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SUSTAINABLE NITROGEN SUPPLY - RESEARCH ON NITROGEN-FIXING BACTERIA AND THE DEVELOPMENT OF PRODUCTS CONTAINING NITROGEN-FIXING ORGANISMS IN CONTEMPORARY LITERATURE

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This work provides a comprehensive overview of research on nitrogen-fixing microorganisms from their discovery to the present, with a particular focus on the development of inoculants containing these organisms. Scientific interest in nitrogen-fixing bacteria began to increase in the late 19th century, with early studies concentrating on their nitrogen-assimilation functions. By the early 20th century, systematic research was underway to clarify the ecological and agricultural significance of these microorganisms, assess the efficiency of their nitrogen-fixing mechanisms, identify their biotic and abiotic requirements, and develop methods to enhance their efficiency. Research in the 1960s shifted its focus toward enhancing the nitrogen-fixing capacity of nitrogen-fixing plants and inoculating them with nitrogen-fixing agents. From the 1970s onward, new research perspectives and approaches emerged. Since the 1980s, plant and soil conditioning preparations containing nitrogen-fixing microorganisms have appeared in the market, and their numbers increased significantly in the 1990s and 2000s, although their nitrogen-fixing capacity could not be quantified at that time. By the end of the 2010s, preparations containing endophytic nitrogen-fixing bacteria emerged. These bacteria multiply by entering the tissues of the host plant through gas exchange pores after spraying, providing a significant amount of 20-40 kg/ha of fixed nitrogen.
These new developments are important for agriculture because they are not species-specific and provide the nitrogen demand of agricultural production in a quantifiable way - partially or completely. From a sustainability perspective, it is important that the use of these products can partially replace nitrogen fertilizers, which are not only harmful to the environment in terms of production but also in terms of their release into nature.

1. Introduction

Plant growth-promoting rhizobacteria (PGPR) are soil bacteria that stimulate plant growth either directly or indirectly. These bacteria mobilize nutrients (e.g., nitrogen and phosphorus), produce plant hormones, and help plants cope with pathogens and environmental stressors. The use of PGPR offers a sustainable alternative to chemical fertilizers and pesticides, contributing to environmentally friendly agriculture. In crop production, microbial inoculants—preparations containing selected bacteria and fungi—have been used for decades. These are typically applied either to the surface of seeds, directly into the upper layer of the soil, or even directly onto the plant. The aims of microbial inoculation are (1) to supply the cultivated plant with nutrients, (2) to stimulate plant growth, e.g., through the production of plant hormones, (3) to inhibit or control plant pathogens, and (4) to improve soil structure (Bona et al., 2016).

For the implementation of environmental protection goals and tasks, microbial inoculants are also applied in bio-/phytoremediation processes or to enhance their efficiency. Such practices include, for example, the biodegradation of organic pollutants through biological inoculation, or the bioaccumulation, immobilization, or microbial-assisted "washing" of inorganic contaminants (e.g., heavy metals, toxic elements) from the soil (Khoso et al., 2024).

Microbial inoculants based on biological nitrogen fixation comprise bacteria capable of converting atmospheric nitrogen into plant-available forms. The agronomic application of nitrogen-fixing inoculants has the potential to reduce reliance on synthetic nitrogen fertilizers, contributing to the mitigation of greenhouse gas emissions and decreasing the risk of soil degradation (Soumare et al., 2020). Scientific studies have demonstrated that the application of carefully selected bacterial strains, adapted to specific environmental and cultivation conditions, can significantly enhance the efficiency of biological nitrogen fixation and increase crop yields across diverse agroecological systems.

Biological nitrogen fixation is a key process in the nitrogen cycle, providing a significant portion of the nitrogen requirements for the terrestrial biosphere. Nitrogen-fixing organisms are highly diverse and may be autotrophic, heterotrophic, chemolithotrophic, photoheterotrophic, or methanogenic (Reed et al. 2011). The physiology and functional biology of nitrogen-fixing organisms are primarily determined by the properties and requirements of the nitrogenase enzyme, which is highly sensitive to oxygen and contains protein subunits with essential cofactors such as molybdenum, iron, or vanadium. Nitrogen fixation is an energy-intensive process, and its activity can be inhibited by high concentrations of available combined nitrogen forms, such as NO3- or NH4+.

Only prokaryotic organisms (Bacteria and Archaea) are capable of fixing atmospheric nitrogen, which can be free-living or plant-associated (symbiotic) microorganisms (Sepp et al., 2023). The best-known symbiotic nitrogen fixers are bacteria that form nodules in the roots of leguminous plants, belonging to several genera within two lineages: Alphaproteobacteria (e.g., Bradyrhizobium, Azorhizobium, Rhizobium, Sinorhizobium, Mesorhizobium) and Betaproteobacteria (e.g., Burkholderia, Ralstonia) (Shamseldin et al., 2017). Bacteria of the genus Frankia (Actinobacteria) live in symbiosis with dicotyledonous plants. Filamentous cyanobacteria from the family Nostocaceae are associated with various plant species, including cycads and the aquatic fern Azolla (Xu et al. 2023). Associative nitrogen-fixing bacteria (e.g., Azospirillum and Herbaspirillum) live in loose associations with plants, contributing to their nitrogen supply, though their efficiency is lower

than that of symbiotic bacteria. Free-living nitrogen-fixing microorganisms can fix atmospheric nitrogen (N_2) without being attached to a host plant, including species of Azotobacter, Clostridium, and some cyanobacteria (Orr et al., 2011).
Researchers have achieved significant results from the late 19th century to the present day, from the discovery of nitrogen fixers to soil inoculation, to the development of bacterial fertilizers and, today, efficient endosymbiont nitrogen fixers. This review article provides a comprehensive overview of the research and methodological advancements concerning microbial products containing nitrogen-fixing bacteria, with a particular focus on studies conducted in Hungary.

The manuscript summarizes commercially available formulations comprising diazotrophs and outlines potential directions for future development.

2. Theories and Early Observations

Cserháti underscored the importance of soil biological processes for soil fertility in his 1894 book Soil Knowledge discussed in (Birkás, 2009). The first use of Rhizobium inoculants in Hungary was conducted by Kerpely in 1896 (Szegi, 1968). Subsequently, the work of Beke, titled "Agricultural Bacteriology", presented numerous soil biological aspects related to the nitrogen cycle and cellulose mineralization in 1908 (Szegi, 1968). In recent years, soybean cultivation has shown significant growth in Hungary, with the sown area reaching 111,000 ha in 2024. Since soybeans are not native plants, inoculation with nodule-forming Bradyrhizobium japonicum is essential.

3. Practical Research

At the beginning of the 20th century, researchers had already explored the presence of Azotobacter in soils and the nitrogen-fixing ability of microbial flora. They established that "the amount of bound nitrogen in the soil depends on the species, prevalence, and the favourable or unfavourable living conditions of nitrogen-fixing microorganisms". Studies by Beyerinck, Berthelo, Remy, Thiel, and Schneidewind indicated that these microorganisms could increase the nitrogen content of soils by 20-40 kg/y and, under optimal conditions, by 80-100 kg/ha. They also discovered that phosphorus fertilization could enhance nitrogen fixation, while nitrogen fertilization could reduce it. Further correlations were identified between pH, moisture content, and the efficiency of nitrogen fixation in soils (Herke, 1913).

4. Soil Inoculation

Hungary has a tradition of using Rhizobium inoculants. The first such experiments were conducted by Kálmán Kerpely, who set up open-field trials with Hiltner's first commercially available Rhizobium inoculant, Nitragin. In Sándor Herke's field experiments, Rhizobium and Azotobacter preparations increased plant yields, though preparations based on root nodule bacterial cultures were not always effective. During the production of the first generation of inoculants, researchers focused on ensuring adequate nutrient supply for host plants.

The initial soil inoculations used soil from leguminous plants, assuming that the bacteria residing in the root nodules of host plants were also present in the soil. However, this method was disadvantageous due to the movement of large quantities of soil; consequently, bacterial cultures isolated and propagated from root nodules were developed as the first root nodule bacterial inoculants. The symbiont "Nitragin" (1896, Germany), associated with Hiltner, and "Azotogén" (Dresden) contained free-living nitrogen-fixers. In England, inoculants were marketed under names like Nitro Bacteria, while in America, products such as Nitro Culture, Farmogen, and Hey's Concentrated Nitrogen Producer emerged concurrently with Nitragin. In Hungary, the National Hungarian Royal Plant Production Experimental Station in Magyaróvár was involved in soil inoculant development. In 1925, Kreybig established a soil biology laboratory in Cserhátsurány, where he investigated the effects of Rhizobium inoculants. During the 1940s, Antal Kerpely became one of Hungary's most significant researchers in the field of Rhizobium. His publications demonstrated that inoculants containing dried tuber meal resulted in yield increases of about 10-20 % in legume plants under local conditions.

More than 70 % of a large number of open-field experiments conducted across diverse climatic and soil conditions in the country yielded positive results. The BAKTOLEG inoculant was developed for the five economically most important leguminous host plants: pea, alfalfa, broad bean, tests, and soybean. At that time, both symbiotic and free-living nitrogen fixers were intensively researched, and efforts were made to quantify the extent of nitrogen fixation. For example, Machay indicated that Azotobacter was present in all soils and was effective regardless of plant species, while Rhizobium was also found in all soils but fixed nitrogen only in the presence of leguminous plants.

The former could bind 5-10 kg/y of nitrogen, while the latter could achieve 30-40 kg/acre (Machay, 1958). The Hungarian inoculant known as Baktonit was developed in the Soil Bacteriological Laboratory under the Danube-Tisza Chamber of Agriculture. Its efficacy was comparable to that of similar foreign preparations. For an extended period, there was no large-scale inoculant production in Hungary. However, from the 1940s onward, production increased significantly, with the aforementioned Hungarian company producing sufficient inoculant for annual domestic use on approximately 50,000 ha of land between 1946 and 1949. Due to its regular and consistent operation, this laboratory can be regarded as the first hub of Rhizobium inoculant production in Hungary.

The Agrobiology Department of the Phylaxia State Inoculant Production Institute was established from this laboratory in 1950, where Baktonit was produced in increasing quantities according to demand. In 1958, the Ministry of Agriculture introduced mandatory controls for Rhizobium inoculants (Manninger, 1966). Subsequently, agar and powder preparations were marketed in Hungary under the name Rhizonit.

In the 1970s, the nitrogenase enzyme responsible for biological nitrogen fixation was isolated (Hoffman et al., 2014). Researchers utilized new methods in bacterial and plant genetics to investigate which genes are "responsible" for the function of the nitrogenase enzyme and whether these genes can be transferred to non-leguminous plants. The goal was to produce cell fusion hybrids between cereal and leguminous plants, enabling the non-leguminous host plant to establish a symbiotic nitrogen-fixing relationship with Rhizobium (Cocking et al., 1981). Modern microbial inoculants are expected to comply with sustainable agricultural practices, exhibiting stability and viability, a broad spectrum of effects, environmental friendliness, and economic feasibility.

Contemporary crop intercropping practices, including legume-cereal intercropping and the utilization of legume-containing cover crops, promote soil regeneration and positively impact the genetic and functional diversity of soil microbial communities, including nitrogen fixers (Biró et al., 2000). The mycorrhizosphere of plants colonized with arbuscular mycorrhizal (AM) fungi is enriched with plant growth-promoting microorganisms. Legume plants particularly benefit from mycorrhizal fungi, as the increased phosphorus uptake facilitated by these fungi significantly contributes to meeting the energy demands required for nitrogen fixation (Takács and Vörös, 2003).

5. Bacterial Fertilizers

In Hungary, the marketing and use of bacterial fertilizers were first authorized in 1987 (Phylazonit). This nitrogen-fixing bacterial fertilizer, developed by the Phylaxia Inoculant Production Company, contains nitrogen-fixing strains of Azotobacter and Rhizobium, along with plant hormones (gibberellin, heteroauxin) and B vitamins. Over the past few decades, multiple microbial bacterial fertilizers have been introduced on the market. In addition to nitrogen-fixing organisms, new-generation inoculants typically incorporate other plant growth-promoting rhizobacteria and fungi that mobilize nutrients, produce antibiotics, and have specific active ingredients suited to particular plant species or environmental conditions.

Following Phylazonit, products such as Fitokondi (containing Azotobacter), Terra-Vita, Bactofil (containing Azotobacter and Azospirillum), EM-1, Biorex, Mikro-Vital, Microbion, Azoter, Bionitrophos, E-2001, and Symbion preparations were introduced. Some of these products remain in use today. In the past nearly 40 y, the application of bacterial fertilizers on arable land has grown, but farmers do not primarily view these products as partial replacements for nitrogen fertilization; rather, they utilize them mainly for their positive physiological, metabolic-stimulating, and possibly yield-enhancing effects.

Recent research is attempting to develop a biological fertilizer using the nitrogen-fixing cyanobacterium Trichormus sp. PCC7120, with the aim of developing a sustainable farming method for growing crops without chemical fertilizers. Two types of rice (Oryza sativa L. 'Sasanishiki' and 'Koshihikari'), broccoli (Brassica oleracea var. italica) and melon (Cucumis melo L.) were grown hydroponically with intracellular extracts of Trichormus sp. PCC7120, which were prepared by heat treatment or acid hydrolysis. The results of the studies showed that the growth of the plants with Trichormus extracts was comparable to that of the fertilizer group (Haraguchi and Shimizu, 2025).

6. Endosymbiont Nitrogen Fixers

In addition to bacteria that closely associate with plants and engage in symbiotic nitrogen fixation, lighter associative systems also exist (Table 1). The common characteristic of these systems is that their nitrogen fixation is less efficient. However, their significant advantage lies in their less strict host specificity, allowing for broader agricultural use. Some bacteria adhere to the plant surface, occasionally attaching to leaves, but they predominantly reside in the root zone. A notable group of microorganisms can settle within the roots of the plant, occupying the spaces between the cells of root tissue and performing nitrogen-fixing activities. Associations with grasses are the most prevalent in nature. The nitrogen fixation efficiency of associative systems typically ranges from 20 to 200 kg/ha/y. Additionally, many nitrogen-fixing bacteria produce growth-stimulating hormones, which, alongside nitrogen supply, positively influence plant health

(Mus et al., 2016). A preparation containing Herbaspirillum (diazotrophic) bacteria was marketed as the first endophyte in Hungary. Species of the genus Herbaspirillum (e.g., H. frisingense, H. rubrisubalbicans, and H. seropedicae) establish symbiotic associations with a range of economically significant Poaceae crops, including maize (Zea mays), rice (Oryza sativa), sorghum (Sorghum bicolor), and sugarcane (Saccharum spp.) (Monteiro et al., 2012).

These bacteria contribute to the enhancement of plant growth and yield through multiple mechanisms, with biological nitrogen fixation being a primary factor. Consequently, the optimization and deployment of these plant growth-promoting bacteria represent a promising strategy for achieving both agronomic and environmental benefits. Herbaspirillum bacteria can survive and multiply within the tissues of host plants post-spraying (Matteoli et al., 2020).

They enter plant tissues through leaf stomata and fix nitrogen from the atmosphere, stimulating crop growth by providing an estimated 30-40 kg of nitrogen per ha. Alves et al. (2015) found that H. seropedicae promotes maize hybrid SHS5050 plant growth and contributes to its development with fixed nitrogen. In this study, Herbaspirillum sp. strains were isolated and selected for field inoculation procedures. The variation in response to inoculation depended on the selected bacterial strain and the genotypes of the plant partners.

Microorganisms that have demonstrated plant growth-promoting activity in previous studies — not necessarily through biological nitrogen fixation — may also represent promising candidates for the development of next-generation inoculants. Bacillus mojavensis KJS-3 (Moja-3), isolated from food waste, has demonstrated antifungal activity against Aspergillus terreus, A. fumigatus, A. flavus, and Fusarium redolens, indicating its plant growth-promoting and biocontrol properties.
According to Serafin-Andrzejewska et al. (2025), foliar application of the bacterial strain Methylobacterium symbioticum had yield-enhancing effects on faba bean that were comparable to, or better than, those achieved through seed inoculation with Rhizobium leguminosarum bv. viciae. Modern cultivations employ preparations containing three nitrogen-fixing bacteria based on the plant growth-promoting activity of Bacillus mojavensis, Methylobacterium symbiothicum (Torres et al., 2024), and Azotobacter salinestris (Chennappa et al., 2017), each displaying different lifestyles.

These products already demonstrate a declared nitrogen yield, replacing the need for artificial fertilizers and ensuring more sustainable crop production. Extensive research is currently focused on the availability of other nutrients crucial for plants in the soil, possibly correlating with nitrogen metabolism (Dorka-Vona et al., 2019). In addition to traditional techniques, researchers are developing and testing novel analytical methods for the cost-effective determination of essential physical and chemical soil parameters (Vona et al., 2020), to achieve tests that can be performed rapidly, providing immediate results beneficial for both practical nutrient supply and research (Kalocsai et al., 2024).
This capability is also particularly significant from a soil microbiological perspective (Giczi et al., 2021).

Table 1: Composition of the nitrogen-fixing microbial plant growth - promoting products approved in Hungary

Nitrogen-fixing bacteria	Others	Form of the bacteria	Function of the product
Azotobacter sp.	Pseudomonas fluorescens, algae	FL	FF
Bacillus mojavensis	-	ES	FF
Herbaspirillum seropedicae	-	ES	FF
Rhizobium leguminosarum	-	S	SC
Bradyrhizobium japonicum (2 products)	-	S	SC
Azotobacter vinelandii, Azospirillum brasilense	Bacillus megaterium, Bacillus polymyxa, Streptomyces albus, P. fluorescens	FL/FS	SC
Azotob. vinelandii, Azospirillum lipoferum	B. megaterium, Bacillus circulans, Bacillus subtillis, Micrococcus roseus, P. fluorescens	FL/FS	SC
Azotobacter chroococcum	+18 microorganisms	FL	FF
Azosp. brasilense, Azospirillum irakense	Pseudomonas jessenii, Arthrobacter crystallopoietes, Pseudomonas chlororaphis, Priestia aryabhattai, Azospirillum largimobile	FS	SC
Azosp. brasilense	Bacillus simplex, Pseudomonas frederikbergensis, Agreia pratensis, Paenibacillus peoriae, Priestia aryabhattai, Azosp. largimobile		
Azosp. brasilense	P. jessenii, A. crystallopoietes, Kocuria rosea, Azosp. largimobile	FS	SC
Br. japonicum	-	S	SC
R. leguminosarum	-	-	SC
Azotob. chroococcum, Azosp. lipoferum	Pseudomonas putida	FL/FS	SC
Methylobacterium symbioticum	-	ES	FF
Derxia lacustris, Gluconacetobacter diazotrophicus, (Bacillus amyloliquifaciens), (Rhizobium agrobacterium)		FL/ES	SC
Azotob. chroococcum, Azosp. vinelandii, Azosp. brasilense, Pseudomonas stutzerii	Cellvibrio sp., P. fluorescens, B. megaterium, Microc. roseus	FL/FS	SC
Gluconacetobacter diazotrophicus	-	ES	FF
Azotobacter sp.	-	FL/FS	SC
Azotob. vinelandii, Azosp. brasilense	P. fluorescens, B.circulans,Cellvibrio sp.,Streptomyces cellulose	FL/FS	SC
Rhizobium etli, Ensifer melioti, Rhizobium pisi, R. leguminosarum	-	S	SC
Bacillus paramycotinoides, Bacillus safensis, Paenibacillus polymixa	-	ES	FF
(Bacillus amyloliquefaciens)	-	-	SC

Table 1 (Continued)

Nitrogen-fixing bacteria	Others	Form of the bacteria	Function of the product
Azosp. brasilense, (B. amyloliquefaciens, Rhizobium agrobacterium)	-	FL/FS	SC
Bacillus sp, Azospirillum sp.	-	FL/FS	SC
(Bacillus megaterium)	-	-	C
Azotob. chroococcum, Azosp. lipoferum	B. subtilis, B. megatherium	FL/FS	SC
Azotob. vinelandii, Azosp. brasilense	B. megaterium, B. subtilis, Clostridium pasterianum, Rodobacter sphaeroides, Lactobacillus plantarum, Trichoderma reseei, Saccharomyces cerevisiae, Streptomyces albus	FL/FS	SC
B. mojavensis	-	ES	FF
Methylobacterium	Arthrobacter sp.	ES	FF
Azosp. brasilense	B. amyloliquefaciens	FL/FS	SC/FF
Paenibacillus polymyxa, Azotob. Croococcum, (Cellvibrio flavescens)	B. megaterium+fungi	FL	SC
Paenib. polymyxa,(Paenibacillus amylolyticus), (Paenibacillus macerans)	Cellvibrio flavescens +fungi	FL	SC
Paenib. Polymyxa, Azotob. croococcum, (Cellv. flavescens)		FL	SC
Paenib. polymyxa, Azotob. croococcum, Br. japonicum, R. leguminosarum, (Cellv. flavescens)	B. megaterium +fungi	FL/S	SC
Azotob. vinelandii, Azosp. lipoferum	B. subtilis, B. megaterium	FL/FS	SC
Azotob. vinelandii, Azosp. lipoferum	Bacillus licheniformis	FL/FS	SC
Br. japonicum	B. megaterium, B. subtilis, Pseudomonas putida, P. fluorescens	S	SC
Azotob. chroococcum	P. putida, B. circulans, B. megaterium	FL	SC
Rhizobium melioti, Ensifer adherens	B. subtilis, P. fluorescens, P. putida, B. megaterium	S/FL/FS	SC
Br. japonicum (5 products)	-	S	C
Azotobacter salinestris	-	ES	FF
( ): the distributor claims nitrogen fixation capacity		*	**

*FL: free-living, FS: facultative symbiont, ES: endosymbiont, S: symbiont;

**: FF: foliar fertiliser, C: coating, SC: soil conditioner;

Azospirillum: Azosp.; Azotobacter: Azotb.; Bacillus: B.; Bradyrhizobium: Br.; Cellvibrio: Cellv.; Pseudomonas: P; Rhizobium: R.

In Hungary, the national regulatory authority has authorized 345 microbiological formulations, among which 48 contain nitrogen-fixing bacteria. Of these products, 32 are classified as soil conditioners, 11 as foliar fertilizers, and 6 as seed treatments. The nitrogen-fixing microorganisms used in these products include 50 % free-living, 39% facultative symbiotic, 30 % symbiotic, and 20 % endosymbiotic strains. Furthermore, 79 % of the registered formulations are suitable for use across multiple plant species, while the remaining products are soil inoculants or seed treatments specifically developed for soybean or other leguminous crops.

7. Conclusions

Research into biological nitrogen fixation has progressed significantly, transitioning from early soil inoculation attempts to the development of contemporary endosymbiont bacterial preparations. In response to the pressing challenges of sustainable agriculture, the research and implementation of biological solutions have gained paramount importance. These developments contribute to the reduction of synthetic fertilizers, alleviating environmental and energy burdens. The scientific evaluation of commercially available microbial products and inoculants is crucial for verifying their beneficial effects. However, research emphasizes the necessity of implementing robust quality control mechanisms to ensure that effective and reliable products are delivered to end users. Microbial inoculants play a crucial role in supporting the ecological adaptation and successful cultivation of legumes and protein crops. The concept of sustainable farming is based on balanced nutrient management, rational nutrient replenishment, and the use of environmentally friendly technologies. Sustainability can be achieved by taking into account ecological, biological, and agrotechnical factors.

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