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Use of Biofertilizers in Agricultural Production

José L. Zambrano-Mendoza, Carlos A. Sangoquiza-Caiza, Diego F. Campaña-Cruz and Carlos F. Yáñez-Guzmán

Abstract

Most of agricultural production in Latin America consists of smallholder farmers who need the development of sustainable technologies, with costs according to their economic condition. Biofertilizers composed of free-living bacteria promote plant's growth, improve productivity through the strengthening of its roots, and reduce the amount of synthetic fertilizer applied to the crops. The aims of this chapter are to highlight the microorganisms commonly used in agriculture as biofertilizers and the main researches carried out in several countries of Latin America, and to describe the development of an experimental biofertilizer for maize, based on strains of *Azospirillum* spp. and *Pseudomonas fluorescens*, in the highlands (Sierra Region) of Ecuador. Seven phylum and 95 genera of microorganism used as biofertilizers or Plant Growth-Promoting Rhizobacteria (PGPR) are summarized, along with the benefits, challenges and future prospect of their use. The effectiveness of the experimental biofertilizer developed in Ecuador was demonstrated through several experiments at the green house and field, in which it was evident the increase of root's size, the amount of crocket, the percentage of dry matter, and the crops' yield. The evaluations, accomplished on farmers' fields showed 30% of increase in yield and 21% of decrease in the cost of production per kilogram; as a consequence of the use of biofertilizer plus 50% of the recommended chemical fertilization, in comparison with standard farming techniques. Farmers can reduce the application of synthetic fertilizers and sustainably increase crop yield through the use of this technology.

Keywords: bioinoculant, corn, family farming, maize, microbiology, plant growth-promoting rhizobacteria, PGPR, sustainability

1. Introduction

In order to supply the growing demand for food, the expansion of the agricultural frontier has been one of anthropogenic activities with greater impact on ecosystems mainly due to: monocultures, watering systems, and use of pesticides and fertilizers. It's probably that the world's population will become 8000 billion people in 2030 and 9 billion in 2050, it means that food production should increase in between 60 and 70% [1–3].

There are two ways in order to achieve the challenge of growing agricultural production for supplying the growing population's food needs, such as:

- Increase the crops areas, with little possibility because it would affect large forest masses and nature reserves, especially in developed countries. In Latin America, the main source of new land for agriculture has been native forests or forests which were already intervened [4]. One of the most important challenges of Latin American countries is to achieve develop with a suitable level of environmental sustainability [5].

or

- Enhance crop yield through seed improvement (plant breeding) and use of environmentally safe and more assimilable fertilizers for plants. This option is the most feasible to choose because there are several ways to nourish plants, either by the development of synthetic or inorganic fertilizers (e.g. urea, ammonium nitrate, among others), organic fertilizers (e.g. humus, compost, leachates, among others), or biofertilizers (e.g. bacteria, fungi, algae), also known in a broad sense as bioinoculants or microbial inoculants.

Since 2005 to 2018, Latin America and the Caribbean increased the consumption of synthetic fertilizers from 15.2 million to 26.3 million tons, which means an increase of 73.59% (**Figure 1**). The use of inorganic fertilizers can pollute (e.g. heavy metals), damage water sources (e.g. nitrates and phosphates), and increase production cost. It means a great difficulty for those production systems based on peasant family farming, which has faced a hard situation in the last decades [7, 8]. Synthetic fertilizers represent in between 30 to 50% of production costs, depending on features of each crop.

This chapter summarizes the microorganisms commonly used in agriculture as biofertilizers or Plant Growth-Promoting Rhizobacteria (PGPR) and the main researches carried out in several countries of Latin America. The literature review was conducting using Google Scholar, identifying research literature published after 2000 using the term “biofertilizer”. Then, only the most recent papers whose study was conducted in Latin America was kept to give examples of their use.

In addition, this chapter describes the development of an experimental bio-fertilizer for maize in the highlands (Sierra Region) of Ecuador. The biofertilizer was developed with native strains of *Azospirillum* spp. and *Pseudomonas fluorescens* collected in the highlands of Ecuador. The strains were first evaluated in green-house and only those isolates that showed a beneficial effect on plant growth

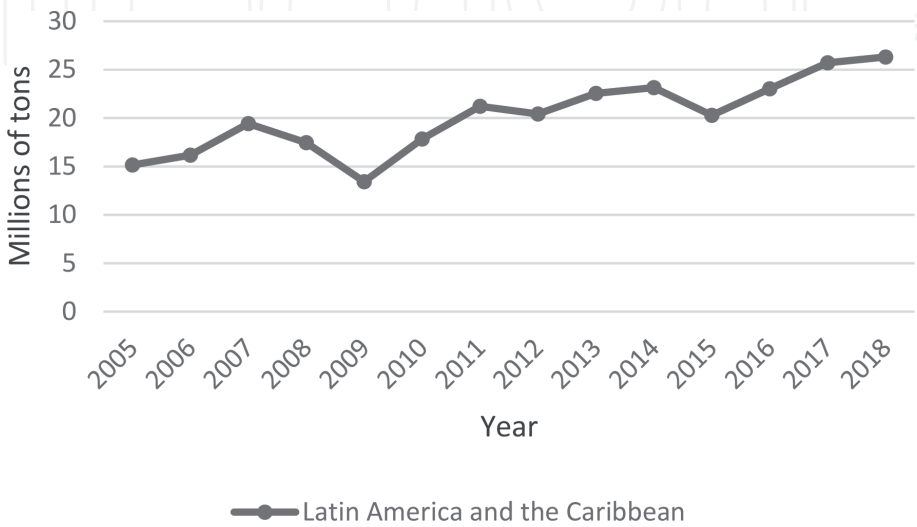


Figure 1. Annual consumption of synthetic fertilizers in Latin America and the Caribbean. Source: Economic Commission for Latin America and the Caribbean (ECLAC), 2021 [6]. Made by: Authors.

were evaluated in the field. This chapter describes the entire protocol used for the development and production of the biofertilizer.

2. Biofertilizers in agriculture

Biofertilizers are made in laboratory with live or latent cells of organisms, either nitrogen fixers, solubilizers of phosphates, cellulite microorganisms, growth promoters, among others, which are applied to seeds or plants in order to boost their growth. In contrast to synthetic fertilizers, biofertilizers have microorganisms that are not a source of nutrients by themselves, but allow the access of available nutrients in the rhizosphere [9, 10].

In the last two decades, the use of biofertilizers or microbial inoculants has increased notably in several parts of the world [11]. Biofertilizers are considered as a feasible and sustainable attractive biotechnological alternative to increase crop yield, improve and restore soil fertility, stimulate plant growth, reduce production costs and the environmental impact associated with chemical fertilization [12–14]. Several microorganisms are commonly used as biofertilizers, including nitrogen-fixing soil bacteria (e.g. *Azotobacter*, *Rhizobium*), nitrogen-fixing cyanobacteria (e.g. *Anabaena*), solubilizing phosphate bacteria (e.g. *Pseudomonas*), and arbuscular mycorrhizal fungi (Table 1). Similarly, the producer bacteria of phytohormones (e.g. auxins) and those cellulite microorganisms are also used as a biofertilizers [10, 15]. In addition, the use of plant growth promoting bacteria can be useful in developing strategies to facilitate plant growth under normal and abiotic stress conditions [21].

It is likely that the market of biofertilizers in Latin America would grow at an annual rate of 10% during the period 2020-2025. Argentina is the largest and fastest growing market, followed by Brazil. The main incentive factors of market in these two countries are: a favorable government policy, easy registration process, and the increase of organic farms [22]. On the other hand, Peru, Colombia, Bolivia, Ecuador, and Venezuela's growth in the biofertilizer market would be slow, because they are ruled by ambiguous regulations.

2.1 Use of biofertilizers in major crops

Biofertilizers and PGPR have been evaluated in a wide variety of crops, including: rice, cucumber, wheat, sugarcane, oats, sunflower, corn, flax, beet, tobacco, tea, coffee, coconut, potato, fan cypress, grass sudan, eggplant, pepper, peanut, alfalfa, tomato, alder, sorghum, pine, black pepper, strawberries, green soybeans, cotton, beans, lettuce, carrots, neem, among others [23].

The most important example of the use and importance of biofertilizers in crop production is in soybean. Soybean production is mainly carried out by inoculating the seed with selected strains of *Bradyrhizobium japonicum*, *Bradyrhizobium diazoefficiens* or *Bradyrhizobium elkanii* (jointly referred to as *Bradyrhizobium* spp.). In Argentina, one of the main soybean producers in the world, there are around 70 companies producing and marketing biofertilizers for this crop [24].

Castro et al. [25] evaluated the benefits of the rice-*Azolla* association for rice cultivation in Cuba. The results showed that this cereal was positively influenced by the use of *Azolla*, which allowed a rise in the number of grains per panicle, panicle per m², and consequently, a significant increase in yields. In addition, it was observed that the association regulated the temperature and pH of the water.

Grageda-Cabrera et al. [26] evaluated the effect of the inoculation of bacterial and fungal isolates on nitrogen use efficiency (NUE) in wheat, using the ¹⁵N

Phylum	Genus	Host	Benefit
Actinobacteria	<i>Arthrobacter</i> , <i>Brevibacterium</i> , <i>Cellulomonas</i> , <i>Corynebacterium</i> , <i>Kocuria</i> , <i>Microbacterium</i> , <i>Micrococcus</i> , <i>Mycobacterium</i> , <i>Rhodococcus</i> , <i>Streptomyces</i> , <i>Streptomonospora</i> , <i>Haloglycomyces</i> , <i>Haloactinospora</i> , <i>Actinopolyspora</i> , <i>Amycolatopsis</i> , <i>Prauserella</i>	Maize, pea, rice, soybean, sugarcane, sunflower, wheat.	Increase plant vigor (growth promoters), and tolerance to biotic and abiotic stress. Improve nutrients use efficiency.
Firmicutes	<i>Bacillus</i> , <i>Paenibacillus</i> , <i>Alicyclobacillus</i> , <i>Aneurinibacillus</i> , <i>Virgibacillus</i> , <i>Salibacillus</i> , <i>Gracilibacillus</i> , <i>Brevibacillus</i> , <i>Amphibacillus</i> , <i>Paralibacillus</i> , <i>Oceanobacillus</i> , <i>Salimicrobium</i> , <i>Halobacillus</i> , <i>Pontibacillus</i> , <i>Thalassobacillus</i> , <i>Sediminibacillus</i> , <i>Alkalibacillus</i> , <i>Tenuibacillus</i> , <i>Ammoniphilus</i> , <i>Salinibacillus</i> , <i>Exiguobacterium</i> , <i>Marinilactibacillus</i> , <i>Alkalinebacterium</i> , <i>Sporosarcina</i> , <i>Planomicrobium</i> , <i>Lysinibacillus</i> , <i>Planococcus</i>	Amaranth, apple, barley, buckwheat, maize, mustard, oat, pepper, rice, sorghum, sunflower, tomato, wheat	Solubilization of phosphorus, potassium, zinc; production of indole acetic acids, hydrogen cyanide, gibberellic acid, and siderophore. Nitrogen fixation, biocontrol.
Proteobacteria	<i>Allidiomarina</i> , <i>Marinobacter</i> , <i>Aquisalimonas</i> , <i>Microbulbifer</i> , <i>Marinobacterium</i> , <i>Pseudomonas</i> , <i>Salicola</i> , <i>Deleya</i> , <i>Halomonas</i> , <i>Marinospirillum</i> , <i>Methylophaga</i> , <i>Achromobacter</i> , <i>Alcaligenes</i> , <i>Rhizobium</i> , <i>Albimonas</i> , <i>Paracoccus</i> , <i>Pantoea</i> , <i>Enterobacter</i> , <i>Kluyvera</i> , <i>Azospirillum</i> , <i>Methylobacterium</i> , <i>Arcobacter</i> , <i>Oceanibaculum</i> , <i>Fodinicurvata</i> , <i>Altererythrobacter</i> , <i>Glycocalis</i> , <i>Xanthobacter</i> , <i>Bradyrhizobium</i> , <i>Amorphus</i> , <i>Sinorhizobium</i>	Amaranth, barley, bean, buckwheat, cotton, cowpea, maize, millet, mustard, oat, pea, rice, soybean, sunflower, tomato, wheat.	Increase plant vigor (growth promoters), nitrogen fixation, solubilization of nutrients, and biocontrol.
Bacteroidetes	<i>Flavobacterium</i> , <i>Shingobacterium</i>	Barley, Millet, Wheat	Plant growth promoting attributes
Ascomycota	<i>Trichoderma</i> , <i>Penicillium</i> , <i>Fusarium</i> , <i>Phoma</i> , <i>Aspergillus</i> , <i>Phomatropica</i> , <i>Acremonium</i>	Horticultural, fruit and forest crops	Biocontrol, biodegradation

Phylum	Genus	Host	Benefit
Glomeromycota	<i>Glomus</i> , <i>Gigaspora</i> , <i>Acaulospora</i> , <i>Scutellospora</i> , <i>Sclerocystis</i> , <i>Laccaria</i> , <i>Pisolithus</i> , <i>Boletus</i> , <i>Amanita</i> , <i>Pezizella</i> .	Horticultural, fruit and forest crops	Phosphate-mobilizing
Cyanobacterias	<i>Asterocapsa</i> , <i>Chroococcus</i> , <i>Aphanothece</i> , <i>Gloeocapsa</i> , <i>Microcystis</i> , <i>Synechococcus</i> , <i>Rhabdoderma</i> , <i>Merismopedia</i> , <i>Aphanocapsa</i> , <i>Coelosphaerium</i> , <i>Leptolyngbya</i> , <i>Pseudanabaena</i> , <i>Komvophoron</i> , <i>Oscillatoria</i> , <i>Lyngbya</i> , <i>Phormidium</i> , <i>Nostoc</i> , <i>Anabaena</i> , <i>Scytonema</i>	Bean, maize, rice.	Fixation of nitrogen, bioremediation, biocontrol.

Source: Modified by the authors from [15–20].

Table 1.
Groups of microbial inoculants for agriculture.

isotopic dilution technique. The inoculation of wheat with arbuscular fungi significantly increased grain yield up to 1 291 kg ha⁻¹, and the NUE up to 11%, in relation with the non-inoculated treatment.

The solubilizing phosphate bacteria *Pseudomonas putida*, *Microbacterium laevaniformans* and *Pantoea agglomerans*, were evaluated in potato to determine the effect of inoculation on growth and crop yield. The mixture of *P. agglomerans* or *M. laevaniformans* and *P. putida* substantially increased biomass and improved tuber growth. The yield was possibly due to the higher supply of phosphorus (P) from the bacteria to the growing plants. Among the microorganisms, *P. agglomerans* significantly improved potato growth and yield by approximately 20-25% [27].

Like several horticultural crops, tomato is influenced by the application of growth promoters. Bernabeu et al. [28] showed that the inoculation of seedlings with *Burkholderia tropica* had an effective colonization of the roots that spread to aerial tissues. This effective colonization led to an increase in tomato production in two growing seasons. Trials carried out by Mirik et al. [29] with pepper and *Bacillus* strains increased yield up to 23.5%.

Garza et al. [30] evaluated the response of annual and perennial crops to the application of biofertilizers in the central region of Mexico, in a series of experiments and plots in which the bacteria *Azospirillum brasilense* and *Rhizobium etli* were tested, as well as the fungus *Glomus intraradices* in cereals, legumes, and citrus. In most of the test locations, increases in production were recorded, that were up to 60% in maize, 85% in wheat, 74% in barley, 25% in oats (biomass), 36% in beans, and 111% in orange, in relation to the non-inoculated treatment.

Biofertilizers are also used in forest species. Inoculum prepared with the ectomycorrhizal fungi *Suillus luteus* and *Rhizopogon luteolus*, and the saprobes *Coriolopsis rigida* and *Trichoderma harzianum*, alone and combined, were evaluated as potential biofertilizers for the growth of *Pinus radiata* seedlings in greenhouse. At the end of the test (after 10 months), it was determined that the inoculants stimulated the growth of the plants compared to the control without inoculation. The inoculant formulated with the mixture *C. rigida* and *R. luteolus* produced *P. radiata* plants with the highest quality indices, being a viable alternative for their use in the production of *Pinus* spp. [31].

3. Mechanism of action of the biofertilizers

Biofertilizers increase the growth and yield of crops in an eco-friendly manner. They show synergistic and antagonistic interactions with the soil native microbiota and participate in many process of ecological importance. Biofertilizers promote plant growth by enhancing biotic and abiotic plant stress tolerance and supporting its nutrition by fixing atmospheric nitrogen and solubilizing soil nutrients [32, 33].

The detailed mechanisms of biofertilizers and PGPR and their specific contribution to plant growth have been reviewed comprehensively [34–37]. The action modes that PGPRs use to benefit plant growth can be classified into direct and indirect mechanisms, which occur inside and outside the plant, respectively. PGPRs directly promote plant growth by enhancing nutrient acquisition and by regulating phytohormones. The indirect effects of PGPRs on plant growth are caused by the induction of systemic resistance of plants against a wide range of pathogenic microbes. Direct action modes include an improvement in plant nutrition by providing nutrients, such as nitrogen, or solubilized minerals from the soil (e.g. P, K, Fe, Zn, among others) and/or stimulating plant growth by regulating the levels of phytohormones (e.g. gibberellins, auxins, ethylene, cytokinins, and abscisic acid). Indirect effects on plant development are given by the suppression of pathogens and other harmful microorganisms through parasitism, competing for nutrients and niches within the rhizosphere, producing antagonistic substances (e.g. antibiotics, hydrogen cyanide and siderophores) and enzymes. lytic (e.g. glucanases, proteases and chitinases), and the induction of plant systemic resistance against a wide spectrum of pathogens [33].

4. Biofertilizers for maize in Latin America

During the last 30 years, several researchers have discovered many species of nitrogen-fixing bacteria associated with cereals and other crops which no generate nodules. Virtually all of these bacteria are microaerophilic, they fix nitrogen only when there is a low oxygen pressure. One of the most important is *Azospirillum* spp. which are associated with the rhizosphere of plants nourish them [38]. Döbereiner et al. [39], mentioned that these bacteria, in tropical and subtropical regions, occur naturally in numbers between 10^3 to 10^6 per gram of soil, and in even higher numbers on the root surface of cereals and forage grasses.

Maize is the most cultivated cereal in the region and its planted area is bigger than other economically important ones, such as rice or wheat [40]. Maize requires intensive use of nitrogen fertilizers for suitable production, which leads to increased costs and possible environmental pollution [41]. Nowadays, it is necessary to preserve the soil's productive capacity. Plant nutrition and soil improvement practices must be integrated to enable an adequate management of nutrients, giving importance to the biodiversity found in soils [42]. An appropriate management of the association *Azospirillum*-maize can result in productivity gains and lower production costs [43].

In Latin America, several cases of use of biofertilizers in maize cultivation have been reported with promising results. Girón Molina and Llallahui Isasi [44], in an experiment with purple maize, conducted in Peru's Ayacucho region, determined substantial performance improvements: treatments with a biofertilizer got better yields, that reached 6376 kg ha^{-1} , while the control achieved just 2225 kg ha^{-1} . Ccente Gaspar [45] in his study "Identification of *Azospirillum* spp. associated with the roots of maize (*Zea mays* L.) in Pomacocha-Acobamba-Huancavelica" demonstrated that the inoculation with *Azospirillum* spp. increased plant height,

dry matter, dry root weight, and root length of inoculated plants, evaluated at 60 days after planting.

Studies in Ecuador reported that the use of 50% of the recommended dose of synthetic fertilizer and *Azospirillum* spp, increased fresh corn harvesting (choclo) by 81% [46], in comparison with the control. Sangoquiza-Caiza [47] in his study reported strains of *Azospirillum* spp. capable of mitigating damage caused by salinity for maize. Subsequently, Sangoquiza et al., [48], reported that inoculation with *Azospirillum* spp. and *Pseudomonas fluorescens*, alone or in combination, promoted greater assimilation of N and P content in the plant tissue of maize plants.

In Venezuela, López et al., [49] studied the effect of nitrogen-fixing bacteria and phosphorus solubilizers (*Azotobacter* sp. and *Bacillus megatherium*) on maize in two contrasting soils (high and low fertility). There was an evident higher plant height, stem diameter, leaf length and width, biomass of roots and aerial parts, and N and P content in the inoculated plants; the greatest effect was evident in the low fertility soil. Later, in Táchira, Valery & Reyes [50] demonstrated that inoculation with two isolations of diazotropic bacteria, individually and in consortium, increased the relative agronomic efficiency of the dry weight of maize grain by 130 and 403%, the N content by 463 and 116%, and the P content by 152 and 376%, respectively. They achieved up to 18% of increase in maize grain production.

Mexico is one of the most advanced countries in research and use of biofertilizers in the Region. Biofertilizers are found in the market with diverse trademarks such as: VOP, Bactiva, Endospor, and Bioraíz [12]. The National Institute of Agricultural and Livestock Forestry Research (INIFAP) has developed biofertilizers for maize with the use of bacterial genera *Azospirillum*, *Azotobacter*, *Pseudomonas*, and *Bacillus*, and arbuscular mycorrhiza fungi. The most widespread products are: "Biofertilizante" (with *Azospirillum* and mycorrhiza) and "Biofertilizante Bacteriano INI2709" (with several strains of *Pseudomonas fluorescens*). The use of these biofertilizers allow the replacement of 20 to 60 kg of N per hectare, depending on the strain and the way of application [51]. In Chiapas, seed inoculation with *Azospirillum* increased grain yield by 28% over the absolute control, with a greater economic benefit [52]. Recently, Hernández-Reyes et al., [53] showed that the use of a cyanobacterias consortium in maize produced a higher plant height, number of leaves, and amount of fodder; while yield was similar to those treated with chemical fertilization. In addition, an increase in the amount of protein in the grain was observed.

For more than 40 years, Brazil has studied the effect of *Azospirillum* spp. on maize's development, not only in terms of crop yield, but also in relation to the physiological causes responsible for it. In addition, there are technology packages that use multiple and efficient bacterial maize varieties and strains. The bacteria can supply more than 50% of the nitrogen needed by the plant, due to the greater ease of assimilation of nitrogen [43]. The answer of several maize genotypes to the inoculation of four strains of *Azospirillum* spp. was evaluated by Salomone and Döbereiner [54], who detected an increase in grain weight of up to 7300 kg ha⁻¹, which was greatly influenced by soil conditions, environment, and the genotypes used. In another study lead by EMBRAPA in the Amazon Region, the inoculation with *A. brasilense* increased maize yield by about 50% compared to plants not fertilized with nitrogen [55]. Recently, Pereira et al., [56] reported increases in grain yield until 39.5% and 34.7%, when maize seed was inoculated with *Bacillus subtilis* and *A. brasilense*, respectively.

Argentina is one of the countries that has developed more biofertilizers containing *Azospirillum* worldwide. There is a great variability in the response of maize yield to this bacterium, which fluctuates in between negative values to more than 100%, regarding to the non-inoculated control [57]. For example, experiments in the province of Buenos Aires have shown a 34% average increase in maize yield

during 15 years, while studies carried out in other regions got 11.5% increase related to the non-inoculated control [58].

5. Development of a biofertilizer for maize in the highlands of Ecuador

In Ecuador, maize is a crop of paramount importance because of the significant role it plays in the food security of the population. Most of the cultivated area in the highlands is managed in small fields by farmers with few economic resources, who characterize by the low use of technology that causes low productivity of the crop (1.1 t ha⁻¹) and low profit [59]. For these reasons, it is advisable to exploit to the maximum the mechanisms of the so-called "Biological Fertilization" through the use of beneficial microorganisms that has the ability to fix nitrogen and solubilize phosphates, as a natural fertilization alternative, which preserves the environment and improves soil quality, with costs available to farmers.

During almost two decades, the National Agricultural Research Institute (INIAP) has leaded research with diazotrophic bacteria through the collection of soil samples and maize roots in the main cultivated areas of the Sierra. Several strains were isolated and characterized, whose effect have been evaluated in green-house and field with liquid and solid inoculation media (Table 2).

Year	Subject	Main Author, Reference
2003	Collection, isolation and characterization of diazotrophic bacteria <i>Azospirillum</i> sp., associated with maize.	Espinosa, [60]
2005	Development of a biofertilizer from strains of <i>Azospirillum</i> sp., for the maize (<i>Zea mays</i> L.), INIAP-102 variety with two chemical fertilizations and two organic fertilizers in the province of Chimborazo.	Molina, [61]
2009	Evaluation of bio-fertilizer based on strains of <i>Azospirillum</i> sp. in maize (<i>Zea mays</i> L.) INIAP 101, in the sector Ainche, province of Chimborazo.	Cool, [46]
2009	"Evaluation of the effect of four inoculation methods of two strains of <i>Azospirillum</i> sp., in maize crops (<i>Zea mays</i> L) INIAP-122 and INIAP-102 in the provinces of Imbabura and Pichincha.	Ortiz, [62]
2009	Evaluation of solid and liquid supports, for the production of a biofertilizer based on <i>Azospirillum</i> sp. applicable to maize cultivation (<i>Zea mays</i> L.)	Pallo, [63]
2010	Selection of strains of <i>Azospirillum</i> sp., such as biofertilizer of <i>Zea mays</i> ,L. under saline stress.	Sangoquiza, [47]
2011	Evaluation of biofertilizer based on strains of <i>Azospirillum</i> sp. in maize (<i>Zea mays</i> L) INIAP-111 Guagal, in addition to three types of fertilization and two methods of inoculation, on Laguacoto II farm, in the province of Bolivar.	Rivadeneira, [64]
2012	Response of maize (<i>Zea mays</i> L.) INIAP 111 to biofertilizer and nitrogen fertilization, on Laguacoto, Guaranda, Bolivar.	Changoluisa, [65]
2013	Characterization and evaluation of <i>Pseudomonas</i> sp. bacteria, phosphate solubizers found in the maize rhizosphere (<i>Zea mays</i> . L) of the provinces of Imbabura, Bolívar, Chimborazo, and Pichincha.	Pincay, [66]
2016	Response of nitrogen and phosphorus absorption of a maize variety when inoculating <i>Azospirillum</i> sp. And <i>Pseudomonas fluorescens</i> .	Sangoquiza, [48]

Table 2. Studies carried out with *Azospirillum* sp. and *Pseudomonas fluorescens* and maize in the highlands of Ecuador.

Ecuador started the research with *Azospirillum* through collection, isolation, and identification of the bacteria in 2003. In this year, a collection of 19 strains of the maize crop rhizosphere was obtained from the highlands of Ecuador [60]. From this collection, four strains were selected because they showed in vitro a greater ability to fix nitrogen. They were evaluated in the field and the C2 strain was selected as the most efficient in promoting higher plant growth. Strain C2, increased plant height by 11.92% relative to the control [61]. A subsequent study verified the beneficial effect of the C2 strain on plant growth [46].

Pallo [63] evaluated solid and liquid supports (carriers) for the production of the biofertilizer. The best solid medium for biofertilizer production was vermiculite peat, evaluated at 180 days of storage at room temperature (average of 20°C), with a production cost of USD 2.11 per 30 ml. For the production of the liquid biofertilizer, the most suitable support was the 2% molasses solution, with a production cost of USD 1.10 per 30 ml. The liquid supports maintained a higher concentration of Colony Forming Units (CFUs) than solids. The liquid inoculum applied to the seed increased the percentage of emergence, plant height, and ear insertion height, showing a more vigorous maize plants with less pest attack, and increases in yield between 14.34 and 26.60%, depending on the variety used [62].

Rivadeneira [64] evaluated several doses of synthetic fertilizers in combination with the biofertilizer, finding that the biofertilizer and 50% of the recommended synthetic fertilization, (55 Kg of N ha⁻¹), produced the highest yield, obtaining 11.21 t ha⁻¹ of fresh corn (choclo), and 4.05 t ha⁻¹ of grain in a floury open pollinate maize variety. This study also determined that the biofertilizer supplemented with 50% of the recommended nitrogen fertilization, obtained the highest marginal rate of return, indicating that for each dollar invested, 9.6 dollars returned. Later, similar results were observed by Changoluisa [65].

In order to complement the biofertilizer with other beneficial species, the isolation, characterization and evaluation of 21 isolates of bacteria of the genus *Pseudomonas* and three of the genus *Acinetobacter*, were accomplished. The isolates were also collected from highlands of Ecuador. With the isolates, tests were carried out to evaluate their ability to solubilize phosphorus in vitro and under greenhouse conditions. In the in vitro evaluation, the aI3 strain (*P. fluorescens*) showed a higher solubility index with an average of 4.8; followed by nP2 (*P. putida*) with 4.5. In the greenhouse evaluation, the strains that demonstrated the greatest capacity to produce a beneficial effect in the maize plants were cnC2, cnI5, caB1, and cnP3, showing higher values in terms of root length (**Figure 2**), percentage of dry matter, foliar area, and phosphorus accumulation in the tissues, with respect to the control treatment not inoculated [66]. A subsequent evaluation selected the *P. fluorescens* strain nI5 to be included in the biofertilizer.

Once the best isolates of *Azospirillum* sp. and *Pseudomonas fluorescens* were identified, field experiments were conducted to test the efficacy of the biofertilizer. Sangoquiza et al. [48] determined that seed inoculated with *Azospirillum* sp. and/or *P. fluorescens*, promoted a significant increase in morphological and agronomic traits, compared to the absolute control (no inoculated and without synthetic fertilizers). Additionally, the inoculated plots showed similar trait values to the plots treated with the conventional doses of synthetic fertilizers. The biofertilized treatments with *Azospirillum* sp. and *P. fluorescens* showed foliar contents of N-total and P-total higher than the controls, evidencing the effect of these rhizospheric microorganisms in the absorption and translocation of nutrients. Seeds inoculated with *Azospirillum* sp. showed the highest foliar absorption of N with 24.49 g plant⁻¹, while seeds inoculated with *Azospirillum* sp. + *P. fluorescens* showed the greatest foliar absorption of P, with 10.86 g plant⁻¹. An economic analysis of the application of the biofertilizer showed that the use of *Azospirillum* sp. + *P. fluorescens* allowed

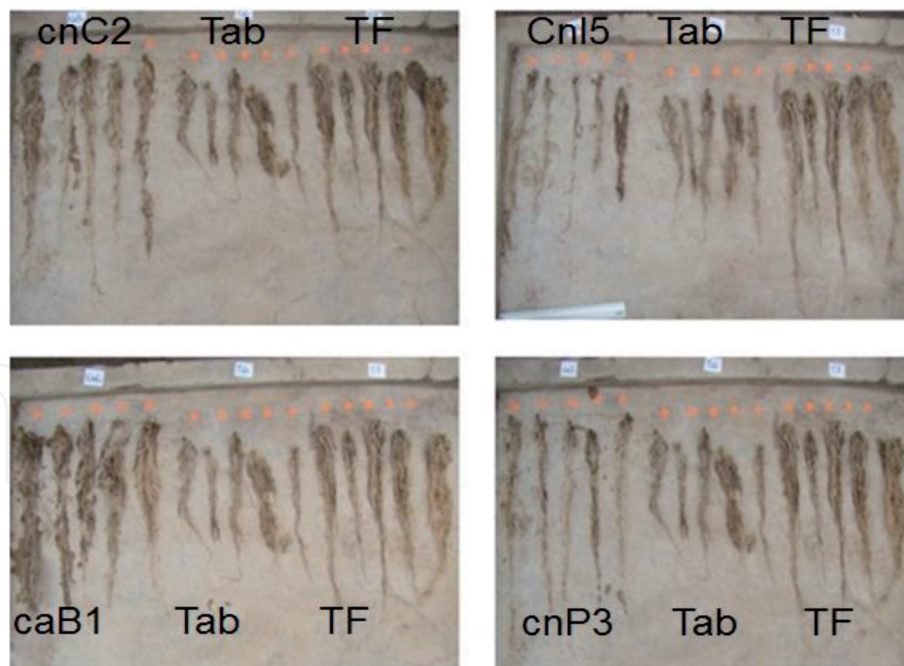


Figure 2.

Root growth of sixty-day-old maize seedlings whose seeds were inoculated with isolates of *Pseudomonas fluorescens*: cnC2, isolate from Chimborazo; cnl5, isolate from Imbabura; caB1, isolate from Bolívar; cnP3, isolate from Pichincha; tab, control not inoculated; TF, control with synthetic fertilizers. Source: [66].

a saving of USD 281.12 ha⁻¹, in relation to the treatment that used conventional synthetic fertilization, which represents a decrease of approximately 16% in the cost of production, without significantly affecting yield.

The validation of the biofertilizer (*Azospirillum* sp. and *P. fluorescens*) with farmers in the provinces of Imbabura, Chimborazo, and Bolívar started in 2019, financed by the Korean Program on International Agriculture (KOPIA). Results of nine locations have shown that the use of the biofertilizer plus 50% of the recommended synthetic fertilization increased on average 30% of the yield, compared to the farmer's plot, and reduced the cost of production per kg of grain produced by 21% [67]. Proposals are currently being made to disseminate and spread the use of this biofertilizer in the highlands of Ecuador and transfer its production and distribution to the private sector.

5.1 Procedure for the production and use of the biofertilizer

The isolates are conserved lyophilized at 4°C in the laboratory of the Maize Program at the Experimental Station Santa Catalina. For reactivation of the isolates, 1000 micro liters (μL) of 1% peptone are added to the Eppendorf tubes containing the lyophilized strains. Then, the tubes are shaken until homogenizing the mixture with the help of a vortex. After that, 50 μL of the strains were taken and placed in Petri dishes containing: solid medium Malic Acid - Congo Red for *Azospirillum* sp., and King B for *P. fluorescens*, with the help of a sterile glass triangle. The isolate was dispersed until dryness, and the Petri dish was placed in incubation at 30°C for seven days. After these days, several colonies of *Azospirillum* sp. and *P. fluorescens* are chosen with the aid of a platinum beam. Pure sections of these strains are transferred to a glass flask containing broth media culture. Subsequently, the strains are placed on a rotary shaking (120 rpm) at 19° C, for 48 hours. Finally, the inoculum was introduced into a liquid support (2% molasses) with a concentration of 1x10⁹ CFU ml⁻¹, and it is incubated for seven days at 30°C. The inoculant is placed in aluminum bags and sealed (**Figure 3**).

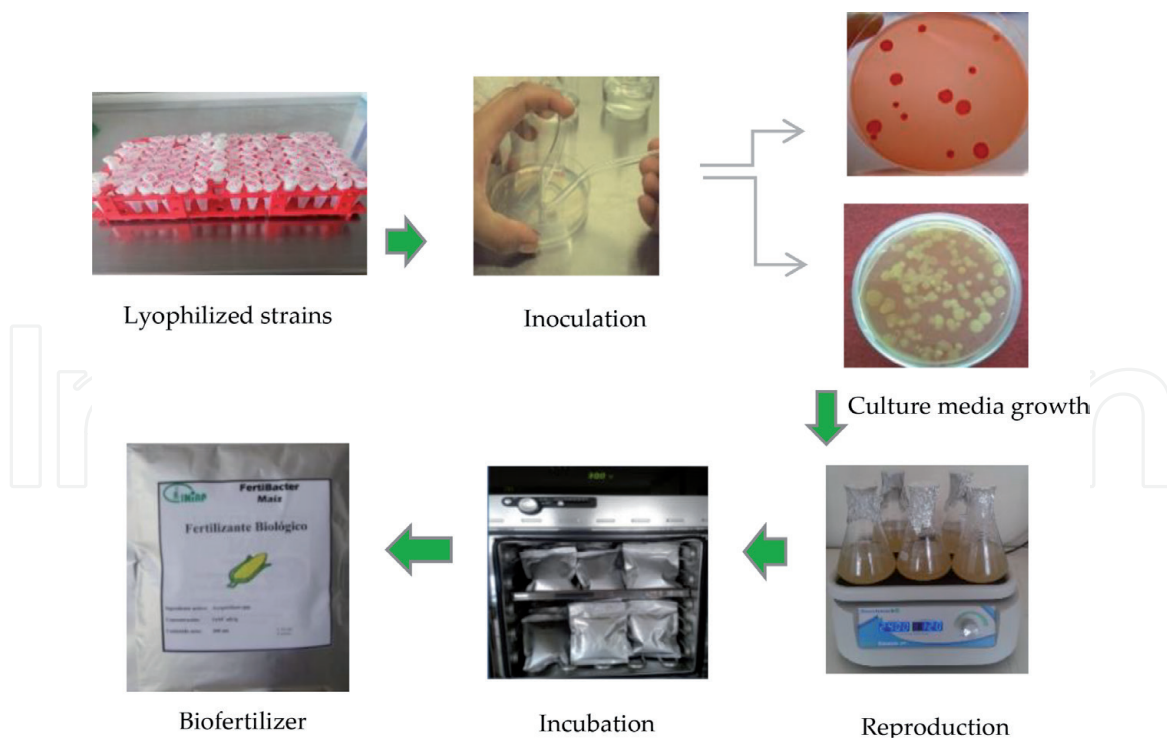


Figure 3.
Production flow of a biofertilizer with Azospirillum sp. and Pseudomonas fluorescens for maize used by the maize program at INIAP, Ecuador. Photo credits: Authors.

Before planting, the inoculant is applied to the seed and homogenized, trying to ensure that the solution comes into contact evenly with the seed. Then, the seed is allowed to stand for 10 to 15 minutes before it is ready to be planted. It is not recommended to store the inoculated seed for more than two days. The bags can be conserved sealed at room temperature (20-26°C) for 180 days.

5.2 Regulations for the registration of biofertilizers in Ecuador

In Ecuador, the Agencia de Regulación y Control Fito y Zoosanitario (AGROCALIDAD) is in charge of regulating the procedure, registration, control, and surveillance of agricultural inputs. To register the biofertilizer the developer has to be considered as an “operator”, which can manufacture, formulate, package, export, import, and distribute fertilizers, soil amendments, and related products for agricultural use. After that, it is required to specify and comply with a series of requirements related to the product to be marketed, such as: type of inoculant, microbial composition, heavy metal content, physical-chemical properties, application methods, security information, among others [53].

6. Challenges of biofertilizers development and use

The use of biofertilizers is a biological approach toward the sustainable intensification of agriculture. However, their application for increasing agricultural yields have several challenges that have to be solved yet. Biofertilizers tend to be susceptible to biotic and abiotic stress and those that perform well in laboratory and greenhouse, often do not perform the same way in the field. Crops are grown under diverse environmental conditions, including diverse ranges of temperature, rainfall, soil type, soil biodiversity, and crop variety. Therefore, such variations

cause inconsistency in the efficacy of the biofertilizers. In addition, biofertilizers act slow compared with synthetic fertilizer, since the inoculum will take time to build its concentration and colonize root [68]. These responses could affect the adoption of biofertilizers by the farmers. To avoid these challenges, potential isolates should be selected based on their performance under field conditions, with several crops, across diverse soil types and environmental conditions [33]. In addition, biofertilizers should not completely replace the other fertilizers, but they can complement them and reduce their use [68].

Another significant challenge encountered during the development of a biofertilizer and the commercialization is its shelf life. Biofertilizers contain live microbial cells, with a short shelf life (approx. 6 months, under 20–25°C.) and their storage and transportation require extra care and precaution, that increase the cost of the product [69]. This situation also causes that the product is not often available in remote rural areas [68].

Regulatory constraints include the challenges in product registration and patent filling. The lack of a standardized legal and regulatory definition for “plant biofertilizer” or “plant biostimulant” is the primary reason behind the lack of a globally coordinated uniform regulatory policy. The process to register a biofertilizer is quite ambiguous or complex, extensive and complicated in most parts of the world [33].

7. Future prospects of biofertilizers

There is a growing demand for agricultural goods produced in a sustainable manner; therefore, the use of eco-friendly inputs for food production, such as biofertilizers, will have a significant increase in the coming years. The global market of biofertilizers is expected to increase from 2.3 billion US dollar in 2020 to 3.9 billion in 2025 [70]. The increase in the market value will be supported by government agencies and industry to create awareness among farmers and consumers about the benefits of the use of biofertilizers, in concordance with the Development Sustainable Goal 12 proposed by FAO: “ensure sustainable consumption and production patterns” [71].

The role of metagenomics to have a better understanding of microbial communities associated with rhizosphere is a growing field of research, and it will continue to increase. Plant rhizosphere is colonized by a large number of microorganisms and this results in a huge number of microbial genes that interact with plant genes. However, more elaborated research has to be conducted, such as meta-transcriptomic and meta-proteomics, and their relation with plant growth [72]. The results of the omics must be translated into the field to have better agronomic practices and new biofertilizer formulations.

An alternative way to improve the growth promoting traits of rhizobacteria is by genetic modification. On this basis, it could be likely that genes of PGPR might be identified by their particular plant-beneficial functions and used for gene editing or transgenic approaches [73]. For instance, certain tumor-inducing *Agrobacterium* strains have the potential to promote plant growth on non-susceptible plant hosts [74], and bacteria genes directly conferring plant-beneficial properties, such as *nif* (nitrogen fixation) or *phl* (phloroglucinol synthesis), have been identified [75].

The use of nano fertilizers in agriculture is growing. Nano fertilizers are non-toxic, minimize production costs, and increase the nutrients use efficiency. Encapsulation of nano biofertilizers will contribute to extend the release of PGPR to target cell by a conjugation of gold, aluminum, and silver nanoparticles [75].

8. Conclusions

The use of biofertilizers have proved to be an efficient way to produce food in a sustainable manner. Many scientific reports assure the benefits of PGPR in the growth and yield of several crops, including: corn, rice, cucumber, wheat, sugar cane, oats, sunflower, flax, beet, tobacco, tea, coffee, coconut, potato, cypress, sudan grass, pepper, peanuts, alfalfa, tomato, sorghum, pine, black pepper, strawberries, soybeans, cotton, beans, lettuce, carrots, among others. The most important microorganisms that have been used in biofertilizer formulations are *Rhizobium*, *Azotobacter*, *Azospirillum*, *Pseudomonas*, *Bacillus*, and vesiculo-arbuscular mycorrhizae.

In Ecuador, the development of the biofertilizer took almost 20 years. The process could have been much faster; however, there were no permanent resources to develop this product. The technological development of the biofertilizer depended on sporadic national founding for science and technology; and currently, it depends on the international cooperation. The development of a biofertilizer for maize containing *Azospirillum* spp. and *P. fluorescens* increased yield and reduced the use of synthetic fertilizers by around 50%, lowering the cost of production. This demonstrated that it is feasible to substitute the use of synthetic fertilizers with biofertilizers, opening the opportunity for a sustainable and environmentally friendly maize production system in the highlands of Ecuador.

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Conflict of interest

We declare no conflict of interest.

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