JOURNAL OF
NATURAL SCIENCES AND MATHEMATICS RESEARCH

Available online at http://journal.walisongo.ac.id/index.php/jnsmr

A glimpse of the Nitrogen-Fixing Wheat possibility

Lee James Watson[1], Tara Puri Ducha Rahmani[2]
[1]Chemtech Environmental Limited, Stanley, County Durham, United Kingdom
[2]Department of Biology, Faculty of Science and Technology, Universitas Islam Negeri Walisongo Semarang, Indonesia

Corresponding author:
ljwatson96@gmail.com
Received : 10 Jan 2022
Revised : 10 May 2022
Accepted : 25 June 2022

Abstracts

As the world's population grows at an exponential rate, greater food production becomes a requirement. Intensive agricultural methods, which are now used to produce the bulk of food, are unsustainable and will be unable to supply demand when natural resources become scarce. The industry faces a significant nitrogen demand, which must be met in order to combat hunger in emerging countries. This study used a literature review technique to investigate the potential for improved wheat crop yield through nitrogen fixation. It has been demonstrated that utilizing microorganisms such as cyanobacteria as biofertilizers or phytopathogen antagonists can boost the development and productivity of non-leguminous agricultural crops. Finally, the possible end result outweighs the costs. It's impossible to argue against the development of nitrogen-fixing wheat at this point in the technology's development. Whether it is possible to develop crops that rely solely on dinitrogen as a nitrogen source, or if the result is only a slight boost in wheat yields, the need to improve global food production needs research like the one described in this article.

Keywords: Nitrogen-fixing; wheat; GMO; biotechnology; microorganism

1. Introduction

As the global population undergoes dramatic increases, improved food production becomes a necessity. At present, the intensive agricultural systems utilised for the majority of food production are unsustainable and will not be able to meet demand as natural resources become scarce [8]. Cereal grains like rice, sorghum, wheat, and maize are some of the most important crops when it comes to human nutrition. Cereals form symbiotic relationships with various bacteria, particularly diazotrophs, also known as nitrogen-fixing bacteria, to foster

their own growth [24]. Despite the fact that nitrogen does not have any direct positive effects on human physiology, it plays a crucial role in the development and productivity of plants [25]. In fact, one of the most prevalent gas elements in the atmosphere of the Earth is nitrogen. It was first identified by Daniel Rutherford in 1772 and makes up roughly 78% of the air on Earth [39] in [26]. Despite being the most prevalent element in the atmosphere, nitrogen is also the essential nutrient that plants lack the most of. However, plants need to develop a symbiotic relationship with some nitrogen-fixing microbes because they are unable to fix nitrogen from the air on their own. Because of the pioneering work of a Dutch botanist and microbiologist named Beijerinck in 1901, plants and nitrogen-fixing microbes have been linked in Europe for almost 114 years now, according to the most recent estimates [40] in [26].

Nitrogen fixation is the process by which nitrogen is changed into ammonia so that plants can make use of it. The term "nitrogen fixation" refers to this process [27]. The biological process of nitrogen fixation is catalyzed by nitrogenase, a metalloenzyme that is extremely complex and sensitive to oxygen [28]. Nitrogenase, a highly oxygen-sensitive metalloenzyme, is the catalyst for biological nitrogen fixation [28]. Because of the increased number and size of leaves, N promotes leaf area (AF) and leaf area index (LAI). It enhances the color of the leaves' green and is a constituent of amino acids, proteins, and nucleic acids, which are vital cellular building blocks. Additionally, it regulates P, K, and other nutrients and raises many crops' succulence. The N promotes photosynthesis by increasing the amount of chlorophyll [41] in [29].

Nitrogenase catalyzes the conversion of dinitrogen (N_2) to ammonia as part of the nitrogen-fixing process (enzyme). The biological nitrogen fixation (BNF) is typically carried out by Leguminosae plants in cooperation with rhizobia, such as those from the genera *Bradyrhizobium, Rhizobium, Azorhizobium, Sinorhizobium, and Photorhizobium*, as well as other genera. Free-living microorganisms called plant growth-promoting bacteria (PGPB) are found in the roots of plants and can promote plant growth by aiding in nitrogen fixation [30]. In the nitrogen cycle, plants' primary natural source of fixed nitrogen is obtained through a process called Biological Nitrogen Fixation (BNF) [31]. Microbial communities can be found both within the roots of a plant and in the space surrounding them, known as the rhizosphere. These microbiomes associated with the roots of plants play an essential role in the nutrition and productivity of plants [32]. Nitrogen-fixing bacteria and the plant they are attached to engage in a nutrient-exchanging relationship with one another, which controls the level of BNF activity in the legume-rhizobial system [33]. There are symbiosomes present in the nodules of legumes; these symbiosomes are home to the endosymbiotic bacteria known as rhizobia. These bacteria acted as acting organelles for the temporary nitrogen fixation process [34]. The rhizosphere microbiome can be affected by a variety of environmental factors, including soil type, plant cultivar, climate change, and anthropogenic activities [35]. It was discovered by Hala [36] that the experiment involving the inoculation of nitrogen-fixing bacteria into upland rice plants could increase both the dry weight of the shoot and the root, in addition to the number of tillers that formed. The cereals' production can make use of a potential alternative source of nitrogen that comes from biological nitrogen fixation (BNF) [37]. According to [38], most of the nitrogen found in the biosphere comes from the BNF produced by diazotrophic bacteria. These bacteria are responsible for contributing between 30 (thirty) and 50 (fifty) percent of the total nitrogen found in crop fields.

Nitrogen availability is of particular concern, as its deficiency is commonly associated with reduced crop yields across the globe. While molecular nitrogen is abundant in the Earth's atmosphere, this atmospheric nitrogen is metabolically unavailable to the majority of higher plants [20]. With cereal cropping contributing significantly to agricultural land use (acting as a staple food for 35% of the world's population), a substantial nitrogen demand is placed upon the industry and must be overcome to combat malnutrition in developing countries [17].

Increasing wheat yields is of great topical significance in the United Kingdom, with the "20:20 Wheat®" programme run by Rothamsted Research and the BBSRC aiming to improve yields to 20 tonnes per hectare before the year 2032. This work in the UK is of great importance and has the potential to be applied to wheat crops worldwide [3].

Cereal crop productivity only reaches approximately 25% of the attainable yield in many developing countries, with the increase in nitrogen application predicted to reach over 50kg.ha^{-1} in order to close any yield gaps [14]. While the productivity of crops is usually improved through the use of chemical fertilisers, their efficiency is low due to losses through volatilisation, denitrification, immobilisation, or leaching. As such, the discovery of alternatives to traditional nitrogen supplementation is becoming a necessity [1]

2. Biological Nitrogen Fixation (BNF) as an Alternative to Traditional Fertilisation Methods

The major source of fixed nitrogen within the natural cycle is provided in the form of ammonium, which is produced from atmospheric nitrogen by diazotrophic microorganisms through the activity of nitrogenase enzymes [8][20]. When nitrogen supplies become limited, these microorganisms form a symbiotic relationship with legumes through the creation of highly specialised organs on the plant's roots or stems. The formation of these so-called nodules and the subsequent endosymbiosis are both controlled by the exchange of signal molecules between the plant and the symbiont. Plants secrete phenolics (such as flavonoids) to induce the expression of rhizobial nodulation genes. At the same time, the symbiont produces Nod-factors to induce cortical cell division, root-hair deformation, and the formation of the nodules themselves. These phenolics act in tandem with the bacterial activator protein NodD to regulate the expression of genes coding for Nod-factor synthesising proteins [7]. Nod-factors are described as elicitors due to their nature as a plant stimulants, inducing a series of genetic responses [15]. The rhizosphere diazotrophs have been studied for decades, giving us a vast understanding of their mechanisms of action [2].

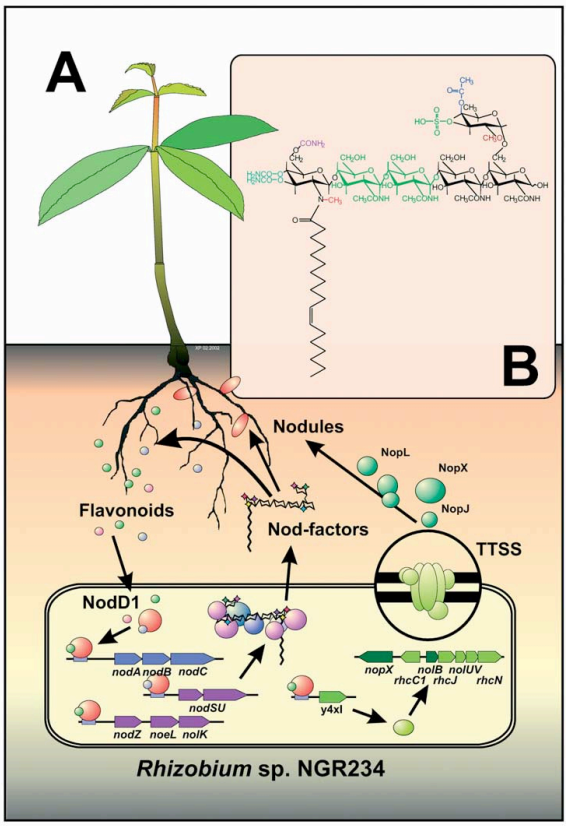

Figure 1. Schematic of the flavonoid-inducible elicitors of root nodulation in Rhizobium sp. NGR234. Section (A) shows the triggering of nodulation gene transcription following the release of flavonoids from the plant roots. NodD1 acts as a transcriptional regulator and promotes the production of Nod-factors (signals for root nodulation). NodD1 also promotes activation of bacterial type III secretion system (TTSS), which leads to secretion of nodulation outer proteins required for host plant nodulation. Section (B) shows the structure of a nod-factor, which in this case is a modified lipo-chito-oligosaccharide comprised of N-acetyl-D-glucosamine oligomers with a fatty acid in place of the N-acetyl group at the non-reducing terminus. Nod-factors are produced by enzymes coded by the nod genes [42].

In legumes, the major method of symbiont infection occurs via intracellular infection

threads [15]. Root nodulation is facilitated via a cascade of developmental steps, initiated by the colonisation of the plant's rhizosphere by the rhizobia. The rhizobia attach to the root hairs and become entrapped in the folds of root hairs, from where entry to the plant cell wall can occur. Plant cortical cells adapt and form so-called nodule primordia, towards which the bacterial route of infection can advance [7]. The bacteria form infection intracellular infection threads inside the plant epidermis [15]. Rhizobia then begin to grow in size and differentiate into a form adapted for nitrogen fixation [7]. Also known to exist is infection via crack entry at the lateral root base, which is specific to subtropical legumes and does not involve the formation of an infection thread [15].

The nitrogenase-driven catalysis of biological nitrogen fixation requires eight electrons and a minimum of 16 ATP equivalents for the fixation of a single gaseous nitrogen molecule [12]. The most commonly found and studied nitrogenase is the molybdenum-dependent enzyme, Mo-nitrogenase, which consists of the dinitrogenase (NifDK) and dinitrogenase reductase (NifH) protein domains [12][23]. NifDK contains an iron and molybdenum cofactor required for the reduction of N_2, the biosynthesis of which requires a number of gene products all encoded within the *nif* cluster [12]. Also contained within NifDK is a metallic P-cluster, which facilitates the transfer of electrons to the iron-molybdenum cofactor [22]. These *nif* clusters have a tendency to be arranged in so-called genomic islands of nitrogen fixation, allowing horizontal gene transfer of every required nitrogen-fixing gene to occur in a single transfer event [23].

This natural symbiosis can be taken advantage of to allow non-leguminous plants to take part in BNF. Mimicry of the symbiotic associations could be applied to wheat, allowing it to benefit from a well-understood natural process [12][16]. Another method of introducing BNF into wheat crops could be the transfer of *nif* clusters into the plant itself, allowing the plant to fix nitrogen of its own accord [12].

3. Creating Artificial Nitrogen-fixing Associations

It has been shown that using inoculations of microorganisms, such as cyanobacteria, as bio fertilisers or antagonists of phytopathogens can increase the growth and yield of even non-leguminous agricultural crops [16]. Some studies have even shown a 12-25% increase in wheat biomass while using cyanobacteria formulations, with inoculation positively correlating with plant growth promotion and stress tolerance in studies with rice [1]. One mechanism proposed for this growth promotion is the ability of cyanobacteria to produce phytohormones, such as the auxin indole-3-acetic acid (IAA). Sergeeva et al. [21] provided evidence that cyanobacteria have the capacity to accumulate and release IAA. Some strains were also shown to possess homologues of *ipdC*, a gene coding for indole pyruvate decarboxylase (involved in the biosynthetic pathway of IAA). This is coupled with their possible role in biocidal activity against fungal pathogens, which provides a critical competitive edge for these bacteria over other soil microorganisms [18].

Other advantages provided by rhizobacterial symbioses include increases in nutrient availability in the rhizosphere, induction of increases in the root area, and benefits to other symbiotic associations that the plant may undergo. In fact, it has been postulated that these effects are the primary modes of action contributing to plant growth promotion and not nitrogen fixation. The fact that most of the plant growth-promoting rhizobacteria are diazotrophs is possible due to there being a distinct advantage provided by nitrogen fixation that allows bacteria to survive in the rhizosphere. It has also been proposed that data showing the benefits of nitrogen fixation may be insignificant at the agricultural scale but is significant in nature. Worthy of note is the idea that researchers in this field commonly utilise media well suited for diazotrophs when attempting to isolate PGPRs of interest, leading to an inherent bias in the data for PGPR diversity [5].

Nodules formed during the creation of artificial nitrogen-fixing symbioses are termed

paranodules and have been successfully produced in wheat in a study by Biabani et al. (2014) [5]. While this study showed that inoculating the wheat plants with soil bacteria increased nitrogen fixation activity of the roots, no significant increase in biomass was observed (and, in fact, there were decreases in biomass in some of their experiments).

Prior to this work, Biabani [4] had managed to produce artificial plant-bacteria symbioses that resulted in increased plant biomass. The study employed the use of various treatments, with the help of an abiogenic nodulation agent (2,4-dichlorophenoxyacetic acid) in tandem with bacterial inoculations (*Xanthomonas sp.* and *Arthrobacter sp*), producing the greatest increase in both plant height and weight. While these results are encouraging for the future of the technology, the lack of consistent results raises concerns about its viability on a larger scale.

This approach to the creation of nitrogen-fixing wheat overcomes the social stigma associated with genetic modification that other methods aim to employ. However, the most significant challenge presented is strengthening the symbiotic associations in order to overcome the need for repeated inoculations [1]. Such a task proves to be daunting, as research into the specific traits required for plant colonisation is limited at present. Some methods are understood, but the overall process is likely to be regulated by hundreds of individual genes and will be difficult to study [13][19].

4. Genetic Modification of Wheat to allow Self-fertilisation

Evidence for naturally occurring horizontal transfer of *nif* genes has been shown by genome analysis, with a *nif* cluster in *Paenibacillus* sp. WLY78 contains a higher G+C content than the average of its entire genome (52.8% in the *nif* cluster compared to 45.1% in the genome). This difference in composition suggests that the genes came from a foreign genome [22]. Whether or not this natural transfer can be replicated in the construction of nitrogen-fixing wheat that does not require a diazotrophic symbiont remains to be seen.

The John Innes Centre (JIC) has been attempting to research ways in which nodulation can be initiated in cereal crops. JIC is also making attempts to insert the nitrogen fixation genes identified in bacteria directly into the plants, effectively creating self-fertilising crops. Research similar to this has already made JIC worth a great deal to the UK economy and worldwide [6]. Such a goal is noble but does not come without risk and will be challenging to achieve.

It has been shown that heterologous expression of *nif* gene products can confer nitrogen fixation to the transgenic host. Xie et al. [23] modified *E. coli* to incorporate nine contiguous genes (*nifBHDKENXhesAnifV*) from the *nif* cluster of *Paenibacillus beijingensis*. *E. coli* modified in this way were shown to incorporate a $^{15}N_2$ isotope supplied to a nitrogen-free medium in gaseous form. The successful creation of nitrogen-fixing *E. coli* shows the potential for other organisms to be given nitrogen-fixing abilities in a similar way, building on ground-breaking work in the 1970s involving the transfer of nitrogenase from *Klebsiella pneumonia* to *E. coli* [13].

The nine genes used represent the minimum number of genes that must be transferred simultaneously to confer nitrogen fixation ability, with the number of required genes varying from 9-20 (all considered essential) [13]. *Nif* gene deletion work carried out by Wang et al. [22] showed that the nine *Paenibacillus* genes *nifBHDKENXhesAnifV* were all necessary for optimal nitrogenase activity in transgenic *E. coli*. The large size of any transgene constructs required for the creation of nitrogen-fixing wheat represents one of the many challenges to overcome [13].

In addition to this, attempts have been made to introduce *nif* genes into eukaryote hosts, working towards the ultimate goal of plant transgenesis. Cheng et al. [11] used homologous recombination to introduce the *nifH* gene into the algae *Chlamydomonas reinhardtii* in place of the native *chlL* gene required for light-independent chlorophyll biosynthesis. Their research aimed to overcome previous issues associated with the transport of

the dinitrogenase reductase subunit into chloroplasts via transit peptides and the proposed lack of compatibility between nitrogen fixation and photosynthetic oxygen evolution. They proposed that functional similarities between NifH and ChlL would allow NifH to replace ChlL in chlorophyllide biosynthesis. They successfully revealed this to be true (using the gene *uidA* as a negative control) and show that *chlL* absence inactivates the light-independent pathway of chlorophyll biosynthesis). As such, the study discovered a potential method for the incorporation of *nif* genes into a plastid genome. The lead author of the paper also proposed that *nif*DK could be introduced as a replacement for *chlN* and *chlB* genes, but no information on this area seems to have become available since then [10]. Despite this research effort, angiosperms rely only on the light-dependent pathways of chlorophyll synthesis, the proteins of which are not related to those of the light-independent pathways. It is not known whether or not suitable electron donors exist in the chloroplasts of cereals to act as homologues to nitrogenase components, and there is no experimental evidence to suggest that homologous mitochondrial electron donors exist [12]. Also worth noting is the fact that expression or co-expression of *nifD* and *nifK* in *Saccharomyces cerevisiae* produced inactive proteins that lacked their metal clusters [12], creating doubt about the viability of this venture.

As previously mentioned, one difficulty in expressing *nif* genes in plants is the interference of oxygen produced through photosynthetic activity with the activity of nitrogenase [12]. Oxygen concentrations as low as 5% are sufficient enough to prevent nitrogenase activity both in natural diazotrophs and engineered *E. coli* [22]. To counter this, it can be argued that many cyanobacteria are diazotrophs and have been able to perform both nitrogen fixation and oxygenic photosynthesis through either spatial or temporal separation of the two processes [9][12]. Wang et al. [22] observed that *Paenibacillus* sp. WLY78 did not produce any subunits of NifDK when not under nitrogen fixation conditions (displaying a potential mechanism that could be used for separation of the oxygen-producing and nitrogen-fixing process). At the same time, transgenic *E. coli* produced nitrogenase components even in the presence of oxygen (though no nitrogenase activity occurred). This suggests that transcriptional regulation of *nif* genes if performed outside of the minimal nine gene cluster. The result of this is that engineering a similar separation in plants could overcome this problem, with particular focus on circadian/light-sensitive rhythms or direction of nitrogenase expression to locations free from photosynthetic oxygen production (such as root cells) [12]. Further study could be dedicated to how *Paenibacillus* regulates nitrogen fixation against oxygen concentration, given that it is a facultative anaerobe and can switch between tolerance to both aerobic and anaerobic conditions [22].

5. Conclusion

The concept of nitrogen fixing wheat as a whole is a noble pursuit that would revolutionise the growth of cereal crops, but comes at a great cost. Despite decades of research, the processes involved in both plant-bacteria symbioses and the nitrogen fixation pathway itself are still relatively poorly understood.

Basal-level research already gives an insight into the possible mechanisms for overcoming problems with both the creation of artificial symbioses and the genetic modification approach to conferring biological nitrogen fixation. However, there is still a large gap between the current successes in model systems and actually making the jump to the introduction of these systems into wheat plants.

It is also unknown as to what extent the proposed methods will improve wheat yields. The work of Wang et al. [22] with transgenic *E. coli* showed a very low nitrogenase activity of around 10% of that in the native *Paenibacillus* sp. WLY78. This is insufficient to allow total diazotrophic growth, but could still wield potential as a means of reducing the amount of nitrogen required from other sources (such as traditional fertilisers).

In conclusion, the potential end result justifies the means. At this stage in the development of the technology, it is difficult to argue against the development of nitrogen-fixing wheat. Whether it is possible to create crops that survive purely on dinitrogen as a nitrogen source, or if the result is merely a minor increase in wheat yields, the need to improve global food production necessitates research such as that mentioned in this review.

Acknowledgments

The authors would like to thank Prof. Gateshead from MSc in Industrial and Commercial Biotechnology, Newcastle University, United Kingdom, for the valuable insight.

References

[1] Babu S, Prasanna R, Bidyarani N, Singh R (2014) Analysing the colonisation of inoculated cyanobacteria in wheat plants using biochemical and molecular tools. Journal of Applied Phycology 27:327-338.

[2] Baldani J, Caruso L, Baldani VLD, Goi SR, Döbereiner J (1997) Recent advances in BNF with non-legume plants. Soil Biology and Biochemistry 29:911-922.

[3] BBSRC (2012) Rothamsted and BBSRC launch "20:20 Wheat ®". http://www.bbsrc.ac.uk/news/food-security/2012/120613-pr-rothamsted-bbsrc-launch-wheat-20-20.aspx. Accessed 23/02/2015

[4] Biabani A (2008) Formation of artificial Nitrogen-fixing bacteria symbioses with wheat. Journal of Applied Biosciences 6:169-172.

[5] Biabani A, Boggs LC, Natasha K, Morad U (2014) Microscopic morphology of nitrogen fixing paranodules on wheat roots. African Journal of Biotechnology 11:2971-2976.

[6] Brookdale Consulting (2013) Impact of the John Innes Centre. Brookdale Consulting, Cheshire

[7] Broughton W, Zhang F, Perret X, Staehelin C (2003) Signals exchanged between legumes and Rhizobium: agricultural uses and perspectives. Plant and Soil 252:129-137.

[8] Charpentier M, Oldroyd G (2010) How close are we to nitrogen-fixing cereals? Current Opinion in Plant Biology 13:556-564.

[9] Chen YB, Dominic B, Mellon MT, Zehr JP (1998) Circadian rhythm of nitrogenase gene expression in the diazotrophic filamentous nonheterocystous cyanobacterium *Trichodesmium* sp. strain IMS 101. Journal of Bacteriology 180:3598-3605.

[10] Cheng Q (2008) Perspectives in Biological Nitrogen Fixation Research. Journal of Integrative Plant Biology 50:786-798.

[11] Cheng Q, Day A, Dowson-Day M, Shen G-F, Dixon R (2005) The Klebsiella pneumoniae nitrogenase Fe protein gene (nifH) functionally substitutes for the chlL gene in Chlamydomonas reinhardtii. Biochemical and Biophysical Research Communications 329:966-975.

[12] Curatti L, Rubio LM (2014) Challenges to develop nitrogen-fixing cereals by direct nif-gene transfer. Plant Science 225:130-137.

[13] Geddes BA, Ryu M-H, Mus F, Garcia Costas A, Peters JW, Voigt CA, Poole P (2015) Use of plant colonizing bacteria as chassis for transfer of N2-fixation to cereals. Current Opinion in Biotechnology 32:216-222.

[14] Mueller ND, Gerber JS, Johnston M, Ray DK, Ramankutty N, Foley JA (2012) Closing yield gaps through nutrient and water management. Nature 490:254-257.

[15] Muñoz V, Ibáñez F, Tordable M, Megías M, Fabra A (2015) Role of reactive oxygen species generation and Nod factors during the early symbiotic interaction between bradyrhizobia and peanut, a legume infected by crack entry. Journal of Applied Microbiology 118:182-192.

[16] Nain L, Rana A, Joshi M, Jadhav SD, Kumar D, Shivay YS, Paul S, Prasanna R (2010) Evaluation of synergistic effects of bacterial and cyanobacterial strains as biofertilizers for wheat. Plant and Soil 331:217-230.

[17] Peoples MB, Herridge DF, Ladha JK (1995) Biological nitrogen fixation: An efficient source of nitrogen for sustainable agricultural production? Plant and Soil 174:3-28.

[18] Prasanna R, Nain L, Tripathi R, Gupta V, Chaudhary V, Middha S, Joshi M, Ancha R, Kaushik BD (2008) Evaluation of fungicidal activity of extracellular filtrates of cyanobacteria–possible role of hydrolytic enzymes. Journal of basic microbiology 48:186-194.

[19] Reinhold-Hurek B, Hurek T (2011) Living inside plants: bacterial endophytes. Current Opinion in Plant Biology 14:435-443.

[20] Saikia SP, Jain V (2007) Biological nitrogen fixation with non-legumes: an achievable target or a dogma. Current science 92:317-322.

[21] Sergeeva E, Liaimer A, Bergman B (2002) Evidence for production of the phytohormone indole-3-acetic acid by cyanobacteria. Planta 215:229-238.

[22] Wang L, Zhang L, Liu Z, Zhao D, Liu X, Zhang B, Xie J, Hong Y, Li P, Chen S (2013) A minimal nitrogen fixation gene cluster from *Paenibacillus* sp. WLY78 enables expression of active nitrogenase in *Escherichia coli*. PLoS genetics 9:e1003865.

[23] Xie J-B, Du Z, Bai L, Tian C, Zhang Y, Xie J-Y, Wang T, Liu X, Chen X, Cheng Q (2014) Comparative genomic analysis of N2-fixing and non-N2-fixing Paenibacillus spp.: organization, evolution and expression of the nitrogen fixation genes. PLoS genetics 10:e1004231

[24] Rosenblueth, M., Ormeño-Orrillo, E., López-López, A., Rogel, M. A., Reyes-Hernández, B. J., Martínez-Romero, J. C., ... & Martínez-Romero, E. (2018). Nitrogen fixation in cereals. Frontiers in Microbiology, 9, 1794.

[25] Rahmani, T. P. D., Kumalawati, D. A., Tyas, D. A., Armanda, D. T., & Rusmadi, R. (2020). Simple Feasibility Analysis Of Nitrogen-Fixing Cereals Project. Al-Hayat: Journal of Biology and Applied Biology, 3(2), 102-110.

[26] Swain, H., Abhijita, S. (2013). Nitrogen fixation and its improvement through genetic engineering. Journal of Global Biosciences. 2 (5), p98-112.

[27] Bano, S. A., & Iqbal, S. M. (2016). Biological nitrogen fixation to improve plant growth and productivity. International Journal of Agriculture Innovations and Research, 4(4), 596-599.

[28] Vicente, E. J., & Dean, D. R. (2017). Keeping the nitrogen-fixation dream alive. Proceedings of the National Academy of Sciences, 114(12), 3009-3011.

[29] Torres-Olivar, T., Villegas-Torre, O.G., Domínguez-Patiño, M.L., Sotelo-Nava, H, Rodríguez-Martínez, A., Melgoza-Alemán, R.M., Valdez-Aguilar, L.A., Alia-Tejacal, I. 2014. Role of Nitrogen and Nutrients in Crop Nutrition. Journal of Agricultural Science and Technology B 4. P29-37

[30] Masood, S., Zhao, X. Q., & Shen, R. F. (2020). Bacillus pumilus promotes the growth and nitrogen uptake of tomato plants under nitrogen fertilization. Scientia Horticulturae, 272, 109581.

[31] Oldroyd, G.E.D. and Dixon, R. (2014). Biotechnological solution to the nitrogen problem. Current Opinion in Biotechnology. 26:19-24. doi:10.1016/j.copbio.2013.08.006

[32] Chen, S., Waghmode, T. R., Sun, R., Kuramae, E. E., Hu, C., & Liu, B. (2019). Root-associated microbiomes of wheat under the combined effect of plant development and nitrogen fertilization. Microbiome, 7(1), 136.

[33] Li, X. G., Jia, B., Lv, J., Ma, Q., Kuzyakov, Y., & Li, F. M. (2018). Nitrogen fertilization decreases the decomposition of soil

organic matter and plant residues in planted soils. Soil Biology and Biochemistry, 112, 47-55.

[34] Coba de la Peña, T., Fedorova, E., Pueyo, J. J., & Lucas, M. M. (2018). The symbiosome: legume and rhizobia co-evolution toward a nitrogen-fixing organelle?. Frontiers in plant science, 8, 2229.

[35] Igiehon, N. O., & Babalola, O. O. (2018). Rhizosphere microbiome modulators: contributions of Nitrogen-fixing bacteria towards sustainable agriculture. International journal of environmental research and public health, 15(4), 574.

[36] Hala, Y. (2020, April). The effect of nitrogen-fixing bacteria towards upland rice plant growth and nitrogen content. In IOP Conference Series: Earth and Environmental Science (Vol. 484, No. 1, p. 012086). IOP Publishing.

[37] Rogers, C., and Oldroyd, G. E. D. (2014). Synthetic biology approaches to engineering the nitrogen symbiosis in cereals. J. Exp. Bot. 65, 1939–1946. doi: 10.1093/jxb/eru098

[38] Ormeño-Orrillo, E., Hungria, M., and Martínez-Romero, E. (2013). "Dinitrogen-fixing prokaryotes," in The Prokaryotes: Prokaryotic Physiology and Biochemistry, eds E. Rosenberg, E. F. de Long, S. Lory,

[39] Cheng Q (2008) Perspectives in biological nitrogen fixation research. J. Integr. Plant Biol.50(7): 784-796.

[40] Wagner S C (2012) Biological Nitrogen Fixation. Nature Edu. Knowl. 3(10): 15.

[41] G. Sedano-Castro, V.A. González, C. Saucedo, M. Soto, M. Sandoval, J.A. Carrillo, Yield and fruit quality of zucchini with high doses of N and K, American TERRA 29 (2) (2011) 133-142

[42] Broughton, W.J., Zhang, F., Perret, X. et al. Signals exchanged between legumes and Rhizobium: agricultural uses and perspectives. Plant and Soil 252, 129–137 (2003). https://doi.org/10.1023/A:1024179717780