Developments in Applied Microbiology and Biotechnology

Trends of Applied Microbiology for Sustainable Economy

Edited by
Ravindra Soni, Deep Chandra Suyal,
Ajar Nath Yadav, and Reeta Goel

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Edited by

RAVINDRA SONI
Department of Agricultural Microbiology, College of Agriculture,
Indira Gandhi Krishi Vishwavidyalaya, Raipur, Chhattisgarh, India

DEEP CHANDRA SUYAL
Department of Microbiology, Akal College of Basic Sciences, Eternal University,
Sirmour, Himachal Pradesh, India

AJAR NATH YADAV
Department of Biotechnology, Dr. Khem Singh Gill Akal College of Agriculture,
Eternal University, Baru Sahib, Sirmour, Himachal Pradesh, India

REETA GOEL
Department of Biotechnology, Institute of Applied Sciences and Humanities,
GLA University Mathura, Mathura, Uttar Pradesh, India

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Plant growth-promoting diazotrophs: Current research and advancements

Chanda Vikrant Berde[a], P. Veera Bramhachari[b], and Vikrant Balkrishna Berde[c]

[a]Marine Microbiology, School of Earth, Ocean and Atmospheric Sciences, Goa University, Taleigao Plateau, Goa, India, [b]Department of Biotechnology, Krishna University, Machilipatnam, Andhra Pradesh, India, [c]Department of Zoology, Arts Commerce and Science College, Lanja, Maharashtra, India

8.1 Introduction

Increasing demands for food supply run parallel with increasing population. To meet the demands of increasing food production, it is necessary to increase the agricultural yields. Application of biofertilizers in the form of diverse nitrogen-fixing microorganisms called diazotrophs will ensure the optimization of agricultural yields. The overall increase of crop plant growth is achieved through plant growth-promoting rhizobacteria (PGPR). The PGPR covers microorganisms such as IAA (indole acetic acid) producers, phosphate solubilizers, potassium mobilizers, etc. Biological nitrogen fixation carried out by the diazotrophic microorganisms contributes to more than 60% of the fixed nitrogen on our planet. Thus, isolating very efficient nitrogen-fixing microorganisms, studying the mechanisms involved in nitrogen fixation, using these microorganisms as formulations for agricultural use for not only the leguminous but also the nonleguminous crops, will help in achieving success in increasing the crop yield (Singh, 2018). Research over the last few years has been handling various approaches to extend nitrogen fixation to crops other than legumes, develop inoculums with diazotrophs for nitrogen-deficient soils, and application of nonleguminous diazotrophs (Saumare et al., 2020). Those studies seek to illustrate diazotrophic agronomic value for the improvement of soil fertility and crop production as a nonpolluting, cost-effective method. The biological fixation of nitrogen produces about 200 million tonnes (Graham, 1992; Peoples et al., 2009).

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With agricultural practices such as mixed cropping patterns, the nitrogen fixed in the soil during one crop can be efficiently utilized by the next crop. Thus, mixed cropping with leguminous and nonleguminous crops such as the soybean-wheat system, or the next season crops in crop rotation, can maximally utilize the fixed nitrogen (Fustec et al., 2010). This chapter focuses on the recent advances in the diazotrophic research apropos to terrestrial as well as diazotrophs from the ocean, the research focused on nitrogen-fixing genes, the enzymes and proteins involved as well as the efforts taken to apply the nitrogen-fixing ability of diazotrophs to nonleguminous plants.

8.2 Nitrogen fixation in diazotrophs

Atmospheric nitrogen fixation by diazotrophs is carried out by the enzyme nitrogenase, which catalyzes the conversion of nitrogen to ammonia (Hoffman et al., 2014; Einsle and Rees, 2020). All diazotrophic microorganisms harbor this enzyme. It is made up of two metalloprotein components, both of which play a role in ammonia formation. Component I, also called dinitrogenase, i.e., molybdo-ferro-protein (Mo-Fe-protein), is 2.2×10^5 Da protein, which reduces nitrogen as well as several substrates such as acetylene, protons, cyanide, isocyanide, and azide, as reported in nonphototrophic bacteria *R. rubrum* (Munson and Burris, 1969). Component II, also called dinitrogenase reductase, is an electron-transfer Fe protein. Catalysis requires a reduction source and Mg-ATP. In a catalytic loop of single-electron transfer and Mg-ATP hydrolysis, two member proteins associate and dissociate. The active substrate binding and reduction site, involving electron transfer from the Fe protein to FeMo-Co, is provided by an iron-molybdenum cofactor. Alternative nitrogenases of the form –V and –Fe, where Mo from FeMo-Co is substituted with V or Fe, were discovered as well. The extension of nitrogenase substrates to include CO and CO_2 has been reported (Seefeldt et al., 2020).

Many diazotrophs have another hydrogenase enzyme that is involved with the elimination of the hydrogen that is formed as nitrogen is fixed. Hydrogenases reuse H_2 for ATP synthesis and increase N_2 fixation speed (Johansson et al., 1983). Hydrogenase also has an oxygen sensitivity as nitrogenase enzyme and contains four iron and four molecule-labile sulfur atoms. Hence, the removal or utilization of hydrogen is important, to prevent the inactivation of the nitrogenase enzyme. In some diazotrophs like *Azotobactor vinelandii* and *A. chroococcum*, nitrogen fixation and hydrogen production may be carried out by the same enzyme complex. Hydrogen evolved is quickly removed from the cells by diffusion.

Nitrogenase enzyme is also inhibited by the higher concentration of ammonia. Hence, ammonia formed during nitrogen fixation needs to be converted to organic nitrogen compounds, to protect the nitrogenase enzyme from getting inactivated. Aerobic diazotrophs, which lack special compartments for nitrogen fixation, demonstrate a high respiratory rate, which is an adaptation to prevent the oxygen from reaching the nitrogenase enzyme and inactivating it. In the case of cyanobacteria, reduction of nitrogen occurs in heterocysts, which are thick-walled cells (Haselkorn, 2003). The O_2-producing photo-system II, ribulose bisphosphate carboxylase, is missing and the photosynthetic biliproteins may be lacking or diminished. In nonheterocystous cyanobacterial species such as *Lyngbya*, the nitrogen fixation occurs in cells in the center of the colony where oxygen penetration is less. In leguminous plants, nitrogen fixation takes place in root nodules that contain leghemoglobin to regulate oxygen tension.

8.3 Terrestrial nitrogen-fixing diazotrophs

The need to satisfy the growing demand for food productivity requires isolation and the efficiency of diazotrophs. The efficient relationship between diazotrophs and the host plant is a major prerequisite for nitrogen fixation, given the phylogenic and ecological richness of diazotrophic bacteria and their hosts. Hye Jia et al. (2014) identified diazotrophs from 576 endophytic bacteria of leaves, stems, and roots, from 10 rice cultivars. Eighty-one percent of these isolates produced ammonia and were classified with the application of special *nif* gene primary set as the diazotrophic bacteria. Diazotroph species of *Bacillus, Penibacillus, Microbacteria,* and *Klebsiella* have been reported as belonging to the genes of *nif*H. This group focused on the ability to fix nitrogen and other properties of the diazotrophs, including the ability to produce auxins and siderophores, solubilize phosphate and induce fungal resistance in plants. Diazotrophic bacterial isolation from maize mucilage has been confirmed by Higdon et al. (2020a) with the potential to fix nitrogen and having other plant growth-promoting attributes. Three large groups of sequences referring to the nitrogen-fixing gene were included in the sequences of the isolates. The sequences were homologous to *nif* genes (*nifHDKENB*) in the Dos Santos model (Dos Santos et al., 2012; Higdon et al., 2020b). Half of the overall diazotrophic isolates revealed the *nif*H gene and 193 isolates among these were belonging to *Enterobacter*, *Klebsiella*, *Metakosakonia*, *Pseudomonas*, *Rahnella*, and *Raoultella* species (Higdon et al., 2020b).

Lateral transfer of chromosomal symbiosis islands was observed in species of the genus *Mesorhizobium*, bearing the ability to nodulate legumes like chickpeas (Laranjo et al., 2014). This trait helps in nodulating new hosts, which is beneficial for the development of bioinoculants with wider host ranges. Thus, understanding the mechanisms of adaptation to new hosts and the symbiosis between the gene bearers and the host plant will enable more effective development of mesorhizobium bioinoculants as biofertilizers.

Recent studies by Elhady et al. (2020) focused on the effect of nodule size on nitrogen-fixing efficiency of *Bradyrhizobium japonicum* and the host soybean crop. Smaller nodules were formed as a result of *P. penetrans* invasion of plant roots. It affected the nodule size as well as the number of bacteroids in the infested roots; however, the number of nodules was higher. Therefore, it can be seen that the successful establishment of the diazotroph in the host plant, and establishment of nitrogen-fixing mechanism in the host, is still affected by external factors, biotic as well as abiotic.

Numerous cyanobacteria contain pale and dense cells called heterocysts in *Anabaena, Nostoc,* and *Cylindrospermopsis* and are filamentous (Ogawa and Carr, 1969; Haselkorn, 2003; Willis et al., 2015; Aly and Andrews, 2016; Zulkefli and Hwang, 2020). These are the nitrogen fixation sites (Haselkorn, 2003; Videau et al., 2016). In the absence of available combined nitrogen, heterocysts are formed, as ammonia prevents the differentiation of heterocysts as well as inhibits the nitrogenase enzyme. Nitrogen fixation takes place in in-house structured cells under reduced conditions; cyanobacteria such as *Lyngbya, Oscillatoria, Plectonomas,* and in bacterium do not produce heterocyst. The nitrogen fixation occurs in root nodules produced by *Rhizobium* (in leguminous plants) and *Frankia* (in nonleguminous plants). The lichens (a symbiotic structure created by cyanobacteria and fungi) are the site of nitrogen fixation in lower community of microorganisms.

Diazotrophism is seen in endophytic microorganisms, especially endophytic bacteria; these intracell colonizers bring about nitrogen fixation in tissues of the plants and promote their growth. The endophytic diazotrophs can be exploited as biofertilizers for sustainable agriculture. A strain of *P. polymyxa* can colonize nonnative hosts and fix atmospheric nitrogen within, promoting plant growth. *Gluconacetobacter diazotrophicus* is another well-studied endophytic diazotroph isolated from sugarcane. It has the nitrogen-fixing ability as well as additional plant growth-promoting traits (Puri et al., 2017). Diazotrophs imparting drought stress resistance to the plants such as strains of genus *Herbaspirillum* offer great hope for agriculture in drought-prone areas (Aguiar et al., 2016).

8.4 Nitrogen fixation in the ocean

Diazotrophs were isolated from the rhizosphere and soil in general, but during recent years, the focus is shifted to endophytic and marine diazotrophic microorganisms. The occurrence of diazotrophs, both bacteria and archaea, has been detected in the Arctic Ocean and found to play a role in the conversion of atmospheric nitrogen to bioavailable ammonia in the marine ecosystem. Both symbiotic cyanobacterial nitrogen fixation and heterotrophic diazotrophs have been reported (von Friesen and Riemann, 2020). The research workers, however, point out some gaps such as the inability to sample larger regions and the nonavailability of enough quantitative data to come to specific conclusions. This calls for future and in-depth research on diazotroph distribution, composition, and their activity in pelagic and sea ice-associated environments of the Arctic Ocean.

Though previously, there were reports of studies being carried out measuring nitrogen fixation and denitrification at the seafloor and pelagic zone using stable isotope technique (Fan et al., 2015). The same group has also worked on the diversity, abundance, and activity of nitrogen-fixing and denitrifying microorganisms at three stations in the southern North Sea. Their genomic analysis studies indicated the presence of *nif*H genes in anaerobic sulfur/iron reducers and sulfate reducers. These results are concomitant with the reports of the discoveries of diazotrophic methanogenic archaea *Methanosarcina barkeri* by ^{15}N radiotracer technique (Murray and Zinder, 1984; Leigh Nitrogen, 2000) and *Methanococcus thermolithotrophicus* by acetylene reduction assay (ARA) technique (Belay et al., 1984; Leigh Nitrogen, 2000).

Noncyanobacterial diazotrophs or heterotrophic diazotrophs are distributed widely in marine waters, including the oxygenated zones; however, the mechanism of nitrogenase protection from oxygen is yet to be understood (Pedersen et al., 2018; Geisler et al., 2019). A major fraction of the aquatic biosphere such as eutrophic estuaries has high ambient nitrogen concentrations and oxidized aphotic water. The diazotrophs found in these zones are closely associated with bacterioplankton and are referred to as planktonic heterotrophic diazotrophs. The prime requirement for the colonization of the diazotrophs onto surfaces is the initial colonization by bacterioplankton (Pedersen et al., 2018). Putative diazotrophs appeared after 80 h of colonization initiation, after the plankton, following the colonization by bacterioplankton. The surfaces for the colonization of diazotrophs can be natural particles that act as the nitrogen fixation loci. The natural particles or aggregates comprise polysaccharides that offer a microenvironment with less oxygen for the activity of the nitrogenase enzyme. It was also pointed out by the authors that resuspension of sediment material can promote pelagic N_2-fixation. Thus, these heterotrophic diazotrophs are responsible for the nitrogen fixation taking place in the ocean waters.

Work on diazotrophs associated with the particulate matter related to their high concentrations of nitrogen fixation rates and the existence of *nif*H genes indicates that they are extremely unique and specific. This relationship was shown for the first time recently with a direct staining approach (Geisler et al., 2019). Earlier such research was conducted mostly through indirect relations and various methodological especially statistical approaches. This new staining technique incorporates fluorescent tagging of active diazotrophs by nitrogenase immunolabeling and Alcian blue or concanavalin-A polysaccharide stain. Nucleic acid staining was used for the total bacteria. This approach provides nitrogen fixing frequencies, bacterial activity, and specific location of heterotrophic diazotrophs on artificial and natural aggregates (Geisler et al., 2019).

Diazotrophs in the marine environment contribute to nitrogen fixation, but these studies have been focusing only a certain hotspots. There is a dearth of knowledge of diazotrophic activities in the open oceans. The reasons are very less volumes being sampled, fewer sampling sites as compared to the vastness of the oceans, less frequency of sampling, and the practical difficulties of having a good geographic coverage. These difficulties have hampered the studies on diazotrophic diversity and distribution; hence, getting global nitrogen budget becomes a failure or is inaccurate. A solution for these inadequacies requires leveraging high spatiotemporal resolution measurements, and failure to employ these has come in the way of measurement methods according to Benavides and Robidart (2020). Increasing the spatiotemporal resolution of diazotroph activity and diversity will provide more accurate quantifications of nitrogen fluxes in ocean waters. A very recent study based on the application of combined values from two established acetylene-based assays was used to study the nitrogen cycling in coral reefs (El-Khaled et al., 2020). This method makes possible studying two processes, i.e., nitrogen fixation and denitrification, simultaneously by analyzing the gases formed during the processes. Gas chromatography is used for ethylene and nitrous oxide analyses formed during nitrogen fixation and denitrification, respectively.

8.5 Genomic and transcriptomics of diazotrophs

Mahmud et al. (2020) in their review have elaborated the need for research focusing on transferring nitrogen-fixing mechanisms to nonlegumes, with emphasis on molecular techniques. This in turn necessitates the importance of genome and proteomic/transcriptomic studies. The last two decades has seen a slow and assuring increase in reports on work pertaining to these aspects of diazotrophic research. From diazotrophic rhizobial genome, numerous symbiotic genes (*nod* genes) encoding for nodulation, and nitrogen-fixing genes (*nif* genes), have been identified.

In symbiotic diazotrophs, especially *Rhizobium* sp., *nif* genes are located on a megaplasmid adjacent to *nod* genes. In the nonsymbiotic diazotrophs like cyanobacteria, these genes are localized on the chromosome. *Nif* genes comprises gene cluster of 24-Kb nucleotides, between the genes encoding for histidine (his) and shikimic acid (shi A). The cluster is organized in seven operons, i.e., transcription units (e.g., QB AL FM VSUX NE YKDH J). These operons transcribe for nitrogenase, Fe-protein, and Mo-Fe-protein. In nonsymbiotic diazotroph *Azospirillum* sp., m/HDK cluster megaplasmid and the sequence homologous to *nod* genes were reported (Acosta-Cruz et al., 2012). The presence of plasmids has also been described for other diazotrophs, including *Anabaena, Azotobacter*, and *Frankia* (Elmerich et al., 1987).

Diazotrophs also harbor hydrogen uptake (or *Hup*) genes that help in the removal of hydrogen formed during nitrogen fixation. The need for the removal of hydrogen is due to the reduced efficiency of nitrogenase in the presence of hydrogen. There are reports of genetically engineered diazotrophic strain by transferring the *Hup* genes of *R. leguminosarum* into *Rhizobium* strain (Lambert et al., 1985). This is the world's first report of interspecific transfer of *Hup* genes. The efficient transfer and expression of *Hup* genes has made it easier for the chick-pea-*Rhizobium* system to improve symbiotic energy efficiency. Transfer of nitrogen-fixing genes to nonleguminous plants is one of the strategies to overcome nitrogen deficiency and also to reduce the use of chemical fertilizers. The best way to improve nitrogen availability in crop plants would be to transfer the genes m/genes into chloroplasts; however, lack of chloroplast transferring techniques and protection of nitrogenase from O_2 evolved during photosynthesis are the drawbacks of the process that need to be addressed (Long, 1989; Báscones et al., 2000).

According to Gaby and Buckley (2011), the diazotrophs are a poorly described group and many more diazotrophic strains and nitrogen-fixing genomes need to be discovered and studied. The authors have reported 16,989 *nif*H sequences so far. Other sequences include nitrogenase genes *nif*D, *nif*K, *nif*E, *nif*N, etc., which make up the total of 32,954 sequences in the database. This database allowed for a comparative study of the symbiotic systems designed to identify core genetic networks that shape the root nodule and to define strategies to transfer the nitrogen-fixing capability of nonlegume crops (López-Torrejón et al., 2016; Wardhani et al., 2019; Mahmud et al., 2020; van Heerwaarden et al., 2018). In a recent study, homologous coding sequences for the *acdS* and *ipdC/ppdC* genes were identified for the diazotrophs grouped in Dos Santos Positive (DSP) (Higdon et al., 2020b). In the case of PQQ genes, approximately 28% of all isolates examined had homologous *pqq*BCDE sequences, whereas 12% had coding sequences equal to *pqq*F and 90% had *pqq*DH matches (Higdon et al., 2020a).

The simultaneous developments in transcriptomics of diazotrophs and the nitrogen-fixing mechanisms have opened up a new era in this field. Mergaert et al. (2003) reported the discovery of the NCR (module-specific cysteine-rich) peptides in nodules of *Medicago truncatula*. Using transcriptome analysis, it was found that these have a signal peptide with a conserved cysteine motif and 300 plus members have been discovered in galeloid legumes as well as other plants (Wojciechowsk et al., 2004; Kondorosi et al., 2013; Pan and Wang, 2017; Kereszt et al., 2018). Further characterization of these NCR peptides has been reported by some workers (Kereszt et al., 2018; Lindstrom and Mousavi, 2019).

8.6 Beneficial mechanisms other than N-fixation provided by diazotrophs

Apart from fixing atmospheric nitrogen for the plants, the diazotrophs have been reported to have multiple other abilities that add to overall growth-promoting attributes. Phosphate solubilization helps in making inorganic phosphorus available, potassium mobilization provides essential potassium for plant growth and functions, the production of plant growth hormones like IAA benefits the plant root and shoot development, protection against plant diseases is provided by the production of secondary metabolites, etc. These are some of the additional properties observed and studied in diazotrophic microorganisms (Unpublished data). Genome mining showed that isolates of all diazotrophic groups possessed marker genes for multiple mechanisms of direct plant growth promotion (PGP). These findings reveal a potential to confer the targeted PGP traits to the host organism and also revealed phenotypic variation among isolates. Diazotrophs belonging to *Rhizobia, Bradyrhizobia, Azotobacter, Azospirillum, Pseudomonas, Klebsiella*, and *Bacillus* genera that harbor the beneficial mechanisms in addition to nitrogen fixation, play a vital role in the overall growth and yield of crop plants.

There is a lacunae of information and research on the other beneficial properties present in diazotrophs forming legume nodules except for the knowledge about their nitrogen-fixing abilities. Table 8.1 summarizes some of the plant growth-promoting attributes reported in some diazotrophs in addition to nitrogen-fixing properties.

Table 8.1 Plant growth-promoting properties found in diazotrophs.

Properties	Diazotrophic microorganism	References
Acetic indole (IAA), hydrocyanic acid (HCN), antibiotics and/or mycolytic enzymes, organic acid, and siderophores	Rhizobia	Gopalakrishnan et al. (2015, 2018)
Inorganic phosphate solubilization, nitrogen fixation, IAA production	Mycorrhizae	Agnolucci et al. (2019)
ACC-deaminase activity and production of IAA, phosphorous solubilization	12 Diazotrophic strains, including *Sphingomonas azotifigens* (JN085438), *Pseudomonas putida* (JN222977), *Herbispirillum* sp. (JF990839)	Laskar et al. (2013)
IAA production, $FePO_4$ solubilization, $AlPO_4$ solubilization, siderophores production	91 Isolates belonging to the Proteobacteria phylum	Zuluaga et al. (2020)
Higher auxin-producing activity, high siderophore-producing activity, high phosphate-solubilizing activity	Two species of *Penibacillus*, three species of *Microbacterium*, three *Bacillus* species, and four species of *Klebsiella*	Hye Jia et al. (2014)

8.7 Application in global agriculture

A variety of diazotroph-based biofertilizers are available globally for agricultural applications. Table 8.2 summarizes the available diazotroph-containing biofertilizers used for different crop cultivars and the difference in yields obtained. Use of diazotrophic biofertilizers impacted the economy of several countries such as Brazil with an economic benefit in terms of N-fertilizer saving over USDA 2.5 billion per year (Bruno et al., 2003). Countries like Canada, Germany, the United Kingdom, Spain, Italy, France China, Japan, Australia, New Zealand, India, and the rest of Asia produce large numbers of biofertilizers that are based on nitrogen-fixing bacteria and contributing to economic growth making around USD 0.284–0.45 billion dollars (Swarnalakshmi et al., 2016). China holds more than 511 biofertilizer products and accounted for 43.2% of the biofertilizer market share for the Asia-Pacific region in 2017 (Market Data Forecast, 2018).

As a result of the availability of biofertilizers, the nitrogen fixed by crops is around 55–60 million tons per year (Vitousek et al., 2013; Figueiredo et al., 2013; Rao and Balachandar, 2017) and the highest contributor is *Bradyrhizobium* species found in the legumes of soybean as microsymbiont (Hungria and Mendes, 2015; Gyogluu et al., 2018). Application of the biological nitrogen-fixing diazotrophs along

Table 8.2 Commercially available diazotrophic biofertilizers and their impact on agricultural yields.

Commercial biofertilizer	Microorganism used	Country	Benefits	Plant cultivar	References
Diazotroph formulated with perlite-biochar carriers	*Rhizobium leguminosarum* bv. *phaseoli* LCS0306	Spain	Increased yield	Common bean	Pastor-Bueis et al. (2019)
Biofix and Legumefix	Rhizobia inoculants	Ghana	Grain yield (12%–19%)	Soybean and cowpea	Ulzen et al. (2016)
Sympal and Legumefix	Rhizobia inoculants	Zambia	Rise in yields from 2000 to 4000 kg/ha	Soybean	Thuita et al. (2018), Mathenge et al. (2019)
Nitragin	Root-associated bacteria	Germany	Three- to fourfold increase in yield	Soybean	William and Akiko (2020)
Maize seeds coated with *Azospirillum*	*Azospirillum*	Mexico, Argentina	Yield increase	Maize	Reis (2007)
Microbin and Azottein	Phosphate solubilizing and nitrogen fixing	Egypt	Grain yield increased	Barley	El-Sayed et al. (2000)
BioGro	BNF	Southeast Asia	Replaced 23%–52% of N chemical fertilizers without the loss of yield	Rice	Rose et al. (2014)
–	Rhizobia	Northern Nigeria	Yield increase by 447 kg/ha	Soybean	Ronner et al. (2016)
Biofertilizer	*Azospirillum brasilense, Azotobacter chroccocum,* and *Trichoderma lignorum*		Contributed to 60% of the nitrogen	Sugarcane	Serna-Cock et al. (2011)
Biofertilizer	*Herbaspirillum seropedicae, Pseudomonas* sp., and *Bacillus megaterium*		Yield increase from 18% to 57.31%	Sugarcane	Antunes et al. (2019)

with vermicompost and other soil conditioners has been observed to further improve the soils, especially poor alkaline soils (Mathenge et al., 2019).

8.8 Future challenges in agriculture: Application of diazotrophs to nonlegumes

Research into the genetics of diazotrophic microorganisms has led to the transfer of the benefits of nitrogen fixation to nonleguminous plants such as wheat, rice, sorghum, or maize. One way of achieving this is by inducing symbiosis between the diazotrophs and the non-nitrogen-fixing plants, resulting in the development of root nodules (Mus et al., 2016; Burén and Rubio, 2018). The plant needs to produce and secrete nodulation signals for the initiation of nodulation by the diazotroph. Another approach is to introduce the nitrogen-fixing genes in the nonleguminous plant itself (Dent and Cocking, 2017; Vicente and Dean, 2017; Burén et al., 2018).

The difficulty of this biosynthesis and the enzyme's oxygen sensitivity are a major challenge for this strategy to be implemented. It is also uncertain if the power and energy required to support nitrogenase catalysis can be provided by the cereal host (van Velzen et al., 2018). There are reports of the successful transfer of *nif* genes to nondiazotrophs such as *E. coli, Saccharomyces cerevisiae, Paenibacillus* sp., tobacco plants (Dixon and Postgate, 1972; Han et al., 2015; Oldroyd and Dixon, 2014; Burén et al. 2017; Burén and Rubio, 2018), as well as research carried out on the nonlegumes, *Parasponi* legumes, and Actinorrhizae, which shows that such a transfer of genes leading to acquiring nitrogen-fixing ability is possible. Significant developments in developing strategies in this regard were reported mostly in transgenic plants, yeast, etc. Ivleva et al. (2016) reported the integration of *nif*H and *nif*M genes into the tobacco chloroplast genome, which could encode active Fe protein of nitrogenase in the transgenic plant. Burén and Rubio (2018) described the transfer of *nif* cassette of nine genes in transgenic yeasts (*Saccharomyces cerevisiae*), which is sufficient for nitrogen fixation. Another report shows that the transfer of the nitrogenase gene to mitochondria and root plastids in eukaryotes can result in nitrogenase expression under a low-oxygen environment (Wardhani et al., 2019; Ivleva et al., 2016).

According to Postgate (1992), the production of a "diazoplast" in plants would allow plants to resolve their genetic and physiological bacterial dependency for nitrogen fixation. The new organelle has diazotrophic properties incorporated in chloroplasts. By adding an endosymbiotic prokaryote to the plant's genome, diazoplast could also be obtained similarly to a chloroplast. Research on the establishment of *Gluconacetobacter diazotrophicus* in root meristem cells indicate that symbiosomal vesicular cytoplasmic compartments are possible locations for diazoplast formation.

A third approach can be making use of the endophytic microbial community that is associated with the plant system, which will enable aerobic nitrogen fixation by microsymbionts, for improved plant growth (Kennedy and Islam, 2001). For example, Yonebayashi et al. (2014) have reported the association of endophyte and tumorous growth on sweet potato. Oliveira et al. (2003) have studied the effect of diazotrophic endophytic inoculants on sugarcane growth. Similarly, the nonsymbiotic diazotrophs also contribute significantly to nitrogen fixation as pointed out by the work done by Pankievicz et al. (2015) and van Deynze et al. (2018).

Nitrogen fixation efficacy and plant growth benefits in rhizobia-legume symbiosis have been successfully demonstrated for *Gluconacetobacter diazotrophicus* (Dent and Cocking, 2017). The development of nitrogen-fixing endophytic bioinoculants that can be useful to all staple food crops and an apt replacement for chemical nitrogen fertilizers, having yield benefits, is significant progress leading to Greener Nitrogen Revolution (Dent and Cocking, 2017).

8.9 Conclusions and future perspectives

The extensive application of synthetic fertilizers to meet the increasing nitrogen demand of agriculture has been seen in the last decades. This has ultimately led to environmental pollution especially due to the runoff of excess nitrates and soils losing fertility. The increasing concern over the deteriorating environment necessitates lowering the dependence on chemical fertilizers. Scientists are investigating bio-inoculants, in particular diazotrophic bioinoculants, in different dimensions to enhance legume and nonlegume plant growth. This is crucial for the future of sustainable farming, in an effort to guarantee food security and also to reduce air-polluting emissions from chemical fertilizers. Research dimensions being targeted include diazotrophs for nonlegumes, endophytic diazotrophs as bioinoculants, transferring *nif* genes to nondiazotrophs as well as crops and organelles of plants, etc. Future in situ studies are needed to establish the identity, activity, and ecology of particle-associated noncyanobacterial diazotrophs (NCDs) and also focus future research on diazotrophs of the aquatic system as well as environments in which diazotrophic research lacks or has been overlooked. The PGP properties of diazotrophs offer promise as effective bioinoculants of the future. By appropriate application of the naturally occurring endophytic diazotrophs, biological nitrogen fixation for cereals and other nonlegumes can be achieved.

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