Society for Advancement of Wheat and Barley Research
ICAR-Indian Institute of Wheat & Barley Research
Karnal - 132 001, India

Journal of Wheat Research

8(2):1-11

Homepage: http://epubs.icar.org.in/ejournal/index.php/JWR

Review Article

Biological nitrogen fixation in cereals: An overview

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Article history

Received: 05 August 2016
Revised : 14 October 2016
Accepted: 08 December 2016

Citation

Sharma P, G Devi, M Sharma, HM Mamrutha, K Venkatesh, V Tiwari, GP Singh and P Sharma. 2016. Biological nitrogen fixation in cereals: An overview. *Journal of Wheat Research*, **8**(2):1-11.

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Abstract

Cereals like rice, wheat, maize, sorghum and millets are the crops with total annual yields of 2000 million tons whereas two-third population consumes only wheat worldwide. Demand for cereals is gradually increasing so there is a need to improve the agronomic and molecular parameters to enhance the quality and productivity of cereals. Nitrogen is one of the essential nutrients required by plants; however, cereals are unable to directly uptake nitrogen from the environment. The nitrogen content of soil is maintained either though fertilizer or organic farming. An excess use of nitrogen compounds in any form like water, air, and soil wreaks havoc on the delicate rhizosphere. An alternative sustainable solution is the incorporation of biological nitrogen fixation into cereals that reduces the undesired effects of chemical nitrogen. In this review article we will discussing how the fertility of soil is maintained using diazotrophs and genetically engineering in nitrogen fixing pathways in cereals.

Keywords: Diazotroph, rhizosphere, biological nitrogen fixation, cereal

1. Introduction

Agriculture covers nearly 40% of the world's land surface with 3.4 billion ha of pastures, 1.4 billion ha of arable land and 136 million ha of permanent crops. There is huge demand for cereals in the global market because it has become the foundation of world food security. Cereals include wheat, rice, maize, sorghum, millet and barley, with total annual yields of 2000 million tons compared with about 700 million tons for root and tuber crops and about 380 million tons for legumes and oilseeds. Wheat and rice are two main crops amongst cereals, which account for maximum production and widely consumed by two third of the population worldwide. Global agriculture relies on chemical fertilizers, which are ecologically as well as economically expensive. In chemical fertilizer, nitrogen is one of the most essential nutrients required by plants; however, it is unable to directly uptake from the environment. Only half of the applied fertilizer nitrogen is used, while remaining is lost from the soil-plant system via leaching, volatilization and denitrification. Due to these factors not only an annual economic loss of US$ 3 billion but also cause pollution to the environment(Westhoff, 2009). However, the higher dependency on chemical based fertilizers leads to decline in the organic carbon level in soil, soil biodiversity and impaired soil fertility. Therefore, chemical based fertilizers should be replaced with ecofriendly alternative molecules that not only fix the nitrogen naturally but also maintain the soil and agricultural sustainability. Some of the prokaryotes are able to utilize the atmospheric nitrogen and convert it into NH_3, required by plants is known as "Biological Nitrogen Fixation". Currently more emphasis on use of biological nitrogen fixation and biofertilizers are being provided in agriculture worldwide. The most effective and peculiar processes for nitrogen fixation involve symbiosis with the root nodule bacteria in legumes and in non-legumes. This occurs by various types of interaction between the host plant and bacterium (Oldroyd and Downie, 2008). It is assumed that, about 20-25% of total nitrogen, requirements are fulfilled by nitrogen fixation in rice and maize crops (Montanez *et al.*, 2012). For instance, *Azospirillum spp*., *Azoarcus spp*. and *Herbaspirillum*, develop associative or endophytic relationships with a wide variety of plant roots including cereals also. Another symbiosis process of nitrogen fixation takes place by cyanobacteria (e.g. *Nostoc sp.* etc) and colonizes different plant organs, either intracellularly or extracellularly(Wagner, 2012).

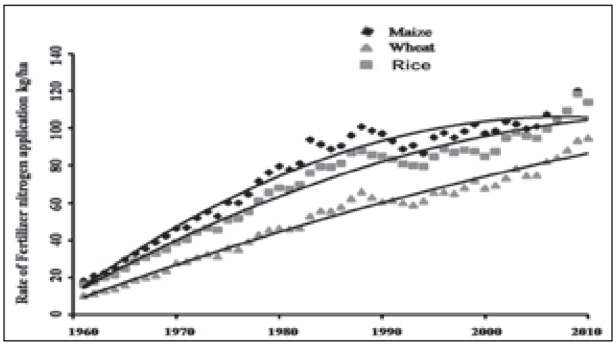

Fig.1 Trends in global averages of fertilizer-nitrogen application rates in maize, rice, and wheat (modified from Ladha *et al.*, 2016).

No doubt there have been thorough research attempts to persuade cereals to fix N after the mid 1970s. However, such efforts are still in debated. Diazotrophs also enhance crop growth and development through other processes. Thus, it is a novel approach by which nitrogen could be fixed directly in soil with the help of microorganisms and beneficial for ecosystem. Now days researchers have a keen interest in introducing root nodule formation in cereals. But nodulation functioning in cereals is tedious task, still if succeeded will be a novel achievement in agricultural world. This review provides an overview of biological nitrogen fixation, its mechanism and different approaches for improving biological nitrogen fixation in cereals.

2. Nitrogen fixing bacteria associated with cereals

In an agriculture eco-system, microorganisms play a vital role in nitrogen fixation, solubilization and mobilization or nutrient recycling. The use of nitrogen by plants involves several steps, including uptake, assimilation, and translocation. Schematic overview in plant cell shown in fig. 2.

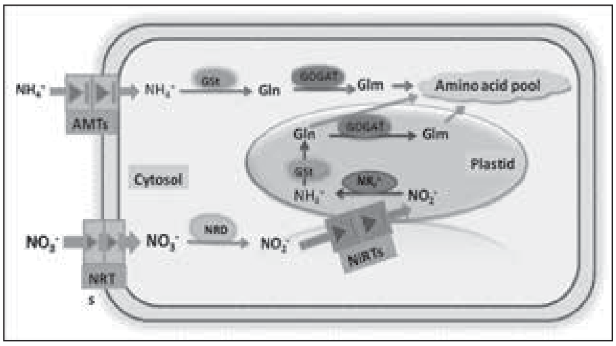

Fig. 2 A general schematic overview of nitrogen uptake and assimilation in plant cell

(Nitrate (NO_3-) uptake into plant cell is facilitated by nitrate transporters (NRTs). Then NO_3- converted in Nitrite (NO_2-) by nitrate reductase enzymes (NRD), these NO_2- transported in the plastid via nitrite transporters (NiRTs), where it is reduced into Ammonium (NH4+) with the help of ferredoxin dependent nitrite reductase (NRf+). NH4+ derived from nitrate reduction or directly from environment to cytosol by Ammonium transporters (AMTs) are assimilated into Glutamine (Gln) by plastidial or cytosolic Glutamine sythases (GSt). Both plastidial or cytosolic glutamine 2-oxoglutarate amino transferase (GOGAT) synthesize Gln into Glutamate (Glm), contributed in amino acid pool in which nitrogen is organically bound and used by further cellular process.)

Nitrogen fixing bacteria present in plant roots that can 'fix' atmospheric nitrogen (N_2) into nitrate known as *diazotrophs.* Similarly, cyanobacteria, (blue green algae), also fix the atmospheric nitrogen. However, these are generally endemic to soil and their efficiency towards nitrogen in rhizosphere is based on behavior, concentrations of organic constituents of exudates secreted by plants as well as their corresponding ability to utilize organic compounds as carbon source(Florence *et al.,* 2016). Therefore, cereals developed multiple solutions to associate and accommodate with diazotrophs in order to acquire atmospheric nitrogen. This led to identifying the two broad categories within cereals, based on the degree of intimacy, interdependency and how they interact with roots of the plants:

3. Types of Nitrogen fixing diazotrophs

Free living nitrogen fixing diazotrophs associative and endophytic symbiotic nitrogen fixing diazotrophs

3.1 Free living nitrogen fixation

Free-living diazotrophs are the bacteria in soil, which are capable to survive and replicate without entering into a symbiotic relationship with plants. They are free from the direct influence of plant roots and do not involve in any structural or morphological accommodation. They might be proven as an alternate source of chemical fertilizer within coming years as cereals plant system showing the phenomena of symbiosis.

In the last decades, the number of free-living diazotrphs in cereals has gained attention due to their nitrogen fixing and other growth promoting ability. Various species of bacteria like*, Azotobacter, Beijerinckia, Derxia* and *Clostridium* have been studied. Crop yield in cereals had been increased by inoculating these strains in many experiments (Bhattarai and Hess 1993, Ozturk *et al.*, 2003; Cakmakci *et al.*, 2001). In corn plants, it was observed that the concentration of nitrogen in the above ground plant-parts increases with the addition of *Azotobacter*, *Beijerinckia* and *Derxia* strains. Moreover, it has been reported that *Azotobacter* can fix annually approx. 0.26–20 kg N/ha and it may be used in crop production as a substitute for a considerable amount of mineral nitrogen fertilizers (Govedarica *et al.*, 1997). The dosages of chemical fertilizer for wheat production can be significantly reduced by using *Azotobacter* and *Pseudomonas* inoculums (Yousefi and Barzegarin, 2014). *Azotobacter* and *Azospirillum sp.* have increased the available nitrogen in the soil, which could enhance the grain number and grain yield in wheat (Chaudhary *et al.*, 2013; Lakshminarayana *et al.*, 2000; Vessey, 2003). Different analogues of *Azotobacter* (Msx1, Msx27, Mal27, Mal30, Mac19 and Mac27) have increased grain yield, which is varying from 10 to 30% under field conditions. The percent of seed germination in rice, maize, wheat cv. Sonalika was stimulated when treated with *Azotobacter sps.*

A cyanobacterium, such as Blue Green Algae (BGA) is one of the major components of the free nitrogen-fixing bacteria in paddy fields. Species of *Nostoc, Anabaena, Tolypothrix, Aulosira, Cylindrospermum, Scytonema* and *aquatic fern (Azolla)* found in rice fields have shown symbiotic relation with BGA and contribute significantly in soil fertility or green manuring. Besides rice, other crops (wheat, sorghum, maize and sugarcane) also show good response against cyanobacterial biofertilizer. The co-cultivation effect of *Nostoc* and *Anabaena* sp. showed the significant enhancement of plant nitrogen, root and shoot length in different wheat varieties (Obreht, 1993). Various efficient nitrogen-fixing strains (*Nostoc linkia, Anabaena variabilis, Aulosira fertilisima, Calothrix sp., Tolypothrix sp.* and *Scytonema sp.)* were isolated from different agro-ecological regions and utilized for rice cultivation (Prasad and Prasad, 2001).

3.2 Associative and endophytic symbiotic nitrogen fixation

It is well known that *Rhizobium* is one of the best examples of symbiotic interaction to fix the nitrogen in legumes (Oldroyd, 2013). However, true symbiosis does not exist in cereal crops. Some of the bacteria are known which grow in the rhizosphere in close contact with the roots and exert natural influence between the true symbionts and free living. Two essential association exhibiits between diazotrophs and cereals to improve the biological nitrogen fixation is discussed below:

3.2.1 Associative symbiotic nitrogen fixing bacterium

Bacterium, which forms a close association with the roots of cereal, not only lives in rhizosphere environment, but also fixes N_2 from the atmosphere and contributes passively to the plant growth. This mutualism type of association is known as associative symbiotic nitrogen fixation. The bacterium grows in the rhizosphere in close contact with the roots; sometimes invade the outer cortical regions of the roots for fixation of nitrogen. Associative nitrogen fixation can supply 20-25 % of total nitrogen requirements in rice and maize (Montanez *et al.*, 2012). The most common example exhibiting the associative nitrogen fixation are the species of *Azospirillum* (Saikia and Jain, 2007) persisting in nature with a wide diversity of plants, including wheat, rice, sorghum, maize and several non-*Poaceae* plant species. Positive effect of *Azospirillum sp.* inoculums in wheat was observed in terms of assimilation of nitrogen and grain yield under field greenhouse conditions (Naiman *et al.*, 2009; Merten and Hess, 1984) .

3.2.2 Endophytic nitrogen fixing bacterium

Endophytic diazotrophs may have an advantage over associative symbiotic nitrogen fixing bacteria, as they colonize in the interior of plant roots and grew in less competitive zone and establish themselves in the region that provide more appropriate conditions for effective nitrogen fixation (Reinhold-Hurek and Hurek, 2011; Sturz and Nowak, 2000). It has been known from previous studies that some nitrogen-fixing endophytic bacteria are independently living in root differentiating structure called nodules or paranodule with cereal crops (Stoltzfus *et al.*, 1997). These nodules predominantly provide a favorable environment for nitrogen fixation. Several species of *Rhizobium, Acetobacter, Klebsiella, Pseudomonas, Herbaspirillum, Gluconacetobacter, Burkholderia* have been reported as endophytes (Baldani and Baldani, 2005) (Table1). Wheat grain yield increased significantly with the addition of wheat-adapted rhizobial strains in field conditions; nevertheless, grain yield also depends upon potential of variety, inoculum and the site-specific environmental conditions. Recently it was evidenced that mixed inoculum contains multiple wheat-adapted *rhizobium* strains performed better than those inocula containing only a single strain . *Rhizobium* strains are potent in developing endophytic association in wheat under natural conditions (Yanni *et al.,* 2016). N_2-fixing *R. leguminosarum bv. trifolii* and clover nodulator forms a natural endophytic association within rice rhizosphere and can successfully promote growth of rice seedlings under genotobiotic conditions and significantly enhance the grain production in field (Dazzo and Yanni 2006). It was reported that the *H. seropedicae* colonizing specifically in *Poaceae* family plants (rice, wheat, maize and sugarcane) (Monteiro *et al.*, 2012; Roncato-Maccari *et al.*, 2003). *Klebsiella pneumoniae*342 (Kp342) is an another endophytic diazotrophs reported in cereal and observed that it relieved nitrogen deficiency in Trenton wheat cultured in the absence of N fertilizer. This nitrogen-fixing capable Kp342 strain originally isolated from a nitrogen-efficient line of maize (Chelius and Triplett, 2000).

Table 1. Efficient Nitrogen fixing bacteria in the rhizosphere of cereals

S. No.	Host plant	Nature of BNF	Bacteria	Reference
1.	Wheat	Free -Living	*Azotobacter spp.*	Chaudhary *et al.,* 2013, Lakshminarayana *et al.,* 2000,
		Symbiotic	*Azospirillum sp.*	Saubidet *et al,*. 2002, Naiman *et al.*, 2009
			Azospirillum brasilense	Dobbelaere *et al.*, 2001, Boddey and Dobereiner 1988
			Klebsiella pneumoniae 342	Chelius and Triplett 2000, Iniguez *et al.*, 2004
			Herbaspirillum seropedicae	Riggs *et al.*, 2001, Komy *et al.*, 2003 Patil *et al.*, 2012
			Rhizobium spp.	Yanni *et al.*, 2016, Yanni *et al.*, 2016
2.	Rice	Free –Living	*Azotobacter sp.*	Singh 2006,
		Symbiotic	*Azoarcus sp.*	
			Azospirillum brasilense	Omar *et al.*, 1989
			Blue Green algae Azolla	Rodriguez *et al.*, 2006, Saadatnia and Riahi 2009, Wilson 2006
			Burkholderia sp.	Baldani *et al.*, 2000
			Gluconacetobacter diazotrophicus	Muthukumarasamy *et al.* 2007
			Herbaspirillum seropedicae	Elbeltagy *et al.*, 2001
			Enterobacter sp.	Alam *et al.*, 2001
			Rhizobium leguminosarum bv. trifolli	
3.	Maize	Free –Living	*Burkholderia sp.*	Riggs *et al.*, 2001
		Symbiotic	*Azospirillum brasilense*	Riggs *et al.*, 2001;; Ribaudo *et al.*, 2001
			Azospirillum lipoferum	Dobbelaere *et al.*, 2001 Fages, 1994
			Herbaspirillum seropedicae	Riggs *et al.*, 2001

4. Signaling mechanism in biological nitrogen fixation

The induction of nodules harbouring nitrogen-fixing bacteria is result of complex interaction between BNF microorganism and plant. It involves several sets of genes and signals from both partners in a coordinated expression(Madsen *et al.,* 2010).

Collectively it may be possible that *NSP1/NSP2*, *NF-YA* and *ERN1* act in combination to regulate the expression of early infection markers(Smit *et al.,* 2005) such as ENOD11 with spatial and temporal patterns as shown in Figure 3. One of the gene, *NAD1* (Nodules with Activated Defense 1) encodes a novel peptide with two trans-membrane domains was highly expressed in the maintenance of rhizobial endosymbiosis in nodules (Cerri *et al.*, 2012). However the exact regulatory pathway, involved in increasing nutrient uptake yet to deciphered.

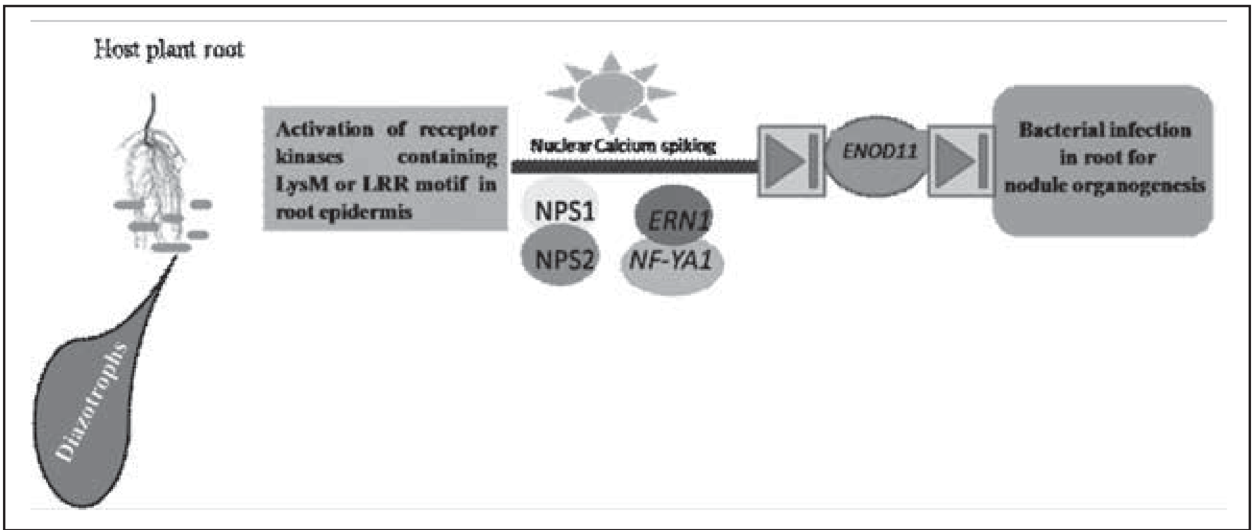

Fig. 3 Signaling cascade pathway essential for coordinating the expression of genes linked to rhizobial infection in the epidermis.

5. Different approaches for improving biological nitrogen fixation in crops

5.1 Conventional approach

5.1.1 Efficient host genotypes and strains used as fertilizer

Identification of diazotrophic bacterial population such as *Legume-Rhizobia*, *Parasponia-Bradyrhizobium* and *Actinorhiza-Frankia* symbiosis system is the fundamental key for efficient biological nitrogen fixation. However, rate of symbiotic N_2 fixation is variable with plant species/cultivar, growing season, and soil fertility. In contrast, members of *Poaceae* do not have such type of symbiotic nitrogen-fixing associations as in legume(Perez-Montano *et al.,* 2014). *Parasponia* and *Actinorhiza*. However, *Rhizobium* has capability to induce at low frequency nodule-like structures on the roots of rice and wheat upon treatment with cell wall degrading enzymes(Reddy *et al.,* 1997). The co-cutivation of *Rhizobium* and associative diazotrophs on legume nodulation could fix 600 kg of nitrogen ha^{-1} $year^{-1}$ (Hungria *et al.*, 2013). Similarly, in maize inoculated with *A. caulinodans* supplement with auxin had higher NPK content in grains and stover (Saikia *et al.*, 2006). In case of wheat when inoculated with *Azospirillium sp.*, it was observed the nitrates uptake capacity improved due to increases the root surface area. The *Azospirillum sp.* strain B510, isolated from a rice plant was shown to enhance rice growth as well as yield (Isawa *et al.*, 2010). Identification of a variety/genotype showing high compatibility with BNF strain is also a challenging task ahead. The ability of non-leguminous plants to stimulate N fixation in their rhizosphere is known as N fixation supportive (NFS) trait. Genetic variability for NFS trait exists as heritable traits and can be used in breeding cereals genotypes with high BNF. However, the genotypes with NFS traits can utilize N supply from the stimulated associative N fixation.

5.2 Molecular approaches to explore the nitrogen fixing genes

The development of transgenic tobacco in the early 1800, have opened a gateway for the development of transgenic crops with improved yield and stress tolerance. Golden rice and Bt-maize are potential example of transgenic which revolutionize in the area of cereal. Engineering nitrogen-fixing symbiosis by adapting existing signaling and developmental mechanisms to facilitate a suitable environment for nitrogenase activity in the plant nodule would be proved best solutions (Oldroyd and Dixon, 2014; Rogers and Oldroyd, 2014).

5.2.1 Molecular markers in biological nitrogen fixation

For nitrogen evaluation, nitrogenase biosynthesis and N_2 fixation, both are cumbersome processes. Thus, the expression of *nif* genes using molecular markers is the preliminary approach of validation (Schmid and Hartmall, 2007). Initially in cyano-bacterium, gene diversity was identified using nif gene probes and PCR fingerprinting using RFLP marker (Plazinski *et al.*, 1985).

Recently Rai *et al.*, 2014, demonstrated that 12 different terminal restriction fragments (TRF) were isolated using *nifH-RFLP* markers analysis from the soil samples. Construction of library is an efficient way to reveal the gene diversity of uncharacterized diazotrophs in rhizosphere. Ueda *et al.*, (1995) identified diazotrophs in rice using PCR-amplified *nif-H* sequences. The major problem using RFLP is pattern of *nif-H* gene was different under cultivation and permanent pasture within same soil sample (Poly *et al.*, 2001) which can be resolved using cluster analysis of *nifH-RFLP* profile. The study could generate the data with a small variation in cluster analysis of *nifH-RFLP* profile in soil community DNA of two species at four different stages of plant development that is correlated with the relative stability of microbial populations in marsh soil (Burke *et al.*, 2002).

Two novel endophytic rhizobial strains having dual symbiosis property (*B. cepacia* and *R. leguminosorum*) were isolated from rice root using 16S rDNA sequences. They are capable to establish PGPR with rice plants and can stimulate nodules in common bean (*P. vulgaris*) roots. It is assumed that this rhizobium strain isolated from rice transferred from the bean-nodulated rhizobium through Horizontal Gene Transfer during the course of evolution (Singh *et al.*, 2006). Besides this, the 16S rRNA is a good sign of molecular marker due to its highly conserved function and ubiquitous distribution. The sequence of 16S rRNA varies from highly conserved to highly variable region. By studying the 16S rRNA sequence of cyanobionts, a single coralloid root of *Cycas revoluta* harbor with more than two cyanobacterial strains and in multiple roots from a single plant diversity was also observed (Gheringer *et al.*, 2010; Yamada *et al.*, 2012).

6. Genomic regions/QTL for nitrogen fixation

Important root architectural traits like root length, diameter, surface area and volume, presence of root hairs and nodulation traits which play key role in BNF are known to be genetically controlled by multiple genes or genomic regions referred to as quantitative trait loci (QTLs). Even though few QTLs have been reported to be playing a dominant effect on one trait, most have been found to have influence on many traits. The identification of major QTLs for these key BNF influencing traits will be an important objective of genetic research and breeding programs aimed at enhancing BNF in cereals. RIL population (157 F2:7) and 105 SSR markers have used to carry out a composite interval mapping and identified two QTLs for shoot dry weight, three QTLs for nodule number and one QTL for nodule dry weight, all QTLs were found to have small effect explained 15.4%, 13.8% and 6.5% of total variation for these three traits respectively (Santos *et al.*, 2013). In *Lotus japonicas*, using a RIL population 34 QTLs controlling key BNF traits such as acetylene reduction activity (ARA) per plant, ARA per nodule weight, ARA per nodule number, nodule number per plant, nodule weight per plant etc. were identified and mapped (Akiyoshi *et al.*, 2012). A novel nitrogen-dependent gene *Ndhrl1* was isolated from wheat and mapped it to the short arm of chromosome 2B which is associated with the lesion mimic trait. This putative gene was further delimited into an interval of 8.1cM flanked by the CAPS/dCAPS markers 7hrC9 and 7hr2dc14 (Li *et al.*, 2016). Similar studies could be of great importance in cereals, for identification of contrasting genotypes, which support BNF, is the first and foremost step in developing mapping populations and further mapping of QTLs.

7. Engineering symbiotic nitrogen fixation

Replacing nitrogen fertilizer globally would require nitrogen fixation in cereals equivalent to the legumes, and it would be extremely challenging. To introduce a symbiosis system in cereals some essential genetic changes would be introduced such as recognition of Nod factors, organogenesis of the root nodule, and establishment of a suitable environment for nitrogenase activity inside the nodule (Curatti *et al.*, 2014). One potential criticism to transfer the legume symbiosis into cereals is the yield penalty associated with the increased demand on photosynthesis required to support nitrogen fixation. There are multiple biotechnological approaches currently being explored that could deliver fixed nitrogen to cereal crops (Oldroyd and Dixon, 2014; Beatty and Good, 2011). Recently a key element that facilitates the movements of calcium in plants was identified which signals to the nitrogen-fixing bacteria and stimulates the development of nodules on roots (John *et al.,* 2007). It has also been reported that the Nod factors are similar to Myc factors (mycorrhizal symbiosis) may leads to the activation of a common symbiosis signaling (SYM) pathway (Maillet *et al.,* 2011). The direct transfer of nitrogen fixation (*nif*) genes into non-legumes has also become more feasible especially six out of the numerous *nif* genes are required for FeMo-Co biosynthesis and nitrogenase activity. Wheat plants inoculated with *nif-H* mutant of *Klebsiella pneumonia* grown in N-deficient media showed unhealthy plant growth in comparison to wild type *K. pheumonia*-inoculated plants (Iniguez *et al.*, 2004). Thus, *nif-H* gene play major role in biological nitrogen fixation and this could be complemented if *nif-H* gene gets possibly transformed in wheat.

8. Future prospects

Biological nitrogen fixation has the potential to reduce chemical fertilizer use thereby greatly alleviating the environmental impact. However, replacing chemical fertilizer would require optimum levels of nitrogen fixation in cereals, which is extremely challenging.

- To improve the N_2-fixation capacity of the cereals through selection and breeding. It would be better if breeding for plant varieties, which are more successfully exploited by strains or already present in soil used as inoculants (Streeter, 1988).
- Lack of reliable techniques for measuring nitrogen fixation in the field is one of the major methodological constraints. If non-fixing genetic isolines or some kind of indicator plant are available, then nitrogen difference method could be easily captured.
- Engineering nitrogen-fixing symbiosis in cereals, either through transferring the legume-rhizobial interaction or by improving pre-existing associations in cereal roots. The nitrogenase enzyme itself could be introduced into organelles of plant cells to create a new nitrogen-fixing capability in cereals.
- Establishing highly efficient transformation procedures in cereals or finding ways to transiently express gene constructs in cereals.
- Indian Council of Agriculture Research (ICAR) launched a combined programme with UK at ICAR-Indian Institute of Soil Science, Bhopal for development of nitrogen-fixation in cereals. The group is also working on rice, which would be more dependent on BNF and reduces the chemical N fertilizer requirement as one of the main objective of this collaboration. The ICAR has also taken initiative in this area in a coordinated project mode involving 11 centers located all over India(Incentinizing Research Agriculture).

Acknowledgement

Authors acknowledge the grant received from "Incentinizing Research in Agriculture- BNF" (No. 1007161) of Indian Council of Agricultural Research, New Delhi. This paper is ICAR-IIWBR contribution no. 138/26.

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