded by Hup genes creates a slight anaerobic condition for nitrogenase activity.

2. Legume Nodulin Genes:
In Rhizobium-legume symbiosis, the plant root secretes some nodule specific polypeptides called nodulins in the root nodules. There are about 20 nodulins in root nodules, but only a few are known in detail. The genes coding for all these nodulins are localized in the chromosomal DNA of plant cells.

Leghaemoglobin Gene:
Leghaemoglobin gene (Lb gene) resides in the chromosomal DNA of legumes and it is present at somewhere away from other nodulin genes. The pseudogenes are necessary for transcription of Lb genes, but they never translate into polypeptides. All these Lb genes have three introns and four exons as in *Phaseolus vulgaris*, but they have a common transcription signal at 5' end of the genes.

Comparative analysis of Nif genes:
Nif genes are the ultimate repository for the nitrogen fixation. Therefore, Nif genes of various nitrogen-fixing micro-organisms are analyzed in order to identify the most probable economical and beneficial microorganism which can be implemented as biofertilizer.

Nitrogen fixation is widely but sporadically distributed among both eubacteria and methanogenic archaea. The current understanding of nitrogenase diversity has been based largely on phylogenetic analyses of nifH and nifD, the nitrogenase structural genes. Genomic analysis of nif genes encoding the core components of nitrogenase, including the NifH, NifD, NifK, NifE and NifN proteins, and proposed five groups: (1) typical Mo–Fe nitrogenases, predominantly composed of members of the proteobacterial and cyanobacterial phyla; (2) anaerobic Mo–Fe nitrogenases from predominantly anaerobic bacteria and several methanogens; (3) alternative nitrogenases, including the Mo-independent Anf and Vnf proteins (except VnfH, which is more similar to NifH rather than AnfH); (4) uncharacterized nif homologues detected only in methanogens and some anoxygenic photosynthetic bacteria; and (5) bacteriochlorophyll and chlorophyll biosynthesis genes common to all phototrophs.

Neighbor-joining phylogenetic tree constructed from 16S rDNA sequences for the microbial genomes used in this analysis. Diazotrophic genomes, as determined by the presence of NifHDKEN operons, are indicated with bold lines. Lineages outlined by dashed bold lines have homologs to NifH and NifD but are not known to fix nitrogen. Also shown are the major bacterial and archaeal phyla, highlighted if nitrogen-fixing lineages are found among them.

It is interesting that the emphasis in genome sequencing towards pathogenic microorganisms has resulted in an inadvertent bias against nitrogen fixers.

(a) Nitrogen-fixing Last Common Ancestor (LCA) hypothesis, showing the three domains and their divergence from the LCA in blue text and dashed blue lines. Solid lines (black and green, depending on hypothesized metal specificity) indicate the evolution of nitrogenase from the base of the tree a group IV ancestor. Also indicated are putative gene duplication (red dots) and horizontal gene transfer (originating at grey boxes, with transfer indicated by grey dashed lines) events. The three nitrogenase phylogenetic groups are indicated at the tips of the tree, as are the predominant organisms in which they are found. According to the LCA model, gene loss has been extensive and accounts for the majority of modern lineages not being able to fix their own nitrogen. (b) Methanogen origin hypothesis, using the same colour scheme and symbols. According to this model, nitrogen fixation was invented in methanogenic archaea and subsequently was transferred into a primitive bacterium, circumventing the necessity for extensive gene loss to explain the paucity of diazotrophic lineages. As with the LCA hypothesis, several relatively recent HGT events must have occurred to explain the distribution and high identities of group II and III nitrogenases.

(b) The interior node values of the tree are clade credibility values, values lower than 100% are indicated.

Branches are coloured blue (Mo-nitrogenase, Nif), green (V-nitrogenase, Vnf), purple (Fe-nitrogenase, nf), light blue (uncharacterized homolog), dark yellow (uncharacterized nitrogenase).

Gene transfer for nitrogen fixation:

Nif genes, Hup genes and Nod genes have been transferred to micro-organisms or plants for manipulating them to have the capacity to fix the atmospheric nitrogen. The genetically engineered bacteria could be employed as additional nitrogen-fixing micro-organisms in agriculture.

1. Transfer of Nif genes to Non-nitrogen fixing bacteria:

Although there are many species of soil-borne bacteria, only a few are capable of fixing the atmospheric nitrogen in the soil. These bacteria contain Nif genes in their cells. Efforts have been taken to transfer Nif genes of *Klebsiella pneumoniae* and Rhizobium to harmless bacteria lacking Nif genes. At first Dixon and Postgate (1972) isolated Nif gene cluster of *Klebsiella pneumoniae* and introduced into *E.coli* through the R-plasmid pDR1. The genetically engineered *E.coli* had the capacity to fix the atmospheric nitrogen in the soil. The Nif gene cluster of *Klebsiella pneumoniae* (M5A1) was transferred to *Klebsiella aerogens, Salmonella typhimurium, Serrata marcescens, Erwinia herbicola, Pseudomonas,* etc. The Nif gene cluster of *Rhizobium trifolii* (T1K) was transferred to *E.coli* in order to make it to fix nitrogen.

2. Transfer of Nif genes to Yeasts:

The Nif gene cluster of *Klebsiella pneumoniae* was transferred to *Saccharomyces cerevisiae* through a plasmid vector. The engineered *S. cerevisiae* retained the Nif genes but the genes hardly expressed. The eukaryotic transcription and translation systems never recognize prokaryotic regulatory sequences. Therefore, nitrogenase had not been produced in the yeast. Because of the absence of nitrogenase, there was no nitrogen fixation in that yeast.

3. Transfer of Nif genes to Plants:

Efforts are made to produce transgenic plants with nitrogen-fixing property. Isolated gene cluster of *Klebsiella pneumoniae* and introduced into protoplast of tobacco through plasmid vectors. Plants regenerated from the protoplasts retained the Nif genes in their cells, but the cloned Nif genes had not been expressed.

Nif genes of Rhizobium were transferred to protoplasts of non-legumes and cereals through Ti plasmid of *Agrobacterium tumefaciens*. Expression of cloned Nif gene in the plants has hardly been known so far. The eukaryotic enzymes might have failed to recognize the prokaryotic regulatory sequences on the Nif genes. In order to overcome this problem, Nif genes were transferred to chloroplasts of leguminous plants and then the chloroplasts were introduced into protoplasts. Chloroplasts show prokaryotic transcriptional and translational enzymes. Besides these, they have a steady ATP generating system and reductants. Even then, the transgenic plants have failed to fix the nitrogen.

4. Transfer of Nod genes:

The nod genes of Rhizobium are responsible for host specificity while establishing symbiotic association. *Rhizobium phaseoli* forms root nodules only in beans such as French bean, kidney bean, etc. It never modulates on peas. Nod genes of *Rhizobium leguminosarum* were transferred to *Rhizobium phaseoli*. The genetically engineered *R. phaseoli* can nodulate on the roots of beans as well as peas. So, it can be used as biofertilizer. The transfer of nod genes increases the host range of Rhizobial strains.

5. Transfer of Hup genes:

In Rhizobial strains that lack hup genes, the rate of nitrogen fixation is low because of the reduction of H^+ ions into hydrogen (H_2) and called Hup^- strains. The transfer of Hup genes of a Hup^+ strain to a Hup^- strain of Rhizobium that nodulates on chickpea roots. The genetically engineered Rhizobial strain produces *uptake* hydrogenase which combines H^+ and O^- into H_2O. Meantime, it generates some ATPs for nitrogen reduction. Therefore, the rate of nitrogen fixation is very high[16].

DISCUSSION:

The study reveals the proximate data analysis of Nif gene. It also confers the development of efficient genetically modified micro-organisms to match the global demand. It is suggested that the improved species along with photoautotrophic micro-organisms would be more beneficial in order to prevent soil contamination, increased fertilizer property and free nutrient and energy supply.

CONCLUSION:

Nitrogen fixation is undoubtedly an ancient innovation that is not only crucial for extant life but played a critical role during the early expansion of microbial life as abiotic nitrogen sources became scarce. By considering histories of multiple genes and operons from completely sequenced genomes, it is possible to understand the influence of paralogy, gene recruitment, and horizontal gene transfer in the evolution of nitrogenase. In light of the intractability often thought posed by such complex genetic events, converging lines of biochemical, geological, and phylogenetic evidence make it possible not only to rectify inconsistencies between gene and

species of trees but also to elucidate the selective pressures dictating the tempo and mode of organismal versus genomic evolution.

Thus biological nitrogen fixation is global natural process which stabilizes the uniform cycling of every ecosystem. The use of chemical fertilizer inhibits the efficacy of biological nitrogen fixation, thus relative research efforts are needed in order to bring about the global demands of nitrogen fixation which stimulates the better environment in future. More research is needed to focus on the present scenario of biological nitrogen fixation and also development of improved natural fertilizers thereby to promote nitrogen fixation globally in large scale.

Table 1. Molecular composition of Nitrogen complex

Property	Nitrogenase reductase	Dinitrogenase reductase
Molecular weight	23KD	70KD
Number of subunits	4	2
Iron atoms	28	8
Molybdenum atoms	2	0

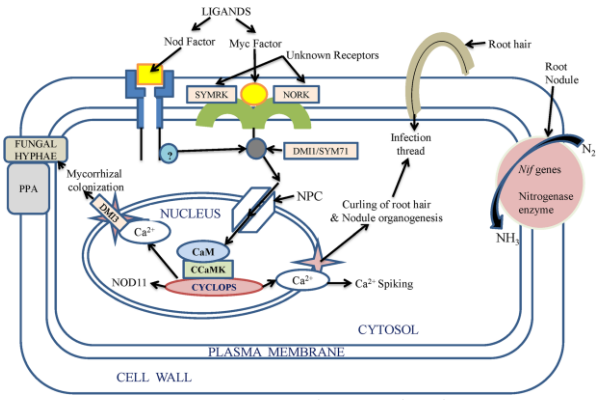

Fig 1. Hypothetical mechanism of action of biofertilizers in the root cell

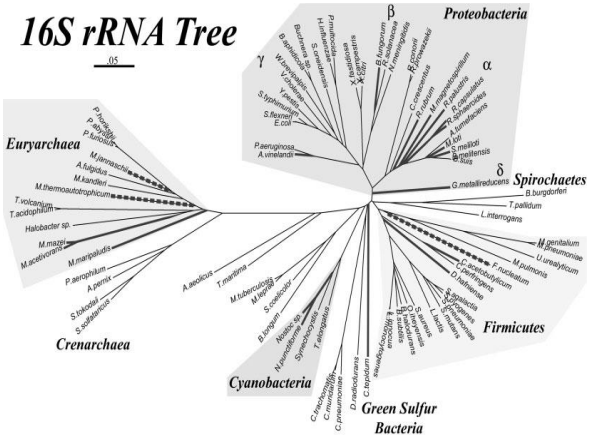

Fig 2. Nif gene cluster in various microbial family

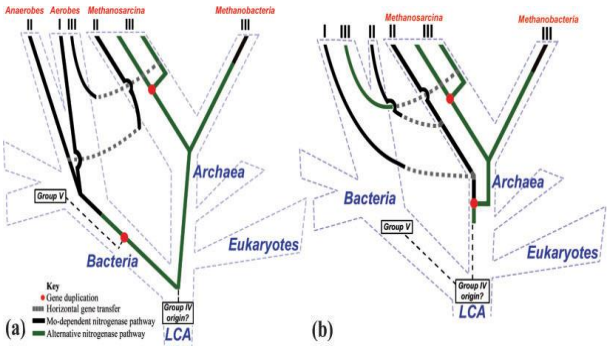

Fig 3. Proposed Nif gene versus species trees

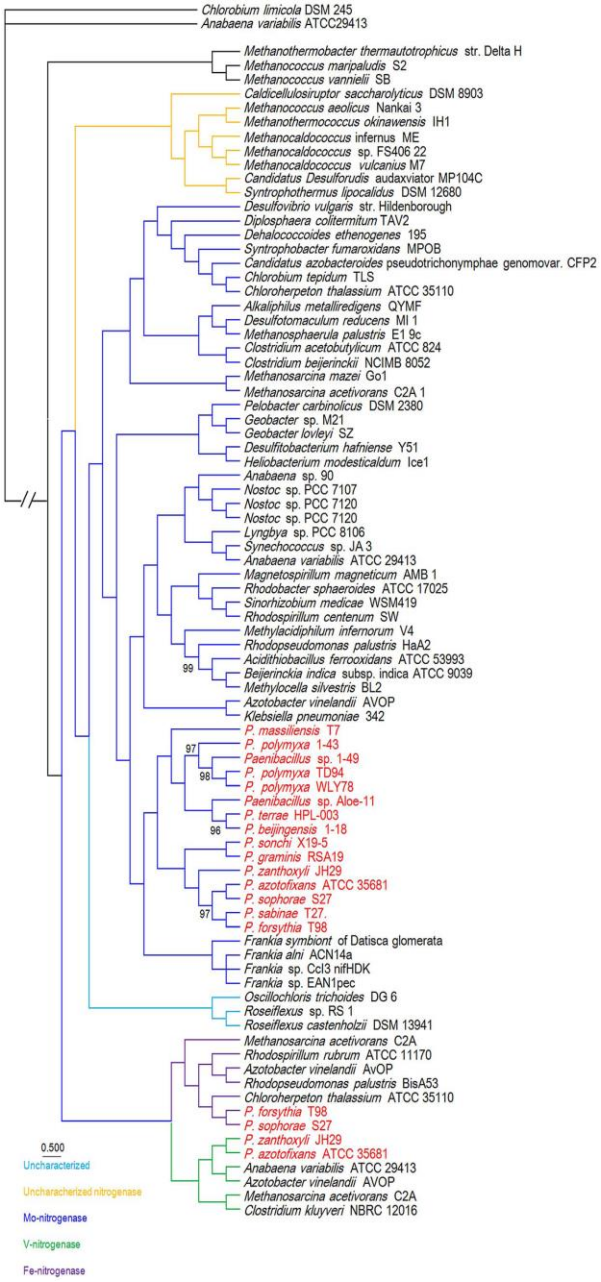

Fig 4. Bayesian-inferred phylogenetic tree of concatenated NifHDK homologs.

Table 2. Strains and GenBank/DDBJ/EMBL accession/protein ID numbers of nif HDKEN sequences used

Species/strain	Code	Accession/protein ID number				
		NifH	**NifD**	**NifK**	**NifE**	**NifN**
Rhizobium etli CFN 42	Retli	U80928	U80928	U80928	U80928	U80928
Mesorhizobium loti MAFF 303099	Mloti	AP003005	AP003005	AP003005	AP003005	AP003005
Sinorhizobium meliloti 1021	Smeli	AE007235	AE007235	AE007236	AE007236	AE007238
Gluconacetobacter diazotrophicusPA15	Gacd	AF030414	AF030414	AF030414	AF030414	AF030414
Rhodobacter capsulatus	nRbcap	M15270	M15270		X17433	X17433
Rhodobacter capsulatus B10S	aRbcap	X70033	X70033	X70033		
Rhodopseudomonas palustris CGA009	nRppal	BX572607	BX572607	BX572607	BX572607	BX572607
Rhodopseudomonas palustris CGA009	aRppal	BX572597	BX572597	BX572597		
Rhodopseudomonas palustris CGA009	vRppal	BX572597	BX572597	BX572597	BX572597	BX572597
Rhodobacter sphaeroides ATH 2.4.1	Rbsph	ZP_00007624	ZP_00007625	ZP_00007626	ZP_00207062	ZP_00207063
Rhodospirillum rubrum	Rsrub	ZP_00269733	ZP_00269732	ZP_00269731	ZP_00267748	ZP_00267747
Azospirillum brasilense ATCC 29145	Asbra	M64344	M64344	M64344	AF361867	AF361867
Herbaspirillum seropedicae Z78	Hsser	Z54207	Z54207	Z54207	AF088132	AF088132
Burkholderia fungorum LB400	Bfun	ZP_00282264	ZP_00282265	ZP_00282266	ZP_00282269	ZP_00282270
Azoarcus sp. BH72	Aasp	AF200742	AF200742	AF200742		
Nostoc sp. PCC 7120	Nossp	BAB73411	BAB73398	BAB73397	BAB73395	BAB73394
Cyanothece sp. PCC 8801	Cthsp	U22146	U22146	U22146	AF003700	AF003700
Trichodesmium erythraeum IMS101	Tder	ZP_00327022	ZP_00327023	ZP_00327024	ZP_00327025	ZP_00327025
Plectonema boryanum M101	Pbor	D00666	D00666			
Klebsiella pneumonia	Kp	X13303	X13303	X13303	X13303	X13303
Azotobacter vinelandii OP	nAbvin	M20568	M20568	M20568	M20568	M20568
Azotobacter vinelandii CA	aAbvin	M23528	M23528	M23528		
Azotobacter vinelandii CA	vAbvin	M32371	M32371	M32371		
Thiobacillus ferrooxidans ATCC 33020	Tfer	M15238	M15238	M15238		
Pseudomonas stutzeri A1501	Pms	Q44044	Q44045	AJ313205	AJ313205	
Magnetococcus sp. MC-1	Mcsp	ZP_00291144	ZP_00291143	ZP_00291142	ZP_00291137	ZP_00291136
Paenibacillus azotofixans ATCC 35681	Pbaz	AJ515294	AJ515294			
Frankia sp. EuIK1	Frsp	U53362	U53362	U53362	AF119361	AF119361
Geobacter sulfurreducens PCA	Gbsul	AE017217-77	AE017217-76	AE017217-75	AE017217-62	AE017217-62
Geobacter metallireducens GS-15	Gbmet	ZP_00300753	ZP_00300754	ZP_00300755	ZP_00300759	ZP_00300759
Desulfitobacterium hafniense DCB-2	Dbh	ZP_00099588	ZP_00099589	ZP_00099590	ZP_00099591	ZP_00099592
Desulfovibrio vulgaris Hildenborough	Dvv	AE017286	AE017286	AE017286	AE017286	AE017286
Chlorobium tepidum TLS	Chtep	AE012909	AE012909	AE012909	AE012909	AE012909
Clostridium pasteurianum W5	Clp	AY603957	AY603957	AY603957	AY603957	AY603957
Clostridium acetobutylicum ATCC 824	Cla	AE007538	AE007538	AE007538	AE007539	AE007539
Clostridium beijerinckii NRRL B-593	Clb	AF266462	AF266462	AF266462	AF266462	AF266462
Methanococcus maripaludis LL	Mcmar	U75887	U75887	U75887	U75887	U75887
Methanobacterium thermoautotrophicum ΔH	Mbt	AE000916	AE000916	AE000916	AE000916	AE000916
Methanobacterium ivanovii	Mbi	X56071				
Methanosarcina acetivorans C2A	nMsa	AE011101	AE011101	AE011101	AE011102	AE011102
Methanosarcina acetivorans C2A	aMsa	AE010788	AE010788	AE010788		
Methanosarcina acetivorans C2A	vMsa	AE010789	AE010789	AE010789		
Methanosarcina barkeri fusaro	nMsb	ZP_00295507	ZP_00295504	ZP_00295503	ZP_00295502	ZP_00295501
Methanosarcina barkeri fusaro	vMsb	ZP_00295459	ZP_00295456	ZP_00295454		
Methanosarcina barkeri fusaro	aMsb		ZP_00297291	ZP_00297289		
Methanosarcina mazei Go1	Msm	AE013297	AE013297	AE013297	AE013298	AE013298
Leptospirillum ferrooxidans L3.2	Lsfer	AF547999	AF547999			
Heliobacterium chlorum DSM 3682	Hbtc	AB196525	AB196525	AB196525	AB196525	AB196525

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