Biotechnological approaches to develop nitrogen-fixing cereals: A review

Asma Boujenna¹ and Luis F. Garcia del Moral²

¹ Université Abdel Malek Essaadi, Faculté des Sciences, Dept. de Biologie, Av. Sebta, Tétouan 93002, Morocco. ² University of Granada, Faculty of Sciences, Institute of Biotechnology, Dept. of Plant Physiology, Avda. Fuentenueva s/n, 18075 Granada, Spain.

Abstract

Agricultural yields are often limited by nitrogen (N) availability, especially in countries of the developing world, whereas in industrialized nations the application of chemical N fertilizers has reached unsustainable levels that have resulted in severe environmental consequences. Finding alternatives to inorganic fertilizers is critical for sustainable and secure food production. Although gaseous nitrogen (N₂) is abundant in the atmosphere, it cannot be assimilated by most living organisms. Only a selected group of microorganisms termed diazotrophs, have evolved the ability to reduce N₂ to generate NH₃ (N-fixing bacteria) or the nitrogenase enzyme responsible for N fixation. This review explores three potential approaches to obtain N-fixing cereals: (a) engineering the nitrogenase enzyme to function in plant cells; (b) engineering the legume symbiosis into cereals; and (c) engineering cereals with the capability to associate with N-fixing bacteria.

Additional key words: biological nitrogen fixation; nitrogenase; nif genes; O; tolerance; plastids; mitochondria; plant growth-promoting rhizobacteria.

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Correspondence should be addressed to Luis F. Garcia del Moral: lfgm@ugr.es

Introduction

Human population is dramatically increasing. The current world population of 7.6 billion is projected to reach more than 9 billion in 2050 (UN-DESA, 2017) and consequently demand for food is rising. On a global scale, up to 70% of mankind’s food depends directly on cereals, mainly rice, wheat, corn and sorghum. According to Food and Agriculture Organization of United Nations (FAO), food production must increase by 70% to feed this larger population, which means an increase of 44 million tons/year; that is, a 38% increase over the historical average since there are records of agricultural productivity (FAO, 2020).

Nitrogen (N) availability is one of the main limiting factors for crop yield, especially in developing countries, where small farmers often do not have access to inorganic fertilizers. In contrast, the application of chemicals in developed nations has allowed high agricultural production, but has led to accumulation of inorganic nutrients in agricultural soils. Typical N-use efficiencies for wheat, rice, and maize indicate that more than 50% of N applied in fertilizers is lost to the environment (Lahda et al., 2016; Anas et al., 2020), either in the form of nitrous oxides, which are potent greenhouse gases, or as soluble nitrates, which causes eutrophication of aquatic systems and problems for human health due to their accumulation in ground water (Galloway & Cowling, 2002; Townsend et al., 2003; Glendining et al., 2009; Erisman et al., 2015; Stokstad, 2016). Globally, almost 100 million tons of N is deposited every year in terrestrial, freshwater and marine environments, i.e., a three-fold increase over preindustrial levels (Galloway et al., 2008; Rockström et al., 2009). Nutrient excesses are especially large in China, northern India, the USA, and Western Europe, leading to widespread nutrient pollution (Foley et al., 2011).

The use of N fertilizers obtained through the Haber-Bosch process dominates the fertilization of crops on a global scale, producing more than 100 million tons of nitrogen fertilizer per year, but it consumes a great quantity of energy. In fact, the energy-intensive production of nitrogen fertilizers is the greatest consumption of fossil
fuels in agriculture, with predictions that this process will consume around 2% of global energy by 2050 (Glendinning et al., 2009). For this reason, considering the future menaces of a decline in petroleum reserves and the low efficiency with which cereal crops use chemical nitrogen fertilizers, the search for new alternative sources of nitrogen to reduce agricultural reliance on nitrogen fertilizers is an urgent need. In the past decades one of the strategies to improve assimilation and use efficiency of nitrogen in cereals has been focused on overexpressing important genes for nitrate and ammonium transporters in the roots of rice, maize and wheat, although with different success (reviewed in Li et al., 2020). In the case of cereal crops under Mediterranean conditions, it has been shown (Ramos et al., 1989; García del Moral et al., 1999) that a foliar application of elemental sulfur could reduce the need for nitrogen fertilizer by 25%, since SO$_2$ generated would increase the methionine content and in turn the formation of ethylene, thus favoring the survival of a greater number of ears/m$^2$ and therefore increasing the grain yield.

Although atmospheric nitrogen represents 78% of the air, due to the stability of the triple bond between the two nitrogen atoms, N$_2$ is inaccessible to eukaryotes, since only a group of bacteria and archaea (called diazotrophos organisms) have developed the ability to fix N$_2$ organic through the enzyme nitrogenase. This biological nitrogen fixation (BNF) is the major contributor to the N economy of the biosphere, accounting for 30–50% of the total N in crop fields (Ormeño-Orrillo et al., 2013) and represents a promising substitute for chemical N fertilizers (Olivares et al., 2013; Dent & Cocking, 2017; Good, 2018). BNF is thought to be one of the most ancient enzyme-catalyzed reactions (Raymond et al., 2004) and depending on their way of life, diazotrophos organisms can be divided into three groups (Table 1): (1) free living; (2) symbiotic, mainly bacteria living within plant root nodules; and (3) those that live in associative or endophytic relationships with other organisms.

There are three biotechnological approaches currently being explored that could deliver fixed N from these diazotrophos organisms to cereal crops (Beatty & Good, 2011; Oldroyd & Dixon, 2014; Burén & Rubio, 2018). One option focuses on directly engineering the nitrogenase enzyme into organelles of plant cells to create a new N-fixing capability. This is an attractive solution, but it would need solving two great challenges. Firstly, the nitrogenase is a highly complex enzyme and would require the coordinated expression in the plant cell of at least 16 $N_2$-fixation ($nif$) genes (Temme et al., 2012; Li & Chen, 2020). Secondly, nitrogenase activity has high energetic demand and is irreversibly denatured by oxygen from photosynthesis (Seefeldt et al., 2009; Curatti & Rubio, 2014). Thus, expressing functional nitrogenase in chloroplasts requires temporal (day/night) separation of photosynthesis and N fixation by confining $nif$ gene expression only to dark periods (nights) or, alternatively, by spatially restricting $nif$ gene expression to non-photosynthetic tissues such as the cereal roots.

Legumes have evolved the capability to associate with N-fixing bacteria, which are housed inside nodules on its roots and this ability offers the second possible scenario, i.e., engineering a N-fixing symbiosis in cereal roots through transferring the legume-rhizobial interaction. However, the process of N fixation through symbiosis is very complex, involving multiple events and their regulation in both the host and the rhizobia Therefore, engineering a N-fixing symbiosis will requires adapting existing signaling and developmental mechanisms to provide a suitable environment for nitrogenase activity in the new cereal nodules (Oldroyd & Dixon, 2014; Burén & Rubio, 2018).

### Table 1. Several types of atmospheric nitrogen-fixing organisms

| SYMBIOTIC ORGANISMS | Host plant |
|----------------------|------------|
| Rhizobium, Sinorhizobium, Bradyrhizobium, Azorhizobium, Mesorhizobium | Leguminous, legumes, Parasporia |
| Frankia | Actinorrhizal: alder, Casuarina, Datisca |
| Nostoc | Gunnera |
| Anabaena | Water fern (Azolla) |
| Acetobacter | Sugarcane |
| Azospirillum | Miscanthus |
| FREE-LIVING ORGANISMS | Genera |
| Cyanobacteria | Anabaena, Calothrix, Nostoc |
| Aerobic bacteria | Azospirillum, Azotobacter, Beijerinckia, Dersia |
| Facultative bacteria | Bacillus, Klebsiella |
| Non-photosynthetic anaerobic bacteria | Clostridium, Methanococcus |
| Photosynthetic bacteria | Chromatium, Rhodospirillum |
A third approach could involve the enhancement of naturally occurring plant-associated diazotrophs by generating strains that release fixed N to benefit the crop (Beatty & Good, 2011; Geddes et al., 2015; Stokstad, 2016; Mus et al., 2016). Manipulation of soil diazotrophs can potentially provide a short-term solution to reduce the use of synthetic N fertilizers in agriculture.

This review provides comprehensive and updated information on these different approaches to reducing the N fertilizers demand through improving BNF: (1) direct transfer of bacterial nif genes for expressing heterologous nitrogenase in cereals; (2) engineering new symbioses between cereals and N₂-fixing bacteria in a similar form to the legume–rhizobium symbiosis; and (3) improvement of N₂-fixing bacterial endophytes naturally associated to cereals.

The nitrogenase enzymatic complex

The molybdenum nitrogenase is a complex enzyme consisting of two proteins (Seefeldt et al., 2009; Hu & Ribbe, 2015; Addo & Dos Santos, 2020) (Fig. 1). The dinitrogenase reductase or NifH protein, also known as the Fe protein, is a homodimer of the nifH gene product that contains one Mg·ATP-binding site in each subunit. The catalytic dinitrogenase component or NifDK, also termed as the MoFe protein, is a heterotetramer of the nifD and nifK gene products. Fe protein accepts electrons from reduced flavodoxin II and acts as obligate electron donor to MoFe protein, whereas substrate reduction takes place at the FeMo-co inside each MoFe subunit. The catalytic process requires 16 moles of ATP for every mol of dinitrogen gas that is converted to 2 moles of ammonia, as well as reduction equivalents that are supplied by the reduced ferredoxin (Seefeldt et al., 2012; Curatti & Rubio, 2014; Taiz et al., 2015; Nag et al., 2019) (Fig. 1). However, the real cost is estimated to be 20–30 ATP, accounting for the production of the nitrogenase complex, the reductive power, and recycling the toxic dihydrogen waste resulting from the process (Lodwig & Poole, 2003).

Nitrogenase contains three metalloclusters. One of them, the iron-molybdenum cofactor (FeMo-co), located within the MoFe protein, provides the catalytic site for N reduction and contains molybdenum, iron, sulphur, a central carbon atom and homocitrate as an organic compound. FeMo-co is one of the most complex heterometallic groups known in biology and its biosynthesis requires several nif products. The other two metalloclusters are a single [4Fe-4S] cluster located at the subunit interface of the Fe protein and one P-cluster at MoFe protein. These metalloclusters are required for inter-protein and intra-protein electron transfer and reduction of N₂, in a process that is energetically coupled to Mg·ATP hydrolysis (Seefeldt et al., 2012; Hu & Ribbe, 2015; Curatti & Rubio, 2014; Sickerman et al., 2017). Both Fe protein and MoFe protein are irreversibly inactivated by oxygen (Fig. 1). Although all diazotrophs studied to date contain the molybdenum-dependent nitrogenase, a subset of diazotrophs also have alternative nitrogenases, namely vanadium dependent and iron-only nitrogenases, as recently revealed by genomic analysis (Addo & Dos Santos, 2020).

Engineering nitrogenase biosynthesis in eukaryotic cells

This strategy involves transfer of prokaryotic nif genes so that the eukaryotic cell synthesizes its own N₂-fixing machinery without the need for bacterial interactions. However, this approach faces three major difficulties: (a) the complexity and fragility of nitrogenase biosynthesis; (b) the high sensitivity to O₂ of nitrogenase and many of the accessory proteins and metal clusters needed for maturation of the nitrogenase components; and (c) the
The genetics of N fixation was initially elucidated in the model N₂-fixing bacteria *K. oxytoca*, where the *nif* genes required for the synthesis of nitrogenase are clustered in a 24 kb region of the chromosome (Arnold et al., 1988). In this bacterium, in addition to the *nifH*, three additional *nif* genes are required to assemble a functional NifH protein: *nifU*, *nifS* and *nifM*. The *nifU* and *nifS* gene products constitute an [Fe-S] cluster assembly machinery specialized in synthesizing clusters for the nitrogenase component proteins, whereas the *nifM* gene encodes a peptidyl-prolyl cis-trans isomerase required for NifH maturation. The synthesis of the functional NifDK protein requires, in addition to the *NifD* and *NifK* gene products, the generation and proper assembly of the FeMo-co metallocluster, which is known to occur outside of apo-NifDK (Dixon & Kahn, 2004; Curatti & Rubio, 2014; Nag et al., 2019; Li & Chen, 2020). To achieve complete FeMo-co synthesis, it is necessary to express, at a minimum, the *nifB*, *nifE*, *nifN*, and *nifH* genes whose products are required for the synthesis of the Fe-S core of FeMo-co. The Nif proteins that are required to assemble FeMo-co are NifU and NifS, which provide two pairs of [4Fe-4S] clusters to the NifDK polypeptides, and the NifH protein that drives the reductive coupling of each pair of [4Fe-4S] clusters to form the P-clusters (Dixon & Kahn, 2004; Curatti & Rubio, 2014; Li & Chen, 2020). Table 2 lists the different known genes involved in the conformation of the nitrogenase enzyme complex and its function in *Klebsiella oxytoca*. The genetic components and arrangement of *nif* genes vary greatly across the diverse range of diazotrophs reflecting the environmental niche and physiology of the particular organisms (Li & Chen, 2020; Jiang et al., 2021). The rapid expansion of microbial genome sequencing in the last few years has offered novel opportunities to reexamine the distribution of N fixation genes among the known diazotrophs and for the identification of a minimum gene set for N fixation. Thus, in a review of 1200 genomes of different species of bacteria and archaea with fully sequenced genomes, Dos Santos et al. (2012) have found that nearly all known diazotrophs contain a minimum of six conserved genes: *nifH*, *nifD*, *nifK*, *nifE*, *nifN*, and *nifB*. The co-occurrence of these six *nif* genes, known to be essential for N fixation in characterized systems, has led to these authors to propose this minimum gene set as an *in silico* tool for the identification both of known diazotrophs as those that could be identified in new research.

Another problem added to the complexity of engineering nitrogenase expression is the level and timing of expression for each individual *nif* gene transferred into the plant cell. Nitrogenase is a very slow enzyme, and a N₂-fixing cereal plant might require the accumulation of considerable amounts of NifH and NifDK component proteins. In fact, for N fixation up to 20% of the total proteins are dedicated to nitrogenase production and maturation (Batista & Dixon, 2019). This is a burden for the cell, diverting energy from other pathways, causing oxidative stress and excess of intracellular NH₄⁺ which can be toxic to the plant cell. However, this can be controlled by expressing the *nif* genes with the help of heterologous promoters and manipulation or fine tuning of the NifL–NifA regulators (Dixon & Kahn, 2004; Curatti & Rubio, 2014; Li & Chen, 2020).

The transfer of *nif* genes to engineering the nitrogenase complex in eukaryotes was initially studied in yeasts (a

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**Table 2.** The different known genes involved in the conformation of the nitrogenase enzyme complex and its function in *Klebsiella oxytoca* (formerly *Klebsiella pneumoniae*).

| Function(s) | NifH (Fe protein) | NifDK (MoFe protein) | 4Fe4S | P-cluster | FeMO-co |
|-------------|-------------------|----------------------|-------|-----------|---------|
| Gene(s) involved in structural components | nifH | nifD, nifK | nifU, nifS, nifD | nifH | nifY, nifE, nifN, nifX, nifV, nifB, nifQ |
| Chaperones involved in maturation | nifM | nifY, nifZ, nifH | nifH | nifH |
| Cofactors involved in biosynthesis | nifU, nifS | nifU, nifS, nifH, nifB, nifE, nifN, nifV, nifQ |
| Proteins involved in electron transfer | nifF, nifJ | nifH | nifK |

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non-photosynthetic organism), where has it been possible to engineer various genes nif from Azotobacter vinelandii to generate an active Fe protein (López-Torrejón et al., 2016) and the successful formation of NifDK tetramer (Burén et al., 2017b), essential first steps in assembling a functional nitrogenase in an eukaryotic cell. However, although all the Nif components have been successfully expressed in yeast cells, the formation of a fully functional nitrogenase complex has not yet been achieved (López-Torrejón et al., 2016; Burén et al., 2017a; Pérez-Gonzalez et al., 2017). In higher plants there are some attempts to express nif genes in chloroplasts and mitochondria. Thus, Ivleva et al. (2016) introduced the nifH gene together with nifM from K. oxytoca into chloroplasts of tobacco plants, generating functional NifH protein, although with low activity. Similarly, Allen et al. (2017) demonstrated the viability of expressing the complete range of biosynthetic and catalytic nitrogenase Nif as mitochondrial proteins in tobacco leaves. Table 3 shows some of the attempts to engineering the nif genes in various eukaryotes organisms.

**Engineering nitrogenase for prevent its inactivation by oxygen**

Regarding the sensitivity of nitrogenase to O₂, it is important to note that filamentous cyanobacteria have been able to reconcile oxygenic photosynthesis and N₂ fixation either by spatial or by temporal separation. Spatial separation is achieved by expressing nitrogenase exclusively inside specific cells termed heterocysts without Photosystem II and therefore O₂ is not generated during photosynthesis (Flores & Herrero, 2010). Temporal separation in cyanobacteria is achieved through control by the circadian rhythm (Chen et al., 1998).

In the plant cell the possible subcellular locations for the nitrogenase proteins are plastids and mitochondria (Liu et al., 2018). The advantages of nif gene expression in plastids are: (a) they possess the prokaryotic-type transcription and translation machineries that allow the use of bacterial promoters and gene clusters in the form of operons; (b) the high levels of gene expression and protein accumulation that can be achieved due to the high degree of ploidy of the chloroplastid genome and (c) the local production of ATP and reducing power required for nitrogenase function (Dixon et al., 1997; Scharff & Bock, 2014; Liu et al., 2018). However, a major disadvantage would be the energy costs of synthesizing nitrogenase proteins every night, as photosynthetic O₂ will result in their irreversible inactivation during the day (Scharff & Bock, 2014).

The knowledge that eukaryotic mitochondria harbor machineries for [Fe-S] cluster assembly highly similar to those involved in the early steps of nitrogenase metallocluster biosynthesis, has recognized these organelles as possible candidates for the transfer of nif genes to the plant cell (Allen et al., 2017; Liu et al., 2018). One of the reasons why the [Fe-S] cluster assembly machinery

| Gene(s) | System | Targeting | Diazotrophic donor | Reference(s) |
|---------|--------|-----------|-------------------|--------------|
| nifH, nifM | Saccharomyces cerevisiae | mitochondria | Azotobacter vinelandii | Lopez-Torrejón et al., 2016; Burén et al., 2017b; Perez-Gonzalez et al., 2017 |
| nifH, nifM | Nicotiana tabacum | mitochondria | A. vinelandii | Ivleva et al., 2016 |
| nifH, nifK, nifD | N. benthamiana | mitochondria | Klebsiella pneumoniae | Allen et al., 2017 |
| nifDK, nifM, nifY, nifZ, nifU, nifE, nifN, nifX, nifQ, nifJ | Chlamydomonas reinhardtii | chloroplast | K. pneumoniae | Cheng et al., 2005 |
| nifD, nifK, nifE | S. cerevisiae | mitochondria | A. vinelandii | Burén et al., 2017b; Perez-Gonzalez et al., 2017 |
| nifU, nifS | S. cerevisiae | mitochondria | A. vinelandii | Lopez-Torrejón et al., 2016; Burén et al., 2017a,b; Perez-Gonzalez et al., 2017 |
| nifB, | S. cerevisiae | mitochondria | A. vinelandii | Burén et al., 2017a,b; Perez-Gonzalez et al., 2017 |
| nifX, nifV, nifQ, nifJ | S. cerevisiae | mitochondria | A. vinelandii | Perez-Gonzalez et al., 2017 |
operates in the mitochondrial matrix seems because respiration results in O₂ depletion inside this organelle, allowing thus the biosynthesis of O₂-sensitive proteins. In addition to respiratory protection, mitochondria can provide the ATP and reducing power required for nitrogenase catalytic activity (Dixon & Kahn, 2004; Scharff & Bock, 2014; Liu et al., 2018). However, the current lack of an efficient system for in vivo transformation of mitochondria is currently a major obstacle to the transfer of nif genes to these organelles. The previous considerations exemplify the complexity of the introduction and expression of N fixation in aerobic plant cells.

**Engineering legume symbiosis in cereals**

A number of species of plants, most notably legumes, have evolved the ability to form intimate N-fixing symbioses with diazotrophs and form specialized organs (nodules). The best studied plant endosymbiosis are those between legumes and rhizobia. Through this mutualistic relationship, plant cells provide nutrients to the bacteria in exchange for ammonia produced by nitrogenase and provide a suitable oxygen-limited environment for N fixation, because inside the nodule the oxygen concentration is maintained at 20 to 40 nM, levels that can support respiration but are sufficiently low to avoid inactivation of nitrogenase (Oldroyd & Dixon, 2014). The most abundant proteins in nodules are oxygen-binding heme proteins called leghemoglobins with a high affinity for oxygen. Leghemoglobins provide a buffer for nodule oxygen inside the nodule and participate to increasing the rate of oxygen transport to the respiring symbiotic bacterial cells (Larraínzar et al., 2020).

The establishment and functioning of this effective symbiosis is dependent on genetic determinants in both plant and bacteria (Oldroyd et al., 2011; Goyal et al., 2021). Accurate mutual recognition in legume-rhizobia systems is accomplished by exchange of biochemical signals. This signaling, the subsequent infection process, and the development of N-fixing nodules involve specific genes in both the host and the rhizobia (Rolfe & Gresshoff, 1988; Oldroyd et al., 2011; Ibañez et al., 2017; Oldroyd & Poole, 2019; Goyal et al., 2021). Plant genes specific to nodules are called nodulin genes and rhizobial genes that participate in nodule formation are named nodulation (nod) genes. The nod genes are classified as common nod genes or host-specific nod genes. The common nodA, nodB, nodC, and nodD genes are found in all rhizobial strains, whereas the specific-host genes nodP, nodQ, nodE, nodE or nodL differ among rhizobial species and determine the host range of legumes that can be infected. Only one of the nod genes, the regulatory nodD is constitutively expressed and its protein product NodD regulates the transcription of the other nod genes (Oldroyd et al., 2011; Goyal et al., 2021). The plant signals that induce nod gene expression are commonly flavonoids and isoflavonoids (e.g., the flavone 7,4 dihydroxyflavone and the isoflavone genistein) and betaines secreted by the roots. These attractants activate the rhizobial NodD protein (activator) which then induces transcription of the other nod genes. The nod genes, which NodD activates, code for nodulation proteins involved in the biosynthesis of lipochitin oligosaccharides or nod factors, molecular signals that triggered nodulation-related changes in the host plant (Oldroyd & Dixon, 2014; Pankievicz et al., 2019; Dong & Song, 2020; Goyal et al., 2021). This activates a downstream gene cascade including those involved in nucleoporin, cation channels, early nodule expression, and cytokinin signaling leading to cortical and pericyclic cell divisions. Rhizobia are then entrapped by root hair curling after the Nod factors has been perceived, which results in initiating the formation of infection thread (a tubular structure) that facilitates the penetration of bacteria into root hair and adjacent cortical cells (Venado et al., 2020; Tsyganova et al., 2021).

The fact that most land plants, including cereals, can form arbuscular mycorrhizal associations but are unable to form root nodules that can fix N, could be a way to transfer symbiotic N fixation to non-legume plants. In fact, recent phylogenomic studies suggest that a small set of genes could convert a species in association with arbuscular mycorrhizal fungi into a N-fixing symbiont (Griesmann et al., 2018; Van Velzen et al., 2018). Interestingly, several components of the legume symbiotic signaling (SYM) pathway also play a role in the arbuscular mycorrhizal symbiosis. The key symbiotic signals produced both by rhizobia (Nod factors) and arbuscular mycorrhizal fungi (Myc factors) are lipochitin oligosaccharides in a process that involve related signaling pathways and receptors (Dénarié et al., 1996; Froussart et al., 2016). Since cereals contain the signaling pathway for arbuscular mycorrhizal associations, nodulation could be established in them by engineering the perception of rhizobial signaling molecules to activate this recognition pathway and to form in roots an oxygen-limited nodule for N fixation (Mus et al., 2016; Oldroy & Poole, 2019). However, to achieve such synthetic N-fixing symbiosis there are several factors that must be taken into account (Oldroyd & Dixon, 2014; Mus et al., 2016): (1) optimization of the colonization process; (2) engineering of symbionts synthetic nif clusters optimized for N fixation; (3) engineering of respiratory protection and O₂-binding proteins to allow aerobic N fixation by symbionts; (4) conditional suppression of ammonium assimilation by symbionts to ensure adequate N delivery to plants; (5) ensured effective uptake of ammonium by plant cells; and (6) optimization of carbon supply from root cells to endosymbiotic bacteria. From the point of view of genetic engineering the main factors lies with the challenge to control the expression of multigene systems
Improvement of N₂-fixation through bacterial endophytes naturally associated to cereals

In this strategy, plant-growth-promoting rhizobacteria (PGPR) with beneficial effects on plant development and already naturally associated with cereals are modified to improve their colonization ability, density, N₂-fixing capabilities and release of NH₃ produced to plant cells (Stoltzfus et al., 1997; Savka et al., 2002; Santi et al., 2013; Ryu et al., 2020). Such PGPR can be loosely associated in close proximity to the plant root or invade and spread within the plant tissue as endophytic diazotrophs (Santi et al., 2013; Mus et al., 2016). These last may have an advantage over root-surface associative diazotrophs, as they colonize the interior of plant roots and can establish themselves in niches that provide more appropriate conditions for effective N fixation and subsequent transfer of the fixed N to the host plant (Reinhold-Hurek & Hurek, 2011). In wheat, rice and maize, various species of the genera Azorarcus, Acetobacter, Pseudomonas, Azospirillum, Glucenobacter and Azotobacter have been identified as PGPR (Santi et al., 2013; Ryu et al., 2020) exerting significant effects in increasing plant height, root length and dry-matter production not only through N fixation, but also through other physiological effects on the growth and development of the host plant (Rosenblueth & Martinez-Romero, 2006; Saharan & Nehra, 2011; Aasfar et al., 2021). Indeed, most of PGPR produces the auxin indole-3-acetic acid (IAA) together with cytokinins such as isopentyladenosine ([9R]iP), two hormones that stimulate root branching and root elongation which in turn favour the uptake of soil water and minerals with a positive effect on plant growth (Baca & Elmerich, 2007). Other effects of PGPR include the production of siderophores that may stimulate plant growth directly by increasing the availability of iron in the soil surrounding the roots, phosphate solubilization and acquisition of other nutrients like calcium, potassium, iron, copper, magnesium, and zinc (Richardson et al., 2009; Pérez-Montaño et al., 2014; Fukami et al., 2018; Pankieievicz et al., 2019). However, since the population density of endophytic bacteria in plant tissues is too low to support adequate N fixation, it is important to design systems that aid greater colonization of diazotrophic endophytes, for instance, by engineering plants to produce a specific metabolite and thus create a “biased rhizosphere” to favor the growth of an introduced diazotroph able to use the novel metabolite (Rosenblueth et al., 2018; Nag et al., 2019). An interesting finding is that some diazotrophs, including Herbaspirillum species, living in mucilage released from the aerial roots of maize landraces from Sierra Mixe, Mexico, can provide up to 82% of the host N at a critical period of the growing season (Van Deynze et al., 2018).

This strategy will involve the identification of appropriate plant and bacterial signals, receptors, and target genes. Rhizopines are a rare group of compounds produced by a few species of rhizobia inside legume nodules and are exuded into the rhizosphere. Among them, the scyllo-inosamine 1 (SIA) and 3-O-methyl-scyllo-inosamine 2 (3-O-MSI) are believed to be suitable as chemical signals between plants and rhizosphere bacteria (Murphy et al., 1995; Gordon et al., 1996; Savka et al., 2013; Goyal et al., 2021) and recently has been reported the successfully transfer of rhizopine biosynthesis genes into barley (Geddes et al., 2019). A step forward in this strategy consists of plants engineered to release an orthogonal chemical signal (as nopaline or octopine) that could be sensed only by a corresponding engineered bacterium with the appropriate sensors, which would have the added benefit of only inducing nitrogenase in the presence of the engineered crop (Ryu et al., 2020).

Conclusions

N₂-fixing cereals would be an enormous biotechnological challenge that might revolutionize world agricultural systems. The most important difficulties involved in the direct transfer of bacterial nif genes into the cereal are the sensitivity of nitrogenase to O₂ and the complexity and fragility of nitrogenase biosynthesis. Nitrogen fixation is a highly energy demanding process, and so chloroplasts and mitochondria are envisaged as potential subcellular locations sites for N fixation since they can meet the energy requirements for nitrogenase, each one showing advantages and disadvantages. Because nitrogenase is extremely sensitive to oxygen evolved by chloroplasts during photosynthesis, expression of functional
nitrogenase in chloroplasts requires temporal (day/night) separation by confining nif gene expression only to dark periods (nights) or, alternatively, by spatially restricting nif gene expression to non-photosynthetic tissues such as the roots. The possibility of developing legume-like root-nodule symbioses in cereals arises from contemporary knowledge that cereals contain the signaling pathway to form arbuscular mycorrhizal associations with diverse diazotrophic rhizobia. However, there is still insufficient knowledge about the genetics and microbiology involved in the formation of an oxygen-limited nodule for N fixation in cereal roots. An alternative option is the use of mixed PGPR and N-fixing bacteria to develop cereals with improved root growth and better exploitation of environmental and nutritional resources. To achieve this, it is critical to design systems that aid greater colonization of diazotrophic endophytes to improve the chances that the inoculated diazotroph will selectively colonize the crop plant, because N fixation is highly variable depending on the associated diazotroph and the plant variety, Batista MB, Dixon R, 2019. Manipulating nitrogen regulation in diazotrophic bacteria for agronomic benefit. Biochem Soc Trans 47: 603-614. https://doi.org/10.1042/BST20180342
Beatty PH, Good AG, 2011. Future prospects for cereals that fix nitrogen. Science 333: 416-417. https://doi.org/10.1126/science.1209467
Burén S, Jiang X, López-Torrejón G, Echavarrí-Erasun C, Rubio LM, 2017a. Purification and in vitro activity of mitochondria targeted nitrogenase cofactor maturase NifB. Front Plant Sci 8: 1-16. https://doi.org/10.3389/fpls.2017.01567
Burén S, Young EM, Sweeney EA, Lopez-Torrejon G, Veldhuizen M, Voigt CA, Rubio LM, 2017b. Formation of nitrogenase NifDK tetramers in the mitochondria of Saccharomyces cerevisiae. ACS Synth Biol 6: 1043-1055. https://doi.org/10.1021/acssynbio.6b00371
Burén S, Rubio LM, 2018. State of the art in eukaryotic nitrogenase engineering. Microbiol Lett 365 fnx274. https://doi.org/10.1093/femsle/fnx274
Chen YB, Dominic B, Mellon MT, Zehr JP, 1998. Circadian rhythm of nitrogenase gene expression in the diazotrophic filamentous non heterocystous cyanobacterium Trichodesmium sp. strain IMS 101. J Bacteriol 180: 3598-3605. https://doi.org/10.1128/JB.180.14.3598-3605.1998
Cheng Q, Day A, Dowson-Day M, Dixon R, 2005. The Klebsiella pneumoniae nitrogenase Fe protein gene (nifH) functionally substitutes for the chlL gene in Chlamydomonas reinhardtii. Biochem Biophys Res Commun 329: 966-975. https://doi.org/10.1016/j.bbrc.2005.02.064
Curatti L, Rubio LM, 2014. Challenges to develop nitrogen-fixing cereals by direct nif-gene transfer. Plant Sci 225: 130-137. https://doi.org/10.1016/j.plantsci.2014.06.003
Dénarié J, Debelle F, Prome J-C, 1996. Rhizobium lipochitooligosaccharide noduleation factors: signaling molecules mediating recognition and morphogenesis. Ann Rev Biochem 65: 503-535. https://doi.org/10.1146/annurev.bi.65.070196.002443
Dent DR, Cocking EC, 2017. Establishing symbiotic nitrogen fixation in cereals and other non-legume crops: The greener nitrogen revolution. Agri Food Secur 6: 7. https://doi.org/10.1186/s40066-016-0084-2
Dixon RA, Postgate JR, 1971. Transfer of nitrogen-fixation genes by conjugation in Klebsiella pneumoniae. Nature 234: 47-48. https://doi.org/10.1038/234047a0
Dixon R, Kahn D, 2004. Genetic regulation of biological nitrogen fixation. Nat Rev Microbiol 2, 621-631. https://doi.org/10.1038/nrmicro954
Dixon R, Cheng Q, Shen GF, Day A, Dowson-Day M, 1997. Nif gene transfer and expression in chloroplasts:

References
Aasfar A, Bargaz A, Yaakoubi K, Hilali A, Bennis I, Zeroual Y, Kadmiri I M, 2021. Nitrogen fixing Azotobacter species as potential soil biological enhancers for crop nutrition and yield stability. Front Microbiol 12: 628379. https://doi.org/10.3389/fmicb.2021.628379
Addo MA, Dos Santos PC, 2020. Distribution of nitrogen-fixation genes in prokaryotes containing alternative nitrogenases. Chem BioChem 21: 1749-1759. https://doi.org/10.1016/cbic.202000022
Allen RS, Tilbrook K, Warden AC, Campbell PC, Rolland V, Singh SP, Wood CC, 2017. Expression of 16 nitrogenase proteins within the plant mitochondrial matrix. Front Plant Sci 8: 287. https://doi.org/10.3389/fpls.2017.00287
Anas M, Liao F, Verma KK, Sarwar MA, Mahmood A, Chen ZL, et al., 2020. Fate of nitrogen in agriculture and environment: agronomic, eco-physiological and molecular approaches to improve nitrogen use efficiency. Biol Res 53: 47. https://doi.org/10.1186/s40659-020-00312-4
Arnold W, Rump A, Klipp W, Priefe UB, Pühler A, 1988. Nucleotide sequence of a 24,206 base-pair DNA fragment carrying the entire nitrogen fixation gene cluster of Klebsiella pneumoniae. J Mol Biol 203: 715-738. https://doi.org/10.1016/S0022-2836(88)90205-7
Baca BE, Elmerich C, 2007. Microbial production of plant hormones. In: Associative and endophytic nitrogen-fixing bacteria and cyanobacterial associations; Elmerich C, Newton WE (eds), pp: 113-143, Kluwer Acad Publ, Dordrecht. https://doi.org/10.1007/1-4020-3546-2/6
Beatty PH, Good AG, 2011. Future prospects for cereals that fix nitrogen. Science 333: 416-417. https://doi.org/10.1126/science.1209467
Burén S, Jiang X, López-Torrejón G, Echavarrí-Erasun C, Rubio LM, 2017a. Purification and in vitro activity of mitochondria targeted nitrogenase cofactor maturase NifB. Front Plant Sci 8: 1-16. https://doi.org/10.3389/fpls.2017.01567
Burén S, Young EM, Sweeney EA, Lopez-Torrejon G, Veldhuizen M, Voigt CA, Rubio LM, 2017b. Formation of nitrogenase NifDK tetramers in the mitochondria of Saccharomyces cerevisiae. ACS Synth Biol 6: 1043-1055. https://doi.org/10.1021/acssynbio.6b00371
Burén S, Rubio LM, 2018. State of the art in eukaryotic nitrogenase engineering. Microbiol Lett 365 fnx274. https://doi.org/10.1093/femsle/fnx274
Chen YB, Dominic B, Mellon MT, Zehr JP, 1998. Circadian rhythm of nitrogenase gene expression in the diazotrophic filamentous non heterocystous cyanobacterium Trichodesmium sp. strain IMS 101. J Bacteriol 180: 3598-3605. https://doi.org/10.1128/JB.180.14.3598-3605.1998
Cheng Q, Day A, Dowson-Day M, Dixon R, 2005. The Klebsiella pneumoniae nitrogenase Fe protein gene (nifH) functionally substitutes for the chlL gene in Chlamydomonas reinhardtii. Biochem Biophys Res Commun 329: 966-975. https://doi.org/10.1016/j.bbrc.2005.02.064
Curatti L, Rubio LM, 2014. Challenges to develop nitrogen-fixing cereals by direct nif-gene transfer. Plant Sci 225: 130-137. https://doi.org/10.1016/j.plantsci.2014.06.003
Dénarié J, Debelle F, Prome J-C, 1996. Rhizobium lipochitooligosaccharide noduleation factors: signaling molecules mediating recognition and morphogenesis. Ann Rev Biochem 65: 503-535. https://doi.org/10.1146/annurev.bi.65.070196.002443
Dent DR, Cocking EC, 2017. Establishing symbiotic nitrogen fixation in cereals and other non-legume crops: The greener nitrogen revolution. Agri Food Secur 6: 7. https://doi.org/10.1186/s40066-016-0084-2
Dixon RA, Postgate JR, 1971. Transfer of nitrogen-fixation genes by conjugation in Klebsiella pneumoniae. Nature 234: 47-48. https://doi.org/10.1038/234047a0
Dixon R, Kahn D, 2004. Genetic regulation of biological nitrogen fixation. Nat Rev Microbiol 2, 621-631. https://doi.org/10.1038/nrmicro954
Dixon R, Cheng Q, Shen GF, Day A, Dowson-Day M, 1997. Nif gene transfer and expression in chloroplasts:
Prospects and problems. Plant Soil 194: 193-203. https://doi.org/10.1007/978-94-011-7113-7_19

Dong W, Song Y. 2020. The significance of flavonoids in the process of biological nitrogen fixation. Int J Mol Sci 21: 5926. https://doi.org/10.3390/ijms21165926

Dos Santos PC, Fang Z, Mason SW, Setubal JC, Dixon R, 2012. Distribution of nitrogen fixation and nitrogenase-like sequences amongst microbial genomes. BMC Genom 13: 162. https://doi.org/10.1186/1471-2164-13-162

Geddes BA, Galloway JN, Dice NB, Sutton MA, Bleeker A, Grizzetti B, et al., 2015. Nitrogen: too much of a vital resource. Science Brief WWF Netherlands 1-48.

FAO, 2020. The state of food security and nutrition in the world 2020. Transforming food systems for affordable healthy diets. FAO, IFAD, UNICEF, WFP and WHO, Rome.

Flores E, Herrero A, 2010. Compartmentalized function through cell differentiation in filamentous cyanobacteria. Nat Rev Microbiol 8: 39-50. https://doi.org/10.1038/nrmicro2242

Foley JA, Ramankutty N, Brauman KA, Cassidy ES, Gerber JS, Johnston M, et al., 2011. Solutions for a cultivated planet. Nature 478: 337-342. https://doi.org/10.1038/nature10452

Froussert E, Bonneau J, Franche C, Bogusz D. 2014. Recent advances in actinorhizal symbiosis signaling. Plant Mol Biol 90: 613-622. https://doi.org/10.1007/s11103-014-0450-2

Fukami J, Cerezini P, Hugo M, 2018. Azosporillum: benefits that go far beyond biological nitrogen fixation. AMB Express 8: 73. https://doi.org/10.1186/s13568-018-0608-1

Galloway JN, Cowling EB, 2002. Reactive nitrogen and the world: 200 years of change. Ambio 31: 64-71. https://doi.org/10.1579/0044-7447-31.2.64

Galloway JN, Townsend AR, Willem J, Erisman JW, Froussart E, Bonneau J, Franche C, Bogusz D, 2016. The significance of flavonoids in the process of biological nitrogen fixation by Rhizobia and extending the scope to cereals. Microorganisms 9: 125. https://doi.org/10.3390/microorganisms9010125

Gates KM, Chen Y, Liu X, Song Y, Haberer G, Crook MB, et al., 2018. Phylogenomics reveals multiple losses of nitrogen-fixing root nodule symbiosis. Science 361: eaat1743. https://www.science.org/doi/10.1126/science.aat1743

Hensel G, 2020. Genetic transformation of Triticaceae cereals - Summary of almost three decade's development. Biotechnol Adv 40: 107484. https://doi.org/10.1016/j.biotechnadv.2019.107484

Hu Y, Ribbe MW, 2015. Nitrogenase and homologs. J Biol Inorg Chem 20: 435-445. https://doi.org/10.1007/s00775-014-1225-3

Ibañez F, Wall L, Fabra A, 2017. Starting points in plant-bacteria nitrogen-fixing symbioses: intercellular invasion of the roots. J Exp Bot 68: 1905-1918. https://doi.org/10.1038/jxb.2016.203

Ivleva NB, Groat J, Staub JM, Stephens M, 2016. Expression of active subunit of nitrogenase via integration of plant organelle genome. PLoS One 11: e0160951. https://doi.org/10.1371/journal.pone.0160951

Jiang X, Payá-Tormo L, Coroian D, García-Rubio I, Castellanos-Rueda R, Eseverri A, et al., 2021. Exploiting genetic diversity and gene synthesis to identify superior nitrogenase NifH protein variants to engineer N2-fixation in plants. Comm Biol 4: 4. https://doi.org/10.1038/s42003-020-01536-6

Jorgensen RBH, 2008. Nitrogen and vegetation dynamics. New Phytol 179: 1-5. https://doi.org/10.1111/j.1469-8137.2007.02328.x

Kamminga E, 1993. Genetic engineering of the nitrogenase: the perspective. Trends Biotechnol 11: 237-243. https://doi.org/10.1016/0167-7799(93)90012-7

Kamei M, 2010. Genetic engineering of nitrogen fixation in plants. Curr Opin Biotechnol 21: 12-19. https://doi.org/10.1016/j.copbio.2009.10.001

Ladha JK, Tirol-Padre A, Reddy CK, Cassman KG, SudhirVerma, Powlson DS, et al., 2016. Global nitrogen budgets in cereals: A 50-year assessment for maize, rice, and wheat production systems. Sci Rep 6: 19355. https://doi.org/10.1038/srep19355

Larrainzar E, Villar I, Rubio MC, Perez-Rontome C, Huertas R, Sato S, et al., 2020. Hemoglobins in the legumes. New Phytol 228: 472-484. https://doi.org/10.1111/nph.16673
Li Q, Chen S, 2020. Transfer of nitrogen fixation \( (nif) \) genes to non-diazotrophic hosts. ChemBioChem 21: 1717-1722. https://doi.org/10.1002/cbic.201900784

Li M, Xu J, Gao Z, Tian H, Gao Y, Kariman K, 2020. Genetically modified crops are superior in their nitrogen use efficiency—A meta-analysis of three major cereals. Sci Rep 10: 8568. https://doi.org/10.1038/s41598-020-65684-9

Liu D, Liberton M, Yu J, Pakrasi HB, Bhattacharyya-Pakrasi M, 2018. Engineering nitrogen fixation activity in an oxygenic phototroph. mBio 9: e01029-18. https://doi.org/10.1128/mBio.01029-18

Ludwig E, Poole P, 2003. Metabolism of Rhizobium bacteria. CRC Crit Rev Plant Sci 22: 37-78. https://doi.org/10.1080/713610850

López-Torrejón G, Jiménez-Vicente E, Buesa JM, Hernandez JA, Verma HK, Rubio LM, 2016. Expression of a functional oxygen-labile nitrogenase component in the mitochondrial matrix of acrobically grown yeast. Nat Comm 7: 11426. https://doi.org/10.1038/ncomm11426

Murphy P, Wexler W, Grzemski W, Rao J, Gordon D, 1995. Rhizopines—Their role in symbiosis and competition. Soil Biol Biochem 27: 525-529. https://doi.org/10.1016/0038-0717(95)98627-Z

Mus F, Crook MB, Garcia K, Garcia Costas A, Geddes BA, Kouri ED, et al., 2016. Symbiotic nitrogen fixation and the challenges to its extension to nonlegumes. Appl Environ Microbiol 82: 3695-3710. https://doi.org/10.1128/AEM.01055-16

Nag P, Shriti S, Das S, 2019. Microbiological strategies for enhancing biological nitrogen fixation in nonlegumes. J Appl Microbiol 129: 186-198. https://doi.org/10.1111/jam.14557

Olivares J, Bedmar EJ, Sanjuan J, 2013. Biological nitrogen fixation in the context of global change. Mol Plant-Microbe Interact 26: 486-494. https://doi.org/10.1094/MPMI-12-12-0293-CR

Oldroyd GED, Dixon R, 2014. Biotechnological solutions to the nitrogen problem.Curr Opin Biotechnol 26: 19-24. https://doi.org/10.1016/j.copbio.2013.08.006

Oldroyd GED, Poole PS, 2019. Engineering transkingdom signalling in plants to control gene expression in rhizosphere bacteria. Nat Commun 10: 1-11. https://doi.org/10.1038/s41467-019-10882-x

Oldroyd GED, Murray JD, Poole PS, Downie JA, 2011. The rules of engagement in the legume-rhizobial symbiosis. Annu Rev Genet 45: 119-144. https://doi.org/10.1146/annurev-genet-110410-132549

Ormeño-Orrillo E, Hungria M, Martínez-Romero E, 2013. Dinitrogen-fixing prokaryotes. In: The prokaryotes: prokaryotic physiology and biochemistry; Rosenberg E et al. (eds). pp: 427-451, Springer, Berlin. https://doi.org/10.1007/978-3-642-30141-4_72

Pankievicz VCS, Irving TB, Maia LCS, Ané JM, 2019. Are we there yet? The long walk towards the development of efficient symbiotic associations between nitrogen-fixing bacteria and non-leguminous crops. BMC Biol 17: 99. https://doi.org/10.1186/s12915-019-0710-0

Pérez-González A, Knievel R, Veldhuizen M, Verma HK, Navarro-Rodriguez M, Rubio LM, Caro E, 2017. Adaptation of the GoldenBraid modular cloning system and creation of a toolkit for the expression of heterologous proteins in yeast mitochondria. BMC Biotechnol 17: 80. https://doi.org/10.1186/s12896-017-0393-y

Pérez-Montaño F, Alias-Villegas C, Bellogin RA, Del Cerro P, Espuny MR, Jiménez-Guerrero I, et al., 2014. Plant growth promotion in cereal and leguminous agricultural important plants: From microorganism capacities to crop production. Microbiol Res 169: 325-336. https://doi.org/10.1016/j.micres.2013.09.011

Ramos JM, García del Moral LF, Molina Cano JL, Salamanca P, Roca de Togores F, 1989. Effects of an early application of sulphur or etephon as foliar spray on the growth and yield of spring barley in a Mediterranean environment. J Agron Crop Sci 163: 129 137. https://doi.org/10.11011/j.j.1439-037X.1989.tb00746.x

Raymond J, Siefert JL, Staples CR, Blankenship RE, 2004. The natural history of nitrogen fixation. Mol Biol Evol 21: 541-554. https://doi.org/10.1093/molbev/ms047

Reinhold-Hurek B, Hurek T, 2011. Living inside plants: bacterial endophytes. Curr Op Plant Biol 14: 435-443. https://doi.org/10.1016/j.pbi.2011.04.004

Richardson AE, Barea JM, McNeill AM, Prigent-Combaret C, 2009. Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. Plant Soil 321: 111-117. https://doi.org/10.1007/s11104-009-9895-2

Rockström J, Steffen W, Noone K, Persson A, Chapin FS 3rd, Lambin EF, et al., 2009. A safe operating space for humanity. Nature 461: 472-475. https://doi.org/10.1038/461472a

Rogers C, Oldroyd GED, 2014. Synthetic biology approaches to engineering the nitrogen symbiosis in cereals. J Exp Bot 65: 1939-1946. https://doi.org/10.1038/jxb.eru098

Rolfe BG, Gresshoff PM, 1988. Genetic-analysis of legume nodule initiation. Ann Rev Plant Physiol Plant Mol Biol 39: 297-319. https://doi.org/10.1146/annurev.pp.39.060188.001501

Rosenblueth M, Martínez-Romero E, 2006. Bacterial endophytes and their interactions with hosts. Mol Plant-Microbe Interact 19: 827-837. https://doi.org/10.1099/mpmi.0-0827

Rosenblueth M, Ormeño-Orrillo E, López-López A, Rogel MA, Reyes-Hermández BJ, Martínez-Romero JC, et
Biotechnological approaches to develop nitrogen-fixing cereals: A review

al., 2018. Nitrogen fixation in cereals. Front Microbiol 9: 1794. https://doi.org/10.3389/fmicb.2018.01794

Ryu MH, Zhang J, Toth T, Khokhani D, Geddes BA, Mus F, et al., 2020. Control of nitrogen fixation in bacteria that associate with cereals. Nat Microbiol 5: 314-330. https://doi.org/10.1038/s41564-019-0631-2

Saharan BS, Nehra V, 2011. Plant growth promoting rhizobacteria: a critical review. Life Sci Med Res 21: 1-30.

Santi C, Bogusz D, Franche C, 2013. Biological nitrogen fixation in non-legume plants. Ann Bot 111: 743-767. https://doi.org/10.1093/aob/mct048

Savka MA, Dessaux Y, Oger P, Rossbach S, 2002. Engineering bacterial competitiveness and persistence in the phytosphere. Mol Plant-Microbe Interact 15: 866-874. https://doi.org/10.1094/MPMI.2002.15.9.866

Savka MA, Dessaux Y, Gardener BBM, Mondy S, Kohler PRA, Rossbach S, 2013. The “biased rhizosphere” concept and advances in the Omics era to study bacterial competitiveness and persistence in the phytosphere. In: Mol Microb Rhzosphere 1, pp: 1145-1161. John Wiley & Sons, Ltd. https://doi.org/10.1002/9781118297674.ch110

Scharff LB, Bock R, 2014. Synthetic biology in plastids. Plant J 78: 783-798. https://doi.org/10.1111/tpj.12356

Seefeldt LC, Hoffman BM, Dean DR, 2009. Mechanism of Mo-dependent nitrogenase. Ann Rev Biochem 78: 701-722. https://doi.org/10.1146/annurev.biochem.78.070907.103812

Seefeldt LC, Hoffman BM, Dean, DR, 2012. Electron transfer in nitrogenase catalysis. Curr Opin Chem Biol 16: 19-25. https://doi.org/10.1016/j.cbpa.2012.02.012

Sickerman NS, Ribbe MW, Hu Y, 2017. Nitrogenase cofactor assembly: an elemental inventory. Acc Chem Res 50: 2834-2841. https://doi.org/10.1021/acs.accounts.7b00417

Stokstad E, 2016. The nitrogen fix. Science 353: 1225-1227. https://doi.org/10.1126/science.353.6305.1225

Stoltzfus JR, So R, Malarvithi PP, Ladha JK, de Bruijn FJ, 1997. Isolation of endophytic bacteria from rice and assessment of their potential for supplying rice with biologically fixed nitrogen. Plant Soil 194: 25-36. https://doi.org/10.1007/978-94-011-5744-5_4

Taiz L, Zeiger E, Moller IM, Murphy A, 2015. Plant physiology and development, 6th ed. Sinauer Assoc, Inc. Sunderland, MA, USA 761 p.

Temme K, Zhao DH, Voigt CS, 2012. Refactoring the nitrogen fixation gene cluster from Klebsiella oxytoca. Proc Natl Acad Sci USA 109: 7085-7090. https://doi.org/10.1073/pnas.1120788109

Townsend AR, Howarth RW, Bazzaz FA, Booth MS, Cleveland CC, Collinge SK, et al., 2003. Human health effects of a changing global nitrogen cycle. Front Ecol Environ 1: 240-246. https://doi.org/10.1890/1540-9295(2003)001[0240:HHEOAC]2.0.CO;2

Tsyganova AV, Brewin NJ, Tsyganov VE, 2021. Structure and development of the legume-rhizobial symbiotic interface in infection threads. Cells 10: 1050. https://doi.org/10.3390/cells10051050

UN-DESA, 2017. World population projected to reach 9.8 billion in 2050, and 11.2 billion in 2100. United Nations Dept of Econ and Soc Aff. https://www.un.org/development/desa/en/news/population/world-population-prospects-2017.html. [March 6, 2021].

Van Deynze A, Zamora P, Delaux PM, Heitmann C, Jayaraman D, Rajasekar S, et al., 2018. Nitrogen fixation in a landrace of maize is supported by a mucilage associated diazotrophic microbiota. PLoS Biol 16: 1-21. https://doi.org/10.1371/journal.pbio.2006352

Van Velzen R, Holmer R, Bu F, Rutten L, Van Zeijl A, Liu W, et al., 2018. Comparative genomics of the non-legume Parasponia reveals insights into evolution of nitrogen-fixing rhizobium symbioses. Proc Natl Acad Sci USA 115: e4700-e4709. https://doi.org/10.1073/pnas.1721395115

Venado RE, Liang J, Marin M, 2020. Rhizobia infection, a journey to the inside of plant cells. In: Regulation of nitrogen-fixing symbioses in legumes. Adv Bot Res 94: 97-118, Frendo P et al. (eds.), Elsevier, TheHague. https://doi.org/10.1016/bs.abr.2019.09.007