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FEASIBILITY ANALYSIS OF NITROGEN-FIXING CEREALS PROJECT

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Abstract

Nitrogen does not directly have advantages in the human physiology system, but it holds one of the most critical roles in plants' life cycles and productivity. Even though Nitrogen is the most abundant element in the atmosphere, it is also the most deficient essential nutrients in plants. The proposed idea of nitrogen-fixing genetically modified (GM) crops, particularly wheat, is aimed to overcome those stated cons of the traditional diculture and nitrogen fertilizer. This study focuses on the overview as well as the pro and cons of genetically modified nitrogen-fixing plants in providing a better agricultural method. The genetically modifying method to generate nitrogen-fixing non-legumes carries a significant chance of failure results and hindrance. The multilevel implication occurs when we need to modify the plants that do not naturally produce nodules in their roots to form the nodules and to modify the Nitrogen-fixing microbes to live in the nodules of non-legumes, which are not their natural dwelling places.

In conclusion, the project to make genetically-modified crops which can fix Nitrogen is feasible, but the difficulties and funds needed still outweigh the future benefits. With all of those limitations, the target goal to eradicate famine in 2050 just by funding the nitrogen-fixing wheat alone seems to be too high to be reached. The funds and efforts should be better spent on other factors and farming methods.

Keywords: nitrogen-fixing microbes, fertilizer, genetically-modified plants, agriculture, legumes

Introduction

The most crucial crops for human nutrition are cereals, such as rice, sorghum, wheat and maize. To promote its growth, cereals also symbioses with various bacteria, especially diazotrophs or nitrogen-fixing bacteria (Rosenblueth et al., 2018). Nitrogen does not directly benefit the human physiology system, but it holds one of the most important roles in plants' life cycle and productivity. Even though Nitrogen is the most abundant element in the atmosphere, it is also the most deficient essential nutrients in plants. The proposed idea of nitrogen-fixing GM crops, particularly wheat, is aimed to overcome those stated cons of traditional agriculture and nitrogen fertilizer. Based on the GM Freeze article (in 2012), US$ 10 million projects from a foundation is allocated for the aim that in 2050, there is a target set to overcome the world's hunger. Some stakeholders thought of developing nitrogen-fixing wheat that will no longer need any nitrogen fertilizer in its growth and development. There is always a pro and contra in overcoming the plan. Still, the biggest question would be whether it is possible to create genetically modified wheat and whether the advantages outweigh the loss. This article focuses on the overview and the pro and cons of genetically modified nitrogen-fixing plants in providing a better agricultural method.

The importance of Nitrogen for plants

In the human physiological system, Nitrogen does not specifically have benefits, but it plays one of the most significant roles in the life cycle and productivity of the plant. All vital processes in plants need proteins, of which Nitrogen is one of the main components of protein. Nitrogen is also a component of chlorophyll and therefore supports the plants'

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photosynthesis process, which will determine the plants' total yields. Plants need combined Nitrogen forms such as Ammonia or nitrate as the primary nutrients to support its growth and productivity. Nowadays, more than fifty per cent of the world population consumes food grown with synthetic fertilizers, including Ammonia and nitrate (Zhang et al., 2015). More than 110 million tonnes of synthetic nitrogen fertilizer is applied annually on a global scale (FAO, 2017).

Nitrogen is, in fact, one of the most abundant gas elements on the earth's atmosphere. It occupies around seventy-eight per cent of the total earth's air element, discovered in 1772 by Daniel Rutherford (Cheng, 2008 in Swain and Abhijita, 2013). Even though Nitrogen is the most abundant element in the atmosphere, it is also the most deficient essential nutrients in plants. However, plants cannot directly fix Nitrogen from the air; they need to form a symbiotic relationship with some nitrogen fixation microbes. The relationship between the plants and nitrogen fixation microbes was first discovered in 1901 by a Dutch microbiologist and botanist named Beijerinck, which means that the nitrogen fixation process has been brought over for nearly 114 years until now (Wagner, 2012 in Swain and Abhijita, 2013). Nitrogen fixation is a term of the process in which Nitrogen is converted into Ammonia, so that it can be used by the plants (Bano and Iqbal, 2016). Nitrogen fixation biologically catalyzed by nitrogenase, a complex and extremely oxygen-sensitive metalloenzyme (Vicente and Dean, 2017).

The NO3 - is the main source of N for most crop species; however, this depends on the plant species and some factors that should be taken into account. Among such factors are the pH, the temperature, and the carbohydrate content in roots. NO3- absorption also varies with the variety and light intensity as might be found in lettuce and other vegetables (Rodrigues, 2002 in Torres-Olivar, 2014). The N promotes leaf area (AF) and leaf area index (LAI), which may be due to a higher number and size of leaves. It intensifies the green colour of the leaves and is a constituent of essential cellular components such as amino acids, proteins and nucleic acids. It is also the controller of P, K and other nutrients, and improves the succulence of many crops. It also promotes photosynthesis because the N increases the amount of chlorophyll (Sedano-Castro et al., 2011 in Torres-Olivar, 2014).

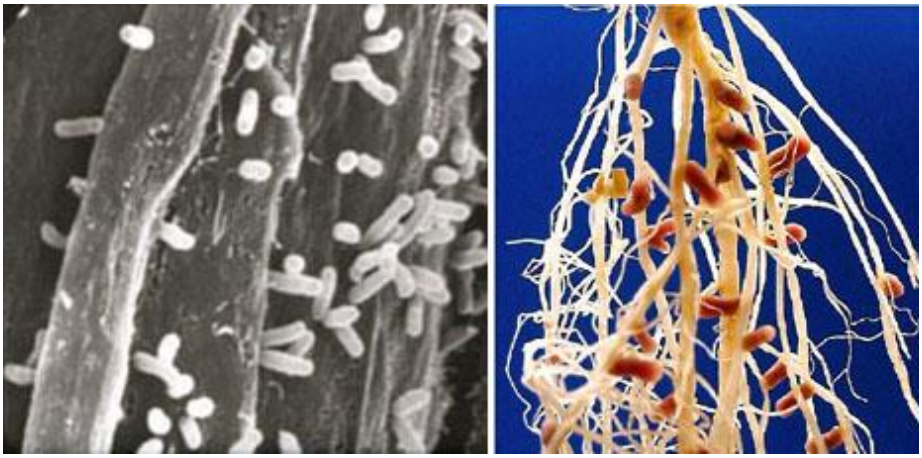

Figure 1. The mutualistic symbiosis between Nitrogen-fixing bacteria and leguminous plants. Left: Nitrogen-fixing *Rhizobium* bacteria found on the root hairs of clover plants. Right: Plant root nodules containing *Rhizobium* bacteria (Kenneth, 2012).

Nitrogen-fixing activity

The nitrogen-fixing activity is the conversion of dinitrogen (N_2) to Ammonia, catalyzed by nitrogenase (enzyme). The biological nitrogen fixation (BNF) is usually carried by Leguminosae plants coordinating with microbes referred to as rhizobia, such as from genera *Azorhizobium*, *Bradyrhizobium*, *Photorhizobium*, *Rhizobium* and *Sinorhizobium* and also found in other genera. Free-living organisms that live in the roots of plants and can stimulate the growth of plants, including help fixing the Nitrogen, is called Plant growth promoting bacteria (PGPB) (Masood, et al., 2020).

Fixed Nitrogen can be obtained via the Biological Nitrogen Fixation (BNF) as a predominant natural source for plants within the cycle of Nitrogen (Oldroyd and Dixon, 2014). Within the roots and in the rhizosphere, plant roots assemble microbial populations. In plant nutrition and productivity, these root-associated microbiomes play a fundamental role (Chen et al., 2019). The activity of BNF in the system of legume-rhizobia is regulated by C and N nutrient exchange between the Nitrogen-fixing bacteria and the host-plant (Li et al., 2018). In the nodules of Legumes, there are symbiosomes which contain the endosymbiotic rhizobial bacteria. These bacteria functioned as temporary organelles for nitrogen fixation (Coba de la Pena et al., 2018). Several environmental factors can affect the rhizosphere microbiome: climate change, type of soil, plant cultivar, and anthropogenic activities (Igiehon and Babalola, 2018).

The mutualistic symbiosis generated when the plants formed nodules in the roots for the bacteria to dwell and take nutrients and carbohydrates from plants to give the plants fixed Nitrogen in return. In rare cases, the BNF process also occurred in the stem. The BNF systems are also found in some non-legumes, such as the symbiosis between Acetobacter *diazotrophicus* and the sugarcane. The Acetobacter even supplies the fixed Nitrogen from the atmosphere and produces auxin as the growth hormone supporting the plants' growth. There are also *Herbaspirillum* organisms that can fix the Nitrogen and occupying the intercellular spaces in maize, rice, sorghum and sugarcane. The *Herbaspirillum* also been found in the roots, stems and leaves of Graminaceae class (Saikia and Jain, 2007). There are also several genera of free-living microbes that can implement the nitrogen fixation without any symbiosis with other organisms, such as *Azotobacter, Bacillus, Clostridium, and Klebsiella*. Li et al. (2017) had studied the genetic diversity of *Pseudomonas* species, *Pseudomonas* also one of the microbe species that can implement the Nitrogen-fixing process. According to Saikia and Jain (2007), those free-living microbes only contribute little to the agricultural crops' nitrogen fixation. One of the last genus species mentioned, *Klebsiella pneumonia*, has been studied for the gene responsible for the nitrogen fixation, which is *nif* genes. Hala (2019) had observed that the experiment of nitrogen-fixing bacteria inoculation into the upland rice plants could increase the dry weight of shoot and root, as well as increasing tiller formation.

Cereal production can utilize the potential alternative Nitrogen source from the Biological nitrogen fixation (BNF) (Rogers and Oldroyd, 2014). The major supply of Nitrogen level in the biosphere is originated from the BNF produced by diazotrophic bacteria, which contributes 30–50% of the crop field's total Nitrogen (Ormeño-Orrillo et al., 2013).

The gene responsible for the nitrogen fixation is *nif* genes. *Nif* gene encodes the enzyme involved in the nitrogen fixation process, which is a nitrogenase complex. The process involves nine nif-related genes: *nifN, nifV, nifB, nifQ, nifE, nifX, nifU, nifS, and nifY*. Many *nif* genes' functions have been detailed at the biochemical level; it is not easy to determine the exact number of genes that can be required to uphold the activity of nitrogenase in plastids or mitochondria. *Nif* gene content supplies comprehensive parts of synthetic biology set for the optimation of nitrogen fixation (Oldroyd and Dixon, 2014). Ouyang et al. (2018) stated that there were several marker genes crucial to the N-cycling process, such as *nif*H (this gene encodes nitrogenase; essential for N-fixation), *amo*A (which encodes ammonia monooxygenase for nitrification), *nir*K and *nir*S (crucial for denitrification, nitrite reductase encoder), and *nos*Z (encoding nitrous oxide reductase, for denitrification).

Since the genes responsible for carrying the nitrogen fixation process had been revealed, the chance to do the gene manipulation for nitrogen fixation is higher over time. Furthermore, the nitrogen-fixing microbe *K. pneumoniae* has a high similarity in structure and physiological processes with *E. coli* as the non-nitrogen fixing microbe. Therefore a gene-transfer between both species is possible to be implemented. Research conducted by Dixon et al. in 1976 had succeeded in transferring genes to non-nitrogen fixing bacteria (Swain and Abhijita, 2013). The next big goal is to transfer such genes to the eukaryotic cells, particularly the non-legumes plants, to fix their own Nitrogen and produce more yield in the end.

The idea of the nitrogen-fixing GM crops

Based on the GM Freeze article (in 2012) US$ 10 million projects from a foundation is allocated for the aim that in 2050, there is a target set to vanish the world's hunger. Some stakeholders had been thinking of developing nitrogen-fixing wheat which will no longer need

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any nitrogen fertilizer in its growth and development. There is always a pro and contra in overcoming the plan. Still, the biggest question would be whether it is possible to create such genetically modified wheat and whether the advantages outweigh the loss.

Naturally, the nitrogen fixation only occurs in legumes. Therefore, another way to enhance the amount of Nitrogen absorbed in non-legumes is by diculture method, which is planting the farm area with legumes before planting the wanted crops so that the Nitrogen in soil is fixed and available for use by the next crops planted. Increasing the diversity of crops can also change soil Nitrogen, the physiochemical properties, and change the soil microbial communities (Linton, 2020). Nitrogen fertilizer is also utilized to enrich the crops with nitrogen supply. However, the excess usage of the nitrogen fertilizer allures many cons which disadvantaging the environment.

The idea of GM crops that can fix their own Nitrogen has arisen for the hope of higher yield to feed the people in undeveloped countries and also for the facts that many cons found in the traditional farming method by nitrogen fertilizer. The practices in agriculture: tillage, cover crops, and Nitrogen fertilization can impact the biological soil parameters, and the physico-chemical properties of the soil (Nivelle et al., 2016). It can alter the soil surface chemistry after a period of time (Obour et al., 2017) and can cause nitrate pollution (Mahmud et al., 2020). Saikia and Jain (2007) had scrutinized some of the adverse effects of the excessive use of nitrogen fertilizers, particularly for the environment. The nitrogen fertilizers applied to the crops do not efficiently absorb; thus, it leaches through the environment causing significant loss financially for the redundant leached materials. The leached nitrogen fertilizers will expose the NO_3 and NO_2 to the surrounding and contaminate the waters and edible crops that can be toxic to plants. For worse, it can cause methemoglobinemia or haemoglobin disorder in infants. The accumulation of the leached nitrogen fertilizer component can also cause the excess of nutrients or eutrophication in surface waters, which can poison the aquatic organisms living in it. In the end, the severely contaminated environment will take years to be restored, either by sophisticated bioremediation, which take more years of research or in any other way which implied more significant potential loss than the advantages. In the farmer point of view, the leaching of nitrogen fertilizer has caused a lot of money wasted to pollute the environment rather than doing the work of being absorbed by the crops. The end yield results will not be optimally increased because of the leached fertilizer to the environment. Researchers noted some negative environmental and human health impacts of the reactive nitrogen leaching from agricultural soil, comprising groundwater contamination, eutrophication of ecosystems, tropospheric pollutions, and nitrous oxide (GHG) accumulations (Zhang et al., 2015). Optimizing nitrogen fertilizer use efficiency to lessen those environmental burdens is a site and crop type-dependent challenge (Jiang et al., 2019). Moreover, the Nitrogen fertilization may affect the balance of the ecosystems by reducing the microbial biomass and decreasing the CO_2 emissions in soil (Treseder, 2008 in Li et al., 2017). Zhao et al. (2019) has found that the utilization of Nitrogen fertilizers affected the Protists more strongly, compared to bacterial and fungal communities. The use of a huge amount of nitrogen fertilizer also makes the price of crop production rises (Guo, et al., 2020).

Nitrogen uptake of plants and Nitrogen transformation (such as nitrification process) in soil affects the increase in soil acidification as well as releases protons (Zamanian et al., 2018). Artificial fertilization in soil can also change the structure of the communities of natural plants (Liu et al., 2017). A study by Ruan et al. (2016) also implied that the use of Nitrogen fertilizer might also interfere with the climate benefits obtained from the production of cellulosic biofuel. Climate change can also be contributed from the release of greenhouse gases, like nitrous oxide, which originated from Nitrogen fertilizers (Ibáñez et al., 2017). Over time, Nitrogen fertilization, along with tillage, can be expected to change the dynamics of micronutrients in the soil and plants (Shiwakoti et al., 2019).

The proposed idea of nitrogen-fixing GM crops, particularly wheat, is aimed to overcome those stated cons of the traditional diculture and nitrogen fertilizer. With the nitrogen-fixing wheat, there must be no need to spend, or waste, more time to plant the legumes and remove the legumes for the crops to grow. There will also no need to buy the costly nitrogen fertilizer and put some effort into fertilizing the crops. The surrounding environment does not need to be contaminated with the leaching of nitrogen fertilizer, means no organisms will be poisoned and no bioremediation project to be researched and implemented to restore the polluted environment. The primary purpose of the whole nitrogen-fixing wheat project is to support crops to produce their own Nitrogen and, in the end, will produce a higher amount of yield. The extra yield produced will eventually bring more profit to the farmer as a financial benefit, and will be enough to feed the people in need to fight famine.

Since there are many hopes and promises offered by the nitrogen-fixing wheat (NFW) project, the next step is to find a path to reaching the goal. The molecular biology scientist plays the most important key role in this project, even before the funding stakeholder. Several approaches had been proposed for the method. The first one is that the research needs to be conducted to find the specific responsible genes for nitrogen-fixing and transfer it from the microbe source to the wheat and make the wheat express the genes to fix its own Nitrogen, such as transferring the *nif* genes or other genes. Untergrasser et al. (2012) had researched a method to transform eight genes essential for Rhizobium symbiotic signalling, which will enable the non-legumes to perform a symbiotic relation with rhizobia in the future, thus will be able to fix their own Nitrogen. The non-legumes plants tested in the study were strawberry, poplar, tomato and tobacco. Those plants studied were transgenic plants that have been "injected" with the nodulation factor (nod) genes to form their own root nodules. Even though the transgenic plants studied expressed the Nod factor signalling genes, the symbiotic responses between the non-legumes studied, and the rhizobia could not be detected, indicating the lack of response.

Further research and method need to be done to modify the non-legumes to form nodules and put the NFW microbes in the nodule to help to fix the Nitrogen. Other than the natural nodule, the plant hormones, such as 2,4 D, NAA, BAP, and zeatin, could also induce the formation of the nodule-like structure, referred to as para-nodules. The para-nodules, found on the hormones-induced cereal roots, differ from the natural legumes nodules as the para-nodules are modified lateral roots (Saikia and Jain, 2007). The para-nodules induced by plant growth hormones could also be a future solution of nodule-like formations in the non-legumes, which can be studied and developed further to enable the nitrogen-fixing microbes to colonialize the nodules in non-legumes, especially in wheat.

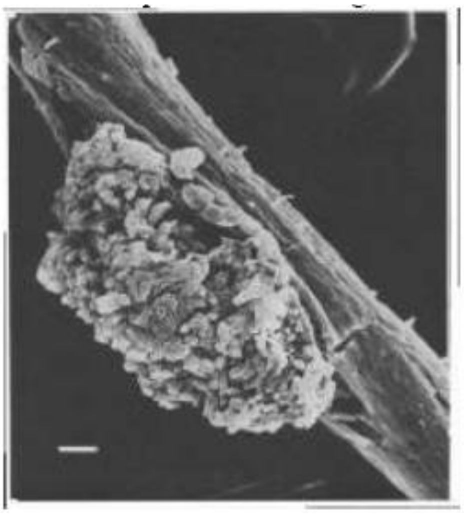

Figure 2. Scanning electron micrograph showing a para-nodule of wheat inoculated with *Rhizobium leguminosarum* (Swain and Abhijita, 2013)

Future Perspective

Symbiotic nitrogen fixation is largely limited to legumes. Still, there is an array of microorganisms, including some diazotrophs that inhabit the rhizosphere of other crop plants, which have been shown to enhance plant growth. The mechanisms involved in plants and microbes that lead to the formation and function of symbioses will help us in transferring these traits and processes to non-leguminous crops, especially in cereals. Advanced understanding of BNF, bacterial association with non-leguminous plants, and the microbial community composition of the rhizosphere population have led to several future research ideas for scientists; for instance, engineering non-legume plants to

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nodulate and establish symbiotic nitrogen fixation, and the formulation of new associations between nitrogen-fixing microorganisms and crop plants. The introduction of nitrogenase-encoding bacterial *nif* genes into non-legumes is challenging due to the complex nature of nitrogenase biosynthesis and the extreme sensitivity of nitrogenase to the presence of oxygen. Extensive genetic and biochemical studies have identified the common core set of genes/gene products required for functional nitrogenase biosynthesis (Rubio and Ludden, 2008 in Mahmud et al., 2020).

Studies of evolutionary genomics suggest that relatively few genetic elements are needed to bestow nitrogen-fixation capabilities from legume to non-legume plants. Transferring nitrogenase to plants requires the concatemerization of bacterial genetic units to create a minimum set of three genes. Advanced understanding of BNF, bacterial association with non-leguminous plants, and the microbial community composition of the rhizosphere population have led to several future research ideas for scientists. Some of the ideas are: to perform genetic engineering toward non-legume plants to nodulate and establish symbiotic nitrogen fixation, and to discover the formulation of new associations between nitrogen-fixing microorganisms and crop plants. In addition, potential subcellular (micro-pockets of air) low-oxygen environments offered via plastids and mitochondria to express active nitrogenase in plants making this engineering strategy feasible (Currati and Rubio, 2014 in Mahmud et al., 2020).

The genetically modifying method to generate nitrogen-fixing non-legumes carries a significant chance of failure results and hindrance. The multilevel implication occurs when we need to modify the plants that do not normally produce nodules in their roots to form the nodules and modify the Nitrogen-fixing microbes to live in the nodules of non-legumes, which are not their natural dwelling places. In terms of funding, there must be very high funding needs to afford the research and experiments until this method proves to be successful. It will surely take more time and effort to reach the successful experiments, while 2050, as the target year to diminish famine, is only thirty years away by the time this study is written. Moreover, although the experiments are successful, it needs to prove that the nitrogen-fixing crops (wheat) will produce a greater amount of yield, which will compensate for the fund and efforts spent. The further possible issue is that the GM plantation will urge the monoculture planting method, which only uses one kind of crop compared to the diculture crop rotations by planting legumes before the main crops. The monoculture method will drain the soil aeration and fertility since no plough or more aeration like the rotation planting. Additionally, the GM crops problem might arise, such as herbicide tolerance and weed resistance problems (GM Freeze, 2012). Into the bargain, the GM seeds would cost so much higher to buy and plant than providing crop rotations and nitrogen fertilizers. On the other hand, more traditional methods offer a higher probability of successfully fulfilling the food security target in 2050. Since the lack of nitrogen fertilizer in the developing country caused the less crop yield which may lead to famine and malnutrition (Good, 2018), the funds could be allocated for the nitrogen fertilizer donation or providing more legumes and crop rotations instead. The funds can also be allocated toward more environmentally friendly fertilizer research, such as the organic fertilizer from fish by-product or waste (Zahroh et al., 2018), or fertilizer from tofu processing waste (Amalia et al., 2018). Even though the nitrogen fertilizers are worried to be leached into the environment and cause contamination, the pollution effect is not that high compared to the benefits obtained, and the cost of bioremediation is must be cheaper than the molecular GM research experiments.

In the term of religious view, the Ecosophy of Islam can be a basis of ethics toward environment preservation (Rusmadi, 2016). A lot of Quranic verses reveal the importance of preserving the environment, some of them are: Al-Baqarah 11, Al Baqarah 60, Al Baqarah 205, Al Maidah 64, Al A'raf 56, Al A'raf 74, Al A'raf 85, Al A'raf 142, Hud 85, Asy-Syu'ara 183, Al Qasas 77, and Al 'Ankabut 36. All of those verses implied how God Asks humans not to damage or abuse the earth. All in all, whichever method or approach is chosen to be implemented to fight famine, it has to give a greater good for both the humans and the environments.

Conclusion

In conclusion, it is feasible to conduct a project to make genetically-modified crops that can fix Nitrogen, but the difficulties and the funds needed still outweigh the benefits obtained in the future. With all of those limitations, the target goal to erase famine in 2050 just by funding the nitrogen-fixing wheat alone seems to be too high to be reached. The funds and efforts should be better spent on other factors and farming methods.

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