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Review

Engineering the Plant Microenvironment To Facilitate Plant-Growth-Promoting Microbe Association

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ABSTRACT: New technologies that enhance soil biodiversity and minimize the use of scarce resources while boosting crop production are highly sought to mitigate the increasing threats that climate change, population growth, and desertification pose on the food infrastructure. In particular, solutions based on plant-growth-promoting bacteria (PGPB) bring merits of self-replication, low environmental impact, tolerance to biotic and abiotic stressors, and reduction of inputs, such as fertilizers. However, challenges in facilitating PGPB delivery in the soil still persist and include survival to desiccation, precise delivery, programmable resuscitation, competition with the indigenous rhizosphere, and soil structure. These factors play a critical role in microbial root association and development of a beneficial plant microbiome. Engineering the seed microenvironment with protein and polysaccharides is one proposed way to deliver PGPB precisely and effectively in the seed spermosphere. In this review, we will cover new advancements in the precise and scalable delivery of microbial inoculants, also highlighting the latest development of multifunctional rhizobacteria solutions that have beneficial impact on not only legumes but also cereals. To conclude, we will discuss the role that legislators and policymakers play in promoting the adoption of new technologies that can enhance the sustainability of crop production.

KEYWORDS: *fertilizer, biomaterials, rhizobacteria, endophytes, seed coating, inoculation*

1. INTRODUCTION

Population growth, climate change, desertification, and salinization of the earth soils have led to the necessity to build resilient food systems while increasing agricultural output.[1−4] Chemically derived synthetic fertilizers and pesticides have been used for decades to boost plant growth.[5,6] It is well-known that plants primarily require nitrogen, phosphorus, and potassium (NPK) for their nutrition. However, these nutrients tend to be the limiting resource in plant growth, thus decreasing the yields.[7] Synthetic fertilizers are responsible for 40−60% of the world's food production and primarily constitute NPK. Stewart et al.[8] reviewed data representing 362 seasons of crop production and reported that a minimum of 30−50% of the crop yields can be attributed to synthetic fertilizer use, highlighting the major importance of fertilizer to humanity.[9] Nitrogen-based fertilizer production accounts for about 1% of the world's energy consumption while emitting about 1.2% of the global anthropogenic CO_2 emissions that reinforce climate change effects.[10,11] In addition, poor fertilizer usage and runoff lead to not only degradation and salinization of soils but also eutrophication of our water sources.[11−14] Therefore, upscaling new means to ensure environmentally friendly and sustainable solutions for soil management and agricultural production is required.[15] Furthermore, phosphate is a non-renewable resource.[16] Morocco hosts by far the largest reserve, holding 80% of global rock phosphate.[16] This makes supply a conceivable problem as China, the U.S.A., and India (the largest food demanders) will runout of phosphate by 2040.[17] Microbes have the potential to increase phosphorus plant intake as most phosphate is held in inorganic insoluble form [e.g., $Ca_3(PO_4)^2$] and organic insoluble/soluble form (e.g., phytate and nucleic acid), which microbes can make available to plants and, therefore, optimize the use of the synthetic phosphorus fertilizer application.[18] The exploitation of microbes has proven to provide environmentally friendly and sustainable solutions that should be pursued, yet it shows some constraints.[14,19]

Chemical fertilizer attributes, such as quick and non-specific action, low-cost production, and ease of storage, made them widely acceptable.[20] However, their detrimental effects to soils, plants, and animals when they are not used efficiently motivate us to find complementary alternatives to optimize their use and, thereby, lower their impact on soil fertility and biodiversity.[21−23] Further, pest resistance and high-concentration use/overuse are unresolved problems that generate an increasing demand for sustainable solutions. Therefore, there is a growing interest in the use of microbial fertilizers as complements to synthetic fertilizers and agrochemicals.[24] Nitrogen and phosphorus are the two most important nutrients to plants and applied nutrients in agriculture. Therefore, to secure food supply and farm sustainability, microbial alternatives are necessary to optimize their use.

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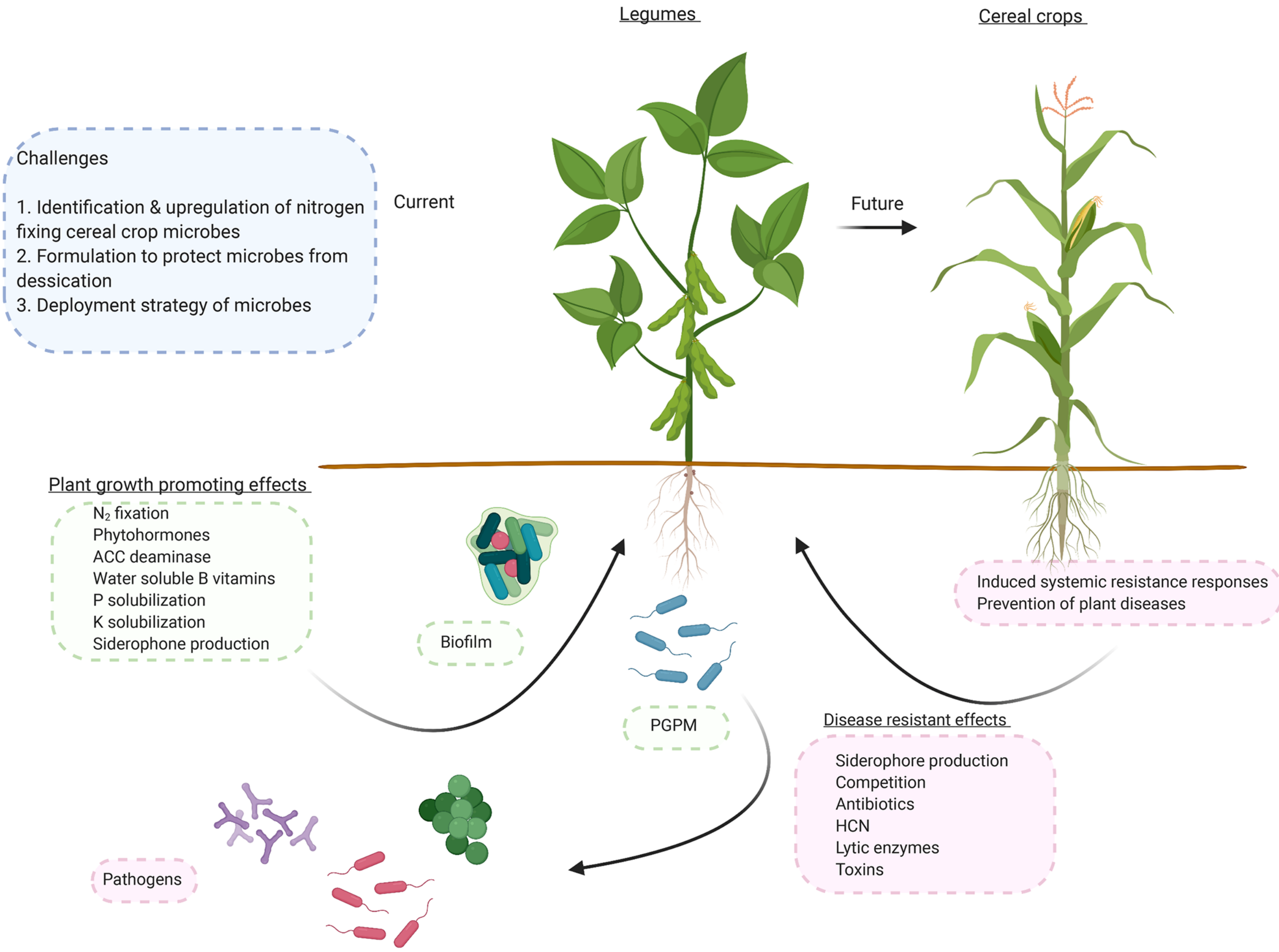

Figure 1. Mechanism of plant-growth-promoting microbes.

Nitrogen-fixing and phosphate-solubilizing microbes can be used in co-inoculations (individually or as consortiums), which result in greater plant growth promotion by providing these essential macronutrients while lowering our carbon footprint.

Naturally derived nutrients and soil stressor alleviators have existed for centuries for integrated nutrient and disease management and soil biodiversity for rhizobia, and now, they are used for other plant-growth-promoting microbes.[25] Initially, farmers knew that the soil taken from a previous legume-sown field to a non-legume field often improved the yield. The soil transfer approach was followed until the end of the 19th century for legume seed inoculation.[26] Advances in the understanding of plant−microorganism interactions are now well-known and have led to the discovery and exploitation of plant-growth-promoting microorganisms (PGPMs), which include archaea, bacteria, and fungi. However, some can be a biohazard.[27] Plant microbes provide the nutrients that plants require and regulate plant growth. PGPMs facilitate this directly through nitrogen fixation, phosphate solubilization, and phytohormone production[28] (Figure 1) and indirectly by preventing the negative effects of phytopathogenic organisms through the production of antimicrobial compounds or the elicitation of induced systemic resistance.[29] PGPMs pertain to the following classes: the rhizospheric microbes found around the soil in the plant rhizhosphere (root system), phyllosphere (aerial parts of plants), and rhizoplane (root surface) and endophytes found inside the plant root, stem, and leaf systems.[30] Implementing solutions that can be used in agricultural practices is crucial. Our focus in this review will be on bacteria, given that archaea are still an underdetected and scarcely studied part of the plant microbiome, while fungi (which are eukaryotic) are only able to obtain fixed nitrogen through symbiotic interactions with nitrogen-fixing prokaryotes and we believe cannot fix nitrogen. Nevertheless, a recent study showed potential for nitrogen fixation in the fungus-growing termite gut.[31−33]

Emerging technologies, such as proteomics, metabolomics, transcriptomics, and next-generation sequencing and data science, have made and will make the discovery of useful compounds, microbe interaction understanding, and identification and characterization of microbial inoculants fast and easier.[27] Microbes are very specific to the plant and use case. Therefore, the gathering of data on microbial interactions and learning from this data are essential in the use and delivery of plant microbes. Furthermore, the interplay of microbes in a consortium needs to be better understood because some have synergistic effects as singular strains but may have detrimental or beneficial effects when used in a consortium. The inoculation of plants with a microbial consortium provides better benefits to a plant than with a single isolate.[34,35] This could be because microbial consortia may have synergistic interactions to provide nutrients, remove inhibitory products,

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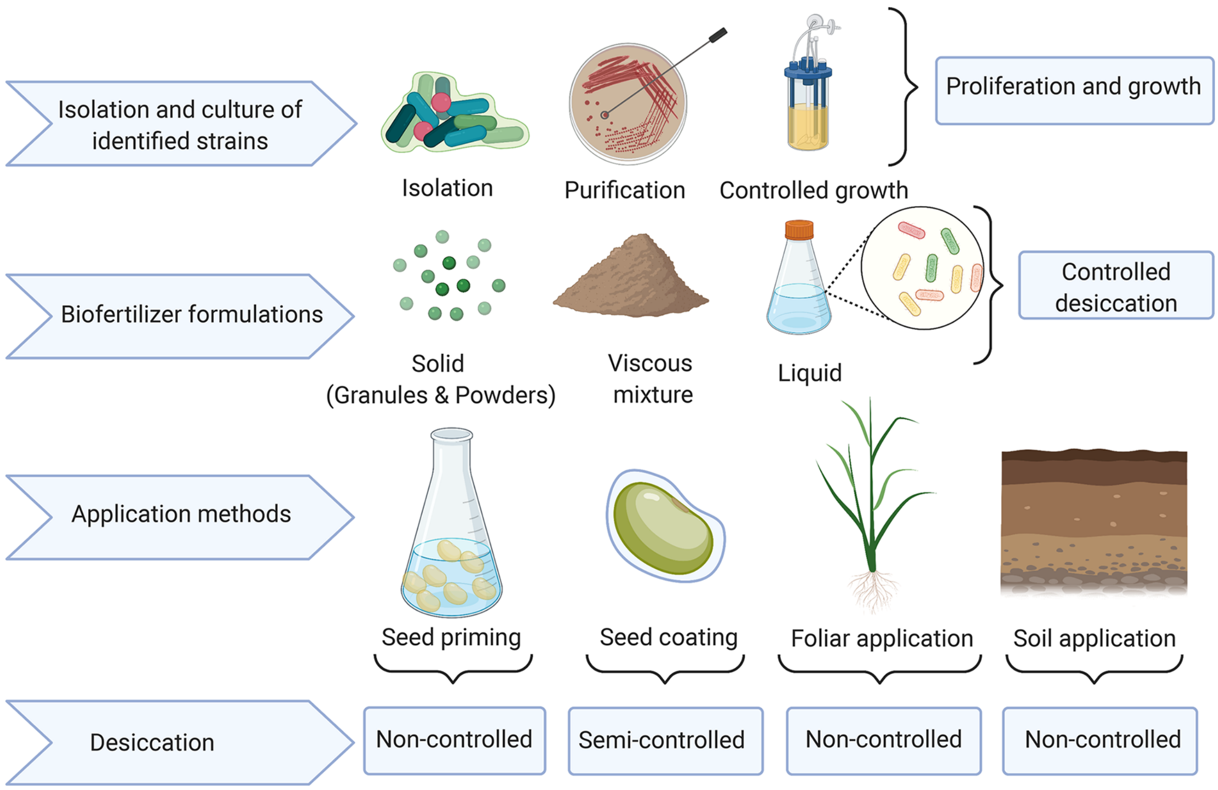

Figure 2. From identification to formulation and application of microbial fertilizers. The application procedure and formulation control the desiccation process.

and trigger each other through biochemical and physical activities that might enhance beneficial effects on plant physiology.[36] Recently, a large-scale genomic comparison of PGPMs discovered that the dominant bacteria associated with plants are Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria, which had also been suggested in previous studies.[37,38] Microbiologists are working on better understanding microbial communities, and this will be essential in understanding how to deliver microbes in different soils that possess different microbial communities and nutrients. It was suggested that inoculated bacteria are actively influenced by the plant genotype, cropping conditions and co-inoculated or residing bacterial populations, which can considerably influence the resulting plant-growth-promoting bacteria (PGPB) effects.[39,40]

Microbes can be classified as either Gram negative or Gram positive. Gram-positive bacteria possess a thick (20−80 nm) cell wall as the outer shell of the cell. In contrast, Gram-negative bacteria have a relatively thin (<10 nm) layer of the cell wall but harbor an additional outer membrane with several pores and appendices.[41] The relatively thin cell wall makes Gram-negative microbes delicate to dry, handle, resuscitate, and deliver. Currently, there are several means to deliver microbes in the soil, but they are not efficient and lack ease of implementation in remote regions of the world, where agriculture practices cannot account for handling of living bacteria.

PGPB are endophytic or rhizospheric and are known to associate with a variety of crops in plant root structures, leaves, and surrounding soils.[42] In an effort to better understand the microbial delivery tools that are currently used to deliver PGPB effectively, it is first necessary to take into account the best strain of microbe or a microbial consortium for the intended effect on the target crop. Then, the formulation of the inoculant should be addressed and, finally, the delivery method (Figure 2).[43] Currently, delivery happens through biopriming, which is a biological process of seed treatment that mixes seed hydration and seed inoculation with plant beneficial microorganisms to improve seed germination and their protection against soil-borne pathogens, achieving seedling and vegetative growth.[44] However, given its labor-intensive nature, this process is mostly appropriate for low−medium volumes of high-value crops.[45] Soil inoculation is also used as an alternative. However, it requires high volumes of inoculant, is labor-intensive and, thus, expensive, and may be restricted by local environmental regulation and health concerns.[46] Seed coating has the potential to be a cost-competitive and time-saving approach for crop production and protection. Nonetheless, microbial seed coating is hindered by low performance and standardization, which limit its broader use.[46]

2. CHALLENGES

Several challenges, such as unpredictability of results, difficulties in the identification and isolation of bacterial strains in field experiments, poor understanding of specific mechanisms that regulate the interplay between microorganisms, plants, and soil, have limited the use and effectiveness of PGPB.[47] In this context, two key aspects that dominate the effectiveness of inoculation are the microbial isolation and the application technologies.[43] The design and delivery of microbial consortia through inoculation are challenging and require the understanding of their modes of interaction, microbial adhesion to seeds, plant root colonization, and antagonistic relationship interactions, if present.[48] Differences

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in root communities have been attributed to plant host effects and microbial host preferences as well as factors pertaining to soil conditions, microbial biogeography, and the presence of viable microbial propagules.[49] The unprotected, inoculated bacteria must compete with the often better-adapted native microflora and withstand predation by soil microfauna.[43] The environmental conditions also affect the inoculant efficacy, and adverse abiotic stresses (hot, dry, and saline conditions) can cause a rapid decrease in PGPB populations.[50,51] The following challenges are important in improving PGPB performance.

2.1. Desiccation. Microbial desiccation affects viability of microorganisms. The number of metabolically or physically active microbes is the leading factor toward the efficacy of PGPB when applied to the seed surface.[52] Desiccation is the process of water removal from (or extreme drying of) an organism; therefore, drought stress affects microbial biodiversity in soils. Microbial viability is important because it increases the effectiveness of microbe infection, permitting PGPB to induce a positive effect in plants. Therefore, desiccation-tolerant microbes are highly desirable because they can remain in soils and inoculant formulations for a longer time than those that are not desiccation-tolerant.[34] A recent study reported that 95% of PGPB do not survive in the time intercurring between inoculation of the seed and planting (considering a 4 h time window) and that 83% of the surviving microorganisms die in soil within 22 h.[53] In nature, there are anhydrobiotic organisms that are able to survive desiccation by going into a dormant state, in which metabolism is undetected. Once rehydrated, they are able to restore their metabolic processes. Learning anhydrobiosis from such organisms will be a beneficial approach in finding ways to mitigate desiccation stress. Some PGPB have acquired desiccation-tolerant mechanisms, such as the production of intrinsic trehalose.[53] Trehalose produced may regulate most of the enzymatic and non-enzymatic responses of the plant by supporting the production of the collection of phytohormones of the plant.[54] Other organisms, called xero−halophiles, are extremophiles and live in areas where soil is very saline and dry. Desiccation is a topical subject in microbial fertilizers because the efficacy of the microbe fertilizer is correlated with viability of the microbes. As the agriculture field looks for opportunities to transition from synthetic fertilizers to microbial fertilizers (also known as biofertilizers), there is an increasing interest in scalable technologies that address desiccation tolerance by providing, for example, a microenvironment that facilitates microbe survival and growth in the form of seed coatings that then degrade in the soil and deliver PGPB. Alternative technologies to boost PGPB performance include the selection of desiccation-resistant strains and the use of synthetic biology tools to provide desiccation-resistant genes.

2.2. Climate Change. Climate change has impacted soil microbial communities, resulting in increased atmospheric CO_2 concentration, temperature, precipitation, and drought.[55] The effects have been both positive and negative. Numerous studies have shown how elevated CO_2 levels increased the abundance of arbuscular and ectomycorrhizal fungi, whereas the effect on PGPB and endophytic fungi were more variable. Mostly, PGPB were beneficial under elevated CO_2,[55] which leads to higher carbon availability in the rhizosphere and may alter root exudation composition. Root exudates play a huge role in the structure and function of microbial communities. This indicates that colonization of plants depends upon compounds produced by plants, which are affected by climate change factors, such as temperature and drought. In these conditions, different microorganisms show potential for different functional activities that lead to altered community structures and may be used to impart different colonization strategies by inoculating microorganisms, such as arbuscular mycorrhizal fungi, to change the composition of the microbial community.[56] Further, at elevated CO_2 concentrations, nitrogen becomes a growth-limiting nutrient, and as such, nitrogen-fixing and -acquiring microorganisms may gain increasing importance.

Temperature effects are coupled with soil moisture and are, thus, difficult to deduce. Soil microorganisms and the processes that they mediate are temperature-sensitive. Decomposition of organic soil matter, soil respiration, and growth of microbial biomass increases with the temperature. It has been hypothesized that temperature effects are transient; as the temperature increases, the soil carbon substrates are quickly depleted by enhanced microbial activity, and because of trade-offs, microbial communities either adjust, shift in composition, or constrain their biomass to respond to altered conditions and substrate availability.[57,58]

Drought leads to soil moisture stress, which impacts the soil microbial community; however, it is less investigated than CO_2 or temperature. Drought amplifies the differential temperature sensitivity of fungi and bacteria.[55] Small changes in soil moisture can shift fungal communities from one dominant member to another, while bacteria remain constant. Typically, drought reduces fungal colonization, although the outcome can be strain-dependent.

2.3. Soil pH. Soil pH is one of the most influential factors affecting the soil microbial community.[59] pH greatly affects abiotic factors, such as carbon availability, nutrient availability, and the solubility of metal ions. Furthermore, pH may affect biotic factors, such as biomass composition of fungi and bacteria in both forest and agriculture.[59] The challenge of studying pH effects is its varied effects on multiple factors. Rousk et al. showed that, as pH drops from 8.3 to 4.5, a 5-fold decrease in bacterial growth and a 5-fold increase in fungal growth were measured. Fungi generally exhibit wider pH tolerance when compared to bacteria, which tend to tolerate narrower ranges.[60] The shift in fungal and bacterial importance as pH drops has a direct negative effect on the total carbon mineralization. Below pH 4.5, there is general microbial inhibition, probably as a result of the release of free aluminum and the decrease in plant productivity. Conversely, studies conducted from soils from North and South America have shown that both the relative abundance and diversity of bacteria increased with soil pH, considering ranges between pH 4 and 8.[60] The relative abundance of fungi was, however, unaffected by pH, and fungal diversity was weakly positively related.[60]

2.4. Competition in the Soil and Microbe Concentration. Inoculated legume root nodules are mostly formed by indigenous microbes present in the soil.[52] Microbe competition is one of the key determining factors for infection effectiveness. Rhizospheric microorganisms connect plants and soils and together develop an ecosystem that provides nutrient life cycle and soil fertility.[61] Technological advances in DNA sequencing, molecular ecology, and data science have provided the tools to study plant-associated and soil microbial diversity and to assess the implication of this diversity on ecosystem functioning.[62] When microorganisms are delivered into the soil, we need to consider the surrounding ecosystem that will

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be in competition with them. The viability, concentration, and delivery method of microbes become vital as a competitive advantage over other microbes as the physiological state of microbes can prevent biomass buildup. Therefore, the microbe release mechanism in soil becomes paramount as it affects the concentration and location of delivery that are impacted by rhizospheric microbe competition. A threshold number of cells, which differs among species, is essential to obtain the intended positive plant response. For example, it has been reported that 10^6-10^7 cells $plant^{-1}$ are necessary for the PGPB *Azospirillum brasilense*.[63] Oliveira et al. showed that a consortium of microbes improved plant growth more than a singular isolate inoculation.[48] Gottel et al. and Shakya et al. found that the ecological niche (endosphere versus root) outperformed other measured factors (soil properties, season, plant genotype, etc.) (upland versus lowland) in shaping microbial communities.[49,64]

2.5. Soil Structure. Soil structure is the arrangement of primary soil particles and the pore spaces between them. Microbe−plant interactions are influenced by the soil type, soils that share a certain set of well-defined properties.[49] Biological linkages between soils, roots, and the atmosphere are poorly characterized. However, Bonito et al. showed that bacterial communities in the root are more tightly structured by plant host species than by soil origin.[49] Plants, soils, and microbiota interact and function in a zone known as the root microbiome,[65] which is characterized by elevated rates of respiration, nutrient turnover, and carbon sequestration, highlighting its importance to the functioning of terrestrial ecosystems.[66] The nutrient concentration, pH, and water content play an active role on microbe colonization. Microbes are very specific and, therefore, have differing niche microenvironments that accommodate them best. The distribution of bacterial and fungal communities and their function vary between different aggregate size classes.[67] Further, compaction of soil has detrimental effects as it affects physical properties of soil, such as bulk density, soil strength, and porosity. Compaction limits the mobility of nutrients, water and air infiltration, and root penetration in soil.[68] Juyal et al. have shown how increasing soil bulk density (compaction) significantly reduced the number of microorganisms in soil and their growth rate. Good soil structure provides an array of niches, such as substrate availability and redox potential, which can house diverse microbial communities.[69] Microbes reside in pores and inner surfaces of aggregates as microcolonies of 2−16 microbes each, and extensive colonization is restricted to microsites with higher carbon availability, e.g., rhizosphere and outer surfaces of freshly formed macroaggregates.[70] The location of aggregates in relation to roots, organic residues, and macropores is more important for determining the microbial community composition and their activity.[69] Understanding the microbe niche environment will help build predictive models and provide skills in shaping the rhizosphere of the plant as microbes are very specific with regard to conditions required for colonization.

2.6. Perspective. PGPB are plant- and soil-specific, which makes them challenging to deploy universally. However, as our understanding of soil structure, soil pH, impact of climate change, soil microbe concentration, and desiccation impact on plant and soil microbe interaction increases, the efficacy of microbe-based fertilizer can be enhanced by precise microbe selection, developing models based on plant, and investigating microbe and soil interactions. All of the extrinsic factors influencing PGPB growth and metabolism are coupled together, and understanding how they all interact will be key to design highly effective techniques to develop and deploy, at scale, biofertilizers.

3. FORMULATIONS

Rhizobia bioformulations have been on the market for centuries in numerous forms. Commercial biofertilizers can be solid carrier-based (organic or inorganic), liquid, synthetic polymer-based, or metabolite-based formulations.[51] The formulation is composed of the microbe, carrier material, and additives. The first commercial nitrogen biofertilizer of rhizobia, "Nitragin", was patented by Nobbe and Hiltner.[51] Initially, the inoculation procedure entailed transferring soil from legume-grown soils to soils that will host plants. Following this first technology, solid-based carriers came into use in the early 1900s. Even today, many of the microbial inoculants all over the world are based on solid-based carriers, mostly peat formulations. This has been true for well-developed legume inoculants based on selected rhizobial strains as a result of peat bacterial protection properties,[71] such as high water holding capacity, chemical and physical evenness, and non-toxic and environmentally friendly nature.[72] However, peat is very inconsistent and a non-renewable resource, making it unusable on a large scale.[73] Thus, interest in substitutes grew, and alternatives, such as lignite, filter mud, coal−bentonite, cellulose, coal, soil, charcoal, manure, compost, powdered coconut shells, ground teak leaves, and wheat straw, have been used as solid carrier materials.[51] Granular carriers were also developed for direct application to the soil, which made handling, storage, and application easier.

Liquid formulations were developed as alternatives to solid carriers as a result of their limitations, such as environmental impact and carbon emissions of peat-made solid carries.[72] Further, liquid formulations are better suited for mechanical sowing in large fields.[43] In 1958, freeze-dried inocula came on the market and then gel-based microbial inoculants that entrapped rhizobia in polymer gels, such as polyacrylamide-entrapped *Rhizobium* (PER), alginate-entrapped *Rhizobium* (AER), and xanthan-entrapped *Rhizobium* (XER), which gave satisfactory results in wet conditions.[51,74] In the early 2000s, the modification of liquid formulations by the addition of additives and cell protectants was proposed. The additives promote cell survival in storage and after application to seed or soil.[75] Commonly used additives for rhizobial inoculants were polyvinylpyrrolidone (PVP), carboxymethyl cellulose (CMC), gum arabic, sodium alginate, and glycerol.[51] PVP protects microbes from desiccation and harmful seed exudates, and the rheological property of CMC increases the gel viscosity of carriers to make it more suitable for viability of rhizobial cells.[51] Further, genetic modification of rhizobia is being developed to improve the efficacy of nitrogen fixation in new formulations, such as upregulating nitrogen fixation.[76] The emerging technique of secondary metabolite addition (flavonoids and phytohormones) to bioformulations increases agricultural productivity by improving the inoculant efficiency.[77] The addition of flavonoids to rhizobial formulations during growth significantly alleviates the effects of adverse conditions,[78] enhances nitrogen fixation,[79] and improves the rhizobial competitiveness and nodulation.[51] The cost associated with flavonoid isolation or synthesis is sometimes justified by the low concentrations used in the final formulation.[80,81]

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Despite, the above-mentioned technologies, bioformulations still face many limitations. Inoculation formulations have improved microbial survival during storage of products, but these efforts have not improved survival on the seed or in soil.[52] Bacterial survival on the seed is mainly affected by three factors: desiccation, the toxic nature of seed coat exudates, and high temperatures.[82] Therefore, there is a need to find biomaterials that could provide a microenvironment to protect microbes from desiccation while also having the mechanical properties to conform around a seed (Figure 3).[83] Biomaterials are biocompatible, biodegradable, and abundant and, thus, have potential in enhancing food security and safety.[84−87]

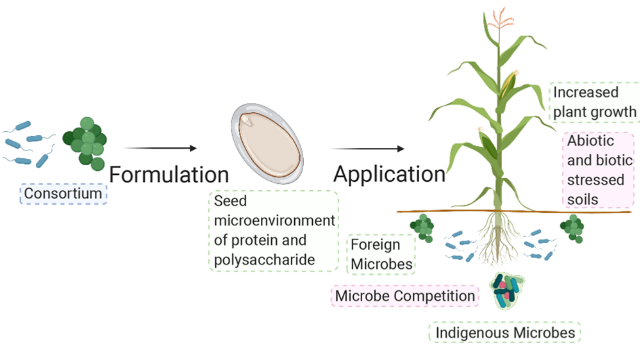

Figure 3. Seed-coating technology encapsulates and protects microbes while providing a targeted *in situ* release of payload to be delivered.

Efficacy of formulations depends upon their shelf life, which depends upon several factors, such as production technology, carrier and packing material used, transport activity, and farmer practices, to sustain the quality of inoculants.[88] Factors related to production processes (quality and marketing standards) are also important for consistency and user uptake. Currently, the storage, preparation, and application of formulations need special facilities and skills, which most farmers and suppliers do not possess.[89] Therefore, an easy to use alternative is necessary for better adoption. The current problems with most formulations are a lack of robust scientific data. According to Brockwell et al.,[90] 90% of inoculants have no impact on the target crop. Further, Herrmann et al.[91] reported that more than 50% of the inoculants have high levels of contamination. Contaminants have detrimental effects on the quality of rhizobial inoculants, and 25% of the contaminants of the commercial inoculants can be opportunistic human pathogens. Therefore, many inoculants produced globally, because of the lack of quality control, tend not to perform well. Thus, there is a requirement for strict regulations for rhizobial bioformulations to overcome the above-mentioned problems related to worldwide production and application of biofertilizers. In the future, emphases should be given to techniques that increase population density and survival of rhizobial strains in inoculants and minimize operator exposure to a high dose of PGBPs whether in solution or in water droplets. Additionally, survival of cells is mandatory for better commercialization of rhizobial inoculants on the global market.[92]

Nano-bioformulation of biofertilizers has emerged as one of the most promising techniques to achieve this goal. It comprises nanoparticles made up of organic or inorganic materials that interact with microorganisms and enhance their survival by providing protection from desiccation, heat, and ultraviolet (UV) inactivation. Applications of nano-bioformulations also include environmental cleanup strategies.[93] In 2015, PGPB, such as *Pseudomonas fluorescens*, *Bacillus subtilis*, and *Paenibacillus elgii*, treated with silver, aluminum, and gold nanoparticles have been shown to support plant growth and increase pathogen resistance.[94] The release of such nano-encapsulated biofertilizers into target cells is operated in a very controlled manner, free from any harmful effects and increasing the adhesion of beneficial bacteria within the root rhizosphere.[95] Additionally, nano-biofertilizers may be considered as an alternative to chemical pesticides,[96] although the deployment of nanoparticles in the environment needs to satisfy stringent requirements imposed by policymakers.

The application of phyto-nanotechnology on agriculture could change the traditional plant production systems, providing the controlled release of agrochemicals (e.g., pesticides, herbicides, and fertilizers) and target-specific transport of biomolecules (e.g., activators, nucleotides, and proteins). Nano-encapsulation using biodegradable materials also makes the assembled active elements straightforward and safe to be handled by the farmers. An advanced understanding of the interactions between nanoparticles and plant responses (uptake, localization, and activity) could transform crop production through improved disease resistance, nutrient use, and crop yield.[97]

The use of polymeric inoculants and alginate beads have already been tested and need more exploration for their future use.[43,51] Furthermore, the use of stress-tolerating microbes/rhizobia in inoculations is also thought to be imperative in developing bioformulations that will survive in stress conditions (high temperature, drought, and salinity).[98,99]

The use of genetically improved rhizobia as inoculants has some legislative constraints because it requires permission from environmental protection agencies to release into the environment and because of the little understanding of microbial ecology.[100] Further, the majority of microbial seed inoculation involves private companies (agrichemical and seed companies) that rarely disclose their data and formulations,[45] although there is compelling need to develop more comprehensive knowledge that integrates academic efforts to speed up advancements and the development of disruptive technologies.

3.1. Perspective. Peat-based formulations have been traditionally used for the delivery of microbe-based fertilizers. These tend to be good at providing the niche for microbe growth when outside the soil and when inoculated. However, because peat is a non-renewable resource, new formulations are required. Liquid-based formulations have been developed; however, performance in microbe preservation can be improved to ensure high efficacy of the inoculant. As we learn new lessons on how microorganisms survive desiccation, e.g., by looking at tardigrade production of trehalose and intrinsically disorder proteins to promote water substitution and vitrification, new strategies can be designed to engineer formulations that better protect and store microbes outside the cold chain and in operational conditions before deployment in the field.

4. RHIZOSPHERE AND ENDOSPHERE

4.1. Rhizobacteria. The rhizosphere is the region of soil directly surrounding the root system that is directly influenced by root secretions and associated soil microorganisms known as the root microbiome.[101,102] Rhizobacteria imply a group of bacteria found in the rhizosphere that can colonize the root

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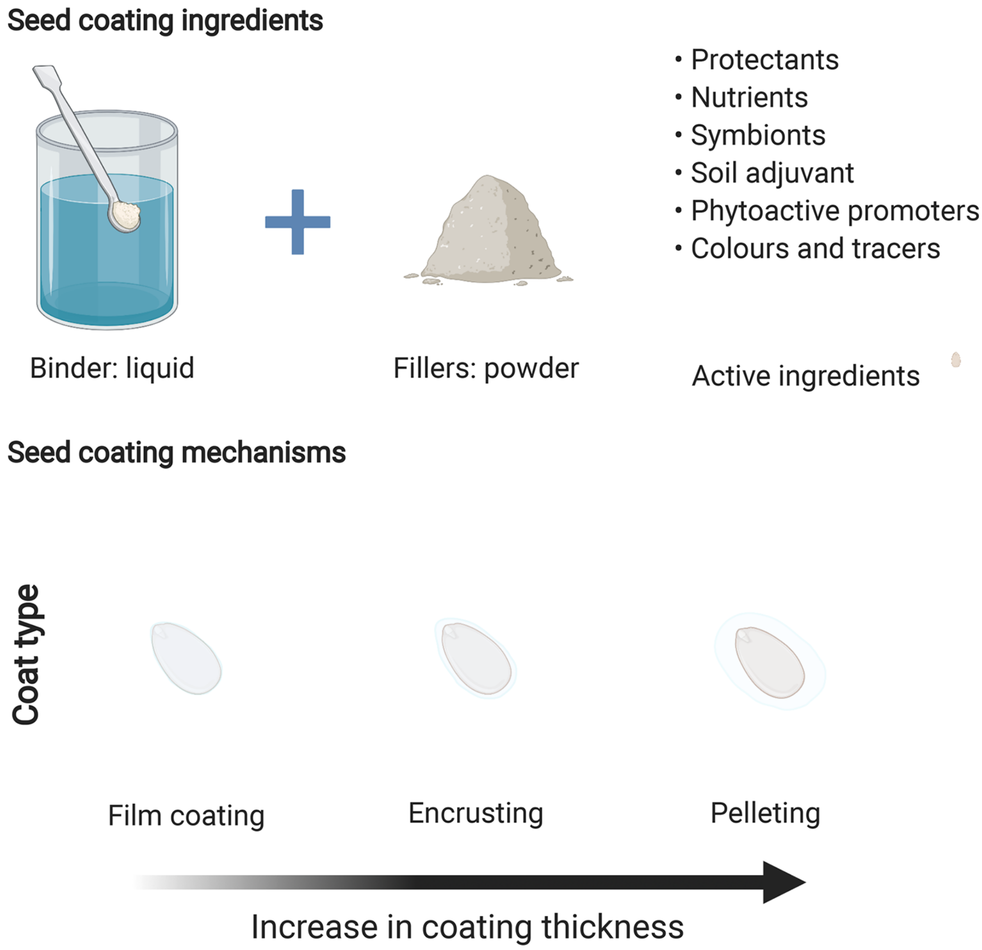

Figure 4. Seed-coating ingredients, process, and types.

system.[103] It has been demonstrated that bacterial cells first colonize the rhizosphere following soil inoculation.[104] Therefore, microorganisms delivered in the soil need to be able to colonize the rhizosphere before they can have an impact on plant health and metabolism. Bacterial cells have been visualized as single cells attached to the root surfaces and, subsequently, as doublets on the rhizodermis, forming a string of bacteria.[105] Colonization then occurs on the whole surface of the rhizodermal cells.[106] For microbes to produce plant-growth-promoting factors, they need to be able to colonize the rhizosphere and/or the rhizoplane during an extended period characterized by strong microbial competition with rhizosphere-competent microbes (microorganisms that have the capacity to effectively build a population of microorganisms on plant roots or in the vicinity).[107] Furthermore, root colonization is complex and non-uniform. This can be explained by different factors, such as varying root exudation patterns released by plants and containing a chemoattractant to promote microbe colonization and growth.[108] Rhizosphere colonization is however a complex system influenced by both microorganism competition during inoculation and rhizosphere competence of the microbe. We have yet to fully understand these interactions, which are soil-specific, as a microbe needs a specific niche to perform optimally.

4.2. Endophytes. There are types of microorganisms that do not only colonize the rhizosphere but also enter and colonize the plant tissue for beneficial effects, i.e., endophytes.[105] Studies have shown how plants host a diverse group of endophytic microbes, and most endophytes are derived from the rhizosphere, e.g., rhizobium.[109,110] Endophytes are a subgroup of rhizobacteria known for entering the endorhiza (the root interior) once the rhizosphere has been colonized. Moreover, they are known to show a more intense plant-growth-promoting behavior when compared to exclusively rhizospheric colonizing microbes.[111] The penetration process does not involve an active mechanism but rather a passive mechanism. Passive penetration can take place at cracks, such as those occurring at root emergence sites or created by deleterious microorganisms, as well as root tips.[112] However, some microorganisms have developed active mechanisms, such as root-nodulating rhizobia. The nodulation mechanism is mediated by root release of chemoattractants (e.g., flavonoid exudes) and microbial signals (nod factors), and as such, it is specific and specialized. Root invasion can happen through fissures that occur at the lateral root base and by cortical intracellular entry.[113,114] Besides, plant−rhizobia endophytic interactions are not well-understood. Further, emerging but limited knowledge exists on endophytes colonizing flowers, fruit, and seeds.[115] In addition, evidence of endophytic microbes found in plant stems and leaves and not in the rhizosphere highlights other potential colonization mechanisms. Bacterial endophytes are carried inside the seed (vertical transmission) and can be equally important for the evolution of the microbial community of the seedling.[116,117]

4.3. Perspective. Microbe identification remains a very important matter as we search for the best performing

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microbes with regard to nitrogen fixation and phosphate solubilization. These remain a matter of interest as we search for nitrogen-fixing microbes for cereal crops. Cereal crops make up a considerable percentage of the foods farmed globally. The diversity of our soils has decreased with modern agricultural practices; however, PGPB play a pivotal role in enhancing the sustainability of the agriculture system and may enable the production of better quality food, thus promoting health and wellness.

5. APPLICATION METHODS

Soil microbe delivery systems, to be effective for field-scale use, have to be designed to provide a dependable source of bacteria that survives in the soil and becomes available to crops, when needed.[43] Rhizobia application can be performed on the seed surface, directly into the soil, or through plant inoculation.[43,46] Seed inoculation outnumbers soil application and depends upon the requirement of the type of inoculant, the seed type, and the inoculant volume. The efficacy of each inoculation technique needs to be taken into account. Effects such as a high temperature of a seed coater and air seeder, high pressure, rapid drying when the inoculant is sprayed into sowing machinery and when inoculated seeds are sown under hot and dry conditions, and when seeds are treated with fungicides and herbicides potentially have large deleterious effects.[43]

5.1. Seed Inoculant: Seed Coating and Biopriming. There is typically limited success from coating seeds with rhizobia because it is difficult to maintain living and active bacterial cells.[118] Factors such as temperature, humidity, and toxic substances all affect the survival of rhizobia in the seed-coating agent.[82] However, this is the most common and practical seed inoculation procedure. This happens because it is the easiest method to use and requires considerably small volumes for inoculation.[82] Additionally, the standard seed-coating technology has not changed in years.

Seed coating is a technique that entails the covering of a seed with a material laden with microbes to enhance the seed performance and plant establishment while reducing cost, to meet the requirements in development for precision agriculture (Figure 4). Historically, coating seeds has been broadly used as a cost-effective way to alleviate abiotic and biotic stresses, thus boosting crop growth, yield, and health.[119] The process is very streamlined; seeds are dusted with peat inoculant, with or without water or adhesive. With small seeds, fillers, such as limestone, are added, with or without adhesive, and allowed to dry.[43] The coated seeds are dried *in situ* or just before sowing. *In situ* coating standardizes the delivery and makes the technology easy to use for farmers but tends to lead to a lower microbial count than coating before sowing. Seeds may be a basic input deciding the fate of productivity of any crop. Commonly, seeds are studied for their germination and distributed to growers. Despite the very fact that the germination percentage registered within the seed-testing laboratory is about 80−90%, these efficiencies can hardly be replicated in the field because of the inadequacy or non-availability of sufficient moisture under rain-fed systems.[120]

One essential condition to seed coating is adding adhesive materials. There is no standardized material used as an adhesive.[121] Adhesives are used to ensure that a threshold of microbes are added and to secure microbes on the seed. Adhesives include gum arabic, carboxymethyl cellulose, sucrose solutions, vegetable oils, and any non-toxic, commercial adhesive that can bind to bacteria and seeds.[43] With regard to seed-coating applications, coating is either performed by hand, rotating drums that are cheap to operate, large dough or cement mixers, or mechanical tumbling machines.[122] Liquid inoculants are directly sprayed onto the seed before being sown once dry. The microbes can be macro- or micro-encapsulated during the process. Microencapsulation leads to smaller particles and, thus, a larger surface area, which enhances controlled release.[123] However, seed coating has several disadvantages. Each seed can only contain a restricted amount of inoculant, which may be a limiting factor because a threshold of bacteria may be needed for successful inoculation with most PGPB.[43] The seed-coating process may damage the natural coating of seeds and alter the water or oxygen absorption properties of the seed, affecting its germination capabilities.[43] Furthermore, release and degradation properties of microbes from seed coating are important parameters to control induction of microbe colonization and combat desiccation in the soil. Some fungicides and insecticides applied to the seeds before coating may be detrimental to the inoculant; therefore, seed treatments need to be carefully streamlined to avoid detrimental effects on the final product.

Biopriming is a process of biological seed treatment that involves the soaking of seeds in any solution containing required biological compound followed by redrying the seeds, which results in the start of the germination process, except the radicle emergence.[124] It allows for the bacterial imbibition into the seed, creating ideal conditions for the bacterial inoculation and colonization in the seed, and reduces the chance of desiccation and the amount of pesticide applied to the field.[124] Soaking of seeds initiates the physiological germination processes, where plumule and radicle emergence is prevented, until the seeds are provided with the right temperature and oxygen after being sown. Microbes in the seed keep on multiplying and proliferate in the spermosphere even before sowing.[124] Biopriming leads to improved germination and seedling establishment; however, it has to be performed on site and can be labor-intensive.[46] Given the effort required for this process, it is most appropriate for low−medium-volume high-value crops, such as vegetable seeds.[45]

5.2. Soil Inoculant. Soil inoculation is used to release high volumes of inoculant into the soil but is time-intensive and expensive and may be limited by threshold number regulations.[46,125] Soil inoculation can be achieved by adding granules in the seedbed or adding a liquid inoculant into the seedbed.[43] This process ensures that no inoculant is lost during seed planting through sowing machines. Besides, small seeds that have limited surface area can be sufficiently inoculated with enough microbes using this technique.[43] In highly mechanized farming, granular inoculants work well because the machinery for seeding commonly includes accessories for application of fertilizer and pesticide and inoculation is just one additional input during seeding.[43]

Granular forms of soil inoculant include peat, marble combined with peat, perlite, charcoal, or soil aggregates. Granular inoculation enhances the chance for the inoculant to be in contact with plant roots, which helps with microbe colonization and, therefore, effectiveness.[43] The method of soil inoculation used depends upon the farmer preference. Nonetheless, it always tends to be more expensive than seed coating. The method of application is determined by the seed size, equipment availability, seed fragility, presence of insecticide and fungicide on the seed surface, and cost that farmer is willing to pay.[43]

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Table 1. Comparison Table between Biofertilizer Application Methods

application method	comparison	reference
	Seed Inoculation	
seed coating	**advantages**	
	seed inoculation is less expensive than in-furrow inoculation, especially for small seeds	135
	can be stored easily	136
	low costs of storage; easy handling and transportation	45
	used for recalcitrant species multiplied by seeds like orchids	137, 138
	controlled release of microorganisms	119
	increase of the microbial shelf life	119
	limitations	
	adapted to microbes compatible with dry formulations	45
	non-sporulating bacteria experience large viable cell losses during dry formulation	75
	affected by storage conditions	139
	affected by the abrasion and seed contact	140
	antagonism between the soil microbiome and the inoculated bacteria	141
biopriming	**advantages**	
	useful to combat the disease problem	142, 143
	improve immediate availability of micronutrients	144
	used for recalcitrant species	145, 146
	limitations	
	immediate application	147
	dependent upon the interaction time	147

application method	comparison	reference
	Soil Inoculation	
	advantages	
	increase of the effectiveness by immobilization of inoculant cells and their embodiment in polymers	148
	limitations	
	antagonism between the soil microbiome and the inoculated bacteria	141
	Plant Inoculation	
root	**advantages**	
	adapted to *in vitro* plants and recalcitrant species	127, 128
	facilitate bacterial root adhesion through formation of biofilm on the root surface	149
	limitations	
	requires large amounts of inoculant and the concentration of the bacterial suspension	150
	dependent upon the exposure time of the root to the bacteria	150
foliar	**advantages**	
	passive colonization through the stomata apertures, plant wounds, or insect feeding	134, 151
	can be combined to nanoparticles to increase the efficiency and effectiveness of the inoculation	152
	limitations	
	unfeasibility in large-scale agriculture	45
	spraying equipment can influence the uniformity of foliar spray	153
	dependent upon the droplet size in terms of microbe concentration and leaf coverage	154
seedling pretreatment	**advantages**	
	can be used in greenhouse vegetables	155
	limitations	
	requires a plasma treatment for immediate and effective bacteria activation	156

5.3. Plant Inoculation. The plant microenvironment is naturally colonized by microorganisms. More than 90% are bacteria.[126] Some of them are PGPB with the ability to enhance plant growth via providing required nutrition or increasing the availability of nutrients in an assimilable form. Plant inoculation involves the inoculation of plants through root dipping or foliar spray.[46] These techniques require large amounts of inoculant, and with regard to root dipping, plant nursery preparation is also required.[46] This highlights that the root-dipping process is very time- and labor-intensive, which makes it unfeasible in large-scale agriculture.[45] PGPB application performed on roots or cuttings to promote *in vitro* rhizogenesis is mainly performed in recalcitrant species.[127,128] They can be applied as a dipping solution or can be added to the rooting media just before transferring the shoots.[129,130]

Exogenous application using foliar spraying is conducted using the inoculum alone or in specific formulations to ensure bacterial cell fixation on the leaves and also to maintain a live bacterial count until colonization through the stomatal apertures.[131] This method of application relies on climatic conditions; increased atmospheric temperature alters the plant microbe interaction by reducing the bacterial charge and inducing intrinsic reactions in the plant by water deficits.[132] To overcome this issue, inoculant screening based on thermotolerance has shown great efficacy. Current findings in greenhouse studies suggest that co-application with *Bacillus cereus* and humic acid can be used in the mitigation of heat stress damage in tomato seedlings and can be commercialized as a biofertilizer.[133] However, the inoculation is also affected by humidity and rain, revealing the unfeasibility of this method in large-scale agriculture with certain microbe and plant types.[45] However, Fukami et al.[134] showed that foliar spray in maize and wheat improved colonization of leaves, while soil inoculations favored root and rhizosphere colonization (Table 1).

5.4. Perspective. Seed coatings provide a targeted, controlled, and low-volume way to deliver beneficial microbes to the plant microbiome. An ideal strategy for future technologies consists of the development of seed-coating techniques that can be streamlined in seed treatment processed and applied during the seed packaging to ensure standardization of seeds for planting. However, inoculation through seed-coating formulations needs to reach performances that are comparable to coating on site or soil inoculation, to have an impact in precision agriculture, despite providing an easier technology.

6. LEGISLATION AND BUSINESS OPPORTUNITY

Regulation and legislation from production on field application of microbial fertilizers will play an important role in their use and eventual success.[157,158] Environmental policies regulate the type and quantities of microbes allowed in their environment but also impose restrictions on the type of carrier used and

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degradation profile permitted for each carrier. In particular, an increasing amount of attention is growing in the use of microplastics in agricultural practices, despite the low quantities involved. One of the toughest challenges for policymakers is the lack of a universally accepted definition for a microbial fertilizer. The different types of microbes used to improve plant growth (fungi or bacteria) and the different mechanisms used to obtain this final effect have created some inconsistencies in the definition of biofertilizers. There is then a need to develop adequate standards and legal provisions to support the production and use of biofertilizers at the global level. Globalization of microbial markets and the need for environmentally friendly and sustainable agricultural activities strengthen this need.

Recently, the European Union (EU) came up with a definition for microbial fertilizers. The new regulations will come into effect in 2022. Prior to these new regulations, the European market was segmented and, now, will become more consolidated. Further, this type of regulation will reduce costs and the administrative burden when launching a product. Europe is the second largest biofertilizer market, with 30% of the industry in 2019, and is expected to grow at 10% per year for the next several years.[159] Further, the EU defined biostimulants by what they do and not by what they are. The European Biostimulant Industry Council defines plant biostimulants as substances and/or microorganisms whose function when applied to plants or to soil is to stimulate natural processes to enhance or benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality.[160] It is projected that this new EU regulation will improve transparency, quality, and safety. Additionally, the EU set out a new procedure for authorizing biostimulants in agriculture, which will ensure conformity and accreditation in all member states. New regulations are stricter, and manufacturers can only declare those benefits derived from their products that have been scientifically proven. These new requirements will provide greater transparency and confidence when defining the limits of the efficacy. However, on the innovation side, only four microorganisms are regulated, meaning any product developed from other microorganisms cannot be marketed in the EU. This highlights the growing need of aligning innovation and regulation.

In the U.S.A., there is no federal law regulating biofertilizers. However, the individual states regulate this type of product through the United States Department of Agriculture.[158] Regulations may differ drastically, where, in some states, only notification is required, while, in some other states, local efficacy trials are required. The fragmented market makes it costly and bureaucratic to operate in the U.S. market.[161] Further, in the U.S.A., there are currently no legal definitions for the term "biofertilizer" or specific legal provisions defining their characteristics.[162]

The global biofertilizer market size was USD 1.34 billion in 2018 and is projected to reach USD 3.15 billion by the end of 2026, showing a compound annual growth rate of 11.3% forecast for 2019−2026.[163] With regard to application, the global fertilizer industry is segmented into seed treatment, soil treatment, and other. Seed treatment has the largest market share[164] (65% in 2014) and is expected to grow by 12.1% per year between 2019 and 2026, therefore making the seed treatment application a lucrative sector to enter. Further, nitrogen-fixing biofertilizers are the leading segment in the market (82%) and are expected to remain the most important biofertilizer segment. North America and Europe account for 55% of the global market revenue. The trade in North America is expanding considerably as a result of the growing number of organic farms in prominent economies, such as the U.S., Canada, and Mexico. Novozymes AS, Rizobacter Argentina S.A., Lallemand, Inc., and BioWorks, Inc. are the key active players in the biofertilizer business. North America is expected to hold the highest market share in the biofertilizer market. The market is highly fragmented, with many small and large players present across different geographical regions. The global biofertilizer commerce being unregulated is the reason why there are many small companies in the market. Once proper regulations are put in place, it is likely that the market will be consolidated among a few companies.

Further, with the recent EU ban on intentionally added microplastics (IAMPs), agriculture-based companies will require to be cognizant on the type of materials manufactured for plant and soil application and, thus, microbial fertilizer application tools.[165] Recently, IAMPs have become an issue of importance because of their ubiquitous presence. However, most research has been focused on the marine environment and not much on soil until of late.[166] Soils may represent a large reservoir of IAMPs, with sources such as sewage sludge applied as fertilizer and fallout from the air. Therefore, IAMPs may pose a threat to soil biodiversity. However, there is still a lack of information.[167] Recent studies show harmful effects of IAMPs on various groups of soil fauna, such as earthworms, snails, collembolans, and nematodes.[168] Nevertheless, the impacts of IAMPs on soil microbial communities have led to inconsistent results.[168]

6.1. Perspective. Farming is a low-margin business; thus, any new strategy suggested requires to be effective and cheap. Numerous effective techniques have been developed in laboratories across the world. However, collaboration between research and business is required to ensure scalability of these exciting ideas. Thus, startups working to scale up and lower costs of farming techniques will be required to bring some of the new technologies and techniques to the farmer. Also, working with the government will be critical to develop supportive legislation for these initiatives.

7. FUTURE PERSPECTIVE

Climate change and rapid population growth combined with the scarcity of resources impose a rapid transformation of agriculture to a more resilient and sustainable infrastructure. Crop production is currently too carbon-intensive, and lowering the carbon footprint of synthetic fertilizers is one of the major goals to enable a more sustainable future for our society. Microbial fertilizers have shown great potential in solving the environmental challenges that we face.[169] Future formulations for microbial inoculants will focus on precise and scalable delivery tools for microbes while also focusing on developing multifunctional microbe solutions that work for a variety of crops. However, we face a two-pronged challenge for the effective use of biofertilizers that will spur large- and small-scale uptake: (1) effective delivery methods, (2a) microbes for cereal crops, and (2b) multifunctional microbe solutions. Furthermore, the cost of microbial inoculants will be key to complementing with synthetic fertilizers.

Engineering the seed microenvironment with microbes in silk and trehalose seed coating has recently shown to effectively deliver plant microbial fertilizers.[83] A protein and polysaccharide mixture that encapsulated microbes was shown to

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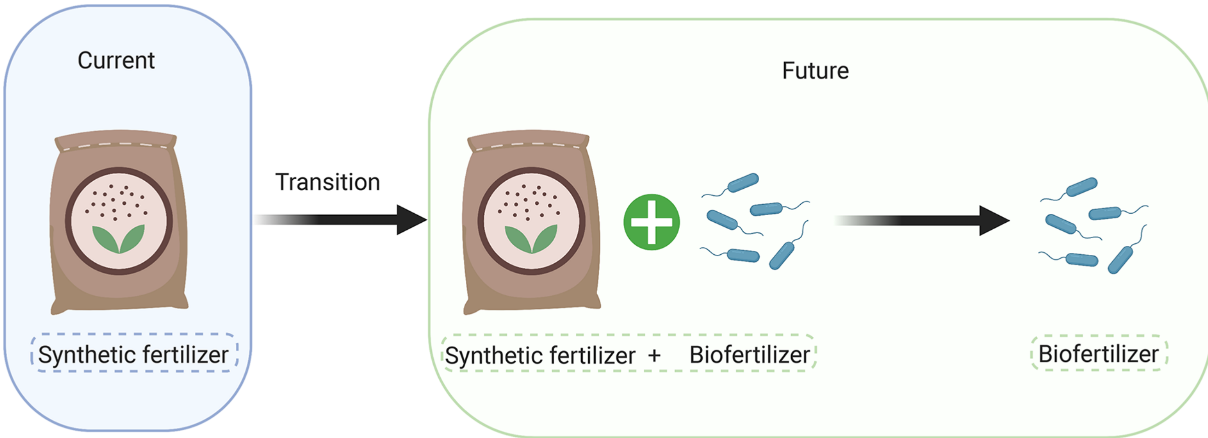

Figure 5. Transition from synthetic to microbe-based fertilizers in synergy with synthetic fertilizers to improve soil health and lower the environmental impact through increasing fertilizer absorption rates, thus minimizing runoff rates, solubilizing phosphates, and fixing nitrogen for the plant.

be able to protect rhizobium from desiccation for over a month and finally deliver in the soil the microbes for colonization.[83] The bioinspired approach that guided the material formulation imparted the appropriate mechanical properties and preservation capabilities required for an effective microbial delivery tool. This may enable the application of the proposed seed-coating technology for both small- and large-scale farmers, independent from their resources, skills, and equipment. Second, the ability to preserve microbes at standard conditions suggests that storage costs can be lowered as most microbial fertilizers to be preserved require to be refrigerated. The framework of the technique of engineering the seed micro-environment can be used at a large scale to solve the most important challenges faced in making microbial fertilizers ubiquitous in agriculture.

Cereal crop production accounts for a large proportion of agricultural production in the world, providing 60% of plant calories for humans.[170,171] Therefore, corn, wheat, and rice are some of the most important crops that will be essential in driving uptake of microbial fertilizers. Nitrogen-based fertilizers account for more than two-thirds of the global revenue.[172] Recently, Pivot Bio commercialized and released nitrogen-fixing microbes for corn that can supply cheaply and environmentally necessary nitrogen in association with synthetic fertilizer, thus lowering the environmental impact (Figure 5). From 2015, several techniques have been explored. One technique mentioned by Geddes et al.[173] is the transfer of nitrogenase and other supporting traits to microorganisms that already closely associate with cereal crops as a logical approach to deliver nitrogen to cereal crops. Ryu et al.[174] show engineering inducible nitrogenase activity in two cereal endophytes (*Azorhizobium caulinodans* ORS571 and *Rhizobium* sp. IRBG74) and the well-characterized plant epiphyte *Pseudomonas protegens* Pf-5, a maize seed inoculant.[174] Such synthetic biotechnology tools have opened up possibilities for rice and wheat nitrogen fixation in the near future, as highlighted by previous literature and Pivot Bio.

Special attention is increasing for microbial inoculants that have multifunctional properties and contain more than one organism.[172] Most biofertilizers to date consist of one inoculant. However, it has been shown that a consortium of microbes confers additional benefits to the plant and soil. Therefore, there is a drive to commercialize multifunctional property and consortium microbe fertilizers. Strains of *Rhizobium*, phosphate-solubilizing bacteria and fungi, arbuscular mycorrhizal fungi, and free-living nitrogen-fixing *Azotobacter* strains improve the nodulating ability, nitrogen content, and herbage yield (up to 2-fold) of subabul seedlings (*Leucaena leucocephala*), in comparison to the independent application of each component of the consortium. This use case has also led to the developing of consortium-based delivery systems, which will be an important technique in enhancing colonization and performance. Further, synthetic biology has led to the development of high-throughput tools to identify elite strains at the single nodule level with the potential to revolutionize the search for elite indigenous rhizobia.[175]

Regulation will also play a huge role in the coming years to ensure standardization of products and easier product market entrance. Because biofertilizers are not yet ubiquitous, innovators will need to work with policy makers worldwide in developing robust policies that encourage product development and protect the environment and farmers.

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Notes

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