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Review Article

Role of Diazotrophic Bacteria in Biological Nitrogen Fixation and Plant Growth Improvement

Wansik Shin, Rashedul Islam[1], Abitha Benson[1], Manoharan Melvin Joe[1], Kiyoon Kim[1], Selvakumar Gopal[1], Sandipan Samaddar[1], Somak Banerjee[1], and Tongmin Sa[1]*

Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Foresty and Fisheries, Anyang-si, Gyeonggi-do 14055, Korea

[1]Department of Environmental and Biological Chemistry, Chungbuk National University, Cheongju, Chungbuk, 28644, Republic of Korea

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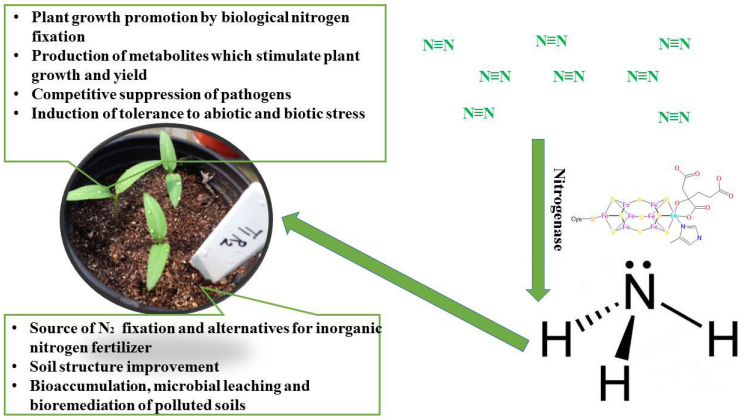
Though there is an abundant supply of nitrogen in the atmosphere, it cannot be used directly by the biological systems since it has to be combined with the element hydrogen before their incorporation. This process of nitrogen fixation (N_2-fixation) may be accomplished either chemically or biologically. Between the two elements, biological nitrogen fixation (BNF) is a microbiological process that converts atmospheric di-nitrogen (N_2) into plant-usable form. In this review, the genetics and mechanism of nitrogen fixation including genes responsible for it, their types and role in BNF are discussed in detail. Nitrogen fixation in the different agricultural systems using different methods is discussed to understand the actual rather than the potential N_2-fixation procedure. The mechanism by which the diazotrophic bacteria improve plant growth apart from nitrogen fixation such as inhibition of plant ethylene synthesis, improvement of nutrient uptake, stress tolerance enhancement, solubilization of inorganic phosphate and mineralization of organic phosphate is also discussed. Role of diazotrophic bacteria in the enhancement of nitrogen fixation is also dealt with suitable examples. This mini review attempts to address the importance of diazotrophic bacteria in nitrogen fixation and plant growth improvement.

Key words: Biological nitrogen fixation (BNF), Diazotrophic bacteria, Plant growth promotion, N_2 fixing prokaryotes, *nif* genes



Biological nitrogen fixation (BNF) can convert atmospheric di-nitrogen (N_2) into plant-usable form, which improves plant growth and yield.

*Corresponding author: Phone: +82432612561, Fax: +82432715921, E-mail: tomsa@chungbuk.ac.kr
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Introduction

Plants require nitrogen large quantities, since it forms essential component of proteins, nucleic acids, and other cellular constituents (Novoa and Loomis, 1981). The abundant availability of nitrogen in the earth's atmosphere (nearly 80% in the form of N_2 gas) cannot be used directly by biological systems to produce chemicals for growth and reproduction. Before being taken up by the plants, N_2 must first be combined with the hydrogen element. Reduction of N_2, commonly referred to as 'nitrogen fixation' (N_2-fixation) may be accomplished chemically or biologically. However, N is continually depleted by some processes such as soil erosion, chemical volatilization, denitrification, soil leaching, and perhaps most importantly, removal of N-containing crop residues from the land (Vitousek and Matson, 2009). The N reserve of agricultural soil should be maintained at an optimal level to maintain an adequate amount for crop production (Tilman et al. 2002). This replacement of soil N is generally accomplished by the addition of chemically fixed N in the form of commercial fertilizers of inorganic nature or by the process of biological nitrogen fixation (BNF).

BNF is a microbiological process that converts atmospheric di-nitrogen (N_2) into plant-usable form, which offers as an alternative to inorganic fertilizers (Buyer and Kaufman, 1996). Because of its potential economic and environmental importance of BNF a lot of researchers (Demba Diallo et al., 2004; Wartiainen et al., 2008) has considered it a very important source of N input in soil. However, N_2-fixation is not exclusively a biological process since abiotic N_2-fixation can be mediated by lightning or fires, and oxidizes N_2 to nitrate (NO_3^-). According to the current knowledge, only prokaryotes (members of the domains *Archaea* and *Bacteria*) are capable of performing BNF. Eukaryotic organisms naturally depend on BNF activity of the N_2- fixing prokaryotes (diazotrophs) for their N supply. Since N is an essential nutrient for all living organisms, N_2-fixation is a key process of the N-cycle (Zehr et al., 1996).

The importance of BNF in any particular ecosystem depends on the system's nutrient status for ecosystem development (Cleveland et al., 1999). BNF by free-living heterotrophs play a major role with high accumulations of carbon sources, e.g. in forest soils with high inputs of litter and decaying plant debris (Giller and Day, 1985). In systems with high concentrations of mineral N, BNF may contribute little to the N-budget (Cleveland et al., 1999). Microbe-mediated mineralization of N to (ammonium) NH_4^+ and its subsequent nitrification to NO_3^- is of major significance to N availability (Fig. 1). Both NH_4^+ and NO_3^- are taken up by plants, although many plants prefer the uptake of one over the other (Marschner, 1995). Under aerobic conditions, NH_4^+ will be rapidly transformed to NO_3^- by nitrifying micro-organisms (ammonium-oxidizers and nitrite-oxidizers). Denitrification, a process of anaerobic respiration that

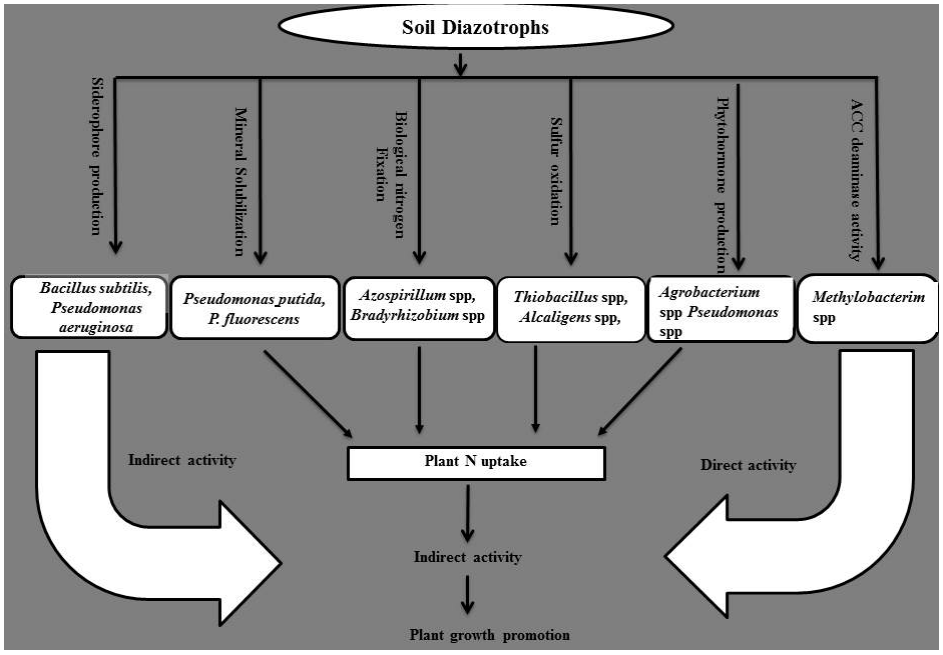

Fig. 1. The plant-soil N cycle and pathways for N transformation mediated by physiological processes (DON = dissolved organic nitrogen) (Richardson et al., 2009).

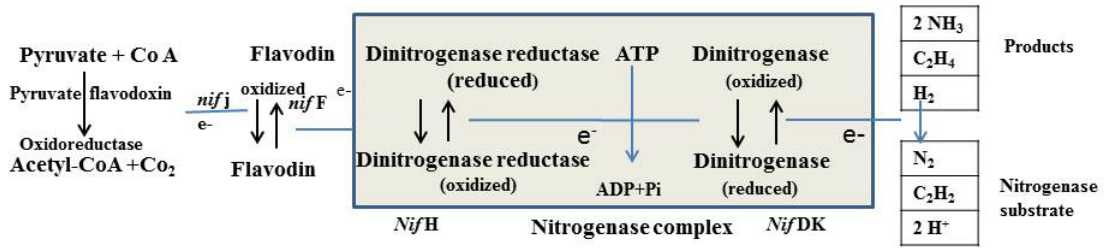

Fig. 2. Electron transport chain for nitrogenase in *Klebsiella pneumoniae*. Nitrogenase reductase (Fe protein, NifH) shuttles electrons to dinitrogenase (MoFe protein, NifD and NifK) under consumption of ATP. Dinitrogenase is the site of reduction of the substrates, most importantly N_2. H_2 production is an obligate part of the process, while acetylene (C_2H_2) is an example for an alternative substrate. Genes involved in the transport chain are indicated on the right (Madigan et al., 2000).

transforms NO_3^- to N_2, thereby completes the N-cycle by returning N to the atmosphere.

Modern agricultural practices and industrialization acts as major sources of fixed N through the production of synthetic N fertilizer and increased NO emissions from fossil fuel combustion. In addition to these new inputs, anthropogenic activities introduced massive changes to the spatial and temporal pattern of N-fluxes, e.g. by transferring N that is contained in the biomass of agricultural crops into urban areas where they are consumed (Delwiche, 1981). Chemical fertilizers guarantee the production of food for a steadily growing population. However, the cost of application, environmental degradation and human health pose major limitations to their excessive usage. Another concern is the decline in crop yields under continuous use of N fertilizers.

Discovery of nitrogen fixers

The discovery of N_2-fixation was attributed to the German scientists Hellriegel and Wilfarth, who in the year 1886 reported that legumes bearing root nodules could use gaseous (molecular) nitrogen. Afterwards, in 1888, Beijerinck succeeded in isolating a bacterial strain later known as *Rhizobium leguminosarum* from root nodules, Beijerinck (1901) was also responsible for the isolation of *Azotobacter* sp., while the discovery of N_2-fixation in blue-green algae was credited to Stewart (1969).

Genetics of BNF

Mechanism involved with biological nitrogen fixation

Nitrogen-fixation is a very costly process in terms of its energy requirements and a very complex mechanism not fully elucidated. BNF can be represented by the following equation (Franche et al., 2009), in which two moles of ammonia are produced from one mole of N_2, at the expense of 16 ATP moles and a supply of electrons and protons (hydrogen ions):

$$N_2 + 16\ MgATP + 8e^- + 8H^+ \rightarrow 2NH_3 + H_2 + 16MgADP + 16Pi$$

The actual reduction of N_2 is performed by the nitrogenase protein complex, which consists of two metalloproteins: the nitrogenase, or nitrogenase molybdenum-iron protein (MoFe protein, *Nif*DK), and the nitrogenase reductase or nitrogenase iron protein (Fe protein, *Nif*H). The molybdenum-iron-sulfur-homocitrate clusters of the MoFe protein are the actual sites of binding and reduction of the substrate N_2 and other alternative substrates, such as acetylene, protons and many others. The Fe protein is responsible for shuttling electrons to the MoFe protein using at least two Mg ATP per electron (Halbleib et al., 2000) (Fig. 2).

The genes responsible for biological nitrogen fixation

The number and arrangement of genes involved in BNF varies between species to species. The MoFe protein is a tetrameric protein ($\alpha_2\beta_2$) encoded by the *nifDK* genes, and the Fe protein is a homo-dimer (α_2) of the *nifH* gene product (Halbleib and Ludden, 2000). These genes, along with regulatory genes and accessory genes coding for enzymes involved in electron transfer and metal cluster synthesis comprise the *nif* regulon (Dean and Jacobson, 1992). The genes *nifD* and *nifK* are usually part of the same operon and appear in the same organization (as *nifDK*). Frequently, *nifH* is also included in the same operon (*nifHDK*) (Fani et al., 2000). The genes *nifE* and *nifN,* which play a role in the biosynthesis of the nitrogenase metal clusters (FeMo-

co), usually appear together and may have arisen from paralogous ancestor of *nifEN* and *nifDK* operon (Fani et al., 2000). These core operons are supplemented by a number of other genes, which code for proteins involved in electron transport (e.g. *nifF* and *nifJ* in *Klebsiella pneumoniae*) or regulation (e.g. *nifA*) (Dean and Jacobson, 1992) or in FeMo-cofactor synthesis (e.g. *nifB, nifV* in *K. pneumoniae* and *A. chroococcum*). "*Fix*" and *"nod"* genes are associated with BNF and nodule formation in rhizobial species, and many do not have homologues in the asymbiotic model organism *K. pneumoniae* or other known free-living diazotrophs (Dean and Jacobson, 1992). In addition to the standard nitrogenase system (*nifDK, nifH*) two important alternative systems have been identified and characterized. The main difference to the standard system is that these enzymes do not contain molybdenum (Mo). Instead, one of these alternative systems contains vanadium (*vnfDK, vnfH*) while the other contains only iron and no unusual metals (*anfDK, anfH*). All three systems are highly homologous but do contain significant sequence differences. The alternative systems are transcriptionally regulated and in most studied organisms, *vnf* and *anf* are only expressed under conditions of Mo limitation (Bishop and Premakumar, 1992). However, it was observed that the diazotroph community in termite gut transcribed *anfH*-like genes even at high Mo concentrations. Little is known about the importance of these alternative nitrogenase systems in the soil environment and *anfH*-type sequences have rarely been detected in soil (Poly et al., 2001). However, it was shown that the alternative systems may be important for N_2-fixation in certain environments, such as termite gut. Only recently a completely novel nitrogenase system was found in *Streptomyces thermoautotrophicus* (Ribbe et al., 1997). The nitrogenase reductase in this system is a manganese superoxide dismutase that shows no homology to *nifH*. Interestingly this Mo nitrogenase is not O_2-sensitive and its energy requirements are significantly lower than the classical nitrogenases (Ribbe et al., 1997).

nifH as a marker gene

While *nifH, nifD* and *nifK* genes have all been analyzed phylogenetically, it is *nifH* that provides the best phylogenetic resolution (Hirsch et al., 1995). Consequently this gene has been used more frequently in ecological studies compared to other *nif* genes. The phylogenetic information content of the gene has been used repeatedly to analyze and describe populations of uncultivated diazotrophs in different environments including forest soils and agricultural soils (Poly et al., 2001), wetland soils (Piceno et al., 1999), and rhizospheres (Ueda et al., 1995). Aquatic environments including estuaries (Affourtit et al., 2001), marine (Zehr and McReynolds, 1989) and freshwater systems (Zani et al., 2000) have been studied in detail. Even the diazotroph communities in termite guts have been described using molecular analysis of *nifH* (Ohkuma et al., 1996).

Molecular studies of the diversity of diazotrophic bacteria thus far have been largely based on the *nifH* gene, which is clustered into four basic groups designated as Clusters I-IV (Chien and Zinder, 1996). The composition of these major clusters consist of the 'conventional' Mo-containing *nifH* and some *vnfH* (Cluster I), the 'second alternative' non-Mo, non-V containing *anfH* and nitrogenases from some Archaea (Cluster II), *nifH* sequences, which are strict anaerobes (e.g. clostridia, sulphate reducers; Cluster III), and a divergent loosely coherent group of *nif*-like sequences from Archaea and distantly related chlorophyllide reductase genes (Cluster IV). It is not clear whether the *vnfH* genes within Cluster I can be distinguished from conventional *nifH*.

Many microorganisms have multiple copies of nitrogenase genes or homologues of nitrogenase genes (Cluster IV). *Clostridium pasteurianum* was shown to have a *nifH* gene family (Wang et al., 1988), and has sequences in Clusters II and III. *Azotobacter* have been observed to have alternative nitrogenases such as *vnfH* and *anfH* (Bishop et al., 1986). Cyanobacteria have possible multiple copies of nitrogenase, including a *vnfH* as well as a second distinct copy of *nifH* (Thiel et al., 1995). Archaea often have two *nifH* homologues, in which two copies are in Cluster IV and Cluster III, and in other cases, the homologues are in Cluster II and Cluster III.

Contribution of biological nitrogen fixation in nitrogen requirement

The prospect of utilizing BNF for agricultural purposes has long been the driving force behind N_2-fixation research. The annual N-demand of the world's human population (1994: 5.3 billion) has been estimated as 23 Tg (tera gram) of Nitrogen per year, a figure that will increase considerably with the growing human population (Vance and Graham, 1995). In symbiotic systems, the NH_4^+ produced by BNF is transferred directly to the plant where it is bound into organic molecules for transport (amino acid amides like asparagine and glutamine or ureides e.g. allantoin). The symbiosis provides the

diazotroph with a steady influx of carbon sources and regulated oxygen supply which provides the basis for highly efficient N_2-fixation. Free-living N_2-fixers (diazotrophs) will mostly incorporate the N they fix into their own biomass and its availability to plant will be indirect through subsequent mineralization of the biomass (Tchan, 1988). The application of free-living diazotrophs to soil as a method to promote plant growth has proven to be less successful in comparison to symbiotic systems, although many questions concerning their utility remain open. On a global scale asymbiotic nitrogen fixation (ANF) contributes to about 30% to the total biologically fixed N, and can play a significant role in many terrestrial ecosystems (Cleveland et al., 1999). However, in an agricultural context, symbiotic BNF by legumes is considered to be by far the most dominant source of biologically fixed N (Kennedy and Islam, 2001). Other symbiotic systems with agricultural applications include the *Azolla-Nostoc* (Vaishampayan et al., 2001), and the *Frankia*-Alder symbioses (Paul and Clark, 1996). All of these symbioses are genetically defined systems that include specific interactions between diazotroph and host-plant (Kennedy and Islam, 2001).

Estimation of nitrogen fixation in the different agricultural systems

There are four methods to estimate nitrogen fixation in plants. They are Acetylene Reduction Assay (Hardy et al., 1973), N-solute Analysis of Xylem Exudate method (Unkovich et al., 2008), total nitrogen difference (TND) method and 15N labeled compounds.

Acetylene Reduction Assay

The nitrogen fixers not only catalyze the reduction of atmospheric N_2 to NH_3, but can also catalyze the reduction of acetylene to ethylene (C_2H_4). The acetylene reduction assay (ARA) is carried out in nodules, de-topped roots, or whole plants in a closed vessel containing 10% acetylene and incubated for few minutes to hours. A gas chromatograph is used to determine the amount of ethylene formed. The amount of ethylene formed is equal to acetylene reduction thereby the amount of nitrogen fixers are estimated. Data are expressed as nanomoles or micromoles of ethylene produced per hour per plant or per weight unit of nodules. This assay provides a quick measure of nitrogenase activity (but not necessarily of N_2 fixed) under experimental conditions. For longer estimates, many measurements are done which include diurnal and seasonal changes. Though this method is cost effective and sensitive, these methods are not used widely, owing to the short-term nature of the assays, and the auto-inhibition of acetylene conversion to ethylene.

Total nitrogen difference (TND) method

The TND method measures BNF on the estimation of the total nitrogen (NT) in the fixing plant accumulated from soil (Ns); the difference in this is attributed to the amount of fixed N (Unkvich et al. 2008). Total N in a control (maintained from non-N_2-fixing plant) will reflect the Ns. This is based on the assumption that both the N_2 fixing and non-fixing control plants absorb equal amounts of soil N for growth. The major limitation of this method is that it is suited only for soil with low N concentrations.

N-solute Analysis of Xylem Exudate

Analysis of N-solute of xylem exudates is a tool for estimating BNF, because it is based on the fact that nitrogen can be transported to the leaves in the form of (1) ureides, allantoin and allantoic acid, or (2) asparagine and glutamine from BNF. In agricultural soils, nitrate is the most readily available form of N for plant growth, the solutes derived from soil mineral N will contain free nitrate and organic products of nitrate reduction in the roots. The methods are simple and have been used successfully in legumes (Unkovich et al., 2008).This technique is also simple and relatively inexpensive (Danso, 1995). With this technique, many samples can be collected and analysed in a day (Unkovich et al., 2008).

^{15}N Methods

In this method, the ^{15}N isotope dilution (ID) method and ^{15}N natural abundance (NA) method have been used to study the estimation. It is generally based on the principle that the concentration of ^{15}N in the atmosphere is different from that of plant and therefore the analyses of ^{15}N of the N_2 − fixing plant and the non - fixing plant is considered as the amount of N fixed. The small variations of ^{15}N natural abundance are usually expressed as $\delta^{15}N$ values denoting the relative deviation from the ^{15}N abundance of

atmospheric N_2. When the $\delta^{15}N$ in soil is significantly different from that of atmospheric N_2, the $\delta^{15}N$ in N_2-fixing plants may be altered compared with non N_2-fixing plants that rely solely on soil N (reference plant). In the case when soil $\delta^{15}N$ is not significantly different from that of atmospheric N_2, soil ^{15}N can be enriched by ^{15}N labeled fertilizer and the difference between soil and atmospheric $\delta^{15}N$ used to estimate contribution of fixed atmospheric N_2 (ID method) is enlarged. There has been an increasing interest in the use of NA method, and quantitative data on N_2-fixation in various large-scale fields, and from natural forests. This method provides an accurate estimate of BNF but it is expensive and requires specialized equipment and skills (Danso, 1995).

The diversity of diazotrophic bacteria

The most primitive N_2-fixing organisms, the anaerobic autotrophs, are the first form of life on earth and have the widest distribution in aquatic and soil ecosystems. Furthermore, N_2-fixation is widely distributed among the two subdivisions of prokaryotes, including green sulphur bacteria, proteobacteria, methanobacteria, and cyanobacteria. N_2-fixation, did not emerge in free-living organisms until atmospheric nitrogen was easily available as a substrate and prior to the accumulation of atmospheric oxygen. Nitrogenase becomes damaged by exposure to atmospheric levels of oxygen. Ability of diazotrophs to fix nitrogen is related to the availability of 'free' nitrogen in their immediate environment. Complicating our understanding of biodiversity among the free-living prokaryotes is the inability to culture them; as a result, many of these organisms remain undiscovered (Ward et al., 1990).

Diazotrophic bacteria are phylogenetically diverse and include organisms with vastly different physiological properties. The capacity to perform BNF has been detected in various phototrophic microorganisms e.g., aerobic phototrophic *Cyanobacteria* (Vaishampayan et al., 2001), anaerobic purple-sulfur phototrophs like *Chromatium*, and green-sulfur phototrophs, e.g., *Chlorobium* (Young, 1992). Despite the large number of known diazotrophic genera, many isolates have never been assessed for BNF capacity. Accordingly, BNF may be even more widespread than currently known (Young, 1992; Madigan et al., 2000). Only a minority of diazotroph species is involved in symbioses, and free-living soil diazotrophs have received comparatively little attention from researchers and still poorly characterized. Free-living aerobic N_2-fixing bacterial genera found in soil include *Azotobacter*, *Beijerinckia* and *Derxia*, but the majorities are microaerophilic (e.g. *Azospirillum*, *Herbaspirillum*) or facultative and obligate anaerobes (e.g. *Klebsiella*, *Clostridium*, *Erwinia*) (Malik and Schlegel, 1981; Hill, 1992).

Diallo et al. (2004) using DGGE, identified forty-four major bands, and when partially sequenced, they yielded 33 different *nifH* sequences in soil under *Acacia tortilis* ssp. raddiana and *Balanites aegyptiaca* cultivation in the dryland part of Senegal. Most sequences were affiliated with the α, β, and *γ-proteobacteria* and among which *nifH* sequences were identical to those of *Pseudomonas stutzeri* and to *Azotobacter vinelandii.* Roesch et al. (2008) studied the diversity of nitrogen fixing bacteria associated with the soil; stem and root of field grown maize based on the *nifH* gene and reported that only two genera, *Azospirillum* and *Azotobacter*, were in all samples at over 1%. The other associated bacteria are *Beijerinckia*, *Geobacter*, *Rhodovulum*, *Methylobacterium*, *Methylocystis*, *Methylosinus*, *Raoultella*, *Gluconacetobacter*, *Methylocella*, *Delftia*, *Dechloromonas*, *Rhizobium*, *Herbaspiril* gene *lum*, *Ideonella*, and *Klebsiella*. Olson et al. (2009) studied the diversity of the nitrogenase (*nifH*) gene in association with *Montipora capitata* and *Montipora flabellate* and reported that these *nifH* sequences were closely affiliated with known taxa in α, β, γ, and δ -proteobacteria, as well as cyanobacteria. These authors also reported Gamma-proteobacteria as the dominant bacterial class represented, and the bacteria were closely related to the genus *Vibrio*.

Shu et al. (2011) studied the bulk paddy soil rhizosphere for diazotrophic bacterial diversity which was maintained under conventional agriculture and with different durations of organic management based on of the *nifH* gene. These authors reported α, β and γ as the dominant class of bacteria. Among the order, *Pseudomonadales* and *Rhizobiales* are the dominant diazotrophs. Fischer et al. (2011) identified the active diazotrophs in a sugarcane field in Seropedica, Brasil through 16S rRNA and *nifH* transcript analyses. Through *nifH* expression transcript analysis, these authors observed *Gluconacetobacter* spp., *Burkholderia* spp., *Herbaspirillum*, *Bradyrhizobium* sp. and *Rhizobium* sp. as the major diazotrophic bacteria from sugarcane.

Barua et al. (2012) isolated diazotrophic bacteria from agricultural lands and coastal saline soils of the Sundarbans, West Bengal, India, to evaluate the influence of salinity on crop productivity. The isolates belonging to genus *Agrobacterium* and *Bacillus* were isolated and identified as diazotrophs based on their ARA activity, dinitrogen fixation ability and the presence of *nifH*

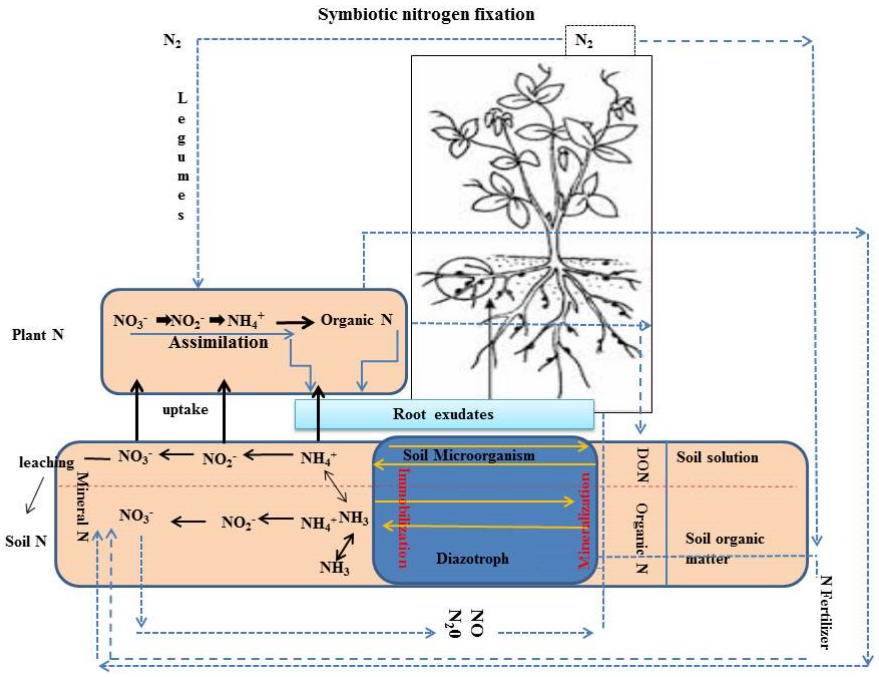

Fig. 3. Biological nitrogen fixing agents in agricultural and terrestrial natural systems (Herridge et al., 2008).

genes. Bahulikar et al. (2014) studied the nitrogen fixing bacterial diversity of Switch grass (*Panicum virgatum* L.) native to North America. Based on the sequences derived from *nifH* RNA these authors reported alpha-, beta-, delta-, and gamma Proteobacter as the major groups associated with roots. Among these, *Rhizobium* and *Methylobacterium* belonging to alphaproteobacteria, *Burkholderia*, *Desulfuromonas* and *Geobacter* belonging to deltaproteobacteria and *Azoarcus* belonging to beta-proteobacteria are the dominant diazotrophic bacterial flora of Switch grass. Wang et al. (2015) analysed the diazotrophic bacterial community in the Dongzhen Reservoir, China with DGGE analysis, constructed clone libraries, did quantitative PCR and quantitative reverse transcription (RT)-PCR and examined microbial communities based on *nifH* gene sequences. Based on these studies these authors reported that Cyanobacteria dominated by *Cylindrospermopsis raciborskii*, and the other class such as alpha proteobacteria and gamma proteobacteria were the most dominant microflora.

The different N_2-fixing organisms and symbioses found in agricultural and terrestrial ecosystems are shown in Fig. 3.

Plant growth promotion by diazotrophic bacteria

Diazotrophic bacteria, by their ability to convert N_2 into NH_3, which can be used by the plant, belong to the plant growth-promoting bacteria (PGPB). Plant growth promotion (PGP) is a complex phenomenon that often cannot be attributed to a single mechanism and, as outlined in Fig. 4. PGPB may typically display a combination of mechanisms (Kuklinsky-Sobral et al., 2004). Apart from fixing N_2, diazotrophs can affect plant growth directly by the synthesis of phytohormones and vitamins, inhibition of ethylene synthesis, improvement of nutrient uptake, enhancement of stress resistance, solubilization of inorganic phosphate and mineralization of organic phosphate (Dobbelaere et al., 2003). Indirectly, diazotrophs decrease or prevent the deleterious effects of pathogenic microorganisms, through the synthesis of antibiotics and/or fungicidal compounds, competition for nutrients through production of siderophores or the induction of systemic resistance to pathogens (Dobbelaere et al., 2003). Many soil bacteria encode *acdS* gene, which degrades 1-aminocyclopropane-1-carboxylic acid (ACC), the direct precursor of ethylene (Glick, 2005; Prigent-Combaret et al., 2008; Yan et al., 2008; Rothballer et al., 2008) and introduction of *acds* gene introduction into *A. brasilense* resulted in increased root elongation of some plants (Holguin and Glick, 2001). Production of N-acylhomoserine lactone may be involved in plant growth promotion as observed in some cases (Rothballer et al., 2008). A particular strain of *A. brasilense* was reported to be antagonistic of the parasitic weed *Striga*, which affect many tropical cereals by preventing *Striga* seed germination (Miché et al.,

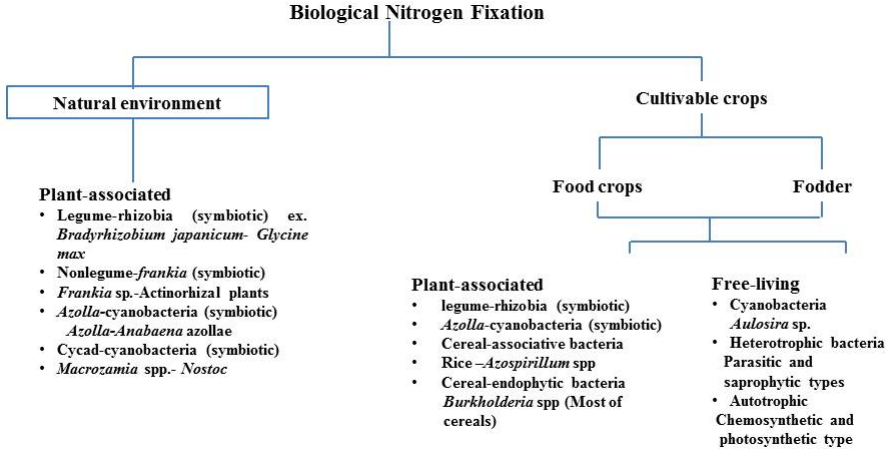

Fig. 4. Plant growth promotion mechanisms of diazotrophic bacteria.

2000).

However, free-living N_2-fixing bacteria were probably the first bacteria used to promote plant growth. *Azospirillum* was first isolated in 1970s (Steenhoudt and Vanderleyden, 2000) and this genus has been studied widely, and the study by Bashan et al. (2004) being the most recent one, reported the latest advances in physiology, molecular characteristics and agricultural applications of this genus. Other bacterial genera capable of N_2-fixation that is probably responsible for plant growth promotion effect are *Azoarcus* sp., *Burkholderia* sp., *Gluconacetobacter diazotrophicus*, *Azotobacter* sp., *Herbaspirillum* sp., and *Paenibacillus polymyxa* (Vessey, 2003). These strains are being isolated from rice, sugarcane, corn, sorghum, other cereals, pineapple and coffee bean. Rondon et al (2007) reported enhanced biological N_2 fixation (BNF) in *Phaseolus vulgaris* L. through the addition of bio-char. These authors reported that the proportion of fixed N increased (50%), when compared to inoculations without bio-char addition (Table 1).

Herridge et al. (2008) reported that the annual N_2 fixation inputs based on BNF are 12−25 million tons (MT) for pasture and fodder legumes, 5 MT for rice, 0.5 MT for sugar cane, <4 MT for non-legume crop lands and <14 MT for extensive savannas. Taule et al. (2011) reported *Pseudomonas*, *Stenotrophomonas*, *Xanthomonas*, *Acinetobacter, Rhanella, Enterobacter*, *Pantoea*, *Shinella*, *Agrobacterium* and *Achromobacter* as the major diazotrophic bacteria associated with Sugarcane grown in Uruguay. Using ^{15}N-dilution technique, these authors reported inputs of N from BNF (34.8−58.8% Ndfa). Mahieu et al. (2013) studied the association of the legume *Anthyllis vulneraria* and the grass *Festuca arvernensis* to be efficient for the phytostabilisation of highly multi-metal contaminated mine tailings. Hassan et al. (2013) compared the efficiencies of AN8 and AN12 having both nitrogen fixing and ACC deaminase producing ability with ACC5 and ACC8 having only ACC deaminase producing ability for its ability to promote plant growth in Maize under lead polluted soil. Hassan et al. (2014) reported in his studies, diazotrophic bacteria TAN1 significantly ($P < 0.05$) increased the root length by 8.25-fold, root fresh weight by 8.36-fold and dry weight by 12.6-fold, shoot length by 6.92-fold, shoot fresh weight by 7.18-fold and dry weight by 6.90-fold, number of leaves by 11.0-fold, chlorophyll a content by 6.25-fold, chlorophyll b content by 10.7-fold and, carotenoid contents by 8.80-fold, when compared to uninoculated control. Based on the results these authors reported that the PGP traits are more effective and resistive against Pb pollution. Puri et al. (2015) reported that the diazotrophic bacteria *Paenibacillus polymyxa* P2b-2R enhanced seedling height (25%) and biomass (30%) after 60 days post inoculation in canola.

Conclusion

Nitrogen is one of the key elements essential for the growth of plants, since they form essential part of proteins, nucleic acids and cellular constituents. In spite of its abundance in the atmosphere, nitrogen cannot be utilized directly by the plants, and to be utilized, it has to combine with elemental hydrogen. This fixation of nitrogen is facilitated by diazotrophic bacteria in a process called as diazotrophic 'N' fixation. Biological nitrogen fixation is facilitated by the nitrogenase enzyme complex at the expense of 16 ATP molecules. The nitrogenase enzyme complex is made up of MoFe protein, which is encoded by *nif*DK genes. Asymbiotic nitrogen fixation contributes to 30% of the fixed N. N_2

Table 1. Yield responses in different crops as influenced by diazotrophic bacterial inoculations.

Name of the diazotroph	Crop	Yield response	Reference
Azospirillum sp. strain B510	Rice	Plant nitrogen content increased by 13-21% in rice as response to inoculation with strain B510	Sasaki et al. (2010)
Gluconacetobacter diazotrophicus	Sugarcane	Bacterial inoculation increased germination (10.89–23.66%), tiller number (16.8–54.16%) and plant height (26.0–0.32%) over control	Suman et al. (2005)
P.fluorescens and *Bradyrhizobium* sp.	*Origanum majorana*	Leaf number increased by 80% and a 3.2-fold increase in shoot fresh weight higher in *P. fluorescens* inoculated than in control. In plants treated with *Bradyrhizobium* and *P. fluorescens*, terpinen-4-ol rose to 73.32 and 66.65%, respectively, compared to control, and *trans*-sabinene hydrate rose to 17.33 and 15.50%, compared to control	Banchio et al. (2008)
Bacillus OSU-142, *Bacillus* M-3, *Burkholderia* OSU-7 and *Pseudomonas* BA-8	*Malus domestica* Borkh	Increased shoot number (up to 30.8%), shoot length (up to 59.2%), shoot diameter (up to 16.3), and fruit yield (up to 137.5%, respectively). of young apple trees with different bacterial strains	Aslantas et al. (2007)
Pseudomonas spp. Four of these isolates, viz. PGPR1, PGPR2, PGPR4 and PGPR7	*Arachis hypogaea*	21-28% increase in pod yield over uninoculated control	Dey et al. (2004)
Asaia bogorensis (strain 219)	Champaka pineapple intercropped with irrigated sapota	*A. bogorensis* (strain AB219) affected the weight of fresh fruit as evident by a 6% increase in productivity	Weber et al. (2010)
Burkholderia vietnamiensis strain AR 1122	Rice Cana Roxa and Cana Forte	Strain AR1122 promoted increase 10 and 29%, respectively, in the grain yield compared to fertilized with 100 kg N ha^{-1} and reference strain ZAE 94	Araujo et al. (2013)
AC32 (*Herbaspirillum* sp.), AG15 (*Burkholderia* sp.), CA21 (*Pseudacidovorax* sp.), and UR51 (*Azospirillum* sp.)	Rice	half-fertilization plus (60 kg of urea ha^{-1})separate inoculation with the these isolates achieved rice growth similar to those achieved by full-fertilization without inoculation (120 kg of urea ha^{-1})	De Souza et al. (2013)
Azospirillum brasilense	Winter wheat and oat	Yield increases of up to 27% in wheat and 6% in oats	Swędrzyńska and Sawicka (2000)
Azospirillum sp. B510	Rice	Increased biomass of the colonized plants (6–12%) than control plants in the Nipponbare, Koshihikari and Kasarat rice cultivars	Isawa et al. (2010)
Azospirillum brasilence	*Pachycereus pringlei*	In the poorest soils of bare areas (BA) shoot dry mass and root length were increased by 60 and 100% as a result of inoculation	Carrillo-Garcia et al. (2000)
Herbaspirillum and Burkholderia strains	Rice	*Herbaspirillum* strains (H18, ZA15) and a *Burkholderia vietaminensis* strain (AR114) increased rice grain yield from 33 to 47% with Tricalcium Phosphate and 18 to 44% with simple superphosphate, respectively	Estrada et al. (2013)

fixation is widely distributed among the prokaryotes such as proteobacteria, cyanobacteria, green Sulphur bacteria, and methanobacteria. Diazotrophic bacteria apart from fixing 'N' are also involved in diverse plant growth promoting activities such as synthesis of phytohormones and vitamins, inhibition of plant ethylene, improvement of nutrient uptake, solubilization of inorganic phosphate and mineralization of organic phosphate. This mini review helps us to understand the role of diazotrophic bacteria in improving soil fertility and plant growth.

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