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Enabling biological nitrogen fixation in agriculture: An eco-industrial perspective

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ABSTRACT

Food production, including cereal crops such as maize, rice, and wheat, and other products like oilseed, tubers, dairy, meat, fish and sugar, accounts for up to 50 % of global crop output and is projected to rise along with population and income growth. As industrial agriculture heavily relies on mineral fertilizers, for sustaining crop growth, the global fertilizer consumption is also projected to rise in the upcoming years. Nitrogen is the most critical nutrient for crops, so that N-based fertilizers are the most widely used worldwide. However, excessive N fertilization leads to remarkable environmental damage and economic losses annually. Enhancing nitrogen use efficiency (NUE) by aligning nitrogen supply with plant demand is crucial for more sustainable agriculture. Biological nitrogen fixation (BNF) presents a promising strategy to reduce synthetic N use. However, integrating BNF into industrial agriculture requires strategies that address both biological and technical challenges. This review discusses the limitations and feasibility of existing strategies to enable BNF in non-leguminous crops. Currently, issues such as consistency and scalability of microbial products, along with regulations, are amongst the main limitations to the adoption of BNF in agriculture. In addition, as the production of bioformulations has not been standardized yet, many products still lack reproducibility, stability and applicability. In this review, relevant factors contributing to the design of effective microbial formulations are discussed, and product design is proposed as alternative valuable strategy which – coupled with appropriate proof of agronomic efficacy – can enable BNF-based solutions as more sustainable fertilization practices. Therefore, the development of effective microbial formulations aiming at reducing N input, losses, and dependence on synthetic fertilizers, is described in the light of both industrial and ecological perspectives. The review remarks the potential of biofertilizers as tools to enable BNF in agriculture and how an eco-design can help developing more effective, stable and hence scalable products. On the other hand, beside the increasing market demand, the regulatory framework is still a major barrier, playing an important role in the identification and formulation of reliable protocols aimed at standardizing the production of microbial products for fertilizing purposes.

1. Introduction

The 2023 Organisation for Economic Cooperation and Development – Food and Agriculture Organization (OECD-FAO) joint report projects an increase in global agricultural production over the next decade, resulting from both population and income growth (OECD/FAO (2023), 2023). Food production is amongst the key agricultural commodities, accounting for up to 50 % of the global crop output, followed by animal feed (26 %), and biofuels and industrial products (8 %). To meet the growing demands of food production, industrial agriculture – henceforth referred to as industrial agriculture – heavily relies on the use of mineral fertilizers, such as nitrogen (N), phosphorus (P), and potassium (K). These nutrients are crucial for crop growth, with nitrogen promoting leafy growth, phosphorus supporting root and flower development, and potassium enhancing overall plant health (Corradini et al., 2010; Gu et al., 2009). In 2022, the global consumption of fertilizers accounted for 190 Mt annually and it is projected to exceed 200 Mt by 2026 (IFA Market Intelligence Service, 2023). Along with the agricultural production, the global demand for fertilizers is also anticipated to increase (IFA Secretariat, 2021).

Mineral N, P, K fertilizers are applied based on crop needs and soil conditions, with the choice of application – either individual or in

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combination – depending on factors such as soil nutrient status, crop type, and local agronomy practices. Quantitatively, N is one of the most important nutrients for crops and its deficiency is a common limiting factor in many agricultural systems, significantly restricting plant growth and development (Francis et al., 2023). Fertilizer use, particularly nitrogen-based compounds, varies by region, crop type, and management system, with N-based fertilizers being among the most commonly applied to address these specific deficiencies (Kraiser et al., 2011).These compounds include urea, ammonia, ammonium nitrate, ammonium sulphate, as well as the phosphorus-potassium salts like ammonium phosphate and potassium nitrate, which can be used individually or in combination. Among these compounds, urea is the most widely used nitrogen fertilizer globally, with an annual production exceeding 50 million tons according to FAO data. Fig. 1 shows the most utilized N fertilizer compounds globally, as according to the International Fertilizers Association – Statistics (IFASTAT) 2023 database (Keshavarz Afshar et al., 2018; Masjedi et al., 2024).

The environmental cost mainly resulting from run off, leaching and gasification, was estimated as 5.1 billion US dollars (USD) in 2007 only in the US, and was projected to reach up to 24.9 billion USD worldwide in 2030 (Good & Beatty, 2011). In more recent assessments, pollution from nitrogen and phosphorus in freshwater systems corresponded to a financial burden of at least $2.4 billion per year on the US economy (Dodds et al., 2009; Wurtsbaugh et al., 2019). Furthermore, nutrient contamination in coastal regions is estimated to result in additional annual costs of approximately $100 million (Davidson et al., 2014). Due to the relatively low nitrogen use efficiency (NUE) in current agricultural practices, N inputs and losses have already exceeded the planetary thresholds estimated to ensure safe rates of terrestrial nitrogen deposition and prevent harmful groundwater and surface water pollution (Conijn et al., 2018; Schulte-Uebbing et al., 2022). Therefore, it would be crucial to both alleviate nitrogen losses and improve NUE to preserve soil quality and meet food production needs within the planetary boundaries (Kopittke et al., 2021; Penuelas et al., 2023). In fact, aligning nitrogen supply with plant demand, thereby reducing nitrogen surplus, is believed to be the most effective way to enhance NUE (Bai et al., 2022). Within this framework, biological nitrogen fixation (BNF) is considered a crucial strategy to decrease dependence on synthetic nitrogen inputs and to optimize nutrient supply according to plant requirements (Soumare et al., 2020). However, the adoption of BNF in agriculture is still hindered by various technological, social and cultural factors such as the lack of infrastructure, limited farmer knowledge, and resistance to changing traditional farming practices.

This review discusses available strategies to overcome these barriers and enable the effective integration of BNF in agriculture, considering the current limitations and opportunities. Specifically, the effective design of microbial formulations is explored as tool to enable free-living nitrogen fixation (FNF) in non-leguminous crops, which do not have the natural ability to fix nitrogen like legumes. By harnessing the potential of FNF, the use of BNF as a nitrogen input could be expanded to a wider range of crops beyond legumes, promoting more consistent adoption and ultimately reducing nitrogen inputs and losses on a larger scale. Therefore, the following chapters focus on soil dynamics and their relevance in the design of microbial formulations to enhance plant-beneficial functions, such as FNF, in agriculture.

In section 2 the main nitrogen dynamics occurring in soil are described and discussed with respect to conventional agricultural practices, with a more detailed comparison of biological and industrial nitrogen fixation processes presented in section 3. Sections 4 and 5 describe the mechanisms and types of plant-microbe interactions in soil and their relevance for agricultural purposes. Therefore, sections 6 and 7 discuss current challenges in the utilization of microbial products and highlight the available strategies for enabling BNF in agriculture, including the development of effective microbial formulations to reduce nitrogen inputs and losses, with respect to both industrial and ecological perspectives. It is highlighted how material design should take into account soil microbial and nutrient dynamics – as the ecological perspective – to improve product efficacy, as well as product reproducibility and ease of application – as the industrial perspective – to achieve scalability and increase chances to favour the wide-spread utilization of microbial products in agriculture. Finally, the existing limitations are discussed in the light of the regulatory and market frameworks concerning biofertilizer industry, which are considered as relevant contributors to the adoption of BNF in agriculture worldwide.

2. Nitrogen dynamics

The chemistry of nitrogen compounds in soil is complex, as various

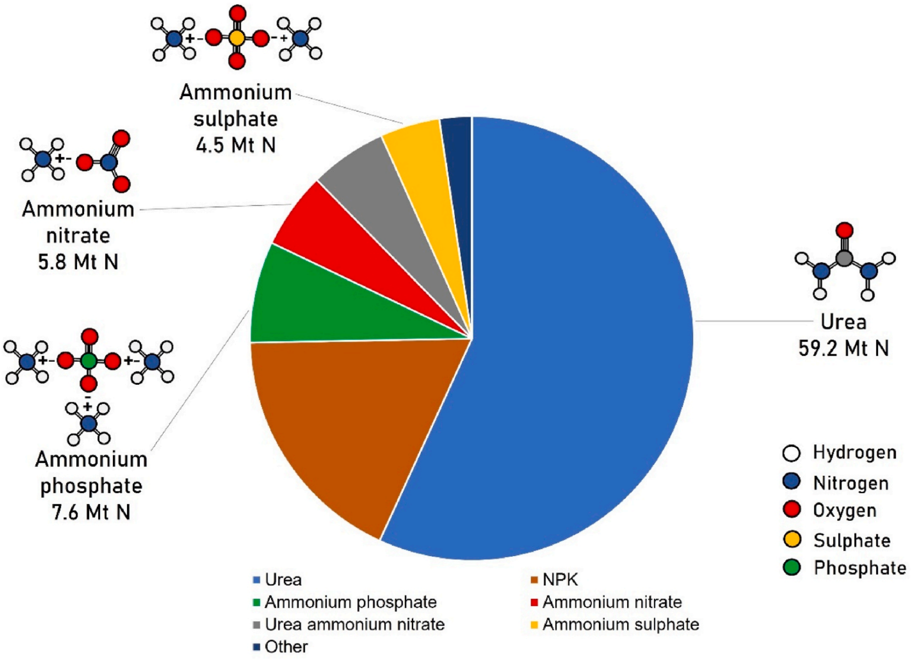

Fig. 1. Global utilization of N fertilizers. Data adapted from International Fertilizer Association online statistical database IFASTAT 2023.

factors contribute to determine which extent of the applied N is eventually assimilated by the crop. Plants can assimilate mineral N in the forms of ammonium (NH_4^+) and nitrate (NO_3^-), with uptake depending on various factors such as availability as well as plant and soil type, climate, and pH (Maathuis, 2009). The influence of each factor is variable and up to date it has not been fully rationalized. Soil pH, for instance, influences the chemistry of N in soil also impacting the conversion of ammonium to nitrate through nitrification, while plant species vary in their preference for ammonium or nitrate uptake. Climate can influence microbial activity, affecting nitrogen mineralization and immobilization rates, while soil type influences the retention and leaching of nitrogen species. Together, these factors interact to shape nitrogen availability in soils.

Once nitrogen is available, plants can uptake N both as ammonium and as nitrates (NO_3^-), which once assimilated are further converted into ammonium within the plant through enzymatic processes and incorporated into amino acids and nucleotides. When nitrogen is applied in soil as urea, it first undergoes hydrolysis breaking down into ammonium and carbon dioxide (CO_2), either spontaneously or facilitated by urease-producing microbes. Thus, the resulting ammonium can either be taken up by plants directly or be converted into NO_3^- through nitrification processes, depending on the soil conditions. Nitrates are more susceptible to leaching (intended as the downward movement through the soil) and discharge (transport into surface waters) than ammonium ions (NH_4^+), which are less mobile in soil due to their positively charged nature (Y. Wang et al., 2019). While ammonium is retained in the soil for longer periods, a portion of it is either converted by soil microbes into nitrates, integrated into microbial biomass, or volatilized as ammonia, depending on factors such as temperature and soil moisture (Kaur et al., 2017). In the presence of oxygen, autotrophic nitrifying bacteria such as *Nitrosomonas* spp., can oxidize ammonia into nitrites (NO_2^-) through a multi-step process catalysed by ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) (Equation (1)). While the general pathway is established, the detailed mechanism of hydroxylamine oxidation, including whether nitric oxide (NO) serves as a key intermediate, remains not completely understood (Caranto & Lancaster, 2017; Choi et al., 2023). It is believed that the oxidation of hydroxylamine (NH_2OH) to nitrites (Equation (2)) passes through an intermediate stage involving the formation of NO (Mills, 2019). Nitrites are further oxidized to nitrates by the enzyme nitrite oxidoreductase (NIO) in the final step of nitrification (Equation (3)). Both fungal and bacterial forms of nitric oxide reductases (NOR) catalyse the reduction of NO to nitrous oxide (N_2O), which can subsequently be reduced to dinitrogen gas (N_2) by nitrous oxide reductase (NOS) during the denitrification process (Equations (4) and (5)) (Lehnert et al., 2018).

$$NH_3 + O_2 + 2e^- + 2H^+ \overset{AMO^*}{\leftrightarrow} NH_2OH + H_2O \tag{1}$$

$$NH_2OH + H_2O \xleftrightarrow{HAO} NO_2^- + 5H^+ + 4e^- \tag{2}$$

$$NO_2^- + H_2O \xleftrightarrow{NIO} NO_3^- + 2H^+ + 2e^- \tag{3}$$

$$2NO + 2H^+ + 2e^- \xleftrightarrow{NOR} N_2O + H_2O \tag{4}$$

$$N_2O + 2H^+ + 2e^- \xleftrightarrow{NOS} N_2 + H_2O \tag{5}$$

$^*AMO$ = ammonia monooxygenase, HAO = hydroxylamine oxidoreductase, NIO = nitrite oxidoreductase, NOR = nitric oxide reductases, NOS = nitrous oxide reductase

Therefore, nitrate (NO_3^-) and its reduced intermediates, such as nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O), can be released into the atmosphere as NO and N_2O, or further reduced to dinitrogen gas (N_2) by denitrifying enzymes, depending on oxygen availability (Equation (5) and Fig. 2) (Tyagi et al., 2022). In fact, it is estimated that up to 50 % of nitrogen applied as mineral fertilizer is lost to the environment through processes like leaching, runoff, and volatilization, which can further result in potential environmental issues, such as eutrophication in water bodies, contamination of aquifers, and the release of greenhouse gases (Diaz & Rosenberg, 2008; Ren et al., 2022). The occurrence of over 400 dead zones in coastal areas has been related to eutrophication caused by fertilizer runoff (Naqvi et al., 2010). For example, N compounds derived especially from agricultural fertilizers in the Mississippi river basin have contributed to seasonal hypoxic "dead zones" in the Gulf of Mexico, threatening marine ecosystems (Pontius & McIntosh, 2024). This condition is projected to intensify with increasing temperature and storm waters expected with climate change. Additionally, hypoxic and anoxic conditions can further contribute to the emission of greenhouse gases such as methane (CH_4) and nitrous oxide (N_2O).

The excessive use of nitrogen fertilizers can affect soil integrity over time. The conversion of nitrogen fertilizers into nitrates and the subsequent release of protons (H^+) often leads to soil acidification which can eventually result in depleted soil quality, mineral loss, reduced biodiversity, and increased availability of heavy metals in soil (Tyagi et al., 2022; Wei et al., 2020).

Various strategies have been explored to reduce nitrogen losses from agricultural soils, including modifications to both the type of fertilizer and the application method. For example, the use of controlled- or slow-release fertilizers, such as polymer-coated urea, has been shown to improve crop yield while reducing nitrogen losses. These fertilizers gradually release nitrogen over time, reducing volatilization and leaching. Studies have reported a reduction of nitrogen losses by up to 20 % compared to conventional urea fertilizers, while also minimizing NO_x and N_2O emissions (J. Chen et al., 2018; Vejan et al., 2021). In addition, precision agriculture technologies, such as GPS-guided systems and variable rate application, can enable farmers to apply fertilizers more accurately, ensuring optimal nitrogen levels for crops and reducing excess application. These techniques reduced up to 30 % the fertilizers input dose in an olive orchard compared to standard application methods (Roma et al., 2023). Other techniques, such as deep urea placement and fertigation, have also proven effective in minimizing nitrogen losses. Deep urea placement, where urea is applied at a greater depth in the soil, reduces volatilization and minimizes the risk of surface runoff, especially in sandy soils. This method is particularly effective in dry regions, where moisture availability is crucial for nitrogen absorption. On the other hand, the application of fertilizers through the irrigation systems, also known as fertigation, can lead to a more controlled and timely nitrogen delivery, optimizing plant uptake and increasing yield on various crops, although without necessarily reducing nutrient losses[30]. Therefore, significant efforts are still needed to counteract the negative impacts related to excessive nitrogen application expected in the years to come (Penuelas et al., 2023).

3. Biological and industrial nitrogen fixation

Following the industrial-scale development of the Haber-Bosch process, nitrogen fertilization has greatly increased during the 20th century leading to an average increase in agricultural yields (Penuelas et al., 2023). The Haber-Bosch process converts atmospheric nitrogen (N_2) and hydrogen (H_2) into NH_3 using an iron-based catalyst under high temperatures and pressures (Appl, 2011; Erfani et al., 2024; Humphreys et al., 2021) (Equation (6)).

$$N_2 + 3H_2 \xleftrightarrow{Fe_xO_x} 2NH_3 \; \Delta H^\circ = -92.44\ kJ/kmol \tag{6}$$

Both the H_2 production – mainly originating from natural gas – and N_2 purification, necessary to enable the Haber-Bosch reaction, are energy intensive processes, so that the overall process contributes significantly to greenhouse gas emission, such as CO_2 deriving from

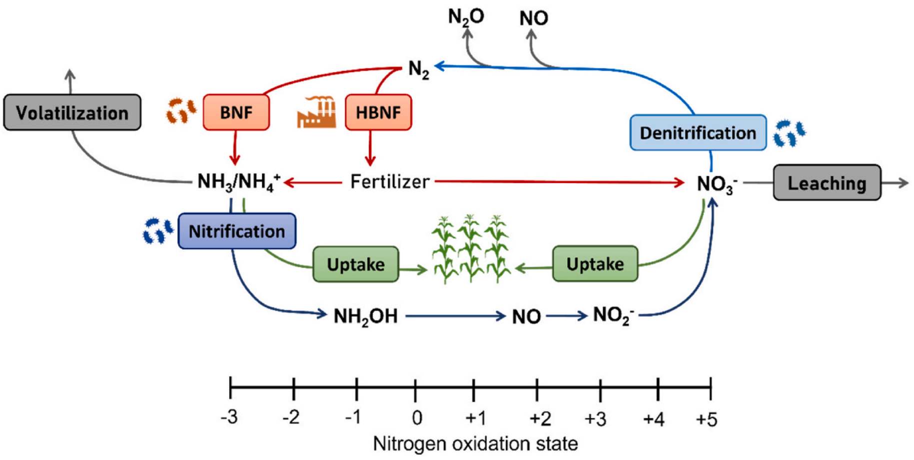

Fig. 2. Nitrogen oxidation states across N cycling processes in soil.

combustion and CH_4 from H_2 production (Song et al., 2018). Currently, up to 80 % of industrially produced ammonia is used to synthesize nitrogen-based fertilizers (Erfani et al., 2024), such as urea, although alternative methods are still being explored (Lv et al., 2021).

Beside lightning, nitrogen fixation occurs naturally in both aquatic and terrestrial ecosystems (Ladha et al., 2022). BNF is mainly carried out by microorganisms known as diazotrophs, including both bacteria and archaea (Shrimant Shridhar, 2012), although it has also been observed in fungi and plants (Mukhin et al., 2018). Bacterial BNF is performed by nitrogenase, an enzyme able to catalyse the reduction of N_2 to NH^{4+} in presence of hydrogen (Equation (7)). This process is estimated to operate the fixation of approximately $\sim 10^8$ Mt of N per year (Einsle & Rees, 2020).

$$N_2 + 8H^+ + 8e^- + 16ATP \xleftrightarrow{Nitrogenase} 2NH_3 + H_2 + 16ADP + 16P_i \qquad (7)$$

Nitrogenase is a multicomponent metalloprotein consisting of an iron (Fe) protein and an iron-molybdenum (Fe-Mo) protein. The Fe-Mo protein can also be found in homologous forms such as vanadium-iron (V-Fe) or iron-only (Fe-Fe) nitrogenases. The Fe-Mo protein contains a metallocluster that acts as the reducing agent and a P-cluster, an asymmetric iron-sulfur [8Fe:7S] cluster, which mediates the electron transfer between the Fe and the Fe-Mo components. Therefore, the Fe protein binds an ATP-binding iron-sulfur [4Fe:4S] cluster, which transfers a single electron to the P-cluster, thereby restoring the original redox state of the active site (Hoffman et al., 2013; Siegbahn, 2019). The crystallographic structure of a reversibly CO-inhibited nitrogenase has provided insights into the reaction mechanism at the nitrogen-binding site and into the role of interstitial carbon in stabilizing the Fe-Mo cluster (Yagishita et al., 2014) (Fig. 3).

Nitrogenase is expressed through the transcription of *nif* genes, a gene cluster responsible for regulating N fixation in various N-fixing bacteria (Poza-Carrión et al., 2014). The transcription of nitrogenase is typically activated under N-deficient conditions by the NifA regulator, which stimulates the expression of two oxygen-sensitive metalloproteins, NifDK and NifH, which then combine to form the enzyme. Since nitrogenase is inactivated by oxygen, BNF primarily occurs either under anaerobic conditions or in the presence of very low levels of oxygen, thus being restricted to specific environments. For example, cyanobacteria produce specialized oxygen-free heterocysts where nitrogen fixation takes place (Böhme, 1998; Zehr, 2011). Leguminous plants (Fabaceae spp.) can establish symbiotic relationship with N-fixing bacteria known as Rhizobia. In this mutualistic association, the plants create oxygen-free root compartments, called nodules, which allow the bacteria to fix nitrogen in exchange for nutrients (Lindström & Mousavi, 2020). Rhizobia are typically Gram-negative bacteria, including various families such as *Rhizobaceae,* Phyllobacteriaceae, *Brucellaceae, Methylobacteriaceae,* Bradyrhizobiaceae, *Xanthobacteraceae, and Burkholderaceae* that interact with plants through the production of Nod factors

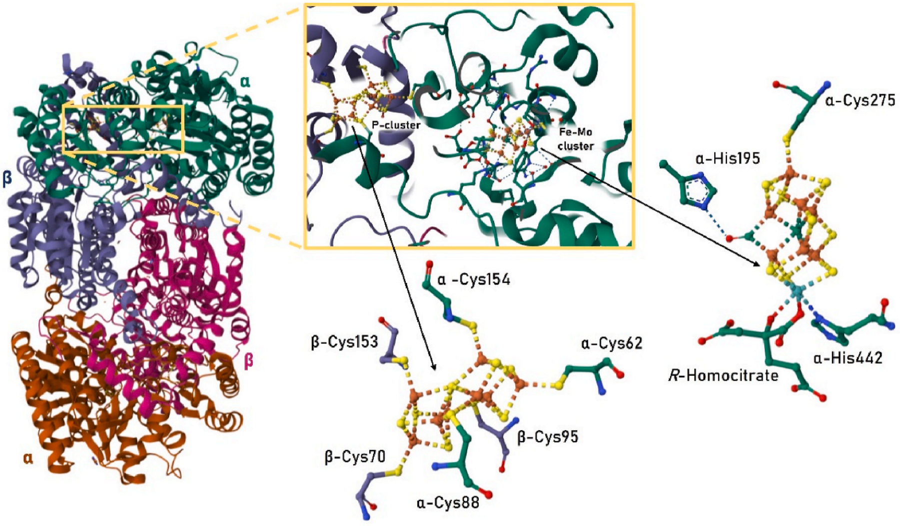

Fig. 3. Structure of the CO-bound nitrogenase Fe-Mo-protein from *A. vinelandii*. Adapted from protein data Bank (PDB) ID: 4TKV (Yagishita et al., 2014).

(Lichtfouse et al., 2009). These factors, which are encoded by *nod* genes, regulate nodulation by initiating the host-specific response required for symbiosis.

In addition to symbiotic N fixation, free-living diazotrophs in soil can also fix nitrogen non-symbiotically. This process is referred to as FNF. The most studied microorganisms able to FNF include bacteria from the genera *Azotobacter, Azotococcus, Azospirillum, Beijerinckia, Derxia*, and *Clostridium*, as well as cyanobacteria such as *Anabaena* and *Nostoc* (Shrimant Shridhar, 2012). Although being efficient, BNF is usually limited to certain plant species, while FNF can form cooperative relationships with a wider range of plant genera, being typically non-specific. In fact, free-living N fixers can establish interactions with plant roots through both epiphytic and endophytic associations (James, 2000). Moreover, the nitrogenases of some aerobic diazotrophs can tolerate the presence of oxygen, enabling N fixation also in aerobic conditions (Bennett et al., 2023; Lery et al., 2010). Although agriculture still consistently relies on industrial nitrogen fixation for N supply, it has been proposed that enabling BNF, and especially FNF, could reduce the need for traditional fertilizers while enhancing nitrogen uptake by plants (Olivares et al., 2013; Wen et al., 2021). Although integrating BNF into agricultural systems is expected to both alleviate the negative impacts of overfertilization and promote more sustainable farming practices, numerous challenges need to be addressed to fully exploit the potential of BNF in this field (Franzen et al., 2023).

Whether symbiotic or free-living, BNF offers a sustainable alternative to synthetic fertilizers. While symbiotic BNF occurs in specialized, oxygen-limited plant structures like root nodules, FNF bacteria can associate with a broader range of plants, sometimes even under aerobic conditions. Despite its ecological potential, the widespread agricultural application of BNF, especially FNF, faces practical and biological challenges that must be overcome to reduce dependence on industrial nitrogen inputs.

4. Plant-microbe interaction as driver for microbial functions

Plants can adapt to varying N supply conditions through genetic and phenotypic adjustments, including to both organic and inorganic forms of N. Plants modulate the uptake of different nitrogen species by regulating the expression of specific transporters for NO^{3-}, NH^{4+}, urea, and amino acid assimilation (Kraiser et al., 2011). Phenotypic adaptations, such as modifications in root architecture and growth patterns contributing to optimize nitrogen assimilation, are typically controlled by phytohormones like auxin (Guan et al., 2019). In addition to these autonomous adaptive mechanisms, plants can form associations with other organisms to enhance nitrogen acquisition, either through symbiotic or non-symbiotic relationships. Naturally occurring soil microbes not only can influence soil fertility and nutrient cycling but also support plant growth and development in various ways (Omae & Tsuda, 2022; Ray et al., 2020).

The interactions between plants and microorganisms primarily take place in the rhizosphere, which refers to the region of soil surrounding plant roots (Bais et al., 2006; X.-F. Huang et al., 2014). This area is impacted by root secretions, and it is densely populated by microorganisms known as rhizo-colonizers (Somers et al., 2004). The compounds released by plant roots can influence soil chemistry, mediate plant competition, and shape the microbial communities populating the rhizosphere. These root secretions, known as root exudates, contain a mixture of ions, simple sugars, amino acids, proteins, organic acids, phenolics, and other organic compounds originating form plant metabolism (Badri et al., 2013). Root exudates can include phytotoxic compounds with allelopathic effects that inhibit the growth of competing plants, and molecules owning antimicrobial properties that help control pathogenic microbes. Other molecules can act as chelating and redox agents to increase nutrient availability, or as growth-promoting compounds able to favour the colonization of the root by beneficial microbes (Bais et al., 2006). Molecules secreted via root exudation can also serve signalling or chemo-attractive functions, influencing recipient microorganisms. In Gram-negative bacteria, these signals are recognized via a two-component signalling system, typically including a sensor kinase (GacS), a membrane protein that initiates a signalling cascade in response to environmental stimuli, and a transcriptional regulator (GacA) that modulates the genetic response (Heeb et al., 2002). These responses may include the activation of metabolic pathways, increased motility, and quorum sensing (Corral-Lugo et al., 2016). Table 1 reports some of the compounds detected in root exudates of various plants and the possible interaction with soil microbes.

The process of root colonization by microorganisms involves three key steps: adhesion, anchoring, and colonization. The collective community of microorganisms present at the root level of a specific plant species is referred to as the plant's microbiome. The composition of plant's microbiome, whose formation begins at the surface of seeds, can change during the plant lifetime, by adapting to various stages of root development (Lugtenberg, 2015) as well as to various environmental condition (Ray et al., 2020; Timm et al., 2018).

Compounds produced by plants and microbes modulate rhizosphere colonization and shape plant-microbe interactions. Microorganisms able to utilize root exudates as a carbon (C) source and to effectively adjust their metabolism have greater advantage in colonizing the rhizosphere (L. Chen & Liu, 2024; A. C. Huang et al., 2019). The rhizo-colonizers considered to be beneficial to plants can enhance nutrient availability and uptake, promote plant growth, improve stress tolerance, or act as biocontrol agents against certain pathogens. For example, arbuscular mycorrhizal fungi (AMF) form mutualistic associations with plants, where the endophytic fungus colonizes the roots and enhances nutrient mobility in the soil, thereby increasing nutrient uptake in exchange for C (Rouphael et al., 2015).

Along with fungi, various bacteria known as rhizobacteria inhabit the rhizosphere by performing beneficial functions for the plant. Generally, the composition of the bacterial community in the rhizosphere differs from that in bulk soil, as root exudates selectively enrich specific microbial taxa (Fonseca et al., 2018; García-Salamanca et al., 2013; Labouyrie et al., 2023). Bacteroidetes and Proteobacteria, which are typically the most abundant bacterial phyla found in the rhizosphere, can have different functions, such as denitrification, cellulolysis, chitinolysis, nitrogen fixation, methylotrophy, ureolysis, ligninolysis, as well as plant pathogenesis. However, the functional composition of these microbial communities is also influenced by the ecosystem type and the plant species involved (Inskeep et al., 2010; Ling et al., 2022). Therefore, if by one hand, soil properties and climate play a relevant role in defining the composition of soil microbes geographically, on the other hand, plants mostly select the microbes colonizing the rhizosphere by functional properties (Kent & Triplett, 2002; Labouyrie et al., 2023; Ling et al., 2022; Tkacz et al., 2015) (Fig. 4). Therefore, in the rhizosphere, root exudates play a central role in shaping microbial communities and facilitating nutrient uptake, stress tolerance, and pathogen defence. Microorganisms that effectively respond to root signals gain a colonization advantage, forming plant-specific microbiomes that evolve over time. These plant–microbe partnerships are crucial for sustainable nutrient acquisition, particularly nitrogen, and are influenced by both plant traits and environmental conditions.

5. Plant-beneficial microbes in agriculture

Plant-beneficial bacteria are commonly referred to as plant-growth-promoting bacteria (PGPB) in a more general sense, and more specifically as plant-growth-promoting rhizobacteria (PGPR) when referring to bacteria able to live in the rhizosphere which can benefit the plant through various mechanisms (Allouzi et al., 2022). Some PGPR can produce phytohormones, such as indole-3-acetic acid (IAA) and cytokinins, which contribute to regulate plant growth, root morphology, and development (Lee et al., 2006). PGPR can enhance the plant's tolerance to both biotic and abiotic stress. For example, PGPR can act as biocontrol

Table 1

Lists of some detected compounds driving plant-microbe interactions in the rhizosphere.

Compound	System	Function	Recipient	Ref.
Flavonoids (4′7-dihydroxyflavone, Apigenin, Naringenin, Isoliquiritigenin and Chrysoeriol)	Leguminous plants	Specifically induce rhizobia chemotaxis and Nod genes expression	Mainly *Rhizobium* spp.	Scheidemann and Wetzel (1997)
Phenolic compounds (Daidzein, 4′,7-dihydroxyflavanone, Genistein, 4′,7-dihydroxyflavone)	Leguminous plants	Chemo-attraction, growth promotion, expression of Nod genes	Mainly *Rzobium* spp.	Badri et al. (2013)
Isoliquiritigenin, 7,3′-dihydroxy-4′-methoxyflavone umbelliferone	Plant (root)	Inhibition of growth, antimicrobial activity	Soil phytopathogens	
Rutin	Plant (Carrot, Parsely, Pepper)	Symbiosis regulation, abiotic stress mitigation	AMF	Shah and Smith (2020)
Fusaric acid	Fusarium oxysporum (fungus)	Toxic compund, induced immune response	Plants	Bouizgarne et al. (2006)
Fusaric acid	Fusarium oxysporum (fungus)	Chemoattraction	Pseudomonas fluorescens WCS365	De Weert et al. (2004)
Malic, aldonic, fumaric, erythronic acids	Plant (root)	Chemoattraction	Soil microbes	Lugtenberg (2015)
Citric, glutaric, oxalic, malonic acids	Plant (root)	Nutrient source	Soil microbes	
Aconitic, pyruvic acids	Plant (root)	*Nod gene activation*	*Rhizobium* spp.	
Vitamins (Biotin, thiamin, ribose, niacin)	Plant (root)	Growth promotion	Plant and microbes	
Adenine, guanine, cytidine, uridine	Plant (root)	Nutrient source	Soil microbes	
Siderophores	Bacteria	Nutrient mobilization, antibacterial activity, chelation	Soil microbes	Kramer et al. (2020)
Phytohormones (auxins, GA, cytokinins)	Bacteria	Development and growth promotion, stimulation of root exudation	Plants	Kudoyarova et al. (2014)
N-acyl-homoserin Lactones (AHL)	Bacteria	Alter root's cell division, activate G-protein and $Ca2+$ signalling, modulate plant defence via oxylipin and salicylic acid, root growth promotion	Plants	Moshynets et al. (2019)
N-acyl-homoserin Lactones (AHL)	Bacteria	QS-induction, biofilm formation, cell density regulation, organic N degardation	Mainly Gram-negative bacteria and Rhizobia	Decho et al. (2011)
Strigolactones	Plant (root)	Adaptation regulation, Microbial recruitment, Biocontrol	Plants, Fungi, Bacteria	Aliche et al. (2020)
Lypopolysaccharides (LPS)	Bacteria	Induced systemic resistance	Plants	(Choudhary et al., 2007; Meziane et al., 2005)
Putisolvins (I and II)	*Pseudomonas putida*	Biofilm formation inhibition	Bacteria (*Pseudomonas* spp.)	Kuiper et al. (2004)
Nod-factors (Lipo-chitooligosaccharides)	Rhizobia	Nod genes activation, nodule modulation, growth promotion	Plants	Prithiviraj et al. (2003)
Rosmarinic acid	Plant (root)	Antimicrobial activity	Pseudomonas aeruginosa PAO1	Corral-Lugo et al. (2016)
Phytoalexins (isoflavonoid)	Plant (root)	Antimicrobial activity	Bacteria	Kuc (1995)
Phenylpropanoids (sinapoyl glucose, coniferyl alcohol and coniferin)	*Arabidopsis thaliana*	Antifungal activity	Verticillium longisporum	König et al. (2014)
Triptophan	Plant (root)	Phytohormone precursor	Soil microbes	Idris et al. (2007)
Acetate and propionate	Plant (root)	Weak chemoattraction	*Rhodobacter sphaeroides*	Hamblin et al. (1997)
Amino acids, sugar and organic acids	Cucumber	Chemoattraction	*Bacillus amyloliquefaciens*	(Feng et al., 2018; Xie et al., 2012)
Proteoglycan and arabinogalactan proteins (AGPs)	Leguminous Plant	Biofilm formation, adhesion	*Rhizobium leguminosarium*	Xie et al. (2012)
Poly-hidroxy-butyrate	*Azospirillum* spp.	Favouring etablishment, proliferation, survival and competition in the rhizosphere	Bacteria	(Okon & Itzigsohn, 1992)
Acetosyringone	Plant (root)	Virulence gene induction and chemoattraction	*Agrobacterium* spp.	Palmer and Shaw (1992)
EPS	Bacteria	Developmental and host defense response trigger, affinity-dependent adhesion, regulation of drought tolerance and nodulation	Plants	Marczak et al. (2017)
CPS, EPS, LPS	Bacteria	Modulate adhesion, aggregation, biofilm formation and root colonization	Bacteria	Saile et al. (1997)
Richadesin, adhesin	Bacteria	Root adhesion and adsorption, regulation of colonization and nodulation	Plants	Bogino et al. (2013)
Methyl 3-(4-hydroxyphenyl) propionate	Plant (Sorghum bicolor)	Inhibition of nitrification	Nitrifying bacteria (*Nitrosomonas europaea*)	Zakir et al. (2008)
Phenazines, hydrogen cyanide, pyrrolnitrin, polyketides 2,4- diacetylphloroglucinol, rhizoxin and pyoluteorin	*P. fluorescens*	Antimicrobial activity	Microbial phythopatogens	Lugtenberg (2015)
Iturins and fengycin	*B. amyloliquefaciens*	Antifungal activity	Phytopathogenic fungi	

agents against plant pathogens and soil-borne diseases by competing for resources or by releasing antimicrobic compounds (Kumari et al., 2019). In addition, some PGPR have been reported to alleviate the negative effects of abiotic stress, like drought and salinity, on plant growth (Ahluwalia et al., 2021; Forni et al., 2017).

Beside alleviating stress, plant-beneficial strains can also support plant development by increasing the bioavailability of macronutrients and micronutrients (Di Benedetto et al., 2019). For example, the bacterial production of organic chelating compounds, like siderophores, can facilitate the mobilization and availability of nutrients – such as Fe – in the soil (Maheshwari et al., 2013; Timofeeva et al., 2022). In addition, bacterial genera like *Bacillus*, *Azospirillum*, *Pseudomonas*, *Serratia*,

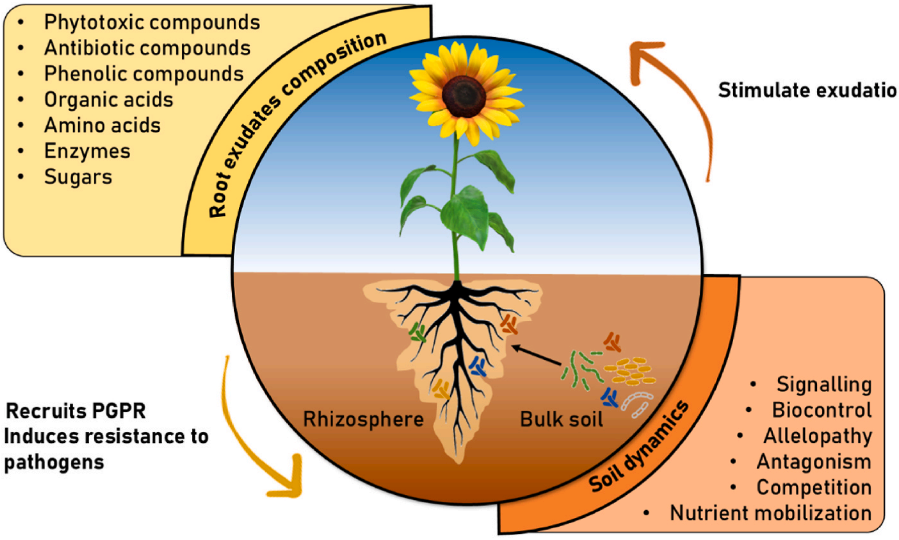

Fig. 4. Soil dynamics related to root exudation. PGPR stands for plant growth promoting rhizobacteria.

Burkholderia, and *Enterobacter* can enhance P availability by producing phosphate-solubilizing enzymes. P in soil is predominantly found in the form of mineral phosphate in phosphatic rocks or as part of organic residues. The concentration of soluble P in soil is typically low, and its mobility is restricted, as available phosphates are often fixed by free oxides, hydroxides, or by calcium and other cations, depending on soil pH (Rodríguez & Fraga, 1999). Microbial-mediated release of phosphates from insoluble sources is thought to occur through the secretion of organic compounds combined with local pH acidification mediated by H^+ release. A similar process likely enables K solubilization from insoluble sources, such as silicate rocks (Bihariirammmaurya & Rammswarooppmeena, 2016; Etesami et al., 2017). Enzymes like phosphohydrolases, including phosphatases and phytases, mediate the hydrolysis of phosphodiesters or phosphor-anhydride bonds under both acidic and alkaline conditions. These enzymes, which can either be secreted outside the cell or function as membrane-bound proteins, contribute to the solubilization of phosphates from organic sources in the soil (Billah et al., 2019; Rodríguez & Fraga, 1999).

Finally, PGPR can enhance N availability in soil through the mineralization of soil organic matter (SOM) or through direct N fixation (Bloch, Clark, et al., 2020; James, 2000). Nutrients mobilized by these microorganisms can either be utilized directly by plants or retained in the soil's microbial biomass, making them available for future use. Some of these nutrients and the related processes are schematically represented in Fig. 5.

PGPR can promote plant development through multiple mechanisms, including hormone production, nutrient solubilization, and stress mitigation. These microbial functions contribute to improved nutrient availability while also helping plants tolerate both biotic and abiotic stress, making PGPR valuable allies in modern agriculture.

For the reasons outlined above, the use of naturally occurring PGPR in agriculture has garnered increasing attention in the recent decades (Rajvir & Sudhir Kumar, 2013). The potential of PGPR to enhance nutrient use efficiency, by improving both crop yield and quality, has been reported for various agricultural systems (Ramakrishna et al., 2019; Shakeri et al., 2016). For example, cytokinins production and local N fixation were attributed to the improved growth observed for rice seedlings inoculated with a N-fixing strain (Ladha et al., 2022; Syaiful et al., 2013). Beneficial strains isolated from durum wheat (*Triticum turgidum* L. subs. *durum*), such as *Bacillus* spp., *Pseudomonas* spp., and *Stenotrophomonas* spp., improved plant growth, with the effect being reconducted to both IAA productions and enhanced N and P use

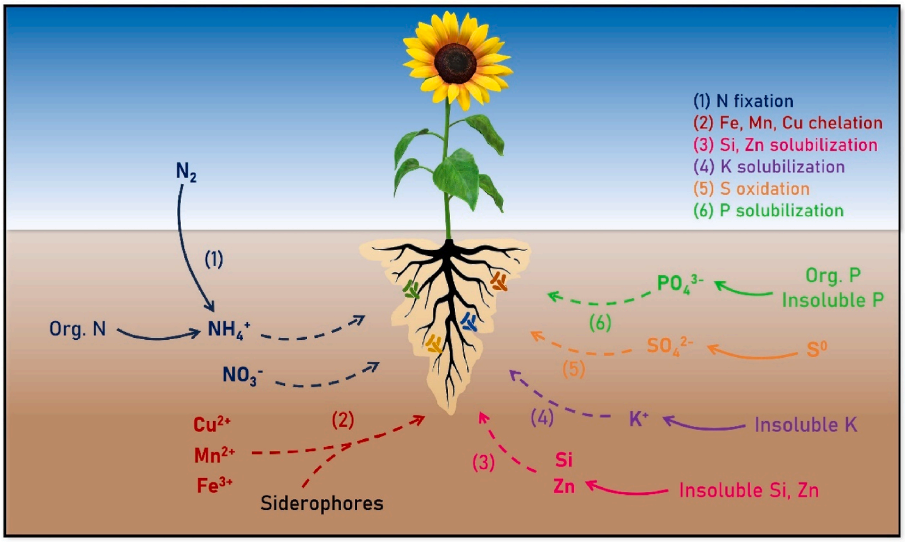

Fig. 5. Some of the nutrients mobilized by microbes in soil and the relative processes.

efficiency [9]. In field trials, inoculating *Azotobacter* spp. improved N utilization and increased the grain yield of oat (*Avena sativa* L.) (Sheoran et al., 2000). The co-application of *Acidithiobacillus* strains and mycorrhizal fungi boosted nodulation and increased yield in cowpeas (*Vigna unguiculata*), by increasing P and K availability in soil (Andrade et al., 2013). In wheat (*Triticum aestivum* L.), improved N accumulation and protein content were linked to increased enzymatic activity in the rhizosphere, following the inoculation with various diazotrophic consortia such as *Azoarcus* spp., *Azospirillum* spp., *Azorhizobium* spp., *Azotobacter vinelandii*, *Rhizophagus irregularis*, and *Bacillus megaterium* (Dal Cortivo et al., 2020). In fact, it has been suggested that multiple strains inoculation can produce synergistic effects and lead to a greater effect (Wani et al., 2007).

It has been hypothesized that the use of N-fixing bacteria could help reduce N fertilization rates (Mirshekari et al., 2012). For instance, combining diazotrophs with urea improved seed quality and yield in sesame (*Sesamum indicum* L.), while reducing overall input of N fertilizer (Shakeri et al., 2016). The use of N-fixing microbial strains showed improved yield and nitrogen uptake in rice crops (*Oryza sativa* L.), with reduced N rates (Choudhury et al., 2014). Hence several microbial formulations have been proposed as alternatives to urea application. The microbial-based products used to supply essential nutrients to plants, or to improve the nutrient use efficiency of crops in agriculture, are also referred to as biofertilizers (BFs). These include living or dead microbial cells applied to seeds, plants, or soil (Raimi et al., 2020). In general, BFs can consist of cyanobacteria, microalgae, algal extracts, N-fixing strains, P solubilizers, siderophores, and phytohormone producers from various species. Some examples of commercially available MFs are outlined in Table 2, which summarizes commercially available BFs, and highlights their microbial composition and intended agronomic functions. The table shows that various strains have been studied and commercialized for both PGPR and biocontrol purposes, and that the available products are mostly in liquid form.

A global meta-analysis highlighted that BFs can improve yield up to 20 % in dry climates and up to 8.5 % in continental climates, often without the need for crop-specific formulations (Schütz et al., 2018). Additionally, some BFs may enhance plant tolerance to abiotic stress, potentially helping to mitigate the effects of global warming (Olivares et al., 2013; Raimi et al., 2020). However, the design and application of effective BFs are complex, especially when concerning N fixation (Aasfar et al., 2021). Viability of microbial strains and storability of BFs, along with climate, soil and plant type, and competitivity with the indigenous microflora are all factors currently limiting both the production and the efficacy of the applied formulation, affecting aspects like stability and persistence of BFs in the applied soil or plant (Raimi et al., 2020). Furthermore, while BFs offer significant potential for promoting agricultural sustainability, their widespread use raises questions about feasibility, scalability, and long-term environmental consequences. The integration of BFs into farming practices requires careful evaluation of these concerns and the development of a robust framework for their practical implementation.

Therefore, BFs demonstrated promising potential to enhance crop yields and improve plant resilience to abiotic stress, supporting climate change mitigation. However, their widespread adoption is constrained by technical challenges such as strain viability, formulation stability, environmental interactions, and nitrogen fixation efficiency. To unlock the full potential of BFs in sustainable agriculture, it is essential to address these limitations through targeted innovation, regulatory frameworks, and strategic implementation. To address these issues, the following chapters will address some of these critical aspects, aiming to pave the way for effective and scalable use of BFs, with a focus on enabling biological nitrogen fixation (BNF) in modern farming systems.

Table 2
List of some commercial biofertilizers analysed for their respective compositions. CBF stands for commercial biofertilizers.

Product	Composition	Form	Function	Ref.
CBF-1	*Enterobacter, Bacillus, Rhizobium, Pseudomonas, Trichoderma, Stenotromonas*	Liquid	PGPR, Biocontrol	Raimi et al. (2020)
CBF-2	*Rhizobium tropica*	Solid	PGPR	
CBF-3	*Azotobacter chrococcum, Bacillus subtilis, Bacillus thuringiensis, Saccharomyces cerevisiae, Pseudomonas fluorescence, Trichoderma harzianum, Lactobacillus* sp.	Liquid	PGPR, Biocontrol	
CBF-4	*Bacillus* sp.	Liquid	PGPR, Biocontrol	
CBF-5	*Bradyrhizobium japonicum*	Solid	PGPR	
CBF-6	*Azospirillum brasilense, Azospirillum lipoferum*	Liquid	PGPR	
CBF-7	*Azospirillum brasilense, Azospirillum lipoferum, Azotobacter chrococcum*	Liquid	PGPR	
CBF-8	*Bradyrhizobium japonicum*	Liquid	PGPR	
CBF-9	*Pseudomonas fluorescence*	Liquid	PGPR, Biocontrol	
CBF-10	*Bravibacillus laterosporous, Paenibacillus chitinolyticus, Lysinibacillus sphaericus, Sporolactobacillus laevolacticus*	Liquid	PGPR, Biocontrol	
CBF-11	*Bacillus* sp.	Liquid	PGPR, Biocontrol	
CBF-12	*Rhizobium phaseolus*	Liquid	PGPR	
CBF-13	Gigaspora gigantea, Funneliformis mosseae, Claroideoglomus etunicatum, Paraglomus occulum, Rhizophagus clarus	Solid	PGPR, Biocontrol	
CBF-14	*Rhizobium* sp.	Solid	PGPR	Husen et al. (2007)
CBF-15	*Azotobacter beijerinckii, Aeromonas punctuata, Azospirillum lipoverum*	Solid	PGPR	
CBF-16	*Lactobacillus* sp., *Azotobacter* sp., *Rhizobium* sp.	Liquid	PGPR	
CBF-17	*Lactobacillus* sp., *Azotobacter* sp., *Bacillus* sp., *Acetobacter* sp.	Liquid	PGPR	
CBF-19	*Azotobacter chroococcum, Pseudomonas fluorescens*	Liquid	PGPR, Biocontrol	Allouzi et al. (2022)
CBF-20	*Bacillus megatherium*	Liquid	PGPR	
CBF-21	*Nitrosomonadales, Rhizobiales, Cantharellales*	Liquid	PGPR	
CBF-22	*Azotobacter chroococcum, Acetobacter aurantis, Pseudomonas striata, Paenibacillus mucilaginosus, Bacillus magaterium* var. *phosphate, Trichoderma virens, Streptomyces gelaticus*	Liquid	PGPR, Biocontrol	
CBF-23	*Azotobacter chroococcum, Azotobacter vinelandii, Paenibacillus durus, Azospirillum lipoferum, Rhizobium japonicum, Herbaspirillum frisingense, Gluconocetobacter*	Liquid	PGPR, Biocontrol	
CBF-24	*Acidithiobacillus thioxidans*	Liquid	PGPR	
CBF-25	*Bacillus megaterium, Bacillus coagulans*	Liquid	PGPR	
CBF-26	*Bacillus coagulans*	Liquid	PGPR	
CBF-27	*Frateuria aurantia, Bacillus mucilaginosus*	Liquid	PGPR	

6. Factors affecting microbial viability in soil

The formulation and application of microbial products for agriculture are challenging, as their effectiveness can be limited by climatic factors (such as temperature fluctuations, moisture levels, and UV radiation), edaphic conditions (including soil pH, nutrient availability, salinity, and texture), and biotic interactions (such as competition with native microorganisms, predation, and plant-microbe specificity), all of which collectively determine the fate and performance of the introduced microbes (Labouyrie et al., 2023).

6.1. Physico-chemical factors

Temperature has a considerable impact on microbial activity in soil, especially if relative to enzymatic, such as nitrogenase, activity (de Vries & Griffiths, 2018; Karhu et al., 2014). Studies have shown that the optimal temperature for N fixation in non-symbiotic systems is around 25 °C, while in symbiotic natural ecosystems, it can range from 29 °C to 37 °C (Bytnerowicz et al., 2022; Houlton et al., 2008). For example, the application of BFs in dry climates resulted in higher yield increase (up to 20 %) compared to the same application in different climatic regions, suggesting that mild temperatures are more likely to favour microbial survival (Schütz et al., 2018). Soil type can also influence microbial activity as functions of various soil factors. Considering that water is necessary for bacterial metabolism, microbial respiration in soil is usually strongly correlated to soil moisture (Cook & Orchard, 2008). In fact, soil moisture drives microbial response to thermal shifts, thus influencing the ability of microbes to adapt to climatic variations (J. T. Li et al., 2023). When water is preponderant, as in the case of paddy soils, pH and soil texture becomes relevant factors for free-living BNF (Yang et al., 2022). On the other hand, excess water often relates to anoxic conditions thereby limiting the biochemical processes deriving from aerobic metabolism, including redox processes and the mineralization of soil organic matter (Borowik & Wyszkowska, 2016). In general, nitrogenase activity occurs optimally at neutral or slightly basic pH (7.0–8.0). Furthermore, the soil silt and clay content can further influence microbial activity by modulating the formation of microbial aggregates (Cosentino et al., 2006). Microbial aggregates in soil can promote specific biochemical processes through the formation of semi-controlled environments with localized micro-climatic conditions (Han et al., 2021). Similarly, calcium (Ca^{2+}) contents can affect microbial activity and adhesions to soil particles, thereby influencing the formation of aggregates and the mineralization of SOM, at the same time (Shabtai et al., 2023). Amongst other factors, nutrients content, especially with respect to C, N and P relative concentrations, contribute to shape both microbial communities' structure and composition (Delgado-Baquerizo et al., 2016; Labouyrie et al., 2023). In general, it has been observed that low soil C:N ratios are associated with increased bacterial diversity and higher microbial respiration, as the relatively higher nitrogen availability can support the proliferation of a greater variety of microbial species (Wan et al., 2015). In contrast, high C:N ratios are more commonly linked to nitrogen cycling through soil organic matter (SOM) mineralization, where microbes break down carbon-rich organic material to release nitrogen in forms that both plants and microbes can utilize. In these conditions, the higher carbon content may lead to slower nitrogen cycling, as microbes first focus on decomposing the carbon before accessing nitrogen (Meyer et al., 2018; Schütz et al., 2018; Smercina et al., 2019).

6.2. Biological factors

Nitrogen concentration in the soil is closely linked to the dynamics between plants and rhizobacteria. The availability of nitrogen influences the pathways through which these microbes interact with plants, ultimately affecting nutrient uptake and plant growth. Therefore, both microbial community composition and activity can be affected by N fertilization rate, along with other agricultural practices. For example, it has been observed that long-term N fertilization with urea reduced the natural N fixation rate in soil by 50 % and decreased the abundance of N fixers (Fan et al., 2019). Ammonium and other N-containing compounds can inhibit the nitrogenase activity in some N_2 fixing microorganisms as metabolic feedback (Ccjudo & Panequc, 1988; Neilson A N & Arrheniuslaboratoriet, 1975). For this reason, BNF is usually favoured when the N availability is low (Vitousek et al., 2013), although the sensitivity of BNF response to N supply can be influenced by other environmental factors (Zheng et al., 2019). In addition, for instance, ammonium resulting from traditional fertilization, can both acidify soil thus shifting microbial populations and recruit ammonia-oxidizing bacteria (Lin et al., 2021). Once inoculated, microbes must be able to survive for an appropriate amount of time and to spatially interact with the plant prior to carry out the plant-beneficial functions. Therefore, the applied microbial strains should be able to survive alongside the endemic soil microflora and to adapt on plant exudates to successfully colonize the rhizosphere, which is the narrow region of soil influenced by root exudates and root-microbe interactions. Fig. 6 summarizes the main abiotic and biotic factors affecting the survival, colonization, and functional performance of microbial formulations within the rhizosphere. In general, plant roots can promote the formation of different niches with quite variable microbial composition. Thus, by enriching functionally distinct bacteria, plants can adapt to various environmental conditions (Ling et al., 2022). In addition, the introduction of non-resident microbial species in soil can change the microbial community composition in a variable – and hardly predictable – extent, dependent on the competitive and cooperative interactions amongst microbial populations (Mawarda et al., 2020). For example, the application of a fungal BFs markedly affected the composition of soil microflora, by suppressing plant-pathogenic fungal strains (Zhao et al., 2014). On the other hand, the addition of *B. subtilis* strain on tomato (*Solanum lycopersicum* L.) rhizosphere had only a transient impact on the plant's microbiome, while still providing detectable benefits such as disease suppression and increased plant biomass (10 %) (Qiao et al., 2017). In this regard, it was observed that a periodic bacterial inoculation would be required to obtain a more marked impact on the resident microbial community (Z. Wang et al., 2021).

The application of microbial strains to soil face various challenges due to both biotic, climatic and edaphic factors. Temperature, soil moisture, pH, and nutrient availability all influence microbial activity, including nitrogen fixation, thereby also likely affecting survival and effectiveness of exogenously inoculated strains. Therefore, to achieve sustained benefits, effective microbial inoculation for agricultural purposes requires strains that can survive in the soil, interact with plants, and adapt to local microbial populations.

7. Enabling BNF in agriculture

Currently, BNF is only partially utilized in agriculture through various techniques such as succession, cover-cropping, or the co-cultivation of plants symbiotically associated with N fixers (i.e. leguminous plants) whose biomass is usually integrated into the soil to provide C and N as green manure (Cherr et al., 2006; Shamseldin, 2022). However, despite its well-known potential to reduce N losses, management complexity and its related risks are still limiting the standardized adoption of green manure by farmers, especially in conventional intensive agricultural systems (Ladha et al., 2022). Similarly, the use of BNF as alternative or additional N input is still mainly related to leguminous plants: biologically fixed N input in cereal cropping systems was estimated to account for 35 Mt of $N \bullet yr^{-1}$ worldwide, of which only about 15 Mt of $N \bullet yr^{-1}$ – less than half – would be ascribed to non-symbiotic N sources, deriving from free-living N fixation and endophytic plant-microbes associations (Ladha et al., 2016).

Despite promising results in controlled environments have been shown and various BFs have been commercialized worldwide (Ansari et al., 2015; Raimi et al., 2020), the translation of N-fixing microbial inoculants to field conditions often lacks consistency and repeatability, which remains a significant barrier to their widespread adoption in industrial agriculture (Franzen et al., 2023; Ibáñez et al., 2023; D. P. Singh et al., 2016).

Different strategies have been proposed to address the challenges currently limiting the exploitation of free-living BNF, particularly in cereals and other industrially important non-leguminous crops. These strategies focus on either enhancing nitrogenase activity or improving

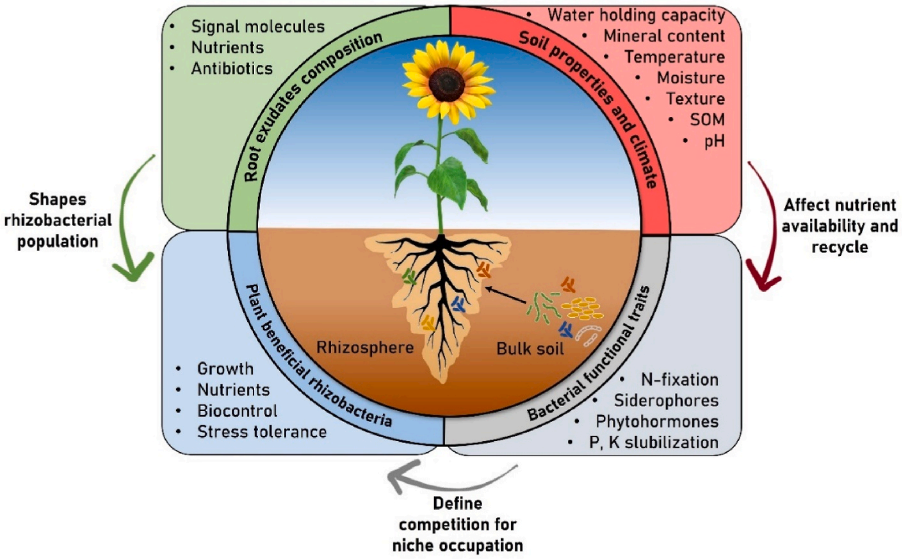

Fig. 6. Main factors and their interactions involved in the determination of bacterial survival in the rhizosphere.

root association with diazotrophs (Nag et al., 2020). Some of the strategies proposed for enabling BNF in agriculture are reported in Table 3. For instance, genetic engineering approaches have focused on either enabling nitrogenase expression via introduction of *nif* genes in plants (Shamseldin, 2022), or on favouring the establishment of symbiotic-like relationships amongst non-leguminous plants and rhizobia (Bloch, Ryu, et al., 2020). Both these strategies could increase N fixation rates in soils and enable BNF in plants such as cereals crops, although their practical implementation is still constrained by substantial technical and biological limitations. The transfer of *nif* genes from model organisms to non-diazotrophic bacteria has also been attempted (Brophy et al., 2018). Although this strategy has the potential to favour the development of stable and reproducible BNF technologies, results still report lower N fixation efficiencies (50 %) compared to N-fixers fixation efficiencies (Shamseldin, 2022). In addition, diazotrophs have been effectively modified to assimilate nitrogen without interference from external nitrogen sources, thereby potentially enabling the co-application of N-fixing BFs with conventional fertilizers (Wen et al., 2021). Nevertheless, although promising, the scalability of transgenic organisms may raise regulatory concerns or face other restrictions which may delay or prevent their adoption in many countries. On the other hand, working on the bio-formulation design, such as improving microbial selection and inoculation modes, can help to address issues like viability, stability of composition, persistence upon application and overall reproducibility (Kumar et al., 2022). Therefore, the optimization of the composition and application of BFs via product design appear to be a more feasible strategy to potentially maximize FNF, especially in the short term (Roper & Gupta, 2016).

Table 3
Some of the explored strategies enabling or enhancing BNF in agriculture.

Strategy	Target system(s)	Method	Ref.
Enable ammonium excretion in N-rich conditions	*Klebsiella variicola* (137–2253) *Kosakonia sacchari* (6–5687)	Non-transgenic gene editing	Martinez-Feria et al. (2024)
Enable ammonium excretion in N-rich conditions	*Klebsiella variicola* (137–1036)	Gene editing via modification of the *nifL* locus	Wen et al. (2021)
Enabling nitrogenase expression in non-diazotrophs	*Escherichia coli*	Transfer of the *nif* genes cluster	L. Wang et al. (2013)
Enabling nitrogenase expression in eukaryotes	*Saccharomyces cerevisiae*	Transfer of *nifH* to mitochondria	López-Torrejón et al. (2016)
Enabling nitrogenase expression in crops	*Oryza sativa* L.	Transfer of *nifH* to plant's mitochondria	Baysal et al. (2022)
Controlling N fixation	*Azotobacter vinelandii*	CRISPR[a] for gene manipulation	Russell et al. (2024)
Enhance N fixation via bacterial biohybrid	*Azotobacter vinelandii*	Membrane intercalation of light-harvesting molecules	Zhang et al. (2023)
Improving nitrogenase activity in non-diazotrophs	*Escherichia coli*	Chromosomal integration of *nifiscA, nifU*, and *nifS* genes	Ito et al. (2024)
Enabling N fixation in aerobic conditions	*Pseudomonas stutzeri* (A1501) *Azospirillum brasilense*	Stimulation of biofilm formation	D. Wang et al. (2017)

[a] Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR).

A rational design approach, along with the standardization of BFs production and application, can help reducing the existing variability of products and outcomes (Franzen et al., 2023; Husen et al., 2007). The development of effective plant-beneficial BFs begins with the selection of the most suitable microbial strain(s). As mentioned above, numerous biotic and abiotic factors may influence the successful application of PGPR in agriculture. Therefore, these factors must be considered during product design. The three main variables affecting microbial survival in soil can be generalized as: (a) plant species, (b) soil properties, and (c) microbial function, which can be also intended as the plant-beneficial function to be performed by the designed BFs and therefore reflecting the goal of the product. Together, these elements determine the suitability of microbial strain(s) and thus can help define the final composition of the bioformulation.

The selected microbes need to interact with the rhizosphere of the crop of interest to exert the beneficial function. The ability of bacterial strains to survive in the rhizosphere is defined as rhizo-competency. As previously discussed, root exudates composition drives the formation of microbial niches, shaping the microbial population at the rhizosphere level. It has been previously suggested that selecting microbial strains for their rhizo-competency can enhance the chances of survival in the rhizosphere (Kamilova et al., 2005). However, a successful colonization is eventually determined by the interspecies interactions resulting from a trade-off between competition and cooperation, from native and inoculated strains. Traits such as pH and T tolerance, endo- and epiphytism, chemotaxis, along with the ability to degrade toxic compounds and to feed on different C sources, can increase the competitiveness of microbial species for niche colonization in a certain soil (Rosenblueth & Martínez-Romero, 2004). Therefore, along with rhizo-competency, the competitiveness of the selected strain is a relevant factor contributing to a successful root colonization.

Beside an appropriate bacterial selection, the MFs must be complemented with the necessary nutrients, to support microbial survival during both storage and inoculation. Therefore, an important step of MFs design consists in the selection of the appropriate inoculation strategy, to successfully mediate the transition of microbial strains form the producing site to the field. The main application strategies consist of either liquid media or solid carriers, in which microbial strains can be dispersed or loaded, respectively (Raimi et al., 2020). As discussed above, the amount and type of carbon source in soil (SOM) and nutrient availability have relevant effect on microbial growth and survival (Strigul & Kravchenko, 2006), so they both should be included in the final formulation, compatibly with the selected bacterial strain and the targeted plant. Surface area, chemical stability, cost-effectiveness, long-term storability, easy applicability, non-toxicity and moderate biodegradability are all desirable features for solid carriers (Xiang et al., 2022).

Even though solid carriers can be scalable and easier to apply by farmers, solid MFs are often challenging to prepare, due to the low bacterial survival rates and low storability generally observed (Husen et al., 2007). For this reason, liquid MFs are currently dominating the market (Ibáñez et al., 2023). Other ingredients can be incorporated into the final bioformulation to both enhance bacterial growth and offer additional benefits to plants. Various polymeric materials have been explored for the immobilization or for the bio-encapsulation of microbial inoculants (Vassilev et al., 2020). Biopolymers such as chitosan, alginate, starch, and cellulose are increasingly being used as biodegradable coatings or as adjuvants in fertilizing products (J. Chen et al., 2018). Chitosan, for example, has been shown to have a priming effect on seeds, protecting plants from both biotic and abiotic stress (Shahrajabian et al., 2021). In addition, these polymers can contribute to chelate soil ions, own antifungal properties, or serve as additional labile source of C for specific microbes (Lopez-Moya et al., 2019; Stasińska-Jakubas & Hawrylak-Nowak, 2022). Biochar has also been proposed as a soil amendment to boost carbon storage and provide a slow, short-term release of nutrients (Gul et al., 2015; Olmo et al., 2016; Wolna-Maruwka et al., 2021). While biochar can stimulate root growth and improve nutrient uptake its impact on plants and on microbes may vary based on factors such as the biomass source and process parameters (Prendergast-Miller et al., 2014), which can influence the carbon content, nutrient profile, porosity, and ash content of the biochar, further affecting its mineral composition, pH, and nutrient retention. Therefore, that the impact of biochar impact can differ based on its feedstock, making its application an area of ongoing research (Gul et al., 2015; Joseph et al., 2021). Moreover, blends with multiple carriers and mineral additives, have also been explored to enhance the performance of materials as microbial supports (Vassilev et al., 2020). A list of previously explored carriers for microbial inoculation purposes is reported in Table 4.

Table 4
Some of the proposed solid carriers for the application of microbial inoculants in agriculture.

Carrier	Advantage	Disadvantage	Ref.
Alginate beads	Stable Versatile Good shelf-life	Limited scalability Not inert Non-porous	Trivedi et al. (2005)
Charcoal	Low-cost Available Naturally rich in C Porosity Biodegradable	Limited bacterial viability Limited bacterial adhesion	
Talc powder	Low-cost Stable Good shelf-life	Limited applicability Low porosity	Parveen et al. (2023)
Vermiculite	Stable Available Porosity	Limited bacterial adhesion Low porosity	(Maheshwari et al., 2015; Su & Jin, 2022)
Peat	Stable Available Low-cost Naturally rich in C Biodegradable	Limited bacterial adhesion Limited bacterial viability Limited scalability	Kravets et al. (2021)
Biochar	Increased survival upon inoculation Low-cost Available Naturally rich in C Porosity Biodegradable	Limited bacterial viability Limited bacterial adhesion	Hale et al. (2015)
Dried compost pellets	Low cost Available Good shelf-life Naturally rich in C Biodegradable	Low porosity Not inert	Pathirana and Yapa (2020)
Zeolite	Stable Available Porosity	Limited applicability Limited bacterial viability	(Aksani et al., 2021; Dandurand et al., 1994)
Kaolin	Stable Available Porosity	Limited applicability Limited bacterial viability Limited bacterial adhesion	

Besides appropriate media and carrier selection, the inoculation of microbial consortia over individual strains can be an alternative to increase the overall microbial survival rate. Microbial consortia consist of a mixture of PGPR strains able to live in the same environment (Mitri & Richard Foster, 2013). Different types if interactions can arise within the same environment, including cooperation (Hernández-Álvarez et al., 2023; Khan, 2022; Nath et al., 2020). In fact, cooperativity can favour microbial resilience through faster adaptation to adverse conditions and higher nutrient use efficiency, as the result of mutualistic relationships amongst the different strains (Machado et al., 2021). The formulation of multi-strain BFs can enhance the success of inoculation – especially when the application does not occur directly on the seeds or on the roots.

The inoculation of multiple strains through microbial consortia can further enhance the plant-beneficial traits of the formulation, in which multi-functional features can co-exist, thereby also improving the colonization success. A meta-analysis suggested that the inoculation of microbial consortia is more effective, leading to over 20 % yield increase compared to single strains inoculation (Liu et al., 2023). The co-inoculation of microbial strains including N-fixers like *Azotobacter* sp., phosphorous and zinc solubilizers, and zinc mobilizing bacteria, increased the wheat (*Triticum aestivum* L.) harvest index by 40 % in field (Jain et al., 2021). Another field experiment showed that the co-inoculation of *F. mossae* and *B. sonorensis* with fertilizers on chilly (*Capsicum annuum* L.) improved the fruit yield (43 %) by reducing the NPK input by 50 % (Thilagar et al., 2016). Similarly, french bean inoculated with a microbial consortium composed of *P. polymyxa* and *P. agglomerans* and *F. mosseae* increased yield by 41 % with 75 % of conventional fertilizers dose (Chauhan & Bagyaraj, 2015). The experimental evidence suggests that the use of microbial combinations can be a strategy to obtain to more robust and effective formulations to be used in real-world agricultural applications.

To summarize, the ideal biofertilizer should contain two or three compatible strains, owning both rhizo-competency and compatibility with the indigenous soil microflora, combined with a solid or liquid carrier able to support microbial survival during storage and ensures their active release after inoculation. A schematic representation of the proposed iterative method for the design of effective MFs is depicted in Fig. 7, reporting four key steps for effective MFs design. As shown in Fig. 7, beside climatic and edaphic factors, microbial survival in the rhizosphere depends on rhizo-competency which drives bacterial selection (1). Therefore, the plant beneficial potential is defined by bacterial functional traits (2) while product also efficacy depends on the agricultural systems of interest and consequently on the application mode (3). All these factors contribute to the design of effective formulations (4), which can be further optimized as iterative function of the mentioned processes.

The successful integration of BNF into industrial agriculture requires a multifaceted approach, addressing both biological and technical limitations. While promising strategies such as genetic engineering, microbial consortia, and innovative carrier materials are being explored, challenges related to consistency, scalability, and regulatory constraints remain. This review proposed a novel systemic eco-design approach to design more effective and applicable MFs, as previously resumed in Fig. 7. In summary, a rational design approach that considers rhizo-competency, microbial competitiveness, and appropriate carrier selection is crucial for enhancing microbial survival and efficacy in field applications. By optimizing microbial formulations through iterative improvements and field validation, BNF-based solutions can move closer to widespread adoption, ultimately contributing to more sustainable and efficient fertilization practices in modern agriculture.

8. Global BFs market and regulatory frameworks

While the potential of BNF as a sustainable solution for nitrogen fertilization is evident, its widespread adoption hinges on both biological advancements and the development of a robust market for BFs. The increasing demand for biostimulants and the growth of the global BFs market provide a promising outlook for the integration of BNF technologies into modern agriculture. This section explores the current state of the BFs market and its regulatory landscape, as well as the key challenges and opportunities for further commercialization.

According to the (DunhamTrimmerR Global, 2020) market report biostimulants demand is predicted to reach $3.9 billion USD by 2025. Consequently, the BFs market is projected to grow, with microbial products accounting for about 10 % of the global market of bio-based agricultural products (Birinchi et al., n.d.), especially for N-fixing strains like *Rhizobium, Azotobacter* and *Azospirillum* spp. (Yadav & Yadav, 2024). International standards regulating biofertilizers production and utilization globally has not yet been introduced. Both Southern and Northern Asia have made significant investments in the development of effective regulations for BFs over the past few decades. In Malaysia, the development of BFs is generally addressed by the National Science, Technology and Innovation Policy (NPSTI) 2021–2030, yet without a specific regulation addressing biofertilizers production and commercialization. The Malaysian market accounts for about 44 products commercialized as both bacterial and fungal BFs. Microbial products for agricultural purposes are diffusely employed in the Philippines, whose commercialization is regulated by the Fertilizer and Pesticide Authority (FPA) upon appropriate registration (Krishnen et al., 2016). In Vietnam, the collection and isolation of microbes for biofertilizer use are standardized and, agriculturally relevant strains are stored in national repositories with periodical updates. The quality of BFs products is regulated by the law on Quality of National Assembly of Vietnam (2008), including specific BFs requirements like appropriate cell density, shelf life, biosafety and plant-beneficial functions. In China, the regulation of BFs products dates back to 1996, with the regulatory framework including both safety and product standards (Ruan et al., 2020). In India, beneficial microbes are regulated as biofertilizers by the Indian Fertilizers Control Order (FCO) since 1985, including prescriptions for quality standards, although clear guidelines for BFs commercialization and development are still being developed (Peter et al., 2020). On the other hand, the regulatory frameworks of Western Asian countries mainly include biopesticides, although still considered under the chemical pesticides regulatory systems.

In North and South America, the BFs regulatory frameworks can broadly differ by country. For examples, in Canada BFs are recognized and regulated as fertilizers by the Canadian Food Inspection Agency (CFIA), including various microbial genera such as *Rhizobium, Bradyrhizobium, Mesorhizobium, Sinorhizobium, Bacillus*, and Mycorrhizal fungi. On the other hand, there is no legally recognized definition of biofertilizers in the United States of America, and the utilization and commercialization of bio-based products, including microbial formulations, are still being developed (Santos et al., 2024). In Brazil, microbial

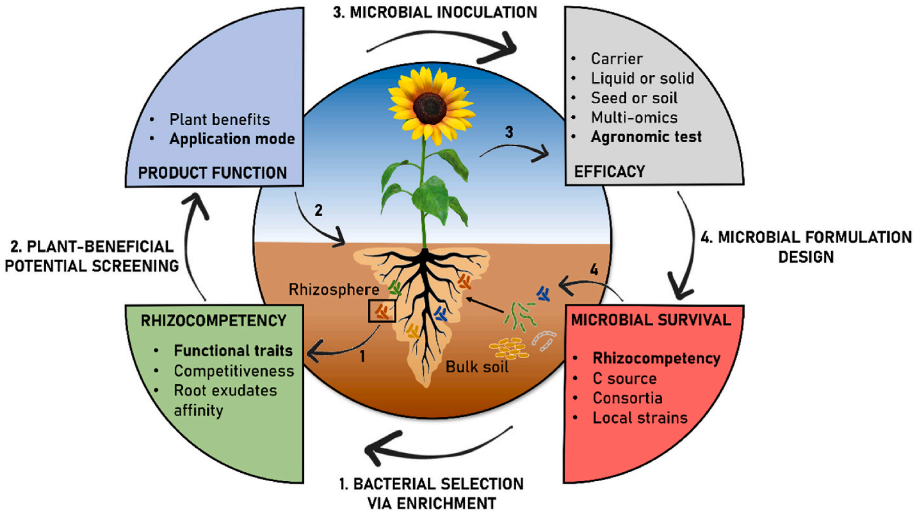

Fig. 7. Key four iterative steps for effective MFs design.

inoculants are also regulated as biofertilizers, with greater interest for the commercialization of N-fixing strains as to enable BNF in agriculture (Bomfim et al., 2021). Very recently, with the Bioinput Law 50.070/2024 Brazil exempted from registration the microbial inoculants produced and utilized for personal, which is expected to favour the utilization of bio-based products by farmers.

Europe's regulatory history for microbial products is relatively recent. As a result, the use of PGPR as BFs allowed in agriculture in Europe include only a few genera limited to *Azotobacter, Rhizobium, Azospirillum* and Mycorrhizal fungi, as outlined in the updated EU Regulation 2009/1009. Guidelines for the development and registration of microbial inoculants allowed for agricultural applications generally requires the identification of plant growth-promoting strains, followed by a proof of their agronomic efficiency. New microbial species can be recognized as valid BFs under the EU framework only once sufficient scientific evidence demonstrating their beneficial properties is provided.

Table 5 summarizes some of the national regulatory standards for BFs production considered by different countries.

By 2025, the global biostimulants market is expected to grow especially in Europe and Asia (Table 5). The global BFs market is anticipated to increase by over 10 % by 2028, with N-fixing BFs demand reaching about 4.5 USD billions by 2026 (Aloo et al., 2022). As the global BFs market is projected to increase, the development of adequate regulatory systems and clear guidelines on BFs design is necessary to standardize the quality and the production of BFs products, and to ease their widespread commercialization and application (Giller et al., 2024).

As long as the progress on BFs technology is limited, the chances to increase N use efficiency through enabling BNF in agriculture will also be limited. In this context, a more effective product design can help achieve more consistent proof of efficacy and increase the adoption of BFs in agriculture in the incoming years. Furthermore, the introduction of protocols for BFs design can help standardize the quality of bioformulations and tailor their function according to agricultural needs, location, and existing regulations, thereby contributing to the development of more sustainable agricultural systems.

9. Final remarks and future perspectives

Table 6 summarized some of the existing limitations and solutions concerning BFs application in agriculture. Product efficacy is often affected by both formulation and strain stability. It was discussed how an appropriate product design may help reducing some of the inconsistencies currently affecting the production of BFs. The presence of contaminants and the reduced total number of viable cells were found to be responsible for the reduced quality of over 60 % of the analysed commercial BFs (Husen et al., 2007; Raimi et al., 2020). It was mentioned that the development of liquid BFs is more widespread. However, liquid formulations are more likely to favour the growth of undesired microbes and might be less practical to be employed by farmers in intensive agricultural systems. On the other hand, solid BFs can offer some advantages in facilitating large-scale microbial application and in providing more effective handling, storage, and application, although features like microbial cell adhesion and survival still need to be properly addressed (Chakraborty & Akhtar, 2021). To this end, a more punctual characterization of the available cost-effective solid carriers and their interaction with living microbes is expected to help addressing these issues (Malusá et al., 2012). In addition, the mode of inoculation must be adapted both to the crop type and to the agricultural system. In irrigated orchards, for example, liquid formulations can better satisfy the need to deliver the microbes closer to the roots via fertigation. In annual and non-irrigated cropping systems, dried formulations can be more easily mixed with soil (Kuzin et al., 2020).

Table 6
Current limitations concerning MFs application along with some possible solutions with the estimated timeframe for their practical realization. The room of improvement for each action is unknown.

Limitation	Source of possible solutions	Expected time frame
Efficacy	Improved knowledge, formulation, mode of application, product design	Short-term
Rhizosphere colonization	Genetic engineering, formulation	Long-term
Inoculant survival	Formulation, management of soil nutrients	Short-term
N-fixation inhibition	Genetic engineering, management of N inputs	Medium-term
Applicability by farmers	Product design	Short-term
Culture	Evidence of agronomic efficacy, product design	Long-term
Regulation	Evidence of agronomic efficacy, safety, standardized production	Long-term

The collected evidence also highlights the need for improved understanding of plant-microbe interactions and microbial dynamics in soil. Improving the understanding of plant and soil microbiomes can help design more effective for the targeted crop and agricultural system (Hernández-Álvarez et al., 2023; Ramakrishna et al., 2019). To this end, multi-omics techniques can be valuable tools to track how soil microflora is affected by agricultural practices (Schütz et al., 2018). Metagenomic, coupled with metabolomic and proteomic technologies, can be utilized to interpret soil nutrient cycling and microbial dynamics as function of soil fertility (K. Li et al., 2014). When possible, these techniques can help increase the understanding of the plant-microbe interactions in the rhizosphere and support planning for an effective

Table 5
Regulatory frameworks and market values of BFs in different countries.

Region	Country	Standard	Regulatory system	Market shares projections by region (Mln USD)[a]
North Asia	China	Product registration according to quality standards	Fertilizers (GB 20287-2006)	$ 936
	India	Quality standards prescriptions	Fertilizers (ECA 10/1955)	
South Asia	Philippines	Product registration according to quality standards	Fertilizers (FPA)	
	Vietnam	Sampling and quality standards	Fertilizers (Circular 09/2019/TT-BNNPTNT)	
	Malaysia	Product registration according to quality standards	Biopesticides (GP 7/2026)	
North America	Canada	Product registration according to quality standards	Fertilizers (CFIA, T-4-130)	$ 659
South America	Brazil	Registration and production	Biofertilizers (50.070/2024)	$ 840
Europe	EU countries	Product registration according to quality standards	Biostimulants (EU 2009/1009)	$ 923

[a] Projected biostimulants marked shares for the 2025, extracted from the DunhamTrimmer®, Global Biostimulant report 2020.

fertilization (Pudake et al., 2021). Additionally, the experimental evidence can be coupled with mathematical modelling and computer simulations to analyse and predict patterns of interaction, being tools often employed in microbial ecology (Wade et al., 2016). Finally, it was suggested that the inoculation of multiple microbial strains to obtain multifunctional products can both increase benefits to the plant and improve the resilience and survival chance of the inoculated microbes, in different environmental conditions (Khan, 2022; Nath et al., 2020).

Applicability also requires that the developed BFs can be handled safely without any harm for human health and for the environment. It was discussed how gene editing approaches can help addressing some of the challenges currently limiting the development of effective microbial formulations, as well as the use of BNF in agriculture. However, the large-scale introduction of genetically edited strains in the environment can lead to potential unintended ecological impacts, such as affecting soil biodiversity and impacting the other related natural ecosystems, so that this strategy is more likely to be subjected to regulatory limitations (Chemla et al., 2025).

Complexity is one of the main constraints to the design of effective BFs, considering the many factors contributing to the success and efficacy of microbial inoculation, as above mentioned. Environmental and biotic factors shaping soil microbial communities are often interdependent, thereby further decreasing predictability on soil dynamics evolution. As example, soil microbial diversity was better explained when vegetation cover, climate and soil properties were considered together (Labouyrie et al., 2023). Similarly, the application of fertilizers could be more effective if accounting for naturally occurring N, P and other elements soil cycling, as well as by adopting the most suitable agricultural practices (Nag et al., 2020; U. Singh et al., 2021). System thinking analyses can help facing the multidisciplinary approach required to better support the industrial design in the face of soil complexity (prevot et al., 2024). Addressing these challenges is required to be able to design more effective BFs products and to standardized composition and production methods.

Finally, the implementation of effective regulatory frameworks and internationally recognized standards will play a relevant role in shaping both the composition BFs and the related technological advancement. The lack of a well-developed regulatory framework can hinder the production of BFs, affect product quality, and delay the integration into existing agricultural systems. In fact, the global BFs market is rapidly expanding, driven by rising demand for sustainable agricultural inputs, particularly N-fixing strains. However, the lack of unified international regulations poses a major barrier to their broader adoption. While some countries have established specific frameworks, regulatory standards vary significantly worldwide. To support the growing BFs sector and enhance nitrogen use efficiency through BNF, harmonized guidelines and robust product design protocols are essential to ensure efficacy, quality, and safe commercialization across diverse agricultural systems.

CRediT authorship contribution statement

Giulia Forghieri: Writing – original draft, Conceptualization. **Elena Ghedini:** Writing – review & editing. **Federica Torrigino:** Writing – review & editing, Formal analysis. **Rita Di Martino:** Writing – review & editing, Methodology. **Fiorella Lucarini:** Writing – review & editing, Formal analysis. **Davide Staedler:** Writing – review & editing, Supervision, Project administration. **Pierdomenico Biasi:** Validation, Supervision, Project administration. **Michela Signoretto:** Writing – review & editing, Validation, Supervision.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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