Isolation and evaluation of beneficial microorganisms for their potential use in agriculture as microbial biostimulants

Miriam Briones Sendra

UNIVERSITAT DE BARCELONA

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Miriam Briones Sendra

Evolutive Biology, Ecology and Environmental Sciences Department

Plant Physiology Section

Universitat de Barcelona

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Thesis presented by Míriam Briones Sendra to obtain the degree of Doctor from the Universitat de Barcelona.

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Summary

Conventional agricultural practices often rely on the extensive application of fertilizers to ensure maximum crop yields, leading to significant economic costs and negative environmental impacts. Nitrogen (N) and phosphorus (P) are critical nutrients for plant growth; however, excessive nitrogen use contributes to ecosystem pollution and disrupts the nitrogen cycle, while phosphorus, typically derived from limited mining sources, necessitates constant applications to maintain nutrient levels and high yields. In this context, microbial biostimulants emerged as a promising alternative to conventional chemical fertilizers, being defined as materials containing one or more microorganisms that, when applied to plants or the rhizosphere, stimulate natural processes to enhance nutrient use efficiency, seed quality, plant growth promotion, and accessibility on confined nutrients in soil. Promising microorganisms for biostimulant development include *Bacillus subtilis, Pseudomonas fluorescens, Azospirillum spp.,* and *Azotobacter spp.,* for their abilities to solubilize phosphorus, fix nitrogen, promote plant growth, and improve soil health. This thesis is part of an industrial PhD program, a scholarship from Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR, Resolution EMC/460/2020), Biocontrol Technologies, S.L. and Universitat de Barcelona. The general objective of the research was to isolate, characterize, and explore the potential application of these genera to plants, with the aim, to develop a microbial biostimulant that complies with Spanish regulations (Royal Decree 999/2017) or European regulations (Regulation (EU) 2019/1009) by combining *in vitro* characterization experiments, plant trials, and soil studies. The isolation and characterization identified several strains of interest, including 16 *Bacillus subtilis*, 16 *Pseudomonas fluorescens*, 4 *Azotobacter spp.*, and 9 *Azospirillum spp.* with some *P. fluorescens* excelling for Indole-3-Acetic acid production (IAA), *B. subtilis* for IAA, siderophores production and phosphorus solubilizing capacities while *Azotobacter spp.* demonstrated higher mineral phosphorus solubilization and *Azospirillum spp.* nitrogen fixing capabilities. Plant trials revealed *Bacillus subtilis* B7 and B17 as promising candidates for the development of biofertilizers according to Spanish regulations when applied at the substrate at a concentration of 10^7 CFU mL^{-1} which significantly enhanced seed germination by 10% and 13%, respectively, and promoted plant growth in cucumber, lettuce, and maize compared to non-inoculated plants. Furthermore, with the inoculation of B7 and B17 applied at the plant rhizosphere at a concentration of 10^7 CFU mL^{-1} the maize phosphorus use efficiency was improved under conditions of soluble phosphorus limitation, where B7 increased maize biomass by 34% and B17 enhanced plant phosphorus accumulation by 59% compared to non-inoculated plants. Moreover, *Azospirillum brasilense* 21F221 and *Azospirillum aestuarii* 21F226 were selected as candidates for the development of microbial biostimulants under European regulations due to their significant increase in nitrogen use efficiency when applied to maize and rice seeds at 10^8 CFU g $seed^{-1}$ cultivated with different N fertilization regimes. Strain 21F221 notably

enhanced plant growth (16% and 4%) and plant nitrogen accumulation (33% and 34%) and 21F226 boosted plant biomass (20% and 11%) and yield (148% and 37%) compared to non-inoculated plants in maize and rice respectively, suggesting the possibility to significantly reduce fertilizer. The study of these *Azospirillum spp*. strains' effects on soil dynamic revealed no nitrogen fixing in the absence of plants and efficient nutrient mining from organic matter, as well as significant impacts on native microbial populations, underscoring the need for tailored application strategies. The studies conducted in the framework of this PhD thesis successfully bridges academic research with industrial application, supporting the initial steps for the development of a microbial biostimulant that meets stringent Spanish and European regulatory standards. Further studies would be necessary focused on development of growth, sporulation and formulation methods of selected strains, optimization of application strategies and field trials to validate these strains under diverse agricultural conditions.

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Glossary

AE: Agronomic efficiency of applied nutrient

API: Analytical profile index

ARA: Acetylene reduction assay

$CaCO_3$: Calcium carbonate

CAS: Chrome Azurol Sulfate

CECT: Colección Española de Cultivos Tipo

CEN: European Committee for Standardization

CFU: Colony forming units

CMC: Component Material Category

DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen

EC: Electrical conductivity

FeEDTA: Iron-Ethylenediaminetetraacetic Acid

FeMo-Co: Iron-molybdenum cofactor

FPR: Fertilizer Products Regulation

HDTMA: Hexadecyltrimethylammonium bromide

HPLC: High-Performance Liquid Chromatography

IAA: Indole-3-acetic acid

IE: Internal utilization efficiency of a nutrient

KOH: Potassium hydroxide

LB: Lennox: Luria-Bertani Lennox

MALDI-TOF: Matrix-assisted Laser Desorption/Ionization

Min-N: Mineral nitrogen

N: Nitrogen

NA: Nutrient agar

NBI: Nitrogen balance index

NBRIP: National Botanical Research Institute's phosphate growth medium

NBS: Nutrient broth solution

NE: Nutrient export

NFB: Nitrogen-fixing bacteria

NH_4+: Ammonium

N-NO_3/ NO_3^-: Nitrate

NO: Nitric oxide

NPK: Nitrogen phosphorus potassium

NR: Nitrate reductase

NUE: Nutrient use efficiency

NVZs: Nitrate Vulnerable Zones

OM: Oxidable organic matter

P: Phosphorus

PCR: Polymerase Chain Reaction

PE: Physiological efficiency of applied nutrient

PFC: Product Function Categories

PFP: Partial factor productivity of applied nutrient

PGPR: Plant Growth-Promoting Bacteria

PSB: Phosphorus-solubilizing bacteria

RC: Red Congo

RE: Recovery efficiency of applied nutrient

RH: Relative humidity

SIR: Substrate induced respiration

SOM: Soil organic matter

SON: Soluble organic nitrogen

TC: Technical committee

TNO: Total oxidable nitrogen

Introduction

Introduction

1. Plant nutrition

1.1. Essential nutrients

Plant nutrition is a central area of study in plant biology that has been developed over decades since Liebig established mineral nutrition as a scientific discipline by identifying essential elements for plant growth. This scientific discipline allowed the commercial production of mineral fertilizers and the crop production improvement early in the 20th century, in the developed countries (Marschner, 2011).

While plants largely meet their basic life needs with light, CO_2, and water, they require a list of elemental nutrients from the soil to develop. Essential nutrients for plants can be classified into macronutrients and micronutrients, based on the amount required by the plant (Singh et al., 2022). Macronutrients include nitrogen (N), phosphorus (P), potassium (K), sulphur (S), magnesium (Mg), and calcium (Ca). Nitrogen represents 1-5% of total plant matter and is integral to various vital components, such as proteins, nucleic acids, chlorophyll, coenzymes, phytohormones, and secondary metabolites (Marschner, 2011; Zewide, 2021). Phosphorus, another crucial nutrient, is part of nucleic acids, membrane lipids, and energy transfer molecules, boosts grain quality and increase resistance to abiotic stress (Havlin, 2016; Poirier & Bucher, 2002). Potassium is the most abundant cation in the cells and plays an essential role in osmoregulation, transport of assimilates, and protein synthesis, significantly influencing fruit quality (Clarkson & Hanson, 1980; Leigh et al., 1984). Magnesium is vital for chlorophyll production, photophosphorylation and protein synthesis, sulphur is found in amino acids, vitamins and phytochelatins, helping regulate oxidative stress (Narayan et al., 2023) and calcium is crucial for cell structure, division, and signalling (Burstrom, 1968).

Micronutrients being iron (Fe), manganese (Mn), copper (Cu), zinc (Zn), molybdenum (Mo), nickel (Ni), and chlorine (Cl) usually are part of enzymatic cofactors, facilitating energy conversion, protein synthesis, and genetic regulation (Ravet & Pilon, 2013). Copper is involved in electron transport and redox control (Andresen et al., 2018); iron is crucial for photosynthesis and cellular respiration; and manganese in redox balance (Pirson et al., 1952; Woo et al., 2000). Molybdenum and nickel are essential in nitrogen absorption (Andresen et al., 2018; Hoffman et al., 2014; Schwarz & Mendel, 2006), and zinc is necessary for protein synthesis and genetic regulation (Noguero et al., 2013; Rowlett, 2010; Tran et al., 2010).

1.2 Plant nutrient uptake

Growth stage of plants and nutrient availability in soil influence the pattern by which plants absorb nutrients. Nutrient absorption usually starts slow at initial stages with rapid increase during peak dry matter production and then decreases as crops approach maturity. During germination, plants require high levels of nitrogen to promote initial growth and leaf development (Weil & Brady, 2017), then during vegetative growth there is usually a gradual daily increase in nutrient uptake that peaks during the major growth spurt and, at later stages, the absorption of phosphorus and potassium becomes crucial for root development and fruit maturation (Bruulsema & Garcia, 2015). These patterns of nutrient absorption are dependent of plant species and influenced by environmental factors such as temperature, soil pH, and microbial activity, which can alter nutrient availability (Epstein, 1972). Understanding and leveraging absorption patterns can lead to more effective fertilization strategies, enhancing both crop yield and soil health over time.

2. Nitrogen and phosphorus

2.2. Nitrogen and phosphorus biogeochemical cycles

The nitrogen and phosphorus cycles are fundamental biogeochemical processes that regulate the availability of different forms of these essential nutrients in ecosystems. It is important to understand these cycles to optimize agricultural practices and ensure sustainable food production, as both nutrients play vital roles in plant growth (Alewell et al., 2020; Robertson & Groffman, 2015).

Until recently, most biologists assumed that the biogeochemical nitrogen cycle followed a straightforward path in which free-living or symbiotic dinitrogen gas-fixing microbes transform nitrogen from the air (N_2) and provide ammonium (NH_4) for assimilation, nitrifying microbes oxidize excess ammonium (NH_4) via nitrite (NO_2) to nitrate (NO_3), and finally, denitrifying microorganisms return the oxidized nitrogen species back to N_2, thereby closing the cycle (Strous et al., 2006) as depicted in Figure 1A. However, in the last decade, extensive research has revealed an enormous biodiversity and metabolic capability of nitrogen conversions by microorganisms, like aerobic ammonium oxidation, nitrate reduction to dinitrogen gas, nitrite-oxidizing phototrophs, nitrite-dependent anaerobic methane oxidation, and hyperthermophilic N_2-fixing methane-producing archaea (Kibret, 2021).

The biogeochemical cycle of phosphorus is a crucial process in natural ecosystems regulating the availability of this essential element for life (Vitousek et al., 2010). The cycle begins with the release of phosphorus from rocks and minerals through physical processes such as erosion and

weathering releasing inorganic phosphate ions into the soil (Turner, 2008). Once in the soil, phosphorus can be absorbed by plants and utilized in various metabolic processes, such as energy transfer through ATP, or in the formation of nucleic acids and cellular membranes (Vitousek et al., 2010). After being absorbed by plants, phosphorus enters into the food chain when herbivores consume the plant biomass and it is transferred to higher trophic levels. Later, when organisms excrete waste or die and decompose, phosphorus is returned to the soil, where it can be taken up by plants again or undergo further microbial transformations, thereby completing the cycle (Vitousek et al., 2010) as described in Figure 1B. The availability of phosphorus to plants is regulated by adsorption and desorption rates; adsorption means the binding process of phosphate ions to soil particles making them less available for plant uptake, while desorption is the release of the bound phosphates back into the soil solution (Barrow & Hartemink, 2023). All of these biogeochemical rates are influenced by the soil chemical composition and microbial activity, as microbes play a key role in transforming phosphorus into the soil (Richardson et al., 2009).

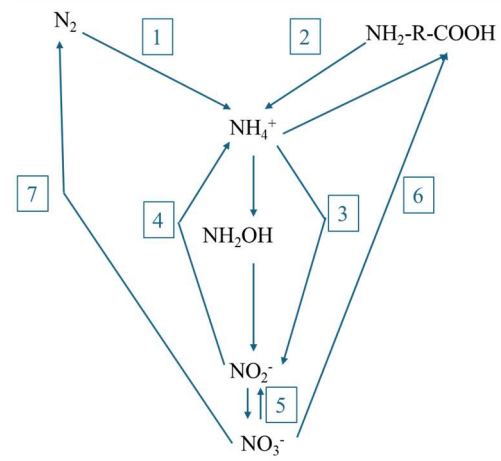

Figure 1A Nitrogen cycle
(1) Nitrogen fixation (2) Ammonification (3) Nitrification (4) Nitrate and nitrite reduction (5) Nitrate oxidation (6) Nitrogen assimilation (7) Denitrification

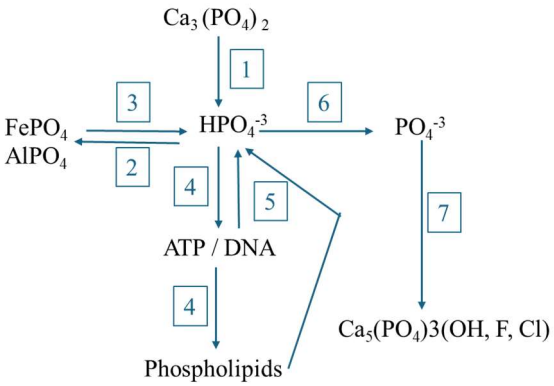

Figure 1B Phosphorus cycle
(1) Weathering and erosion (2) Adsorption (3) Desorption (4) Plant uptake and herbivore consumption (5) Decomposition (6) Leaching (7) Sedimentation

2.2 Nitrogen and phosphorus plant deficiency

Advances in our understanding of plant nutrient requirements have led to substantial progress in agricultural food production, as a result, most farmers in Western Europe and the USA routinely apply N, P, and K fertilizers. However, despite these efforts, nutrient deficiencies regularly occur even in fertilized fields due to the chemical and physical properties of the soil, which can lead to reduced mobility, absorbance, or leaching of individual nutrients (Amtmann & Blatt, 2009). These nutrient deficiencies have various consequences at molecular and plant phenotypic levels (Gong et al., 2020; Singh et al., 2022). In natural soils, for instance, only a small fraction of P in soils

and bedrocks is available to plants because HPO_4^{-3}, the form of P which can be absorbed by plants, is produced by weathering which is a very slow process (Alewell et al., 2020),

Nitrogen (N) and phosphorus (P) are crucial nutrients that significantly affect crop yield and plant development. Nitrogen is a primary constituent of proteins, nucleic acids, phytohormones, and chlorophylls and its deficiency severely impacts the photosynthesis process, first leading to the yellowing of lower leaves which hinder plant development, while severe deficiency causes spots on the tips and edges of leaves, stunted growth, and early flowering and fruiting affecting the yield and quality of the crops (Mu & Chen, 2021; Zhao et al., 2005). Similarly, phosphorus deficiency presents a significant impact on plant growth, showing stunted plants, narrow leaf insertion angles, dark green opaque colour, chlorotic points in internerval regions, necrosis, upward curved leaf edges and inhibited root development, phosphorus deficiency also delays and reduces flowering and fruiting, prolonging the time needed for crops to ripen and affecting the shape and size of fruits (Chen et al., 2021; Dar et al., 2017; Khan et al., 2023).

3. Fertilizers

3.1 Mineral and organic fertilizers

Fertilizers are defined as materials of natural or synthetic origin used to supply nutrients to plants, either applied directly to plant tissues or indirectly to the soil (Scherer et al., 2009). Fertilizers are classified based on the number of nutrients they supply as straight fertilizers providing a single nutrient and multi-nutrient or complex fertilizers which provide two or more nutrients. The primary nutrients provided by fertilizers are the three essential macronutrients N, P, and K (Yahaya et al., 2023). Additionally, fertilizers can be also categorized by their origin into mineral and organic fertilizers (Jones, 2012).

Mineral fertilizers, also known as inorganic fertilizer, are a type of fertilizers produced industrially from natural minerals, containing essential nutrients in forms that plants can quickly absorb (Havlin., 2016). Mineral fertilizers have played a transformative role in global food production, leading to an 800% increase in output between 1961 and 2019 although they have also caused serious environmental issues (Mbow et al., 2020). Mineral nitrogen fertilizers began to be produced following the discovery of the Haber-Bosch synthesis in 1909, which allows the conversion of atmospheric nitrogen into ammonia (Vicente & Dean, 2017). Since then, mineral nitrogen fertilizers have been used and the most used forms today are amide, nitrate, and N-reduced forms (Kumar et al., 2013; Sinha & Tandon, 2020). Regarding mineral phosphorus fertilizers, all forms are produced from rock phosphate subjected to high temperatures or through acidulation, to release P in the form of P_2O_5 (Blaise et al., 2014). The most common phosphate

fertilizers used are single superphosphate (SSP), monoammonium phosphate (MAP), triple superphosphate (TSP) and diammonium phosphate (DAP) (Sinha & Tandon, 2020).

Organic fertilizers are a type of fertilizers derived from natural organic matter such as plant and animal residues (Havlin, 2016). They provide essential nutrients in smaller quantities compared to synthetic fertilizers but offer significant benefits for soil health and the environment (Dhaliwal et al., 2019). These benefits include the slow release of nutrients, which can help reduce nutrient leaching and improve long-term soil fertility, and contribute to the build-up of soil organic matter, enhancing soil carbon sequestration and mitigating climate change (Lal, 2004). The most common organic sources used are manure, compost, and bone meal (Shepherd et al., 2002).

3.2 Biofertilizers / Microbial biostimulants

Microbial biostimulants or biofertilizers comprise a group of microorganisms such as bacteria and fungi which alone or combined, applied to soil, seeds or plants, can supply or make more accessible essential nutrients and promote plant growth (Mahanty et al., 2017; Mahmud et al., 2020). This arises as a promising instrument to upgrade horticultural efficiency while decreasing reliance on agrochemicals, thereby enhancing agricultural yields, and promoting sustainable agricultural development (Stewart & Roberts, 2012).

The microorganisms found in biofertilizers use a variety of strategies, either nitrogen fixation, phosphate solubilization, or production of substances to stimulate plant growth or all such traits working simultaneously (Mahmud et al., 2020). Biological nitrogen fixation and phosphate solubilization are two critical processes driven by soil microorganisms that impact nutrient availability for plants and, consequently, crop productivity (Bashan et al., 2014). Kumar et al. (2022) categorizes nitrogen-fixing microbes in three main types based on their ecological niches being “symbiotic nitrogen-fixing microbes” collective termed *Rhizobium* which are the ones that form symbiotic structures with legume roots; “free-living nitrogen-fixing bacteria” including those that fix nitrogen without root association, such as *Azotobacter spp.* found in neutral to alkaline soils and in non-legume crops rhizosphere (cotton, wheat, rice, and vegetables) (Jain et al., 2021) and the “associative nitrogen-fixing microbes” being the ones that live in close proximity to plant roots within the rhizosphere or loosely interact with root surfaces, which include *Azospirillium spp.* This genus is commonly isolated from the rhizosphere of diverse plants, particularly grasses, and which is prevalent in both agricultural and non-agricultural soils (Steenhoudt & Vanderleyden, 2000). Additionally, *Pseudomonas fluorescens* and *Bacillus subtilis* are also described, as phosphate-solubilizing/mobilizing microbes, *P. fluorescens* inhabiting water, soil and plant tissues (Garrido-Sanz et al., 2017), while *B. subtilis*, known for its versatility, is commonly found in soil and associated with the rhizosphere of diverse plant species (Madika et al., 2019). Several studies have shown that inoculation with these nitrogen-fixing and

phosphate-solubilizing bacteria can enhance crop yields under a variety of soil and climatic conditions (Bashan et al., 2014; Bhattacharyya & Jha, 2012).

However, challenges remain in developing effective bioinoculants formulations suitable for diverse applications and environmental conditions (Cassán et al., 2020). These include inconsistent performance influenced by environmental factors, short shelf-life demanding precise storage conditions, formulation complexities, competition with native soil microorganisms, regulatory and quality control issues, limited awareness among farmers, economic concerns, and technological and research gaps. Overcoming these obstacles necessitates a multifaceted approach involving scientific research, technological innovation, policy development, and comprehensive education and support programs for farmers (Cassán & Diaz-Zorita, 2016).

4. Environmental challenges of fertilizer use

4.1 Environmental impact

The extensive use of fertilizers has had significant environmental and ecological consequences, as the misuse of chemical fertilizers disrupted soil pH, promoted pest infestations, caused soil acidification and crusting, reduced soil organic carbon and beneficial organisms and consequently these changes have affected plant growth, decreased yields, and contributed to greenhouse gas emissions (Krasilnikov et al., 2022). Among the most significant pollution processes resulting from the application of fertilizers, we find nitrate and phosphorus leaching into groundwater, nitrous oxide emissions and contamination by heavy metals in soils (Chien et al., 2009) being agriculture the responsible for the 10.3% of the European greenhouse gas emissions (European Commission, 2020).

Over the past century, the overuse of synthetic nitrogen fertilizers has led to a doubling of nitrogen compounds in water, soil and air, making nitrogen a serious pollutant that damages ecosystems, endangers human health, and fuels climate change by releasing powerful greenhouse gases like nitrous oxide (Galloway et al., 2004). Additionally, the synthesis of mineral nitrogen fertilizers relies on fossil fuels, emitting carbon dioxide and nitrogen oxides that pollute the air (Galloway et al., 2004; Socolow, 1999).

Providing an optimal phosphorus fertilization is another critical challenge in modern agriculture, as despite phosphorus being very abundant in the lithosphere, the form available for plants, inorganic orthophosphate, is insoluble and diffuses slowly in soils, leading to widespread deficiency, as a result, the 43% of the world's cultivated area is lacking in phosphorus (Chen et al., 2021; Turner & Blackwell, 2013). Moreover, phosphorus fertilization is highly inefficient,

with only 15–25% of the applied phosphorus being absorbed by plants, and the remainder leaches into water bodies, causing eutrophication, and contributes to soil degradation (Sharpley, 1995) Additionally, rock phosphate supplies are finite, potentially leading to a shortage of phosphorus fertilizers by the end of the century (Cordell et al., 2009).

To address these issues, sustainable agricultural practices must be developed, including the use of organic fertilizers and bio-fertilizers that improve soil properties and enhance crop productivity without the adverse effects of synthetic fertilizers (Krasilnikov et al., 2022).

4.2 European Union response to mitigate fertilizers pollution

In response to the excessive use of fertilizers and the pollution produced, Europe exercises regulation on fertilizer use, the Nitrates Directive (91/676/EEC) and the Water Framework Directive (2000/60/EC) particularly addressing the use of nitrogen (N) and phosphorus (P) in agriculture. Furthermore, the Farm to Fork Strategy was presented in 2020 by the Commission to the European Parliament, the Council, the European Economic and Social Committee and the Committee of the Regions also regulating fertilization inputs (European Commission, 2020).

The Nitrates Directive sets mandatory standards to reduce water pollution caused by nitrates from agricultural sources, designating Nitrate Vulnerable Zones (NVZs) where specific action programs must be implemented to manage and limit the use of nitrogen fertilizers to protect water resources from nitrate contamination and improve water quality. Examples of NVZs include intensive agricultural areas such as the Netherlands or Belgium, and certain regions of France, Italy, and Spain, where intensive fertilizer use and high livestock density increase the risk of nitrate pollution. Specific regions in Spain, such as Catalonia, the Valencian Community, Andalucia, Castilla y León, Murcia, Aragón, and Galicia, have also been designated as NVZs (European Council, 1991).

The Water Framework Directive (WFD, 2000/60/EC) is a regulation which affects N and P fertilization as aims to achieve good ecological and chemical status for EU waters by implementing measures to control diffuse pollution from nutrients. This directive requires Member States to develop river basin management plans that limit fertilizer application and improve agricultural practices (European Parliament and Council, 2000).

Farm to Fork Strategy is an essential element from the European Green Deal which sets out how to make Europe climate-neutral continent by 2050, assessing a climate target plan for 2030, to reduce emissions. With a specific objective to reduce the excess of nutrients losses in the environment, especially nitrogen and phosphorus, by at least 50%, while ensuring that there is no deterioration in soil fertility, reducing the use of fertilizers at least 20% by 2030. By developing

an integrated nutrient management action plan with the Member States and work to extend the precise fertilization techniques and sustainability agricultural practices.

5. Microbial biostimulant regulation

5.1. European biostimulant regulation

The market for microbial inoculants is constantly evolving, facing both regulatory and commercial challenges. In Europe, the legislation on microbial biostimulants is framed by the EU Fertilizer Products Regulation 2019/1009 (FPR), which came into effect on July 16, 2022. This regulation establishes harmonized provisions for fertilizer commercialization products within 27 EU Member States from the European Union (European Parliament and Council, 2019).

Article 47, section 2, defines a plant biostimulant as "an EU fertilizer product whose function is to stimulate plant nutrition processes independently of the product's nutrient content, with the sole aim of improving one or more of the following plant or plant rhizosphere characteristics: nutrient use efficiency, tolerance to abiotic stress, quality traits, or availability of confined nutrients in the soil or rhizosphere". Microbial biostimulants are further defined in Annex I, which outlines the Product Function Categories (PFC) under section 6, subsection A and in Annex II under the Component Material Category 7 (CMC 7), which is defined as a plant biostimulant formulated with one or many microorganisms. In PFC (A) it is specified the constitution of the product, the maximum number of human pathogens allowed (*Salmonella spp, Esterichia coli, Listeria monocitogenes, Vibrio spp., Shigella spp., Staphylococcus aureus, Enterococcus)* and pH specifications for liquid formulations. In CMC7 defines that an EU fertilizer product belonging to PFC 6 (A) may contain microorganisms, including dead microorganisms or empty cells, and non-harmful residues from their growth medium, provided they have undergone no treatment other than drying or lyophilization and the microorganisms accepted, at the present moment include *Azotobacter spp*., mycorrhizal fungi, *Rhizobium spp*., and *Azospirillum spp*. The regulation also defines labelling characteristics for these products (Annex II part II) (European Parliament and Council, 2019).

The efficacy of microbial biostimulant products must be supported by solid scientific evidence and is meticulously reviewed during the certification process. The European Committee for Standardization (CEN) and specifically the CEN/TC 455 which focuses on plant biostimulants sets the procedures for demonstrating agronomical claims (e.g., nutrient use efficiency, tolerance to abiotic stress, crop quality traits) through the standardization of sampling, denominations, specifications including safety requirements, marking, and test methods that verify product claims for plant biostimulants, including microorganisms. In this thesis, we will focus on the draft

document Fertilising Products Regulation Committee for Standardization (FprCEN/TS 17700), published by the Slovenian standard kSIST-TS committee, which defines the scope, normative references, terms and definitions, claim terminology, assessment indices to validate claims, and performance specifications for agronomic traits (Slovenski standard kSIST-TS committee, 2021). This document has five parts corresponding to general specifications and the claims defined.

Part 1 (FprCEN/TS 17700-1) defines the general specifications, the necessary information to demonstrate efficacy, the types of tests to be conducted, the specifications, labelling terminology, quality evaluation criteria, and data to be presented, it is notable that to justify a claim, at least three tests with positive results are required. These must include a control treatment (not inoculated) under the same management practices, and all treatments should be irrigated with the same concentration of water. Additionally, the number of replicates should be four under controlled conditions and in a minimum plot of 20 m² under field conditions. Likewise, in the evaluation criteria section, given the variable nature of the effects of plant biostimulants, a confidence level of 90% ($p < 0.1$) is recommended under controlled conditions and 85% ($p < 0.15$) in the field, between the treatment and the control, regardless of this last detail, in the studies of this thesis, we have used a more stringent confidence level ($p < 0.05$) to identify highly effective microorganisms performing an ANOVA and Tukey's tests for the trials *in vitro* and Dunett's test for the experiments on plant to compare each treatment and the control (non-inoculated plants). Part 2 (FprCEN/TS 17700-2) establishes the framework to validate the claim regarding the biostimulant's ability to improve nutrient use efficiency and Part 5 (FprCEN/TS 17700-5) the biostimulant's ability to improve the availability of confined nutrients in the soil or rhizosphere. These sections define various agronomic indices and ways to calculate them, as well as mentions that each index can be used independently to justify a claim (Slovenski standard kSIST-TS committee, 2021).

5.2. Spanish regulation

In Europe, beside the mentioned Regulation (EU) 2019/1009, there are different national regulations among the Member States. So far, the companies willing to commercialize a microbial biostimulant can chose to follow the national or the European regulation.

In Spain, the regulatory framework for microorganism-based fertilizers is defined by Royal Decree 999/2017, published on November 24, 2017. The decree includes annexes that introduce new product types (Annex I), update identification and labelling provisions (Annex II), modify tolerance margins (Annex III), establish analysis methods for these new products (Annex VI), and correct an error in the instructions for including a new type of fertilizer (Annex VII) (Gobierno de España, 2017).

Among the fertilizer product types, group 4.4 includes "special products based on microorganisms," defined as non-mycorrhizal microorganism-based products. To register a product in this category, the minimum necessary information includes the identification of microbial strains (molecular sequences) and a minimum inoculum for each microorganism present, which must be at least 10^7 CFU ml^{-1} or 10^7 CFU g^{-1} unless efficiency is demonstrated according to the protocol defined in Annex VIII. Additionally, items in section 4 of group 4 of Annex I must submit a technical report at the time of application, as referenced in Article 24.1. This report must be produced by an independent organization and according to a standardized model and should contain two sections: Identification and characterization of the microorganisms and the demonstration of the agronomic efficiency of the product to be registered (Gobierno de España, 2017).

The microorganism identification and characterization must include the genus and species based on the 16S gene sequence in prokaryotes, a description of isolation and quantification methods, growth conditions in the laboratory, and PCR conditions for amplifying the sequence, including primer sequences. The demonstration of the product's agronomic efficiency must be specific to the formulation being registered signed by a field-experienced trial manager from an independent organization, following the testing protocol approved by the Directorate General for Agricultural Production and Markets. This protocol includes a favorable conclusion on the product's agronomic efficiency, summarizing the conditions of use; authorization for specific crop groups where agronomic efficiency has been demonstrated (horticultural, herbaceous, woody, or plant production); a complete product composition description and field trials conducted in Spain (Gobierno de España, 2017).

6. Crops

6.1. Maize

Maize (*Zea mays*) is an annual plant from the *Poaceae* family and correspond to the C4 pathway group, first dated from Mesoamerica before 5000 B.C. (Ranum et al., 2014). This plant is characterized to have a robust and erect stem that can reach up to three meters tall with broad leaves arranged alternately along the stem. Maize plants reproduce through monoecious cross-pollination and has male flowers (tassels) at the top of the plant and female flowers (ears) which are covered in protective green bracts; inside each ear numerous flowers develop into rows of kernels along the rachis (Nuss & Tanumihardjo, 2010). Maize types are classified based on the size and composition of the kernel endosperm being dent, flint, waxy, flour, sweet, pop, Indian, and pod corn (Ranum et al., 2014).

Maize, along with wheat, rice and barley in 2022 accounted for 90 percent of total world cereal production as it is cultivated on 156 million hectares annually in nearly 100 countries with almost 1.2 billion tonnes produced. Maize production accounted for 44 million tonnes with a 4% decrease from 2021 to 2022 due to drought in European counties (Brown et al., 1988; FAO, 2023). In Spain on 2021 maize cultivated surface was 458.3 thousand hectares with a production of 4.6 million tonnes, being the principal producing regions Castilla - León, Extremadura and Aragón (MAPA, 2022). Maize is a very demanding plant and requires significant levels of NPK fertilizer, specifically for N the recommended application is about 150 to 200 kg ha^{-1} during vegetative growth stages (Fageria et al., 2010) and for P applications of 40 to 60 kg ha^{-1} are necessary to prevent deficiencies (Havlin., 2016).

6.2. Rice

Rice (*Oryza sativa*) is an annual plant also from the *Poaceae* family but contrary to maize from the C3 pathway group. This plant is characterized to have round, hollow, and jointed stems that support panicles, when mature the plant typically has a main stem and several lateral branches (tillers). It is a diploid plant and is normally self-pollinated (Izawa & Shimamoto, 1996). Rice growth is divided into three stages: vegetative (from germination to panicle initiation) where the shoot apical meristem produces leaves, and tillers emerge from leaf axils; reproductive (from panicle initiation to flowering) that involves stem elongation, panicle initiation and differentiation, flowering, maturation and grain filling or maturation (from flowering to maturity) where rice grains increase in size and weight, accumulating sugars, starches, storage proteins, and other compounds (Wang & Li, 2005).

In 2024, over 167 million hectares of rice were cultivated globally, producing approximately 530.1 million tonnes corresponding to one of the most important food crops, feeding more than half population. In the world, the major rice-producing regions include Asia, Latin America, and Africa, notably being Vietnam the fifth world largest producer with 43.5 million tonnes production in 2023 with the major field surface in Mekong and Red River Delta (FAO, 2024). Spain is also a rice productor with 84 thousand hectares cultivated in 2021 with 72 thousand tonnes produced in 2022, the mayor autonomous communities' producers of rice are Andalusia, Extremadura, and Catalonia, significantly contributing to the national rice production (MAPA, 2022).

In rice production nitrogen and phosphorus are vital for achieving high rice yields, being N important in the vegetative growth, with recommended applications of 100 to 150 kg ha^{-1} (Fageria et al., 2010) and P is essential for root development and grain maturation, with recommended applications of 30 to 60 kg/ha (Havlin., 2016).

7. Biocontrol Technologies S.L.

This doctoral research is conducted within the framework of the Industrial Doctorate supported by the Catalan Government (Generalitat de Catalunya) with the Universitat de Barcelona, and Biocontrol Technologies S.L. This company is specialized in biological control of crop diseases, with the *Trichoderma asperellum* strain T34 (T34) CECT No. 20417 being its leading product, commercialized against several soil and foliar diseases, in example *Fusarium oxysporum, Rhizoctonia solani, Pythium aphanidermatum, Sclerotinia spp.,* and *Botrytis cinerea.* The company was founded in 2004 as a spin-off from the University of Barcelona, this emerged from the discovery of the efficiency of *T. asperellum* strain T34 to supress *Fusarium* wilt of tomato by the Professor and Doctor M. I. Trillas and the doctoral student L. Cotxarrera (Cotxarrera et al., 2002). In 2002, the patent for this strain was registered in Spain (ES 2188385 B1) and later was registered to several other countries in the EU (EP 1400586 B1) and the United States (US 7553657 B2). This product has been authorized as microbial Plant Protection Product in different countries, USA, Canada, Peru, Egypt, Morocco, and in several EU States forming part of the Annex I by the Commission Implementing Regulation No. 1238/2012 and with Article 80(1)(a) of Regulation (EC) No 1107/2009.

Since then, the company has been dedicated to the development of new uses of *T. asperellum* strain T34 and the research of other microorganisms for biological crop control. Additionally, it has been working on the development of new products, including microbial biostimulants. In 2023, the company funded a chair with the University of Barcelona, the UB Biocontrol Technologies Chair of Microorganisms for Agriculture. The objective is promoting research, training, and dissemination activities on biological control and plant nutrition using microorganisms, to achieve a more sustainable and secure agriculture, aligning with the European Green Deal (European Commission, 2019) and the Sustainable Development Goals (United Nations, 2015).

Aims of this work

Aims of this work

This thesis is part of an Industrial PhD program from Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR, Resolution EMC/460/2020) in collaboration with Biocontrol Technologies, S.L. and Universitat de Barcelona. As part of the research project with an industrial perspective, the general aim of this thesis was to isolate, characterize, and evaluate the *in vitro* and *in vivo* the functionality and capacities of the plant growth-promoting rhizobacteria isolated, in order to develop a microbial biostimulant that complies with Spanish regulations (Royal Decree 999/2017) and/or European regulations (Regulation (EU) 2019/1009).

The specific aims were:

1. Reviewing effective methodologies and stablishing protocols for the isolation, preservation, growth and classification of plant growth promotion rhizobacteria.

2. Develop a new collection of isolates with nitrogen-fixing and phosphorus-solubilizing capabilities, specifically focusing on *Bacillus subtilis* and *Pseudomonas fluorescens* in compliance with Spanish regulations, as well as *Azospirillum spp. and Azotobacter spp.* strains adhering to European regulations.

3. To evaluate the *in vitro* capacities of interest by performing various techniques being indole-3-acetic acid and siderophore production, phosphorus solubilization and nitrogen fixing capacity.

4. To evaluate the *in vivo* efficacy of the top-performing *in vitro* candidate strains in enhancing seed quality traits, promoting growth, and improving phosphorus and nitrogen use efficiency across various plant species, with a primary focus on maize and rice, under both greenhouse and field conditions.

5. The biochemical and ecological characterisation of the selected strains, on soils with different fertilization background (organic and mineral), to gain deeper knowledge for its better performance in soil.

Case Studies

Chapter 1: *Bacillus spp.* and *Pseudomonas spp.* as potential candidates to develop a biofertilizer

Introduction

During their growth and development, plants establish continuous interactions with soil-dwelling microorganisms. Plant Growth-Promoting Bacteria (PGPR) represent a diverse group of microorganisms inhabiting the rhizosphere of plants, where they play a critical role in influencing plant growth and development through various mechanisms. By enhancing the availability of essential nutrients for plants, reduce heavy metals, and produce phytohormones (Goswami et al., 2016; Shailendra Singh, 2015; Singh et al., 2019). Additionally, can aid in disease control by producing substances that trigger systemic resistance and enhance competition for nutrients and niches (Bjelić et al., 2018; Di Salvo & García de Salamone, 2019; Etesami & Maheshwari, 2018; Vejan et al., 2016), as well as, promote soil health (Etesami, 2018; He et al., 2019).

PGPR among their notable multifaceted mechanisms that contribute to their beneficial effects on plants, is the capacity to enhance the availability of essential nutrients. For instance, PGPR possess the capacity to fix atmospheric nitrogen, converting it into a form that plants can utilize effectively, thereby enhancing soil fertility and plant growth (Dobbelaere et al., 2003). As well as some PGPR can solubilize inorganic phosphate, making it more accessible to plants, thus bolstering the absorption of this vital nutrient (Ahmed & Shahab, 2009). Another key aspect of the beneficial interaction between PGPR and plants is their ability to synthesize and release plant growth regulators, these are organic substances very similar to the phytohormones synthesized by plants, such as auxins, cytokinins, and gibberellins (Vejan et al., 2016) and act as chemical signals that regulate a variety of growth and development processes in plants, including cell elongation, cell division, and root formation (Cassán et al., 2014).

The capacity of PGPR to enhance nutrient availability, promote plant growth and development, and protect against soil pathogens makes them promising candidates in the quest for more efficient and environmentally friendly food production solutions. However, various studies describe that their effectiveness can vary among different plants and varieties, this specificity between PGPR and genotype underscores the importance of understanding the specific interactions between strains and particular crops, as well as testing their effectiveness across different species and varieties (Bhattacharyya & Jha, 2012; Glick, 2012). This targeted approach can significantly improve the overall efficacy of PGPR and their use as biofertilizers.

Biofertilizers comprise live formulations of beneficial microbes, such as PGPR or arbuscular mycorrhizal fungi, among others, that assist in nutrient availability and promote plant growth (Mohanty et al., 2021; Vejan et al., 2016). These products when applied to seeds, plant surfaces, or

soil, colonize the rhizosphere or the interior of the plant, promoting plant growth by increasing the supply or availability of primary nutrients for the host plant (Etesami & Maheshwari, 2018; He et al., 2019; Rao & Kishore, 2006; Vessey, 2003). The use of biofertilizers is becoming increasingly important in organic agriculture and plays a crucial role in the economy and agricultural production on a global scale, as they provide a sustainable solution for improving soil health and enhancing crop productivity.

In Spain, the production and commercialization of biofertilizers are subject to two fundamental regulations: the European regulation established by the EU Fertilizer Regulation (2019/1009) of the European Parliament and the Council, and the national regulation, specifically through Real Decreto 999/2017, of November 24th. This regulatory framework establishes guidelines and requirements for the manufacturing, registration, and proper use of biofertilizers, ensuring their effectiveness and safety in agriculture. In the Spanish regulation, Royal Decree 999/2017 amends the previous 506/2013 and redefines the regulatory framework for the production, registration, and commercialization of microbial fertilizers. Article 18 bis specifies that only microorganisms that have demonstrated, alone or mixed with fertilizers, their capacity to improve nutrient absorption, tolerance to abiotic stress, or crop quality can be registered in the Registro de Productos Fertilizantes. In this regulation, it is permissible to register any microorganism that meets the requirements specified in Annex VIII, which demands the identification and characterization of the microorganisms, as well as a demonstration of their agronomic efficiency (La Presidencia y para las Administraciones Territoriales, 2017). However, European regulation is more restrictive, allowing the registration of only four specific genera: *Rhizobium spp.*, mycorrhizae, *Azotobacter spp.*, and *Azospirillum spp.*

The selection of *Bacillus subtilis* and *Pseudomonas fluorescens* as subjects of our study to evaluate their characteristics as plant growth-promoting rhizobacteria (PGPR) and their subsequent use in creating a biofertilizer is based on a series of fundamental reasons. *B. subtilis* is one of the most studied and utilized genera among plant growth-promoting bacteria (PGPR). This microorganism plays a fundamental role in improving plant growth and development through various mechanisms. For instance, *B. subtilis* can promote plant growth and control pathogens by enhancing nutrient availability, modulating phytohormones, and producing antimicrobials (Arkhipova et al., 2005). Additionally, its ability to form resistant spores makes it a remarkably versatile microorganism that exhibits a wide variety of states and behaviours, enabling it to cope with diverse environmental challenges, as it can withstand abiotic stresses such as drought, temperature, and nutrient limitation (Losick, 2020; Schisler et al., 2004). This adaptability and plasticity make it an attractive candidate for studying its potential as a PGPR and its application in agriculture. The interaction between plants

and *B. subtilis* constitutes a symbiotic relationship, wherein both bacteria and the host plant mutually benefit; on one hand, plants secrete organic substances (approximately 30% of the carbon fixed through photosynthesis is secreted through root exudates), and these substances serve as a nutrient source for bacteria associated to the rhizosphere and in return, plants receive compounds and bacterial activities that promote their growth and shield them against stress. This exchange occurs through the root surface, where *B. subtilis* forms a thin biofilm for long-term colonization of the rhizosphere (Hashem et al., 2019). Moreover certain species of *Pseudomonas* for example *P. fluorescens*, have been described to have the ability to increase the absorption of essential plant nutrients (nitrogen, phosphorus, and potassium), produce phytohormones in the rhizosphere such as indole-3-acetic acid (IAA) and cytokinins, promoting plant growth and can act as biocontrol agents against phytopathogenic fungi (Santoro et al., 2016). Its versatility in promoting plant growth and protection make it a valuable component as PGPR in sustainable agriculture and improving crop yields.

The production of phytohormones and its impact on the development and growth of plants is an area of research of great relevance (Vejan et al., 2016). Auxins play a fundamental role in regulating a variety of processes such as cell division, elongation, differentiation, tropisms, and flowering, among others (Bajguz & Piotrowska-Niczyporuk, 2023). Microorganisms present in the rhizosphere have the ability to synthesize and release auxins in response to the presence of root exudates. PGPR, such as *B. subtilis* or *P. fluorescens*, play a crucial role in actively altering the homeostasis of plant growth hormones, by the induction of their production in plants through secreted compounds (Arkhipova et al., 2005; Cassán et al., 2014). One of the most studied phytohormones in plants, being part of the auxins group, is indole-3-acetic acid (Merzaeva & Shirokikh, 2010; Suansia & Senapati, 2023; Tsavkelova et al., 2006). Although IAA, is not necessary for seed germination, studies on the expression of genes related to auxins indicate that IAA is present at the radicle tip during and after seed germination (Miransari and Smith, 2014).

Furthermore, PGPR have phyto-stimulating effects by intervening in biochemical cycles and enhancing nutrient availability, including iron uptake and phosphorus solubilization, processes relevant to counteract the loss of efficiency of fertilizers due to biochemical processes such as sorption, fixation, and immobilization (Bargaz et al., 2021; Stutter et al., 2012). By rendering these nutrients accessible to plants, these bacteria contribute to greater nutrient use efficiency and reduce environmental pollution associated with excessive fertilizer application. Iron uptake efficiency by PGPR is determined by their capacity to produce siderophores, low molecular weight compounds that bacteria produce to chelate ferric iron [Fe(III)], facilitating its transport and absorption by microorganisms (Mohamed & Gomaa, 2012). The transport and absorption is different between gram

positives and gram negatives, in the case of *P. fluorescens,* a gram-negative bacterium, iron is transported through receptors in the outer membrane that recognize iron-siderophore complexes and are released into the plasmatic space (Krewulak & Vogel, 2008). In *B. subtilis,* a gram-positive bacterium, there are no outer membrane receptors, instead the iron-siderophore complexes are directly recognised by the periplasmic siderophore binding proteins of the periplasmatic membrane and brought into de cell (Fukushima et al., 2013).

Phosphate solubilization by PGPR can significantly improve the availability of this essential nutrient for plants, resulting in more vigorous plant growth and higher crop productivity (Stutter et al., 2012). Some PGPR solubilize inorganic phosphorus by producing a variety of organic compounds that can solubilize inorganic phosphorus present in the soil (Bargaz et al., 2021; Saeid et al., 2018). These organic acids, such as citric acid, acetic acid, and malic acid have the ability to chelate phosphate ions, converting them into soluble forms that plants can easily absorb (Bargaz et al., 2021). This process is of utmost importance, especially in soils with low phosphorus availability, as phosphorus is an essential macronutrient for plant growth and plays a vital role in photosynthesis, energy transfer, and organic compound synthesis.

Objectives

The general objective was to isolate *B. subtilis* and *P. fluorescens* strains, characterize and evaluate them *in vitro* and *in vivo* to determine if they have PGPR properties in order to develop microbial-based fertilizers

For this purpose, the following specific objectives were stablished:

1. Creating a new collection of isolates along with establishing protocols for their conservation and growth.
2. Evaluate the characteristics of interest of the new collection of isolates *in vitro*: i) the phytohormone production (IAA), ii) siderophore production capacity iii) phosphorus solubilization capacity from different sources.
3. Test on plants the isolates with the best *in vitro* results; i) improvement in germination ii) plant growth promotion iii) plant phosphorus use efficiency.

Materials and methods

1. Isolation

Several strains of *Bacillus spp.* (22 in total) and *Pseudomonas fluorescens* were isolated from rhizosphere or bulk soil of wheat (*Tricum aestivum*) or maize (*Zea mays*) plants growing in different soils from Catalonia, named A, B, C, D and Compost. Non-rhizospheric microorganisms were isolated mixing 10 g of soil with 90 mL of saline solution during 30 min with a rotatory shaker (150 rpm). In addition, rhizospheric microorganisms were isolated from the soil attached to the roots by dipping and gentle shaking in water under aseptic conditions.

To isolate *Bacillus spp.* strains, both type of suspensions were diluted (10^{-2} and 10^{-4}) and placed in 80ºC for 10 min and then NA (peptone 5 g L^{-1}, meat extract 10 g L^{-1}, agar powder 15 g L^{-1} and pH adjusted to 7) plates were inoculated. *P. fluorescens* were isolated by diluting the solutions at 10^{-2} and 10^{-4} colony forming units (CFU) mL^{-1} in King's B plates (Proteose peptone 20 g L^{-1}, glycerol 10 mL L^{-1}, K_2HPO_4 1.5 gL^{-1}, MgSO4 1.5 g L^{-1} and Agar 15 gL^{-1}) at 30 ºC after 24 h and only the ones showing florescence were selected.

As a first screening to select isolates belonging to the *B. subtilis* group and to exclude members of *B. cereus* a PCR with primers Bsub5F and Bsub3R was performed following Wattiau et al., (2001) methodology. Those primers are designed to amplify a fragment only in the case of *B. subtilis* group. DNA was extracted through a simple extraction by boiling the microbial solutions and immediately putting them in ice. The quality and concentration of DNA were determined on a Nanodrop spectrophotometer. The protocol for the PCR was: initial denaturation set at 95ºC for 2 min, 30 cycles of denaturation at 95ºC for 30 s, annealing at 59ºC for 30 s and extension at 72ºC for 60 s, followed by a final elongation step at 72ºC for 5 min. The PCR products were observed in an agarose gel after electrophoresis. To confirm our results the selected strains were also sent to the Laboratory of Instrumental Techniques at University of León where they were classified taxonomically by either Matrix-assisted Laser Desorption/Ionization (MALDI-TOF) analysis or sequencing of the 16S gene and 13 strains were selected from 22 in total.

Each microorganism of the collection was grown in triplicate in liquid growth media NA for *P. fluorescens* and LB (Tryptone 10 g L^{-1}, yeast extract 5 g L^{-1}, NaCl 5 g L^{-1}) for *Bacillus spp.* in a rotatory shaker (150 rpm) at 30ºC for 24h. After that time, each aliquot was measured spectrophotometrically at 600 nm and serial dilutions were made (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} in sterile saline solution (NaCl 9 g L^{-1}) and plated in solid media NA and LB (same described above

with agar 15 g L^{-1}) for 24h at 30ºC, then colonies were counted. The correlation between the microorganism concentration and the absorbance at 600 nm, was calculated.

2. Assessment of biochemical capacities *in vitro*

2.1 Idole-3-acetic acid determination

The presence of IAA-like substances was detected and quantified following the method of Gordon & Weber (1951) in L-tryptophan liquid medium. *P. fluorescens* stains were grown in NA solid plates for 48 h and *B. subtilis* were grown in LB plates for 24 h at 30ºC. Flasks containing 5 mL of liquid growth medium (NA for *P. fluorescens,* LB for *B. subtilis)* enriched with 0.1% (m v^{-1}) with L-tryptophan and inoculated with one full loop of each isolate in triplicate and incubated in a rotatory shaker (150 rpm) at 30ºC for 24 h. Then 1.5 mL of the liquid culture was centrifuged at 10000 x g for 5 min. IAA-like substances in the supernatant were determined by the freshly prepared Salkowski reagent (Gang et al., 2020) and incubated in the dark for 30 min for development of pink color. Each aliquot was measured spectrophotometrically at 530 nm using IAA as standard.

The amount of bacterial protein was quantified from the inoculated liquid growth medium. Proteins were quantified using Bradford reagent (Bradford, 1976) with bovine serum albumin as standard. The absorbance was measured at 595 nm. The quantity of indole acetic acid was expressed as mg mg^{-1} protein. To determine the auxin production peak and growth rate it was performed the same procedure as it is described above after 24 h, 48 h, 72 h, and 96 h.

2.2 Siderophore production

The siderophore production was performed using a modified technique of the Chrome Azurol Sulfate (CAS) composed of Chrome Azurol S 60.5 mg L^{-1}, Hexadecyltrimethylammonium bromide (HDTMA) 72.9 mg L^{-1}, $FeCl_3 \cdot 6H_2O$ 10 mg L^{-1}, HCl 10 mL L^{-1}, PIPES buffer 30.24 g L^{-1} and Agar 15 g L^{-1}. CAS medium was prepared in Petri dishes as described by (Neilands, 1987). In this method, the Petri plates were divided into two sections when solidified: one half with CAS medium and the other half with LB or NA growth medium to avoid the toxicity of HDTMA that affects *Bacillus spp.* Each medium was placed next to the other avoiding any space between them.

Microorganisms were placed in triplicate in the growth medium as far as possible form de borderline between the two media. Bacteria were incubated in the dark at 30ºC for 7 days. Microorganisms capable of producing siderophores caused a colour swerve in CAS medium from blue to orange, and then the length between the colony border and the colour swerve in CAS was measured.

2.3 Phosphorus solubilizing capacity

The isolated strains were cultivated in different liquid media enriched with four different P sources in order to examine de capacity of each microorganism to mobilize phosphate. *Bacillus spp.* strains and *P. fluorescens* strains were cultivated in National Botanical Research Institute's Phosphate (NBRIP) composed of glucose 10 g L^{-1}, $MgCl_2.6H_2O$ 5 g L^{-1}, $MgCl_2.7H_2O$ 0.25 g L^{-1}, KCl 0.2 g L^{-1}, $(NH_4)_2SO_4$ 0.1 g L^{-1} and adjusted to pH of 7 (Nautiyal, 1999).

Each media was enriched with four different sources of P corresponding to 4 different treatments. P was applied at 0.23 g L^{-1} as either monopotassium phosphate (KH_2PO_4), hydroxyapatite, phytate and iron phosphate (III). *Bacillus spp.* and *P. fluorescens* strains were then inoculated at the initial concentration of 10^8 CFU mL^{-1} per tube in triplicate and placed in a rotatory shaker (150 rpm) at 30ºC. After 7 days of the inoculation, the media were passed through a cellulose nitrate filter of 0.22 µm pore size and the molybdate reactive P was determined according to Murphy & Riley (1962). A solution of KH_2PO_4 (1.15 mg L^{-1}) was used as a standard and the absorbance was measured at 882 nm.

2.4 Quantification of organic acids production

B. subtilis strains B7 and B17 were cultured by quadruplicate in liquid NBRIP media containing hydroxyapatite at a concentration of 0.23 g L^{-1}. Inoculation was performed by introducing one full loop of bacteria into a tube with 15 mL of NBRIP and placing it on a rotatory shaker (150 rpm) at 30ºC. Additionally, a control (NBRIP with insoluble phosphate) without inoculation was included in quadruplicate for this experiment.

After 7 days, a 2 mL sample was extracted to determine the organic acids, the remaining aliquot was used to assess the concentration of microorganisms in each tube and their pH. The 2 mL samples were passed through a cellulose nitrate filter with a pore size of 0.45 µm and then used to quantify citric acid, malic acid, acetic acid, succinic acid, gluconic acid, lactic acid, oxalic acid, fumaric acid, propionic acid, and glycolic acid by Furlani et al., (2006) methodology. By injecting 100 µL of filtered samples in the HPLC with an Aminex HPX-87H column (300 x 7.8) from BioRad and detected with a Diode Array Detector DAD (UV) fixed at 210 nm. The eluent used was H_2SO_4 0.01 M, with a flow rate of 0.8 mL min^{-1} at a temperature of 60ºC. Organic acid peaks were determined based on the characteristic spectra of standards and calibration curves were built by injection of known concentrations of pure standards.

3. Assessment of plant growth-promoting capacity tested in vivo

3.1 Improvement in germination

The role of *B. subtilis* (B7 and B17) on the improvement of germination was evaluated using seeds of *Solanum lycopersicum* cv "Roma" (tomato). To prepare the microorganism inoculum, a fully colonized Petri dish was used to inoculate 300 mL of LB Lennox medium in a 500 mL bottle. The glass bottles were placed in a rotatory shaker at 150 rpm with a temperature of 30ºC for 72 h. the bacterial concentration was measured by spectrophotometry at 600 nm and compared with a calibration curve previously calculated. The aliquots were then centrifuged at 4000 g for 10 min to get rid of the growing media and resuspended to a final concentration of 10^8 CFU mL^{-1} with saline solution (9 g NaCl L^{-1}). The microorganism were prepared on the same way for all experiments in this chapter (section 3.2 and 3.3), that is for media, incubation time and evaluation of spectrophotometry concentration.

The setup of the experiment was performed in the greenhouse of the Faculty of Biology of the Universitat de Barcelona (41°23'06.4"N 2°07'12.8"E) being a Latin square of 4 factors corresponding to non-inoculated substrate (control), inoculated with strain B7, inoculated with strain B17 and the combination of both, with 4 replicates per each. The replicates corresponded to 4 aluminium trays with 500 g of plant growth substrate inoculated and 20 tomato seeds. Inoculation of the substrate was performed with 50 mL of each inoculum for 500 mL substrate to a final concentration of 10^7 CFU mL^{-1} of substrate. The trays were watered every other day with 100 mL of tap water per each tray.

The plant growth substrate used in this study was a Jiffy peat composed of a mixture of peat moss and black peat (Jiffy GO M8) with a pH of 5.5 and EC of 0.5 dS m^{-1} with a basal nutrient content (160-200 mg L^{-1} of N, 180-280 mg L^{-1} of P_2O_5, 200-350 mg L^{-1} of K_2O, 80-150 mg L^{-1} of Mg and 1.5 g L^{-1} of KCl) and prehydrated with 100 mL of distilled water per litter. The substrate and the hydration were used for all germination experiments but also for growth promotion assays described in the next section 3.2.

The same experiment was performed three times (A, B and C) following the exact same procedure explained above, each of the experiments lasted one week. (A) Performed from the 14th to the 22ond of March 2022 with temperature average of 17.03 ± 3.61 ºC and humidity 65.48 ± 12% RH. (B) Performed from the 9th to the 16th of May 2022 with temperature average of 24.24 ± 6.29 ºC and humidity of 47.38 ± 15% RH. (C) Performed from the 29th of June until the 6th of July 2022 with temperature average of 26.87 ± 4.13 ºC and humidity of 57.14 ± 13.29% RH.

At sampling point, the number of germinated plants and the development stage of the seedlings were tracked every day. The development stages were set according to BBCH Monograph (Meier, 2001), being defined as 007 when the hypocotyl with cotyledons was breaking through the seed coat, 009 when the seedling was emerging, and the cotyledons were breaking through the soil surface and 100 when the cotyledons were totally unfolded. The plant was considered germinated when reached stage 009 and 100 when the cotyledons were observed, and plants were pulled out once half of the seeds were at a development stage of 101: the first true leaf on the main shoot was completely unfolded. The measurements performed were the height, the fresh weight for each development stage and treatment and the total fresh weight.

The parameters calculated were related to seed quality traits as percentage of germination, time to reach the 50% of germination, seed vigour, and seedling establishment. The seed vigour was calculated as the percentage of germination multiplied by the height of the seedling. The seed establishment was calculated as the number of seedlings divided by the number of seeds sown per cent, and the seedling weight was calculated with a precision scale. In all cases, a seedling corresponded to plants at a development stage of 101.

3.2 Plant growth promotion capacity

The microorganisms' inoculum were prepared, according to descriptions mentioned on point 3.1, using the media previously mentioned, incubation time and cell concentrations. As well as the same plant growth substrate (Jiffy GO M8).

Three experiments (i, ii and iii) were performed to analyse the effect of inoculation in plant growth promotion all conducted in the greenhouse of the Faculty of Biology in the Universitat de Barcelona. The setup of the experiments was performed as following:

(i) The first experiment was performed with the leading producers of IAA in various crops and was composed of four treatments corresponding to non-inoculated substrate and substrate inoculated with *P. fluorescens* (P10) and two *B. subtilis* (B7 and B17) at a final concentration of 10^7 CFU mL^{-1} of substrate. It was used five different plants species: native *Zea Mays* cv "Tía María" (corn), *Helianthus annuus* cv "Russian Giant" (sunflower), *Cucumis sativus* cv "País" (cucumber), *Lactuca sativa* cv "Morella" (lettuce), and *Glycine max* cv Azurra (soya) non-treated ecological seeds from Les Refardes SCLL (Mura, Spain). The plants were seeded in trays with 16 seeds per treatment and plant species, each with 250 mL of substrate, randomly distributed into two blocks with 8 plants per each, trays were rotated every two days to alleviate the environmental positional effect, and plants were watered every other day with tap water with a volume of 250 mL per tray. The experiment lasted 3

weeks (15th of June until the 6th of July 2022) within the average temperature 26.02 ± 3.83 ºC and average relative humidity 63.94 ± 13.89% RH.

(ii) In this experiment it was assess the second-best IAA producers in maize plants (*Zea Mays* cv "Tía María"). The *experiment* design was composed of eight treatments corresponding to non-inoculated substrate, substrate inoculated with *P. fluorescens* (P2a and P7) and *B. subtilis* (B3, B7, B9 and B12) at a final concentration of 10^7 CFU mL^{-1} of substrate. Another treatment was added: *Trichoderma asperellum* (T34) from the commercial formulation T34 Biocontrol® (Biocontrol Technologies S.L) at the concentration of 10^4 CFU L^{-1} of substrate. The plants were seeded in trays with 12 seeds per treatment and plant species, each with 250 mL of substrate, randomly distributed into two blocks with 6 plants per each, the trays were rotated every two days to alleviate the environmental positional effect. Plants were watered every other day with tap water with a volume of 10 mL per plant. The experiment lasted 3 weeks (6th until the 20th of September 2022) within the average temperature 26.71 ± 3.72 ºC and average relative humidity 63.65 ± 13.28% RH.

(iii) In this experiment it was assessed the growth promotion effect of *B. subtilis* strain B7 in four different maize cultivars and two-inoculum concentrations (10^7 and 10^8 CFU mL^{-1}). The experiment design was composed of three different treatments corresponding to non-inoculated substrate and substrate inoculated with *B. subtilis* strain B7 with four different maize cultivars, "Hatay" (Fitó S.L), "Palomero" (Batlle S.A), "Tía María" and "Cruz" (Les Refardes S.L). Twelve seeds were sown for treatment and B7 concentration in pots of 125 mL and placed in a random distribution; also, plants were rotated every two days to alleviate the environmental positional effect. Plants were watered every other day with tap water with a volume of 10 mL per plant. The experiment lasted 18 days (16th of June until the 4th of July 2022) within the average temperature 27.4 ± 4.07 ºC and average relative humidity 59.14 ± 15.08 % RH.

The same growth measurements, including plant length and fresh and dry shoot weight, were conducted in all three experiments. However, root weight measurements were only performed in the first experiment. The plant length was measured from the plant base to the tip of the last leaf and the stem length from the base to the first node. The shoot fresh weight was measured at harvest and the shoot dry weight, after plants were left at 90ºC for 72 h. To measure root fresh and dry weight the roots were meticulously washed 3 times, the excess of moisture was removed, and roots were weighed for fresh weight and the dry root weight was performed after drying at 90ºC for 72 h.

3.3 Effect of inoculation on phosphorus solubilization and plant uptake

Microorganisms' inoculums were prepared on the same way as described above in section 3.1 with a final concentration of the inoculum 10^8 CFU mL^{-1}. Although in this assay, the inoculation was performed directly to the plant and not mixed with the substrate.

In these experiments the substrate used was a mix of coconut fibre and perlite (2:1 v:v) mixed with 100 mg L^{-1} of insoluble rock phosphate (Fertiagro S.L). The substrate was fertilized with 100 mL per litre of the substrate with a complete Hoagland solution for non-inoculated plants corresponding to the positive control treatment (C+) and a P-free Hoagland solution for non-inoculated plants as negative control and inoculated plants. The complete Hoagland solution was prepared according to Hoagland & Arnon (1950) and the P-free Hoagland solution was prepared with KCl instead of KH_2PO. In this case, seeds were surface sterilized by soaking them in ethanol 70% for 3 min and rinsing them with distilled water for 6 times. Then the substrate was placed in plastic pots of 1L and two seeds per pot were sown to guarantee that at least one germinates then were distributed randomised on the greenhouse table, and temperature and humidity were monitored. After 2 weeks, plants were inoculated adding 1 ml of each microorganism aliquot by adding it to the base of the plant with an automatic pipette. For control treatments (C+ and C-), 1 mL of saline solution was added instead of microorganisms. Plants were watered with 40 mL of tap water twice a week and with 40 ml Hoagland solutions with and without P once a week. The measurements performed in the plants were the same as in section 3.2 trials, as described above.

Three experiments (I, II and III) were performed to analyse the effect of inoculation on phosphorus solubilization and plant uptake, all were conducted in the greenhouse of the Faculty of Biology in the Universitat de Barcelona. The setup of the experiments was performed as following:

(I) To assess the effect of inoculation by the leading phosphorus solubilizers in various crops, the experiment design was composed of six treatments corresponding to non-inoculated (C+ and C-), *P. fluorescens* (P2b)*, B. subtilis* (B7, B17) and *B. megaterium* (MB18) at a final concentration of 10^8 CFU mL^{-1}, and five different plants species: maize, sunflower, lettuce, cucumber, and soya, the same species as defined in section 3.2 (experiment i). For each treatment and plant species, 10 pre-sterilized seeds were sown and 5 were left for inoculation. The experiment lasted 6 weeks (5^{th} of July 2021 to the 16^{th} of August 2021), when the lack of phosphorus was observed. The average temperature was 27 ± 4 ºC and the relative humidity was 67.47 ± 13%. In this experiment macronutrients (Ca, Fe, K, Mg, P, S and Si) content in plants were analysed for the whole plant. Plants were grinded to obtain plant particles ≤ 2 mm, then a representative subsample of 100 mg was further grinded with the help of 3 stainless steel balls in a rotor ball mill (Mixer Mill MM 400) for 3 min at 1500 oscillations min-

[1]. A 45 mg of sample was attacked overnight in Teflon reactors with 1 ml HNO_3 and 0.5 ml H_2O_2 at 90 ºC and analysed by an inductively coupled plasma optical emission spectrometry (ICP-OES), using a Perkin–Elmer apparatus, model Optima-3200RL.

(II) To confirm the effect of phosphorus solubilization and phosphorus uptake on maize (*Zea mays* cv "Tía María") plants by the inoculation of *B. subtilis* B7 and B17 strains an experiment design was composed of four treatments corresponding to non-inoculated plants (C+ and C-) and plants inoculated by two *B. subtilis* at a final concentration of 10^8 CFU mL^{-1}. For each treatment, 20 pre-sterilized seeds were sown and then 10 plants were left for inoculation. The experiment lasted 7 weeks (11^{th} of October 2021 to the 29^{th} of November 2022), within the average temperature 17 ± 5 ºC and relative humidity 63 ± 17% RH.

(III) To assess the effect of inoculation of strains B7 and B17 on radish, the experiment design was composed of four treatments corresponding to non-inoculated plants (C+ and C-) and plants inoculated by two *B. subtilis* at a final concentration of 10^8 CFU mL^{-1}. For each treatment, 24 pre-sterilized seeds were sown and 12 plants of *Raphanus sativus* cv. Rabanito (Fitó SL) were inoculated and grown. In this case, plants were pregerminated in the greenhouse and after a week were transplanted in pots of 0.5 mL, inoculated and left in a growing chamber. The experiment lasted 5 weeks (2^{nd} of May 2022 until the 9^{th} of June 2022), plants grew under constant controlled environmental conditions, with a photoperiod of 14 h at 23ºC / 25ºC day/ night temperature, 70% RH, and 22 W m^{-2} light intensity.

4. Statistical analysis

All data was analysed with the SPSS software package version 27.0. For the assessment of biochemical capacities *in vitro*, analysis of variance (one-way ANOVA) and the post- hoc Tukey's test were used with a significance level of 95% for each parameter analysed to assess differences between isolated strains. In molybdate reactive phosphate content also a more general statistics was performed by a two-way ANOVA (isolated strains and phosphorus source as fixed factors) and post hoc Tukey's test to determine differences between insoluble phosphorus sources with a significance level of 95%.

For the experiments in assessment of plant growth-promoting capacity tested *in vivo* Dunnett's test was performed making the comparison of the different inoculation treatments with the non-inoculated control plants and, using a significance level of 95%.

Results

1. Isolation and analysis of soil samples

Table 1 provides a comprehensive characterization of the soils used for the isolation of *Bacillus subtilis.* and *Pseudomonas fluorescens*. The selected soils were evaluated based on reference values from Chapman & Pratt (1973). In terms of pH levels, the soils were identified as alkaline with a pH range: 7.6- 8.5, except for Compost being categorized as very alkaline (9.1 pH value). For electrical conductivity (EC), the soils were generally non-saline (reference range: 0- 2 dS m^{-1}), except for Compost, which was considered slightly saline (2- 4 dS m^{-1}). Regarding oxidable organic matter (OM), all soil types were deemed suitable for intensive irrigated crops like maize (reference range: 2.0- 3.2% OM), while Soil C and the compost were regarded as excessive (> 4.5% OM). When examining calcium carbonate equivalent content ($CaCO_3$), Soil C exhibited low values (< 5% $CaCO_3$), Soil A and Compost fell within the normal range (5- 25% $CaCO_3$), and Soil B and D showed high carbonate levels (> 25% $CaCO_3$). For nitrate content ($N\text{-}NO_3$), none of the soils were within the appropriate range (20- 25 mg kg^{-1}), Soils B and D, along with Compost, had low nitrate content (< 20 mg kg^{-1}), whereas Soil A and Soil C showed very high values (> 25 mg kg^{-1}).

Table 1 Characterization of soil samples for nitrogen-fixing bacteria isolation

Soil Type	pH	EC[a] (dS m^{-1})	OM[a] (%)	$CaCO_3$[a] (%)	$N\text{-}NO_3$[a] (mg kg^{-1})	Texture
Soil A	7.90	0.461	2.98	8	28	Loam-Clay-Sandy
Soil B	7.93	0.427	2.50	34	7	Loam- Sandy
Soil C	7.13	0.530	4.60	< 5	69	Loam-Clay-Sandy
Soil D	8.00	0.528	2.78	26	14	Loam-Clay-Sandy
Compost	9.10	3.0	6.3	7	18	-

[a] Parameters analysed meaning; EC to electrical conductivity, OM to oxidable organic matter, $CaCO_3$ to calcium carbonate equivalent and $N\text{-}NO_3$ to nitrate content

The majority of isolated *B. subtilis* strains were found in Compost (69%), with a few from different soil types, specifically Soils A, B, and D, with most strains being found in bulk soil (85%) except for strain B20 and B21, which were isolated from maize rhizosphere (Table 2). For *P. fluorescens* slightly more than half of the isolated strains were found in Soil B (54%), with the remainder in Compost (31%) and Soil A (15%), being equally found between bulk soil and the maize and wheat rhizosphere (Table 2). No strains were isolated from Soil C (Table 2).

Table 2 Comprehensive list of all microbial isolates included in this chapter, with soil type and their sources and associated plant species.

Pseudomonas florescence			*Bacillus subtilis*			*Bacillus megaterium*	
ID[a]	Soil Type[b]	Isolation site	ID[a]	Soil Type[b]	Isolation site	ID[a]	Collection[c]
P1	Compost	Bulk soil	B1	Compost	Bulk soil	MB2	Biocontrol
P2a	Compost	Bulk soil	B3	Compost	Bulk soil	MB10	Biocontrol
P2b	Compost	Bulk soil	B4b	Compost	Bulk soil	MB16	Biocontrol
P2c	Compost	Bulk soil	B5	Compost	Bulk soil	MB17	Biocontrol
P3	Soil B	Bulk soil	B7	Compost	Bulk soil	MB18	Biocontrol
P4a	Soil A	Bulk soil	B9	Compost	Bulk soil	MB19	Biocontrol
P4b	Soil A	Bulk soil	B10	Compost	Bulk soil		
P5	Soil B	Rhizosphere (wheat)	B12	Soil A	Bulk soil		
P6	Soil B	Rhizosphere (maize)	B13	Soil A	Bulk soil		
P7	Soil B	Rhizosphere (maize)	B16	Compost	Bulk soil		
P8	Soil B	Rhizosphere (maize)	B17	Compost	Bulk soil		
P10	Soil B	Rhizosphere (maize)	B20	Soil D	Rhizosphere (maize)		
P20	Soil B	Rhizosphere (maize)	B21	Soil B	Rhizosphere (maize)		

[a] Code for the identification of each of the isolated strains

[b] Soil type from which the strain was isolated being Soil A, B, C and D and Compost

[c] Isolates from Biocontrol Technologies S.L Collection

2. Assessment of biochemical capacities in vitro of the isolated strains

2.1 Indole-3-acetic acid, siderophores production and phosphorus solubilizing capacity

Table 3 shows that *B. subtilis* strains B5 and B7, followed by B17, as well as the *P. fluorescens* strains P10 and P7, yielded promising outcomes as IAA producers. Among the *Bacillus strains*, B5 stood out by demonstrating a mean production of 34.5 µg mL^{-1}. However, the *Pseudomonas* strains P7 and P10 exhibited the highest levels of production, ranging between 46 µg mL^{-1} and 61 µg mL^{-1}, respectively, after the incubation period.

Table 3 *In vitro* evaluation of indole-3-acetic acid production, phosphate solubilizing capacity and siderophores production of *Bacillus spp.* and *P. fluorescens.*

ID[a]	Indole-3-acetic acid (µg mg protein^{-1})	Phosphate solubilizing capacity (µg molybdate reactive phosphorus mL^{-1})			Siderophores (cm colony halo)	
		Hydroxyapatite	Phytate	Iron Phosphate (III)	5 DAI	10 DAI
B3	10.34 ± 2.9 abc	10.95 ± 1.4 ab β	0.00 ± 0.1 a α	0.38 ± 1.3 ab α	nd	nd
B4b	9.60 ± 3.1 ab	10.71 ± 1.1 ab β	0.00 ± 0 a α	0.72 ± 1.4 abc α	nd	nd
B5	**34.50 ± 1.2 c**	7.35 ± 1.1 ab β	**0.12 ± 1.2 b** α	0.80 ± 1.5 abc α	nd	nd
B7	**30.63 ± 3.5 c**	16.29 ± 2.1 ab β	0.00 ± 0.3 a α	0.31 ± 1.3 ab α	0.23 ± 0.06 a	0.25 ± 0.08 a
B9	11.94 ± 1.4 abc	5.45 ± 10.4ab β	0.00 ± 0.1 a α	**0.90 ± 1.5 bc** α	nd	nd
B10	9.08 ± 2.1 ab	nd	nd	nd	nd	nd
B12	9.84 ± 0.6 abc	**16.93 ± 7.6 b** β	0.00 ± 0.2 a α	0.62 ± 1.4 abc α	0.17 ± 0.03 a	0.45 ± 0.05 ab
B13	0.67 ± 0.2 a	0.00 ± 0.4 a β	0.00 ± 0.1 a α	0.45 ± 1.4 ab α	nd	nd
B16	3.48 ± 0.1 ab	6.60 ± 2.9 ab β	0.00 ± 0.8 a α	0.11 ± 1.2 a α	nd	nd
B17	**21.45 ± 2.1 bcd**	13.99 ± 1.7 ab β	0.00 ± 0.2 a α	0.28 ± 1.3 ab α	0.2 ± 0.08 a	0.45 ± 0.12 ab
B20	nd[a]	14.31 ± 1.8 ab β	0.00 ± 0.1 a α	0.51 ± 1.4 ab α	0.2 ± 0 a	0.48 ± 0.04 ab
MB2	nd	1.18 ± 1.7 a β	0.00 ± 1.2 a α	0.55 ± 1.4 b α	nd	nd
MB10	nd	15.77 ± 1.1 b β	**0.01 ± 1.2 ab** α	0.05 ± 1.2 b α	nd	nd
MB16	nd	16.05 ± 0.9 b β	0.00 ± 0.1 a α	0.00 ± 0 a α	nd	nd
MB17	nd	**22.43** ± 1.5 **bc** β	0.00 ± 0.8 a α	0.20 ± 1.3 b α	nd	nd
MB18	nd	**33.08** ± 5.9 **c** β	0.00 ± 0.8 a α	**5.08 ± 2.9 c** α	nd	**0.98 ± 0.07 c**
MB19	nd	19.95 ± 0.6 b β	0.00 ± 0.7 a α	0.81 ± 1.5 b α	nd	**0.85 ± 0.12 bc**
P1	5.07 ± 1.1 a	14.16 ± 5.5 abc δ	0.00 ± 0.6 a β	0.00 ± 0.1 a α	0.2 ± 0.0 a	0.78 ± 0.14 a
P2a	10.94 ± 1.6 a	**23.48 ± 3.5 cd** δ	0.00 ± 0.1 a β	0.00 ± 1.3 a α	0.5 ± 0.0 a	0.83 ± 0.02 a
P2b	4.11 ± 0.5 a	**21.61 ± 1.4 cd** δ	0.00 ± 0.4 a β	0.00 ± 0.2 a α	1.1 ± 0.12 a	**1.93 ± 0.03 b**
P2c	5.88 ± 0.6 a	17.23 ± 5.2 bcd δ	0.00 ± 0.7 a β	0.00 ± 0.1 a α	nd	nd
P3	4.64 ± 0.3 a	8.54 ± 2.3 abc δ	0.00 ± 0.7 a β	0.00 ± 0.4 a α	nd	nd
P4a	3.52 ± 1.4 a	8.62 ± 2.3 abc δ	0.00 ± 0 a β	0.00 ± 0.2 a α	nd	nd
P4b	10.20 ± 1.0 a	**33.51 ± 2.9 d** δ	0.00 ± 0.2 a β	0.00 ± 2.3 a α	0.1 ± 0.0 a	0.88 ± 0.43 a
P5	6.80 ± 1.0 a	0.35 ± 1.1 ab δ	0.00 ± 1.2 a β	0.00 ± 0.1 a α	nd	nd
P6	nd	6.85 ± 0.7 abc δ	0.00 ± 0.1 a β	0.00 ± 0.1 a α	nd	nd
P7	**46.53 ± 0.4 ab**	0.00 ± 0.6 a δ	0.01 ± 1.3 a β	0.00 ± 0.1 a α	nd	nd
P8	11.20 ± 0.6 a	7.05 ± 3.7 abc δ	0.00 ± 0.1 a β	0.00 ± 0.2 a α	nd	nd
P10	**61.41 ± 5.6 b**	6.59 ± 1.3 abc δ	0.00 ± 0.1 a β	0.00 ± 0.2 a α	nd	nd
P20	0.88 ± 0.8 a	15.81 ± 7 abcd δ	0.00 ± 0.9 a β	0.00 ± 0.1 a α	nd	nd

Values are means ± SE (n= 3). Values marked with lowcase letter are significantly different between isolates (ID) and values marked with a greek letter are significant different between phosphorus sources determined through a two-way ANOVA and Tukey's analysis test (with p-value < 0.05). [a] Non-determined (nd.)

The response of phosphorus solubilization by the microorganisms also varied depending on the source of insoluble phosphorus, higher solubilization values were observed in the hydroxyapatite-enriched NBRIP medium compared to the phytate and iron phosphate-enriched media (Table 3). In terms of hydroxyapatite solubilizing capacity stand out *B. megaterium* strains MB18 and MB17 and *B. subtilis* strain B12 which exhibited notable phosphorus solubilizing capacity after 7 days, showcasing an increment of molybdate reactive phosphorus of 14%, 9.7% and 8.6% respectively. Among phytate solubilizing capacity of the strains, the highest values were observed in strains B5 and MB10 with increments of 0.05% and 0.004%, and in iron phosphate (III) higher values were obtained with MB18 and B9 strains by increasing a 2% and 0.3% molybdite reactive phosphorus, respectively. In contrast, for *P. fluorescens* strains, no significant differences were noted in phosphorus solubilization nor in phytate or iron phosphate (III) enriched NBRIP medium, although in hydroxyapatite-enriched media a significant higher solubilization was observed in some strains such P4b, P2a and P2b sowing a 14%, 10% and 9% increment of molybdate reactive P, compared to others.

Regarding siderophore production values *Bacillus* spp. strains MB18 and MB19 did not exhibit apparent siderophore production effects within the initial 5 days, however, after 10 days, a noticeable halo appeared, with substantial diameter of 0.98 cm and 0.85 cm, respectively. As well as *P. fluorescens* strain P2b displayed halo colour change with 1.93 cm halo diameter after 10 days.

2.2 Rate of indole-3-acetic acid production by *B. subtilis* and *P. fluorescens* top producers

The peak of IAA production in *B. subtilis* strains B7 and B17 was observed at 72 hours, as indicated by the black dots in Figure 1. In contrast, the *P. fluorescens* represented strains did not show a well-defined peak, specifically strain P7, while strain P10 displayed a continuous increase in IAA production after 72 and 96 hours. Further time points were not sampled, so the exact timing of the production peak remains undetermined.

The progression of protein concentration identified in the medium is depicted in Figure 1, represented by white dots. The increase in protein follows a different pattern compared to IAA production. In *B. subtilis* strains, protein levels showed a consistent increase from 48 to 96 hours. For *P. fluorescens* strains, there was an increase in protein concentration after 48 hours (strain P10), followed by a stabilization over time. In cultures of *P. fluorescens* and *B. subtilis*, the IAA content typically ranged between 5 µg mL^{-1} and 20 µg mL^{-1}, but for P10 strain of *P. fluorescens* exhibited a remarkable increase in IAA content, from 20 µg mL^{-1} to 100 µg mL-1 after 96 hours. These results highlight the uniqueness and potential impact of the P10 strain on auxin production.

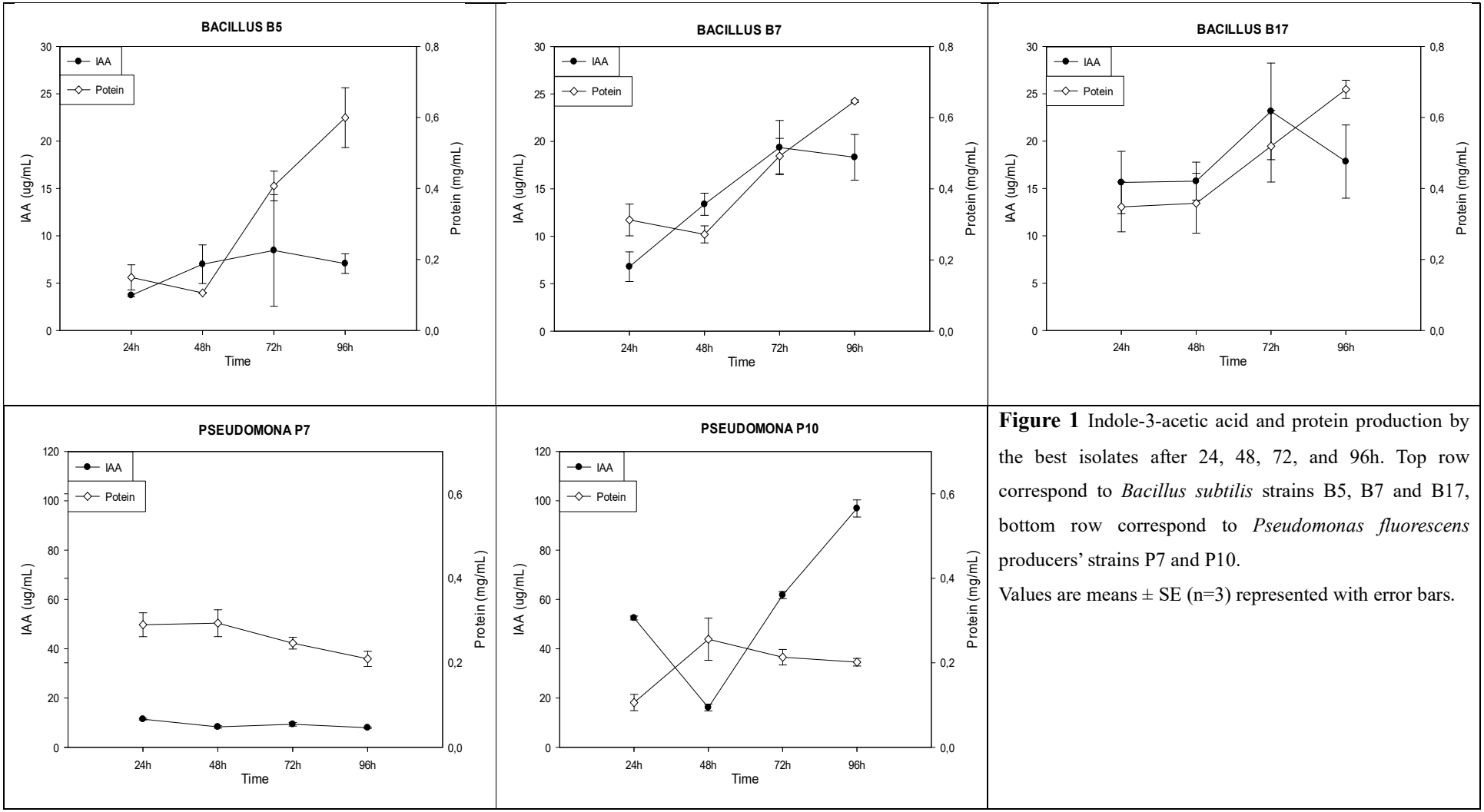

Figure 1 Indole-3-acetic acid and protein production by the best isolates after 24, 48, 72, and 96h. Top row correspond to *Bacillus subtilis* strains B5, B7 and B17, bottom row correspond to *Pseudomonas fluorescens* producers' strains P7 and P10.

Values are means ± SE (n=3) represented with error bars.

2.3 Phosphorus solubilizing capacity, organic acids and pH determination

B. subtilis strains B7 and B17 exhibited high levels of molybdate reactive phosphorus in the medium enriched with hydroxyapatite, along with a lower pH compared to non-inoculated medium. Specifically, in NBRIP media inoculated with strain B7 the pH decreased from 6.8 to 5.7 and in the medium inoculated with strain B17 it decreased to 5.9 (Table 4). Among the organic acids identified, malic acid was the most abundant, followed by gluconic and acetic acid in both strains. Additionally, fumaric acid was significantly more abundant in the medium inoculated by *B. subtilis* strain B7 compared to B17 (Table 4).

Table 4 Phosphorus solubilizing capacity of *B. subtilis* strains B7 and B17 and pH and organic acid content in National Botanical Research Institute's Phosphate medium enriched with hydroxyapatite

ID[a]	PSC[b]	pH[c]	Organic acid production (mg L^{-1})						
			Malic	Gluconic	Oxalic	Glucuronic	Fumaric	Acetic	Lactic
B7	16.29 ± 2.1	**5.7 ± 0.03 ***	26.36 ± 6	0.01 ± 0	3.09 ± 0.9	14.74 ± 2.1	**0.07 ± 0 ***	9.60 ± 0.9	8.27 ± 0
B17	13.99 ± 1.7	5.9 ± 0.05	26.03 ± 8	-	1.32 ± 0.3	5.28 ± 0.7	0.01 ± 0	9.26 ± 1	3.11 ± 7

Values are means ± SE (n= 4). Asterisks represent significant differences between B7 and B17 strains determined through a t-student test (p-vale < 0.05)

[a] ID microorganisms

[b] Phosphorus solubilizing capacity (PSC) by increment of molybdate reactive in the medium with units mg L^{-1}

[c] pH compared to non-inoculated medium pH (6.8)

3. Assessment of isolates' efficacy as plant growth-promoting rhizobacteria (PGPR) in plant

3.1 Improvement in germination

B. subtilis (B7) showed a significant increase in germination percentages of 11% and 17% in Experiments A and C, respectively, compared to non-inoculated plants (C). Similarly, *B. subtilis* (B17) exhibited higher germination percentages of 10% and 13% in Experiments B and C, respectively (Figure 2). Regarding seed quality traits, there were no discernible differences in the days to reach 50% seed germination between treatments. However, noteworthy variations were apparent across experiments; for instance, the germination period in Experiment A was comparatively longer, ranging from 4 days in Experiments B or C to over 7 days in Experiment A. The same pattern was observed in seed vigour values and seedling weights, with higher values recorded in Experiments B and C (Table 5). On the other hand, seedling establishment values exhibited consistency across both experiments and treatments, except for combination treatment at experiment A (lowest values). No significant improvement for any of the studied parameters and experiment were observed for the combination of B7 and B17 strains.

The most notable improvement in quality traits among the plants was observed in the seed vigour values, in this regard, *B. subtilis* strain B17 demonstrated significantly higher values compared to non-inoculated seeds (C), showing a remarkable increase of 60% in Experiment B (Table 5).

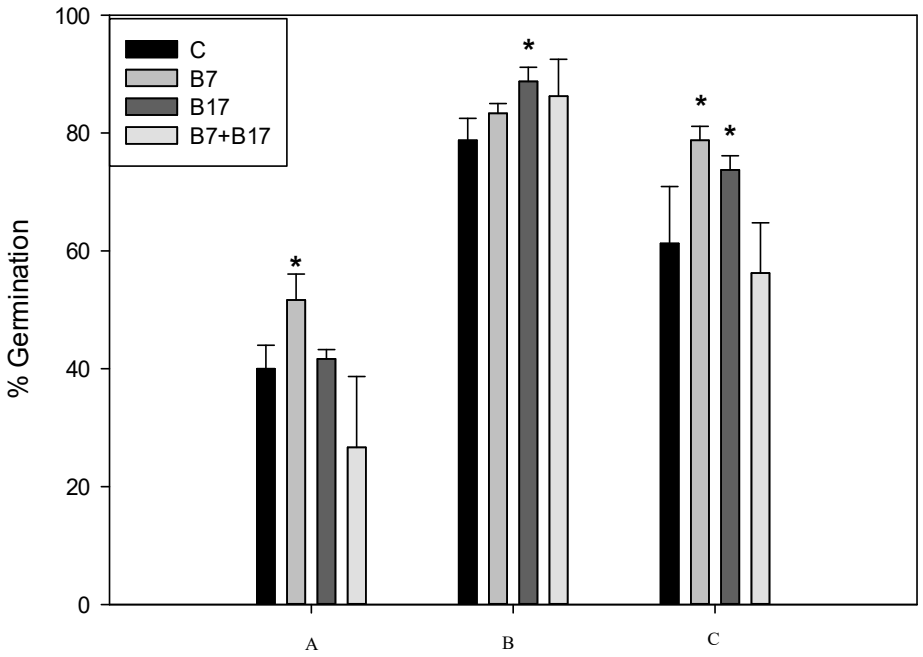

Figure 2: Effect of bacterial treatments on *Solanum lycopersicum* cv "Roma" seeds germination. Data is organized in tree experiments conducted A (March 2021), B (May 2021) and C (June 2021). In each set, black bars represent non- inoculated control plants, while the other bars indicate plants inoculated with *Bacillus subtilis* strains B7, B17 and the combination of both (B7+B17) at a concentration of 10^7 CFU mL^{-1} of substrate. Values are means ± SE (n=3) represented with error bars. Values marked with asterisks are significantly different compared to the control (p-value < 0.05), determined through Dunnett's analysis test (p-value < 0.05) between inoculated and non-inoculated plants (Control).

Table 5 Seed quality traits of *Solanum lycopersicum* cv. "Roma" as defined in the technical guide Bio-stimulants Regulation CEN/TS 17700-2

Exp[a]	Treatment[b]	Time 50% (days)	Seed vigour	Seedling establishment (%)	Seedling weight (g)
A	Control	>7	80.00 ± 5.2	56.67 ± 4.4	0.022 ± 0
	B7	>7	92.50 ± 10.4	51.67 ± 4.4	0.025 ± 0
	B17	>7	57.29 ± 11.9	41.67 ± 1.7	0.023 ± 0
	B7+17	>7	21.19 ± 11.8	28.33 ± 0.1	0.021 ± 0
B	C	4.25 ± 1	181.13 ± 17.1	60.00 ± 4.6	0.120 ± 0
	B7	6.25 ± 0.8	185.55 ± 38.9	42.50 ± 10.5	0.099 ± 0
	B17	3.25 ± 0.6	**286.98 ± 13.4 ***	68.75 ± 10.5	0.153 ± 0
	B7+17	3.50 ± 0.9	251.23 ± 28.3	67.50 ± 5.2	0.150 ± 0
C	C	4.25 ± 0.3	236.56 ± 24.3	52.50 ± 6.6	0.954 ± 0.1
	B7	4.00 ± 0	217.81 ± 25.6	57.50 ± 6	1.100 ± 0.2
	B17	4.00 ± 0	221.25 ± 25.6	58.75 ± 10.5	0.861 ± 0.1
	B7+17	4.75 ± 0.3	205.31 ± 37	51.25 ± 9.7	0.888 ± 0.2

Values represent means ± SE (n=3 blocks). Significantly different values are marked with an asterisk based on Dunnett's analysis test (p-value < 0.05) between inoculated and non-inoculated plants (Control) for each experiment (A, B and C)

[a] Data organized across three experiments (A - March 2021, B - May 2021, C - June 2021)

[b] Treatments are as follows: Control (no inoculation), *Bacillus subtilis* B7 and B17 strains at 10^7 CFU mL^{-1} substrate and the combination of both (B7+B17)

3.2 Growth promotion capacity

3.2.1 Growth promotion by the leading producers of indole-3 acetic acid in various crops

In cucumber plants, significant differences were observed in the aerial height of plants between the control and those treated with B7 and B17 (Table 6). Specifically, B17-treated plants displayed a remarkable 14.17% increase in shoot length, while B7-inoculated plants exhibited a 10.50% rise compared to the control. Moreover, plants treated with *B. subtilis* strain B17 demonstrated a higher shoot fresh weight with an increase of 21.7% compared to non-treated plants.

In maize plants, there were significant differences in shoot length between inoculated and non-inoculated plants (Table 6). All microbial inoculations (P10, B7, and B17) contributed to increased plant height. For shoot weight, significant differences were noted in B7 compared to the control plants, both in fresh and dry weight, with an increase of 31.55% in fresh weight and 15.30% in dry weight. However, no differences were observed in root fresh and dry weight.

In lettuce plants, significant differences between non-treated and inoculated plants were observed in plant length and shoot fresh and dry weight (Table 6). In particular, the inoculated lettuce plants

showed significant improvements in these parameters compared to the non-treated ones. Conversely, no notable differences were noted in root measurements.

Table 6 Effect of bacterial treatments on plant growth in *Zea mays* cv. "Tía María", *Helianthus annuus* cv "Russian Giant", *Glycine max* cv "Palafolls", *Cucumis sativus* cv "del país" and *Lacttuca sativa* cv "Morella."

Plant specie	Treatment[a]	Shoot length (cm)	Shoot fresh weight (g)	Shoot dry weight (g)	Root fresh weight (g)	Root dry weight (g)
Cucumber	Control	11.93 ± 0.3	1.38 ± 0.1	0.09 ± 0.0	0.77 ± 0.01	0.04 ± 0.003
	P10	12.62 ± 0.4	1.48 ± 0.1	0.10 ± 0.1	0.88 ± 0.01	0.04 ± 0.002
	B7	**13.33 ± 0.3 ***	1.55 ± 0.1	0.11 ± 0.1	0.88 ± 0.01	0.04 ± 0.004
	B17	**13.9 ± 0.3 ***	**1.68 ± 0.1 ***	0.11 ± 0.1	0.87 ± 0.01	0.04 ± 0.003
Maize	Control	29.26 ± 1.1	1.54 ± 0.1	0.13 ± 0.1	1.93 ± 0.01	0.16 ± 0.010
	P10	**34.53 ± 0.9 ***	**1.96 ± 0.1 ***	0.17 ± 0.1	2.06 ± 0.01	0.16 ± 0.008
	B7	**34.56 ± 1.2 ***	**2.20 ± 0.1 ***	**0.18 ± 0.1 ***	2.08 ± 0.01	0.16 ± 0.009
	B17	**33.92 ± 1.3 ***	**1.89 ± 0.1 ***	0.17 ± 0.1	1.80 ± 0.01	0.14 ± 0.011
Lettuce	Control	7.1 ± 0.3	0.46 ± 0.04	0.02 ± 0.02	0.17 ± 0.002	0.01 ± 0.002
	P10	**7.93 ± 0.2 ***	**0.67 ± 0.03 ***	**0.03 ± 0.02 ***	0.21 ± 0.002	0.02 ± 0.002
	B7	**8.58 ± 0.2 ***	**0.76 ± 0.04 ***	**0.03 ± 0.02 ***	0.20 ± 0.002	0.03 ± 0.014
	B17	**8.15 ± 0.2 ***	**0.68 ± 0.04 ***	**0.03 ± 0.02 ***	0.19 ± 0.003	0.01 ± 0.001

Values represent means ± SE (n=16). Significantly different values are marked with an asterisk based on Dunnett's analysis test (p-value < 0.05) between inoculated and non-inoculated plants (Control)

[a] Treatment as follows: non-inoculated (Control) and inoculated with *Pseudomonas fluorescens* (P10) and *Bacillus subtilis* (B7 and B17) at a concentration of 10^7 CFU mL^{-1} substrate.

3.2.2 Growth promotion of the second-best auxin producers in maize plants (*Zea mays* cv. Tía María)

Notable differences were observed in shoot fresh and dry weight between the control plants and those inoculated with *B.subtilis* (B7) (Table 7). Plants inoculated with B7 exhibited a 20% and 7.86% higher shoot fresh and dry weight, respectively, compared to the non-inoculated plants. No significant differences were observed in root biomass or plant height for all the other tested *B. subtilis* and *P. fluorescens* strains, as well as by the inoculation with *T. asperellum* T34.

Table 7 Effect on *Zea mays* cv. "Tía María" plant growth by the second-best auxin producers

Treatment[a]	Shoot lenght (cm)	Plant lenght (cm)	Shoot fresh weight (g)	Shoot dry weight (g)	Root fresh weight (g)	Root dry weight (g)
Control	9.44 ± 0.7	40.77 ± 1.5	2.08 ± 0.1	0.13 ± 0.01	1.86 ± 0.1	0.16 ± 0.02
P2a	9.36 ± 0.5	38.09 ± 1.3	2.16 ± 0.1	0.14 ± 0.01	1.83 ± 0.2	0.13 ± 0.01
P7	8,22 ± 0.7	33,88 ± 1.4	1.77 ± 0.1	0.11 ± 0.01	1.49 ± 0.1	0.14 ± 0.01
T34	8.8 ± 0.5	35.4 ± 1.4	1.87 ± 0.1	0.12 ± 0.01	1.94 ± 0.1	0.13 ± 0.01
B3	10.66 ± 0.4	39.44 ± 1.2	2.26 ± 0.1	0.15 ± 0.01	1.67 ± 0.1	0.11 ± 0.01
B7	10.25 ± 0.5	41.8 ± 1	**2.50 ± 0.2 ***	**0.18 ± 0.01 ***	2.02 ± 0.1	0.11 ± 0.01
B9	8.22 ± 0.7	33.88 ± 1.4	1.77 ± 0.1	0.11 ± 0.01	1.49 ± 0.1	0.14 ± 0.01
B12	9.95 ± 0.4	40.5 ± 0.7	2.28 ± 0.1	0.15 ± 0.01	2.11 ± 0.1	0.14 ± 0.01

Values represent means ± SE (n=12). Significantly different values are marked with an asterisk based on Dunnett's analysis test (p-value < 0.05) between inoculated and non-inoculated plants (Control)

[a] Treatment as follows; non-inoculated (Control) and inoculated with *Bacillus subtilis* (B3, B7, B9 and B12), with *Pseudomonas fluorescens* (P2a and P7) at a concentration of 10^7 CFU mL^{-1} substrate and with *Trichoderma asperellum* (T34) at a concentration of 10^4 CFU mL^{-1}

3.2.3 Growth effect of *B. subtilis* strain B7 in four different maize cultivars

In the "Hatay" maize cultivar differences in plant length were noted after 18 days between non-inoculated (control) and *B. subtilis* B7-inoculated plants, particularly at a concentration of $1 \cdot 10^7$ CFU mL^{-1}, resulting in a 7.6% increase. Similarly, for "Palomero" maize cultivar, significant differences were observed in the shoot fresh weight by inoculation of strain B7 at a concentration of $1 \cdot 10^7$ CFU mL^{-1}, leading to a 17.5% increase (Table 8). For the "Tía María" cultivar, differences were observed in both fresh and dry shoot biomass between untreated plants and those treated with the highest concentration of *B. subtilis* ($1.55 \cdot 10^8$ CFU mL^{-1}). In the "Cruz" maize cultivar, there was a 10% increase in shoot dry weight at a B7 concentration of 10^7 CFU mL^{-1} (Table 8).

Table 8 Effect of inoculation of *Bacillus subtilis* strain B7 in different *Zea mays* varieties

Cultivar [a]	Treatment [b]	Plant lengh (cm)	Shoot fresh weight (g)	Shoot dry weight (g)
Hatay	Control	3.37 ± 0.1	0.54 ± 0.05	0.03 ± 0
	B7 10^7	**3.95 ± 0.2 ***	0.57 ± 0.05	0.04 ± 0
	B7 10^8	3.54 ± 0.2	0.52 ± 0.05	0.04 ± 0
Palomero	Control	2.77 ± 0.1	0.4 ± 0.02	0.04 ± 0
	B7 10^7	2.95 ± 0.1	**0.47 ± 0.04 ***	0.03 ± 0
	B7 10^8	3 ± 0.2	0.44 ± 0.04	0.03 ± 0
Tía María	Control	9.86 ± 0.3	2.31 ± 0.09	0.24 ± 0.01
	B7 10^7	10.08 ± 0.3	2.2 ± 0.15	0.22 ± 0.01
	B7 10^8	9.83 ± 0.3	**2.39 ± 0.09 ***	**0.28 ± 0.01 ***
Cruz	Control	11.08 ± 0.4	2.22 ± 0.22	0.23 ± 0.02
	B7 10^7	10.04 ± 0.7	2.27 ± 0.16	**0.25 ± 0.01 ***
	B7 10^8	10.61 ± 0.4	2.22 ± 0.13	0.21 ± 0.01

Values represent means ± SE (n=12). Significantly different values are marked with an asterisk based on Dunnett's analysis test (p-value < 0.05) between the two B7 concentrations inoculum and Control plants.
[a] Different maize cultivars being "Hatay," "Palomero," "Tía María," and "Cruz".
[b] Treatment as follows; non-inoculated (Control) and inoculated with *Bacillus subtilis* strain B7 at two different concentrations 10^7 CFU mL^{-1} and $1.55 \cdot 10^8$ CFU mL^{-1}

3.3 Phosphorus solubilizing capacity

3.3.1 Impact of top isolates (strains P2b, B7, B17 and MB18) in maize and sunflower fertilized with P-free nutrient solution

When comparing non-inoculated plants fertilized with a phosphorus-free Hoagland solution (C-P) to those fertilized with a complete Hoagland solution (C+P), significant differences were observed (Table 9). In sunflower plants, the C+P treatment resulted in higher shoot fresh and dry weights, while in maize, there were significant increases in plant length, shoot fresh and dry weights, and root fresh and dry weights. These findings highlight the critical role of phosphorus in plant development, as the complete Hoagland solution significantly enhanced plant growth. Additionally, a significant increase in sunflower shoot fresh weight when inoculated with all tested isolates, with P2b, B7, B17, and MB18 resulting in increases of 13%, 44%, 52%, and 32%, respectively, compared to non-inoculated plants without phosphorus fertilization (C-P). Additionally, differences were observed in shoot dry weight between non-inoculated plants and those inoculated with B7 and B17, showing increases of 46% and 53%, respectively. For root dry weight, only plants inoculated with strain B17 exhibited a notable difference, with values 68% higher than those of non-phosphorus fertilized sunflower plants (Table 9).

In the evaluated maize parameters, various differences were observed concerning the effect of inoculation compared to the values obtained from non-inoculated maize plants fertilized without soluble phosphorus (C-P) (Table 9). Firstly, significant differences were noted in leaf number due to the inoculation with strains P2b, B7, and MB18, also differences in plant length were also observed, particularly with plants inoculated with *B. subtilis* strains B7, B17, and *B. megaterium* MB18, showing increases of 30%, 28%, and 26%, respectively. Additionally, variations in biomass values were evident, significant differences were noted in shoot fresh weight due to the inoculation with strains P2b, B7, B17, and MB18 with increases of 61%, 81%, 84% and 57% respectively compared to non-inoculated plants (C-P), which were later reflected in substantial differences in shoot dry weight, by inoculation with P2b, B7, and B17 which led to increases of 70%, 48%, and 91%, respectively, compared to non-inoculated maize plants. Furthermore, differences were observed in underground biomass values, with B7 inoculation resulting in increases of 95% and 110% in fresh and dry root weight, respectively, and B17 inoculation resulting in a 93% increase in dry root weight compared to non-inoculated plants (C-P).

Table 9 Effect in *Zea mays* cv "Tía María" and *Helianthus annuus* cv "Russian Giant" plant growth by the phosphorus solubilizing top isolates

Plant specie	Treat.[a]	Number of leaves	Plant length (cm)	Shoot fresh weight (g)	Shoot dry weight (g)	Root fresh weight (g)	Root dry weight (g)
Sunflower	C+P	**14,6 ± 0.6 ***	82.8 ± 4.1	**36.01 ± 1.4 ***	**3.85 ± 0.1 ***	11.21 ± 1	0,83 ± 0,1
	C-P	12,5 ± 0.4	72.5 ± 5.3	16.79 ± 1.5	2.28 ± 0.2	10.57 ± 0.5	0,73 ± 0
	P2b	12,2 ± 0.8	74.7 ± 4.7	**18.98 ± 1.4 ***	2.51 ± 0.2	10.48 ± 0.3	0,78 ± 0
	B7	13,4 ± 0.2	82.4 ± 3.4	**24.20 ± 1.5 ***	**3.32 ± 0.2 ***	10.74 ± 1	0,74 ± 0,1
	B17	12,6 ± 0.6	79.8 ± 3.8	**25.59 ± 1.4 ***	**3.49 ± 0.4 ***	10.72 ± 0.9	**1,23 ± 0,2 ***
	MB18	14,4 ± 0.4	84.8 ± 2.7	**22.14 ± 1 ***	2.85 ± 0.1	10.50 ± 0.5	0,79 ± 0,1
Maize	C+P	9,2 ± 0.5	**140.4 ± 6 ***	**70.38 ± 5.2 ***	**12.74 ± 2 ***	**24.79 ± 3.7 ***	**2,01 ± 0,3 ***
	C-P	8,4 ± 0.4	97.6 ± 10	25.22 ± 5.3	3.47 ± 1	11.30 ± 3.3	1,03 ± 0,3
	P2b	**10,0 ± 0.4 ***	120.7 ± 2.3	**40.71 ± 1.4 ***	**5.89 ± 0.9 ***	18.41 ± 1.1	1,61 ± 0,1
	B7	**10,0 ± 0.3 ***	**126.8 ± 4.8 ***	**45.69 ± 2.5 ***	**5.13 ± 0.2 ***	**22.09 ± 1.2 ***	**2,17 ± 0,2 ***
	B17	8,6 ± 0.3	**125.3 ± 8 ***	**43.92 ± 1.9 ***	**6.62 ± 1 ***	19.58 ± 0.6	**1,99 ± 0,1 ***
	MB18	**10,2 ± 0.2 ***	**123.0 ± 5.8 ***	**39.71 ± 2.4 ***	4.01 ± 0.1 a	14.58 ± 0.7	1,47 ± 0,1

Values represent means ± SE (n=5). Significantly different values are marked with an asterisk based on Dunnett's analysis test (p-value < 0.05) between inoculated and non-inoculated plants (C-P)

[a] Treatment as follow: to non-inoculated plants fertilized with P-free Hoagland (C-P), plants non-inoculated and fertilized with complete Hoagland solution (C+P), and plants fertilized with free-P Hoagland and inoculated with *Pseudomonas florescens* strain P2b, *Bacillus subtilis* strains B7 and B17 and *Bacillus megaterium* strain MB18 at a concentration of 10^8 CFU mL^{-1}

Regarding the analysed nutrient content in maize plants (Table 10) inoculated with *B. subtilis* strain B17 exhibited a significant increase of 59% in phosphorus per plant compared to non-

inoculated plants, also the calcium content was significantly higher (44%) than in the inoculated plants.

Conversely, in sunflower plants, there was no significant increase in phosphorus content per plant between inoculated and non-inoculated groups, although a tendency to increase phosphorus content was observed in plants treated mainly with *B. subtilis* strains B7 and B17 (Table 10). Although significant differences are observed in potassium content between non-inoculated plants without phosphorus fertilization (C-P) and plants inoculated with *B.subtilis* strain B17 with a content increase of 47% (Table 10).

Table 10 Effect of nutrient content in *Zea mays* and *Helianthus annuus* inoculated with the phosphorus solubilizing top isolates

Plant specie	Treat.[a]	Nutrient content (mg plant^{-1})					
		Potassium (K)	Calcium (Ca)	Magnesium (Mg)	Iron (Fe)	Phosphorus (P)	Sulphur (S)
Sunflower	C+P	**329 ± 16.5 ***	31.67 ± 3.2	**15.02 ± 1.4 ***	0.22 ± 0.03	**10.94 ± 0.6 ***	**8.36 ± 0.8 ***
	C-P	153 ± 4.4	20.65 ± 4	8.55 ± 0.9	0.20 ± 0.06	1.31 ± 0.2	4.09 ± 0.5
	P2b	171 ± 19.7	23.80 ± 2.3	9.14 ± 0.3	0.16 ± 0.02	1.64 ± 0.1	4.59 ± 0.2
	B7	197 ± 12.7	25.87 ± 2.2	9.94 ± 1.3	0.29 ± 0.07	2.10 ± 0.4	4.97 ± 0.7
	B17	**225 ± 27.8 ***	30.64 ± 5.2	11.97 ± 1.3	0.22 ± 0.03	2.02 ± 0.3	6.05 ± 1.5
	MB18	191 ± 11.1	25.52 ± 0.9	10.08 ± 0.7	0.14 ± 0.02	1.65 ± 0.1	4.45 ± 0.2
Maize	C+P	**664 ± 98.9 ***	**26.29 ± 4.7 ***	**26.59 ± 4.7 ***	**1.04± 0.22 ***	**21.32 ± 2.7 ***	**15.32 ± 1.7 ***
	C-P	217 ± 56.5	7.03 ± 1.5	7.64 ± 2	0.40 ± 0.04	2.23 ± 0.3	4.50 ± 1.0
	P2b	297 ± 57.7	10.18 ± 2.1	10.89 ± 2.3	0.51 ± 0.23	3.27 ± 0.6	6.40 ± 1.3
	B7	292 ± 9.8	9.56 ± 0.7	10.14 ± 0.2	0.31 ± 0.02	2.95 ± 0.2	5.84 ± 0.4
	B17	347 ± 49.06	**12.17 ± 0.8 ***	12.67 ± 1.7	0.51 ± 0.09	**3.55 ± 0.6 ***	6.91 ± 0.8
	MB18	234 ± 13.53	7.91 ± 0.7	8.90 ± 0.7	0.28 ± 0.05	2.19 ± 0.1	4.85 ± 0.4

Values represent means ± SE (n=5). Significantly different values are marked with an asterisk based on Dunnett's analysis test (p-value < 0.05) between inoculated and non-inoculated plants (C-P)[a] Treatments as follow: non-inoculated plants fertilized with P-free Hoagland (C-), and inoculated with *Pseudomonas fluorescens* strain P2b, *Bacillus subtilis* strains B7 and B17 and *Bacillus megaterium* strain MB18 at a concentration of 10^8 CFU mL^{-1}

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3.3.2 Confirmatory effect of *Bacillus subtilis* strain B7 and B17 in phosphate uptake in maize fertilized with P-free nutrient solution.

In the context of plants fertilized without soluble P, significant differences emerged between non-inoculated and inoculated maize plants in terms of shoot fresh and dry weight, notably, plants inoculated with *B subtilis* strain B7 exhibited a 5% higher value in fresh shoot weight and a 9% higher value in dry weight compared to non-inoculated plants (Table 11). Interestingly, there were no discernible differences in plant length among treatments, including between plants fertilized

without soluble P and those with a complete Hoagland solution. Maize plant length exhibited a consistent pattern of increase across all plants, irrespective of inoculation status (Table 11).

Table 11 Plant growth promotion of maize inoculated with *B. subtilis* strains B7 and B17

Treat.[a]	Shoot lenght (cm)[b]				Shoot fresh weight (g)	Shoot dry weight (g)
	T1	T2	T3	T4		
C+P	16.15 ± 0.1	20.75 ± 0.2	29.90 ± 0.2	40.35 ± 0.2	**68.33 ± 0.6 ***	**6.41 ± 0.06 ***
C-P	15.65 ± 0	19.80 ± 0.1	27.75 ± 0.3	38.85 ± 0.3	54.55 ± 0.3	5.50 ± 0.04
B7	16.95 ± 0	21.40 ± 0.1	30.05 ± 0.2	40.20 ± 0.2	**57.77 ± 0.2 ***	**6.07 ± 0.05 ***
B17	16.35 ± 0.1	20.55 ± 0.2	28.80 ± 0.4	40.45 ± 0.6	55.67 ± 0.7	5.44 ± 0.06

Values represent means ± SE (n=10). Significantly different values are marked with an asterisk based on Dunnett's analysis test (p-value < 0.05) between inoculated and non-inoculated plants (C-P)

[a] Treatments as follow: non-inoculated plants fertilized with P-free Hoagland (C-), non-inoculated plants and fertilized with complete Hoagland solution (C+), and plants inoculated with *Bacillus subtilis* strain B7 and strain B17 at a concentration of 10^8 CFU mL^{-1}.

[b] Stem length at first week (T1), second week (T2), third week (T3) and fourth week (T4) after inoculation

3.3.3 Effect of *Bacillus subtilis* strain B7 and strain B17 in phosphate uptake in radish plants fertilized with P-free nutrient solution

To assess the impact of microbial inoculation on radish plants, we compared non-inoculated plants fertilized with a complete Hoagland solution (C+) to those treated with a P-free Hoagland solution (C-). The results revealed a significant increase in plant growth when the complete Hoagland solution was applied, indicating a phosphorus deficit in radish plants (Table 12). When evaluating differences in shoot dry weight between non-inoculated plants and those inoculated with *B. subtilis* strain B7 at a concentration of $1x10^8$ CFU mL^{-1}, a notable increase of 20% in plant values was observed (Table 12). However, no significant difference in radish root weight was observed between inoculated and non-inoculated plants.

Table 12 Effect of inoculation of *B. subtilis* strains B7 and B17 in *Raphanus sativus* cv "Rabanito" plant growth

Treatment[a]	Leaves number	Shoot fresh weight (g)	Shoot dry weight (g)	Root fresh weight (g)	Root dry weight (g)
C+P	6,45 ± 0,2	**11,67 ± 0,5 ***	**1,17 ± 0,09 ***	**20,08 ± 1,3 ***	**1,54 ± 0,06 ***
C-P	5,91 ± 0,2	5,19 ± 0,2	0,56 ± 0,02	13,94 ± 0,8	1,02 ± 0,06
B7	5,5 ± 0,1	5,58 ± 0,3	**0,69 ± 0,05 ***	13,9 ± 0,4	1,09 ± 0,03
B17	5,58 ± 0,2	5,35 ± 0,1	0,59 ± 0,05	14,89 ± 0,6	1,08 ± 0,05

Values represent means ± SE (n=12). Significantly different values are marked with an asterisk based on Dunnett's analysis test (p-value < 0.05) between inoculated and non-inoculated plants (C-P)

[a] Treatment as follow: to non-inoculated plants fertilized with P-free Hoagland (C-), plants non-inoculated and fertilized with complete Hoagland solution (C+), and plants fertilized with free-P Hoagland and inoculated with *Bacillus subtilis* strain B7 and strain B17 at a concentration of 10^8 CFU ml^{-1}

Discussion

1. Isolates and sampling site

Bacillus subtilis strains were predominantly isolated from compost samples, with some strains of *Pseudomonas fluorescens* also identified. These findings align with previous research highlighting the fundamental role of these species in the composting process and also in promoting plant growth and soil health (Cariello et al., 2007; Lin et al., 2014). Compost, with its alkaline pH, high electrical conductivity, and abundant organic matter, creates an optimal environment for the growth and activity of bacteria involved in organic material decomposition (Ancuţa & Renata, 2011), specially Falcón et al., (1987) found that the majority of microorganisms that were identified during the initial mesophilic phase of composting belonged to *Bacillus spp*., with *B. subtilis* being particularly prominent.

In addition, our study revealed that the maize rhizosphere harbours an abundant presence of *B. subtilis* and *P. fluorescens* highlighting the significance of this environment in agricultural contexts. These microorganisms, find a conducive niche for their development and activity in the maize rhizosphere (Mumtaz et al., 2017) as previous research has underscored notable differences between the microbiota present in the maize rhizosphere and that in the surrounding soil (Niu et al., 2017). These differences not only reflect the direct influence of host plants on the microbial composition of their environment but also highlight the complexity of plant-microorganism interactions in agricultural soils.

2. Assessment of biochemical capacities *in vitro*

Our study elucidates the *in vitro* capabilities of *B. subtilis* and *P. fluorescens* isolates in the production of IAA, consistent with the findings documented in the literature, these species possess the remarkable capability to secrete IAA, one of the most physiologically active phytohormones in soil, thus contributing to plant growth (Spaepen et al., 2007). Egorshina et al., (2012) showed that the *B. subtilis* 11BM strain can synthesize indole compounds *in vitro* and stimulate the growth of wheat plants by seed inoculation. Similarly, the study by Ribeiro et al., (2018) revealed that the *B. subtilis* B2084 strain produced 24.4 µg mL^{-1} of IAA *in vitro*, while strain B2088 produced 55.8 µg mL^{-1}. These results are aligning with those acquired in our study by strains B5, B7 and B17.

The IAA production pattern, where the concentration of IAA in suspension is maximum and then decreases are consistent with the studies of Khianngam et al. (2023) who reported that IAA production in the VR2 and MG9 strains of *B. subtilis* began at 24 hours after incubation, peaked at 48 hours, and then gradually decreased. Similarly, Panigrahi et al. (2020) and Wagi & Ahmed (2019) observed that IAA production gradually decreased after 24 hours in the *B. cereus* So3II and *B. subtilis* Mt3b strains, respectively. Furthermore Patten & Glick, (2002) confirms the ability

of *P. fluorescens* to produce IAA, and Sethia et al., (2015), observed that the *P. fluorescens* FP10 strain reached a maximum production of 70 μg mL^1 after 72 hours. These results are in agreement with those obtained by our P10 strain.

The ability of *Bacillus spp.* and *P. fluorescens* to produce siderophores as it is observed in our results has been extensively documented in the scientific literature. *Bacillus spp.* is recognized for its ability to produce siderophores when grown on CAS medium agar plates, as evidenced by studies by Di et al. (2023), Ahmad et al. (2021) and Ribeiro et al. (2018). Specifically, *B. megaterium* is been described as a siderophore producer associated with the ability to chelate heavy metals such as Mn(II), Zn(II), and Cu(II) (Yin et al., 2022) as observed in our study by MB18 and MB19 strains. There are also studies demonstrating the siderophore production capacity of *P. fluorescens*, where the main siderophore described is pyoverdine (De Vleesschauwer et al., 2009; Mohamed & Gomaa, 2012; Trapet et al., 2016) in accordance with the results obtained by strain P2b.

The ability of *Bacillus spp*. and *P. fluorescens* to mobilize sparingly available phosphorus through solubilization and mineralization, has been extensively documented in the scientific literature (Alori et al., 2017; Babalola et al., 2005; Manzoor et al., 2017). It is remarkable the capacity for phosphate solubilization using hydroxyapatite ($Ca_5(PO_4)_3OH$) as the mineral phosphate source observed in our study by *B. subtilis, B. megaterium*, and *P. fluorescens*. As it is described in Wang et al., (2020) who defined the capacity of the *B. subtilis* BPM12 strain isolated from *Z. mays* rhizosphere to solubilize mineral phosphate and Rodríguez & Fraga, (1999) that described an increase in soluble phosphate in media enriched with hydroxyapatite by *B. megaterium*. These results are in agreement with the values obtained in our study with the MB17 and MB18 strains.

Other literature describes the ability to solubilize insoluble inorganic phosphate compounds, particularly strains isolated from the rhizosphere of *Z. mays* (Li et al., 2017; Rodríguez & Fraga, 1999; Mumtaz et al., 2017). *Pseudomonas spp.* has also been described in the literature as having the ability to solubilize insoluble inorganic phosphate and promote P absorption (Rezakhani et al., 2019). Likewise, Li et al., (2017) describe the ability of *P. fluorescens* strain B10 isolated from *Z. mays* to solubilize both inorganic and organic phosphate. Regarding the results obtained with iron phosphate (Fe-P), our observation does not reveal a significant effect on solubilization by either *B. subtilis.* or *P. fluorescens* a conclusion consistent with the findings of Wang et al., (2020). However, the *B. megaterium* MB18 strain exhibited the highest Fe-P solubilization and also produced the most siderophores, thus underscoring the potential role of siderophores in iron phosphate solubilization, as pointed out by Cui et al. (2022).

Concerning the ability to mineralize organic phosphate (phytate) by the *Bacillus spp.* and *P. fluorescens* strains, there were no indications of solubilization in our study. Therefore, we

hypothesize that his phenomenon could be attributed to the inactivation of alkaline phosphatase enzyme activity which is influenced by temperature and pH (Mingmongkolchai & Panbangred, 2019). In our case the temperature was 30ºC and the pH 6,8, since the optimal temperature for enzyme production is 27ºC, it is possible that exceeding this temperature affected enzyme activity (Li et al., 2013).

The technique used in our study to measure phosphate solubilization, through the molybdate reactive phosphorus method in suspension in culture broth enriched with insoluble phosphate sources, is generally considered more reliable than culturing on Pikovskaya's agar medium, however, it is important to note that this estimation has a significant limitation in not accounting for the phosphorus used by the microbial cells during growth (Rodríguez & Fraga, 1999).

The main mechanisms involved in phosphorolysis include the production of organic acids. Manzoor et al., (2017) observed that the maximum drop in pH was correlated with the highest phosphate solubilization, consistent with the results obtained in our study. This is due to a decrease in the concentration of surrounding metal ions or a reduction in the pH of the substrate; finally, hydrogen ions replace various metallic elements or Ca^{2+}, resulting in the release of phosphorus (Glickmann & Dessaux, 1995; Seshachala & Tallapragada, 2012). Among the organic acids, Di et al., (2023) observed the production of acetic and malic acids as part of the mineral phosphate solubilization process by *B. subtilis*. Similarly, Alori et al., (2017) observed the production of gluconic acid. In our research, we also observed the ability of *B. subtilis* strain B7 and B17 to produce several organic acids mainly malic acid for both strains and glucuronic, fumaric and lactic acids for B7 strain.

3. Effect of inoculation in plant

The genus *Bacillus*, particularly the species *Bacillus subtilis*, has been the subject of numerous studies investigating its impact on the germination of various seeds. Luna Martínez et al., (2013) and Mehta et al., (2015) demonstrated that different strains of *Bacillus* significantly increase the germination percentage in tomato seeds ranging from 6.1% to 14.56%. These results are in agreement with those obtained in our study where the inoculation of both *B. subtilis* tested (B7 and B17) increased tomato germination within the same percentage range, additionally Girish & Umesha, (2005) demonstrate that the *B. subtilis* strain GBO3 not only enhanced tomato germination but also increased seed vigour. The impact of *B. subtilis* inoculation on seed germination has also been investigated in various other plant species, such as radish (Kaymak et al., 2009), lettuce (Malkoclu et al., 2017) or in pearl millet seeds (Raj et al., 2003).

Although there are studies where the inoculation of *B. subtilis* did not affect tomato seed germination, a faster growth in seedlings and a significant increase in stem and root length was observed (Cabra Cendales et al., 2017; Ozaktan et al., 2017). Within this context Rojas-Badía et

al., (2020) and Ajilogba et al., (2013) agree in that certain strains of *B. subtilis* can improve stem diameter and the fresh weight of roots and aerial parts of tomato plants. The diversity in the impact of *B. subtilis* inoculation on plant germination and seedling physiological aspects was also evident in our study across three distinct experiments. This variability could be attributed to the varying temperature and light conditions observed during March, May, and June 2021.

When examining the plant growth promotion effect of *B. subtilis* and *P. fluorescens* isolates inoculated in lettuce, cucumber, and maize, a consistent positive impact of *B. subtilis* inoculation on plant growth and yield is evident in the literature. Malkoclu et al., (2017) and De Leon et al., (2020) observed a significant increase in lettuce growth, specifically in terms of dry shoot biomass increment, attributed to *B. subtilis* inoculation yields findings that are consistent with those garnered in our study by B7 and B17 strains. Additionally, Sahin et al., (2015) not only noted a substantial growth enhancement but also reported elevated nutrient content, relative water content, and stomatal conductance. In the case of cucumbers, in our studies, the inoculation of *B. subtilis* strains B7 and B17 exhibited a significant impact on plant growth. This effect was underscored by Li et al., (2023) where the *B. subtilis* strain K424 notably enhanced photosynthetic capacity and nutritional content, further supported by Xu et al., (2022). In our investigation, we also observed a promotion in maize growth by inoculation of *B. subtilis* strains B7 and B17. These findings align with existing literature, where the application of various *B. subtilis* strains has consistently led to significant enhancements in shoot and root length, as well as fresh and dry weight of plants (Misra & Chauhan, 2020; Ouhaddou et al., 2023).

Regarding the impact of *P. fluorescens* inoculation on maize and lettuce growth, our findings are consistent with existing literature. In the case of maize, Chavéz-Díaz et al., (2022) reported that *P. fluorescens* inoculation enhanced the length of the aerial parts and the fresh weight of the seedlings, similarly, Sandini et al., (2019) noted improvements in plant growth and grain yield. Regarding lettuce, studies by Someya et al., (2018) and Cipriano et al., (2016) corroborate these effects, demonstrating increased plant length and shoot weight, similarly to observations made in or studies with the P10 strain.

Moreover, the investigation into how the inoculation of the same *B. subtilis* strain affects different varieties of the same plant species remains relatively unexplored in current research. Similarly, De Leon et al., (2020) observed diverse responses to the inoculation of arbuscular mycorrhizae across six distinct wheat cultivars, underscoring the significance of considering varietal disparities in microbial interactions. Nevertheless, we encountered a study by Singh et al., (2023) that parallels to our findings, showcasing distinct growth promotion effects when inoculating a *B. subtilis* strain in different maize varieties. Moreover, in our case there was not a dosage effect.

In our research, we operated under the assumption that higher biomass correlates with increased final plant yield production. This assumption is supported by recent studies that have established a strong linear relationship between biomass production and crop yield (Kang et al., 2017; Munns et al., 2010; Wu et al., 2022). Specifically, in maize studies, Ghassemi-Golezani, (2012) demonstrated there was a tight association between crop yield and both aerial and total plant biomass, while (Singh et al., 2023) by a long-term study demonstrate that, under adequate water conditions, increased biomass accumulation is the primary factor contributing to improve yield. All this research underscores the validity of conducting short-term trials that assess biomass without reaching harvest, as a tool for screenings.

In our studies of the effects of isolate inoculation on phosphorus uptake and accumulation in plants grown in environments with limited or insoluble phosphate, we observed significant growth enhancements in maize, sunflower, and radish plants due to the action of *B. subtilis* strains B7 and B17, moreover, B17 inoculation led to an increase in the total phosphorus concentration in plants. These findings are consistent with prior research indicating the positive impact of specific *B. subtilis* strains on phosphate solubilization and phosphorus assimilation in maize plants, which can result in increased plant biomass in environments with limited phosphorus availability (Li et al., 2017; Lobo et al., 2019). Furthermore, in the study by Pereira et al., (2020), not only demonstrate the beneficial effect of *B. subtilis* inoculation on phosphorus plant use efficiency, but also in plant grain yield consequently.

The findings from these studies underscore the consistent impact of *B. subtilis* inoculation on various aspects of plant growth and phosphorus uptake. Shehzad et al., (2014) demonstrated a remarkable increase in root growth in maize plants grown with phosphate rock, mirroring our own results where strain B7 inoculation led to a 52% increase in root dry weight. Similarly, Ahmad et al., (2021) confirmed a significant enhancement in plant phosphorus concentration following *B. subtilis* inoculation by a 43%, aligning closely with our observations of strain B17's effect, which resulted in a 59% increase in phosphorus plant accumulation. Moreover, the literature consistently highlights the role of *B. subtilis* as a phosphorus solubilizing bacterium, with documented effects on the growth of sunflower (López-Valdez et al., 2011) and radish (Mohamed & Gomaa, 2012), which are in line with the outcomes of our study involving strain B7.

Conclusions

A new collection of isolates was successfully created, most of the strains of interest were free living bacteria isolated from compost, resulting in 26 strains of interest: 13 *P. fluorescens* and 13 *B. subtilis*, which are added to the existing list of 75 bacteria already in our collection.

Regarding the evaluation of *in vitro* characteristics, the *P. fluorescens* strain P10 stood out for its capacity to produce IAA, although the *B. subtilis* strains B5, B7, and B17 also presented good values. Several strains also exhibited the ability to produce siderophores. Additionally, referring to the ability to solubilize phosphate in the form of hydroxyapatite, the best results were obtained from *B. megaterium* strains MB17 and MB18, along with *P. fluorescens* strains P2a and P4b. Furthermore, the phosphate solubilizing capacity in B7 and B17 strains was associated with a decrease in the pH of the medium, along with the production of organic acids.

The strains that achieved a combination of the best *in vitro* results were inoculated in plants. These trials revealed a positive effect on tomato germination and seed quality traits due to the inoculation with *B. subtilis* (B7 and B17). Additionally, growth promotion effects were observed with *B. subtilis* strain B7 at a concentration of 10^7 CFU mL^{-1} in various crops, with the effect also being associated with the cultivar used. Similarly, in experiments evaluating the effect of inoculation on plants with soluble phosphorus deficiency, it was observed that *B. subtilis* strain B17 at a concentration of 10^8 CFU mL^{-1} exhibits profound capabilities as a phosphorus solubilizer associated with the plant, thereby enhancing phosphorus uptake in maize plants, leading to a consequential increase in biomass and phosphorus accumulation in the aerial parts of the plants.

In the present study, the findings shed light on the potential of *Bacillus subtilis* strains B7 and B17 as biofertilizers. The multiple plant growth-promoting (PGP) traits exhibited by these strains reveal promising results for further exploration in agricultural practices, particularly in enhancing yield and minimizing fertilizer usage.

Chapter 2: *Azospirillum spp.* and *Azotobacter spp.* isolation and characterization *in vitro*

Introduction

The diversity of bacterial populations in soil is remarkable, with significant variations in their composition observed at different locations or points within the same soil (Torsvik & Ovreas, 2002). A considerable portion of these soil microbes, however, remains uncultivable under laboratory conditions. Despite this challenge, understanding the native bacterial population, their relative abundance in soil, and optimizing their growth conditions are crucial to comprehending the performance and diversity of indigenous bacteria in specific crop soils (Yaghoubi et al., 2021). This knowledge is fundamental for harnessing the potential of soil microbial communities to support sustainable agriculture and enhance crop productivity.

In the rhizosphere, the space surrounding roots, microbial communities harbour beneficial members known as plant growth-promoting rhizobacteria (PGPR) (Wang & Song, 2022). As described in Chapter 1, these bacteria can regulate plant growth and development by multifaceted mechanisms, including phytohormones production (Richardson et al., 2009) and the enhancement of plant nutrition through nitrogen fixation and phosphate solubilization (Zhou et al., 2024). In agricultural practices, PGPR are commonly used for inoculation purposes as microbial biostimulants.

At European level, beneficial microorganisms which are not plant protection products can be commercialized under national regulations or according to the Fertilizers Regulation (EU) 2019/1009 as microbial biostimulants. According to the European Regulation, eligible microorganisms for inclusion as microbial biostimulants include *Azotobacter spp*., mycorrhizal fungi, *Rhizobium spp.*, and *Azospirillum spp.*, as these genera are considered harmless to human health and certification procedure simply requires efficacy information to support the claims stated in the product label as well as formulation information. Following this regulation, our research has focused on these microbial genera for subsequent commercialization as microbial biostimulants at European level.

The nitrogen cycle and the phosphorus cycle encompass fundamental biological processes that significantly contribute to soil health and crop productivity, where microorganisms play a key role in making nutrients available to plants (Elmerich & Newton 2007). Diazotrophic bacteria from genera such as *Azospirillum*, *Azotobacter*, *Bradyrhizobium,* and *Rhizobium* are typically utilized as biostimulants to boost plant nitrogen levels. Additionally, some of these nitrogen-fixing bacteria, as well as strains of *Bacillus* and *Pseudomonas*, are commonly employed for phosphate-solubilizing purposes.

Nitrogen fixation ranks among the most important biological processes and is regarded as a crucial microbial activity on Earth's surface as it provides a means of recycling nitrogen (N) and

plays a critical role in nitrogen homeostasis in the biosphere (Wani et al., 2017). Nitrogen fixation occurs via nitrogen-fixing bacteria (NFB) such as *Rhizobium*, *Azospirillum*, and *Azotobacter*, which are soil free-living or establish symbiotic associations with plant roots (Vessey et al., 2004; Elmerich & Newton, 2007). The process of nitrogen fixation in bacteria operates by reducing carbon from carbohydrates and lipids, providing the electrons and energy required for N_2 fixation, that is transferred to nitrogenase to stepwise the reduction of N_2 to ammonia (NH_3), most of which is instantly converted to ammonium (NH_4+) at typical intracellular pH (Inomura et al., 2020). Consequently, the amounts of fixed nitrogen between different nitrogen-fixing bacteria species vary, but, in simple terms, free-living N_2-fixers fix much less nitrogen than nodule-forming bacteria systems where host plants directly provide the microsymbiont with energy and shield the nitrogenase enzyme from being deactivated by oxygen (Jehani et al., 2023).

The measurement of nitrogen fixation is usually carried out using the acetylene reduction assay (ARA) technique, which analyses the activity of the nitrogenase enzyme by measuring the reduction of acetylene (C_2H_2) to ethylene (C_2H_4) (Crews et al., 2001). However, ARA has limitations such as the manual labour involved and the difficulty in monitoring ethylene reduction in real time (Payá-Tormo et al., 2022). Currently, other techniques are used, such as sequencing of the nifH gene, which encodes for the iron-molybdenum protein subunit of nitrogenase known as iron-molybdenum cofactor (FeMo-Co), although these techniques are often costly (Cassán & Diaz-Zorita, 2016). Another technique which is rapid and economic, is analysing the ammonium concentration produced by a pure bacterial strain cultured in a free N-growth medium, through the variation of absorbance caused by the colour change of bromothymol blue (Chalk, 2016; Cordova-Rodriguez et al., 2022; Smercina et al., 2019).

In addition to nitrogen fixation, NFB can also play a crucial role in phosphorus recycling cycle. Phosphorus (P) is an essential nutrient for plants involved in various biochemical processes, including lipid metabolism and the biosynthesis of nucleic acids and cell membranes (Ha & Tran, 2014). However, P is one of the most limiting nutrients in global agricultural ecosystems (Lin et al., 2016), applied to soils can accumulate in non-labile forms due to its high-affinity chemical reactions and occlusion to soil minerals and organic matter, leading to insoluble phosphorus (Gatiboni et al., 2020). This situation presents an ecological paradox: while there are limited quantities of soluble phosphorus for plant growth, there are substantial amounts of non-labile forms, even in native soils. This contradictory scenario in phosphorus availability has driven the natural selection of microorganisms capable of solubilizing phosphorus into forms usable by plants, as a survival strategy (Goldstein, 1986). These microorganisms are known as phosphate-solubilizing bacteria and they play a critical role accumulating and transforming P and accounting for 68–78% of total P in biomass (Fan et al., 2018) and used as biostimulants may be considered

a cost-effective, environmentally friendly, and long-term biological solution to address soil P deficiency.

Phosphate-solubilizing bacteria participate directly in the increase of soluble and absorbable phosphorus by plants through two processes: P mineralization and inorganic P solubilization. As well as, indirectly, influencing the structure of the rhizosphere microbial community and the configuration of the root system (Al-Ali et al., 2018). Phosphorus mineralization is the process by which organic phosphorus present in organic matter such as plant residues, animal remains, and other organic detritus, is converted into inorganic forms of phosphorus through the action of decomposing microorganisms (Al-Ali et al., 2018). Inorganic P in soil is typically present bond to calcium under neutral to alkaline soil conditions and bound to iron or aluminium under acidic conditions (Kumar et al., 1999). The solubilization of inorganic phosphorus involves breaking down inorganic phosphate compounds, converting them into more plant-available soluble forms of phosphorus. This can occur through the production of organic acids or other compounds that dissolve inorganic phosphates in the soil (Cheng et al., 2023). Organic acids are low molecular weight compounds produced by phosphorus-solubilizing bacteria through fermentation, respiration, and oxidation of organic compounds. Their involvement in the phosphorus solubilization process is characterized by the release of hydrogen ions (H+), effectively reducing the pH of the medium, thereby promoting the dissolution of insoluble phosphorus compounds and enhancing phosphorus availability for plant uptake (Rawat et al., 2021).

Azospirillum are gram-negative vibrio or spirillum-shaped bacteria of 1 μm diameter, possessing peritrichous flagella with short wavelengths used for swarming and polar flagellum used for swimming. Poly-β-hidroxybutirate granules fill most of the bacteria cell that's why colonies develop a pink pigment and usually, they proliferate under both anaerobic and aerobic conditions but are preferentially microaerophilic (Okon & Labandera-Gonzalez, 1994). They are considered a nitrogen fixing bacteria (NFB) recognized as PGPR, in agronomic contexts, they are of particular interest as biostimulants due to their capacity to enhance plant growth through nitrogen fixation, production of phytohormones, polyamines, and trehalose potentially boosting crop productivity and reducing reliance on synthetic nitrogen fertilizers (Bashan & de-Bashan, 2010; García et al., 2017; Santos et al., 2017b). The genus *Azospirillum,* belonging to the α-subclass of the proteobacteria, encompasses 21 species with varying genome sizes (Lin et al., 2016; Martin-Didonet et al., 2000; Miransari, 2016) (Figure 1), notably, recent research by Xu et al., (2023) has identified a new species, *A. aestuarii*. The *Azospirillum* nitrogen fixation ability is explained as the ability to convert atmospheric nitrogen into ammonium under microaerobic conditions and low nitrogen levels, through the nitrogenase complex (Burris & Roberts, 1993). Although the nitrogen fixation from *Azospirillum spp* is a recognized processes involved in promoting plant

growth it is not clear if it is the main reason of growth promotion, as the transfer of fixed nitrogen to the plant is limited (Çakmakçi et al., 2007; Steenhoudt & Vanderleyden, 2000).

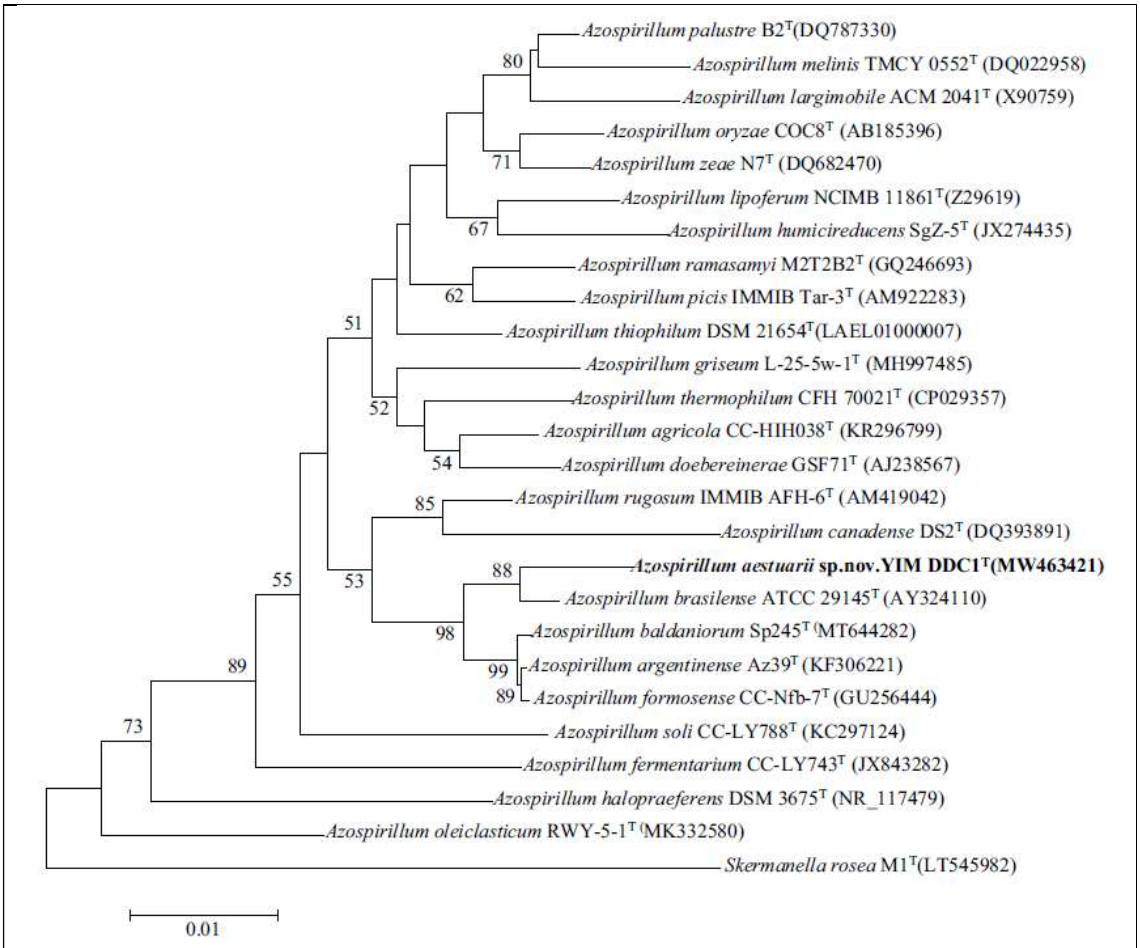

Figure 1 Phylogenetic analysis of species of the genus *Azospirillum* based on 16S rRNA gene sequences. Source (Xu et al., 2023)

Azotobacter spp. are gram-negative oval-shaped bacterium of 1-3 µm wide and 2–10 µm long some species produce yellow-green, or red-violet, or brownish-black pigments (Das, 2019). They are considered a genus of free-living NFB also recognized as PGPR and utilized as biostimulants to enhance the yield of non-leguminous crops, owing to their diverse array of plant growth-promoting attributes. These attributes encompass nitrogen fixation, production of growth hormones, fungicidal compounds, siderophores, and the ability to solubilize phosphate (Narula et al. 2000). Such characteristics serve to booster nutrient availability for plants, thereby contributing to heightened crop productivity. The genus *Azotobacter* belongs to the γ-subclass of the proteobacteria and includes 7 species corresponding to *A. armeniacus, A. beijerinckii, A. chroococcum, A. nigricans, A. paspali, A. salinestri, A. tropicalis, and A. vinelandii* (Özen & Ussery, 2012) (Figure 2). This genus is characterized for producing exopolysaccharides, which are involved in processes such as encystment induction, protection of nitrogenase from oxygen, and support for biofilm formation, contribute to salt stress tolerance, desiccation tolerance, and

tolerance to pesticides/insecticides, furthermore, these exopolysaccharides can also provide nutritional benefits by acting as surface-active agents, serving as a carbon source, promoting soil aggregation, and facilitating nutrient solubilization (Gauri et al., 2012).

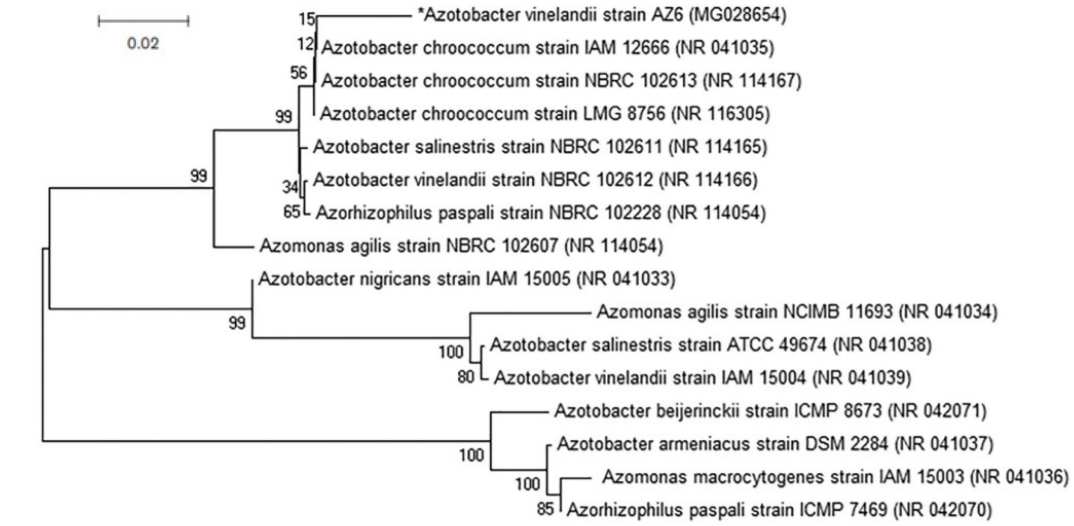

Figure 2 Phylogenetic analysis of species of the genus *Azotobacter* based on 16S rRNA gene sequences. Source: (Shahid et al., 2019)

Objectives

The general objective was to develop a collection of isolates capable of nitrogen fixation and phosphorus solubilization, particularity isolates from the *Azospirillum* and *Azotobacter* genus, with the aim of characterizing their *in vitro* potential as biostimulants.

The specific objectives were:

1. Establish a new collection of isolates belonging to the species accepted under the European Union Fertilizer Regulation (2019/1009).
2. Characterize their nitrogen-fixing capacities by analysing growth and ammonium production in nitrogen-free media.
3. Characterize their phosphorus-solubilizing capacities by studying the conversion of insoluble phosphorus to soluble forms, monitoring pH changes, and measuring the production of organic acids.

Material and methods

1. Isolation of strains with nitrogen fixing capacity

To isolate nitrogen-fixing bacteria, two methods were employed: microorganisms were isolated from plants grown in pots or from field soils.

In the first method, the pot experiment, specific plant species were grown in various soil types (Soil A, B, C, D and Compost) the same as described in Chapter 1 (Material and Methods, 1-Isolation). In this case the plants used for isolating nitrogen-fixing bacteria included *Solanum tuberosum* (potato), *Oryza sativa* (rice), *Beta vulgaris* (beetroot), *Triticum aestivum* (wheat), *Zea mays* (corn), *Cynodon dactylon* (grass), *Sorghum bicolor* (sorghum), *Olea europea* (olive tree), and *Vicia faba* (broad bean). The seeds were sown, and the pots were placed in a greenhouse at the Campus de l'Alimentació de Torribera from the Universitat de Barcelona (41°27'47.9"N 2°12'52.7"E). The plants received water through a drip irrigation for 2 minutes, three times a week. Isolation was carried out when the plants were fully developed and had set fruit, typically one or two months after planting, depending on the specific plant. Strains were isolated from rhizospheric and bulk soil as well as from plant roots.

In the second method, field soil samples were collected from various sampling points, specifically targeting areas where the plants of interest were growing. At each location, both bulk soil and plants with their rhizosphere were carefully gathered to isolate strains of interest. The sampling points included soil from Mediterranean forests, specifically from Tarradell (41°52'01.3"N, 2°17'21.7"E) and Canonges (42°11'52"N, 1°37'48"E); soil from conventional agricultural fields in Sevilla (37°29'49.0"N, 5°59'26.4"W) and Segarra (41°42'27.1"N, 1°28'18.3"E); soil and grass from an urban park in Lleida (41°37'43.3"N, 0°38'33.7"E); and from organic garden practicing regenerative agriculture in Bossòst (42°47'15"N, 0°41'37"E), and Collserola (41°26'44.0"N, 2°09'26.6"E). In Collserola, samples were taken from four closely located points: north (41°26'45.7"N, 2°09'28.4"E), south (41°26'43.4"N, 2°09'27.9"E), east (41°26'44.4"N, 2°09'29.0"E), and west (41°26'44.3"N, 2°09'24.7"E).

To isolate free-living bacteria and rhizosphere bacteria from bulk and rhizosphere soil Baldani et al., (2014) method was used, 10 g of soil were mixed with 90 mL of sterile saline solution (9 g NaCl L^{-1}). The mixture was then placed in a rotary shaker at 150 rpm for one hour. With the resulting aliquot three dilutions being 10^{-1}, 10^{-2}, 10^{-3} colony forming units (CFU) mL^{-1}, were prepared and placed in a selective semisolid media. To isolate nitrogen-fixing bacteria likely to be *Gluconacetobacter diazotrophic* and *Azospirillum brasilense* two types of selective semisolid media was used LGIP (Glucose 100 g L^{-1}, NH_4Cl 0.25 g L^{-1}, K_2HPO_4 0.25 g L^{-1}, $MgSO_4$ ($7H_2O$) 0.25 g L^{-1}, KH_2PO_4 0.6 g L^{-1}, $CaCl_2$ ($2H_2O$) 0.02 g L^{-1}, $FeCl_3$ ($6H_2O$) 0.01 g L^{-1}, Bromothymol

blue 0.5% 5 mL, Na_2MoO_4 ($2H_2O$) 0.002 g L^{-1}, and agar 1.75 g L^{-1}) and NFb (D-L Malic acid 5 g L^{-1}, NH_4Cl 0.2 g L^{-1}, K_2HPO_4 0.5 g L^{-1}, $MgSO_4$ ($7H_2O$) 0.2 g L^{-1}, NaCl 0.1 g L^{-1}, $CaCl_2$ ($2H_2O$) 0.02 g L^{-1}, 4 mL FeEDTA solution at 1.64%, 2 mL Bromothymol blue 0.5%, 2 mL microelement solution, and agar 1.75 g L^{-1}) respectively.

To isolate endophytic bacteria from roots, samples were sterilized in chloramine solution 1% (w/v) for 15 minutes, then dipped in sterilized distilled water for 5 min, in phosphate buffer (50 mM, pH 7) for 5 min and washed with sterilized distilled water for 5 more minutes. The roots, stems, and leaves were separated using a sterilized scalpel and transferred to a microtube, then grounded in a ball mill for 6 minutes at 3000 rpm, with 2-minute intervals. After grinding, 1 mL of saline solution was added, and the mixture was vortexed. The resulting mixture was let stand for 1 hour, then mixed with a rotary shaker for 5 min at 150 rpm and 100 µL were inoculated in the corresponding selective semisolid media. All microorganisms were grown in an incubator for 4 days at 30ºC and then placed in a fresh semisolid (LGIP and NFb) media for 4 more days, the different strains were then separated by placing them in a selective solid media enriched with yeast extract (0.5 g L^{-1}) and each colony morphology was inoculated in a new fresh semisolid media.

A total of 158 strains from 399 bacterial samples were selected as good candidates based on their pellicle growth in semisolid media and colony morphology, these strains were then coded and stored at -70ºC.

To isolate species of the genus *Azospirillum spp.* and *Azotobacter spp.* we followed the methodology proposed by Caceres (1982) and Martinez-Toledo et al. (1985), respectively. Soil and roots obtained from both methodologies (pot experiment and agricultural soil experiment) defined in the above section were used.

To isolate *Azospirillum spp.,* roots were previously washed twice and sterilized as described before. On the other hand, bulk and rhizospheric soil samples were weighed (2 g) and transferred to 18 mL of NFb semisolid media and placed in the incubator at 37ºC for 72 hours until a dense pellicle formed approximately 1-4 mm below the surface. Once the pellicle was grown, one mL of each sample was transferred to 9 mL of saline solution (9 NaCl g L^{-1}), and serial dilutions were made (10^{-1} to 10^{-5}) and placed in semi-selective solid media RC (K_2HPO_4 0.5 gL^{-1}, $MgSO_4(7H_2O)$ 0.2 g L^{-1}, NaCl 0.1 g L^{-1}, yeast extract 0.5 g L^{-1}, $FeCl_3(6H_2O)$ 0.015 g L^{-1}, DL-malic acid 5 g L^{-1}, KOH 4.8 g L^{-1} and agar 20 g L^{-1}). After 48 h, *Azospirillum spp.* colonies appeared as small pink colonies, turning scarlet after 72 h. These scarlet colonies were confirmed to be Gram negative using the potassium hydroxide (KOH) method (Silva Romeiro, 2001), involving placing a drop of 3% KOH and full loop of each colony on a glass slide. After 30 seconds, if no mucus appeared when the slide was separated about 3 cm from the glass plate it denoted Gram-negative bacteria.

Additionally, colonies were observed under the microscope (Olympus optical 50,60Hz) at 100x magnification with immersion liquid (MOIL- T02- 100 from Labkem S.L), revealing that *Azospirillum spp.* bacteria exhibited motility and a bacilli shape.

To isolate *Azotobacter* spp. soil and roots were transferred to tubes with 18 mL of liquid and semisolid Burk's media (glucose 5 g L^{-1}, K_2HPO_4 0.64 g L^{-1}, KH_2PO_4 0.16 g L^{-1}, NaCl 0.2 g L^{-1}, $MgSO_4(7H_2O)$ 0.2 g L^{-1}, $CaSO_4$ $(2H_2O)$ 0.05 g L^{-1}, $NaMoO_4$ $(2H_2O)$ 0.01 g L^{-1}, $FeSO_4$ 0.003 g L^{-1}) and agar 1.75 g L^{-1}. Tubes were left 48 hours at 28-30ºC at 150 rpm for liquid media, then 2 mL of the cloudy aliquots were transferred to a new Burk's liquid tube and left for an additional 48 hours at 28-30ºC. This process was repeated four times until a loop of the aliquot was transferred to Burk's solid plate and incubated at 28-30ºC for 48 hours until white mucous colonies appeared. These mucous colonies were confirmed to be Gram-negative using the KOH method (Silva Romeiro, 2001) and observed under the microscope (Olympus optical 50/60Hz) at 100x magnification with immersion liquid (MOIL- T02- 100 from Labkem S).

A total of 84 strains from 227 bacterial isolates were selected as good candidates based on their pellicle growth in semisolid media and colony morphology, these strains were then coded and stored at -70ºC.

2. *In vitro* analysis

2.1 Evaluation of nitrogen fixing capacity

2.1.1 Growth in nitrogen free medium

To assess the nitrogen-fixing capacity, 158 strains that grew in nitrogen-free media (indicating potential nitrogen fixation) and 84 colonies selected through semi-specific isolation protocols for *Azospirillum spp.* and *Azotobacter spp.* were evaluated. To assess the nitrogen-fixing capacity of isolated strains, an estimative method was employed based on measuring growth through the pellicle formed by these strains in a nitrogen-free semi-solid medium. Isolates were streaked on NBS growth medium and incubated for 48 hours at 30°C. Subsequently, a selected colony was picked and transferred to a tube containing semi-solid medium, NFb enriched with 0.02 % of NH_4Cl medium for other isolates, the colony was positioned on the medium surface and with Burk's medium used for *Azotobacter spp.* like colonies, and the tubes were covered before being incubated at 30°C. Measurements of the pellicle formed by each isolate were taken after 1, 3, and 5 days. The thickness of the pellicle was measured, and its appearance was recorded as very thin, thin, intense, or very intense with a score of 1, 2, 3 or 4, respectively. Based on these data, a value was generated by multiplying the thickness in centimetres by the score ranging from 1 to 4 corresponding to the appearance. These measurements allowed extrapolating greater growth to a

higher nitrogen-fixing capacity, thus creating a ranking of candidates with potentially higher nitrogen-fixing ability.

After performing this trial, 34 strains from the 158 strains that grew in nitrogen-free media and 29 strains from 84 selected through semi-specific isolation protocols for *Azospirillum spp.* and *Azotobacter spp.* were sent to the Laboratory of Instrumental Techniques at University of León, for taxonomical identification. Samples underwent either Matrix-assisted Laser Desorption/Ionization (MALDI-TOF) analysis or sequencing of the 16S genes.

2.1.2 Ammonium production

To assess the nitrogen-fixing ability of strains corresponding to the species *Azospirillum spp.* and *Azotobacter spp.,* we conducted an analysis of ammonia production in nitrogen-free liquid medium, specifically using Burk's medium for *Azotobacter spp.* and Nfb for *Azospirillum spp.*

The isolates were placed on NBS growth medium and incubated for 48 hours at 30°C. Subsequently, two selected colonies were picked and transferred to a tube containing 10 mL of nitrogen-free liquid medium, followed by vortexing for 5 seconds. This process was repeated three times for each strain. The tubes were then sealed and agitated for 72 hours at 140 rpm at 28°C. After the incubation period, tenfold dilution plates were prepared to determine the concentration of each bacterium.

For the ammonium measurement Sparks et al. (1996) methodology was applied. Several reagents were prepared as follows: Reagent A consisted of 7 g phenol and 34 mg sodium nitroprusside, made up to 100 mL with distilled water. Reagent B was prepared by mixing 1.48 g NaOH with 4.96 g $NaHPO_4$, adding 70 mL distilled water and 20 mL NaClO, and then adjusting the volume with distilled water to 100 mL. Reagent C was prepared by mixing Sodium EDTA and adjusting the pH to 7 with distilled water. Additionally, a standard solution (1000 ppm NH_4) was prepared using 2.97 g ammonium and diluted to 100 mL with distilled water. This standard solution was further diluted to create a stock solution of 1 ppm to construct a standard curve of 0, 0.5, 1, 1.5, 2, and 2.5 ppm. For sample preparation, 2 mL aliquots of the growth media of each microorganism cultivated for 72 h were centrifuged at 800 rpm for 10 minutes, and 1 mL of the supernatant was transferred to a test tube to create the samples, subsequently, under an extractor cabinet 0.5 mL of each sample was mixed with 2 mL of distilled water with 0.5 mL of Reagent C, 1 mL of Reagent A, and 2 mL of Reagent B. The tubes were left in darkness for 30 minutes, after which the optical density was measured at 636 nm using a spectrophotometer. The resulting colour was blue and remained stable for 20 minutes. The ammonium concentration in the control without inoculation was subtracted from the concentration measured in the inoculated samples.

2.2 Phosphorus solubilizing capacity

2.2.1 Mineral phosphorus solubilizing in National Botanical Research Institute's Phosphate medium

To assess the capacity to mobilize phosphorus by the nitrogen-fixing bacteria isolates, these were cultivated in liquid media National Botanical Research Institute's Phosphate (NBRIP) composed of glucose (10 g L^{-1}), $MgCl_2.6H_2O$ (5 g L^{-1}), $MgCl_2.7H_2O$ (0.25 g L^{-1}), KCl (0.2 g L^{-1}), $(NH_4)_2SO4$ (0.1 g L^{-1}) with a pH adjusted to 7 (Nautiyal, 1999) enriched with tree different forms of insoluble phosphate at 0.23 g L^{-1} of hydroxyapatite, phosphate rock, tri-calcium phosphate and iron phosphate (III).

First, the nitrogen-fixing bacteria were tested to check if they could survive in liquid NBRIP media. Then each microorganism strain was cultivated in triplicate in NBRIP with the different phosphate sources. The inoculation was performed by introducing one loop in 10 mL of each NBRIP medium and placed in a rotary shaker (150 rpm) at 30ºC. In addition, a control (NBRIP with insoluble phosphate) without inoculation was included in this experiment in triplicate. After 3, 5 and 7 days 2 mL were extracted from each sample and passed through a cellulose nitrate filter of 0.22 µm pore size. To calculate the solubilized phosphate produced on the medium the filtered solutions were processed according to the protocol Murphy & Riley (1962) where the molybdate reactive P was measured. The standard curve was prepared with KH_2PO_4 and the absorbance was measured at 882 nm. On the last sampling (7 days after the inoculation of the growth medium), the pH was also measured and serial dilutions from (10^{-5} to 10^{-7}) were made to determine the final concentration of the bacteria.

2.2.2 Organic acids production

Nitrogen-fixing bacterial isolates were cultured in liquid NBRIP media containing hydroxyapatite at a concentration of 0.23 g L^{-1}. Inoculation was performed by introducing a full loop into a tube with 15 mL of NBRIP and placing it on a rotary shaker (150 rpm) at 30ºC. Additionally, a control without inoculation was included in triplicate for this experiment. After 7 days, 2 mL were extracted from each sample and passed through a cellulose nitrate filter with a pore size of 0.45 µm. The remaining sample was used to assess the concentration of microorganisms in each tube and their pH.

To quantify the production of organic acids (citric, malic, acetic, succinic, gluconic, lactic, oxalic, fumaric, propionic, and glycolic acids) produced by de isolates, the Furlani et al., (2006) method was used. Samples were analysed by an HPLC with an Aminex HPX-87H column (300 mm x 7.8 mm) from BioRad equipped with a diode array detector (UV) at 210 nm. The eluent used was H_2SO_4 0.01 M, with a flow rate of 0.8 ml min^{-1} at a temperature of 60 ºC. Peaks that presented an

absorbance spectrum incompatible with that typical of organic acid spectrum were not considered. The filtered NBRIP samples (inoculated or not with the bacteria) were injected in the HPLC system (100 µL) and peaks were selected based on the retention times and spectra of the standards of each organic acid. Once the detection peaks were obtained, the areas were obtained, and the concentration of organic acids for each sample was calculated based on the calibration curves prepared with the standards.

2.3 Statistical analysis

All data were analysed using the SPSS software package version 27.0. For the assessment of biochemical capacities *in vitro*, regarding ammonium production, and organic acids quantification, were analysed by a one-way ANOVA and Tukey's post-hoc test with a significance level of 95%, considering as factor each strain. Additionally, a Pearson correlation analysis was performed between the concentration of these strains and ammonium production, with a significance level of 95%. Data from molybdate reactive phosphate content was analysed by a three-way factorial repeated measures ANOVA followed by Tukey's post-hoc test at significance levels of p-value < 0.05, considering as factors days after inoculation, strain and insoluble phosphorous sources (hydroxyapatite, tricalcium phosphate and phosphate rock) and the interaction between factors.

Results

1. Isolation and analysis of soil samples

1.1 Isolation from soil and plants grown in pots

Soils used isolate microorganisms are characterized in Table 1 and described in Chapter 1 (Results, 1-Isolation and analysis of soil samples).

Table 1 Characterization of soil samples for nitrogen-fixing bacteria isolation

Soil Type	pH	EC[a] (dS m^{-1})	OM[a] (%)	$CaCO_3$[a] (%)	$N\text{-}NO_3$[a] (mg kg^{-1})	Texture
Soil A	7.90	0.461	2.98	8	28	Loam-Clay-Sandy
Soil B	7.93	0.427	2.50	34	7	Loam- Sandy
Soil C	7.13	0.530	4.60	<5	69	Loam-Clay-Sandy
Soil D	8.00	0.528	2.78	26	14	Loam-Clay-Sandy
Compost	9.10	3	6.3	7	18	-

[a] Parameters analysed meaning; EC to electrical conductivity, OM to oxidable organic matter, $CaCO_3$ to calcium carbonate equivalent and $N\text{-}NO_3$ to nitrate content

Figure 1 illustrates the percentage of nitrogen-fixing isolates selected being the best grown in nitrogen free medium and stored (34 total) obtained from the rhizosphere of potted plants according to each soil type and plant species. Concerning soil type (Figure 1A), most nitrogen fixers were isolated from Soil A constituting 32.96 % of the isolates. The soil type that yielded the fewest nitrogen fixers was Compost (14.48 %), but values are fairly balanced across different soil types. Regarding the origin of the isolated microorganisms (Figure 1B), all microorganisms were isolated from the rhizosphere of plants; no microorganisms were isolated from bulk soil or endophytic microorganisms through this methodology. The microorganisms that were isolated were associated with the rhizosphere of various plants, as summarized in Figure 1B. Predominantly, nitrogen-fixing microorganisms were obtained from the rhizosphere of gramineous plants, comprising 27.47 % from grass and 23.08 % from rice, followed by maize with 13.74 % of isolates. Sorghum contributed only a small proportion of selected microorganisms, accounting for merely 3.85 % of isolates. Interestingly, even in the rhizosphere of beans, the presence of nitrogen-fixing organisms was scarce, constituting only 3.85 %.

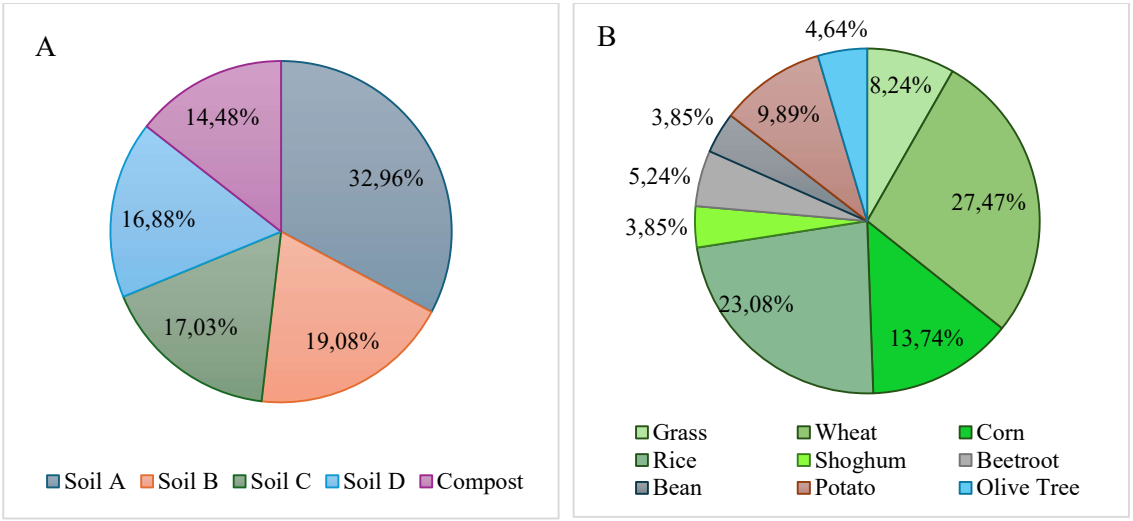

Figure 1: Percentage of nitrogen-fixing isolates obtained from the rhizosphere of potted plants according to: (A) different soil types and (B) plants species.

1.2 Isolation from soil and plants grown in fields

The selected soils were evaluated based on reference values from Chapman & Pratt (1973). Concerning pH of the various soils sampled, levels ranged from neutral to alkaline, varying from 6.6 in Tarradell soil to 8.3 in Sevilla and Segarra soils (Table 2). In terms of EC, the analysed soils are predominantly non-saline (0-2 dS m^{-1}), except for the soil from Lleida which falls into the slightly saline category (2.3 dS m^{-1}). It was observed variability in organic matter among the different soil types; the levels are relatively low in Segarra (2.19 %), Sevilla (3.14 %), and West Collserola (3.22 %), while they are high in Lleida (10.76 %), Canonges (10.02 %), East Collserola

(9.65 %), and North Collserola (9.36 %), the remaining values fall within the moderate range (Table 2).

In relation to calcium carbonate ($CaCO_3$) content, values are highly variable, with low levels in Tarradell and Bossòst soils (0 and < 3 %, respectively), normal levels in soils from West, South and East Collserola (7.06, 9.49 and 14.10 %, respectively), and elevated levels in the other soil types. Additionally, nitrate ($N\text{-}NO_3$) levels are high in all soil types except for soil from West Collserola (9.6 mg kg^{-1}), which is considered to have adequate values and in reference to sodium content (Na), low values (< 46 mg kg^{-1}) are observed in Canonges, West Collserola, Tarradell and Bossòst soils, being high (230- 690 mg kg^{-1}) in Lleida soil and moderated (46-230 mg kg^{-1}) in all other soil types. Regarding soil texture, it was observed that almost all of them contained a combination of loam and sandy textures, except for Tarradell soil, which was sandy, and Sevilla soil, classified as loam-clay (Table 2).

Table 2 Characterization of soil samples for nitrogen-fixing bacteria isolation

Soil Type	pH	EC[a] (dS m^{-1})	OM[a] (%)	$CaCO_3$[a] (%)	$N\text{-}NO_3$[a] (mg kg^{-1})	Na[a] (mg kg^{-1})	Texture
South Collserola	7.7	0.25	6.82	9.40	27	55	Loam
North Collserola	7.8	0.30	9.36	20.17	38	69	Loam-Sandy
East Collserola	7.6	0.43	9.65	14.10	110	131	Loam-Sandy
West Collserola	8.1	0.16	3.22	7.06	9.6	28	Loam-Sandy
Tarradell	6.6	0.34	5.94	0	160	15	Sandy
Canonges	7.7	0.60	10.02	29.54	> 200	41	Loam-Sandy
Sevilla	8.3	0.36	3.14	41.64	28	147	Loam-Clay
Segarra	8.3	0.27	2.19	32.34	29	48	-
Lleida	7.5	2.30	10.76	31.55	>200	323	-
Bossòst	7.2	0.44	5.19	< 3	190	13	-

[a] Parameters analysed meaning; EC to electrical conductivity, OM to oxidable organic matter, $CaCO_3$ to calcium carbonate equivalent, $N\text{-}NO_3$ to nitrate content and Na to sodium content

Figure 2 illustrates the percentage of nitrogen-fixing isolates selected being the best grown in nitrogen free medium and stored (29 in total) from each agricultural field location and whether it was isolated from the bulk soil or the rhizosphere of the plant. Examining Figure 2A the majority of nitrogen-fixing bacteria were isolated from Tarradell soil constituting 17.88% of the total isolates, and from South Collserola soil accounting for 15.53% of the total isolates. Both locations exhibited moderate to high concentrations of organic matter (MO) and low concentrations of calcium carbonate ($CaCO_3$). Following closely, 12% of the isolates were found in Lleida a soil rich in high electrical conductivity (EC), oxidizable organic matter, and nutrient concentrations ($CaCO_3$, $N\text{-}NO_3$, and Na). Among the other types of soil used for isolation, the relative percentage is evenly distributed. When examining whether the isolates were free-living or associated with the rhizosphere (Figure 2B), we observed that the majority of the isolates were associated with

the plants (83%). Among these, 16% were endophytes and 84% were part of the soil attached to the roots (rhizosphere). Additionally, most nitrogen-fixing isolates were obtained from meadows where grass was growing, constituting 37.68% of the isolates, following this, a significant proportion of nitrogen-fixing isolates (15%) were free-living, isolated directly form the soil. In third place, in the rhizosphere of wheat, sweet potato, and beans, an average of 5- 13% of the isolates were obtained. The percentage of isolates in the rhizosphere of other analysed crops was much lower.

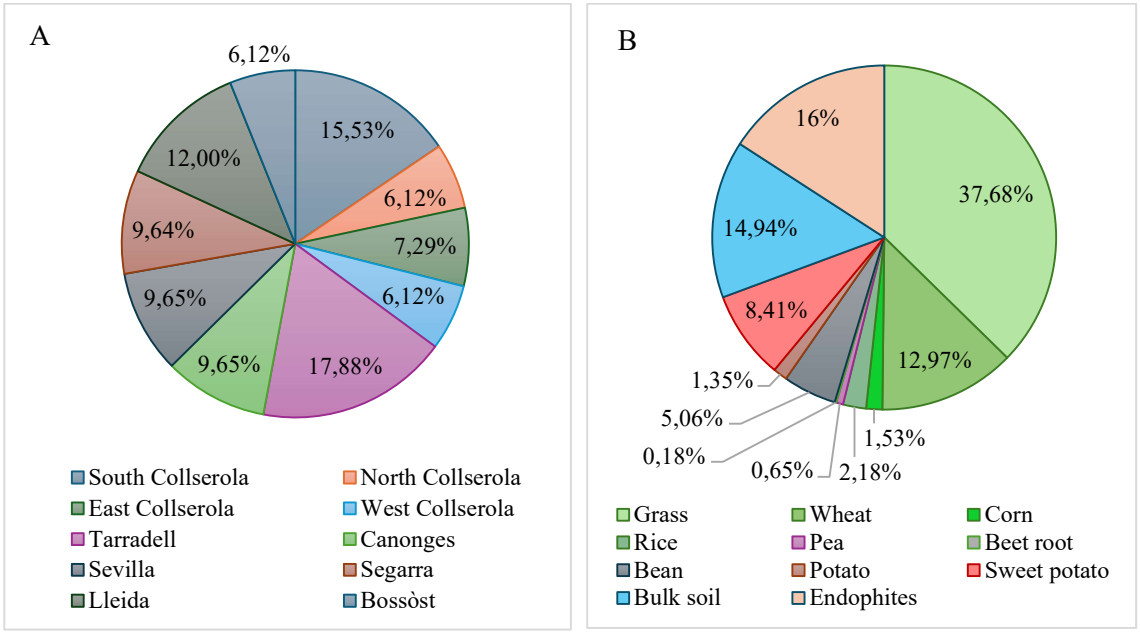

Figure 2: Percentage of nitrogen-fixing isolates distribution in: (A) different soil types from which microorganisms were isolated (B) site from the rhizosphere of plants.

1.3 Selected *Azospirillum spp.* and *Azotobacter spp.* strains

After sequencing of the 16S gene and MALDI-TOF analysis, 9 and 4 isolates were identified as belonging to the species *Azospirillum spp* and *Azotobacter spp.,* respectively form the 63 strains selected and stored. The most effective method for isolating these species was from agricultural field crops (76.92 %), compared to isolation from plant pots (23.08%) (Table 3). On one hand, from agricultural field crops, Collserola soils had the highest percentage of *Azospirillum spp.*, constituting 30.8 % of the total isolates selected (21F221, 21F222, 21F224, and 21F226). The soil with the highest percentage of *Azotobacter spp.* isolated was the Sevilla soil, representing 15.4% of the total isolates selected (21F200 and 21F201). The majority of the selected *Azospirillum spp*. were found in the rhizosphere of plants (67%) compared to free-living forms, and the majority were associated with grass rhizosphere (21F220, 21F221, 21F224, and 21F226). All *Azotobacter spp.* were associated with plant rhizosphere with a 50 % associated with maize and the other 50 % associated with wheat. On the other hand, regarding the potted soil method only three microorganisms from the selected genus were isolated, one from Soil D (*Azotobacter*

chroococcum strain 21F209) from wheat rhizosphere and two from Soil B (*Azospirillum brasilense* strain 21F210 and *Azotobacter salinestris* strain 21F213) from soil rhizosphere of wheat and in bulk soil, respectively.

Table 3 Strains of *Azospirillum spp* and *Azotobacter spp* isolated and taxonomically identified.

ID[a]	Specie	Soil Type	Isolation site
21F200	*Azotobacter salinestris*	Sevillla	Rhizosphere (maize)
21F201	*Azotobacter salinestris*	Sevilla	Rhizosphere (maize)
21F203	*Azospirillum brasilense*	Segarra	Rhizosphere (wheat)
21F205	*Azospirillum brasilense*	Canonges	Bulk soil
21F209	*Azotobacter chroococcum*	Soil D	Rhizosphere (wheat)
21F210	*Azospirillum brasilense*	Soil B	Bulk soil
21F213	*Azotobacter salinestris*	Soil B	Rhizosphere (wheat)
21F220	*Azospirillum oryzae*	Lleida	Rhizosphere (grass)
21F221	*Azospirillum brasilense*	South Collserola	Rhizosphere (grass)
21F222	*Azospirillum brasilense*	West Collserola	Rhizosphere (maize)
21F224	*Azospirillum oryzae*	East Collserola	Rhizosphere (grass)
21F226	*Azospirillum aestuarii*	North Collserola	Rhizosphere (grass)
21F227	*Azospirillum sp.*	Tarradell	Bulk soil

[a] Identification of the strains

2. *In vitro* analysis

2.1 Evaluation of nitrogen fixing capacity by ammonium production

The nitrogen fixing capacity of the isolates was measured by ammonium production *in vitro* being the most effective ammonium producers *Azospirillum spp*. strains 21F221 and 21F226, followed by strains 21F224 and 21F227 (Table 4). While *Azotobacter spp.* exhibited lower ammonium production values.

Figure 3 illustrates that the *Azospirillum spp.* strains that exhibited higher ammonium production also showed a high concentration after 72 hours, being 21F221, 21F224, 21F226, and 21F227 indicated by the upper-right cluster of white data points on the graph. These findings suggest a strong and significant relationship ($\rho = 0.611$, p-value < 0.001) between the bacterial concentration of the isolated strains and their biochemical capacity for ammonium production, supporting their potential in nitrogen-fixing capacity.

Table 4 Ammonium production capacity and microorganism concentration after 72h

ID	Ammonium (μg mL^{-1})	Concentration ($\cdot 10^8$ CFU mL^{-1})
21F200	22.14 ± 1.5 a	1.13 ± 0.76 ab
21F201	24.98 ± 2.4 a	1.20 ± 0.44 ab
21F203	31.93 ± 7.4 ab	1.70 ± 0.29 ab
21F205	6.76 ± 0.6 a	1.93 ± 0.43 ab
21F209	23.47 ± 1.6 a	0.49 ± 0.16 a
21F210	18.42 ± 2.5 a	2.64 ± 0.16 ab
21F213	21.61 ± 1.4 a	1.22 ± 0.55 ab
21F220	29.48 ± 3.1 ab	1.94 ± 0.18 ab
21F221	**72.06 ± 7.5 c**	3.73 ± 0.16 ab
21F222	18.01 ± 2.9 a	0.68 ± 0.08 ab
21F224	**58.55 ± 13.0 bc**	2.15 ± 0.21 ab
21F226	**69.60 ± 10.7 c**	**3.36 ± 0.44 b**
21F227	**56.50 ± 0.7 bc**	1.99 ± 0.12 ab

Values are means ± SE (n=3). Values marked with different letters are significantly different (p-value < 0.05), determined through a one-way ANOVA and Tukey's analysis test.

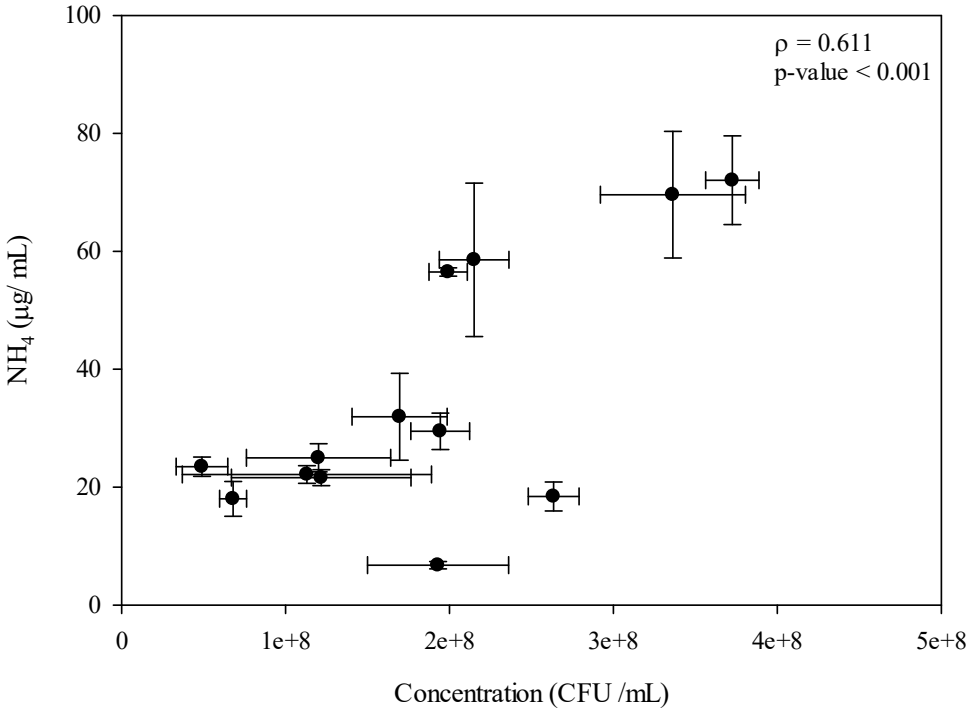

Figure 3: Scatterplot depicting *Azospirillum spp*. and *Azotobacter spp.* strains (black points in the graphic), where the x-axis represents the concentration of the different strains, and the y-axis represents the ammonium concentration produced in nitrogen-free media (Burk's or Nfb) after 72h.

Values are means ± SE (n=3) and analysed by Pearson's correlation value (ρ value) being a positive correlation between parameters (p-vale < 0.05)

2.2 Phosphorus solubilizing capacity

2.2.1 Phosphorus solubilization in National Botanical Research Institute's Phosphate medium

The soluble phosphate concentration (molybdate-reactive P), calculated by subtracting the values of the non-inoculated media from the inoculated media, shows significant differences obtained by a three-way ANOVA, between days after inoculation, treatments and insoluble phosphorus sources, as well as, in all the doble interactions (Table 5).

The increase in soluble phosphate concentration, was notably pronounced in the media enriched with hydroxyapatite and tricalcium phosphate when compared to those with phosphate rock, except for the medium inoculated with 21F201 with higher values in phosphate rock solubilization compared to tricalcium phosphate (Table 5). This variation among insoluble P sources could be attributed to the natural leaching of phosphorus by phosphate rock, as in the non-inoculated medium containing phosphate rock, as 3, 5, and 7 days passed, the concentration of molybdate-reactive phosphorus increased by 35.51 µg mL^{-1}, 41.91 µg mL^{-1}, and 44.30 µg mL^{-1} respective. In contrast, for hydroxyapatite, the baseline values of soluble phosphorus in media without inoculation remained within the range of 2.16-3.21 µg mL^{-1}.

The microorganism with a higher phosphorus solubilization capacity *in vitro* was *Azotobacter salinestris* strain 21F213, this was observed across all three insoluble substrates (hydroxyapatite, tricalcium phosphate, and phosphate rock) over 3, 5 and 7 days after inoculation. With the most significant increase occurring in the hydroxyapatite medium after 3 days post inoculation (Table 5), however, the highest solubilization in tricalcium phosphate was observed after 5 days, and in phosphate rock after 7 days. Additionally, some differences are observed in the solubilization of hydroxyapatite after 7 days and phosphate rock after 3 days by strain 21F200, as well as phosphate rock solubilization by strain 21F201 after 3 days.

Table 5 Phosphorus solubilization supplied as various forms of insoluble P by *Azospirillum* and *Azotobacter spp.* isolates in *in vitro* conditions

ID[a]	Days after inocuation	Phosphate solubilizing capacity (µg molybdate reactive P mL^{-1})					
		Hydroxyapatite		Tricalcium Phosphate		Phosphate Rock	
21F200	3	33.33 ± 2.8	cαA	0.00	aαA	**11.28 ± 0.3**	**bβB**
	5	53.3 ± 3.2	bβA	40.29 ± 9.6	abαA	9.55 ± 0.9	aβA
	7	**147.38 ± 7.2**	**bγB**	38.09 ± 35.8	aα	0.00	aαA
21F201	3	20.07 ± 5.2	bαA	0.00	aαA	**15.57 ± 2.7**	**bαB**
	5	29.49 ± 0.8	cβA	0.00	aαA	10.64 ± 2	bαA
	7	39.15 ± 4.8	bβA	0.00	aαA	15.01 ± 8.4	bαAB
21F203	3	4.27 ± 0.1	bαA	0.00	aαA	0.00	aαA
	5	12.64 ± 1.6	bβA	0.00	aαA	3.6 ± 5	bαA
	7	18.14 ± 7.3	bβA	0.00	aαA	39.91 ± 7	bβAB
21F205	3	3.39 ± 0.4	aαA	95.68 ± 7.2	bαA	0.92 ± 2.6	aαA
	5	3.42 ± 0.3	aαβA	120.47 ± 13.9	bβA	5.22 ± 2.5	aβA
	7	13.78 ± 2.5	abβA	92.58 ± 2.2	bαAB	9.7 ± 2.4	aβAB
21F209	3	14.04 ± 1.3	bαA	19.41 ± 1.4	cαA	0.00	aαA
	5	34.76 ± 2.6	bβA	53.22 ± 1.1	cβAB	0.00	aαA
	7	67.35 ± 4.2	bβAB	77.42 ± 4	bβAB	27.18 ± 25	aβAB
21F210	3	4.3 ± 0.3	aαA	4.18 ± 0	aαA	6.16 ± 4.7	aαAB
	5	4.48 ± 0.4	aαβA	3.56 ± 1	aαβA	18.49 ± 9.2	aαβA
	7	15.8 ± 1.1	aβA	7.99 ± 4.9	aβA	25.08 ± 2.6	bβAB
21F213	3	**119.54 ± 1.8**	**bαB**	10.45 ± 1.3	aαA	6.41 ± 1.2	aαAB
	5	**145.31 ± 10.7**	**aαB**	**108.28 ± 43.6**	**aβB**	42.47 ± 32	aαβA
	7	**179.5 ± 12.3**	**aαB**	**156.27 ± 4.9**	**aβB**	**91.44 ± 9.9**	**aβB**
21F220	3	12 ± 2.4	aαA	4.29 ± 1.1	aαA	3.48 ± 1	aβAB
	5	46.93 ± 4.7	bβAB	13.95 ± 1.7	abβA	0.00	aαA
	7	20.07 ± 5.2	aαA	18.21 ± 10.1	aβAB	0.91 ± 2.7	aαA
21F221	3	28.33 ± 3.6	bαA	7.24 ± 0.6	aαA	2.81 ± 1	aαAB
	5	34.67 ± 4.2	bαA	7.79 ± 2.7	aαA	5.3 ± 3.3	aαA
	7	89.2 ± 29.2	bαAB	9.03 ± 19.2	aαA	0.00	aαA
21F222	3	0.00	aαA	1.27 ± 0.1	aαA	1.74 ± 0.5	aαA
	5	41.7 ± 2.4	bαβA	30.36 ± 21.1	bαβA	4.07 ± 1.8	aαβA
	7	22.95 ± 7.8	bβAB	23.16 ± 2.9	bβAB	5.4 ± 0.6	aβA
21F224	3	22.95 ± 7.8	bαA	32.89 ± 6.1	bαA	0.79 ± 1.2	aαA
	5	39.29 ± 2.9	bαβA	54.21 ± 2	bαβAB	1.32 ± 1.4	aαβA
	7	89.2 ± 29.2	bβAB	52.42 ± 1	bβAB	4.84 ± 7.4	aβA
21F226	3	24.29 ± 2.3	bαA	52.42 ± 1	bαA	0.00	aαA
	5	20.27 ± 3.9	bαA	16.67 ± 1.7	bαA	0.00	aαA

	7	19.88 ± 2.7	bαA	25.23 ± 1.7	bαAB	0.86 ± 0	aαA
21F227	3	22.14 ± 1.9	bαA	22.03 ± 1.4	bαA	4.38 ± 2.3	aαAB
	5	31.93 ± 3.6	bαA	27.61 ± 1.4	bαA	3.51 ± 2	aαA
	7	45.24 ± 7.2	bαAB	32.05 ± 1.7	bαAB	6.8 ± 1.6	aαA

Values are means ± SE (n=3). A three-way factorial ANOVA was performed and Tukey's tests (p-value<0.05); lowercase letters (a, b) correspond to differences among phosphorus sources, greek letters (α, β) differences between time and capital letters (A, B) between strains determined through an ANOVA and Tukey's test (p-value < 0.05). Significant differences were obtained in all factors analysed (phosphorus sources, time and strains) as well as, in double interactions (time*sources; time*strains, sources*strains).

[a] Identification of the strains as follow: *Azospirillum brasilense* (strains 21F203, 21F205, 21F210, 21F221, 21F222), *Azospirillum aestuarii* (21F226), *Azospirillum spp.* (strain 21F227), *Azotobacter salinestris* (strains 21F200, 21F201, and 213), *Azotobacter chroococcum* (21F209) and *Azotobacter oryzae* (strains 21F220 and 21F224)

2.1.2 Organic acids production

Table 6 presents the data on the production of organic acids by isolated nitrogen-fixing microorganisms (*Azospirillum spp*. and *Azotobacter spp.*) in relation to the pH reduction of the NBRIP medium enriched with hydroxyapatite, with an initial pH of 6.8 ± 0.1. In this scenario, *Azotobacter spp.* exhibited a more pronounced decrease in pH compared to *Azospirillum spp*. Specifically, two *Azotobacter salinestris* strains, 21F200 and 21F213 demonstrated the most substantial reduction with 44 % and 37 %, respectively, transforming the pH from 6.8 to 3.8 and 4.3 respectively. Upon examining the specific concentration of organic acids by these two strains, they exhibit high production of gluconic, glucuronic and acetic acid, with acetic acid concentration being the highest. Furthermore, the production of other types of acids such as of malic acid is also shown in *Azotobacter salinestris* 21F213 strain and oxalic acid in *Azotobacter salinestris* strain 21F200.

Table 6 Concentration of the isolates, pH and organic acids production by *Azotobacter spp.* and *Azospirillum spp.* cultivated in National Botanical Research Institute's phosphate medium enriched with hydroxyapatite for 72 h.

ID[a]	Concentration (x10^8 CFU mL^{-1})	pH[b]	Organic acids production (µg mL^{-1})						
			Malic	Gluconic	Oxalic	Glucuronic	Glicolic	Acetic	Lactic
21F200	2.76 ± 1.6	**3.8 a**	0 ± 0.2 a	**50.4 ± 9.3 b**	**10.20 ± 3.4 b**	**151.90 ± 5.4 b**	**-**	**501.82 ± 58 b**	**-**
21F201	6.45 ± 0.5	**4.7 b**	-	-	-	-	-	-	-
21F203	1.56 ± 1.3	6.6 de	-	-	-	-	-	-	9.50 ± 1.4 a
21F205	0.94 ± 0.3	6.0 cd	0.17 ± 0.9 a	0.02 ± 0.01 a	0.37 ± 0.04 a	-	-	9.86 ± 1.8 a	-
21F209	7.53 ± 0.6	**4.8 b**	-	-	-	-	-	-	-
21F210	0.80 ± 0.1	6.5 cde	-	0.05 ± 0.04 a	-	-	3.57 ± 0.5 a	-	-
21F213	5.12 ± 0.3	**4.3 ab**	**465.8 ± 74 b**	**53.8 ± 8.4 b**	**-**	**246.3 ± 54 b**	**-**	**460.05 ± 56 b**	**-**
21F220	0.0005 ± 0.0003	6.7 de	1.14 ± 0.8 a	-	-	-	-	-	-
21F221	0.01 ± 0	6.3 cde	-	-	2.35 ± 0.04 a	17.60 ± 10.2 a	-	14.57 ± 2 a	-
21F222	0.001 ± 0	6.8 e	-	-	0.53 ± 0.4 a	-	4.42 ± 0.3 a	-	-
21F224	0.57 ± 0.4	6.0 c	50.54 ± 21 a	0.46 ± 0.3 a	-	-	16.0 ± 6.2 a	-	-
21F226	0.57 ± 0.4	6.6 cde	-	-	-	-	-	-	-
21F227	1.32 ± 0	6.4 cde	73.80 ± 17 a	-	-	47.21 ± 7.5 a	-	-	-

Values are means ± SE (n=3). Values marked with a letter are significantly different values between isolates measured in each organic acid produced (p-value < 0.05), determined through a one-way ANOVA and Tukey's analysis test.

[a] Identification of the strains as follow: *Azospirillum brasilense* (strains 21F203, 21F205, 21F210, 21F221, 21F222), *Azospirillum aestuarii* (21F226) *Azospirillum spp.* (strain 21F227), *Azotobacter salinestris* (strains 21F200, 21F201, and 213), *Azotobacter chroococcum* (21F209) and *Azotobacter oryzae* (strains 21F220 and 21F224)

[b] pH referenced to pH non-inoculated media (6.8)

Discussion

1. Nitrogen-fixing isolate sampling site

Recent studies have demonstrated that soil properties significantly influence microbial populations, including nitrogen-fixing bacteria (Fierer, 2017). In our experiment the higher concentration of nitrogen-fixing bacteria isolated from plants cultivated in potted soil was observed in Soil A which was characterized by an alkaline pH and a moderate concentration of organic matter and nutrients (calcium carbonate, and nitrate). As highlighted by Inomura et al., (2020) maintaining an optimal nutrient balance can foster a favourable environment for microbial activity, encompassing nitrogen-fixing bacteria. Similarly, all three soil types (A, B and D) which generated the highest percentage of nitrogen-fixing bacteria isolates, had the presence of clay particles. As proposed by Liu et al., (2016), the inclusion of clay in the soil improves water and nutrient retention, ultimately enhancing soil structure which leads to a higher concentration of microorganisms, and consequently, nitrogen-fixing bacteria.

In our study, we observed that field soil samples yielded a higher number of nitrogen-fixing strains compared to other potted soil samples. Notably, soils from Taradell and Lleida, which are undisturbed agroecosystems, demonstrated this trend. Undisturbed soils, such as those in forest ecosystems, tend to promote extensive colonization of the rhizosphere, potentially leading to greater diversity within the bacterial community (Dong et al., 2019). Additionally, the soil from South Collserola, which also yielded a high number of nitrogen-fixing bacteria, is managed under regenerative agricultural practice, this include avoiding tillage and incorporating carbon-rich compost, which are known to increase the soil's carbon content (Schmidt & Martínez, 2019) and higher carbon content in soil promotes microbial growth (Khanghahi et al., 2019), likely explaining the increased presence of nitrogen-fixing bacteria.

In the context of isolation areas, most of the identified nitrogen-fixing organisms were predominantly located in the rhizosphere of non-leguminous plants, being the 100% of the isolates from plant pots and the 69% isolates from field samples while the others were endophytes isolated from the root interior (16%) or not associated with any rhizosphere (15%) aligning with the findings described by Elmerich & Newton (2007) and Neyra & Dobereiner (1977) respectively.

The majority of nitrogen-fixing bacteria were isolated from gramineous plants, predominantly from maize and grass rhizosphere samples, associations extensively studied in both natural and cultivated ecosystems (Baldani & Baldani, 2005). These findings are supported with specific references; where nitrogen-fixing bacteria associated with maize plants is studied by Castellano-Hinojosa et al., (2018); Pelapudi et al., (2021) and Zahid et al., (2015), also the presence in grass rhizosphere correlates with previous studies like the ones performed by Wright & Weaver, (1981)

and Nelson et al., (1977), as well as research on the association with other perennial grasses such as dune grasses (Dalton et al., 2004) or switchgrass (Bahulikar et al., 2014).

With regards to the specific isolation of *Azospirillum spp.* and *Azotobacter spp.,* Ahmad et al., (2008) and El Zemrany et al., (2007) meticulously categorize them as nitrogen-fixing bacteria with associative and endophytic attributes in plants. This classification supports our findings, where these genera were effectively isolated from the soil rhizosphere in association with various grass and cereal crops, such as maize and wheat. Numerous studies in the literature have documented the isolation of *Azospirillum spp*. from maize, grass, and wheat (Eckert et al., 2001; Mehnaz et al., 2007; Stets et al., 2015) along with *Azotobacter spp*. isolated from maize and wheat (Martinez-Toledo et al., 1985; Qaisrani et al., 2019; Stets et al., 2015). Additionally, it is important to highlight that some strains were also isolated from bulk soil, verifying that *Azospirillum spp.* species can also live independently of plant association, thus classifying them as free-living N_2-fixing diazotrophs (Jehani et al., 2023 and Steenhoudt & Vanderleyden, 2000) alongside *Azotobacter* species (Ahmad et al., 2008; Fallah et al., 2023 and Wang et al., 2020).

2. Assessment of biochemical capacities *in vitro*

Our study aligns with the extensive literature supporting the nitrogen-fixing capacity of *Azospirillum spp*. (Cassán & Diaz-Zorita, 2016; Okon & Labandera-Gonzalez, 1994.; Steenhoudt & Vanderleyden, 2000) and *Azotobacter spp.* (Prajapati et al., 1970; Boddey et al., 1995; Sumbul et al., 2020) specifically, *Azospirillum brasilense's* nitrogen-fixing ability *in vitro* is well-documented through other methods, as detailed in Okon et al., (1983) and Wang et al., (2017) and similarly, the nitrogen-fixing capacity of *Azospirillum oryzae* has been described by Xie & Yokota, (2005). However, while these and other studies on *Azospirillum spp.* and *Azotobacter spp.* species do not determine the nitrogen-fixing capacity through ammonium released in the medium, this technique has been used for other nitrogen-fixing species such as *Stenotrophomonas maltophilia, Kosakonia oryzae,* and *Acinetobacter pittii,* yielding similar ammonium production ranges, 42.06, 35.48, and 18.33 $\mu g\ mL^{-1}$, respectively (Cordova-Rodriguez et al., 2022).

While ammonium measurement may suggest nitrogen-fixing activity, it's crucial to recognize that it does not directly quantify the amount of fixed nitrogen, as it does not account for the nitrogen used by bacteria for their own growth (Cordova-Rodriguez et al., 2022; Das & De, 2018). In our study, the measurement of nitrogen fixation was not aimed at direct quantification, but rather at detecting the fixing capacity, and ultimately, and most importantly, evaluating its associated effect on plant development and nitrogen use efficiency. This effect on plant development and nitrogen use efficiency is studied and discussed in the following chapter (Chapter 3).

It is also crucial to acknowledge that while several studies have indicated a potential increase in nitrogen availability to plants through nitrogen fixation by *Azospirillum spp.,* the boosted yield of inoculated plants is predominantly linked to enhancements in root development and the efficiency of water and mineral absorption by roots, with a lesser contribution from biological nitrogen fixation (Bashan & de-Bashan, 2010; Kennedy et al., 1997; Okon et al., 1983; Giller, 2003). Hence in our study, despite conducting *in vitro* measurements, all isolates were deemed suitable candidates for evaluation in plants; however, it is important to note that some isolates performed better than others in *in vitro* conditions, indicating various degrees in their effectiveness and providing a valuable ranking that supports their potential for plant application. Therefore, to gain a clearer understanding of these strains' capabilities, additional tests in plant systems were performed, which will allow us to assess the actual impact of bacterial strains on plant growth and determine their effectiveness as plant growth-promoting rhizobacteria (PGPR) and their potential application as microbial biostimulants, according to the European fertilizers regulation.

In the context of *Azotobacter's* biochemical capacities *in vitro*, previous studies have predominantly focused on its biological nitrogen fixation, although some researchers have delved into its potential to solubilize phosphate compounds (Aasfar et al., 2021; Cheng et al., 2023; Farajzadeh et al., 2012; Garg et al., 2001; Kumar et al., 1999), as highlighted in our research. Specifically, *Azotobacter salinestris* has been noted in a few articles as a phosphorus solubilizer, consistent with our findings with strain 21F213 (Chennappa et al., 2016; Omer et al., 2016).

During the growth of the culture, the concentration of soluble phosphate exhibits diverse patterns. While some strains display a linear increase over time (21F213), others demonstrate an oscillating behaviour (ex. 21F205 and 21F220), characterized by peaks and valleys. This phosphorus solubilization behaviour aligns with observations by Illmer & Schinner, (1992), Coutinho et al., (2012) and Saber et al., (2009) who attribute this variability to factors such as phosphate precipitation or disparities in the rate of phosphate release and uptake. In summary, when the uptake rate surpasses the solubilization rate, a decline in phosphate concentration in the medium can occur (Rodríguez & Fraga, 1999).

The solubilization in our study was analysed using three sources of insoluble phosphate minerals corresponding to hydroxyapatite, tricalcium phosphate, and phosphate rock. Several reports have examined the ability of different bacterial species to solubilize insoluble inorganic phosphate compounds, such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate (Goldstein, 1986; Kumar et al., 1999). For instance, Reyes et al., (2006) showcased that *Azotobacter spp.* species efficiently dissolve hydroxyapatite when provided with glucose, sucrose, and/or mannitol as carbon sources, and ammonia and/or nitrate as nitrogen sources, however, Jokkaew et al., (2022) found no evidence of hydroxyapatite solubilization capacity in

A. vinelandii. Despite this, the *A. salinestris* hydroxyapatite solubilizing capacity by 21F213 strain, observed in our research, remains undefined in current literature. Concerning to *A. salinestris* tricalcium phosphate and phosphate rock solubilizing capacity Rashad et al., (2023) describe its capacity to solubilize phosphorus by measuring the acid phosphatase activity and Chennappa et al., (2018) describe tricalcium phosphate solubilization by *A. salinestris* with by halo measurement in Pikovskaya agar medium (Nautiyal, 1999). Regarding the solubilization values of *A. salinestris* in suspension method, in the article by (Janati et al., 2022) obtained solubilization values very similar and comparable to those obtained in our study, from strain 21F213, for instance, in NBRIP enriched with tricalcium phosphate they obtained phosphate solubilization values of 75.61-147.62 µg mL^{-1}, and for rock phosphate the solubilization ranged from 15.41-25.16 µg mL^{-1}.

Azotobacter salinestris strain 21F213 exhibited an enhanced phosphorus solubilization, concomitant with a decline in pH and the synthesis of organic acids, including malic, gluconic, acetic, and glucuronic acids. These findings are in agreement with prior studies emphasizing the role of phosphorus-solubilizing bacteria in releasing organic acids, acidifying the medium, and promoting phosphorus solubilization (Azaroual et al., 2020; Marra et al., 2012). Specifically, this solubilization mechanism has been described in *Azotobacter spp*. (Gauri et al., 2012), *Azotobacter chroococcum* (Kumar et al., 1999; Yi et al., 2008), and *Azotobacter vinelandii* (El-Badry et al., 2016; Hafez et al., 2016). While no literature explicitly demonstrates the capacity for organic acid production related to phosphorus solubilization in the species *Azotobacter salinestris*, that is the species of strain 21F213, it is worth noting that articles exist in reference to the production of organic acids in other contexts, such as calcite-solubilizing capacity (Rashad et al., 2023).

The correlation between phosphorus solubilization and the synthesis of organic acids is ascribed to the formation of chelates with cations, primarily protons (H+) or calcium (Ca), through their hydroxyl and carboxyl groups, which bind to phosphate, thereby converting it into soluble forms (Glick, 2012; Nobahar et al., 2017). Our findings regarding the significant production of malic acid (465 µg mL^{-1}) and gluconic acid (246 µg mL^{-1}) by strain 21F213 align with the observations of Kpomblekou-A & Tabatabai, (2003) who highlighted the enhanced efficiency of dicarboxylic and tricarboxylic acids in phosphorus solubilization and Krishnaraj & Dahale, (2014) which observed that along with 2-ketogluconic acid, gluconic acid appears to be the most frequently observed acid produced during mineral phosphate solubilization.

Contrary to this, strains 21F200 (*Azotobacter salinestris*) and 21F209 (*Azotobacter chroococcum)* reduced the pH without a corresponding increase in phosphorus solubilization. This result suggests that, while reduction of pH by organic acids is important, other factors may influence phosphorus solubilization (Yi et al., 2008). For instance, the activity of acid phosphatase, could

contribute to the reduction of the medium's pH through dephosphorylation actions, and so affect indirectly the solubilization of inorganic phosphorus (Achal et al., 2007).

It is noteworthy that each strain within the same species behaved differently in terms of its phosphate-solubilizing activity and the amount and type of acid secreted. This highlights the remarkable strain-specific nature of phosphate-solubilizing activity, prompting a deeper consideration of the distinctive characteristics exhibited by each strain. Such discernment becomes pivotal when evaluating their respective contributions to phosphorus solubilization and soil nutrient dynamics (Chen et al., 2006).

Conclusion

A new collection of nitrogen-fixing bacterial isolates that is compatible with the European Union Fertilizer Regulation (2019/1009) was successfully established. This collection comprises 4 strains of *Azotobacter spp.* and 9 strains of *Azospirillum spp.* of a total 242 nitrogen-fixing strains. Our findings consistently show that the majority of isolates from the genera *Azospirillum spp.* and *Azotobacter spp.* were sourced from the rhizosphere of gramineous plants, specifically grass (31%), wheat (23%), and maize (23%), indicating a preferential association with this group of plants.

Regarding *in vitro* nitrogen-fixing capacity, we found that *Azospirillum spp.* strains, especially strains 21F221, 21F224, 21F226, and 21F227, showed more promising results in terms of growth and ammonium production after 72 hours with 72, 59, 70 and 56 µg NH_4 mL^{-1} produced respectively. In the context of phosphorus solubilization, isolates from the genus *Azotobacter spp*., especially strain 21F213, stood out as the most effective, solubilizing hydroxyapatite (119 to 179 µg mL^{-1}), tricalcium phosphate (10 to 156 µg mL^{-1}), and phosphate rock (6 to 91 µg mL^{-1}) after 3, 5, and 7 days of incubation. This strain also exhibited a remarkable ability to reduce the pH of the medium from 6.8 to 4.3 and produce high levels of organic acids, specifically gluconic acid and malic acid.

Chapter 3: Efficacy of *Azospirillum spp.* and *Azotobacter spp.* isolates as biostimulants in greenhouse and field experiments

Introduction

Agriculture is at a crucial point, where the need to produce food sustainably and efficiently is becoming increasingly clear. Among the key crops to sustain global food supply are wheat (*Triticum aestivum L.*), maize (*Zea mays L.*), which along with rice (*Oryza sativa*), account for over 60% of the calories in the human diet (FAO, 2024). In the Spanish context, in 2022, wheat, maize and rice occupied approximately 35, 11 and 1 %, of the total cultivated area respectively. Specifically in Catalonia, cereals dominate the agricultural landscape, covering an area of 338661 hectares, with maize responsible for 12 %, rice for 6 % and wheat for 5 % of the total cultivated area, in this region, maize primarily is grown in Lleida and Baix Ebre, rice is concentrated in the Ebro delta and wheat in Lleida (DARP, 2022). These crops, essential for the agricultural economy, play a crucial role in both animal and human nutrition, as well as being key components in the food industry and biofuel production.

Cereal crops have the ability to associate with numerous beneficial bacteria, commonly known as plant growth-promoting rhizobacteria (PGPR) these can generate direct and indirect beneficial effects on plant growth by different mechanisms, already described in Chapter 1. In this context, live formulations of beneficial microorganisms for agricultural crop inoculation, known as microbial biostimulants, emerge as a promising tool to enhance agricultural productivity and reduce dependence on agrochemicals (García de Salamone et al., 2012). Although to fully harness the potential of PGPR, it is crucial to understand the mechanisms through which they influence agricultural systems, which entails not only identifying desirable characteristics of growth-promoting bacteria but also ensuring their environmental safety and ability to tolerate abiotic stress (Mohanty et al., 2021)

The legal framework regulating the commercialization of microbial biostimulants in the European Union is defined in the EU Fertilizer Products Regulation 2019/1009 (FPR), which came into effect on July 16, 2022. This regulation defines microbial biostimulant as a formulation of one or many microorganisms whose function is to stimulate plant nutrition processes, with the sole aim of improving nutrient use efficiency (NUE), tolerance to abiotic stress, production quality traits, or availability of confined nutrients in the soil or rhizosphere. This regulation through the CEN/TC, specifically CEN/TC 455, sets the procedures for demonstrating agronomically relevant properties of the microbial biostimulants. In this chapter, we will focus to express the results obtained to align with the terms and definitions, terminology, validation indices, and performance criteria for the claim “Improvement in nutrient use efficiency” described on the draft document Fertilising Products Regulation Committee for Standardization (FprCEN/TS 17700) written by Slovenski standard kSIST-TS committee (2021).

In this context, research into the application of beneficial microorganisms in agriculture for commercialization as microbial biostimulants has garnered increasing interest, being Bayer Crop Science AG the leading biofertilizer company in the world and Symborg S.L. the most important biofertilizer company in Spain (Singh & Kumar, 2024). Some examples of commercial formulations of microbial biostimulants include Nitragin®, a product based on *Rhizobium leguminosarum* used in soya plants (Furtak et al., 2020), products formulated with the *Azospirillum brasilense* strain Az39 for wheat, maize, and sorghum like; Azovisdas® from Microvidas S.R.L, Nitragin Wave® from Novocynes or AzobioMax® from Forbio S.L. (Cassán & Diaz-Zorita, 2016; Coniglio et al., 2019; Grellet Naval et al, 2017) or Twin N® from Mapleton Agri Biotec Pvt. Ltd. composed of a combination of *Azospirillum* and *Azotobacter* (Singh & Kumar, 2024). In Spain there are 72 products registered as microbial based fertilizers, of which 15 contain *Azospirillum spp.,* 5 contain *Azotobacter spp.,* and 33 contain *Bacillus spp.,* with notable examples including Nutribion® from Ceres Biotics S.L., a formulation of *Azotobacter salinestris* and Contribute ibN® from Alltech S.L., formulated with *Azospirillum brasilense* with similar applications to those we aim to develop and the remaining 19 products corresponded to other microbial genera. Regarding the European market, it is important to note that while *Azotobacter spp*. and *Azospirillum spp.* are permitted under the new fertilising product regulation (FPR 2019/1009), specific certified products containing these bacteria may still be limited, representing an opportunity for researchers and companies to develop new microbial biostimulants that comply with the updated regulatory framework.

Varius strains form the genus *Azospirillum spp.* and *Azotobacter spp.* have demonstrated significant capabilities which may promote plant growth and productivity, including nitrogen fixation, phosphorus solubilization, production of plant growth substances, as well as, effect on enhancing plant resistance to biotic and abiotic factors (Barnawal et al., 2017; Cassán et al., 2020; Cassán & Diaz-Zorita, 2016). Specifically, the inoculation of plants with *Azospirillum spp*. has been shown to increase root development, improve nutrient and water uptake, and positively impact crop yields, with notable increases observed in winter cereals (14.0%), summer cereals (9.5%), and legumes (6.6%) (Cassán & Diaz-Zorita, 2016). Similarly, *Azotobacter spp.* have been linked to improved plant growth and significant yield enhancements in various crops, including cereals and pulses, with yield increases of up to 40% (Aasfar et al., 2021). These benefits underscore the successful use of these genera as microbial biostimulants to boost crop yields, particularly in cereals (Cassán & Diaz-Zorita, 2016; Gassmann et al., 2016; Okon & Labandera-Gonzalez, 1994).

However, research on the inoculation of PGPR in agricultural fields reveals a significant discrepancy between theoretical potential and practical results (Bashan, 1999). Although this technique promises to increase agricultural production at a lower cost than conventional chemical

fertilizers, its effectiveness is influenced by a series of interrelated factors. Among these factors are the complex interactions between native and inoculated microorganisms, plant genotypes and environmental factors (Di Salvo et al., 2018; Dobbelaere et al., 2001; García de Salamone et al., 2012; Rani & Goel, 2013). In this context, several studies have demonstrated a significant interaction between inoculated strains of *Azospirillum* and plant genotype in crops such as maize, rice, and wheat (García de Salamone et al., 2012; Garcia De Salomone et al., 1996; Naiman et al., 2009) and complex interaction among bacteria, plants, inoculation methods, and cultivation conditions, soil type, crop rotation, and nutrient management (Díaz-Zorita & Fernández-Canigia, 2009). For example, the response to *Azospirillum sp*. inoculation may be greater in wheat and maize crops grown in less fertile soils and rotated with sunflower or maize compared to soils richer in organic matter or rotated with pasture and/or the inoculation response can be lower in soils with high nitrogen levels (Cassán & Diaz-Zorita, 2016). Despite these challenges, some strains of *Azospirillum* and *Azotobacter* show promising potential to enhance both aerial and root biomass production recurrently in various cereal crops (Aasfar et al., 2021; Escobar Ortega et al., 2021; Kapulnik et al., 1983; Naiman et al., 2009).

Objectives

The main objective was to determine the effect of the inoculation with the best isolates of *Azospirillum spp.* and *Azotobacter spp.* selected based on the *in vitro* results, on extensive crops such as maize and rice, observing the effect on growth parameters, nutrient accumulation, and crop production. The specific objectives were:

1. Evaluate the effectiveness of *Azotobacter salinestris* 21F213 on phosphorus solubilization and phosphorus use efficiency in greenhouse experiments
2. Evaluate the effectiveness of selected *Azospirillum spp.* strains in enhancing nitrogen utilization efficiency and increasing yield at different nitrogen fertilization regimes under controlled and field conditions.
3. To study the results under the point of view of the European Fertilizer Regulation and specifically align with the claims outlined in the Technical Specifications CEN/TC 455 for microbial biostimulants.

Material and methods

1. Assay on the effect of the microbial inoculation on phosphorus solubilization

1.1 Plant inoculation method

In this experiment, it was measured the capacity of *Azotobacter salinestris* strain 21F213 to solubilize phosphate in combination with maize plants, as it had shown the best results *in vitro*. Microorganisms were stored in glycerol in the freezer (-80ºC), to prepare the inoculum one full loop of the microorganism was placed in a liquid growth medium NBS (Beef extract 3.0 g L^{-1}, Peptone 5.0 g L^{-1} and Sodium chloride 5.0 g L^{-1}). The inoculum was placed on a rotary shaker at 150 rpm with a temperature of 30ºC for 72 h, after that time the absorbance was measured in a spectrophotometer to determine the cell concentration and the volume necessary to have the inoculum at 10^8 colony forming units (CFU) mL^{-1}. Then the cells were centrifugated at 4000 *g* for 10 min to wash the growth medium and then resuspended with saline solution (9 g L^{-1} NaCl).

1.2 Set up of the experiment

The experiment was set up in the greenhouse in the "Serveis de Camps Experimentals" at the Faculty of Biology, Universitat de Barcelona (41.385018, 2.120436), for 35 days from the 20th of June 2022 until the 25th of July 2022 with a temperature and humidity average of 27.66 ± 4ºC and 60 ± 13 % RH respectively.

The experiment was performed with *Zea Mays* cv. "Tía María" from Les Refardes S.L (Mura, Spain). The design consisted of a Latin square with three treatments concerning a positive control, a negative control and plants inoculated with *A. salinestris* strain 21F213 with 10 replicates per treatment corresponding to 10 pots with one plant each. Plants were sown in pots of 0.8 L, the substrate used was peat, vermiculite, and perlite (2:1:1 v/v/v) mixed with hydoxyapatite (1g L^{-1} of substrate) and pre-hydrated with 100 mL of tap water. Two seeds per pot were sown and before inoculation only one plant was left in each pot. The microbial treatments were applied on the substrate next to the base of plantlets, one week after sowing with a pipette with 5 mL of inoculum suspension and 5 mL of saline solution (9 g L^{-1} NaCl) for non inoculated treatments. Serial dilutions and plating were made to confirm that the inoculum concentration was adjusted to 10^8 CFU mL^{-1}.

Plants were watered twice a week with tap water and once a week with complete or P-free Hoagland depending on the treatment (Hoagland & Arnon, 1950). The positive control corresponded to non-inoculated plants fertilized with complete Hoagland while the negative control corresponded to non-inoculated plants fertilized with P-free Hoagland solution and all

inoculated plants received P-free Hoagland solution. The P-free Hoagland was prepared with KCl (0.05 mmol L^{-1}) instead of KH_2PO.

1.3 Plant growth parameters

The number of leaves and the length of each plant were tracked once a week by measuring from the base of the plant until the last leaf junction.

On harvest day, plants were pulled out and cut at the base of the plant, just after cutting, the plants were weighted to have the fresh weight. Then the samples were kept in paper bags in an oven at 60 ºC for 72 h and then weighted to have the dry weight.

2. Experiments to assess the impact of inoculation on nitrogen use efficiency and plant production

2.1 Preparation of the microbial inoculum and seed inoculation

The 13 nitrogen-fixing *Azospirillum spp.* and *Azotobacter spp* isolates were selected to asses their impact in plant nitrogen use efficiency, corresponding to three *Azotobacter salinestris* (21F200, 21F201, and 21F213), five *Azospirillum brasilense* (21F203, 21F205, 21F210, 21F221 and 21F222), one *Azotobacter chrococcum* (21F209), two *Azotobacter oryzae* (21F220 and 21F224), one *Azospirillum aestuarii* (21F226) and one *Azospirillum spp.* (21F227). Additionally, two reference strains of *Azospirillum brasilense* were included being CECT 590T from the CECT (Colección Española de Cultivos Tipo) and DSM 1843 from the DSMZ (German collection of microorganisms and cell cultures) corresponding to AP1 and AP2, respectively. Also, for the 75% N experiments, the Vietnamese formulation tested was S(04), a combination of two different mycorrhiza genera 68,56% *Glomus spp.* and 31,36% *Acaulospora spp*. In addition, in all experiments non-inoculated seeds were added as a negative control (Control).

When preparing the seed inoculum for 0% N and 60% N experiments two steps were performed as follow: preparation of the inoculum and seed inoculation. First, to prepare the inoculum one loop from each strain stored at -80ºC as glycerol stocks, was placed in Burks solid plates (Burks, 1956) for *Azotobacter spp.* and RC solid plates (Caceres, 1982) for *Azospirillum spp.* and left at 30ºC for 48 h, the grown colonies were transferred to tubes with 20 mL of NBS liquid media and left with adequate aeration and agitation (150 rpm) for 72 h at 30 ºC. After that time, cell concentration was measured by spectrophotometry at 600 nm and compared with a calibration curve. Second, for seed inoculation, aliquots were centrifugated (Hettich model UNIVERSAL 320) at 4000 *g* for 10 min and resuspended in sterile NBS liquid medium to obtain a concentration of 10^{10} CFU mL^{-1}. The seed inoculum was performed by mixing 10 g of maize seeds (*Zea mays* cv. “Tía María”) with 0.1 g of Jiffy GO M8 peat (sieved with a 0.71 mm mesh), 0.1 mL of bacterial

inoculum adjusted at 10^{10} CFU mL^{-1} and 0.35 mL of 1% (w/v) carboxymethyl cellulose (CMC) to have a final concentration of 10^8 CFU g $seeds^{-1}$. Serial dilutions and plating were made to confirm the purity and the concentration the inoculum. Additionally, in one experiment fertilized with 60% N, an inoculum consisting of a mixture of strains 21F221 and 21F226 was applied, inoculating the seeds with a concentration of $0.5 \cdot 10^8$ CFU g^{-1} seeds for each strain.

When preparing the seed inoculum for 75%N five steps were performed as follows; determination of seed germination rate, preparation of the inoculum, seed sterilization, seed pre-germination and inoculation. First, the seed germination rate was assessed to calculate the number of seeds needed to obtain 60 pre-germinated seeds per treatment. Second, the inoculum was prepared as described for the 0% and 60% N experiments (explained in the previous paragraph) once the concentration of the bacterial culture was calculated, it was centrifuged (Hettich model UNIVERSAL 320) at 4000 *g* for 10 min and re-suspended in LB liquid media at a concentration of $5{,}5 \cdot 10^8$ CFU mL^{-1} for maize and $1{,}5 \cdot 10^9$ CFU ml^{-1} for rice, to have a final concentration of 10^8 CFU g^{-1} seeds. Third, the seeds were sterilized by soaking them in ethanol (70% v/v) for 3 minutes, after the excess moisture was removed and then soaked in NaClO (1% v/v) for 10 minutes and then washed 3 times with sterilized distilled water. Fourth, to pre-germinate the seeds, the already sterile maize and rice seeds were soaked in sterilized distilled water at a temperature of 50 ºC for 3 hours for maize and 12 hours for rice, and then placed in a box with a humid sterile cloth for 48 hours at darkness and room temperature. Fifth, for seed inoculation pre-germinated and sterilized maize (*Zea mays* cv "hybrid waxy maize MX10") and rice seeds (*Oryza sativa* cv "OM5451") were placed inside the bacterial inoculum tubes, in agitation at 150 rpm for 6 h in darkness before sowing them in the pots at the greenhouse or field, for non-inoculated plants (Control treatment), sterilized pre-germinated seed were soaked in LB liquid media previously sterilized.

2.2 Set up of the experiments

2.2.1 Assays performed with nitrogen-free fertilization regime (0% N fertilization)

For the assays performed with no nitrogen fertilization three different assays were performed in the same way (A, B and C) but in different moments. The experiments were all conducted in the greenhouse of the "Serveis de Camps Experimentals" from the Universitat de Barcelona with maize plants (*Zea Mays*) cv "Tía María" from Les Refardes S.L. (Mura, Spain) non-treated and ecological seeds. The experiment design was composed of 16 different treatments; non-inoculated plants as control, AP1, AP2, and the abovementioned 4 *Azotobacter spp*. and 9 *Azospirillum spp.* strains with 10 replicates per treatment corresponding to 10 pots with one plant each.

Seeds were inoculated as described in section 2.1. Plants were sown in pots with a mixture of peat, vermiculite, and perlite (2:1:1 v/v/v) pre-hydrated with 100 mL of tap water per litre of the

substrate, two inoculated seeds per pot were sown and after a week only one plant was left in each pot. Seeds were sown in pots of 392 mL and watered twice a week with tap water and once a week with N-free Hoagland for all treatments except for positive control plants that were fertilized with complete Hoagland solution (Hoagland & Arnon, 1950). The N-free Hoagland was prepared with $CaCl_2$ and KCl instead of Ca $(NO_3)_2$ and KNO_3.

There were performed a set of three experiments. Experiment (A) lasted 28 days, from the 23rd of May to the 20th of June 2022, the average temperature during that period was 24.84 ± 2.84 ºC and the humidity was around 68.3 ± 3.14 % RH. The experiment (B) lasted 21 days, from the 18th of July to the 8th of August 2022, the average temperature during that period was 28.53 ± 3.25 ºC and the humidity was 63.63 ± 12.57 % RH. The experiment (C) lasted 27 days, from the 5th of August to 1st of September 2022, the average temperature during that period was 28.30 ± 3.83 ºC and the humidity was 61.68 ± 12.6 % RH.

2.2.2 Assays performed with 60% nitrogen fertilization regime

For the assays performed with 60% nitrogen fertilization two different assays were performed (A and B). Seeds were inoculated as described in section 2.1 and the experiments set up procedure was performed as for the experiments with N-free fertilization regime described at section 2.2.1 with some differences. Plants were sown in 1 L pots and were watered twice a week with tap water and with 60% N Hoagland once a week during the first 3 weeks, twice a week for the following 2 weeks and three times a week for the weeks before harvest. The 60% N Hoagland was prepared with $(NO_3)_2$ (47.2 mmol L^{-1}) and KNO_3 (4 mmol L^{-1}), $CaCl_2$ (11.8 mmol L^{-1}) and KCl (1 mmol L^{-1}).

There were performed a set of two experiments. Experiment (A) lasted 35 days, from 18th of August until the 22th of September 2022, the temperature average during that period was 27 ± 4 ºC and the humidity was 62.78 ± 13.4 %RH. The experiment (B) lasted 50 days, from 1st of August until the 19th of September 2023, the temperature average during that period was 27.96 ± 3.7 ºC and the humidity was 62.45 ± 12.87 %RH.

2.2.3 Assays performed with 75% nitrogen fertilization regime

The experiment was conducted in the greenhouse of the Agricultural Faculty at Can Tho University, Vietnam (10.028510, 105.766676) with maize variety plants, *Zea mays* cv "hybrid waxy maize MX10" from Southern Seed Corporation SSC, (Ho Chi Minh, Vietnam) and Vietnamese rice variety plants, *Oryza sativa* variety long grain white rice "OM5451". The experiment design was composed of 10 different treatments; non-inoculated plants as Control, S(04), AP1, AP2, 21F210, 21F213, 21F220, 21F221, 21F222, and 21F226, the seed inoculum was performed as described at section 2.1. For maize, 5 replicates per treatment corresponding to 5

pots with one maize plant each was performed and for rice 3 replicates per treatment corresponding to 3 pots with five rice plants each was performed.

For maize four pre-germinated and inoculated seeds were sown in pots of 8 L with 6 kg of a mixture of sand: soil (1:1 w/w) pre-hydrated with 2 L of water two days before sowing, and for rice ten pre-germinated and inoculated seeds were sown in pots of 6 L with 6 kg of a mixture of sand: soil (1:1 w/w) pre-hydrated at the saturation point of water (4L) two days before sowing then pots were placed in the greenhouse in a random distribution. After a week just one maize plant per pot and five rice plants per pot were left.

The sand used in this experiment was washed for 7 days 3 times a day with tap water, due to a high content of aluminium. After washing the mixture, it was measured by triplicate: the pH value, electrical conductivity (EC), the organic content (OC %), the organic matter (OM %), the phosphorus available (P_2O_5) and the total nitrogen (N total %) (Table 1).

Table 1 Analysis of the soil sample and the mixture sand: soil

Sample	pH	EC[a] (dS m^{-1})	OC[a] (%)	OM[a] (%)	P_2O_5[a] (%)	N[a] (%)
Soil	5.44	1550	5.3	9.14	0.022	0.19
Mixture soil:sand (1:1)	4.78	793	1.99	3.43	0.002	0.125

[a] Parameters analysed meaning: EC, electrical conductivity; OC, organic content; OM, oxidable organic matter; P_2O_5, total phosphorus; N, total nitrogen content

Plants were watered twice a week with tap water and the fertilization regime was topdress by solid application with a reduced nitrogen regime equivalent to 75% N of the standard fertilization regime. For maize 75 kg ha^{-1} of nitrogen by urea (46%N), 60 kg ha^{-1} of P_2O_5 by super phosphate (18% P_2O_5), and 90 kg ha^{-1} of K_2O by potassium chloride (60% K_2O) were applied as follows: at sowing day total content of P_2O_5 with 1/3 K_2O was applied, after 11 days 2/10 N was applied, at 19 days after sown 1/2 N was applied and after 37 days after sown 3/10 N with 2/3 K_2O was applied. For rice 75 kg ha^{-1} of nitrogen, 60 kg ha^{-1} of P_2O_5, and 30 kg ha^{-1} of K_2O were applied as follows: at sowing day total content of P_2O_5, after 8 days 1/5 N was applied, at 19 days after sowing 2/5 N with 1/2 K_2O was applied and after 42 days after sown 2/5 N with 1/2 K_2O was applied.

The maize experiment lasted 80 days in total, from the sowing of the seeds until harvest, from the 8th of December 2022 to 16th of February 2023 and the average temperature during that period was 28.67 ± 1.7 ºC and the humidity 71.3 ± 1 %RH, respectively. Rice experiment lasted 90 days in total from sowing the seeds until harvest from 1st of December 2022 to 28th of February 2023 and the temperature average during that period was 27.33 ± 2.1 ºC and the humidity was around 66.6 ± 12.2 %RH.

2.2.4 Assays performed in field conditions

The experiment was conducted in an agronomic field in the countryside of Can Tho city, Vietnam (10.059059, 105.690437). The experiment design was composed of 10 different treatments; non-inoculated plants as Control, S(04), AP1, AP2, 21F210, 21F213, 21F220, 21F221, 21F222, and 21F226, the seed inoculum was performed as described at section 2.1. Each treatment had 3 replicates corresponding to 3 plots of with 500 g rice seeds per each treatment. The plots were built one week before sowing and the total surface per plot was 25 m^2 from which 20 m^2 corresponded to the sown surface, excluding the borders. The plots were divided in the field into three rows with 10 plots per row; plots were saturated with water before sowing, and treatments were distributed randomly. The field soil was characterized corresponding to values expressed in the first row of Table 1 corresponding to "Soil" (Section 2.2.3)

Plants were watered twice a week with tap water for the first 3 weeks and three times a week for the rest, the fertilization regime was by topdress according to the agronomical fertilization doses with a 25% reduction in N fertilization with 75 kg ha^{-1} of nitrogen, 60 kg ha^{-1} of P_2O_5, and 30 kg ha^{-1} of K_2O were applied as follows At sowing day total content of P_2O_5, after 8 days 1/5 N was applied, at 19 days after sowing 2/5 N with 1/2 K_2O was applied and after 42 days after sown 2/5 N with 1/2 K_2O was applied. The experiment lasted 90 days from 9th of December 2022 to 8th of March 2023, during that time plants were exposed to the weather conditions of temperature, humidity, and rain, the average temperature during that period was 27.37 ± 1.8 ºC and the humidity was 65.7 ± 10.7 % RH, also, the precipitation average ranged 9.15 ± 1.7 mm with 61.2 ± 9.9 rainy hours.

2.4 Plant growth, nutrient content and yield analysis

For the N-free fertilization and 60% N experiment, plant length was measured by tracking the distance from the base to the last leaf node, while chlorophyll content and the nitrogen balance index (NBI) were assessed using Dualex on the first emerged non-dry leaf of each plant, i.e. the first counting from the base of the plant, once a week. Upon harvest, plant length and shoot fresh and dry weight were measured. The rhizosphere samples were collected from the plant with the highest and lowest weight values for each treatment to determine the microbial populations

For the assays conducted under the 75% N fertilization regime for maize, growth measurements were taken at 15, 30, 45, 60, and 80 days after sowing, including parameters such as plant height, leaf count, and stem diameter. For rice, measurements were recorded at 15, 30, 45, 60, 75 and 90 days post-sowing, focusing on plant height and stem count. On the day of harvest, maize yield parameters such as ear length, fresh ear weight, total kernels per ear, fresh kernel weight per ear, yield, 100-kernel dry weight at 14% humidity, and fresh weight of 100 kernels were measured also biomass measurements included shoot dry weight and root length, and root dry weight. For

rice, yield parameters included number of panicles per pot, number of panicles per plant, number of spikelets per panicle, panicle length, 1000-grain weight at 14% humidity, wet grain weight, grain humidity percentage, grain weight at 14% humidity, yield at 14% humidity based on filled grain weight, weight of unfilled grain at 14% humidity, unfilled spikelet percentage, filled spikelet percentage, estimated number of plants per 1 m^2, estimated yield based on component parameters, and dried straw biomass.

For samples showing significant differences with the non-inoculated plants, nitrogen and carbon content was analysed. Whole plant samples were ground using a coffee grinder, with careful washing after each sample, a representative sample of 100 mg was taken and placed in a 1.5 mL Eppendorf tube. A second grind was performed by adding 3 stainless steel balls to the Eppendorf tubes and placing them in a rotor ball mill (Mixer Mill MM 400) for 3 min at 1500 rpm. Nitrogen (N) and carbon (C) analyses were conducted using elemental Analysis, for Isotope Ratio Mass Spectrometry (EA-IRMS Delta V) from Thermo Fisher Scientific.

With the values obtained and the nutrient parameters established in the experimental setup, the commercially relevant agronomic parameters (potential claims to be stated in the label of commercial microbial biostimulants) were calculated following Dobermann (2007) as described in FprCEN/TS 17700. These parameters include: apparent crop recovery efficiency of applied nutrient (RE), partial factor productivity of applied nutrient (PFP), internal utilization efficiency of a nutrient (IE), physiological efficiency of applied N (PE), agronomic efficiency of applied nutrient (AE) and nitrogen export (NE). All these parameters were calculated based on the total plant nutrient uptake in aboveground biomass at maturity in plants that received fertilizer (U) and those that did not receive fertilizer (U_0), crop yield with applied fertilizer (Y) and without fertilizer (Y_0), the amount of fertilizer nutrient applied (F), and the concentration of the plant nutrient in the part of interest (C). The formulas used for each index of nutrient use efficiency were: RE=(U – Uo)/F; PE=(Y– Yo)/(U– Uo); IE=Y/U; AE=(Y – Yo)/F; PFP=Y/F and NE=Y*C.

3. Statistical analysis

All data were analysed using the SPSS software package version 27.0.

To assess the effect of inoculum on phosphorus solubilization, a Dunnett's test was conducted to compare non-inoculated plants fertilized with P-free Hoagland solution (Control -) with plants inoculated with *A. salinestris* strain 21F213, using a significance level of 95%. Additionally, data from non-inoculated plants fertilized with complete Hoagland solution (Control +) were included in the statistical test to provide an indication of phosphorus deficiency in the plants.

To analyse the data from the assays on the effect of inoculation on plant nitrogen use efficiency under different nitrogen fertilization regimes (0% N, 60% N, and 75% N), the following approaches were taken:

For the experiments with free-N Hoagland solution (0% N) the data from the three experiments (A, B, and C) were treated together as a one-way ANOVA with the experiment as a fixed factor was conducted, evaluating five parameters: NBI, shoot fresh and dry weight, and nitrogen and carbon content and no significant experiment effect was observed. Subsequently, a Dunnett's test was performed to compare the non-inoculated plants (Control) with the different inoculation treatments, using a 95% significance level. Additionally, a Pearson's correlation analysis was performed between the plant shoot dry weight data from experiment A and the rhizosphere strains population, with a significance level of 95%.

For the experiments with 60% N, the data from the two different experiments performed (A and B) were treated separately as they were not comparable as a two-way T-student test with the experiment as a fixed factor showed significant differences (p-value < 0.05), primarily due to differences in sampling times, with plants in experiment B being more developed at the time of sampling. Subsequently, a Dunnett's test was performed to compare the non-inoculated plants (Control) with the different inoculation treatments, using a 95% significance level for both A and B data separately.

For the pot and field experiments with a 75% N fertilization regime were analysed using a Dunnett's test, comparing the non-inoculated substrate (Control) with the different inoculation treatments, using a 95% significance level.

Results

1. Effect of microbial inoculation on phosphorus solubilization

When evaluating the effect of inoculation with the *Azotobacter salinestris* 21F213 strain on maize plants fertilized with P-free Hoagland solution, no significant differences were observed compared to non-inoculated Control (-) plants, either in terms of plant height or weight (Table 2).

On the other hand, significantly higher height and weight were observed in non-inoculated plants fertilized with soluble phosphorus (Control (+)) compared to non-inoculated plants fertilized without soluble P (Control (-) (Table 2).

Table 2 Effect of *A. salinestris* inoculation on *Zea mays* cv. "Tía María" growth fertilized with soluble phosphorus free fertilization

Treatment[a]	Height (t1)[b] (cm)	Height (t2)[b] (cm)	Shoot fresh weight (g plant^{-1})	Shoot dry weight (g plant^{-1})
Control (+)	**20.75 ± 0.6 ***	**40.35 ± 0.7 ***	**68.34 ± 1.9 ***	**6.41 ± 0.2 ***
Control (-)	19.80 ± 0.5	38.85 ± 1.2	54.56 ± 1	5.51 ± 0.1
21F213	20.40 ± 0.7	39.50 ± 1.4	55.16 ± 2	5.61 ± 0.1

Values are means ± SE (n=10). Values marked with asterisks are significantly different (p-value < 0.05), determined through a Dunnett's test between Control (-) and the other treatments (Control +, and 21F213)

[a] Treatments are as follows: Control (+) non inoculated with complete Hoagland; Control (-) non inoculated, and *Azotobacter salinestris* strain 21F213 inoculated at 10^8 CFU mL^{-1} with P-free Hoagland grown with hydroxyapatite mixed in the substrate at a concentration 1 g L^{-1}

[b] Shoot length expressed in cm measured 2 weeks (t1) and 4 weeks after inoculation (t2)

2. Effect of microbial inoculation in plant nitrogen use efficiency

2.1 Inoculation effect on plants with no nitrogen fertilization (0% N)

Table 2 illustrates the main outcomes derived from three experiments (A, B, and C) with fertilization lacking N (0% N). Concerning the nitrogen balance index (NBI), no significant differences were noted in NBI values (Table 3). In terms of biomass, plants treated with *A. brasilense* strain 21F221 demonstrated superior biomass compared to their non-inoculated counterparts, displaying 20% and 9% higher values in shoot fresh and dry weight, respectively (Table 3). Similarly, those treated with *A. aestuarii* strain 21F226 exhibited increased shoot fresh and dry weight, surpassing non-inoculated plants by 20% and 17%, respectively. Furthermore, N content was significantly higher in plants treated with strain 21F221 and 21F226 compared to non-inoculated plants (Control), demonstrating increases of 28% and 25%, respectively. Similarly, this trend extended to C content, where values were significantly higher in plants inoculated with 21F221 and 21F226 strains compared to non-inoculated plants, displaying increases of 30% and 31%, respectively. It is noteworthy that plants inoculated with the reference strains AP1 and AP2 did not show any significant difference respect to non-inoculated control plants in any of the evaluated parameters (Table 3).

The final rhizosphere *Azospirillum* and *Azotobacter* population after 4 weeks in experiment A, revealed that all microorganisms exhibited a rhizosphere population of approximately 10^6 CFU mL^{-1} (Table 4). Interestingly, no correlation was found between shoot dry weights and its rhizosphere isolates population (p-value > 0.05) (Table 3).

Table 3 Effect of microbial inoculations on *Zea mays* cv. "Tía María" fertilized with no N supply at the harvest day

Treatment[a]	Nitrogen balance index	Shoot fresh weight (g)	Shoot dry weight (g)	N content (mg plant^{-1})	C content (mg plant^{-1})
Control	4.76 ± 0.3	2.9 ± 0.2	0.47 ± 0.03	2.52 ± 0.1	191.88 ± 6.9
AP1	5.24 ± 0.3	3.21 ± 0.1	0.48 ± 0.02	2.35 ± 0.1	173.08 ± 1.6
AP2	5.29 ± 0.2	2.96 ± 0.1	0.45 ± 0.02	nd[b]	nd
21F200	5.33 ± 0.3	3.13 ± 0.2	0.51 ± 0.03	nd	nd
21F201	5.41 ± 0.3	3.03 ± 0.2	0.49 ± 0.02	nd	nd
21F203	5.27 ± 0.3	2.87 ± 0.2	0.47 ± 0.02	nd	nd
21F205	5.1 ± 0.3	3.04 ± 0.1	0.52 ± 0.04	2.67 ± 0.1	198.72 ± 6.9
21F209	5.4 ± 0.2	2.94 ± 0.2	0.47 ± 0.02	nd	nd
21F210	5.55 ± 0.3	2.75 ± 0.2	0.44 ± 0.03	nd	nd
21F213	5.42 ± 0.3	3.04 ± 0.2	0.48 ± 0.02	nd	nd
21F220	5.38 ± 0.3	3.24 ± 0.2	0.51 ± 0.03	nd	nd
21F221	5 ± 0.3	**3.47 ± 0.2 ***	**0.56 ± 0.02 ***	**3.22 ± 0.3 ***	**248.61 ± 18.1 ***
21F222	5.83 ± 0.4	3.24 ± 0.2	0.53 ± 0.03	2.95 ± 0.2	216.18 ± 13.6
21F224	5.47 ± 0.4	2.8 ± 0.1	0.44 ± 0.02	nd	nd
21F226	5.33 ± 0.2	**3.4 ± 0.2 ***	**0.55 ± 0.03 ***	**3.16 ± 0.3 ***	**251.83 ± 21.5 ***
21F227	5.83 ± 0.3	2.97 ± 0.2	0.5 ± 0.03	nd	nd

Values are means ± SE (n=30) concerning to 3 experiments with 10 replicates each (Experiment A, B and C). Values marked with asterisks are significantly different compared to non-inoculated plants with a p-value < 0.05, determined through a Dunnett's test

[a] Treatments correspond to non-inoculated plants (Control) inoculated with *A. brasilense* reference strains (AP1 and AP2) and *Azospirillum* and *Azotobacter* isolates inoculated at 10^8 CFU g^{-1} of seeds

[b] Non determined (nd)

Table 4 Rhizosphere population of the isolates in *Zea mays* cv. "Tía María" and their corresponding shoot dry weight values in experiment A

Treatment[a]	Shoot dry weight (g)		Isolates rhizosphere population (10^6 CFU mL^{-1})
	Mean	Plant[b]	
21F209	0.50 ± 0.03	(+) 0.68	6.30 ± 2.65
		(-) 0.37	4.85 ± 0.21
21F210	**0.53 ± 0.03 ***	(+) 0.69	5.00 ± 0.05
		(-) 0.39	3.00 ± 0.15
21F213	**0.57 ± 0.03 ***	(+) 0.67	4.70 ± 0.70
		(-) 0.38	4.85 ± 1.15
21F220	**0.54 ± 0.04 ***	(+) 0.81	0.50 ± 0.01
		(-) 0.37	1.00 ± 0.10
21F221	**0.60 ± 0.04 ***	(+) 0.79	2.50 ± 1.50
		(-) 0.37	0.25 ± 0.08
21F226	0.50 ± 0.03	(+) 0.69	1.80 ± 0.20
		(-) 0.36	0.71 ± 0.11

Values are shoot dry weight means ± SE (n=10) and concentration means ± SE (n=3). Values marked with asterisks are significantly different compared to non-inoculated plants with a p-value < 0.05, determined through a Dunnett's test. Correlation analysed by Pearson's test with no significance (ρ= 0.081 and p-value > 0.05)

[a] Treatment correspond to inoculation with *A. chroccocum* strain 21F209, *A. salinestris* strain 21F213, *A. oryzae* strain 21F220, *A. brasilense* strains 21F210, 21F221 and *A. aestuarii* 21F226 inoculated at 10^8 CFU g^{-1} of seeds

[b] Shoot fresh weight referred to the plant with higher (+) and the plant with lower (-) weight within each treatment

2.2 Inoculation effect on plants with reduced nitrogen supply (60% N)

In Experiment A, a higher fresh and dry weight was observed in maize plants inoculated with *A. brasilense* strains 21F205, 21F221 and *A. aestuarii* 21F226 and in dry weight only in the case of plants inoculated with 21F224 and 21F227 compared to non-inoculated maize plants (Table 5). The increase for dry weight for those inoculated with *A. brasilense* strains 21F205, 21F221, 21F227, *A. aestuarii* 21F226 and *A. oryzae* strain 21F224, was of 37%, 22%, 29%, 23%, and 21%, respectively, compared to the control (Table 5). Simultaneously, maize plants inoculated with *A. brasilense* strain 21F221 exhibited a higher nitrogen concentration, showing an 11% increase compared to non-inoculated plants (Control). Additionally, plants inoculated with 21F222, 21F205, 21F226 and 21F221 strains presented a significant higher carbon concentration per plant with an increment of 41%, 48%, 38% and 25% respectively (Table 5).

Table 5 Effect of microbial inoculations on *Zea mays* cv. "Tía María" fertilized with 60% N regime at the harvest day

Exp[a]	Treatment[b]	Shoot fresh weight (g)	Shoot dry weight (g)	N content (mg plant^{-1})	C content (mg plant^{-1})
A	Control	42.05 ± 2.7	3.5 ± 0.3	62.18 ± 1.9	1405.3 ± 57
	AP1	37.41 ± 2.5	2.93 ± 0.2	60.58 ± 2.3	1338.7 ± 201
	AP2	39.93 ± 2.6	3.1 ± 0.3	nd	nd
	21F200	43.01 ± 2.9	3.53 ± 0.3	nd	nd
	21F201	40.94 ± 3.2	3.52 ± 0.3	nd	nd
	21F203	41.56 ± 2.4	3.53 ± 0.3	nd	nd
	21F205	**52.18 ± 1.7 ***	**4.81 ± 0.3 ***	67.04 ± 2.1	**1982.8 ± 164 ***
	21F209	45.74 ± 3.2	4.23 ± 0.3	nd	nd
	21F210	46.13 ± 3.3	4.15 ± 0.4	nd	nd
	21F213	45.86 ± 3.3	3.97 ± 0.4	nd	nd
	21F220	43.93 ± 3.5	3.96 ± 0.4	nd	nd
	21F221	**48.69 ± 2.5 ***	**4.24 ± 0.2 ***	**68.88 ± 5.5 ***	**1751.5 ± 87 ***
	21F222	47.17 ± 3.5	4.06 ± 0.4	54.56 ± 10.7	**2093.4 ± 133 ***
	21F224	46.13 ± 2.5	**4.29 ± 0.3 ***	nd	nd
	21F226	**47.92 ± 1.7 ***	**4.54 ± 0.3 ***	62.67 ± 2.3	**1939.1 ± 60 ***
	21F227	45.30 ± 2.3	**4.33 ± 0.2 ***	nd	nd
B	Control	91.98 ± 9.0	8.75 ± 0.6	85.64 ± 3.9	3538.95± 117
	21F221	96.70 ± 5.4	**10.36 ± 0.5 ***	**95.09 ± 1.4 ***	**4189.26 ± 197 ***
	21F226	96.68 ± 7.6	9.88 ± 0.9	**100.42 ± 3.2 ***	**4057.54 ± 82 ***
	21F221+21F226	102.19 ± 6.9	**10.58 ± 0.6 ***	**100.49 ± 3.9 ***	**4369.74 ± 166 ***

Values are means ± SE (n=16). Values marked with asterisks are significantly different p-value < 0.05, determined through Dunnett's test compared to the non inoculated control

[a] Data corresponding to two experiments (A – August/September 2022, B – August/September 2023)

[b] Treatments correspond to non-inoculated plants (Control) inoculated with *A. brasilense* reference strains (AP1 and AP2) and *Azospirillum* and *Azotobacter* isolates inoculated at 10^8 CFU g^{-1} of seeds, as well as the combination of *A. brasilense* (21F221) and *A. aestuarii* (21F226) both at $0.5 \cdot 10^8$ CFU g^{-1} of seeds (21F221+21F226)

During Experiment B, the plants that exhibited differences in biomass compared to the control were those inoculated with *A. brasilense* strain 21F221 and plants inoculated *A.aestuarii* strain 21F226 together showing an 18% and 21% increase in shoot dry weight, respectively compared to the control (Table 5). Furthermore, all plants inoculated with any of the strains individually and with the combination of both presented significant higher N and C content per plant compared to non-inoculated plants, with increases of 16% and 18% respectively by inoculation with 21F221, increases of 17% and 15% by 21F226 and increases of 17% and 23% by the combination of both (21F221+21F226). In this experiment (60% N) as in experiment 0% N, the references strains AP1

and AP2 did not show any significant difference respect to non- inoculated control plants (Table 5).

2.3 Inoculation effect on plant yield with reduced nitrogen supply (75% N)

2.3.1 Effect on maize and rice under controlled conditions

Regarding the inoculation effects observed in maize and rice plants fertilized with a 75% nitrogen regime, notable differences were observed among the effect of inoculation of almost all microbial strains tested, particularly in terms of yield, root length, and nitrogen content in maize, and aerial biomass, yield, and nitrogen content in rice (Table 6).

For maize, the inoculation with all tested microbial strains resulted in notable increases in root biomass and yield compared to non-inoculated plants. The highest increase in root dry weight was observed with the standard strain *A. brasilense* AP1, the Vietnamese formulation S(04), and *A. aestuarii* strain 21F226, each showing a 50-51% increase. Additionally, root length was significantly enhanced by the Vietnamese formulation S(04), *A. brasilense* strain 21F221, and *A. aestuarii* strain 21F226, with increases ranging from 60% to 67%. In terms of grain yield, maize plants inoculated with *A. brasilense* AP1, strain 21F222, and *A. aestuarii* strain 21F226 showed the highest grain weight and production values, with grain weights increasing by 120%, 128%, and 112% respectively compared to non-inoculated plants, and grain production increases of 165%, 155%, and 148% respectively, additionally, the harvest index was similarly improved with increases of 7%, 6%, and 8% respectively compared to non-inoculated plants. The treatments with *A. brasilense* AP1, strain 21F221, *A. aestuarii* strain 21F226, and the Vietnamese formulation S(04) also significantly affected shoot nitrogen accumulation in plants, with increases of 66%, 53%, 8%, and 53% respectively in shoot nitrogen content, and strain AP1, strain 21F221, and strain 21F226 affected in a higher nitrogen seed accumulation by 133%, 65%, and 106%, respectively.

In rice, the most notable inoculation effect was obtained with the inoculation of strain S(04), which showed significant differences in shoot dry weight with increases of 22%, grain weight with increases of 7%, grain production with increases of 72%, and nitrogen accumulation in shoots with increases of 28% and in seeds with increases of 41%. Additionally, strain *A. brasilense* 21F221 showed significant differences compared to non-inoculated plants, with higher values in yield with increases of 3% and 32% in grain weight and grain production, in the harvest index, and in nitrogen content in shoots and seeds with increases of 35% and 52%, respectively.

Taking in account all the strains analysed, regarding the effect of inoculation on the shoot dry biomass of rice, differences were observed compared to non-inoculated plants in strains AP2, S(04), 21F222, and 21F226. In terms of yield, the differences were more widespread, with

differences in grain weight observed in all strains compared to non-inoculated plants, except for strain 21F213, and in grain production except for strains AP2, 21F210, and 21F220. Regarding nitrogen content, only strains S(04) and 21F221 showed differences compared to non-inoculated plants, as mentioned earlier.

Table 6 Effect of microbial inoculation in maize (*Zea mays* cv. "hybrid waxy maize MX10") and rice (*Oryza sativa* cv. "OM5451") fertilized with 75% N regime at harvest

Plant species	Treatment[a]	Biomass			Yield[b]			N content	
		Shoot dry weight (g)	Root dry weight (g)	Root lenght (cm)	Grain weight (g)	Grain production (g plant^{-1})	Harvest Index	Shoot (mg plant^{-1})	Seed (mg 100/1000 seed^{-1})
Maize	Control	40.26 ± 7	6.0 ± 0.3	50.96 ± 0.3	7.32 ± 0.3	363.73 ± 12.5	0.90 ± 0.01	507.35 ± 1.8	116.14 ± 6.5
	AP1	38.17 ± 2.2	**12.22 ± 0.2 ***	**73.62 ± 0.7 ***	**16.13 ± 0.7 ***	**958.35 ± 10.9 ***	**0.96 ± 0 ***	**840.96 ± 16.6 ***	**271.11 ± 0.6 ***
	AP2	41.17 ± 1.4	**9.97 ± 0.1 ***	**73.24 ± 2.1 ***	**10.43 ± 0.9 ***	**552.72 ± 14 ***	0.93 ± 0	nd	nd
	S(04)	36.02 ± 1.1	**12.01 ± 0.2 ***	**81.76 ± 1.4 ***	**12.72 ± 0.8 ***	**713.97 ± 49.7 ***	**0.95 ± 0 ***	**840.51 ± 2.6 ***	124.81 ± 29.1
	21F210	29.74 ± 0.8	**8.58 ± 0.2 ***	**63.62 ± 1.4 ***	**10.76 ± 1.1 ***	**584.18 ± 6 ***	**0.95 ± 0 ***	nd	nd
	21F213	35.2 ± 0.6	**9.31 ± 0.2 ***	**64.34 ± 2.6 ***	**10.92 ± 1.4 ***	**553.68 ± 17.4 ***	**0.94 ± 0 ***	nd	nd
	21F220	35.05 ± 0.6	**10.76 ± 0.2 ***	**63.58 ± 1.9 ***	**11.37 ± 2.5 ***	**564.99 ± 24.4 ***	**0.94 ± 0 ***	nd	nd
	21F221	50.6 ± 0.2	**11.26 ± 0.2 ***	**81.48 ± 0.1 ***	**11.11 ± 1.2 ***	**586.43 ± 7.3 ***	**0.95 ± 0 ***	**776.0 ± 6 ***	**191.84 ± 15.4 ***
	21F222	36.25 ± 1	**11.51 ± 0.2 ***	**79.44 ± 0.9 ***	**16.71 ± 0.9 ***	**928.71 ± 11.9 ***	**0.96 ± 0 ***	nd	nd
	21F226	50.96 ± 1.3	**12.07 ± 0.9 ***	**85.94 ± 1.3 ***	**15.54 ± 0.2***	**903.77 ± 21.8***	**0.97 ± 0***	**550.6 ± 8.4 ***	**239.33 ± 7 ***
Rice	Control	49.39 ± 0.5	nd[c]	nd	18,50 ± 0,1	22.74 ± 0.8	0.32 ± 0.01	391.87 ± 6.2	353.84 ± 3.3
	AP1	55.22 ± 20.8	nd	nd	**19.78 ± 0.1 ***	**36.35 ± 0.5 ***	**0.37 ± 0**	412.6 ± 12.9	130.28 ± 6.2
	AP2	**58.68 ± 19.9 ***	nd	nd	**19.28 ± 0.1 ***	37.37 ± 1.9	0.35 ± 0	nd	nd
	S(04)	**63.2 ± 20.2 ***	nd	nd	**19.88 ± 0 ***	**39.19 ± 0.8 ***	0.35 ± 0	**500.9 ± 21.7 ***	**500.72 ± 19.4 ***
	21F210	49.44 ± 2.2	nd	nd	**19.52 ± 0.2 ***	32.24 ± 1	0.34 ± 0	nd	nd
	21F213	**61.02 ± 1.8 ***	nd	nd	18.89 ± 0.4	**35.79 ± 1.2 ***	0.31 ± 0	nd	nd
	21F220	48.97 ± 0.7	nd	nd	**20.45 ± 0.2 ***	30.96 ± 0.5	0.34 ± 0	nd	nd
	21F221	51.64 ± 0.9	nd	nd	**19.16 ± 0.1***	**30.12 ± 1.8 ***	**0.37 ± 0.01 ***	**530.01 ± 14.4 ***	**536.58 ± 10.5 ***
	21F222	**57.45 ± 0.8 ***	nd	nd	**20.5 ± 0.1 ***	**40.1 ± 1.4 ***	0.35 ± 0	nd	nd
	21F226	**54.89 ± 0.5 ***	nd	nd	**20.57 ± 0.2 ***	**34.57 ± 0.4 ***	**0.39 ± 0.00 ***	408.32 ± 8.8	355.55 ± 6.3

Values are: means ± SE (n=10) in maize and (n=9) in rice. Values marked with asterisks are significantly different to non-inoculated plants (Dunnett's test p-value < 0.05).

[a] Treatments: non-inoculated plants (Control), inoculated with *A. brasilense* reference strains (AP1 and AP2), Vietnamese formulation tested S(04) and *Azospirillum* and *Azotobacter* isolates at 10^8 CFU g^{-1} seed

[b] Yield measurements being 100-filled maize and 1000-filled rice grain weight , grain production at 14% of humidity and the harvest index (grain production/ shoot dry weight)

[c] Non-determined (nd)

2.3.2 Rice under field conditions

The results of field-grown rice plants are summarized in Table 6, which includes data from both non-inoculated plants (Control) and rice plants inoculated with *A. brasilense* strains 21F221 and *A. aestuarii* strain 21F226, as well as reference strains AP1, AP2 and S(04) and other strains from the collection 21F210, 21F213, 21F220, and 21F222. In terms of biomass parameters, significant differences were observed in shoot dry weight between 21F210, 21F213, 21F220, AP1 and AP2 strains and non-inoculated plants with increases of 23%, 17%, 17, 16% and 13% respectively. Also significant variations were noted in the number of panicles and the percentage of filled spikelet compared to non-inoculated plants with strain 21F221 with value increments of 8% and 3%, also on number of panicles differences were noted in inoculation with strains AP1, AP2, S(04), 21F210, 21F213, 21F220 and 21F226 with higher values in inoculation with *A. aestuarii* with 13% increment compared to non-inoculated plants (Table 7). Regarding yield parameters corresponding to weight of 1000 rice grains and grain production differences between non inoculated plants and plants inoculated with S(04) and 21F226 are observed with higher values of 3% and 6% in gran weight and 19% and 23% in grain production respectively, also differences were observed in grain production between non inoculated plants and plants inoculated with all strains tested except for 21F222. Observing the estimated yield values, some differences were noted compared to non-inoculated plants, with the highest values in plants inoculated with AP1 and 21F226, showing a 19% increase, followed by 21F221, 21F213, 21F220, AP2, and S(04). In terms of nitrogen accumulation, significant differences between inoculated plants and control plants were observed in plant shoot nitrogen accumulation with differences in *A. brasilense* strain AP1, 21F221 and *A. aestuarii* strain 21F226 with increments of 27%, 17% and 29% respectively (Table 7).

Table 7 Effect of microbial inoculation in rice plants (*Oryza sativa* cv. "OM5451") cultivated in the field and fertilized with 75% N regime

Treament[a]	Biomass			Yield[b]				N content	
	Shoot dry weight (kg plot^{-1})	Number of panicles (plant^{-1})	Filled spikelet (%)	Grain weight (g)	Grain production (kg plot^{-1})	Estimated yield (ton ha^{-1})	Harvest index	Shoot (mg plant^{-1})	Seed (mg 1000 seeds^{-1})
Control	12.9 ± 0.3	5 ± 0	93.68 ± 0.9	21.02 ± 0.2	9.61 ± 0.03	5.81 ± 0.21	0.43 ± 0.01	258.85 ± 8.3	353.16 ± 15.1
AP1	**14.58 ± 0.2 ***	**5.27 ± 0.03 ***	95.6 ± 0.4	20.53 ± 0.5	**10.84 ± 0.21 ***	**6.91 ± 0.09 ***	0.43 ± 0	**328.46 ± 0.9 ***	344.84 ± 11.2
AP2	**14.95 ± 0.3 ***	**5.33 ± 0.03 ***	96.64 ± 0.2	20.33 ± 0.3	**10.43 ± 0.17 ***	**6.58 ± 0.1 ***	0.41 ± 0.01	nd[c]	nd
S(04)	11.31 ± 0.1	**5.5 ± 0.06 ***	93.36 ± 1	**21.63 ± 0.1 ***	**11.42 ± 0.09 ***	**6.28 ± 0.16 ***	**0.5 ± 0 ***	243.02 ± 4.5	322.62 ± 11.1
21F210	**15.92 ± 0.5 ***	5.25 ± 0.08	95.8 ± 0.4	21.17 ± 0.2	**10.8 ± 0.09 ***	**6.6 ± 0.04 ***	0.4 ± 0.01	nd	nd
21F213	**15.15 ± 0.3 ***	**5.29 ± 0.08 ***	96.11 ± 0.3	21.32 ± 0.3	**11.42 ± 0.15 ***	6.55 ± 0.13	0.43 ± 0.01	nd	nd
21F220	**15.15 ± 0.1 ***	5.29 ± 0.12	96.11 ± 0.5	21.32 ± 0.3	**11.42 ± 0.18 ***	6.55 ± 0.28	0.43 ± 0	nd	nd
21F221	13.33 ± 0.2	**5.41 ± 0.08 ***	**96.99 ± 0.1 ***	21.37 ± 0.3	**11.59 ± 0.41 ***	**6.67 ± 0.09 ***	**0.46 ± 0.01 ***	**303.98 ± 4.6 ***	319.13 ± 14
21F222	12.16 ± 0.1	5.6 ± 0.06	93.91 ± 1.5	21.14 ± 0.1	10.6 ± 0.03	6.77 ± 0.11	0.47 ± 0	nd	nd
21F226	13.79 ± 0.2	**5.63 ± 0.09 ***	94.82 ± 0.7	**22.25 ± 0.1 ***	**11.79 ± 0.06 ***	**6.9 ± 0.04 ***	**0.46 ± 0.01 ***	**334.95 ± 8.6 ***	352.86 ± 10.1

Values are means ± SE (n=3 plots with 160-170 g seeds plot^{-1}). Values marked with asterisks are significantly different to non-inoculated plants p-value < 0.05, determined through Dunnett's test.

[a] Treatments correspond to non-inoculated plants (Control), inoculated with *A. brasilense* reference strains (AP1 and AP2) and *Azospirillum* and *Azotobacter* isolates at 10^8 CFU g^{-1} seed

[b] Yield measurements being 1000-filled rice grain weight, grain production at 14% of humidity and the harvest index calculated as grain production/ shoot dry weight

[c] Non-determined (nd)

3. Inoculation effects of selected isolates on relevant agronomic indices related to nitrogen use efficiency

3.1. Regarding plant biomass

The Table 8 presents agronomic indices to assess nutrient use efficiency (Dobermann, 2007), calculated based on the fertilization regime, values of the aerial plant biomass (shoot dry weight) and plant nitrogen accumulation from maize grown with N free fertilization (0% N) and data form 60% N fertilization regime. These indices are shown for both non-inoculated maize and maize inoculated with *A. brasilense* strains 21F221 and *A. aestuarii* 21F226. Significant differences were observed in the values of apparent crop recovery efficiency of supplied nitrogen (RE) between inoculated with 21F221 and 21F226 and non-inoculated plants, with an increase of 19% and 20%, respectively. Additionally, differences were noted in agronomic efficiency (AE) and partial factor productivity (PFP) of supplied N, in plants inoculated with *A. brasilense* strain 21F221 compared to non-inoculated plants, with increments of 24% increase in AE and a 20% increase in PFP, as well as, for inoculation with *A. aestuarii* strain 21F226 with increases in AE by 34% and PFP by 29%. However, in the data related to physiological efficiency of acquired N (PE) and internal utilization efficiency of N (IE), no significant differences were observed between non-inoculated and inoculated maize plants (Table 8).

Table 8 Agronomic indexes of maize plants (*Zea mays* cv Tía María) fertilized with N-free Hoagland and reduced N Hoagland solution (60%N).

Treatment[a]	Recovery efficiency of supplied N	Physiological efficiency of acquired N	Internal efficiency	Agronomic efficiency of supplied N	Partial factor productivity of supplied N
Control	185,79 ± 7	0,089 ± 0,002	0,100 ± 0,001	16,64 ± 0,9	19,03 ± 0,7
21F221	**219,99 ± 12 ***	0,094 ± 0,001	0,102 ± 0,002	**20,57 ± 1,2 ***	**22,96 ± 1,2 ***
21F226	**222,76 ± 7 ***	0,100 ± 0,004	0,108 ± 0,004	**22,24 ± 0,6 ***	**24,62 ± 0,7 ***

Values are means ± SE (n=12). Values marked with asterisks are significantly different to non-inoculated plants p-value < 0.05, determined through Dunnett's test.

[a] Treatments correspond to non-inoculated plants (Control) and inoculated with *A. brasilense* strain 21F221 or *A. aestuarii* inoculated at 10^8 CFU g^{-1} seeds.

3.2 Regarding grain production

The Table 9 presents agronomic indices to assess nutrient use efficiency (Dobermann, 2007), calculated based on the fertilization regime and the values grain yield and nitrogen seed accumulation data of maize and rice plants grown with a fertilizer concentration lower than the agronomic standard (75%N). These indices are shown for both non-inoculated maize and maize inoculated with *A. brasilense* strains 21F221 and *A. aestuarii* 21F226 (Table 9). Significant differences were also observed in the internal nitrogen utilization efficiency, particularly in plants

inoculated with *A. aestuarii* strain 21F226, which showed a 38% increase in maize and a 28% increase in rice compared to non-inoculated plants.

Additionally, notable differences were found in nitrogen export due to 21F221 and 21F226 inoculation, this parameter measures the amount of absorbed nitrogen allocated to the agronomic part of the plant, in maize, inoculation with strains 21F221 and 21F226 resulted in 70% and 103% higher values, respectively, while in rice, the increases were 76% and 17%, respectively, compared to non-inoculated plants. These findings underscore the positive impact of *A. brasilense* strain 21F221 and *A. aestuarii* strain 21F226 inoculation on nutrient use efficiency in crops under reduced nitrogen fertilization (Table 9).

Table 9 Agronomic indexes of maize plants (*Zea mays* cv Tía María) and rice plants (*Oryza sativa* cv. OM5451) fertilized with reduced N Hoagland solution (75%N).

Plant species	Treatment[a]	Partial factor productivity of supplied N	Internal efficiency	Nutrient export
Maize	Control	101,9 ± 2,6	13,97 ± 0.3	20,09 ± 2.1
	21F221	**161,1 ± 1,5 ***	13,72 ± 0.6	**34,23 ± 2.5 ***
	21F226	**207,9 ± 3,7 ***	**19,24 ± 0.4 ***	**40,88 ± 1 ***
Rice	Control	4,88 ± 0,12	7,83 ± 0.3	19,99 ± 0.4
	21F221	**5,86 ± 0,28 ***	7,21 ± 1.2	**35,11 ± 1.4 ***
	21F226	**6,33 ± 0,07 ***	**9,99 ± 0.2 ***	**23,41 ± 0.4 ***

Values are means ± SE (n=5) in maize and means ± SE (n=6) in rice. Values marked with asterisks are significantly different to non-inoculated plants p-value < 0.05, determined through Dunnett's test.
[a] Treatments correspond to non-inoculated plants (Control) and inoculated with *A. brasilense* strain 21F221 or *A. aestuarii.* inoculated at 10^8 CFU g^{-1} seeds

Discussion

1. Impact of *Azotobacter* inoculation on phosphorus solubilization and nutrient use efficiency

Despite observing a notable capacity for phosphate solubilization in *in vitro* studies, in *Azotobacter salinestris* strain 21F213, a significant increase in maize growth was not evident when fertilized with P-free Hoagland solution in the presence of the hydroxyapatite which is an insoluble source of P. These findings suggest that the effectiveness of phosphate solubilization in the laboratory may not fully reflect its efficacy in the soil as observed in other research (Richardson et al., 2009; Wakelin et al., 2006). The variability in the efficacy of *Azotobacter* strains in phosphate solubilization and plant growth has been documented previously (Bashan et al., 2013; Kumar et al., 1999). Several reasons could explain this discrepancy: first, it is possible that the phosphate solubilization mechanisms identified under laboratory conditions may not

operate similarly in soil (Whitelaw et al., 1999); second, it is important to consider the influence of environmental and edaphic factors, such as soil pH and the availability of other nutrients (Hinsinger et al., 2003; Hodge, 2004; Ryan et al., 2000) and third, unlike the specific associations between rhizobia and their host plants, phosphate solubilizers do not exhibit specific natural associations with host plants, which may lead to greater diversity in plant-microorganism interactions (Richardson, 2001). Therefore, to gain a more accurate and applicable understanding of how *A. salinestris* strain 21F213 works in association with maize plants, further experiments considering these factors are needed.

2. Impact of *Azospirillum* inoculation on nitrogen uptake and utilization

Based on maize plants measurements performed in N-free fertilized plants (experiment A, B and C), no significant effects were observed in plant height or the nitrogen balance index (NBI) following inoculation with *A. brasilense*, the lack of observed differences in plant length can be attributed to the high heritability of this trait, as documented in studies such as those conducted by Ermindo Cavallet et al., (2000) and Rural & Maria, (2000). The no significant differences in NBI between inoculated and non-inoculated plants may arise from the limited representativeness of the sample, as only a small part of the plant is analysed for measurements. However, despite the absence of differences in this traits, significant differences between non-inoculated plants and inoculated plants were noted in N-free fertilized maize (0%N), where it is highlighted the clear impact of *A. brasilense* strains 21F221 and *A. aestuarii* 21F226 on growth and nitrogen accumulation. These findings are consistent with previous results in maize outlined in the literature, where similar experimental parameters, such as seed inoculation with *A. brasilense* at 10^8 CFU mL^{-1}, with N-free fertilization, resulted in a significant increase in nitrogen concentration in plants and grain cob (Oliveira et al., 2018). Moreover, the observed increase in stem dry matter ranging between 11% and 20% by inoculation of strains 21F221 and 21F226 are similar to those obtained performing similar experiments with another strain of *A. brasilense* with seed inoculation at $2 \cdot 10^8$ CFU mL^{-1} without nitrogen fertilization (Marini et al., 2015). Furthermore, a 5% increase in shoot dry weight by effect of strain of *A. brasilense* on 26 maize genotype cultivated with low nitrogen input by seed inoculation at 10^8 CFU mL^{-1} has been documented in the literature (Zeffa et al., 2019). This increase in dry weight due to the inoculation of has also been observed in other crops, such as sugarcane (Moutia et al., 2010).

Additionally, in experiment A from in N-free fertilized plants, the *Azospirillum spp.* population in maize rhizosphere remained stable at 10^6 CFU mL^{-1} after 28 days post-seed inoculation, demonstrating its resilience, which align with Urrea-Valencia et al., (2021) research, which noted

a stabilization in *Azospirillum* after 15 days at a concentration around 10^5 CFU g^{-1} of fresh root post-inoculation of the seed at $2 \cdot 10^8$ CFU mL^{-1} and during whole experiment, this stabilization of the colonies is likely due to the genus's ability to colonize the root surface with the possibility of also penetrating the interior of root hairs as described in Santos et al., (2017a). Our findings also underscore the significance of microbial persistence in the rhizosphere for eliciting beneficial plant effects (El Zemrany et al., 2006) although the presence of *Azospirillum* in the rhizosphere does not necessarily guarantee effects on the plant as observed in our data.

Based on maize plants data obtained from A and B experiments performed at 60% N fertilization regime significant differences were noted between non-inoculated plants and inoculated plants, with increases in biomass and nitrogen accumulation due to inoculation, notably for strains 21F221 and 21F226 aligning with other results in the literature, such as Naiman et al., (2009) which described a 26% increase in maize biomass after *A. brasilense* inoculation, while Quadros, (2009) reported up to a 53% increase in stem dry matter yield. The effect of *Azospirillum* inoculation increasing nitrogen and other macro and micronutrient accumulation in maize plants is also well-documented in the literature (Fonseca Breda et al., 2019; Moutia et al., 2010; Picazevicz et al., 2017). However, in contrast to our findings, Hungria et al., (2010) did not observe effects on grain nitrogen content due to microbial inoculation at a similar fertilization regime.

The effect of inoculation on biomass increases and nitrogen accumulation at 75%N fertilization regime, potentially could be attributed to the *Azospirillum*'s ability to alter maize root morphology by increasing the number of roots and root hairs, thus enhancing root-soil contact surface and, consequently, improving water and nutrient absorption by the plant (Baldani et al., 1997; Bashan et al., 2004; Dobbelaere et al., 2001; Kapulnik et al., 1983; Steenhoudt & Vanderleyden, 2000) these observations are consistent with the observed in our experiment where the effect of inoculation increased maize root dry weight and length. Historically, this effect on biomass and root morphology has been attributed to phytohormone production (Bashan & de-Bashan, 2010; Okon & Labandera-Gonzalez, 1994), however, in recent years, it has also been linked to *A. brasilense's* ability to stimulate root exudation of carboxylates, which affects the rhizosphere microbial community and promotes increased root growth (D' Angioli et al., 2017; Liebersbach et al., 2004; Skonieski et al., 2017). Even though in our experiment we noted a significant increase in maize root biomass, there was no significant differences observed in aboveground biomass, this discrepancy could be attributed to the timing of sampling, as previous research has suggested that aboveground growth tends to decrease as crops develop, generally being less than root growth (Dobbelaere et al., 2001; Naiman et al., 2009). Although in some cases, rice inoculated with AP2, S(04), 21F213, 21F222 and 21F226 showed higher aerial biomass compared to non-inoculated

plants, this could be due to the sampling moment of rice plants which did not capture the full maturity phase.

The benefits in crop grain yield are primarily attributed to the increase in the quantity of grains produced in response to enhanced vegetative growth, suggesting a highly linear relationship between biomass production and crop yield (Cassán & Diaz-Zorita, 2016; Ghassemi-Golezani, 2012; Kang et al., 2017; Munns et al., 2010; Wu et al., 2022). The ability of members of the genus *Azospirillum* to increase yields has been studied and described for several decades, consistently demonstrating the positive impact of inoculation with *Azospirillum* on grain and forage yields (Pereg et al., 2016; Rozier et al., 2017). Specifically, the effect of *A. brasilense* inoculation on increasing maize yield is well-documented in the literature (Cassán & Diaz-Zorita, 2016; Di Salvo & García de Salamone, 2019; Hungria et al., 2010; Mala et al., 2010), with typical increases ranging from 16% to 30% compared to non-inoculated plants. However, our findings in maize experiment at 75%N fertilization, reveal significantly higher increases (52-104%), consistent with the results reported by Garcia et al., (2017), who recorded increases of 46% and 102% in yield through the inoculation of a commercial *A. brasilense* strain under conditions of high nitrogen availability (90%).

Regarding the results obtained in rice at 75% N fertilization, we observed an increase in grain yield due to the inoculation with strains 21F221 and 21F226. The positive effects of *A. brasilense* inoculation on grain production are well-documented in the literature, both in greenhouse-grown plants (Majumdar & Sahg, 2007) and in field conditions (Razie & Anas, 2008). For instance, Ferreira et al., (2015) documented comparable production results, with increases in yields ranging from 40% to 108% compared to non-inoculated controls. In another line of experimentation, for production values obtained in paddy rice fields fertilized with 75% N, studies by Salamone et al., (2010) reported yield increases within the same range as those observed in our study (20% - 22.5%) as well as, the impact on the number of filled spikelets in rice plants cultivated in the field by strain 21F221 was observed in Lakzadeh et al., (2015), suggesting that inoculation enhance plant biochemical condition and this leads to increased flower and pollen production, which could contribute to the observed differences in spikelet filling. The different values obtained between both lines of experimentation in rice, greenhouse and paddy field, could be attributed to multiple factors, including variability in environmental conditions, the complex interaction between microorganisms and plants, as well as differences in nutrient availability and competition with other soil organisms in field conditions (Mehnaz, 2015).

Also, the variability in results among the measures on the different experiments, especially in terms of the response of maize plants to inoculation with *Azospirillum spp*, is a phenomenon previously observed in scientific literature (Marini et al., 2015; Skonieski et al., 2017). However,

there is no single explanation for this variability, some authors suggest it could be attributed to the nature of the association between *Azospirillum* and the plant, as it does not involve the formation of symbiotic structures, these bacteria are more vulnerable to the environment (Gyaneshwar et al., 2002), making them susceptible to variations in environmental factors and plant conditions such as pH, humidity, water activity, oxygen, temperature, and the plant's genotype (Bashan et al., 2004; Hungria, 2010; Joe et al., 2012; Oliveira et al., 2004). Examples of this environmental sensitivity have been observed under stress conditions, where responses to inoculation treatments are less consistent (Lana et al. 2012; Mehnaz et al., 2010; Naiman et al., 2009). Despite this variability, it is important to highlight that there was a general trend towards increased growth, improved nutrition, and higher production in plants inoculated with strains 21F221 and 21F226 compared to the control group.

While the variability in results from different experiments highlights the complex nature of *Azospirillum*-plant interactions, it also underscores the importance of understanding the optimal conditions for inoculation. In the literature, several inoculation methods for *Azospirillum spp*. are commonly reported, including seed coating, soil application, and foliar spray (Bashan et al., 2014). In our experiments, two different seed inoculation methods were employed: seed coating for maize in the 0% and 60% N experiments and seed imbibition for maize and rice in the 75% N experiments. Seed coating, which consist of dusting seeds with peat inoculant with or without water or adhesive, is often considered the most practical and commonly used inoculation technique because it is easy to use and requires relatively small amounts of inoculant (Bashan et al., 2014). In our study, peat was used, also described as an effective carrier for *A. brasiliense* in maize seeds (Barbosa et al., 2022) and carboxymethyl cellulose (CMC) as an adhesive to ensure each seed received a sufficient number of bacteria, also described in Souza et al., (2022) as effective at preserving the viability of *Azospirillum spp*. (Souza et al., 2022). Despite its benefits, seed coating has limitations, particularly for small seeds, where the amount of inoculant that can be applied is limited, which may be insufficient to meet the bacteria threshold needed for successful inoculation (Bashan et al., 2014). Given these constraints, in experiments conducted under a 75% nitrogen fertilization regime, seed inoculation was performed by imbibing maize and rice seeds in the microbial inoculum, a method already reported as effective for inoculating PGPR in rice grown in paddy fields (Nguyen et al., 2021) and for *Azospirillum spp.* inoculation in maize (Bashan et al., 2014; Casanovas et al., 2000). In both cases seed inoculation was effective in increasing plant nitrogen use efficiency, however, to determine which method, seed coating or seed imbibition, yields better results in terms of biomass and production further specific experiments are needed. These experiments should compare both inoculation methods under the same experimental conditions including moment of sampling, plant genotype, environmental factors and substrate.

3. Agronomic indices related to nutrient use efficiency

In order to express the results in terms established by the CEN/TC 55 guidelines, several agronomic indices were calculated to demonstrate the beneficial effects of the selected strains 21F221 and 21F226 on nutrient use efficiency, a claim that can be expressed on the label of a commercial biostimulant according to the European Fertilizer Regulation, indicating that both strains had a positive effect on increasing nitrogen use efficiency in rice and maize, in terms of biomass production and yield. This effect manifested in a higher conversion of applied nitrogen into biomass, suggesting that strains 21F221 and 21F226 facilitate better nitrogen absorption and utilization, results being consistent with previous studies showing that inoculation with *A. brasilense* can significantly improve nutrient absorption and plant growth (Cassán et al., 2009; Hungria, 2010).

Additionally, inoculation was observed to improve nitrogen export efficiency, indicating that inoculated plants are more efficient in translocating this essential nutrient for biomass production and in mobilizing nitrogen towards the grains. These results align with the characteristics of *A. brasilense* described in the literature, which report increases in nitrogen use efficiency, contributing to greater biomass accumulation and yield (Bashan & de-Bashan, 2010; Okon, 1994). A notable difference between the two strains evaluated is that only strain 21F226 shows an improvement in internal nitrogen utilization efficiency (IE). This result suggests that strain 21F226 not only improves nitrogen absorption and translocation but also optimizes the internal conversion of nitrogen into productive biomass. Studies such as those by Dobbelaere et al., (2001) and (Vessey, 2003) have demonstrated that different strains of *Azospirillum* can have varying effects on nutrient utilization efficiency, highlighting the importance of selecting specific strains for agricultural applications.

Conclusions

Although *Azotobacter salinestris* strain 21F213 exhibited considerable phosphate solubilization capabilities *in vitro*, this did not lead to a significant increase in maize growth under P-free Hoagland solution conditions.

Despite the observed variability in some experiments, the overall findings support the potential efficacy of strains 21F221 and 21F226 in enhancing maize and rice growth and nitrogen plant accumulation particularly when supplemented with 25-40% reduction in mineral nitrogen which demonstrates that the use of this strains could imply a potential savings in the use of fertilizers. In maize, inoculation with *Azospirillum brasilense* strain 21F221 resulted in a 20% increase in plant growth, a 26% increase in nitrogen accumulation, and a 61% increase in yield, meanwhile inoculation with *Azospirillum aestuarii* strain 21F226 showed a 24% increase in plant growth, a

13% increase in nitrogen accumulation, and a remarkable 149% increase in yield. Additionally, for rice, strain 21F221 led to a 4% increase in growth, a 26% increase in nitrogen accumulation, and a 26% increase in production, also strain 21F226 resulted in a 7% increase in growth, a 17% increase in nitrogen accumulation, and a 37% increase in yield compared to non-inoculated rice plants.

The ability of these strains to improve multiple agronomic indices related to nutrient use efficiency (NUE) as defined by the microbial biostimulants CEN/TC 455 standards, suggests they could serve as promising candidates for developing of microbial biostimulants compliant with EU fertilizer regulations and standards for future CE marking, thus promoting more sustainable and efficient agricultural practices.

Chapter 4: Biochemical and ecological characterization of selected strains

Introduction

The potential of *Azospirillum spp*. as a biostimulant has been the subject of considerable research over the decades, primarily focusing on its effect on large-scale cereal production such as rice, maize, sorghum, wheat, and millet (Okon & Labandera.Gonzalez, 1994). However, it would also be interesting to explore its effectiveness and applicability in other productive sectors, such as vegetable production and both annual and perennial plants (Vendruscolo & de Lima, 2021). In terms of application *Azospirillum spp.* has been used in a range of formulations, from seed inoculation to the direct application of live cell cultures via fertigation or spraying (Fukami et al., 2017). However, challenges remain in developing formulations that are consistently effective, particularly where seed inoculation may not be feasible or when multiple inoculations are required during the season (Bashan et al., 1995). Therefore, understanding the colonization process of *Azospirillum spp.,* its effects, and its survival in the soil is crucial to improving its effectiveness in these contexts.

The colonization pattern of *Azospirillum spp.* on plant roots has been extensively investigated, particularly in grass species, since the work of Lucia Baldani, (1980). The root colonization by *Azospirillum spp.* involves two phases: adsorption and anchoring, adsorption is a rapid and reversible process, is likely controlled by bacterial protein compounds, while anchoring, which is stronger and takes several hours to form, involves bacterial surface polysaccharides that permanently connect the bacteria to the root surface (Bashan et al., 2004). As colonization progresses, *Azospirillum spp.* colonies position themselves in different root sites; beginning with an increase in the number of cell aggregates at lateral branching points three days after inoculation, by day seven, cell groups are observed throughout the entire root system, and by day twelve, colonization focuses on young root areas such as the elongation and differentiation zone (Santos et al., 2017a).

Detailed characterization of *Azospirillum spp.* strains regarding their sugar metabolism, fermentation, and enzymatic production is also essential for understanding the mechanisms through which it interacts with plants exudates and soil, which in turn can provide insights into its formulation and its potential as a biofertilizer in sustainable agriculture. Steenhoudt & Vanderleyden, (2000) provided a comprehensive overview of *Azospirillum spp.* physiology and biochemistry, highlighting its ability to utilize a wide variety of sugars as carbon and energy sources. Furthermore, Van Bastelaere et al., (1999) demonstrated that *Azospirillum spp.* exhibits chemotaxis toward some sugars, such as D-fucose, L-arabinose, and D-galactose, suggesting an adaptation to actively seek nutrients in the soil. Referring to the biochemical activities, Idris et al., (2007) investigated the production of specific enzymes, such as the ones involved in synthesizing indole-3-acetic acid (IAA), and Mehnaz (2015) described the capacity to produce

substances that influence rhizosphere structure and growth promotion, such as nitric oxide (NO), nitrite, nitrate reductase (NR), and lectins.

The transition from studying the effect of *Azospirillum spp.* application under controlled laboratory or greenhouse conditions to analysing its impact in field conditions poses a significant challenge. From Okon & Labandera-Gonzalez, (1994) to the present day, numerous studies across various crops have concluded that, worldwide, in almost all cases inoculating *Azospirillum spp.* under natural conditions, yields a positive but very variable response in plants, largely influenced by environmental conditions and crop management practices, such as organic and mineral fertilization (Cassán et al., 2020; Pariona-Llanos et al., 2010). Additionally, agricultural practices can significantly affect soil microbial communities, favouring specific autochthonous microorganisms and microbial life strategies, which underscores the complexity and the need for an integrated approach to understanding and optimizing the use of *Azospirillum spp.* in the field (Degrune et al., 2017).

Organic fertilization practices harness natural materials such as manure, compost, crop residues, and kitchen waste to enrich soil fertility. These materials decompose gradually in the soil, facilitated by microbial activity, releasing vital nutrients like nitrogen, phosphorus, potassium, and micronutrients (Sharma, 2017), The decomposition process is influenced by factors like the quantity and quality of residues, soil characteristics and environmental conditions (Dail & Fitzgerald, 1999; Hamel et al., 2004). In organic fertilization, usually the soils are low in available nitrogen and microbes decompose soil organic matter (SOM), in order to access nitrogen for their nutritional needs to growth. This way, they can incorporate the mineral N into organic chains to produce amino acids, proteins, nucleic acids and other organic compounds which they use to live, and which eventually could be released to plants after the death of soil microbes (Wang et al., 2022). This microbial activity is a key component of the priming effect, when microbes are stimulated by fresh organic inputs, they increase their metabolic activity, leading to enhanced decomposition of existing SOM (Gunina & Kuzyakov, 2022), This process not only mobilizes nutrients such as nitrogen and phosphorus but also contributes to the formation of more stable forms of organic matter, which can improve soil structure and fertility in the long term (Fontaine et al., 2011; Kuzyakov et al., 2000). Organic fertilization then offers several advantages as it not only fertilizes plants but also improves soil structure, enhances water retention capabilities, and fosters beneficial microbial communities, thereby promoting overall soil health (Naveed et al., 2014).

Mineral fertilization involves the use of synthetic or mineral chemicals specifically designed to provide nutrients to plants. These products, such as ammonium nitrate, di-ammonium phosphate, and potassium sulphate, contain nutrients in inorganic form, which are rapidly released into the

soil and easily absorbed by plants (Herencia et al., 2007). While mineral fertilization can provide nutrients quickly and in a controlled manner, it does not contribute to increase the levels of organic matter to the soil and can result in salt accumulation and leachate pollution if overused (Magdoff & Van Es, 2021).

In natural ecosystems, microbial communities play a crucial role in supporting global ecosystem services and agricultural sustainability, serving as the drivers of ecosystem functioning and homeostasis (Augelletti et al., 2019). To understand how soil microorganisms function in nutrient recycling and their role in making essential nutrients available to plants, such as nitrogen, phosphorus, and potassium, it is crucial to understand the cycles of these elements. It is also important to study the microbial communities present in the soil with different functional traits by methodologies such as substrate induced respiration (SIR) which is a measurement of microbial respiration of samples after amending them with an excess of a readily nutrient source to trigger microbial activity (Aira & Domínguez, 2010). With this approach, it is possible to establish the metabolic characterization of soil microbial communities quickly, economically, and *in situ* (Campbell et al., 2003) and consequently, obtain the catabolic index which is the microbial community capacity to metabolize a selection of carbon substrates with contrasting chemical characteristics.

Objectives

This chapter is focused to characterise *Azospirillum brasilense* strain 21F221 and *Azospirillum aestuarii* strain 21F226 and understand their performance in soil, which could influence in the biostimulant's effectiveness and application strategies.

The specific objectives were:

1. To study *Azospirillum* strains 21F221 and 21F226 colonization dynamics in maize rhizosphere, encompassing the determination of strain concentration during plant development and the capacity to penetrate within root structures.
2. To characterize both strains by evaluating the ability to metabolize various sugar compounds and to display diverse enzymatic activities.
3. To evaluate the influence of both bacterial strains on the soil nitrogen forms content and the indigenous microbial community in field soils with different fertilization background, fertilized with organic or mineral inputs.

Material and methods

1. Rhizosphere and root colonization

To monitor the rhizosphere concentration dynamics of *A. brasilense* strain 21F221 and *A. aestuarii* 21F226 over time as well as to ascertain the stabilization of their concentration and potential root penetration ability, bacterial suspensions were prepared following the same protocol as used for the plant experiments.

Microorganisms were stored in glycerol in the freezer (-80ºC). When preparing the plant inoculum one loop from each strain was placed in RC solid plates (Caceres, 1982) and left at 30ºC for 48h. The grown colonies were transferred to tubes with 20 mL of NBS liquid media (Beef extract 3.0 g L^{-1}, Peptone 5.0 g L^{-1} and Sodium chloride 5.0 g L^{-1}) and left with adequate aeration and agitation (150 rpm) for 72 h at 30 ºC. After that time, cell concentration was measured by spectrophotometry at 600 nm and compared with a calibration curve. Then the microbial suspensions were centrifugated (Hettich model UNIVERSAL 320) at 4000 *g* for 10 min and resuspended in sterile NBS liquid medium to obtain a concentration of 10^{10} CFU mL^{-1}.

Maize seeds (*Zea Mays* cv "Tia Maria" from Les Refardes S.L) were used for the experiment, seeds were added to sterile plastic tubes. Each tube received 0.2 g of Jiffy GO M8 (peat previously sieved at 0.71 mm), followed by 0.2 mL of each inoculum and 0.70 mL of 1% (w/v) CMC (Carboxymethyl cellulose), obtaining an inoculum of 10^8 CFU g $seeds^{-1}$. The tubes were shaken by hand to ensure homogeneous distribution of the treatment to the seeds.

The experiment was conducted in the greenhouse of the Faculty of Biology at Universitat de Barcelona (41.385018, 2.120436) and followed a Latin square design with 3 treatments: a control without inoculation and seeds inoculated with *A. brasilense* strains 21F221 and *A. aestuarii* 21F226. Each treatment included 12 replicates, with each replicate consisting of one pot containing a single plant. Four pots were assigned to each sampling time point (7, 15, and 36 days post-sowing)

Pre-inoculated seeds were sown in 1 L pots containing a mixture of peat, vermiculite, and perlite (2:1:1 v/v/v) pre-hydrated with 100 mL of tap water. Two seeds per pot were sown and after a week, only one plant was left in each pot. Plants were fertilized on demand with 60% N Hoagland solution prepared with $(NO_3)_2$ (47.2 mmol L^{-1}) and KNO_3 (4 mmol L^{-1}), $CaCl_2$ (11.8 mmol L^{-1}) and KCl (1 mmol L^{-1}) modifying Hoagland & Arnon, (1950) solution.

To collect rhizosphere samples, plants were carefully removed from the pots, and substrate not attached to the root system was excluded. Rhizosphere samples were brought to the laboratory, there the substrate attached to the rhizosphere and roots were carefully separated. In the case of

soil attached to roots, 10 g from each pot were subjected to an initial dilution in 90 ml of NaCl saline solution (9 g L^{-1}) and stirred at 150 rpm for an hour. Then, serial dilutions (10^{-2} to 10^{-7} CFU mL^{-1}) were made and plated onto RC plates, incubated for 72 hours at 30°C, and *Azospirillum*-like colonies (small, round colonies that turned scarlet over time) were counted.

For root colonization assessment, roots were sterilized with 98% ethanol during 1 min, and 70% ethanol during 2 min and rinsed by sequential washing with distilled water for 2 minutes. Root segments with the most root hairs were mashed in a sterilized mortar and plated onto RC media plates. After 72 hours of incubation at 30°C, colonies with morphological characteristics resembling *Azospirillum* were selected and further purified on RC media plates. Root samples that had been previously surface-disinfected and showed colonization with *Azospirillum*-like microorganisms were considered to be colonized internally.

2. Biochemical characterization of the strains

For the biochemical tests, Analytical Profile Index (API)® 20E and 50CHB strips (Scharlab SL, Spain) were used following the manufacturer instructions. *A. brasilense* strain 21F221 and *A. aestuarii* strain 21F226 were transferred from a frozen stock to RC media plates and incubated at 30°C for 72 hours. Subsequently, they were streaked onto LB media plates (Tryptone 10 g L^{-1}, yeast extract 5 g L^{-1}, NaCl 5 g L^{-1}, 15 g L^{-1} agar) using the Scottish streak technique, allowing growth for 48 hours at 30°C in the incubator. Once isolated colonies were observed, one colony was selected and gently emulsified with an ampoule of 0.85% NaCl API medium (5 ml) to obtain a homogeneous bacterial suspension.

The API® 20E strip consists of 20 microtubes containing dehydrated substrates which during incubation are subjected to colour changes due to the metabolism. The enzyme reactions studied by this strip are; β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, H_2S production, urease, tryptophane deaminase, indole production, acetoin production, gelatinase, fermentation-oxidation of glucose, mannitol, inositol, rhamnose, saccharose, melibiose, amygdalin and arabinose. On the other hand, the API® 50CHB strip allows to determine the fermentation of 49 carbohydrates: glycerol, erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, β-methyl-xyloside, galactose, glucose, fructose, mannose, sorbose, rhamnose, dulcitol, inositol, sorbitol, α-methyl-mannoside, α-methyl-glucoside, N-acetyol-glucosamine, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, threalose, inuline, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, D-turanose, D-arabiol, L-arabitol, gluconate, 2-keto-gluconate and 5-keto-gluconate.

For inoculating the microtubes, a pipette was used to distribute the bacterial suspension into the tubes of the strip, avoiding the formation of bubbles at the base of the tubes. The incubation box was then closed and incubated at 36°C for 120 hours. During incubation of the API® 20E strip, bacterial metabolism produces colour changes that are either spontaneous or revealed by the addition of reagents. For the API® 50CHB strip, during incubation, carbohydrates are fermented to acids, resulting in a decrease in pH also detected by the change in colour of the indicator.

3. Impact of the selected strains inoculation on two soils with different fertilization regimes

Agricultural soils from the Parc Agrari del Baix Llobregat, specifically from the Cal Notari (41°19'06.2"N 2°03'07.0"E) and Cal Mitjà (41°19'10.6"N 2°03'11.1"E) plots, were utilized in this study. The soil characterization details are provided in Table 1. These soils were chosen due to their proximity and differing crop management practices. In the Cal Notari soil, organic fertilization methods are used, incorporating organic material from green cover crops and pruning residues, introducing approximately 4 kg/m²/year applied as mulch. Conversely, in Cal Mitjà soil conventional management practices are used, relying on mineral NPK fertilization, particularly using "Nitrosulfate 26" (Fertiberia S.A., Spain) as a nitrogen source at a rate of 200 kg ha^{-1}. This mineral fertilizer consists of ammonium nitrate and ammonium sulphate, with nitric nitrogen concentration at 6.5 % and total sulphur at 15 %.

To investigate the impact of inoculating strains 21F221 and 21F226 with the two soils with different fertilization management, two trials were conducted; (i) Inoculated soils were incubated and nitrogen forms were determined at various time points, (ii) Inoculated soils were used to measure the substrate induced respiration (SIR) using the MicroResp™ kit (The James Hutton Institute, Scotland).

Table 1: Characterization of soil samples with organic (Cal Notari) and mineral (Cal Mitjà) fertilization used for incubations

Soils[a]	pH	EC[b] (dS m^{-1})	OM[b] (%)	Total nitrogen (mg kg^{-1})	Pa Olsen (mg kg^{-1})	C/N Ratio[b]	Texture
Cal Notari	8.0	0.44	6.10	0.42	17.6	8.47	Loam-Silt
Cal Mitjà	8.11	0.98	1.17	1.03	16.8	6.56	Sandy-Loam

[a] Soils corresponding to Cal Notari (organic-fertilized) and Cal Mitjà (mineral-fertilized)
[b] Parameters analysed meaning: EC, electrical conductivity; OM, oxidable organic matter; Pa Olsen, phosphorus available detected by Olsen method; and C/N Ratio, carbon/nitrogen ratio.

For both trials, the microorganisms were inoculated in the same manner into the soils as described in section 1. The strains 21F221 and 21F226, preserved in glycerol at -80°C, were seeded on RC medium plates then incubated at 30°C for 72 hours. Subsequently, on LB agar medium, and after 48 hours of growth at 30°C, the colonies were transferred to glass tubes containing liquid LB medium. After 72 hours of incubation at 30°C with agitation, the cell concentration was measured using spectrophotometry at 600 nm and compared with a calibration curve. Aliquots were centrifuged at 4000 *g* for 10 minutes, and the resulting pellet was resuspended to achieve a concentration of $5 \cdot 10^5$ CFU mL^{-1}. Serial dilutions were performed to confirm the purity and concentration of the microorganisms.

(i) The impact of strains inoculation on nitrogen forms concentration in both soils (Cal Mitjà and Cal Notari) was examined using a randomized experimental design with three independent variables: soil fertilization types (organic and mineral fertilization), sampling time pre- and post-inoculation (3 and 7 days), and inoculation treatment being a control without inoculation, and inoculation with *A. brasilense* strain 21F221 and *A. aestuarii* strain 21F226 at a concentration of $5 \cdot 10^5$ CFU mL^{-1}. Soil sample preparation involved sieving to remove large particles of organic matter and stones. Then fifty-millilitre vials containing 30 g of soil were prepared, and inoculation was performed with *A. brasiliense* bacterial suspension for the inoculated samples and saline solution for controls, totalling 5 vials for each combination of soils, sampling time, and with and without inoculation. The vials were kept covered in darkness for the duration of the 7-day experiment. At each sampling point, 30 g of soil were extracted; 10 g were used for dilutions to determine the concentration of *Azospirillum*-like microorganisms on RC medium plates and the remaining 20 g were used to perform nutrient extractions. Five g of soil were mixed with 50 mL of deionized water or potassium sulphate (KCl 2M) and agitated for 1 hour at 120 rpm, after which the samples were centrifuged at 4000 *g* for 5 minutes. The resulting extraction solution was used to calculate the concentration of ammonium (NH_4) following Sims et al., (1995) methodology; nitrate ($N\text{-}NO_3$) according to Cataldo et al., (1975) and total oxidable nitrogen (TNO) by Kjeldahl method with the autoclave as described in Bremner (1965). The soluble organic nitrogen (SON) was derived from nitrate and ammonium values using the formula: SON = TNO - (nitrate + ammonium), while mineral nitrogen (Min-N) was calculated as the sum of nitrate and ammonium. Furthermore, the rates of change in nitrogen forms were calculated by substracting the concentration values after 7 days to the concentration at the moment of inoculation, then dividing it by the number of incubation days.

(ii) In the second experiment, the CO_2 SIR, the catabolic index and the alpha diversity were determined using the MicroResp™ kit. Initially, sample preparation involved sieving to remove large particles of organic matter and stones, using approximately 100 g of sample. Subsequently, the water field capacity of the samples was determined according to Keen & Raczkowski (1921).

Then, the samples were inoculated with the microorganisms at a concentration of 5 x 10^5 CFU mL^{-1} in both types of soil, using a saline solution (9 gL^{-1} NaCl) to reach 50 % of the field capacity. Additionally, a control treatment without inoculation was included, using only saline solution. The MicroResp™ plates contain 48-wells for each treatment, each well was filled with the exact volume of soil (1.5 mL) and then the plates were weighed to calculate the added soil weight in the wells. Three wells per treatment and substrate were used. These plates were covered with parafilm and incubated for 5 days in darkness within a sealed and humid container, along with a pot of soda lime to capture the produced CO_2. Simultaneously, detection plates were prepared with 3% agar and an indicator solution composed of 18.75 mg cresol red, 16.77 g KCl, and 0.315 g $NaHCO_3$ dissolved in 1 litre of water. These plates were stored in darkness within a sealed container in a CO_2-free environment. After 5 days of incubation, the absorbance at 570 nm of the detection plates was measured, and the coefficient of variation was verified (< 5 %). Once the detection plates were verified, 25 µL per well of carbon sources were added at a concentration of 30 mg mL^{-1} in triplicate, along with controls of distilled sterile water, to evaluate microbial metabolism. The carbon sources used in this study were sugars (glucose, galactose, fructose, lactose, arabinose), amino acids (alanine, cysteine, lysine, aminobutyric acid and glutamic acid) and organic acids (citric acid, phytic acid, maleic acid and oxalic acid). The initial absorbance (t=0) was measured before assembling the MicroResp™ structure, which was left in darkness for 6 hours, finally, after 6 hours, absorbance (t= 6 h) was measured again to complete the process, then the SIR as µg CO_2-C $hour^{-1}$ could be calculated per each well.

Simultaneously with the MicroResp™ process, a portion of the inoculated soil sample was retained to calculate the concentration of *Azospirillum* present at the beginning and after 5 days of incubation, using serial dilutions and plating on RC media plates.

4. Statistical analysis

All data were analysed with the SPSS software package version 27.0. Data from *Azospirillum spp.* rhizosphere populations were analysed with a Dunnett's test in comparison with the non-inoculated plants (control), using a significance level of 95% for each sampling day (7, 15 and 36 days after inoculation).

Data from the effect of inoculation in nitrogen forms were firstly treated together with a three-way factorial repeated measures ANOVA followed by Tukey's post-hoc test at significance levels of p-value < 0.05 for each nitrogen form determined (ammonium, nitrate, total oxidable nitrogen, soluble organic nitrogen and mineral nitrogen), the factors used in the ANOVA were time, soil and inoculation, and the interactions. Additionally, to analyse the rates of nitrogen forms accumulation a two-way factorial repeated measures ANOVA followed by Tukey's post-hoc test

at significance levels of p-value < 0.05 was performed for each rate (ammonium, nitrate, soluble organic nitrogen and mineral nitrogen accumulation), considering as factors soil and treatment and the interaction.

For SIR values obtained a three-way factorial repeated measures ANOVA followed by Tukey's post-hoc test at significance levels of p-value < 0.05 was performed, data from carbon source was treated inside groups regarding to sugar, amino acids and organic acids. The factors, then in the statistical analysis were soils fertilization, treatment and carbon source, as well as, the interactions For catabolic index and alpha diversity index a two-way ANOVA followed by Tukey's post-hoc test at significance levels of p-value < 0.05 was performed, considering as factors soils fertilization and inoculation treatment and the interaction between factors.

Results

1. Rhizosphere colonization dynamics

The two microorganisms used in this experiment were inoculated into the seeds at a concentration of $7 \cdot 10^8$ and $3 \cdot 10^8$ CFU g seed^{-1}, equivalent to $2.40 \cdot 10^8$ and $1.10 \cdot 10^8$ CFU seed^{-1} for 21F221 and 21F226, respectively.

Table 2 *Azospirillum spp.* strains 21F221 and 21F226 maize rhizosphere population and root inner colonization in plants fertilized with 60%N Hoagland solution

Days after inoculation	Treatment[a]	*Azospirillum* population (10^6 CFU g^{-1} substrate)	Inner root colonization
0	Control	-	-
	21F221	240.2 ± 37[b]	-
	21F226	110.2 ± 28[b]	-
7	Control	0.02 ± 0	-
	21F221	**2.08 ± 0.3 ***	-
	21F226	**1.33 ± 0.6 ***	-
15	Control	0.03 ± 0.0	-
	21F221	**0.34 ± 0.1 ***	-
	21F226	**0.49 ± 0.1 ***	+
36	Control	0.14 ± 0.03	-
	21F221	**0.68 ± 0.12 ***	-
	21F226	**0.44 ± 0.12 ***	+

Values are concentration means ± SE (n=4) and presence within the root colonization (n=3). Values marked with asterisks are significantly different compared to non-inoculated plants for each day, with a p-value < 0.05 determined through a Dunnett's test.

[a] Inoculated isolates corresponding to isolates *A. brasiliense* strains 21F221 and *A. aestuarii* 21F226 and non-inoculated (Control)

[b] These values are expressed in 10^6 CFU seed^{-1} as the inoculation compared to the others that are expressed in 10^6 CFU mL of substrate^{-1}

After seven days, the population in the soil rhizosphere *Azospirillum spp.* was $2.08 \cdot 10^6$ and $1.33 \cdot 10^6$ CFU g^{-1} substrate for strains 21F221 and 21F226, respectively (Table 2). Subsequently, over the course of 15 and 36 days, the *Azospirillum* spp. population found in the maize rhizosphere decreased within one magnitude order and remained stable, ranging from 0.34 to 0.68 $\cdot 10^6$ CFU g^{-1} substrate for strain 21F221 and from 0.44 to 0.49 $\cdot 10^6$ CFU g^{-1} substrate for strain 21F226. The ability to colonize inside the root was only demonstrated for *A. aestuarii* strain 21F226 after 15- and 36-days post inoculation.

2. Biochemical strain characterization

Evaluating the data obtained from the API® 20E strip (Table 3) suggests that both isolates exhibited cytochrome oxidase activity and nitrate reductase activity. In addition, a very weak reaction for acetoin production was observed in both *Azospirillum* strains. Regarding β-galactosidase, distinct reactions were observed between the two strains, yielding a positive result for strain 21F221 and a weak positive result for strain 21F226.

Table 3 Biochemical characteristics of *A. brasilense* strain 21F221 and *A. aestuarii* strain 21F226 by Analytical Profile Index (API) ® strip 20E

Biochemical Tests	21F221[a]	21F226[a]
Acetoin production	**ww+**	**ww+**
Arginin dihydrolase	-	-
ß-galactosidase	**+**	**w+**
Citrate utilization	-	-
Cytocrome oxidase	**+**	**+**
Gelatinase	-	-
H_2S production	-	-
Indole production	-	-
Lysine decarboxilase	-	-
Nitrate reduction	+	+
Ornithine decarboxilase	-	-
Tryptophane de aminase	-	-
Ureasa	-	-

Values are means ± SE (n=3)

[a] –, no reaction; +, positive reaction; w+, weak positive; ww+, very week positive

Referring to the carbohydrate fermentation capacity of both strains 21F221 and 21F226 obtained in the API® strip 50CHB (Table 4), both strains presented similar results, demonstrating the ability to ferment esculin and fructose, and to a lesser extent, L-arabinose, D-fucose, glycerol, and D-xylose. However, a difference was observed between the two strains in their ability to ferment D-arabinose, with a positive reaction observed in strain 21F226 and a negative reaction in strain 21F221.

Table 4 Phenotypic characteristics of *A. brasiliense* strain 21F221 and *A. aestuarii* strain 21F226 by Analytical Profile Index (API)® strip 50CHB

Biochemical Test	21F221[a]	21F226[a]	Biochemical Test	21F221[a]	21F226[a]
Control	-	-	Glycerol	w+	w+
N-acetyol-glucosamine	-	-	Inositol	-	-
Adonitol	-	-	Lactose	-	-
Amygdalin	-	-	Maltose	-	-
D-arabinose	-	+	Mannitol	-	-
L-arabinose	w+	w+	Mannose	-	-
D-arabitol	-	-	Melezitose	-	-
L-arabitol	-	-	α-methyl-glucoside	-	-
Arbutin	-	-	α-methyl-mannoside	-	-
Cellobiose	-	-	β-methyl-xyloside	-	-
Dulcitol	-	-	Rhamnose	-	-
Esculin	+	+	Ribose	-	-
Erythritol	-	-	Salicin	-	-
Fructose	+	+	Sucrose	-	-
D-Fucose	w+	w+	Sorbose	-	-
L- Fucose	-	-	Sorbitol	-	-
Galactose	-	-	Treahalose	-	-
Glucose	-	-	D-xylose	w+	w+
Gluconate	-	-	L-xylose	-	-

Values are means ± SE (n=3)

[a] –,no reaction; +,positive reaction; w+, weak positive

3. Soil incubations

3.1. Effect of *Azospirillum* inoculation in nitrogen forms in organic and mineral fertilized soils

The statistical analysis of nitrogen forms in soils, both organically and mineral-fertilized, inoculated with strains 21F221 and 21F226, as well as non-inoculated controls, revealed significant differences (Table 5). For the factor soil type, significant differences were observed in ammonium (NH_4), nitrate ($N\text{-}NO_3$), total oxidizable nitrogen (TON), soluble organic nitrogen (SON) content all with p-values <0.05. In terms of days after inoculation, significant differences were only noted in NH_4 and TON and for the factor treatment just showed significant differences $N\text{-}NO_3$ and Min-N content. Significant interaction effects were found between soil type and days after inoculation for NH_4, $N\text{-}NO_3$, TON, and Min-N and between soil type and treatment for NH_4 content.

Table 5 Effect of *Azospirillum spp.* strains 21F221 and 21F226 inoculation in different nitrogen forms in incubated soils and *Azospirillum* population in the soil

Soil type	Days after inoculation	Treatment[a]	Nitrogen forms content (µg/g soil)					*Azospirillum* population (10^5 CFU mL^{-1})
			Ammonium (NH_4)	Nitrate ($N\text{-}NO_3$)	Total oxidable nitrogen (TON)	Soluble organic nitrogen (SON)	Mineral nitrogen (Min-N)	
Soil with organic fertilization	0	Control	6 ± 0.14 bβA	62.2 ± 12.39 bαB	131.17 ± 6.3 bαA	62.86 ± 7.13 abαβA	68.3 ± 12.51 bαB	-
		21F221	5.3 ± 0.14 bβA	57.97 ± 8.16 bαAB	139.28 ± 6.47 bαA	76.01 ± 7.11 abαβA	63.26 ± 8.16 bαAB	4.5 ± 1.3
		21F226	5.55 ± 0.16 bβA	**47.12 ± 3.06 bαA**	119.06 ± 4.56 bαA	66.39 ± 4.77 abαβA	**52.66 ± 2.94 bαA**	3.0 ± 0.6
	3	Control	5.24 ± 0.14 bβA	74.49 ± 9.47 abαβB	139.50 ± 4.48 bαA	59.74 ± 13.45 abαA	79.76 ± 9.47 abαβB	-
		21F221	5.24 ± 0.04 bβA	67.12 ± 5.3 abαβAB	127.94 ± 5.82 bαA	55.58 ± 4 abαA	72.36 ± 5.16 abαβAB	1.5 ± 0.7
		21F226	4.94 ± 0.08 bβA	**71.53 ± 11.79 abαβA**	137.5 ± 6.45 bαA	61.03 ± 8.5 abαA	**76.47 ± 11.8 abαβA**	1.5 ± 0.1
	7	Control	5.35 ± 0.12 bαB	94.41 ± 7.69 abβB	170.47 ± 11.15 bβA	70.25 ± 17.32 bβA	100.23 ± 8.25 abβB	-
		21F221	**4.86 ± 0.09 bαA**	74.58 ± 4.43 abβAB	155.61 ± 7.28 bβA	76.17 ± 8.69 bβA	79.44 ± 4.45 abβAB	1.4 ± 0.6
		21F226	**4.76 ± 0.06 bαA**	**59.66 ± 16.85 abβA**	154.06 ± 4.86 bβA	89.63 ± 18.35 bβA	**64.43 ± 16.83 abβA**	1.4 ± 0.1
Soil with mineral fertilization	0	Control	3.29 ± 0.14 aαA	93.14 ± 6.36 aβB	144.78 ± 9.28 aαA	48.36 ± 6.39 abαA	96.42 ± 5.64 aαB	-
		21F221	3.33 ± 0.11 aαA	106.1 ± 14.21 aβAB	128.94 ± 6.34 aαA	19.51 ± 17.61 abαA	109.43 ± 14.17 aαAB	5.0 ± 0.5
		21F226	3.24 ± 0.06 aαA	**97.63 ± 7.24 aβA**	135.06 ± 4.36 aαA	38.66 ± 11.59 abαA	**100.87 ± 7.19 aαA**	2.8 ± 1
	3	Control	3.3 ± 0.08 aαA	91.44 ± 8.67 abαβB	119.08 ± 5.11 aαA	24.38 ± 11.38 abαA	94.71 ± 9.73 abαB	-
		21F221	3.53 ± 0.12 aαA	70.17 ± 15.98 abαβAB	114.17 ± 30.17 aαA	40.47 ± 31.77 abαA	73.7 ± 15.94 abαAB	2.5 ± 1.2
		21F226	3.7 ± 0.26 aαA	**77.63 ± 19.55 abαβA**	129.39 ± 8.8 aαA	48.06 ± 21.87 abαA	**81.33 ± 19.65 abαA**	1.1 ± 0.2
	7	Control	4.13 ± 0.18 aβA	118.31 ± 14.79 abαB	162.43 ± 25.36 aαA	39.99 ± 25.11 abαA	122.43 ± 17.17 abαB	-
		21F221	4.3 ± 0.23 aβA	50.85 ± 18.61 abαAB	141.03 ± 5.94 aαA	76.71 ± 22.89 abαA	55.15 ± 18.71 abαAB	0.7 ± 0.3
		21F226	3.99 ± 0.13 aβA	**45.08 ± 10.39 abαA**	119.36 ± 4.46 aαA	67.08 ± 12.91 abαA	**49.08 ± 11.58 abαA**	0.9 ± 0.3

Values are means ± SE (n=5) in nutrient analysis and means ± SE (n=4) in concentration. A three-way factorial ANOVA was performed and Tukey's tests (p-value<0.05); lowercase letters (a, b) correspond to differences between soils, greek letters (α, β) differences between time and capital letters (A, B) between treatments. Significant factors; soil for NH_4, $N\text{-}NO_3$, TON, SON, Min-N, time for NH_4 and TON and treatment for $N\text{-}NO_3$ and Min-N. Significant interactions: soil*time for NH_4, $N\text{-}NO_3$, TON, Min-N and soil*treatment for NH_4

[a] Treatment inoculation with *Azospirillum brasilense* strain 21F221 and *Azospirillum aestuarii* strain 21F226 at a concentration of $5 \cdot 10^5 \cdot$ CFU mL^{-1} and non-inoculated (Control)

Comparing the two soils with different fertilization practices (organic and inorganic fertilization) we observed that ammonium (NH_4) and in total oxidable nitrogen (TON) values in soil with organic fertilization are higher than those in soil with mineral fertilization. As well as, in soluble organic nitrogen (SON) content after 7 days, which is significantly higher in organic fertilized soil compared to mineral fertilized soil.

Regarding changes in the N forms concentration significant differences were observed between inoculated and non-inoculated soils. In the organically fertilized soil, there was a significant reduction in ammonium by *Azospirillum* strains (21F221 and 21F226) inoculation at day 7, with reductions of 9% and 11%, respectively. In mineral fertilization the reduction of ammonium at day 7 was found in all treatments. Conversely, $N\text{-}NO_3$ and Min-N contents were less abundant in soils inoculated with *A. aestuarii* strain 21F226, regardless of the type of fertilization (organic or mineral) or the sampling time.

As for the concentrations of TON, SON and NH_4 in mineral fertilized soil, no significant differences are observed between inoculated and non-inoculated, although there is a tendency for an increase in SON values and a decrease in Min-N values after 7 days comparing non-inoculated soil compared to soil inoculated with 21F226, and less pronounced in 21F221 in both organically and mineral fertilized soil.

Regarding the *Azospirillum* populations found in the soil, the initial concentration was $4.5 \cdot 10^5$ CFU mL^{-1} for 21F221 strain and $3.0 \cdot 10^5$ CFU mL^{-1} for 21F226 in organic-fertilized soil, and $5.0 \cdot 10^5$ CFU mL^{-1} for 21F221 strain and $2.8 \cdot 10^5$ CFU mL^{-1} for 21F226 strain in mineral-fertilized soil respectively. Over time, in organically fertilized soil, the concentration of both strains remained at magnitude order of 10^5 CFU mL^{-1} being $1.5 \cdot 10^5$ CFU mL^{-1} and $1.4 \cdot 10^5$ CFU mL^{-1} on days 3 and 7 for 21F221 inoculation, respectively, and $1.5 \cdot 10^5$ CFU mL^{-1} and $1.4 \cdot 10^5$ CFU mL^{-1} for 21F226 inoculation on days 3 and 7, respectively. While in mineral-fertilized soil, the microorganism concentration decreased to $0.7 \cdot 10^5$ CFU mL^{-1} in 21F221 and $0.9 \cdot 10^5$ CFU mL^{-1} in 21F226 CFU mL^{-1} after 7 days.

3.2. Rates of nitrogen forms accumulation in two different soils influenced by microbial inoculation

The rate of nitrogen forms concentration change is shown in Table 6, where the changes are expressed in rates (µg/g soil/day). According to the global analysis of results (two-factorial ANOVA), differences are observed between organically fertilized soil and mineral fertilized soil in nitrate and mineral nitrogen accumulation rate and between inoculation treatments for accumulation rates of nitrate, soluble organic nitrogen, and mineral nitrogen, as well as, the interaction between soil type and inoculation treatments in accumulation rate of nitrate, SON and Min-N.

The accumulation rate of mineral nitrogen (N-Min) was higher in organically fertilized soil, and ammonium (NH_4) accumulation rate was higher in mineral fertilized soil. No differences were observed in nitrate ($N\text{-}NO_3$) and soluble organic nitrogen (SON) accumulation between soils.

Regarding the overall difference between treatments, significant differences are observed in the rate of $N\text{-}NO_3$, SON and Min-N accumulation. In organically fertilized soil this difference is observed in soil inoculated with 21F226 strain were a lower rate of nitrate accumulation and mineral nitrogen accumulation is observed, the negative values from these rates represent that there is a decreasing rate, transforming, or disappearing of these nitrogen forms. Contrary regarding the rate of soluble organic nitrogen accumulation, higher values are observed when inoculated with 21F226. In mineral fertilized soil the differences in $N\text{-}NO_3$, SON and Min-N accumulation rates are observed by inoculation of both strain in comparison with non-inoculated substrates, with lower rates values in nitrate accumulation and mineral nitrogen accumulation rate and higher values in of soluble organic nitrogen accumulation rate.

Table 6 Rates of nitrogen forms accumulation in two different soils during a seven-day incubation with and without *Azospirillum* inoculation

Soil	Treatment[a]	Rates (µg /g soil /day)			
		Ammonium (NH_4) accumulation	Nitrate ($N\text{-}NO_3$) accumulation	Soluble organic nitrogen (SON) accumulation	Mineral nitrogen (Min-N) accumulation
Organically fertilized soil	Control	0.02 ± 0.06 aA	5.93 ± 1.23 aB	7.26 ± 2.41 aA	5.96 ± 1.18 bB
	21F221	-0.11 ± 0.01 aA	3.10 ± 0.63 aB	9.90 ± 1.24 aAB	2.99 ± 0.64 bB
	21F226	-0.12 ± 0.01 aA	**-1.27 ± 1.14 aA**	**14.11 ± 1.66 aB**	**-1.39 ± 1.13 bA**
Mineral fertilized soil	Control	0.11 ± 0.03 bA	4.13 ± 2.02 aB	-2.34 ± 2.63 aA	4.54 ± 2.01 aB
	21F221	0.14 ± 0.03 bA	**-5.68 ± 2.59 aA**	**3.24 ± 2.10 aB**	**-5.21 ± 2.59 aA**
	21F226	0.09 ± 0.02 bA	**-7.55 ± 1.70 aA**	**4.30 ± 1.70 aB**	**-7.17 ± 1.70 aA**

Means ± SE (n=5). A two-way factorial ANOVA was performed and Tukey's tests (p-value<0.05); lowercase letters (a, b) correspond to differences between soils and capital letters (A, B) between treatments. Significant factors; soils for NH_4 and Min-N accumulation; treatment for $N\text{-}NO_3$, SON and Min-N accumulation and interaction soil*treatment in $N\text{-}NO_3$, SON and Min-N accumulation.

[a] Treatment: Inoculated isolates corresponding to isolates *Azospirillum brasilense* strain 21F221 and *Azospirillum aestuarii* strain 21F226 at a concentration of $5 \cdot 10^5 \cdot$ CFU mL^{-1} and non-inoculated (Control)

3.3. Substrate induced respiration and alpha diversity index

The values of substrate induced respiration (SIR) expressed as µg CO_2-C/g soil/ hour in organically and mineral fertilized soils inoculated with 21F221 and 21F226 and non-inoculated soils are sown in Table 7. Significant differences are observed between different fertilized soils and carbon source group, as well as, in the interaction between both factors and the triple interaction between different fertilized soils, carbon source group and treatment.

The substrate-induced respiration (SIR) values were generally higher in organically fertilized soils compared to mineral-fertilized soils, regardless of whether the soils were inoculated. When examining the response to different carbon source groups, higher SIR values were observed for organic acids in both organically and mineral-fertilized soils, compared to sugars or amino acids, with exception of organically fertilized soil inoculated with the 21F221 strain, where no significant differences were observed between the carbon source groups.

Concerning the effect of inoculation on SIR, significant differences were noted when using organic acids as the carbon source. In organically fertilized soil, inoculation with *A. aestuarii* strain 21F226 led to reduced CO_2 respiration when organic acids were utilized, with reductions recorded for aminobutyric acid (50%), phytic acid (4%), glutamic acid (49%), malic acid (35%), and oxalic acid (45%) although, no significant effect on SIR was observed with *A. brasilense* strain 21F221 compared to non-inoculated soils. Conversely, in mineral-fertilized soil, a significant increase in SIR was observed when organic acids were used as the carbon source in soils inoculated with both strains 21F221 and 21F226, compared to non-inoculated soils, with higher SIR values recorded for citric acid (266% for both strins), phytic acid (183% and 108% respectively), malic acid (85% and 27%), maleic acid (275% and 208%), and oxalic acid (100% and 50%).

For catabolic and alpha diversity index significant differences were observed by a two-way ANOVA and a post-hoc Tukey test between soils and inoculation treatment, the interaction between soil and treatment was also significant for both parameters analysed with a significant value of 95% (Table 7).

Comparing soils, the catabolic activity is significant higher in organic fertilized soil compared to mineral fertilized soil, the same difference is also observed in alpha diversity with higher values in organic fertilized soil. Additionally, when examining the catabolic activity, we see a significant decrease of 21% in catabolic activity in organic-fertilized soil inoculated with *A. aestuarii* strain 21F226 compared to non-inoculated soil. In mineral-fertilized soil, significant differences are also noted between non-inoculated soil and soil inoculated with *A. brasilense* strain 21F221, in this context, inoculation has a positive impact, generating a marked increase of 147% in catabolic activity.

Regarding alpha diversity value, in organic-fertilized soil, significant differences are highlighted between soil inoculated with *A. aestuarii* strain 21F226 and non-inoculated soil, where lower values are exhibited by an 8% reduction. In contrast, in mineral-fertilized soil, significant differences are also noted between non-inoculated soils and inoculated with *A. brasilense* strain 21F221, in this context; inoculation has a positive impact, generating marked increase of 51 %.

Table 7 Effects of carbon sources on Soil Induced Respiration and microbial activity after 5 days incubation with and without *Azospirillum* strains 21F221 and 21F226 inoculation

Carbon sources[a]		Soil Induced Respiration in organic-fertilized soil (µg CO_2-C/ g soil/ hour)			Soil Induced Respiration in mineral-fertilized soil (µg CO_2-C/ g soil/ hour)		
Group	Substance	Control[a]	21F221[a]	21F226[a]	Control[a]	21F221[a]	21F226[a]
Sugar	Glucose	1.7 ± 0.07 bαβA	1.74 ± 0.1 bαA	1.42 ± 0.12 bβA	0.16 ± 0.05 aαA	0.14 ± 0.03 aαA	0.08 ± 0.03 aαA
	Galactose	0.63 ± 0.19 bαβA	0.92 ± 0.06 bαA	0.78 ± 0.04 bβA	0.02 ± 0.02 aαA	0.03 ± 0.01 aαA	0.03 ± 0.01 aαA
	Fructose	1.4 ± 0.22 bαβA	1.82 ± 0.38 bαA	1.09 ± 0.06 bβA	0.04 ± 0.02 aαA	0.08 ± 0.04 aαA	0.08 ± 0.01 aαA
	Lactose	0.56 ± 0.08 bαβA	0.58 ± 0.03 bαA	0.71 ± 0.04 bβA	0.01 ± 0.01 aαA	0.02 ± 0.01 aαA	0.04 ± 0.01 aαA
	Arabinose	0.9 ± 0.03 bαβA	0.82 ± 0.07 bαA	0.65 ± 0.14 bβA	0.01 ± 0.01 aαA	0.03 ± 0.01 aαA	0.04 ± 0.01 aαA
Amino acids	Alanine	1.01 ± 0.09 bαA	1.53 ± 0.23 bαA	0.84 ± 0.05 bαA	0.09 ± 0.03 aαA	0.12 ± 0.01 aαA	0.08 ± 0.01 aαA
	Cysteine	0.39 ± 0.07 bαA	0.52 ± 0.07 bαA	0.23 ± 0.05 bαA	0.01 ± 0 aαA	0.09 ± 0.02 aαA	0.1 ± 0.02 aαA
	Lysine	0.17 ± 0.04 bαA	0.2 ± 0.01 bαA	0.23 ± 0.01 bαA	0 ± 0.01 aαA	0.03 ± 0.01 aαA	-0.02 ± 0.01aαA
	Aminobutyric acid	0.51 ± 0.05 bαA	0.51 ± 0.03 bαA	0.25 ± 0.02 bαA	0.02 ± 0 aαA	0.03 ± 0 aαA	-0.01 ± 0.01aαA
	Glutamic acid	1.53 ± 0.13 bαA	1.13 ± 0.01 bαA	0.93 ± 0 bβA	0.16 ± 0.04 aαA	0.32 ± 0.07 aαA	0.13 ± 0.04 aαA
Organic acids	Citric acid	1.64 ± 0.11 bβB	1.25 ± 0.08 bαAB	**1.3 ± 0.05 bβA**	0.12 ± 0.02 aβA	**0.44 ± 0.12 aβB**	**0.44 ± 0.1 aβB**
	Phytic acid	0.91 ± 0.05 bβB	0.53 ± 0.09 bαAB	**0.54 ± 0.07 bβA**	0.12 ± 0.04 aβA	**0.34 ± 0.05 aβB**	**0.25 ± 0.02 aβB**
	Malic acid	2.02 ± 0.39 bβB	1.81 ± 0.19 bαAB	**1.72 ± 0.21 bβA**	0.41 ± 0.09 aβA	**0.76 ± 0.15 aβB**	**0.52 ± 0.1 aβB**
	Maleic acid	1.15 ± 0.08 bβB	0.59 ± 0.23 bαAB	**0.75 ± 0.08 bβA**	0.12 ± 0.05 aβA	**0.45 ± 0.1 aβB**	**0.37 ± 0.09 aβB**
	Oxalic acid	0.47 ± 0.07 bβB	0.04 ± 0.07 bαAB	**0.26 ± 0.02 bβA**	0.02 ± 0.01 aβA	**0.04 ± 0.18 aβB**	**0.03 ± 0.05 aβB**
Catabolic activity (µg CO_2-C/g/ h)		14.83 ± 0.35 bB	14.24 ± 0.27 bB	**11.69 ± 0.27 bA**	1.32 ± 0.33 aA	**3.27 ± 0.36 aB**	2.43 ± 0.25 aAB
Alpha diversity (Shannon index)		3.79 ± 0.03 bB	3.82 ± 0.04 bB	**3.48 ± 0.07 bA**	0.91 ± 0.09 aA	**1.38 ± 0.07 aB**	1.16 ± 0.07 aAB
Azospirillum populations (10^5 CFU mL^{-1})		-	4.07 ± 4.6 bB	1.78 ± 5.3 bA	-	0.727 ± 1.6 aA	0.395 ± 0.4 aA

Means ± SE (n=3) for soil respiration, catabolic activity, and the Shannon index; means ± SE (n=4) for microorganism concentration. A three-way factorial ANOVA was performed and Tukey's tests (p-value<0.05); lowercase letters (a, b) correspond to differences between soils, greek letters (α, β) differences between carbon source groups and capital letters (A, B) between treatments. Significant factors; soil, carbon source group and interactions; soil*carbon source group and triple interaction

[a] Control (non-inoculated and inoculated isolates corresponding to *Azospirillum brasiliense* strains 21F221 and *Azospirillum aestuarii* strain 21F226 inoculated at $5 \cdot 10^5$ CFU ml^{-1}

The populations of strains 21F221 and 21F226 were maintained in the same range of inoculation (2 to $4 \cdot 10^5$ CFU mL^{-1}) for organic fertilized soil and decreased 10 times for mineral fertilized soils, values for strain 21F221 were slightly higher than strain 21F226 for both types of fertilized soils, being significantly higher in organically fertilized soils.

Discussion

1. Rhizosphere colonization dynamics

The stability of *Azospirillum* populations observed in our study, specifically for strains 21F221 and 21F226, in the rhizosphere of maize fertilized with a nitrogen-reduced Hoagland solution (60% N), aligns with previous findings in the literature. After 15 days, populations stabilized at approximately 10^5 CFU g^{-1} substrate and remained consistent throughout the experiment. This pattern is consistent with the study by Fukami et al., (2016), where maize seeds inoculated with *A. brasilense* at 10^5 CFU $seed^{-1}$ and treated with a nutrient solution containing a 25% reduction in nitrogen content resulted in a similar rhizosphere concentration of about 10^5 CFU g^{-1} soil after 53 days. Furthermore, Urrea-Valencia et al., (2021) reported a comparable outcome in a field study, where inoculating maize seeds at $2 \cdot 10^5$ CFU g^{-1} seed initially led to a decline in the population, which then stabilized after 13 days within the range of 10^5 CFU g^{-1} soil. These stable population levels were associated with positive effects on maize yield, a result that corroborates our observations, suggesting that maintaining *Azospirillum* populations within this range is beneficial for plant growth under reduced nitrogen conditions.

In the literature, *Azospirillum* is recognized as a facultative endophyte, sometimes capable of residing inside plant tissues (Baldani, et al., 1997). The fact that strain 21F226 penetrates the root interior, while strain 21F221 behave as rhizospheric, remaining in the outer regions of the root can be related to the literature describing that some specific strains of *Azospirillum* possess distinct mechanisms to penetrate and colonize the root interior, while others primarily inhabit the mucigel layer or injured root cortical cells (Steenhoudt & Vanderleyden, 2000, Baldani et al., 1986; Patriquin et al., 1978; Shelud'ko et al., 2010). Moreover, the inside root presence of strain 21F226 in samples collected at 15- and 36-days post-inoculation, corresponds with the observations made by Santos et al., (2017a), who reported *A. brasilense* colonies inside barley roots after the same time intervals. Despite the precise mechanism by how *Azospirillum* penetrate the maize root cortex intercellular spaces remains unclear, it is theorized that could be by enzymatic degradation of the host cell wall, supported by its pectinolytic and cellulolytic activities (Atmodjo et al., 2013). Recent research by Sharifsadat et al., (2023) defines a cross-signalling mechanism between rice roots and *A. brasilense*, facilitating endosymbiosis through cell wall

loosening and biochemical changes induced by increased hydrogen peroxide levels. Future studies should be performed, to confirm the ability of 21F226 strain to colonize the inner part of the root and to determine the specific entry mechanism.

2. Biochemical tests

The APIS strips used to identify strains based on their metabolic characteristics, is commonly employed in the literature to identify potential new species of *Azospirillum* (Eckert et al., 2001; Lin et al., 2012; Reinhold et al., 1987; Young et al., 2008). Strains 21F221 and 21F226 exhibit the ability to ferment L-arabinose, D-xylose, and fructose, which is consistent with previous studies in *Azospirillum spp*. (Yang et al., 2019). According to the results, strain 21F221 best match in terms of biochemical profile was as *Azospirillum brasilense* strain N8 or ATCC 29729, except for some discrepancies in urease activity and D-maltose fermentation tests (Mehnaz & Lazarovits, 2006). Furthermore, molecular sequencing of the 16S gene results confirm the identification of strain 21F221 as *Azospirillum brasilense*, according to our results then, we speculate that different strain may also give the variability in the isoenzymatic patterns or fermentation profiles as observed among other different *A. brasilense* strains (Mehnaz & Lazarovits, 2006).

Regarding strain 21F226, differences were observed in the fermentation of D-arabinose and lower beta-galactosidase activity compared to strain 21F221. Despite some similarities with other *Azospirillum* species such as *A. formose*, *A. lipoferum*, *A. thiophilum* or *A. zeae,* the D-galactose and D-glucose fermentation capacity and gelatine hydrolysis activity does not match with our results (Lavrinenko et al., 2010; Lin et al., 2012; Mehnaz et al., 2007). Moreover, genetic identification of strain 21F226 through comparison with NCBI 16S rRNA sequences reveals a 99% query cover with 99.93% similarity to *Azospirillum aestuarii*. Of note, *A. aestuarii* corresponds to a recently described species in the literature by Xu et al., (2023), with no existing records characterizing this strain using API strips, although in the article, within other methods, *A. aestuarii* is shown to have positive response to nitrate reduction and negative response to indole production, gelatinase activity and urease, the same results obtained in our study for 21F226. Given the proximity of this novel species and its apparent similarity to *A. brasiliense*, we thus compare strain 21F226 with *A. brasilense* for our purposes.

Among the enzymatic activities observed in strains 21F221 and 21F226, the positive activity of nitrate reductase stands out. This enzyme catalyses the conversion of nitrate (NO_3-) to nitrite (NO_2-), affecting the composition of inorganic nitrogen forms present (Bertero et al., 2003) and thereby contributing to the nitrogen cycle. In contrast, processes such as acetoin production, beta-galactosidase activity, and cytochrome oxidase activity are not directly involved in nitrogen cycle. The acetoin production is related with the secondary metabolite synthesis and the energetic

metabolism, while the cytochrome oxidase is involved in the electron channel in the cellular respiration and the galactosidase role is directly related with sugar hydrolysis (Juers et al., 2012; Li et al., 2023; Watson & McStay, 2020).

3. Incubations

The observed reduction in ammonium and nitrate content in soil incubations inoculated with *Azospirillum brasilense* strain 21F221 and *Azospirillum aestuarii* strain 21F226, rather than an increase, suggests an absence of atmospheric nitrogen fixation. This phenomenon could be attributed to the fact that nitrogen fixation is a highly energy-demanding process. In our study it seems that the absence of rhizosphere and therefore root exudates, appears to have prevented *Azospirillum* from acquiring the necessary energy from soil organic matter (SOM) or other available energy sources present in the soil (simple organic compounds, microbial exudates, or other substances), to fix nitrogen. This observation aligns with previous studies indicating that *Azospirillum* engages in a symbiotic relationship with plants, primarily colonizing the rhizosphere where it can access the nutrients and energy sources essential for its metabolic processes, including nitrogen fixation (Pereg et al., 2016; Steenhoudt & Vanderleyden, 2000) and outside this root-associated environment, the availability of energy and nutrients is insufficient to support nitrogen fixation (Bashan & de-Bashan, 2010), as demonstrated in our experiment where we inoculated strains 21F221 and 21F226 into soil without plants, a condition more akin to activity in bulk soil than that associated with the rhizosphere.

Inoculation with strains 21F221 and 21F226 in organically fertilized soil resulted in a reduction in ammonium concentration after 7 days, similarly, in both soils (mineral fertilized and organically fertilized soils) inoculation with *Azospirillum aestuarii* strain 21F226 led to a decrease in nitrate concentration, although the total oxidizable nitrogen (TON) remained unchanged. Several mechanisms could independently or simultaneously occur in the soil to explain these results (Bashan & de-Bashan, 2010). Firstly, the findings suggest a potential influence of *Azospirillum* on the consumption or transformation of ammonium and nitrate compounds (Cassán et al., 2020; Van Dommelen et al., 1998) as well as its capacity to mobilize nitrogen retained in organic matter (N mining), also the ability of *Azospirillum spp.* to reduce nitrate through an assimilative pathway, using nitrate (NO_3^-) as a nitrogen source for synthesizing nitrogenous compounds necessary for its growth and reproduction (De Souza & De Oliveira Pedrosa, 2015) or the ability to use and metabolize ammonium to produce glutamate via glutamate dehydrogenase (Arcondéguy et al., 2001; De Souza & De Oliveira Pedrosa, 2015)

Secondly, another possible influence of the inoculation with *Azospirillum spp.* strains 21F221 and 21F226 is its impact on the native soil bacteria involved in nitrogen conversion processes

(Ferrarezi et al., 2023; Florio et al., 2019). This hypothesis could also explain the observed changes in soil respiration rate, catabolic activity, and diversity index. The alteration of both the composition and activity of the native microbial community by *Azospirillum* has been previously described in the literature. Banerjee et al., (2018) and Ferrarezi et al., (2023) observed that following *A. brasilense* inoculation, the native microbial community changed, increasing the abundance of *Acidobacteria, Solirubrobacterales, Actinobacteria,* and *Latescibacteria*, similarly, Renoud et al., (2022b) observed shifts in *Actinobacteria, Cyanobacteria, Firmicutes,* and *Proteobacteria* after *A. lipoferum* inoculation. Changes in microbial community could explain the alterations in nitrogen biochemical forms compared to non-inoculated soils, as certain species may engage in nutritional competition and microbial displacement in the soil (Renoud et al., 2022a). In this sense, results obtained in our investigation on the respiration rates, would suggest that some phyla were capable of metabolizing sugars and organic acids used as carbon sources or *Azospirillum spp.* may be inhibiting or affecting populations of microorganisms responsible for metabolizing those substrates (Demirkan et al., 2014; Gulati et al., 2007; Hopper et al., 1970; Liu et al., 2016; Macias-Benitez et al., 2020). However, microbiome specific studies, will be necessary to establish a correlation between these phyla and changes in nitrogen biochemical forms and soil respiration.

The populations of 21F221 and 21F226 exhibited higher survival rates in organically fertilized soil compared to mineral fertilization. These results are consistent with Bashan et al., (1995) who reported comparable concentration levels (10^4 to 10^5 CFU mL^{-1}) after 10 days, albeit with a notable decline in viability after 30 days. In addition, Bashan et al., (1995), linked the survival of *A. brasilense* to soil characteristics, indicating that factors like clay content, nitrogen levels, organic matter, and water-holding capacity positively affect bacterial viability, characteristics which aligns with the organically fertilized soil used in our assay. Furthermore, the higher SIR observed in organic-fertilized soil compared to mineral-fertilized soil in our study imply a greater microbial activity in organically managed soil. This relationship, as it is described on the literature, can be attributed to addition of SOM in organic-fertilized soil which serves as an organic carbon source that fosters the growth and metabolic activity of bacteria (Pariona-Llanos et al., 2010). Conversely, practices involving the use of chemical nitrates as in mineral-fertilized soil, have been shown to negatively impact soil bacterial populations (Li et al., 2023).

The impact of *Azospirillum spp.* strains inoculation on soil biochemical properties such as the catabolic index differs notably depending on soil fertilization. In organically fertilized soils, the addition of *A. aestuarii* strain 21F226 led to a decrease in substrate-induced respiration (SIR) when organic acids were used as the carbon source leading to a lower catabolic index, however no differences were observed with sugars or amino acids application. This could be explained by the capacity of organic acids to release bound nutrients from soil organic matter, a process known

as the "unbutton model" (Clarholm et al., 2015), and the prioritization of *A. aestuarii* strain 21F226 for nitrogen acquisition over carbon in nitrogen-rich soils through the priming effect (De Souza & De Oliveira Pedrosa, 2015; Eisenhauer et al., 2013; Pardo-Díaz et al., 2021) effectively sequestering a significant portion of the available nitrogen in the soil. The application of organic acids in organically fertilized soil facilitates the availability of easily respirable organic matter increasing the amount of available organic nitrogen, thereby reducing nitrogen competition between *A. aestuarii* strain 21F226 and native soil microbiota. Consequently, the combination with *Azospirillum* inoculation does not lead to a nitrogen shortage that would otherwise elevate microbial activity; instead, it reduces it (Gunina & Kuzyakov, 2022; Steenhoudt & Vanderleyden, 2000). Another possibility is that inoculation inhibits microorganisms that respire these substrates. In contrast, in mineral-fertilized soil, easily respirable materials are scarce, and microbial activity is constrained by carbon availability, in this scenario, the solubilization of organic matter by organic acids, along with *Azospirillum* inoculation strains 21F221 and 21F226, is crucial for mobilizing nitrogen retained in soil organic matter to meet *Azospirillum's* demands, enhancing or maintaining SIR. Also resulting in a higher catabolic index, likely due to the competitive activity of introduced *Azospirillum* strain 21F221 and 21F226, which metabolizes these substrates, along with native microbes competing for the available nutrients (Ambrosini et al., 2016).

This aligns with the hypothesis that free-living *Azospirillum* does not fix nitrogen but instead mines the soil for it, suggesting a strategic allocation of resources based on nutrient availability (Eisenhauer et al., 2013; Pardo-Díaz et al., 2021). The ability of organic acids to mobilize nutrients such as nitrogen from soil organic matter supports the observed decrease in SIR, indicating that *Azospirillum* efficiently uses these compounds to meet its metabolic needs without relying heavily on respiration.

The Shannon index emerges as a valuable tool for understanding biological alpha diversity in an ecosystem (Pla, 2006). Exploring the effect of *Azospirillum* inoculation it also reveals distinctive patterns in organic and mineral fertilized soils decreasing in organically fertilized soil and an increasing in mineral-fertilized soil. Literature on the effects of N fixing microorganism inoculation on native microbial communities shows discrepancies, with some studies reporting a decrease in biodiversity when inoculating with *Herbaspirillum sp.* and *Azospirillum brasilense* (Pardo-Díaz et al., 2021), while others indicate an increase when inoculating *Azotobacter spp.* in combination with rice straw mulch (Mazuecos-Aguilera et al., 2024). The observed discrepancies in alpha diversity between mineral and organically fertilized soils may stem from differences in the richness of the native microbial community. In soils with a more abundant indigenous microbial community, such as organic fertilized soil, inoculation could lead to a reconfiguration of the microbial community, potentially resulting in decreased diversity (Eisenhauer et al., 2013). Conversely, in soils with a less abundant native microbial community, such as mineral fertilization

soil, inoculation may promote increased biodiversity due to the greater impact of the introduced microorganism (Ambrosini et al., 2016; De-Bashan et al., 2010). For instance, in a metagenomic study conducted by inoculating bulk soil with *A. brasilense*, the main genes changing by the inoculation effect were positively correlated with six taxa that were more abundant, including three groups of *Acidobacteria, Solirubrobacterales*, *Actinobacteria*, and *Latescibacteria* (Ferrarezi et al., 2023). These results suggest that the effect of *Azospirillum* inoculation on biodiversity indices like Shannon can be complex and context-dependent, influencing various microbial groups differently based on environmental factors and plant interactions.

Conclusions

In conclusion *A. brasiliense* strain 21F221 and *A. aestuarii* strain 21F226 colonization dynamics in maize rhizosphere, demonstrate a successful establishment and persistence of the inoculated strains at a concentration of 10^5 CFU g^{-1} soil in maize rhizosphere after 15 days through all the duration of the study, a concentration described to show positive impact on plant growth. Notably, strain 21F226 exhibited the potential to colonize and penetrate maize roots, suggesting endophytic capabilities.

The use of APIS strips for metabolic characterization, combined with molecular sequencing of the 16S rRNA gene, confirms the identification of strain 21F221 as *Azospirillum brasilense* and strain 21F226 as *Azospirillum* aestuarii. Despite minor discrepancies in 21F221 in urease activity and D-maltose fermentation and lack of extensive specific studies in the literature concerning 21F226 strain as this species has been recently described (2023). Both strains showed significant nitrate reductase activity, indicating its potential role in the nitrogen cycle.

While nitrogen fixation outside the rhizosphere appears unlikely, both strains of *Azospirillum* demonstrate a significant capacity to mobilize nutrients, particularly nitrogen, through priming effects in organically fertilized soils. This resulted in decrease in nitrate concentrations in organically and mineral-fertilized soil by strain 21F226 and decrease of ammonium concentrations in organic-fertilized soil after 7 days because of both strains inoculation, with total nitrogen oxide (TON) concentrations remaining constant and a higher soil organic nitrogen (SON) accumulation rate. This suggests that while *Azospirillum* strains may not fix nitrogen outside the root environment, they may employ alternative strategies to obtain essential nutrients, thereby influencing the composition and activity of the soil microbial community. Regarding the effect of inoculation on soil respiration (SIR), significant effects were observed only in organically fertilized soil where the SIR decreased significantly by inoculation 21F221 and 21F226 with organic acids acting as carbon sources, reducing the catabolic index. Conversely, in mineral-

fertilized soils, the presence of easily respirable materials was limited, and *Azospirillum* inoculation helped mobilize nitrogen, thereby maintaining or enhancing SIR and increasing the catabolic index.

Inoculation with *Azospirillum spp.* affected alpha diversity (Shannon index) differently depending on soil type, decreasing alpha diversity in organically fertilized soils while increasing it in mineral-fertilized soils, highlighting a context-dependent influence on biodiversity. Moreover, differences between soil fertilization type were observed in *Azospirillum spp.* population which higher values in organically fertilized soil suggesting it provides more favourable conditions for their viability compared to mineral fertilization.

General Discussion

General Discussion

In the early 20th century, advancements in chemical technology revolutionized agriculture by enabling the synthesis of essential nutrients, such as nitrogen and phosphorus, into synthetic fertilizers, thereby boosting global food production (Rahman Farooqi et al., 2021). However, the excessive use of these fertilizers has led to significant environmental pollution and highlighted the need for more sustainable agricultural solutions. In this context, microbial biostimulants/biofertilizers have emerged as promising alternatives. These formulations may consist of plant growth-promoting rhizobacteria (PGPR) from the *Bacillus* and *Pseudomona*s genera, as well as nitrogen-fixing species such as *Azotobacter, Azospirillum*, and *Rhizobium* (Bulgari et al., 2015; Mandal et al., 2023). These bacteria are capable of enhancing plant growth, nutrient use efficiency, and tolerance to abiotic stress, all while being effective and environmentally friendly (Baltazar et al., 2021; Lau et al., 2022).

Despite their potential, the use of biostimulants faces challenges, including the complexity of plant physiological effects and the effectiveness of biostimulant formulations in enhancing crop growth and stress tolerance (Jardin, 2015; Yakhin et al., 2017). Therefore, this thesis aims to isolate, characterize, and evaluate the functionality of PGPR isolates in various crops, focusing on maize and rice. This will be achieved through a combination of *in vitro* experiments, plant assays, and soil studies to develop an effective microbial biostimulant that complies with Spanish regulations (Real Decreto 999/2017) or European regulations (Regulation (EU) 2019/1009). This work aspires to advance scientific knowledge and offer innovative solutions for sustainable agriculture. It is remarkable that at the present moment, only strains of *Azotobacter*, *Azospirillum*, *Rhizobium* and mycorrhizae are accepted as microbial biostimulants under the European Regulation while in the Spanish Regulation there is no limitation in the genera of microorganisms to be used.

Pseudomonas fluorescens as a candidate to develop a biofertilizer

Regarding the isolation of *Pseudomonas fluorescens*, the strategy employed was efficient in isolating 13 strains from bulk soil and from maize rhizosphere, the presence of this specie in bulk soil and associated with plant rhizosphere have been previously documented in the literature. Also most of the strains were from Soil B and Compost, although they have been described as present in a variety of environments (Muriel Rhodes, 1959). In the analysis of the *in vitro* capabilities of

the isolated strains, we observed their ability to produce indole-3-acetic acid (IAA), siderophores, and to solubilize phosphate, with different strains standing out for each characteristic.

The production of IAA by *P. fluorescens* is well-documented in the literature (Patten & Glick, 2002), among the strains tested, P7 and P10 exhibited the highest levels of IAA production with 46 and 61.41 µg mg protein^{-1} respectively. Given that plant growth promotion is closely associated with IAA production (Cheng et al., 2023), these two strains were selected for further plant growth promotion experiments. The results indicated significant growth improvements in maize and lettuce with P10 inoculation compared to non-inoculated plants, aligning with findings in some studies(Cipriano et al., 2016; Sah et al., 2021; Someya et al., 2008).

The capacity to produce siderophores and solubilize phosphate of the isolated P. *fluorescens* strains was evaluated in relation to their potential effect on enhancing plant nutrient uptake. As described, *P. fluorescens* can produce extracellular siderophores that sequester ferric oxides to convert them into forms available to the roots (Krewulak & Vogel, 2008; De Vleesschauwer et al., 2008) and solubilize insoluble inorganic phosphate compounds from mineral sources (Rodríguez & Fraga, 1999). Strain P2b demonstrated significant siderophore production on CAS medium, forming a halo diameter of 1.93 cm, consistent with values reported by Soltani et al., (2011) who observed siderophore production capacity from 25 *P. fluorescens* isolated within 0.6 to 1.27 cm halo diameters. Additionally, strains P2a, P2b, and P4 solubilized hydroxyapatite, increasing soluble phosphorus concentrations by 23.5, 21.6, and 33.51 µg mL^{-1}, respectively, although these findings are noteworthy, comparable studies, such as those by Blanco-Vargas et al., (2020) and Li et al., (2017) reported even higher hydroxyapatite solubilization levels (66.2 µg mL^{-1} and 593, 494, 209 or 307 µg mL^{-1} respectively) by different *Pseudomonas spp.* isolates. However, despite promising *in vitro* results, *P. fluorescens* P2b did not significantly enhance plant growth or phosphorus uptake in maize and sunflower under low phosphorus conditions, indicating that solubilization capacity alone may not translate directly to improved plant nutrition under field conditions.

Bacillus subtilis as a candidate to develop a biofertilizers

Bacillus subtilis is one of the most widely used and studied PGPR and a highly promising candidate for agricultural applications. The genus *Bacillus* is one of the most abundantly isolated genera in the soil, among which *B. subtilis* has been isolated in the rhizosphere of various plants (Earl et al., 2008; Sivasakthi et al., 2014). This aligns with our study, where we isolated 13 strains of *B. subtilis* from bulk soil and rhizosphere source within different soil types: compost, Soil A, Soil B, and Soil D.

Regarding the results of the *in vitro* experiments, the observed capabilities are consistent with those described in the literature, which defines *B. subtilis* as having the ability to produce IAA (Khianngam et al., 2023), siderophores (Ahmad et al., 2021; Ribeiro et al., 2018), and solubilize mineral phosphorus (Rodríguez & Fraga, 1999; Manzoor et al., 2017) through medium acidification by releasing organic acids (Saeid et al., 2018) such as glucuronic, malic, oxalic, acetic and lactic acids.

Similar to *P. fluorescens*, the capability of each strain *in vitro* varied, with *B. subtilis* strains B5, B7, and B17 standing out as IAA producers showing values of 34, 31, and 21 µg mg protein^{-1}, respectively, results comparable to those observed by Wagi & Ahmed, (2019) who reported an IAA production of 19.79 µg mL^{-1} by *B.subtilis.* However, these values were lower compared to those obtained in our study for *P.fluorescens* strains P7 and P10. Strains B7, B17, B3, B9, and B12 were selected to observe their effect on tomato germination and growth promotion. Several articles demonstrate the ability of *Bacillus spp.* to increase germination (Malkoclu et al., 2017; Raj et al., 2003; Kaymak et al., 2009) along with our study we observed that strains B7 and B17 increased the germination percentage of tomato seeds, and strain B17 also increased seed vigour. In terms of growth promotion, strains B7 and B17 were capable of increasing cucumber, maize, and lettuce plants biomass compared to non-inoculated ones (Sahin et al., 2015; Xu et al., 2022), with notable variations depending on the maize cultivars used in the case of the inoculation effect with B7, this impact of plant genotype on the effect of inoculation with beneficial microorganisms has been previously reported in the literature (Singh et al., 2023).

For siderophore production *in vitro, B. megaterium* strains (MB18, MB19) were highlighted as best producers in CAS medium (0.98 and 0.85 cm halo diameter) and *B. subtilis* strain B12 and strains MB18 and MB19 demonstrated a high capacity to solubilize hydroxyapatite in suspension, (17, 22 and 33 µg mL^{-1} respectively) with values similar those obtained in (Saeid et al., 2018) for *B. subtilis* (33.1 µg mL^{-1}) and *B . megaterium* (37 µg mL^{-1}). In the plant trials aimed at assessing the impact of inoculation on phosphorus uptake, notable increases in both aerial and root growth were observed in sunflower and maize plants inoculated with strains B7 and B17 fertilized with limited application of soluble phosphorus. Specifically sunflower shoot dry weight increased 46% by B7 and 53% by B17 inoculation, while maize showed increases of 48% and 91% respectively, as well as, root dry weight also rose significantly, with a 68% increase in sunflower by B17 inoculation, and in maize, increases of 110% by B7 and 93% by B17 values very similar to those obtained in maize plants inoculated with *B. subtilis* in Lobo et al., (2019) study. Notably, strain B17 markedly enhanced phosphorus accumulation in maize, resulting in a 47% increase in phosphorus content compared to non-inoculated plants, a phenomenon previously described (Oliveira-Paiva et al., 2024). Regarding *B. subtilis* stains B7 and B17 capacity to improve phosphorus availability to plants may vary depending on soil type, these bacteria may work better

for improving phosphorus availability in calcareous soils compared to acidic soils, as usually P immobilizes to calcium phosphate complex in calcareous soils and bind with aluminum or iron in acidic soils (Sundra et al., 2002).

The advantages of *B. subtilis* strains B7 and B17 as plants biostimulants presents significant potential for improving phosphorus availability in soils. Given that up to 90% of applied phosphate fertilizers become immobilized in soils (Sundra et al., 2002), these strains could mobilize this "fixed P" reserves increasing the bioavailability of phosphate to plants, especially critical in crops like maize (*Zea mays L.*) (Zhang et al., 2021), as well as, the inoculation combined with mineral P fertilizers could maintain phosphate solubility for extended periods, thus maximizing the efficiency of fertilizer use and potentially reducing P input costs. In terms of fertilizer savings, B7 and B17 could theoretically reduce P fertilizer requirements, potentially reducing the environmental impact of excessive P accumulation and preserving phosphate rock reserves (Withers et al., 2014; Beltran-Medina et al., 2023).

Taking in account the results obtained, *Bacillus subtilis* strains B7 and B17 were selected as candidates for biofertilizer development that would comply with Spanish regulations (Real Decreto 999/2017), unlike *Pseudomonas fluorescens* strain P10, which exhibited lower and inconsistent effects in plant bioassays.

The efficacy of inoculation methods and formulations is crucial for the successful application of these *B. subtilis* strains. In this study, inoculation via substrate application at a concentration of 10^7 CFU mL^{-1} and direct plant application at 10^8 CFU mL^{-1} demonstrated significant effectiveness. For agronomical application is necessary to develop a formulation with substances, known as coformulants, that protect strains until use. In this sense, alginate-based formulations seem to be effective (Trivedi et al., 2005) or applications and encapsulation techniques, such as Ca^{2+}-amended alginate with humic acid (Khan et al., 2023). Future formulation studies should be performed to further develop the selected bacterial strains into commercial products.

Azotobacter spp. as candidates to develop a microbial biostimulant

The genus *Azotobacter* is described as free-living nitrogen-fixing bacteria with various plant growth promotion traits often associated with nitrogen fixation, production of growth hormones, fungicidal substances, siderophore production, and phosphate solubilization (Narula et al., 2000) making it a good candidate for developing a microbial biostimulant. This genus can be isolated from a multitude of different environments, possibly due to its ability to form cysts that makes them resistant to environmental changes and can make associations with plants and live in the rhizosphere of non-leguminous plants. The isolation of *Azotobacter* from maize and wheat

rhizosphere, as in our study, has also been reported in the literature (Martinez-Toledo et al., 1985; Stets et al., 2015).

As mentioned, the growth promotion capacity of *Azotobacter* is often associated with nitrogen fixation and phosphate solubilization (Chen et al., 2018; Narula et al., 2000). To investigate these traits, we performed two *in vitro* assays to evaluate the capacity of our isolates to release ammonium and solubilize mineral phosphate. Our findings indicate that the *Azotobacter spp*. isolates exhibited significantly lower ammonium release compared to *Azospirillum spp*. isolates, however *Azotobacter spp*. isolates demonstrated a superior ability to solubilize phosphate. The limited impact of our *Azotobacter spp.* isolates on nitrogen use efficiency was further substantiated by inoculation of strains in maize plants grown in nitrogen-limited environments which revealed inconsistent effects on plant growth, yield, and nitrogen accumulation, with meaningful improvements observed only in 75% N fertilization regime experiments, involving strain 21F213.

Regarding the phosphate solubilization studies, strains 21F213 and 21F200 showed the highest hydroxyapatite solubilization, followed by 21F209 with tricalcium phosphate, in line with other studies (Reyes et al., 2006; Kumar et al., 1999). The mechanism was found to be the acidification of the medium through the production and release of organic acids (Azaroual et al., 2020; Marra et al., 2012; Kumar et al., 1999), specifically malic, gluconic, glucuronic, and acetic acids by strain 21F213, and gluconic, oxalic, glucuronic, and acetic acids by strain 21F220 (Krishnaraj & Dahale, 2014; Rashid et al., 2004). Considering these results, we tested the effect of inoculation of the *Azotobacter* strains on phosphate solubilization and accumulation in maize cultivated without soluble phosphorus. However, no significant differences in biomass were observed in plants inoculated with strain 21F213, likely because solubilization mechanisms identified in the lab may not function similarly in soil (Whitelaw et al., 1999) or environmental factors like soil pH and nutrient availability are affecting phosphorus solubilizing capacity (Hinsinger et al., 2003; Hodge, 2004; Ryan et al., 2000). This variability in the efficacy of *Azotobacter* strains in phosphate solubilization and their ability to promote plant growth has already been documented previously (Bashan et al., 2013; Kumar et al., 1999). Thus, further trials are needed to assess the potential of this microorganism as a candidate for developing a microbial biostimulant aimed at enhancing phosphorus use efficiency, particularly when applied directly to the root at a concentration of 10^8 CFU mL^{-1}.

Azospirillum spp. as candidates to develop a microbial biostimulant

Azospirillum sp. is probably the most studied genus of PGPR due to its ability to colonize many plant species. Multiple and complex mechanisms in the microbe-plant interaction have been described, such as nitrogen fixation, phytohormone biosynthesis, and phosphate solubilization (Santos et al., 2017b; Bashan & de-Bashan, 2010). These traits make it a promising candidate to develop a microbial biostimulant (Cassán & Diaz-Zorita, 2016; Gassmann et al., 2016; Okon & Labandera-Gonzalez, 1994) specially for gramineous plants (Eckert et al., 2001; Mehnaz et al., 2007; Stets et al., 2015). Aligning with the preference of *Azospirillum* to stablish interactions with gramineous plants most *Azospirillum* strains in our study were isolated from the rhizosphere of grass and maize or root surface, although is also described that some strains can colonize root tissues (Baldani et al., 1986) as observed in strain 21F226.

To determine the nitrogen-fixing capacity of *Azospirillum* strains (Okon et al.,1983 Steenhoudt & Vanderleyden, 2000; Cassán & Diaz-Zorita, 2016), we analysed their ability to produce ammonium in suspension and their growth in nitrogen free culture media, being strains 21F221, 21F224, 21F226 and 21F227 the ones with best results. However, low mineral phosphorus solubilizing capacity was observed among the strains. Interestingly the isolates with best ammonium production were isolated from soils non-saline, rich in organic matter (7-9 %) but low in nitrogen (27-3), suggesting a relationship between the survival of *Azospirillum* and organic matter (Bashan & Vazquez, 2000) and the potential nitrogen fixing capacity and nitrogen present in the soil.

Even though various mechanisms in which *Azospirillum spp.* stimulates plant growth have been described, none have been individually identified as solely responsible; rather, it is the combination of these mechanisms that provides plant growth benefits (Bashan & de-Bashan, 2010; Kennedy et al., 1997; Okon et al., 1983; Giller, 2003). This is reflected in our plant experiments, where our selected *Azospirillum spp.* strains were seed inoculated at a concentration of 10^8 CFU g $seeds^{-1}$ in maize and rice plants grown with a nitrogen-limited fertilization. Inoculation with *A. brasiliense* strain 21F221 and *A. aestuarii* strain 21F226 inoculations affected plants increasing shoot biomass and improving root development, as well, improved plant nitrogen accumulation, and crop yields in agreement with other studies (Cassán & Diaz-Zorita, 2016; Di Salvo & García de Salamone, 2019). The plant effect by 21F221 and 21F226 inoculation was incremented when combined with nitrogen fertilization obtaining higher differences between inoculated and non-inoculated plants in experiments with 60%N and 75%N fertilization compared to those with 0%N, in agreement with study of Zeffa et al., (2019).

Additionally, some differences between strains were observed: strain 21F221 was more associated with higher and more consistent nitrogen accumulation in different parts of the plant, while strain 21F226 had a greater effect on nitrogen internal utilization efficiency (IE) and root development, thereby enhancing more the plant biomass and productivity. Based on these results, agronomic indices as described in FprCEN/TS 17700-2 showed significant effects on agronomic efficiency (RE and AE), partial factor productivity (PFP), and nitrogen export (NE), therefore, strains 21F221 and 21F226 were selected as suitable candidates for developing a microbial biostimulant under European Regulation (EU Fertilizer Products Regulation 2019/1009).

The application of *Azospirillum* strains 21F221 and 21F226 demonstrates considerable benefits in terms of nitrogen fertilizer savings and crop yield enhancements. Regarding nitrogen fertilizer field savings, applying a nitrogen regime of 75%N of the recommended nitrogen dose, equivalent to 163 kg ha^{-1} of urea (46% N), led to a cost savings of approximately 48.62 USD ha^{-1} on commercial urea purchases. As well as energy production savings of 40 MMBtu ha^{-1} (million British thermal unit), which corresponds to a total cost saving of 23.79 USD ha^{-1} related to natural gas consumption for urea production (European Commission, 2019). In terms of yield in maize assuming optimal conditions similar to those in greenhouse trials inoculation with strain 21F221 resulted in a 55% yield increase, while strain 21F226 achieved a remarkable 148% increase translated to additional yields of 10 tons ha^{-1} and 27 tons ha^{-1}, respectively, compared to non-inoculated controls. Given the European maize market price of 279.65 USD ton^{-1} (FAO, 2023), farmers could potentially earn an additional 2796.5 USD ha^{-1} with 21F221 application and 7550.55 USD ha^{-1} with 21F226 application. And for rice, trials showed yield increases of 15% with 21F221 and 19% with 21F226 corresponding to additional production of 0.86 tons ha^{-1} and 1.09 tons ha^{-1}, respectively. With rice priced at 438 USD ton^{-1} at the Vietnam market (FAO, 2023), these results suggest potential additional earnings of 376.7 USD ha^{-1} with 21F221 application and 477.4 USD ha^{-1} with 21F226 application under a 75% nitrogen fertilization regime.

To have a successful application of *Azospirillum* 21F221 and 21F226 strains the method of inoculation is crucial. In our study, we used a seed coating technique involving nutrient-rich peat (Jiffy GO M8) and 1% (w/v) carboxymethyl cellulose (CMC) as a sticker, this method has proven effective in ensuring good microbial adhesion, uniform distribution of the inoculant and ensured plant growth effect. The most widely used method for biofertilizer application involves seed coating with microbial formulations (Nagpal et al., 2021) although this method has potential drawbacks, including seed coat damage and desiccation, which can reduce inoculant viability (Bashan et al., 2014), this can be improved with other formulations such as ZnO nanoparticles as carriers (Awan et al., 2021), and algal extract-based formulations (Iparraguirre et al., 2023). As

well as using other inoculation method such as solid formulations, including granules, powders, and water-dispersible granules which have given good results for *Azospirillum* application, specifically, talc-based and biochar-based formulations have been reported to support root growth and overall plant development effectively (Prasad & Babu, 2017; Saranya et al., 2011).

To be able to develop an effective application with microbial biostimulants it is also important to study the interaction with soil native bacterial population. For this reason, we studied the effect of inoculation on soil dynamics under different fertilization practices (organic and mineral) in incubation experiments, revelling a complex interplay among strains and soil nitrogen forms. Apparently no nitrogen fixation was observed by the selected strains of *Azospirillum spp* outside the rhizosphere, in organically fertilized soil instead they were found to effectively use the priming effect to stimulate the decomposition of existing organic matter to access limiting nutrients such as nitrogen and phosphorus (Fontaine et al., 2011; Kuzyakov et al., 2000), nutrients necessary for their acquisition and growth (De Souza & De Oliveira Pedrosa, 2015; Eisenhauer et al., 2013; Pardo-Díaz et al., 2021). This is traduced in higher accumulation of soluble organic nitrogen (SON) —a water-soluble fraction of soil nitrogen that includes amino acids, proteins, nucleic acids, and other organic compounds— as observed in our study by strains 21F221 and 21F226 inoculation effect in soil. A similar effect is described by Bacilio et al., (2003), highlighting *Azospirillum's* nutritional and metabolic interactions with humic substances, suggesting its potential to decompose and utilize complex organic compounds.

Additionally, inoculation with selected strains of *Azospirillum* spp. was found to alter microbial communities, as evidenced by changes in soil-induced respiration (SIR), the catabolic index, and alpha diversity (Banerjee et al., 2018; Ferrarezi et al., 2023) with different effect on the organically and mineral-fertilized soils. In organic-fertilized soil, inoculation with strain 21F226 resulted in lower substrate-induced respiration (SIR) when organic acids when applied as carbon source, as in mineral fertilized soil inoculation with both strains resulted in a higher substrate-induced respiration (SIR), although no differences were observed in sugar or amino acids as carbon sources. This SIR values affected the catabolic index demonstrating a 21% decrease when inoculated with *A. aestuarii* strain 21F226 in organically fertilized soil and a 147% increase when inoculated with *A. brasilense* strain 21F221 in mineral fertilized soil. Regarding alpha diversity index 21F226 inoculation led to an 8% reduction in organic-fertilized soil, whereas 21F221 inoculation increased alpha diversity by 51% in mineral-fertilized soil, likely due to differences in the richness of the native microbial community in organically and mineral-fertilized soils (Eisenhauer et al., 2013).

Conclusions

Conclusions

Regarding the specific aims of this thesis

1. Protocols for isolation, preservation, cultivation and classification were performed resulting in a new collection of 35 plant growth promoting rhizobacteria (PGPR) strains, including 13 *Bacillus subtilis* and 13 *Pseudomonas fluorescens*. *B. subtilis* was predominantly isolated from bulk soil (85%) and in Compost soil samples (69%) and *P. fluorescens* isolates were equally found between bulk soil and the rhizosphere of maize and wheat, with most isolates found in Soil B (54%).

2. Protocols for isolation, preservation, cultivation and classification were performed resulting in a new collection of 243 nitrogen fixing bacteria (NFB), there were molecular classified 9 *Azospirillum spp*. and 4 *Azotobacter spp.* The main sources were the rhizosphere of gramineous plants, specifically grass (31%), wheat (23%) and maize (23%).

3. The *in vitro* determination identified notable strains according to specific characterisations. In this regard, the best performance for indole-3-acetic acid (IAA) were*: Pseudomonas fluorescens* strain P10 and *Bacillus subtilis* strains B5, B7 and B17. *Bacillus megaterium*, strain MB18 showed the highest siderophore production, as well as phosphate solubilisation (iron phosphate III). Moreover *B. subtilis* strains B12 and P4b, as well as, *Azotobacter salinestris* strain 21F213 showed strong capacity to solubilize hydroxyapatite.

4. The *in vitro* ammonium production study revelled *Azospirillum* species the better producers compared to *Azotobacter* spp., being the best performers *A. brasilense*, strain 21F221; *A. aestuarii*, strain 21F226; *A. oryzae*, strain 21F224 and *Azospirillum spp.* strain 21F227.

5. The plant trials demonstrated that some isolates could effectively improve seed quality, promote growth and enhanced phosphorous and nitrogen efficiency, although the best performing *in vitro* strains did not always were the best plant outcomes.

6. The *in vivo* studies assessing effects in tomato germination revelled *B. subtilis* B7 and B17 at a concentration of 10^7 CFU mL^{-1} of substrate as effective to increase the germination by 14% and 11.5% respectively, compared to non-inoculated controls, as well as B17 strain to increase the seed vigour by a 60%. Additionally, B7 and B17 strains also affected plant growth, increasing the shoot dry weigh in cucumber (14.17% and 10.5%), lettuce (50%) and maize to different extend according to the plant cultivar, compared to non-inoculated plants. Furthermore, B7 and B17 inoculation at a concentration of 10^8 CFU mL^{-1} also improved

phosphorus use efficiency, boosting biomass growth in maize and sunflower without soluble phosphorus fertilization by 46% and 53% in sunflower, and 81% and 84% in maize, respectively. Strain B17 also increased phosphorus accumulation in maize by 47% compared to non-inoculated plants.

7. The *in vivo* studies for NFB demonstrate the best performance of *Azospirillum brasilense* strain 21F221 and *Azospirillum aestuarii* strain 21F226 at a concentration of 10^8 CFU g^{-1} of seeds, on plant nitrogen use efficiency across different fertilization regimes (0% N, 60% N and 75%N) significantly enhancing maize and rice performance. That is, increasing maize shoot dry weight and nitrogen accumulation when grown in 0% N and 60%N compared to non-inoculated seeds, as well as, at 75%N fertilization regime the 21F221 and 21F226 inoculation increased maize yield by 61% and 148%, maize nitrogen accumulation by 59% and 8.4%, and for rice increased shoot dry weight by 4% and 7% respectively, yield by 26% and 37%, and nitrogen accumulation by 26% and 17% compared to non-inoculated seeds.

8. Based on claims outlined in the Technical Specifications CEN/TC455, *Azospirillum aestuarii*, strain 21F226 and *Azospirillum brasilense* strain 21F221 are proposed as promising candidates improving plant nitrogen use efficiency, notably boosting nitrogen accumulation in plant in maize for both strain and in rice for the first one.

9. The inoculation of *Azospirillium* strains 21F221 and 21F226 significantly influenced nitrogen dynamics in soils with different fertilization background (organic or mineral) resulting in lower nitrate ($N\text{-}NO_3$) and mineral (Min.N) levels, while increasing soluble organic nitrogen (SON). Both strains significantly affected substrate induced respiration (SIR), specifically when organic acids were applied as carbon source, strain 21F226 reduced SIR in organically fertilized soil and strain 21F221 enhanced SIR in mineral fertilized soil.

10. Both *Azospirillum* strains significantly altered native microbial communities, with effects varying according to the soil fertilization background, 21F226 reduced diversity in organically fertilized soil, while 21F221 increased diversity in mineral fertilized soil.

11. *Bacillus subtilis* strains B7 and B17 were selected candidates for developing a biofertilizer compliant with Spanish regulations (Royal Decree 999/2017) and *Azospirillum brasilense* strain 21F221 and *Azospirillum aestuarii* strain 21F226 were selected candidates for developing a microbial biostimulant compliant with European regulations (Regulation (EU) 2019/1009).

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