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Thesis:

Characterization of rhizobacteria isolated from wild plants of *Physalis* sp. and study of its biofertilizing effect *in vitro*, and in seedlings and fruits of tomatillo (*Physalis ixocarpa* Brot.).

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Oaxaca de Juárez, Oax., January 2023.

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ACTA DE REGISTRO DE TEMA DE TESIS
Y DESIGNACIÓN DE DIRECTOR DE TESIS

Ciudad de México, a 13 de diciembre del 2022

El Colegio de Profesores de Posgrado de **CIIDIR UNIDAD OAXACA** en su Sesión
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Referente al registro de su tema de tesis; acordando lo siguiente:

1.- Se designa al aspirante el tema de tesis titulado:

"Characterization of rhizobacteria isolated from wild plants of *Physalis* sp. and study of its biofertilizing effect *in vitro*, and in seedlings and fruits of tomatillo (*Physalis ixocarpa* Brot.)".

Objetivo general del trabajo de tesis:

To evaluate the biofertilizing effect of rhizospheric bacteria on the growth and mineral content of tomatillo seedlings and the effect of a bacterial strain on agronomic yield, fruit quality, and mineral content in tomatillo plants and fruits (*Physalis ixocarpa* Brot).

2.- Se designa como Directores de Tesis a los profesores:

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3.- El Trabajo de investigación base para el desarrollo de la tesis será elaborado por el alumno en:

La Universidad Tecnológica de la Mixteca (UTM) y en el Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional, Unidad Oaxaca.

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Aspirante

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"Characterization of rhizobacteria isolated from wild plants of Physalis sp. and study of its biofertilizing effect in vitro, and in seedlings and fruits of tomatillo (Physalis ixocarpa Brot.)".

del alumno:

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En la Ciudad de México el día 20 del mes de diciembre del año 2022, el que suscribe **Ramírez Cariño Heriberto Fortino** alumno del programa de **Doctorado en Ciencias en Conservación y Aprovechamiento de Recursos Naturales** con número de registro **B180120**, adscrito al Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional, Unidad Oaxaca manifiesta que es autor intelectual del presente trabajo de tesis bajo la dirección de **Dr. Isidro Morales García y el Dr. Rogelio Valadez Blanco** y cede los derechos del trabajo intitulado **"Characterization of rhizobacteria isolated from wild plants of *Physalis* sp. and study of its biofertilizing effect *in vitro*, and in seedlings and fruits of tomatillo (*Physalis ixocarpa* Brot.)"**., al Instituto Politécnico Nacional, para su difusión con fines académicos y de investigación.

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UNIDAD OAXACA

Ramírez Cariño Heriberto Fortino

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List of abbreviations

ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylate
AOAC	Association of Official Analytical Chemist
CFU	colony forming units
DAT	days after transplanting
DNA	deoxyribonucleic acid
HCN	hydrogen cyanide
IAA	indole acetic acid
ICP-OES	inductive coupled plasma with optical emission spectroscopy
K	potassium
KSB	potassium solubilizing bacteria
LB	Luria Bertani
NBRIP	National Botanical Research Institute's phosphate
NFB	nitrogen free medium
NH_3	ammonia
P	phosphorus
PAR	photosynthetically active radiation
PCR	polymerase enzyme chain reaction
PGPR	plant growth promoting rhizobacteria
pH	hydrogen potential
PSB	phosphate solubilizing bacteria
UTMR	Universidad Tecnológica de la Mixteca rhizobacteria

Abstract

The use of plant growth promoting rhizobacteria (PGPR) is a sustainable way in agriculture to reduce the use of chemical fertilizers which have caused a deterioration in the composition and structure of the soil and as well as contamination of water reservoirs. Rhizobacteria have shown a set of characteristics that they use for their survival, but that directly and indirectly help improve some agronomic parameters in crops. The most reported mechanisms include: nitrogen fixation, phosphorus (P) and potassium (K) solubilization, ammonia and indole acetic acid (IAA) synthesis, siderophore and 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme production, biocontrol of pathogens in the soil, and the induction of resistance and/or tolerance to biotic and abiotic stress. On the other hand, the production of the tomatillo crop in Mexico has shown an increase in recent years due to the fact that it is part of the consumption of the Mexican diet, mainly as a base for the preparation of sauces and typical dishes. This research work consisted of 2 stages: 1) Isolation of 30 bacteria from the rhizosphere soil of wild tomatillo plants, which were characterized cellular and morphologically and by 4 growth promotion mechanisms (P and K solubilization, and ammonia production and IAA). Based on their results, 6 bacteria were chosen for identification and of these, 4 were evaluated on the growth of tomatillo seedlings; 2) The biofertilizing effects of 1 bacterium (*Acinetobacter calcoaceticus*) in combination with different doses of K, were evaluated in plants and tomatillo fruit quality and mineral content. The results in 1) showed that the bacterium identified as *Atlantibacter* sp. showed activity in all biofertilization mechanisms evaluated, with the best results in phosphorus solubilization and indole acetic acid synthesis. In the seedlings experiment, the seedlings with bacterial treatments presented higher leaf weight (>349%) and root length (>11%) than the control. Seedlings treated with *Priestia megaterium* resulted in the highest height of seedlings, 140% compared to the control. In addition, *P. megaterium* and *Acinetobacter calcoaceticus* were significantly higher in the concentration of three minerals: K (54 and 37%), Ca (88 and 80%), and Mg (89 and 81%). The results in 2) indicated that the inoculant increased the chlorophyll content, dry weight and height at low K fertilizer doses (0 and 50%) compared to the non-inoculated treatments. The application of the inoculant increased the yield of fruits (g plant^{-1}) and number of fruits per plant in the treatment with no K fertilization, which was similar to the

treatments with high K doses without inoculation (75 and 100%). The protein content was higher in K fertilizer 100% doses combined with the inoculant in comparison to K fertilizer 100% doses treatment non-inoculated. The content of K, P, Ca, Mg, and Mn in the inoculated tomatillo plants combined with low K fertilizer doses (0 and 50%) was higher in comparison with the non-inoculated. Tested strains: *Priestia megaterium*, *Acinetobacter calcoaceticus* and *Atlantibacter* sp. demonstrated outstanding biochemical and agronomical characteristics *in vitro* and *in vivo*, making them excellent biofertilizers candidates for tomatillo crop production. *A. calcoaceticus* also showed benefic traits in the physiological and agronomic parameters, and in the mineral content in plant and fruit when was combined at low K fertilization doses. This rhizobacteria can be used to decrease the application of the K fertilizer in tomatillo crop.

Keywords: PGPR, *Atlantibacter*, *Acinetobacter calcoaceticus*, biofertilization, tomatillo plants.

CHAPTER I

1. General introduction

1.1. Overview

The rise of the world's human population have caused an increase in food demand. To meet this demand for food, agriculture based on the use of chemical fertilizers has been implemented (Pathania et al., 2020), but their indiscriminate use is damaging not only the ecological balance but also human health, while the provision of fertilizers has become too expensive for farmers to afford (Fasusi et al., 2021). In addition, the excessive use of this agrochemicals causes greenhouse gas emissions and physical and chemical soil degradation (Shah et al., 2021). The unrestrained use of agrochemicals to increase yields on farmland has brought with it a variety of negative environmental consequences throughout the world. For example, excessive addition of phosphates and nitrates has caused eutrophication of freshwater lakes and streams, as well as seas, and acidification of soils (Zhang and Zhang, 2007). It has been projected that the consumption of mineral fertilizers in the world will reach 225 million tons in 2030, with horticultural production systems being the ones with the highest demand. Dependence on synthetic mineral fertilizers threatens the environment because high amounts of energy are required for their production through limited and non-renewable natural sources (Mpanga et al., 2018).

On the other hand, the tomatillo (*Physalis* spp.), green tomato, husk tomato, milpa tomato or miltomate, is one of the most cultivated species nationwide. This vegetable is a frequent component in the Mexican diet, mainly in the form of sauces, which improve the flavor of meals (Montes-Hernández and Aguirre-Rivera, 1987). Tomatillo (*Physalis* spp.) is of American origin and Mexico is the center of origin and domestication. In this country there are 70 wild species and only *P. philadelphica* Lam (Synonym of *P. ixocarpa* Brot) and *P. angulata* L. are cultivated. The fruits of different species of wild tomatoes are collected for self-consumption or for sale in local markets (Vargas-Ponce et al., 2015).

Currently, fertilization alternatives that are friendly to the environment have been sought. The physical-chemical properties and biological composition of the rhizosphere are important factors in plant nutrition. The interaction of rhizospheric microorganisms with plant roots helps improve the availability of nutrients for plants and reduce the dependence on chemical fertilizers (Jamal et al., 2018). Among the soil microorganisms that promote

plant growth, rhizobacteria in the soil rhizosphere are of the most important. These microorganisms are called plant growth promoting rhizobacteria (PGPR) (Lucas-García et al., 2004). Rhizobacteria use the nutrients released by plants for growth, but also secrete metabolites into the rhizosphere, which can act as signaling compounds for host plant root cells (Van Loon, 2007). In addition, rhizobacteria have the ability to colonize the root surface of the plant, solubilize phosphorus, produce hydrocyanic acid, siderophores, ammonia, and indole-3-acetic acid (Jamal et al., 2018). Rhizobacteria also induce the growth through the production of stimulant volatile compounds and phytohormones, lowering the level of ethylene in the plant, release of phosphates and micronutrients, non-symbiotic nitrogen fixation, and stimulation of resistance mechanisms (Jafari et al., 2014). The use of these microorganisms can help to capitalize synergies aimed at development sustainable agriculture.

Works *in vivo* using rhizobacteria in plants and in seedlings have been reported. In a study Delgado-Ramirez et al. (2021) found that rhizobacteria of the genera *Arthrobacter*, *Bacillus*, *Paenibacillus*, *Pseudomonas*, and *Streptomyces* presented plant-growth promoting traits and improved several agronomic parameters of tomato plants such as length, weight of roots and stem compared with the control. In similar study, Cueva-Yesquen et al. (2021) reported that nine bacterial strains increased growth parameters of Cape gooseberry (*Physalis peruviana*), being the *Leclercia adecarboxylata* strain which showed the best results in the shoot and root length (55.4 and 24.5%), and in the chlorophyll levels compared with the control non-inoculated. In other study, Moreno-Velandia et al. (2019) reported that *Bacillus velezensis* BS006 strain stimulated plant growth in Cape gooseberry (*Physalis peruviana*) in the post-transplant phase at different bacterial suspension concentrations. Specifically, concentration at 1 x 10^7 cfu mL^{-1}, increased the leaf area and the shot dry weight compared to the lowest concentration. On the other hand, Hernandez-Pacheco et al. (2021) found several strains isolated of Mexican husk tomato plants (*Physalis ixocarpa*) increased the length and weight of the root, total biomass, and chlorophyll content in *Physalis ixocarpa* seedlings in comparison with the control. The *Neobacillus drentensis* CH23 strain showed activity in all plant compartments, across exhibited antagonist capacity against fungal pathogens. In another study, Rojas-Solis et al. (2016) reported that a consortium of *Bacillus thuringiensis* and *Pseudomonas fluorescens*

improved the total fresh weight of tomatillo seedlings, increased hypocotyl and root length, and showed beneficial interaction with the plant, while separately showed a beneficial effect on seedlings development, and broad potential for colonizing the rhizosphere and promoting tomatillo plant growth.

Even though Mexican tomatillo production is of utmost importance, a little studies regarding the effect by the rhizobacteria inoculation as biofertilizers in tomatillo seedlings and plants, as well as fruit quality have been reported. It is possible that the use of rhizobacteria as biofertilizers in tomatillo cultivation may decrease the use of chemical fertilizers and increase the yield and quality of the fruit. In this study, our objectives were: a) to isolate and characterize bacteria associated with rhizospheric soils of wild *Physalis* sp. plants, to evaluate their *in vitro* plant growth promoting mechanisms, and their biofertilizing effect on tomatillo seedlings in terms of agronomic parameters and mineral content in the plant leaves; b) to evaluate the effect of the application of the rhizobacteria *Acinetobacter calcoaceticus* combined with different doses of potassium in the agronomic parameters, fruit quality and mineral content on tomatillo plants (*Physalis ixocarpa* Brot).

1.2. Objectives

General objective

To evaluate the biofertilizing effect of rhizospheric bacteria on the growth and mineral content of tomatillo seedlings and the effect of a bacterial strain on agronomic yield, fruit quality, and mineral content in tomatillo plants and fruits (*Physalis ixocarpa* Brot).

1.2.1. Particular objectives

1. Isolate and identify associated rhizobacteria in the soil rhizosphere of wild tomatillo plants and characterize *in vitro* their biofertilizing mechanisms for plant growth.
2. To determine the effect of biofertilization with rhizobacteria on the growth and mineral content of tomatillo seedlings.
3. Evaluate the biofertilizing effect of a bacterium on the agronomic yield of tomato, fruit quality and mineral content in tomatillo plants and fruits.

1.2.2. Goals

Of the particular objective 1

Goal 1.1. Isolation of bacterial strains in three culture media: LB, Nfb and King's B and their morphological and cellular characterization.

Goal 1.2. Characterization of growth promotion mechanisms: solubilization of phosphorus and potassium and production of ammonia and the phytohormone indole acetic acid.

Goal 1.3. Bacterial DNA extraction, PCR amplification and purification for shipment to the company to Macrogen for sequencing and identification.

Of the particular objective 2

Goal 2.1. Determine the growth curves and the bacterial concentration at the time of greatest growth (cfu L^{-1}) in LB medium at 37 °C.

Goal 2.2. Prepare the bacterial suspensions of 4 rhizobacteria in LB broth at 37 °C and adjust it to its maximum cell yield to use it as an inoculant.

Goal 2.3. Sowing of tomatillo seeds in germination trays and inoculation twice with the bacterial suspension.

Of particular objective 3

Goal 3.1. Sow the tomatillo seeds in germination trays, transplant them into bags of 18 kg capacity with a mixture of local soil substrate and river sand (60:40 v/v), and inoculation with the bacterial suspension twice.

Goal 3.2. Determination of the physiological, agronomic, and yield parameters, as well as the physical-chemical and quality characteristics of the tomatillo fruit.

Goal 3.3. Acid digestion of samples for the quantification of mineral content (P, K, Ca, Na, Mg and Mn) in plant and fruit by ICP-OES.

1.3. Hypothesis

1. The rhizosphere soil associated with wild tomatillo plants is a suitable environment to find bacteria that have potential through of different mechanisms as plant biofertilizers.

2. Biofertilization with rhizospheric bacteria, has a positive effect on seedling growth and mineral content, as well as increase agronomic parameters and fruit quality of tomatillo (*Physalis ixocarpa* Brot).

CHAPTER II

2. Theoretical background and literature review

2.1. Fundaments

Microbial communities contribute to soils improvement through organic matter decomposition, nutrient cycling, and plant nutrition. Plant growth-promoting rhizobacteria (PGPR) are a subset of these microbial community and can be isolated from different type of soils (Alemneh et al., 2022). The techniques for isolation initially require that the number of organisms in the inoculum be reduced. The streak-plate method is a rapid qualitative isolation method. It is essentially a dilution technique that involves spreading a loopful of culture over the surface of an agar plate (Cappuccino and Sherman, 2014) (Figure 1). The agar media most commonly used for bacteria isolation are: Luria Bertani, (LB), King B, and nitrogen free Nfb agar supplemented with malic acid (Angulo et al., 2014) (Peña et al., 2016).

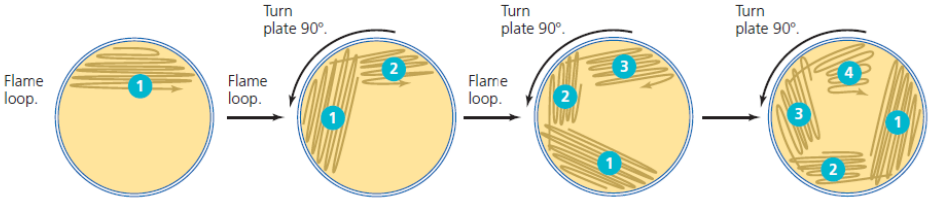

Figure 1. Four-way streak-plate technique (Cappuccino and Sherman, 2014).

The growth of the microorganisms is observed by its cultural characteristics (shape, border, size, color, and appearance), which are used for separating into different groups (Figure 2).

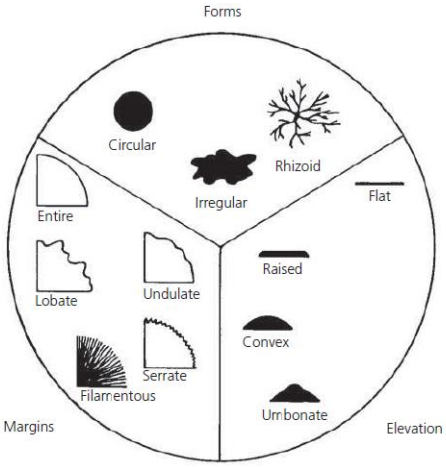

Figure 2. Cultural characteristics of the bacteria

To study the properties of rhizobacteria for the purpose of grouping, a commonly used technique is Gram staining, with which it is possible to observe the morphological form (cocci, bacilli, and spirilla) and the arrangement (chains, groups, pairs, and tetrads). of the bacteria. This Gram stain allows the separation of Gram positive (purple color) and Gram negative (pink color) bacteria. Gram-positive cells have a thick layer of the polysaccharide peptidoglycan, while the peptidoglycan layer in gram-negative cells is much thinner and is surrounded by lipid-containing outer layers. Also, to know the number of cells, the basic method for counting viable cells is to use agar plate dilutions. (Cappuccino & Sherman, 2014).

PGPR colonize the plants rhizosphere and positively contribute plant growth through of one or more growth-promoting mechanisms such as phytohormones synthesis, nitrogen fixing, ammonia production, and solubilizing nutrients (P, K) for which PGPR can be used as biofertilizers (Arif et al., 2017). Phosphorus (P) is one of the essential elements required for plant development and growth, because is a structural component of nucleic acids (Behera et al., 2017). However, only 0.1 % of P is available for plant use, so its deficiency is addressed by applying chemical P fertilizers (Alori et al., 2017). Phosphate Solubilizing Bacteria (PSB) convert phosphate into a bioavailable form through biological processes such as solubilization and mineralization.. Generally, National Botanical Research Institute's phosphate (NBRIP) medium, is used for screening P-solubilizing bacteria, containing the following ingredients: glucose, 10 g; Ca_3 $(PO_4)_2$, 5 g; $MgCl_2 \cdot 6H_2O$, 5 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; KCl, 0.2 g; and $(NH_4)_2$ SO_4, 0.1 g (per liter of double distilled water), with a pH 7 before sterilization (Behera et al., 2017). In the *in vitro* tests, phosphate solubilization is shown when a clear zone develops around the colony. (Ogata et al., 2017). The solubilization of inorganic phosphorus is caused by the action of organic acids such as gluconic and citric acid, which are synthesized by rhizobacteria (Oteino et al., 2015). The quantitative test the P solubilization is determined by a colorimeter method in which the chloromolybdic acid and tin chloride are used. A color changeover of the mixture to blue indicates the presence of soluble phosphates (Chandra, 2018). The change of color is for the combination of phosphate ion with the ammonia molybdate in acid conditions forming a complex known as ammonium phosphomolybdate. The molybdate present in the o-

phosphate is reduced to forming a complex of blue color which is proportional to phosphorus amount present in the medium (Londoño-Carvajal et al., 2010).

Regarding the ammonia production, the Nessler's reagent is used when is added to grown culture in peptone water (4%). Nessler's reagent is prepared as following: potassium iodide, 50 g; saturated mercuric chloride, 35 ml; distilled water, 25 ml; and potassium hydroxide (40%), 400 ml. 50 µL of bacterial cell suspension is inoculated in the peptone water and incubated at determined conditions of time and temperature. After that, 1 mL Nessler's reagent is added. The formation of yellow to dark brown color indicate the ammonia production (Dinesh et al., 2015).

Potassium (K) is one of the most important macronutrients required for the plants. This element intervenes in various plant physiological and metabolic processes such as photosynthesis, plant growth and development, metabolism, and sugar accumulation (dos Santos et al., 2020). Due that in the soil the unavailable form of K comprises approximately 90-98% of the total soil K, the identification of microbial strains capable of solubilizing potassium from insoluble K-bearing minerals such as micas minerals can contribute to the conservation of this resources and decreasing the use of chemical fertilizers (Khanghahi et al., 2018). The capacity of K solubilization for bacterial isolates in Aleksandrow medium is determined by measuring clear zone around of the bacterial colony. This method requires 7-15 days of incubation, although is relatively less sensitive because of the poor visualization of the clear zone (Parmar and Sindhu, 2018).

The indole-3-acetic acid (IAA) is a phytohormone which intervenes in the modulating plant growth and development. Several rhizobacteria are capable of synthesizing IAA (Keswani et al., 2020). The IAA production by rhizobacteria is estimated using a colorimetric technique. The use of nitrite and ferric chloride-sulfuric acid has been proposed for the quantitative estimation of indoleacetic acid (IAA) in aqueous solutions. According to this method, nitrite is sensitive to 10 µg. IAA/ml, and develops a red color that is stable after two hours. If the nitrite concentration is reduced, the red color becomes persistent enough to be read (Gordon and Weber, 1950). On the other hand, the colorimetric technique derived from Salkowski for indole detection has been used. This method is simple, rapid, and cheap and allows the daily analysis of numerous bacterial supernatants. In this technique the

reagent is prepared with 4.5 g of $FeCl_3$ per liter in 10.8 M H_2SO_4. (Glickmann and Dessaux, 1995).

Agronomic parameters are used to evaluate the biofertilizing effect of rhizobacteria on plants. Plant height, dry and fresh weight, and stem thickness are widely reported variables in different studies. Ali et al. (2021) reported that the growth parameters such as plant height, fresh weight, and yield were recorded as result of the effect of the *Bacillus cereus* strain on potato plants. Similarly, Flores et al, (2020) evaluated the stem thickness, shoot height, yield in weight of fruits, and number of fruits per plant as result of the inoculation of *Bacillus thuringiensis* on plants of cucumber (*Cucumis sativus* L.). In the same line, Martínez et al. (2019) reported the biofertilizing effect of *Pseudomonas fluorescens* in the agronomic parameters (yield, axial and equatorial diameter, and weight) on melon plants (*Cucumis melo* L.). Finally, the biofertilizing effect of nine bacterial strains was reported by Katsenios et al. (2021), agronomic parameters (dry weight and yield per plant of tomato plants) were evaluated.

Fruit quality parameters are important characteristics to consider when the biofertilizing attributes of rhizobacteria are evaluated in plants. The proximal analysis includes the moisture content, protein, ash, total fiber, titratable acidity, pH and electricity conductivity.

Protein content according to García-Martínez and Fernández-Segovia (2020) is determined through of the Kjeldahl method, which measures the nitrogen content of a sample. The protein content can be calculated assuming a ratio of protein to nitrogen for the analyzed food. This method can be divided into three stages: digestion, distillation and valuation. The procedure to be followed is different depending on whether in the distillation stage the nitrogen released is collected on a boric acid solution or on a known excess of standard hydrochloric or sulfuric acid. In the digestion stage a treatment with concentrated sulfuric acid, in the presence of a catalyst and boiling converts organic nitrogen into ammonium ion. In this stage, protein nitrogen is transformed into ammonium sulfate by action of hot sulfuric acid. In the distillation stage the digested sample is made alkaline and nitrogen is released in the form of ammonia. The distilled ammonia is collected over an unknown excess of boric acid. In the valuation stage, the quantification of ammoniacal nitrogen is carried out by means of an acid-base titration of the borate formed ion, using hydrochloric or sulfuric acid and an alcoholic solution of a mixture of methyl red and methylene blue as

an indicator. The acid equivalents consumed correspond to the ammonia equivalents distilled. On the other hand, crude fiber is by definition, the residue obtained after the treatment of vegetables with acids and alkalis, that is, it is a more chemical than biological concept. Plant fiber refers mainly to the fibrous elements of the plant cell wall. Dietary fiber includes all kinds of substances, whether fibrous or not, and therefore includes cellulose, lignin, pectins, gums, etc. To identify of the fiber content, the AOAC methods is used (AOAC., 1997).

Identifying the moisture content in the food is an activity very important, although its accurate determination is very difficult. Water into food is found in two forms, as bound and free water. The bound water includes molecules of water chemical united through of hydrogen bonds to ionic or polar groups, while the free water is not united to the food matrix and can be frozen by dried or evaporation. The air oven method is used for moisture determination. The samples are placed in capsules in the oven at 100 °C for 6 hours.

pH determination. The basic principle of potentiometric pH determination is the measurement of hydrogen ion activity by potentiometric measurements using a standard hydrogen electrode and a reference electrode. On the other hand, the acidity of a solution is a measure of its ability to react with strong bases at a given pH, that is, it is the ability to donate protons. The measurement values can vary significantly with the end point, acidity is a measure of the aggregate properties of the solution and can be interpreted in terms of the specific substances, only when the chemical composition of the sample is known.

The minerals quantification in fruit and plant is a method that is based on the measurement of atomic emission by means of an optical spectroscopy technique. The samples are nebulized and the resulting aerosol is transported to the plasma torch where electronic excitation takes place. An inductively coupled radiofrequency plasma (ICP) generates the corresponding atomic emission line spectra. The light beams are scattered by a diffraction grating spectrometer and the detectors are responsible for measuring the intensities of the lines. The signals originating from the detectors are processed and controlled by a computer system. The method used is an adaptation of method 3052, described by the U.S. EPA (Environmental Protection Agency) (González Terreros et al., 2018). Wherein the samples of plant material (300 mg) dried and pulverized, are subjected to acid digestion at reflux with 9 ml of nitric acid, 2 ml of 30% hydrogen peroxide, and 1 ml of hydrochloric acid. At

a temperature of 180 °C for approximately 1.5 hours or until complete digestion of the plant material is observed (Ramirez-Cariño et al., 2023).

For PGPR identification, for a long time research has focused on various biochemical and inoculation-based methods. Genomic analysis of PGPRs can be divided into two broad categories (a) whole-genome sequencing analysis of PGPR species where the entire chromosome and plasmid are sequenced and annotated, and (b) partial/targeted genome or specific gene sequence analysis where a part of the genome is studied and used for characterization and comparison. (Swarnalakshmi et al., 2020). The application of molecular techniques begins with DNA extraction which have be an extraction of intact and pure DNA. Extraction consists of the isolation and purification of DNA molecules and is based on the physicochemical characteristics of the molecule. Phosphate groups of the chain of the DNA have a strong tendency to repel each other, due to its negative charge, which allows to dissolve to DNA in aqueous solutions and form a hydrating layer around the molecule. But, in the presence of ethanol, the moisturizing layer breaks down, leaving exposed phosphate groups. Under these conditions, union with cations such as Na^+ reduce the repulsive forces between the chains of nucleotides and allow DNA to precipitate. On the other hand, the net negative charge of DNA allows it to bind to molecules and matrices positively charged inorganics (Alejos-Velazquez et al., 2008).

Phenol/chloroform/isoamyl alcohol DNA extraction method is one of the most commonly used methods for the isolation and concentration of DNA. In the DNA extraction, first is the lysis of the outer and nuclear membranes of the cells; it is carried out in an alkaline medium, by the action of detergents and proteolytic enzymes, such as proteinase K to release the genetic material. After that, organic extraction is carried out, contaminating proteins are denatured and deposited at the interface between the aqueous phase and the organic phase; nucleic acids remain in the aqueous phase. Phenol is used at pH 8 to prevent oxidative products that damage nucleic acids. The phenol/chloroform mixture is used for the extractions, since it facilitates the denaturation of proteins and their precipitation at the interface. Finally, in ethanol precipitation, the salts remain in solution, while the nucleic acids form a white, mesh-like precipitate that can be separated by centrifugation. This procedure requires a relatively longer time compared to other extraction techniques, but it is

compensated by the efficiency in the yield and the purity of the product (Gallegos Velasco et al., 2019).

2.2. The role of beneficial rhizobacteria in agriculture

To address the food demand of people in the world, current agricultural systems in both developed and under-development countries have turned into intensive production systems as a means to increase global food production. Thus, there is an excessive use of chemical fertilizers with the purpose of increasing agricultural productivity. Environmental effects in the soil, water and air are difficult to reverse. Thus, the development of environmentally friendly ways to reduce the extensive use of these fertilizers is one of the greatest ecological and technological challenges at present (Han et al., 2019). In this regard, the utilization of beneficial microbes is considered a promising alternative for sustainable agriculture and an effective means to face two of the greatest current challenges of humans: food security and environmental wellness (Shah et al., 2021). According to this, different microorganisms (mainly fungi and bacteria) have been evaluated and reported as having great potential for the implementation of sustainable agriculture which aims not only for the reduction of the negative environmental impact of agronomic systems but also for the improvement in agronomic productivity and food quality.

In the rhizosphere, the effects of the microbiome on the plant and soil are determined by its composition, diversity, and microbial activity. Microorganisms can colonize or associate with plant roots or are also found as free-living bacteria in the rhizospheric soil. These microbiota contribute to the improvement of the growth and health of the plant host by utilizing a range of mechanisms which includes: N_2-fixation, solubilization of P and K, production of siderophores to increase Fe availability, synthesis of ACC deaminase, defense against pathogens, stimulation of root growth, modulation of phytohormones in plant tissues, modulation of the antioxidant equilibrium to improve the plant´s tolerance to biotic or abiotic stress factors, and enhancement of the biological diversity in the rhizosphere (Figure 3). The utilization of beneficial microorganisms in agricultural systems has been widely reported; however, in-field studies under high-production cultivation systems are still limited (Shah et al., 2021). Considering the above, the application of rhizobacteria in current agriculture systems constitutes an area of opportunity, because they

cannot only reduce the negative impact in the environment of intensive agriculture based on chemical fertilizers but also aid the growth, development, and production of agronomic crops, as well as improve product quality.

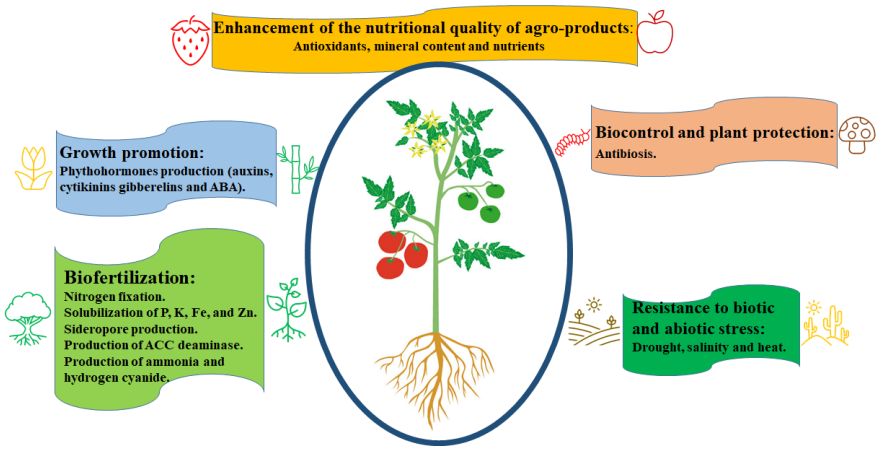

Figure 3. Beneficial traits of rhizobacteria towards plant-growth promotion and biofertilization.

2.3. Contribution to the microbial diversity of the rhizosphere

2.3.1 Regulation of rhizosphere microbial communities

Microbial biodiversity refers to the heterogeneity of living microorganisms and the relationships among them. Microbial communities in the rhizosphere contribute to the health of cultivated plants through fostering nutrients´ cycling in the soil. Recently, microbes have been exploited in sustainable agriculture for their positive effects on crop productivity, environmental impact, and human health (Yadav et al., 2017). Different relationships take place in the microbiota present in soil and in the plant´s rhizospheric soil, such as symbiosis, antagonism, mutualism, etc. Some rhizospheric bacteria and fungi exert antagonism against plant pathogens. The antagonistic mechanisms involve the synthesis of antimicrobial compounds, competition for nutrients and biological space, and induction of the plant´s systemic resistance (Kashyap et al., 2017). Therefore, rhizospheric microbial diversity is a main biological factor which affects not only antagonistic activity against plant pathogens, but also plant nutrient assimilation and growth. Many rhizobacterial

species exert benefits to agricultural products by modifying the microbial diversity of the rhizosphere. This modulation produces metabolic changes which in turn are helpful to reduce the population of pathogenic organisms, and indirectly favors plant growth (Figure 4 and Table 1).

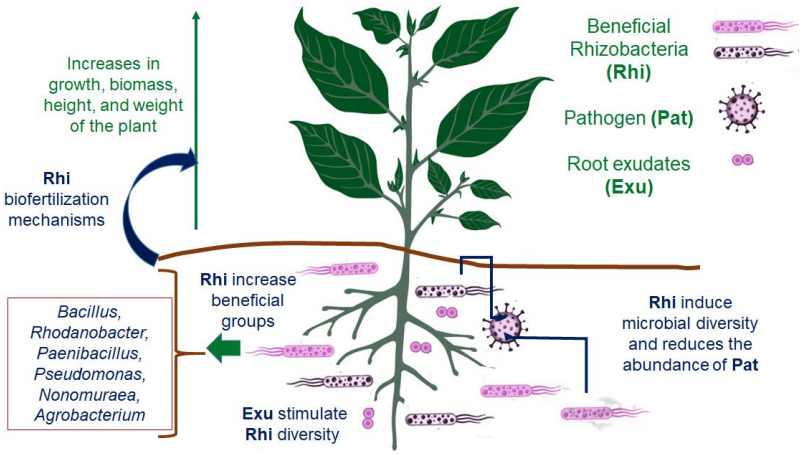

Figure 4. Interaction between rhizobacteria and root exudates and their effect on microbial diversity, and plant´s growth and protection.

Table 1. Rhizobacteria´s beneficial traits in cultivated crops.

Host plant/ Rhizosphere	Microbes evaluated	Observed effects	Reference
Chili (*Capsicum annum)*	*Streptomyces* species	*Cyanobacteria* was enriched, *Devosi*a, *Promicromonospora*, *Kribbella*, *Microbacterium*, *Amylocolatopsis*, and *Pseudomonas* genera were antagonist against pathogen.	Sakineh et al. (2021)
Cucumber (*Cucumis sativus*)	*Bacillus amyloliquefaciens*	Decrease abundance of *Fusarium*. Increase in the genera: *Acidovorax*, *Rhodanobacter*, *Sediminibacterium*, *Dongia*, *Streptomyces*, *Rhizobium*, *Mesorhizobium*, *Burkolderia-Paraburkholderia*, *Asticcacaulis* and *Rhizoscyphus*.	Han et al. (2019)
Tomato (*Solanum lycopersicum*)	*Pseudomonas* sp.	Changes in the diversity of the microbial community and increase in the relative abundance of rare taxa. Beneficial effects on plant growth characteristics.	Hu et al. (2021)
Bean (*Vicia faba* L.)	*B. pumilus*	Increment of soil´s catalase activity by microbial activity of dominant populations.	Kang et al. (2013)
Cucumber (Cucumis	*Streptomyces pactum* and *S.*	Increased the abundance of *Chitinophaga*, *Dokdonella*, *Pseudoxanthomonas* and *Coprinellus*	Li et al.

sativus), Tomato (*Solanum lycoperscum*)	*rochei*	and decreased that of phytopathogenic taxa (*Cladosporium* and *Gibberella*).	(2022)
Tomato (*Solanum lycopersicum*)	*B. velezensis*	High-carbon contents by the rhizospheric microbial community in the less effective treatments of biocontrol against *Fusarium*	Másmela-Mendoza and Moreno-Velandia (2022)
Cucumber (*Cucumis sativus*)	*B. amyloliquefaciences*	Higher presence of beneficial rhizosphere species such as *Bacillus*, *Rhodanobacter*, *Paenibacillus*, *Pseudomonas*, *Nonomuraea*, and *Agrobacterium.* Higher availability of mineral elements.	Qin et al. (2017)
Cucumber (*Cucumis sativus*)	*Paenibacillus polymyxa*	Decrease in the abundance of *Fusarium* and increase in the *Bacillus*, *Actinobacteria*, *Streptomyces*, *Actinospica, Catenulispora* and *Pseudomonas* genera.	Shi et al. (2017)
Apple (*Malus hupehensis*)	*B. velezensis*	Increase in biomass and nutrient absorption. Change in the soil microbial community, suppressing the pathogen *Fusarium*.	Wang et al. (2020)
Cucumber (*Cucumis sativus*)	*B. amyloliquefaciens*	Increase cucumber seedlings growth and *Proteobacteria*. Decreased *Acidobacteria*, *Actinobacteria, Cloroflexi, Plantctomycetes and Verrucomicrobia*.	Wang et al. (2021)
Arabidopsis thaliana and soil	*Bacillus* and *Pseudomonas*	Pathogen suppression and plant-growth promotion. Higher diversity of volatile organic compounds.	Wang et al. (2021)

Strain genera abbreviations: B.: *Bacillus*; P.: *Pseudomonas*.

Suppression of plant diseases has also been attributed to diverse rhizosphere microbial communities, which affect pathogen survival, soil enzymatic activity and root colonization (Shi et al., 2017). A large number of rhizobacterial strains are able to exert positive effects in the soil´s microbiome, increasing the presence of beneficial microbes and decreasing phytopathogenic strains (Figure 2). For instance, the application of *Bacillus velezensis* in lettuce cultivation incremented the diversity and richness of the microbiome structure and reduced the diversity of fungal communities through the production of substances that modulate the rhizospheric microbiota (Wang et al., 2020). In a work on tomato biofertilization, Hu et al. (2021) concluded that the introduction of a bacterial consortium based on *Pseudomonas* spp. as an inoculant caused improvements in different growth parameters of the treated plants. The authors explained attributed the results to changes in

the following factors: the diversity of the resident microbiota and its composition, an increase in the biofertilizer consortium abundance, and in the relative abundance of unique taxa. On the other hand, the presence or absence of fungal phytopathogens affects the microbial structure in the rhizosphere. Jamil et al. (2022) reported that Proteobacteria strains were significantly more abundant in a *Fusarium*-infected banana rhizosphere, while the diversity indices were consistently higher in the infected rhizosphere as compared with the non-infected treatment. A greater abundance of the strains *Burkholderia* spp. and *Streptomyces* spp. was reported in the healthy rhizospheric soil.

2.3.2 Abundance of some plant beneficial taxa

Bacterial diversity is known to influence the physicochemical and biological traits of the soil. There is a great biodiversity of microbes related to agricultural products, which depends of many factors such as climate, soil, agronomic parameters, fertilization regime, crop type, etc. The main bacterial phyla reported in agricultural crops are the following: *Actinobacteria*, *Bacteroidetes*, *Balneolaeota*, *Basidiomycota*, *Cyanobacteria*, *Firmicutes*, *Proteobacteria* and *Spirochaetes*; with the members of the phylum *Firmicutes* and *Proteobacteria* being the most dominant (Yadav et al., 2017). Wang et al. (2021) reported that the utilization of a *B. amyloliquefaciens* strain in cucumber cultivation reduced the presence of 18 phyla, mainly *Acidobacteriota, Chloroflexi, Planctomycetota* and *Verrucomicrobia*, and increased that of *Proteobacteria*. The authors reported that the inoculants modified the soil´s physicochemical characteristics, and induced changes in the microbial community which had an effect on increasing beneficial strains, decreasing pathogen colonization in the rhizosphere, and reducing the occurrence of cucumber *Fusarium* wilt. In a similar study, Qin et al. (2017) concluded that the application of *B. amyloliquefaciens* modified the microbiota related to cucumber seedlings, by increasing the abundance of benefic rhizospheric genera such as *Bacillus, Rhodanobacter*, *Paenibacillus*, *Pseudomonas*, *Nonomuraea*, and *Agrobacterium* with respect to the control. As a result, the bacterial inoculant increased the growth of cucumber plants and the amount of available mineral elements. In a similar study, the application of a strain of the same species in the rhizosphere of cucumber plants reduced the abundance of *Fusarium* sp. and significantly increased bacterial diversity with resulting increments in the presence of the genera

Streptomyces, *Rhizobium*, *Burkolderia*, among others. The abundance of the inoculant strain was related to both plant growth and the abundance of several beneficial bacteria, whereas it was negatively correlated with the plant´s disease index (Han et al., 2019).

In a study of a banana rhizosphere infected with *Fusarium oxysporum*, 18 bacterial genera were predominant, with *Xanthomonadaceae*, *Sphingomonas*, *Azospira*, *Pseudomonas*, and *Acinetobacter* being the most representative groups. On the other hand, in the healthy rhizosphere, the more abundant genera were *Acidobacteriaceae*, *Burkholderia*, *Streptomyces*, *Paraburkholderia*, *Actinospica*, *Bradyrhizobium*, and *Conexibacter* (Jamil et al., 2022). However, the application of rhizobacterial inoculants does not necessarily causes changes in the rhizospheric microbiome; Gomez-Lama et al. (2022) reported that the presence of *Pseudomonas simiae* on banana rhizosphere did not significantly alter neither the relative abundance nor the diversity of the rhizospheric microbiome; however, substantial changes in the interactions between microorganisms were observed. In a study on asymptomatic oil palm trees infected with the phytopathogenic fungi *Ganoderma* sp., Anothai and Thanunchanok (2022) reported an increment in the presence of the phyla *Actinobacteria* and *Firmicutes*, although the diversity with respect to symptomatic oil palm trees was similar. These bacterial groups include a number of genera widely known as biocontrol and biofertilization agents. In addition, corn rhizosphere can be considered as an important source of genes, mainly from abundant rhizospheric bacterial groups such as the phylum *Proteobacteria*, which catalyze the transformations of nutrimental elements; e.g., C, N, P and S (Li et al., 2014). Within these taxa, γ-*Proteobacteria* is the most important class considering its microbial diversity, which includes the well-documented biofertilizer rhizobacteria of the genera *Pseudomonas* and *Azotobacter* (Yadav et al., 2017).

2.3.3. Plant-microbiota interaction mechanisms

Many types of compounds are secreted by plant roots, which include sugars, organic acids, amino acids, phenolic compounds, and a wide number of secondary metabolites. These root exudates constitute a supply of nutrients for rhizospheric bacteria. The presence of secondary metabolites in root secretions has a substantial relevance in plant´s metabolism and growth. Zou et al. (2016) evidenced that the root secretions of *Arabidopsis thaliana* significantly promoted the growth of *B. cereus*. Interestingly, in the study, some root

exudates induced the production of chitinase and siderophores by *B. cereus*, while others inhibited such synthesis. Similarly, Tian et al. (2021) reported that higher concentrations of quercetin exudated by the plant roots of the invasive plant *Triadica sebifera* caused an effect on colonization and plant biomass of arbuscular mycorrhizal fungi (AMF). The authors concluded that root-exuded flavonoids modulate the interactions between the plant and rhizobacteria.

2.4. Biofertilization mechanisms

Bacteria have different growth promotion mechanisms for plants, there may be different mechanisms between strains or species. Among the direct mechanisms used by beneficial rhizobacteria to enhance plant´s nutrition and growth are the following (Figure 5): N_2-fixation, ACC deaminase, mineral solubilization (e.g., P, K, Zn), siderophore and phytohormone synthesis, and other biofertilization mechanisms (Kang et al., 2020). In addition, as indirect biofertilization mechanisms, the biocontrol of pathogenic organisms and pests, in addition to the increase in the plant´s tolerance to abiotic stress have been widely documented in the literature. Ideally, the rhizobacterial effect on crop cultivation would reflect on the enhancement of the nutritional quality of the harvested agro-products.

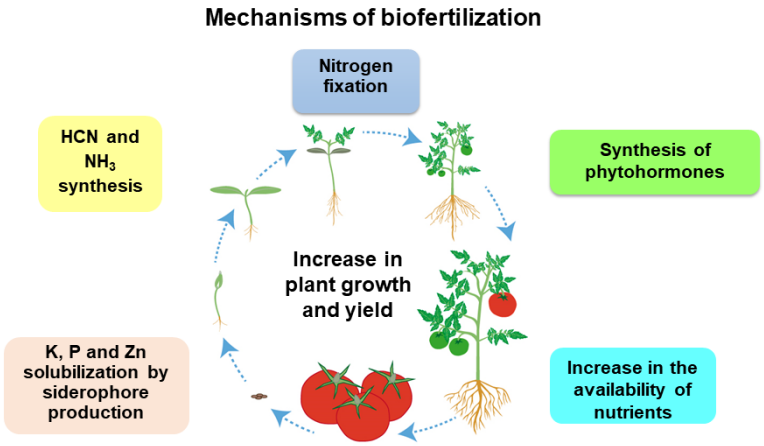

Figure 5. Representation of the most relevant biofertilization mechanisms involved in the application of beneficial rhizobacteria in food crops.

The biofertilizing effect of rhizobacterial inoculants isolated from wild *Physalis* sp. plants on the cultivation of tomatillo seedlings (*Physalis ixocarpa* Brot) was studied. As a result,

the bacterial strains *Atlantibacter* sp. UTMR4, *Priestia megaterium* UTMR3, and *Acinetobacter calcoaceticus* UTMR2 caused increments in the plant´s height, and weight of the plant´s leaves and roots in comparison with the non-inoculated control (Ramírez-Cariño et al., 2023) (Figure 6).

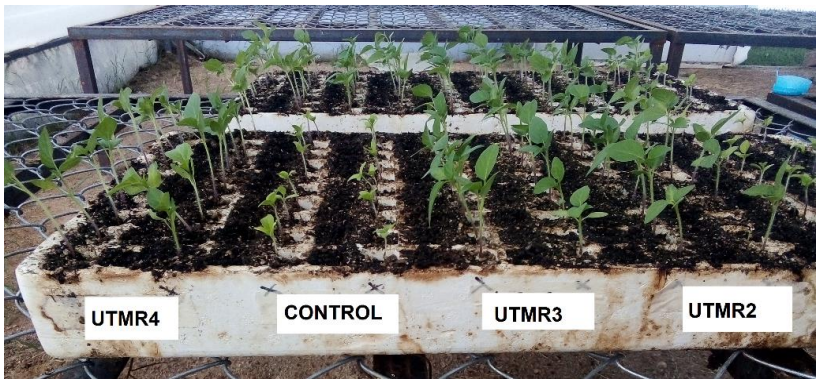

Figure 6. Rhizobacterial inoculation causing an increase in the growth of tomatillo seedlings (*Physalis ixocarpa* Brot.) with respect to the non-inoculated control. Treatments: UTMR4 (*Atlantibacter* sp.) UTMR3 (*Priestia megaterium*, UTMR2 (*Acinetobacter calcoaceticus*) and control (non-inoculated treatment).

2.4.1. Nitrogen fixation

The principal N_2-fixing bacterial symbionts that can colonize legumes include *Rhizobium*, *Bradyrhizobium, Ensifer*, *Azorhizobium*, and *Mesorhizobium*, *Sinorhizobium* (Hakim et al., 2021). On the other hand, bacterial genera which do not live in symbiosis with plants include *Arthrobacter, Azoarcus, Azospirillum*, *Azotobacter, Enterobacter, Mitsuaria*, and *Pseudomonas* (Naqqash et al., 2020). The use of N_2-fixing bacteria as biofertilizers has been widely studied. Ke et al. (2019) found that the inoculation of endophytic strain *P. stutzeri* A1501 in corn cultivation under greenhouse conditions improved the growth and nitrogen content of the plants. The authors reported that the bacterial inoculant also increase the populations of the N_2-fixing and ammonia-oxidizing bacteria. In another study, Gopalakrishnan et al. (2017) inoculated two chickpea cultivars with putative N_2 fixing bacterial strains of the genera *Pseudomonas, Chryseobacterium, Stenotrophomonas,* and *Pantoea*, reporting that the biofertilized treatments increased shoot biomass and grain yield, and the plant´s contents of total nitrogen and phosphorus. Renganathan et al. (2018)

assessed the application of a rhizobacterial isolate of *B. amyloliquefaciens* in tepary bean plants (*Phaseolus acutifolius*) grown under salinity and greenhouse conditions. The authors reported that the inoculation treatment provided stimulating effects on seed germination, shoot length, root length, biomass, and foliar area, while resulting in increments in the chlorophyll and protein content of the plants. In a tomato biofertilization study, Cervantes-Vázquez et al. (2021) assessed the separate use of the inoculants *B. paralicheniformis, Acinetobacter guillouiae, Aeromonas caviae,* and *P. lini* during the seedling stage in greenhouse conditions, reporting that the plants treated with the strain *P. lini* presented a 40% average increase in plant height, stem diameter, root volume, and weights of the root, leaf, and stem. Nitrate reductase activities were quantified considering four different reaction mechanisms, in which *B. paralicheniformis* presented an endogenous nitrate reductase activity 16% higher than the control. In addition, a number of inoculated treatments presented nitrogen contents which were similar to the chemical fertilized control, with the highest contents observed for the inoculants *Acinetobacter guillouiae* and *P. lini*.

2.4.2. ACC Deaminase

Another way by which plants acquire ammoniacal nitrogen is through bacterial strains that produce the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase. This enzyme favors crop growth under stressing conditions by hydrolyzing the precursor of ethylene into carbon (alpha-ketobutyrate) and nitrogen (ammonia) sources, which are used as nutrients for plant growth. Among the most prominent bacterial taxa that produce this enzyme are: C*hromobacter, Alcaligenes, Azospirillum, Bacillus, Burkholderia, Rhizobium, Rhodococcus, Enterobacter, Klebsiella, Methylobacterium, Mesorhizobium, Pseudomonas y Sinorhizobium* (Orozco-Mosqueda et al., 2020).

Danish and Zafar-ul-Hye (2019) assessed *in vitro* the ACC-deaminase activity of several rhizobacterial isolates, along with other biofertilization mechanisms, thus selecting four ACC-deaminase producing rhizobacteria. The strains were applied separately with the addition of timber-waste biochar for the cultivation of wheat under drought conditions. The authors reported that with respect to the chemically-fertilized control, the strain *B. amyloliquefaciens* increased total chlorophyll content (x 1.2), photosynthetic rate (x 1.2),

transpiration rate (73%), grain weight (59%), as well as grain mineral content (N, 58%; P, 18%; and K, 23%). In a similar study on corn cultivation under low-water irrigation, Danish et al. (2020) assessed the separate application of four strains which synthetize the enzyme (*P. aeruginosa, Enterobacter cloacae, Achromobacter xylosoxidans* and *Leclercia adecarboxylata*) with the addition of biochar as a soil amendment. The authors reported that under severe drought stress conditions, the strain *Achromobacter xylosoxidans* enhanced grain yield (x 2), photosynthetic rate (x 2.1), stomatal conductance (x 1.1), total chlorophyll (x 1.5) and carotenoids (x 2.8), with respect to the chemically-fertilized control. In addition, Ji et al. (2020) reported the biofertilization of rice using the rhizobacterial isolate *Glutamicibacter* sp. which presented *in vitro* ACC deaminase and indole acetic acid (IAA) production activities. The authors reported that the inoculated plants under saline conditions presented less Na^+ concentration, more electrolyte losses, and lower ethylene production in comparison with the treatment without inoculant. In addition, it was concluded that the biofertilization treatment enhanced the ACC content of the plant, as its well as ACC oxidase activity.

2.4.3. Phosphorus solubilization

Phosphorus is abundant in soil; however, the available phosphorus (HPO^{2-}_4 or H_2PO_4) for plant absorption is very low (approximately 0.1%) (Umar et al., 2020). Some bacteria are capable to solubilize phosphorous, thus making this essential element more available to the plant. The following rhizobacteria genera have been reported as effective phosphate solubilizers: *Agrobacterium, Arthrobacter, Azospirillum, Azotobacter, Bacillus, Brevundimonas, Chryseobacterium, Delftia, Enterobacter, Flavobacterium Gordonia, Klebsiella, Microbacterium, Pantoea, Phyllobacterium, Pseudomonas, Rhizobium, Rhodococcus, Serratia, Vibrio,* and *Xanthomonas* (Umar et al., 2020). These phosphate solubilizing bacteria (PSB) have the ability to produce a wide range of organic acids; e.g., acetic, adipic, formic, etc. (Kumar et al., 2018). In addition, PSB strains can use other less effective mechanisms for phosphorus solubilization such as the synthesis of chelating compounds and inorganic acids (Alori et al., 2017).

A few studies have addressed the utilization of PSBs in crop cultivation. Cavite et al. (2021) assessed the application of rhizobacterial isolates together with different doses of

inorganic fertilization in rice cultivation under screenhouse conditions. The authors reported that in the in vitro studies some isolates exhibited IAA and siderophore production, as well as ACC deaminase activity, with seven out of 25 isolates displaying tricalcium phosphate solubilization activity. In another study, the application of the rhizobacterial isolate *Acidovorax delafeldii* in rice cultivation using 50% of the NPK recommended dose yielded similar results than the fully fertilized treatment without inoculation in terms of weight of the plant´s shoot and root, plant height, and the yield and NPK contents of the grain. Similarly, Maldonado et al. (2020) tested phosphorus-solubilizing strains (*Erwinia* spp.), which were native to the Atacama Desert in the cultivation of lettuce under greenhouse conditions. The authors reported that some bacterial isolates had high phosphate solubilization capacity for $CaPO_4$ (608.9 to 781.4 mg/L). In addition, the inoculants exhibited *in vitro* production of IAA (23.5-35.8 mg/L), siderophores, as well as phosphatase enzymes (alkaline and acid). In the plant´s cultivation experiments, the bacterial isolates enhanced seed germination and stimulated early root elongation and seedling development, leaf number, as well as the yield parameters of the harvested lettuce: area and weight of roots and leaves with respect to the control. Furthermore, the phosphorus content in plants increased for the bacterial inoculation treatment using 50% of the recommended fertilization dose with respect to the fully-fertilized treatment without inoculation.

Shirinbayan et al. (2019) studied the phosphate solubilization capability of the rhizobacteria *Azotobacter salinestris* and *chroucoccum* reporting the solubilization of both inorganic (117.74 and 133.33 µg/mL) and organic (28.95 and 22.48 µg/mL) phosphorus, respectively. The authors reported that the bacteria were capable to synthetize IAA and siderophores, and to solubilize potassium. The inoculation of these bacteria in corn cultivation under greenhouse conditions increased shoot dry weight, plant height, as well as the plant´s contents of chlorophyll, nitrogen and phosphorus (10% with respect to the control). In a work on rice biofertilization, Rasul et al. (2019) conducted the inoculation of bacterial inoculants of the belonging to the taxa *Enterobacter, Acinetobacter, Pseudomonas, Klebsiella, Bacillus and Rhizobium* in Basmati rice cultivation under net-house conditions. The authors reported that all the strains were capable to solubilize phosphorous, with P solubilization ranging from 27 to 354 µg mL^{-1}. The carboxylic acids

acetic (12-180 µg mL^{-1}) and gluconic (5-130 µg mL^{-1}) were produced in the tested strains using insoluble tricalcium phosphate. Notably, the strains with the highest solubilizing capacity, *P. putida* and *Acinetobacter soli* secreted larger amounts of gluconic acid (130 and 117 µg mL^{-1}, respectively). Regarding crop cultivation, the authors reported that the treatments with a 20% reduced-dose of chemical fertilization inoculated with the strains *Acinetobacter soli* and *P. putida* had the greatest agronomical and nutritional results in comparison with the non-inoculated fully-fertilized control: grain yield (maximum 55%) and P content both in grain and straw (max. 67%). Using gene sequencing, the authors concluded that P solubilizing activity was mainly obtained by the production of gluconic acid.

In another report, Li et al. (2020) conducted the application of a rhizospheric consortium composed of *Providencia rettgeri, Advenella incenata*, *Acinetobacter calcoaceticus*, and *Serratia plymuthica* in the cultivation of oat, alfalfa and cucumber seedlings. The authors reported that all the inoculants were able to solubilize phosphate both inorganic (between 131.26 to 438.35 µg/mL) and organic (between 13.36 to 35.46 µg/mL), and fix nitrogen. In addition, the strains *Providencia rettgeri,* and *Advenella incenata* were capable to synthetize IAA. The multi-bacterial inoculant increased the crops´ growth parameters, chlorophyll content, and activity of antioxidant enzymes (peroxidase, catalase, and superoxide dismutase). In addition, the application of the inoculant on the rhizospheric soil was assessed in the study, resulting in an increase in the catabolic enzymatic activities (urease, invertase, alkaline phosphatase, and catalase), as well as available N, K, and P, with a 33.8 and 52.1% increase of the latter in oat and cucumber, respectively.

2.4.4. Potassium solubilization

Potassium is ubiquitously present in soil; however, only 1 to 2% of this element is available to plants. Potassium solubilizing bacteria (KSB) are capable to transform insoluble K compounds to soluble species. It has been reported that the use of KSB inoculants in the cultivation of a variety of crops enhance the plant´s growth and yield. Among rhizospheric bacteria, strains of the *Bacillus* genus have been described as the most effective K solubilizers. Other reported KSB genera include *Burkholderia*, *Acidithiobacillus*,

Arthrobacter, Enterobacter, Paenibacillus, Cladosporium, Aminobacter, Sphingomonas, Burkholderia (Etesami et al., 2017).

The principal mechanisms for K solubilization include complexation, chelation, ion exchange reactions and proton production (Pattnaik et al., 2021), in which organic acids play a predominant role (Alori et al., 2017; Etesami et al., 2017). Mounir et al. (2020) applied *B. mucilaginosus* with different doses of fertilization in garlic cultivation. The solubilization indexes of the bacterial strain for both soluble and insoluble potassium were 8.8 and 34.5 mm, respectively. The treatments with bacterial inoculation combined with 75% of the recommended K fertilization dose caused an increase in the crop agronomical parameters, such as bulb diameter and weight, as well as yield and storage stability as compared with the treatment without inoculation. In addition, the inoculated treatment presented the highest concentration of total carbohydrates, free amino acids and protein content in the bulb. In a similar study on potato cultivation, Elkhatib et al. (2019) evaluated the application of *B. circulance* together with chemical fertilization, finding that the inoculation of the bacteria not only increased plant growth, but also the contents of chlorophyll, carotene, and the elements N, P and K in the leaves (avg.: 20.8, 21.1, and 15.5%, respectively) and tubers (avg.: 16.9, 17.9 and 27.7%, respectively) with respect to the control treatment. The use of bacterial inoculant with 67% of the recommended K fertilization dose resulted in similar nutritional quality and productivity of potato tubers (number, weight and yield) in comparison with the fully-fertilized treatment without inoculation. In another report, Khanghahi et al. (2018) inoculated *Pantoea agglomerans*, *Rahnella aquatilis* and *P. orientalis* in rice plants using different fertilization doses of K. The solubilization activity of the bacteria were 35.4, 76.0 and 56.6 µg/mL of K release respectively, using from mica as the mineral source. The results indicated that the rhizobacterial inoculants combined with half of the K fertilization resulted in grain yield and productivity as well as K content of the plant, comparable with the fully-fertilized treatment without inoculation.

2.4.5. Siderophore production

Iron is a very abundant element in rhizospheric soils; however, large amounts of this mineral are found in a form not available to plants (Hakim et al., 2021). Beneficial

rhizobacteria synthetize siderophores as a strategy to get some nutrients from soil, thus providing the plant with available iron. These microorganisms are capable of producing siderophores in Fe-limiting conditions which have special affinity for Fe^{3+}, acting specifically as chelating agents to sequester Fe in the presence of other metals, thus causing its reduction to Fe^{2+} (Shah et al., 2021), which is a much more soluble form for the nutrition of rhizobacteria (Hakim et al., 2021). Siderophores can be categorized into the following chemical groups: hydroxamates, catecholates, and carboxylates (Khan et al., 2018). The most relevant rhizobacteria that produce these compounds include the following genera: *Pseudomonas*, *Klebsiella*, *Bacillus*, *Bradyrhizobium*, *Streptomyces*, *Serratia*, and *Rhizobium* (Mustafa et al., 2019). Patel et al. (2018) assessed the application of *Pantoea dispersa* and *P. putida* in mungbean cultivation, reporting in vitro siderophore activities of 89.9 and 85.3%, respectively, for Fe concentrations below 15 μM. It was determined that *Pantoea dispersa* synthetized catecholate, whereas *P. putida* produced hydroxymate-type siderophores. The application of the bacterial inoculant increased plant´s germination and growth parameters, as well as the contents of the harvested mungbean´s protein, carbohydrates and Fe; the latter resulting in 3.4 and 2.8-fold increases, respectively, with respect to the non-inoculated control. Similarly, Ghavami et al. (2017) investigated the application of *Micrococcus yunnanensis* and *Stenotrophomonas chelatiphaga* in corn and canola crops under greenhouse conditions. The authors reported in vitro siderophore activity of 13.8 and 12.7% for the rhizobacterial strains, respectively. Regarding the type of siderophore to which Fe-solubilization was related, it was determined that the strains synthetized hidroxamate and carboxylate, respectively. The inoculation of the rhizobacterial strains caused increments in the weights of shoot and root of both crops in comparison with the non-inoculated treatment. Additionally, *Micrococcus yunnanensis* caused increases of iron content in root and shoot, respectively, of 43.1 and 14.8% for corn, and of 38.3 and 212.7% for canola cultivation. Similarly, the application of *Stenotrophomonas chelatiphaga* increased the iron content of root and shoot of corn plants in 65.0 and 10.6%, and in 8.97 and 462.18% in canola crop, respectively, as compared with the control plants.

In a study on date palm cultivation, AbdElgawad et al. (2019) evaluated a consortium of actinobacterial isolates (*Streptomyces* spp. and *Nocardiopsis* sp.), reporting that *in vitro* the

strains were able to produce IAA (from 0.5 to 5.0 mg/L) and siderophores (from 0.5 to 9.2 mg/L). In addition, the authors performed the inoculation of the consortium in five palm cultivars reporting that the inoculants improved the chemical properties of the rhizospheric soil, with increments in the concentrations of the elements N, P, Mg, Ca, K, and Fe (between 11 and 79%) and organic matter, as well as in the fruit yield (from 13 and 46%) with respect to the control treatments. In general, a significant mineral enhancement for both macro and micronutrients was observed in the date fruits cultivated with the inoculants for the different palm cultivars as compared with the controls. Interestingly, the amount of Cu in the fruits was remarkably enhanced presenting increases from 2.9 to 14.5-fold as compared to the treatments without inoculation.

2.4.6. Synthesis of phytohormones

Benefic rhizobacteria have a substantial contribution towards the synthesis of plant-growth hormones, named phytohormones. These substances favor plant growth by different mechanisms: i) improving seed germination; ii) modulating the balance of hormones in the plants and their reaction to stress; iii) development of lateral and adventitious roots for better nutrient uptake; iv) facilitating root nodulation; v) enhancing of overall plant growth: development of vascular tissue, shoot elongation, as well as flowering and product yield (Kim et al., 2018). Plant hormones can be classified into the following types: auxins, cytokinins, gibberellins, abscisic acid (ABA), and ethylene (Oleńska et al., 2020).

Auxins are the phytohormones that controls apical dominance, cell´s differentiation and division, seed germination, and root´s development. They also contribute to plant´s physiological processes; e.g., photosynthesis, synthesis of bioactive compounds, and induction of the plant´s tolerance to stressing conditions. IAA is the more abundant auxin in nature which can be synthetized by both rhizobacteria and plants. The main bacterial genera that produce auxin include: *Azospirillum*, *Priestia* and *Pseudomonas*. In addition, cytokinins are signaling phytohormones that induce plant´s growth through the enhancement of apical predominance, seed germination, root elongation, nodule production, as well as vascular, flower and fruit growth. The most relevant rhizobacterial genera with the capacity to synthetize these compounds are *Bacillus*, *Escherichia*, *Agrobacterium*, *Methylobacterium*, *Proteus*, *Pseudomonas*, and *Klebsiella* (Pavlů et al.,

2018). On the other hand, gibberellins are phytohormones involved in most phenological stages of plants, from embryogenesis to fruit ripening (dos Santos et al., 2020). Among the wide number of bacterial genera that produce gibberelins, the most reported are *Acetobacter, Azotobacter, Herbaspirillum, Azospirillum, Bacillus and Pseudomonas* (Swarnalakshmi et al., 2020).

A few studies have reported *in vivo* applications of rhizobacterial inoculants that produce phytohormones. Kang et al. (2019) applied *B. tequilensis* in the cultivation of soybean. From *in vitro* assays, the authors reported that the strain had a strong ability to produce seven different types of gibberellins ranging from 0.24 to 2.25 ng/100 mL, as well as IAA (0.42 ng/100 mL) and ABA (0.43 ng/100 mL). Additionally, considering *in vivo* hydroponic experiments under heat-stress conditions, it was concluded that the inoculant improved the plant´s shoot length, biomass, leaf area, and chlorophyll. As related to the phytohormone content in the aerial part under normal cultivation conditions, the content of ABA was increased 20% with respect to the control without bacterial inoculation. In another study, Agarwal et al. (2019) applied *B. amyloliquefaciens* in rice cultivation and obtained the expression of an ASR plant-regulating gene, OsASR6, from the RNA of plant roots. This gene is reported to respond to abscisic acid levels, as well as to dehydration, and cold stress stimuli. The authors conducted a genetic study in *Arabidopsis thaliana* for the evaluation of phenotypic and growth parameters, reporting that the expression of the gene improved the growth of primary roots and root density, as well as plant growth and seed yield in genetically-modified plants under unstressed conditions, due to an increased auxin responsiveness.

2.4.7. Other biofertilization mechanisms

Some rhizobacteria are able to secrete hydrogen cyanide (HCN), which is an important metabolite related to the enhancement of plant growth and biocontrol. The most prominent rhizobacterial taxa in terms of their HCN production capability are: *Pseudomonas*, *Azotobacter*, *Rhizobium* and *Bacillus* (Sendi et al., 2020). HCN forms complexes with mineral salts such as calcium, iron, or aluminum phosphates by sequestering the cations, and consequently increasing the availability of phosphorus for the plant (AbdEl-Rahman et

al., 2019). Up to date, there are no studies that report the evaluation of HCN-producing bacteria in plant or in field cultivation.

Another factor for plant-growth promotion is ammonia (NH_3) production, one of the reactive nitrogen forms. Several rhizobacteria are able to produce NH_3 which serves to provide nitrogen to the plant roots, thus promoting the plant´s aerial growth (Bhattacharyya et al., 2020). In addition, NH_3 indirectly favors plant development by inhibiting plant pathogenic microbes (Kang et al., 2020). Although the production of NH_3 under *in vitro* conditions by rhizobacteria has been extensively reported, there have not been reports of *in vivo* or in-field experiments which assess the effect of NH_3 synthesis by these bacteria on plant´s nutrition and growth promotion.

2.5. Effect of bacterial biofertilizers on the nutritional quality and bioactive compounds of harvested products

2.5.1. Introduction

As stated in the previous sections, microbial inoculants can foster plant nutrition by making nutrients provided in soil more available for plant assimilation. Most bacterial inoculants are endophyte or rhizospheric microorganisms, which is important since the purpose of using as biofertilizers is that they establish symbiotic and synergistic relationships with the plant. Different mechanisms are employed by bacterial biofertilizers, among which the most relevant are: nitrogen fixation, ammonia and HCN production, mineral chelation by siderophores (Fe and Zn), mineral solubilization (K, P, Se). In addition to this, these microbes can benefit plant development through different mechanisms such as the synthesis of phytohormones and ACC deaminase.

A number of literature reports attest the effectiveness of different bacterial genera, species and strains for improving plant nutrition and growth. Most of these studies report the biofertilization mechanisms and growth promotion characteristics of the strains, and the influence of the inoculants on the agronomical parameters of the seedlings or the plants (such as germination time, plant height, weight, root length, product yield, etc.). Therefore, the beneficial effects of plant rhizobacteria have been well established; however, there are not many reports on the effect of these inoculants in the nutritional and biochemical composition of the crop final products. On the other hand, different microorganisms have

been used as biofertilizers among which the ones that have been more successfully utilized are the rhizospheric bacteria. In addition, in order to attest the actual effects of the bacterial inoculants on the harvested products, the crop cultivation experiments should be made in sterilized or disinfected soil; however, few reports consider this in their experimental procedures (Ochoa-Velasco et al., 2016). The reason for this is that on one hand the soil sterilization is cumbersome and not easy to apply; on the other hand, sterilized soil systems are not practical in actual agriculture systems. Furthermore, bacterial colonization assessment is barely reported, which can introduce some uncertainty in the biofertilization studies; therefore, with the aim to promote rhizobacterial colonization repeated inoculation doses are commonly applied. Imra et al. (2022) reported the use of plating bacterial count and confocal laser scanning microscopy (CLSM) combined with fluorescence in situ hybridization (FISH) for the assessment of viable bacteria in the plant rhizobacteria. Similarly, Pagnani et al. (2018; 2020) used scanning electron microcopy (SEM) to attest bacterial root colonization by scanning electron in hemp and wheat cultivation, respectively (Figure 7).

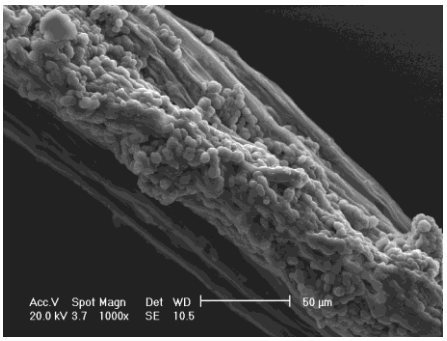

Figure 7. Colonization of a beneficial rhizobacteria consortium on the surface of *Cannabis sativa* (Finola) roots and bacterial penetration inside the root tissues.

The use of bacterial biofertilizers as inoculants in the production of different crops has been reported to cause benefits in the nutritional quality and in the bioactive compounds of the harvested products. A wide range of products have been reported which includes forage, oilseeds, fruits, cereal grains, vegetables, medicinal and aromatical herbs, and forages. The biochemical composition reported in the studies depends on the nutritional aspects that are important for the particular crop product; for example, in the case of fruits, emphasis is put in the mineral content as well as vitamins, and bioactive compounds, such as antioxidants. In this section, a critical survey on the effect of rhizobacterial biofertilizers in the

nutritional composition of the harvested product is presented, no seedling studies were considered (Table 2).

Table 2. Effect of rhizobacteria in the nutritional composition of the harvested products.

Crop	Biofertilizers	Nutritional quality parameters	Reference
Tomato (*Solanum lycopersicum)*	*Bacillus paralicheniformis*	P.M.: fiber, TSS; B.C.: flavonoids, A.A.	Ochoa-Velasco et al. (2016)
	B. amyloliquefaciens *B. methylotrophicus* *B. subtilis*	B.C.: carotenoids, phenols, A.A. VOC.	Ruiz-Cisneros et al. (2022)
	Azospirillum brasilense, Arthrobacter oxydans, Pseudomonas stutzer	B.C.: carotenoids Minerals.: N, P.	Imran et al. (2022)
Chili pepper (*Capsicum annuum*)	Same as above	B.C.: carotenoids Min.: N, P.	Imran et al. (2022)
Potato (*Solanum tuberosum*)	Biofertile	P.M.: protein, starch; Min: N, P, K, Ca.	Abdebayum et al. (2019)
Asparagus (*Asparagus officinali*)	Azoriz *(Rhizobium* sp., *Azotobacter* sp.)	Macro: total aminoacids B.C.: aminoacid profile	Xekarfotakis et al. (2021)
Okra (*Abelmoschus esculentus*)	*Bacillus* sp.	P.M.: protein Min.: N, P, K	Nazir et al. (2017)

Abbreviations: P.M.: Primary metabolites; TSS: total soluble solids; B.C.: Bioactive compounds; AA: antioxidant activity; VOC: volatile organic compounds; C.B.: Commercial biofertilizer; AMF: Arbuscular mycorrhizal fungi; Strain genera abbreviations: *A.*: *Azospirillum*; *B.*: *Bacillus*; *P.*: *Pseudomonas*; Min.: Minerals.

2.5.2. Effect of rhizobacteria inoculation in different harvested products

Rhizobacteria inoculants have been applied in different vegetables, such as tomato, potato, chili pepper, spinach and okra; however, just few works have been reported recently in fruit products such as apples and dates (Table 3). Most of the studies include mineral analysis,

protein as the main primary metabolite, total soluble solids. Among the bioactive compounds, phenols, flavonoids, tannins, vitamin C, and antioxidant activity. Tomato is one of the most studied products probably because of the commercial importance of this crop worldwide. In a test where different mineral fertilizer doses were used, the inoculant *B. paralicheniformis* had a positive effect on flavonoid, fiber, and antioxidant activity (Ochoa-Velasco et al., 2016) in comparison with the non-inoculated treatment. In addition, Ruiz-Cisneros (2022) reported that *Bacillus* spp. strains used in a composted and fully fertilized soil for tomato cultivation had a positive effect on carotenoids, phenols and antioxidant activity with respect to the control in which no inoculant was applied. In terms of mineral analysis, Imran et al. (2022) investigated the influence of a biocompost enriched with a rhizobacterial consortium on the mineral content of tomato and chili pepper, reporting increases of 32.8 and 22.8% in N, respectively and 23% in P, with additional increases of 26 and 40% in carotenoid contents, respectively; which attests the effectiveness of tomato biofertilization.

Nazir et al. (2017) employed a *Bacillus* sp. strain as an inoculant in okra cultivation using nitrogen as 50% biogas slurry and 50% urea fertilizers, reporting maximum increases in the protein and mineral content (N, P, K) in okra harvested fruits with respect to the control (100% urea fertilization without inoculation) and other inoculated fertilization doses.

The effect of biofertilizers in the mineral content of the harvested products have been reported for some vegetable crops. The use of a multi-genera rhizobacterial commercial consortium had a positive effect in most of the primary metabolites and mineral content (N, P, K, Ca) in the biofertilization of potato using a farm compost as soil fertilizer (Abdeldaym et al., 2019). Increased K contents in apple fruits were reported by Bokszczanin et al. (2021) for *B. subtilis* and *Streptoymyces* spp. added with arbuscular mycorrhyzal fungi. Similarly, high increases in Cu and N concentrations, were obtained for the inoculation of date plants with a consortium compraised of the strains *Streptomyces* sp. and *Norcadiopsis* sp.

One of the main aspects considered for assessing the nutritional quality of vegetables and fruits is its content of bioactive compounds, such as phenols, flavonoids, vitamins, as well as their antioxidant activities. According to the literature the different biofertilization inoculants were capable of increasing the contents of the following groups of compounds:

i) carotenoids: tomato (Ruiz-Cisneros et al., 2022; Imran et al., 2022), chili pepper (Imran et al., 2022); ii) phenols: tomato (Ruiz-Cisneros et al., 2022; Imran et al., 2022; iii) flavonoids: tomato (Ochoa-Velasco et al., 2016); and iv) antioxidant activity: tomato (Ochoa-Velasco et al., 2016; Ruiz-Cisneros et al., 2022). These reports attest the efficiency of the rhizobacterial inoculants to participate in the plant nutrition process making more available the mineral compounds to the plant, but also to moderate the secondary metabolism of the plant to synthetize bioactive compounds that are significant for human nutrition considering their antioxidant properties.

2.5.3. Reduction of the chemical fertilization dose by using biofertilizers

Despite that most of the fertilization studies use full fertilization doses, one important trait of biofertilization is the capacity of rhizobacteria to reduce the utilization of agrochemicals. Several works have reported the effectiveness of rhizobacterial inoculation on the reduction of the chemical fertilization dose without affecting the agronomical yields and nutritional quality. Ochoa-Velasco et al. (2016) evaluated the use of the inoculant *B. paralicheniformis* in tomato cultivation. The authors recommended a 25% reduction in the N-fertilizing dose without affecting the nutritional quality of the fruit, considering that the fruits of the 75% N-dose treatment presented a higher antioxidant activity and flavonoid content than the non-inoculated treatment supplied with full N-fertilization. Bilal et al. (2017) studied the cultivation of oat as a forage source with the rhizobacterial composed of the N_2-fixing rhizobacteria *Azotobacter* sp. and *Azospirillum* sp. with different doses of N chemical fertilization. The biofertilization treatment caused an increase in protein, fiber, as well as in total mineral content, allowing a reduction of 33% in N fertilization dose for optimum protein and forage yield with respect to the control. In a similar study, a 50% reduction in the N-P fertilization was possible in the application of a N_2-fixation rhizobacterial consortium to sesame cultivation, while maintaining the nutritional quality of the product, while enhancing the content of oleic acids (Nosheen et al., 2019). In another work, Bokszczanin et al. (2021) concluded that in apple cultivation, the use of rhizobacteria with a 50% reduction in the N dose provided the same nutritional quality than the fully fertilized control, with the highest K content. Remarkably, Pagnani et al. (2018) studied the effect of rhizobacterial consortium in the cultivation of industrial hemp “Finola” without the use of

chemical fertilizers, reporting not only an increase in bioactive compounds but also preserving the plant growth of the fully fertilized treatment.

These reports confirm the beneficial effect of rhizobacterial biofertilizers on the reduction of N and K chemical fertilization, with N reductions from 25 to 50%, while maintaining the product nutritional quality and yield. An alternative approach has been the use of composts together with biofertilizer inoculants, with positive impacts in the productivity and quality of the harvested products. Patra et al. (2022) tested the inoculation of rice cultivation with the application of an enriched rice compost using an N_2 fixing inoculant, reporting an increase in grain mineral micronutrient contents with a reduction of 50% of the N chemical fertilization full dose. Similarly, Imran et al. (2022) applied a wheat/rice compost fortified with Zn, Mg and Fe, together with a multi-genera consortium to the cultivation of tomato and chili pepper, reporting positive effects on the nutritional quality of the horticulture products. The applications of farm compost combined with rhizobacteria inoculants for the cultivation of potato was reported as beneficial in terms of both primary metabolites and macrominerals (Abdeldaym et al., 2019).

2.6. Tomatillo crop

2.6.1. Introduction

Tomatillo (*Physalis ixocarpa* and *Physalis philadelphica*) are native fruits to Mexico and Central America. However, the tomatillo plant grows in tropical and subtropical regions around the world. The *Physalis* genus belongs to the widely distributed Solanaceae botanical family. The *Physalis* genus comprises around 100 species and is recognized by the presence of a permanent calyx in the fruit (Souza et al., 2017). *Physalis ixocarpa* is a species native from Mexico, where it has been consumed by the native population since pre-Hispanic times. The fruits of this species are known as tomatillo or husk tomato (Cobaleda-Velasco et al., 2013). The tomatillo fruits can come in a wide variety of colors ranging from green, purple, yellow to orange. The size of the fruit is highly variable in wild and cultivated species. The fruits can be globose, spherical or oblong and have a sweet, semi-acid, acid and bitter flavor (Vargas-Ponce et al., 2015). *Physalis* species have been shown to have significant antioxidant potential. Plants of this genus have been used as a treatment for urinary and skin diseases, gonorrhea, ulcers, and wounds (Bergier et al.,

2012). Due to its unique and slightly acid flavor, tomatillo fruit is the base for preparing sauces and various typical Mexican dishes, although it is also used as an ingredient in gourmet sauces, stews, and condiments. Its consumption is related to its green color and/or its acidity (Bock et al., 1995). Tomatillo fruit is an important source of phosphorus, calcium, iron, mineral salts and vitamins. The increase in cultivation and demand for the tomatillo fruit is due to its sensory, nutritional and functional properties (Pedro and Aguirre, 2016)

2.6.2. Plant and fruits characteristics

Tomatillo crop is grown best at low altitudes, between 18 and 25 ◦C, slightly acid soils, and areas with 15–120 cm annual rainfall. Tomatillo fruits are small, spherical or slightly oblate and surrounded by an enlarged calyx, which helps to protect the fruit from insects, birds, diseases and extreme climate conditions like heavy rain, hailstorms, extreme temperature changes or radiation (Shenstone et al., 2020). The fruit size has been reported between 27.42 to 54.57 mm in equatorial diameter and 25.0 to 44.32 mm in polar diameter. The pericarp color of mature fruits is usually green, yellow, bright orange-red, purple, or purple-green. Tomatillo fruit can be harvested at mature or immature stage. For commercial marketing, the fruit is harvested when it reached its maximum size (when the calyx is completely filled or broken by the fruit) (González-Pérez and Guerrero-Beltrán, 2021).

2.6.3. Cultivation practices

The most common cultivation practices in tomatillo crop are direct seeding and transplantation. The first one consist in the spreading of seeds directly into fields. Commonly, seeds germinate in about 7-10 days. Transplanting technique consist in the previous seed germination and growing the crop indoors before transplanting outdoors. Some advantages of this technique are the saving of seeds, reduction of weeds and the possibility of starting the growing cycle when the outside temperature is below 18 °C (González-Pérez and Guerrero-Beltrán, 2021).

2.6.4. Economic importance

Tomatillo is one of the industrial crops with greater economic importance within Mexican horticulture: in 2020, this crop was the sixth vegetable with the largest planted area in

Mexico with 40,117 ha and 766,515-ton production (SIAP, 2021). The importance of tomatillo fruit in Mexican diet is due to its flavor and that is the base to prepare a great variety of traditional food.

2.7. Perspectives

The biofertilizing characterization of rhizobacteria isolated from wild tomatillo plants and evaluated in the growth parameters of tomatillo seedlings is a study required for future research..

CHAPTER III

3. Characterization and biofertilizing effect of plant growth promoting rhizobacteria *in vitro* and in tomatillo seedlings (*Physalis ixocarpa* Brot.)

3.1. Introduction

Plants from the genus *Physalis* (family Solanaceae) are native to warm and subtropical regions of Central and South America. Despite the great diversity of *Physalis* in Mexico, only *P. ixocarpa* Brot, and *P. philadelphica* Lam. are cultivated to date (Hernández-Pacheco et al., 2021). Tomatillo fruit (*Physalis ixocarpa* Brot.), also known as Mexican husk and green tomato, is the main ingredient for preparing sauces and various typical Mexican dishes. Additionally, this fruit is an important source of phosphorus, calcium, iron, vitamins, carotenoids, polyphenols, among other phytochemicals (Golubkina et al., 2018).

Tomatillo is one of the industrial crops with greater economic importance within Mexican horticulture: in 2020, this crop was the sixth vegetable with the largest planted area in Mexico with 40,117 ha and 766,515-ton production (SIAP, 2021). Considering tomatillo fruits are highly demanded in the Mexican market, it is a common practice for producers to apply substantial amounts of chemical fertilizers to maintain high crop yields. However, excessive use of these fertilizers not only increases production costs but also produces an undesirable environmental impact, such as soil and water contamination (Zhang et al., 2017). Biofertilizers are an environmentally friendly alternative to enhance the absorption of both water and mineral nutrients by the plant. Rhizobacteria based biofertilizers play an important role in maintaining soil fertility (Qin et al., 2017). Over 95% of the bacteria in plants live attached to the plant roots or in the plant rhizosphere. In many cases, bacteria establish symbiotic relations with the host allowing the plant to obtain nutrients through the bacteria´s metabolism (Ji et al., 2014). The use of plant growth-promoting rhizobacteria (PGPR) can be helpful to reduce chemical fertilizer utilization, improve soil quality, and increase crop yields through various direct and indirect mechanisms (Ma et al., 2018). Direct biofertilization mechanisms include: stimulation of root growth by phytohormones, phosphorus and potassium solubilization, nitrogen fixation, siderophore production, and auxin synthesis (Dinesh et al., 2015).

Nitrogen (N) is the most vital nutrient for plant growth and productivity. The atmospheric N_2 is converted into plant-utilizable forms by biological fixation, converting molecular nitrogen to ammonia by symbiotic and non-symbiotic N_2-fixing bacteria. Ammonia production by rhizobacteria constitutes an important biofertilization mechanism in plants. Antil et al. (2022) reported that *P. megaterium* and *B. cereus* showed important ammonia production (4.7 and 3.2 μg mL^{-1}, respectively) and other plant growth promoting attributes such as phytohormone synthesis and hydrogen cyanide production. In a similar study, Xu et al. (2016) reported two *Bacillus* spp. strains producing ammonia**.**

Another nutrient important to the plant growth-limiting factor is phosphorus. It is abundantly present in soils in both organic and inorganic forms. Despite the large abundance of phosphorus in nature, the available forms of this element for plants is generally low since this mineral is found mainly in its insoluble forms in soil (Ahemad, 2014). Solubilizing microorganisms can provide available phosphorus to the plants which make biofertilizers a viable substitute to chemical phosphate fertilizers (Khan et al., 2006).

Potassium (K) is also an important element for plant nutrition (Aallam et al., 2021). Although plants are able to readily absorb the soluble form of potassium, soil contains only 2% of available K (Parmar and Sindhu, 2018). Potassium-solubilizing bacteria play a vital role in making potassium accessible to plants (Ashfaq et al., 2020) by improving plant growth and reducing the environmental impact of the agricultural process. On the other hand, according to Li et al. (2021) the ability of microorganisms to produce indole acetic acid (IAA) serves as a phytohormone to the plants, thus fostering growth.

Several studies have conducted *in vitro* and *in vivo* tests to assess the biofertilization attributes of rhizobacteria strains. Cervantes-Vázquez et al. (2021) reported that *Pseudomonas lini* showed higher values for plant height, stem diameter, and fresh weight of root, leaf, and stem of tomato seedlings whereas *Aeromonas caviae*, *Bacillus paralicheniformis*, and *P. lini* increased the plant's P content. In a similar a study, Delgado-Ramirez et al. (2021) reported that two *Bacillus* sp. strains, three *Pseudomonas* sp., and a *Paenibacillus* sp. strains showed a strong positive effect on the growth of tomato (*Solanum lycopersicum*) plants. The strains resulted in an increase in the growth parameters such as fresh weight, dry weight, stem length, thickness, as well as root's fresh and dry weight, compared to the control.

Bacterial diversity in the roots and rhizosphere of Mexican tomatillo plants has been reported. Hernández-Pacheco et al. (2021) screened the biofertilization capacity of 315 strains isolated from roots, stems, and leaves from *Physalis ixocarpa* plants. The authors reported that some of the most abundant genera were *Bacillus*, *Microbacterium*, *Pseudomonas*, and *Stenotrophomonas*. The endophyte strains exhibited outstanding *in vitro* biofertilizing activity traits such as IAA and siderophores production, as well as solubilization of phosphates. In seedlings tests, 8 isolates showed similar or greater growth-promotion activities that the *Pseudomonas fluorescens* strains used as control endobiome of tomatillo. In this accord, Rojas-Solis et al. (2016) reported that the combination of *Bacillus thuringiensis* and *P. fluorescens* significantly improved the total fresh weight of tomatillo seedlings and increased hypocotyl and root length. In a similar study, Cueva-Yesquén et al. (2021) reported that the inoculation of *Leclercia adecarboxylata* in Cape gooseberry plants (*Physalis peruviana*) exhibited higher shoot and root lengths (55.4 and 24.5%, respectively), whereas *Priestia megaterium* increased shoot and root lengths (52.7 and 24.5%, respectively) in comparison with the control. In a similar study, Moreno-Velandia et al. (2019) reported that the application of *Bacillus velezensis* on Cape gooseberry plants stimulated their height after 30 days of growth in the nursery.

Even though Mexican tomatillo production is of utmost importance, to date there have not been many studies regarding the application of rhizobacteria as biofertilizers in tomatillo seedlings. The application of effective biofertilizers in tomatillo cultivation can lessen the use of chemical fertilizers leading to reduced costs and detrimental environment effects of the agricultural process. The objective of this study was to isolate and characterize bacteria associated with rhizospheric soils of wild *Physalis* sp. plants, to evaluate its *in vitro* plant growth promoting mechanisms, and their biofertilizing effect on tomatillo seedlings cultivated under greenhouse conditions in terms of agronomic parameters and mineral content in the plant leaves.

3.2. Materials and methods

3.2.1. Isolation of bacteria from wild *Physalis* sp. plant rhizosphere

Through a zig zag sampling, 8 healthy samples of wild plants of *Physalis* sp. in flowering and productivity stages were collected from 8 sites of agricultural plots under

traditional farming systems located in San Juan Mixtepec, Oax., Mexico (17°14'36" N and 97°52'53.2" W and 2229 m.a.s.l altitude). The plants were dug out, and the rhizospheric soil was recovered and homogenized. Subsequently, a soil sample of each plant was processed for bacterial isolation according to the methodology reported by Mohd Nor et al. (2017). Briefly, 1 g of soil sample was added to 9 mL of peptonated water. Subsequently, 100 µL aliquots of the 10^{-4} dilution were inoculated in the following selective agar media for rhizobacteria isolation: Luria Bertani (LB), King B (Angulo et al., 2014), and nitrogen-free NFb agar supplemented with malic acid (Peña-Yam et al., 2016). Petri dishes with LB and NFb media were incubated at 37 °C, whereas those with King B's medium were incubated at 28 °C. All the cultures were incubated for 48 h. The colonies with different morphologies (shape, border, size, color, and appearance) were isolated, and further cultured on the same medium in which they were obtained. All the bacteria isolated were stored in LB broth containing 30% (v/v) glycerol at -20 °C.

3.2.2. Cellular characterization of bacterial isolates

The bacterial isolates were further characterized by their Gram stain and cell morphology. Additionally, the growth curves of the isolates were obtained in LB medium at 37 °C. The bacterial growth was measured at 12 h intervals, from 12 to 96 h, in a Genesys 50 UV–Vis spectrophotometer (Thermoscientific, USA) at 600 nm (OD_{600}), using fresh broth as the blank. Cell concentrations (CFU L^{-1}) were determined for the bacterial strain at their highest growth, by bacterial culturing in LB broth at 37 °C. Serial dilutions were conducted, and the enumeration of the strains was performed in duplicate by cultivation in LB plates at 37 °C.

3.2.3. *In vitro* characterization of biofertilizing mechanisms

Isolated bacterial strains were subjected to four plant growth-promoting mechanisms: ammonia and IAA production, and phosphorus and potassium solubilization.

Ammonia production

The capability of ammonia production *in vitro* by the isolates was determined with the method described by Chandra et al. (2018). Bacterial strains were grown in LB broth for 72

h at a temperature of 37 °C, then 20 µL of the bacterial cell suspension was inoculated in 10 mL of 4% peptone water and incubated at 37 °C for 72 h. After incubation, 0.5 mL of the Nessler reagent was added to each tube. The formation of a yellow-brown precipitate was considered as an indicator of the presence of NH_3 in the culture medium.

Phosphate solubilizing ability

Bacterial isolates were screened for phosphate solubilization using the NBRIP medium (Nautiyal, 1999). The medium was comprised of the following ingredients: glucose, 10 g; $Ca_3 (PO_4)_2$, 5 g; $MgCl_2 \cdot 6H_2O$, 5 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; KCl, 0.2 g; and $(NH_4)_2 SO_4$, 0.1 g (per liter of double distilled water), with a pH 7 before sterilization. Bacterial strains were grown in LB broth at 30 °C by 48 h. Subsequently, a cell suspension (2 µl) of each strain was inoculated in Petri dishes supplied with NBRIP medium. Phosphate solubilizing bacteria were detected by the formation of clear halo zones around colonies caused by the production of organic acids after 7 d of incubation. The solubilization index (SI) was calculated according to the following equation (Ramesh et al., 2014):

$$SI = \frac{Ds + Dc}{Dc};$$

where: Ds is the solubilization diameter; and Dc is the diameter of the colony.

For quantitative analysis, the pure cultures were grown in the NBRIP broth with $Ca_3(PO_4)_2$ added at a concentration of 5 g L^{-1} (Kulimushi et al., 2018). The cultures were grown in 5 mL of the broth incubated at 30 °C for 3 d at 250 rpm. Medium inoculated with double-distilled sterile water was used as the control test. Then, the samples were centrifuged at 9,800 x *g* for 15 min and the supernatant was recovered to quantify the solubilized phosphorous. A colorimetric method was used to measure the amount of dissolved phosphate. One mL of the supernatant was added to 10 mL of chloromolybdic acid (12 mM) and 1 mL of 5 mM tin chloride. This volume was adjusted to 50 mL with double-distilled water. A color changeover of the mixture to blue indicates the presence of soluble phosphates. Estimated amounts solubilized phosphorus was determined by measuring the absorbance at 610 nm in a UV-vis spectrophotometer. A standard calibration curve was conducted using KH_2PO_4 (mg L^{-1}) as the reference standard.

Potassium solubilization assay

For determining potassium solubilization, the isolated bacteria were grown on plates containing the Aleksandrow medium (Glucose, 3.5 g; $MgSO_4.7H_2O$, 0.5 g; $CaCO_3$, 0.1 g; $FeCl_3 \cdot 6H_2O$, 0.0005 g; $Ca_3(PO_4)_2$, 2.0 g; insoluble mica powder as the potassium source, 1.0 g; agar, 15 g; double distilled water, 1 L). The strains were inoculated by mottling a Petri dish, followed by incubation at 30 °C for 15 d (Khanghahi et al., 2018). The formation of a clear halo around the colony was considered as an indicator of potassium solubilization activity. The potassium solubilization efficiency (KE) was calculated according to the following equation:

$$KE = \frac{Ds + Dc}{Dc};$$

where Ds is the solubilization diameter; and Dc is the diameter of the colony.

Auxin activity test

The production of auxin, indole acetic acid (IAA), by the isolates was estimated using a colorimetric technique, in which the isolates were grown in King B's broth supplemented with 1.0% (w/v) L-tryptophan (Glickmann and Dessaux, 1995). The tested strains were incubated for 72 h at 37 °C. The supernatant was collected by centrifugation at 7,840 x *g* for 10 min and 2 mL of the Salkowski reagent (10.8 M H_2SO_4 and 4.5 g of $FeCl_3$ L^{-1}) was added to 1 mL of the supernatant. The mixture was left in the dark for 30 min before the absorbance of the mixtures was measured at 540 nm in a UV-vis spectrophotometer. An IAA (Sigma, USA) standard curve was constructed for the determination of the IAA concentration of the samples (µg mL^{-1}).

3.2.4. Genetic identification and phylogenetic analysis

Phylogenetic analyses of the 16S RNA of six strains, from a total of 30 bacterial isolates, which presented at least two plant growth-promoting mechanisms in the *in vitro* assays were selected for identification. Genomic DNA was extracted from 48 h cultures using the phenol chloroform isoamyl alcohol method (Mauti et al., 2013).

The extracted DNA was amplified using a PCR protocol with the Taq PCR Solution Master Mix Kit (Qiagen, Germany). The primers 27F and 1492R (Macrogen Inc., South Korea) were used for PCR and sequencing. The PCR reaction mixture was prepared according to the manufacturer's instructions and was performed in a Thermal Cycler 2720 (Applied biosystems, USA) according to Ercole et al. (2021). The PCR products were purified with the purification Kit GeneJet^{TM}, #K0702 TC (Fermentas, EUA) following manufacturer's recommendations. The ADNr 16S concentration was quantified using a Genova Nano spectrophotometer (Jenway, UK). The nucleotide sequences were determined through 16S rDNA gene sequencing (Macrogen, Korea). The acquired sequences were compared with the most closely related sequences available in the GenBank database using the BLAST software (NCBI, USA) and in the EzBioCloud 16S database for species identification (Yoon et al., 2017). The 16S sequence of the identified isolates were deposited in GenBank and an accession number was obtained for each one of them. A phylogenetic tree based on the 16S rDNA sequences was constructed by using the software MEGA 11.0 with the Maximum Likelihood method and Bootstrap method with 1000 repetitions (Tamura et al., 2021). DNA sequences containing missing data were eliminated. A sequenced *Metallosphaera sedula* strain was used as an external group, which has a basal position within the phylogenetic tree.

3.2.5. Evaluation of the effect of rhizobacterial inoculants on tomatillo seedlings

Four strains (UTMR1, UTMR2, UTMR3 and UTMR4) were selected from the initial isolates with the highest positive results from the *in vitro* biofertilization assays. To prepare the bacterial inoculants, the strains were cultured in LB broth and incubated at 37 °C in an orbital shaker at 150 rpm. Different incubation times were used to achieve the late exponential phase for each strain according to the culture optical density at 600 nm (OD600). The bacterial suspensions of the selected strains were adjusted to 10^6 or 10^9 CFU mL^{-1} depending on the maximum cell yield of each strain.

Tomatillo seeds (*Physalis ixocarpa*) of the variety "cáscara morada" (Golden Vegetables Seeds, Mexico) were selected for the experiment. The seeds were disinfected with 1% sodium hypochlorite in an orbital shaker at 150 rpm for 15 min. The chlorinated solution was removed from the seeds with three rinses of sterile RO water. The substrate for

tomatillo seedling cultivation was a mixture of peat moss, perlite, and vermiculite in a 3:1:1 ratio. The substrate was moistened and autoclaved at 120 °C for 40 min. The germination trays (200 cavities), previously disinfected with 2% sodium hypochlorite, were filled with the substrate mixture, which was further irrigated at field capacity with sterile water. A singe tomatillo seed was placed in each cavity at an approximate depth of 1-1.5 cm. A row of cavities was left without sowing to avoid agglomeration of plants and to prevent mixing between the treatments. After sowing, irrigation was performed with a sterile atomizer by applying 5 mL of sterile distilled water per cavity. Two bacterial inoculations with 5 mL of the tested strain were applied to the seedlings at 11 and 24 d after sowing. For the blank treatment, 5 mL of sterile distilled water was added to the seedlings instead of the inoculant. No fertilizer or agrochemical was used in this experiment.

The germination trays were kept in the greenhouse of the Universidad Tecnológica de la Mixteca (UTM) at an average temperature of 27 °C, with maximum and minimum average temperatures of 38 and 16 °C. The average relative humidity was 50%, with maximum and minimum of 81 and 19%, respectively. The photosynthetically active radiation (PAR) was measured with a Light Scout® Mod. 3415FSE quantum light meter (Spectrum Technology Inc. USA) between 10:00 and 15:00 h, with an average PAR measurement of 42.1 mol m^{-2} d^{-1}. Sowing of the seeds was conducted on September 8, 2020, and the experiment was completed on October 8, 2020, 30 days after sowing, according to Qin et al. (2017). The experiment consisted of four inoculant treatments (UTMR1, UTMR2, UTMR3, and UTMR4) and a blank, with 20 seedlings per treatment. The experiment was performed in a randomized complete block design with three replications.

The height of the seedlings was measured from the base to the apex, any substrate adhered to the root was first removed and then the main root was measured from the base to the tip. Both measurements were made with a digital caliper (TTC, USA). Fresh and dry weight was measured using an analytical scale (Ohaus, USA). The dry weight of the seedlings was after drying an oven at 60 °C for 48 h. Dry samples were stored in glass vials previously treated with 5% nitric acid for further analysis.

3.2.6. ICP-OES analysis

The content of minerals (P, K, Ca, Na, Mg, Fe, Mn, Cu, and Zn) from samples of the aerial part of the seedlings was quantified using an inductive coupled plasma with optical emission spectroscopy (ICP-OES) equipment (Optima 7000, USA). The aerial part of the tomatillo seedlings was oven-dried at 60 °C for 48 h, followed by particle reduction with a porcelain mortar. The samples were subjected to acid digestion with reflux and 300 mg of the plant sample was weighed on an analytical scale then placed in 25 mL glass bottles. The following reagents were added to each sample: 9 mL of concentrated nitric acid (Baker, USA), 2 mL of 30% hydrogen peroxide, and 1 mL of concentrated hydrochloric acid (Baker, USA). The mixture was heated at a constant temperature of 180 °C, until complete digestion of the plant material. The digest was filtered through a Whatman No. 40 filter paper into a 25 mL volumetric flask, which was filled with ultrapure water. The digested samples were emptied into high-density polypropylene bottles, that had been previously rinsed in a 5% nitric acid solution and dried in an oven at 65 °C for 24 h. The samples were kept refrigerated for further analysis (González-Terreros et al., 2018).

Elemental analysis was conducted using a Spectroblue instrument that was equipped with the Spectro Smart Analyzer program for data processing and was used for ICP-OES analysis. The operation condition of the ICP-OES was: power 1300 W; flow rate of sample 1.5 mL min^{-1}; and gas flow rates: plasma 15 mL min^{-1}; auxiliary 0.2 mL min^{-1}; nebulizer, 0.8 mL min^{-1}. The analysis optical was adjusted with manganese at 1 mg L^{-1} to axial view (for low mineral concentration samples) and 10 mg L^{-1} to radial view (for high mineral concentration samples). The main operating parameters of the ICP-OES equipment and limits of detection and background equivalent concentration (BEC) for elements are given in the Table 3 and 4. The calibration curves for the elements (P, K, Ca, Na, Mg, Fe, Mn, Cu, and Zn), were obtained using individual standard solutions (Perkin Elmer, USA). The lineal correlation coefficient was 0.9999 for each element. The results from the measurements are presented as the mean value ± standard deviation and expressed as mg kg^{-1} of dry weight.

Table 3. The main operating parameters of the ICP-OES spectrometer.

Instrument	**SPECTROMETER-ICP-OES**

	Model: OPTIMA 7000
RF power	1400 W
Auxiliary Ar flow	0.2 L/min
Nebulizer flow	0.8 L/min
Plasma flow	15.00 lL/min
Sample introduction system	Manual through peristaltic pump
sample flow rate	1.5 mL/min
Washing time between samples (Prefulsh)	60 sec
Plasma view	Axial/Radial
System rinsing solution	HNO_3, *v*/*v* = 2 %
Standard Definition	Blank (0 ppm); Std Na y Mg 0.005, 0.25, 0,5, 0.75, 1, 1.5, 2.0, 3.0, 4.0, 5.0 ppm; Std S, P 0.005, 0.02, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20 ppm; Std Cu, Zn, Fe, Mn , 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2, 5, 10, 20, ppm; Std K, 0.1, 0.5, 1, 1.5, 2, 5, 10, 20, 50, 100 ppm Std Ca 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 10 ppm
Element Wavelengths	Cu 327.393 Zn 206.200 Fe 238.204 Mn 257.610 Na 589.592 K 766.490 Ca 317.933 Mg 285.213 P 213.617

Table 4. Method limits of detection (LOD) and limits of quantification in the background equivalent concentration (BEC) for elements.

Element	LOD	BEC
	(mg/L)	
Ca	0.0100	0.33
Cu	0.0097	0.32
Fe	0.0046	0.15
K	-	7.10
Mg	0.0016	0.05
Mn	0.0014	0.05
Na	0.0690	2.33
P	0.0760	2.56
Zn	0.0059	0.2

3.2.7. Statistical analysis

To check the normality of data, the Shapiro-Wilk and Kolmogorov-Smirnov normality tests were performed considering a 95% confidence level. An analysis of variance (ANOVA) was performed to determine the significant differences among the treatments. For all the statistical analysis carried out in this study, a comparison was made using Tukey's honestly significant difference test ($p < 0.05$). SAS v.9.00 (SAS 2002) statistical software (SAS Institute Inc., USA) was used.

3.3 Results

3.3.1. *In vitro* characterization of the isolates biofertilizer potential

Six bacterial strains were successfully isolated from the rhizosphere of wild tomatillo plants, and their cellular morphology, growth rate, and four plant growth-promoting mechanisms were further characterized. The morphological characteristics, Gram staining, and maximum cell concentration of the isolates are detailed in Table 5.

Table 5. Characterization molecular of bacterial isolates.

Strain	Size	Color	Shape	Border	Elevation	Gram stain	Time of greatest growth (h)	UFC mL^{-1}
UTMR1	S	Y	Pointed	Wavy	Flat	+	48	1.5×10^9
UTMR2	M	W	Rounded	Wavy	Convex	-	72	8.0×10^8
UTMR3	L	W	Irregular	Whole	Convex	+	72	1.9×10^6
UTMR4	M	W	Pointed	Whole	Elevated	-	72	3.7×10^8
UTMR5	L	W	Rounded	Wavy	Convex	+	48	2.0×10^6
UTMR6	S	Y	Pointed	Whole	Elevated	+	48	1.2×10^8

Size: small (S), medium (M), and large (L); color: white (W), and yellow (Y).

Ammonia production

The ammonia production test resulted in three isolates forming a yellow precipitate when the Nessler reagent was added, thus confirming ammonia production. The strain UTMR4 presented the highest precipitation intensity.

Phosphate and potassium solubilizing activity

The formation of a clear halo around the bacterial colony in the phosphate-solubilizing assay was indicative of phosphorus solubilization (Figure 8). As a result of the experiment, 6 isolates tested positive for phosphorus solubilization. The halo index of the bacterial strains presenting some solubilization activity was measured (Table 6).

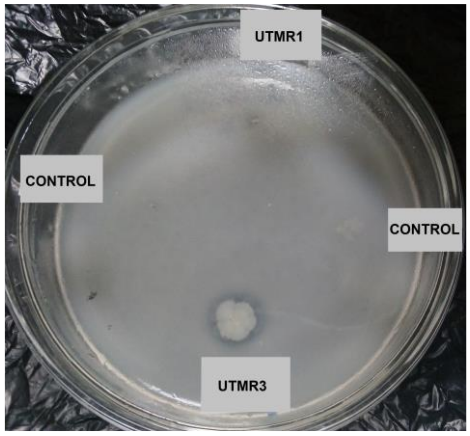

Figure 8. Example of the clear halo in solid NBRIP medium showing phosphorus solubilization by bacterial strain UTMR3.

Table 6. Analysis of biofertilization activity of rhizobacterial isolates.

Strain	Index of P solubilization (mm)	Solubilized P (mg L^{-1})	IAA production (µg mL^{-1})
UTMR1	1.2 ± 0.03 [b]	1.0 ± 0.02 [b]	ND
UTMR2	2.1 ± 0.27 [b]	1.0 ± 0.05 [b]	4.5 [b]
UTMR3	1.7 ± 0.61 [b]	0.2 ± 0.03 [c]	3.1 [b]
UTMR4	4.9 ± 0.48 [a]	5.0 ± 0.12 [a]	34.8 [a]
UTMR5	1.8 ± 0.15 [b]	1.0 ± 0.06 [b]	ND
UTMR6	1.4 ± 0.1 [b]	ND	ND

Notes: [*]Mean with different letters are significantly different at $p < 0.05$ according to the HSD Tukey test procedure (n = 3).
Mean +/- standard deviation.
ND = Not detected.

The strain UTMR4 showed the largest size of the halo index and was statistically superior to the other isolates ($p < 0.05$). The quantification of solubilized phosphorus by the colorimetric assay showed that the bacterial strains with the highest solubilization activity were UTMR4 and UTMR2. However, the UTMR4 strain was statistically superior in relation to the other strains tested. Potassium-solubilizing activity, among all the bacterial strains resulted in UTMR4 being the only strain with activity when tested on semi-solid culture media, with a solubilization index of 1.7 ± 0.1.

Production of auxin

Only three isolates, UTMR4, UTMR2, and UTMR3, caused IAA production. The auxin concentrations determined for these bacteria were 34.8, 4.5, and 3.1 μg mL^{-1} of IAA, respectively. The isolated strain UTMR4 showed the highest IAA production ($p < 0.05$).

Overall biofertilization results

All the strains reported in this work presented activity in at least two of the biofertilization mechanisms evaluated (Table 7). However, the strains UTMR2 and UTMR4 had more biofertilization mechanisms than the others. In addition, the latter presented higher phosphate solubilization and IAA production than all the other strains.

Table 7. Summary of the biofertilizing activities presented by the rhizobacterial isolates from wild tomatillo plants.

Strain	Ammonia production	Phosphorus solubilization	Potassium solubilization	IAA production
UTMR1	+	+	-	-
UTMR2	+	+	-	+
UTMR3	-	+	-	+
UTMR4	++	++	+	++
UTMR5	-	+	-	-
UTMR6	-	+	-	-

Note: (++) indicates a strong effect, (+) indicates a medium effect, and (-) indicates a null effect.

3.3.2. Molecular identification and characterization of rhizospheric isolates

Based on the molecular characterization of the strains by the 16S rRNA gene sequencing, the isolates were identified as listed in Table 8. The phylogenetic tree of the putative growth promoting bacterial strains is shown in Figure 9.

Table 8. Identification of bacteria strains isolated from rhizospheric soil of wild *Physalis* sp. by 16S rRNA according to database available in GenBank-NCBI using the BLAST software and EzBioCloud.

Strain	Sequence size (pb)	Nearest bacterial species	Accession number GenBank (reference strain)	Identity (%)	Accession number GenBank (isolated strain)
UTMR1	1531	*Cellulosimicrobium cellulans*	CAOI01000359	99.9	OL589187
UTMR2	1543	*Acinetobacter calcoaceticus*	AIEC01000170	100	OL589188
UTMR3	1549	*Priestia megaterium*	JJMH01000057	99.9	OL589193
UTMR4	1547	*Atlantibacter* sp.	JN175345	98.5	OM112278
UTMR5	1409	*Priestia aryabhattai*	EF114313	100	OL589198
UTMR6	1280	*Arthrobacter koreensis*	AY116496	99.5	OL589203

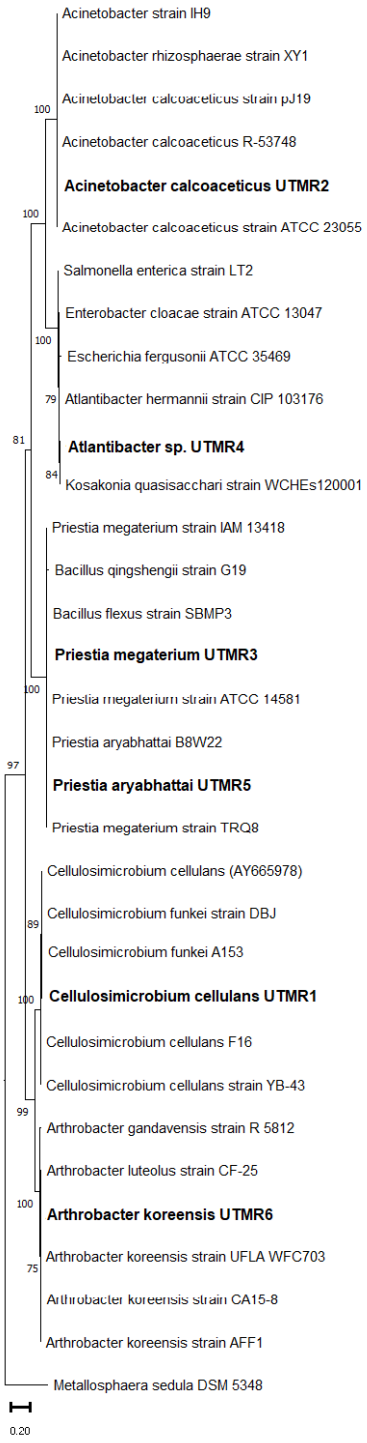

Figure 9. Phylogenetic tree based on partial 16S rRNA gene sequences of the isolate strains from wild tomatillo plants and reference sequences from EzBioCloud. Sequences were aligned using MEGA 11.0 and the tree was constructed using the Maximum Likelihood method. Bootstrap values based on 1000 replications are indicated at branch points.

3.3.3. Effect of rhizobacteria on the growth promotion of tomatillo seedlings

Based on the *in vitro* results, UTMR1, UTMR2, UTMR3, and UTMR4 were further studied for their biofertilization activity in tomatillo seedlings. Four days after the first inoculation, seedlings inoculated with bacterial treatments presented greater growth than the control plants and presented their first true leaves. At the end of the experiment (day 30) the seedlings treated with bacterial inoculants had larger leaf areas than those of the control treatment (Figure 10). Also, the leaves of the tomatillo seedlings treated with inoculants presented between three and four true leaves that were large and well developed, while the untreated control seedlings had between two and three leaves that were smaller underdeveloped.

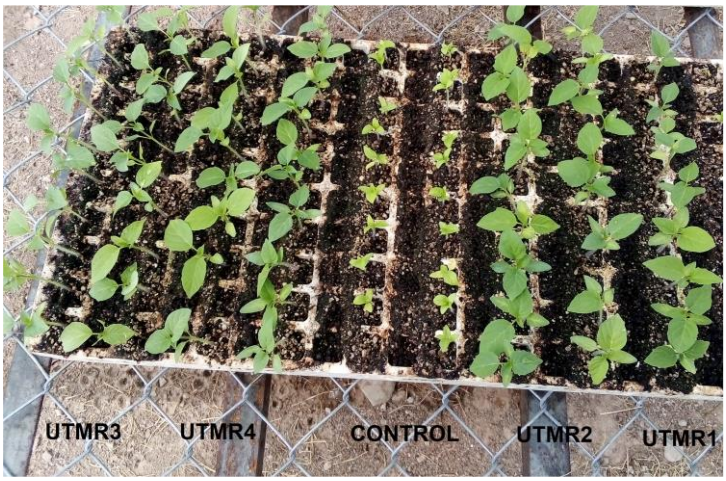

Figure 10. Tomatillo seedlings at 30 days-old from sowing grown in germination trays with different rhizobacterial inoculation treatments and the control.
Treatments: UTMR3: *Priestia megaterium*; UTMR4: *Atlantibacter* sp.; UTMR2: *Acinetobacter calcoaceticus*; UTMR1: *Cellulosimicrobium cellulans*; CONTROL: No treatment.

All the seedlings treated with bacterial inoculants presented greater growth than those belonging to the control group (Table 9). Also, the treatments inoculated with the strains *P. megaterium*, *Atlantibacter* sp., and *Acinetobacter calcoaceticus* presented a greater plant growth with average height increases of 142, 123, and 112%, respectively. The tomatillo seedlings inoculated with *P. megaterium*, *A. calcoaceticus*, and *Atlantibacter* sp. had an increase in the dry weight of 413, 407, and 362% compared to the control group. In addition, all the inoculants caused a remarkable increment in the dry-weight of the

seedling´s root from 413 to 573%; furthermore, three of the treatments (*C. cellulans*, *A. calcoaceticus* and *P. megaterium*) considerably increased the seedling´s root length from 11 to 15%, as compared to the control treatment.

Additionally, the inoculated seedlings showed a larger quantity of secondary roots and root hairs and a greater adherence of the substrate to the roots of the inoculated seedlings (Figure 11).

Table 9. Effect of rhizobacterial-strains inoculation on the growth parameters of tomatillo seedlings 30 days after sowing.

Treatment	Seedlings height (cm)	Foliar weight		Root	
		Fresh (mg)	Dry (mg)	Length (cm)	Dry weight (mg)
C. cellulans UTMR1	4.9 ± 0.0 [b]	395.0 ± 10.0 [a]	40.0 ± 2.0 [a]	8.9 ± 0.4 [a]	34.4 ± 5.0 [a]
A. calcoaceticus UTMR2	5.5 ± 0.4 [ab]	425.0 ± 15.0 [a]	45.1 ± 3.6 [a]	8.7 ± 0.4 [a]	42.1 ± 6.1 [a]
P. megaterium UTMR3	6.0 ± 0.4 [a]	458.3 ± 27.5 [a]	45.7 ± 3.0 [a]	9.0 ± 0.3 [a]	38.8 ± 2.0 [a]
Atlantibacter sp. UTMR4	5.3 ± 0.4 [ab]	418.3 ± 57.1 [a]	41.1 ± 3.4 [a]	8.7 ± 0.2 [b]	45.1 ± 6.1 [a]
Control	2.5 ± 0.3 [c]	173.3 ± 2.9 [b]	8.9 ± 1.3 [b]	7.8 ± 0.2 [b]	6.7 ± 0.5 [b]

Notes: [*]Mean with different letters are significantly different at $p < 0.05$ according to the HSD Tukey test procedure (n = 3).
Mean +/- standard deviation.

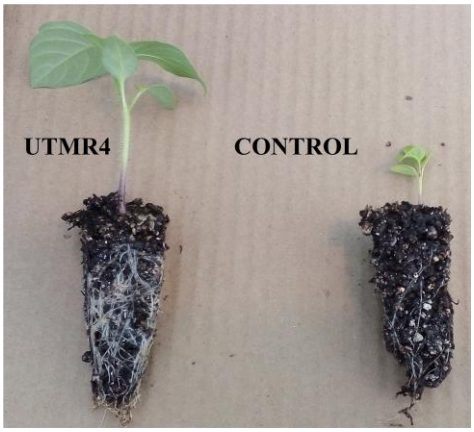

Figure 11. Example of the root-growth comparison between tomatillo seedlings inoculated with rhizobacteria and the control. Treatment UTMR4 (*Atlantibacter* sp.); CONTROL: No treatment.

3.3.4. Mineral content in tomatillo seedlings

The treatments inoculated with *P. megaterium* and *A. calcoaceticus* had significantly higher concentrations of K (54 and 37%), of Ca (87 and 80%), and of Mg (89 and 81%), compared to the other treatments. All the bacterial treatments showed an increase in Mn concentration from 87 to 142% while compared to the control, but there was no significant difference between them. However, there was no difference in P and Na contents between the rhizobacterial treatments and the control. Even though the presence of low Fe concentrations was measured in the treatments inoculated with *P. megaterium* UTMR3, *A. calcoaceticus* UTMR2, and *C. cellulans* UTMR1, no significant differences were estimated for these values compared to the control group, where the presence of Fe was not detected. Finally, the minerals Cu and Zn were not detected in any of the treatments, including the control (Table 10).

Table 10. Mineral content in tomatillo seedlings inoculated with bacterial strains.

Strains	Essential minerals (mg kg^{-1} sample)								
	P	K	Ca	Mg	Na	Cu	Zn	Fe	Mn
C. cellulans	71.2 ± 15.9 [a]	1951.5 ± 144.2 [b]	30.0 ± 2.0 [b]	427.0 ± 30.4 [a]	95.2 ± 15.9 [a]	ND	ND	0.2 ± 0.3 [a]	2.1 ± 0.2 [a]
A. calcoaceticus	104.7 ± 23.9 [a]	1986.8 ± 37.9 [ab]	35.5 ± 1.5 [a]	445.7 ± 55.7 [a]	95.4 ± 14.9 [a]	ND	ND	0.3 ± 0.5 [a]	2.1 ± 0.2 [a]
P. megaterium	75.9 ± 6.3 [a]	2229.3 ± 9.1[a]	37.0 ± 1.4 [a]	466.3 ± 47.5 [a]	102.6 ± 15.6 [a]	ND	ND	2.2 ± 3.3 [a]	2.1 ± 0.3 [a]
Atlantibacter sp.	77.1 ± 5.6 [a]	1809.8 ± 145.5 [b]	34.6 ± 1.7 [a]	286.5 ± 46.8 [b]	86.1 ± 26.6 [a]	ND	ND	0.0 [a]	1.6 ± 0.3 [a]
Control	106.0 ± 10.6 [a]	1450.8 ± 94.1 [c]	19.7 ± 0.8 [c]	246.7 ± 9.7 [b]	126.0 ± 44.7 [a]	ND	ND	0.0 [a]	0.9 ± 0.1 [b]

Notes: [*]Mean with different letters are significantly different at $p < 0.05$ according to the HSD Tukey test procedure (n = 3).
Mean +/- standard deviation
ND = Not detected

3.4. Discussion

3.4.1. Biofertilizing mechanisms *in vitro*

In this study, 6 bacterial strains were isolated from rhizospheric soil of native *Physalis* sp. plants. The strains were identified through the 16S rRNA sequence analysis as belonging to the genera *Priestia*, *Acinetobacter*, *Atlantibacter*, *Cellulosimicrobium*, and *Arthrobacter*. The identification of our bacterial isolates was performed using a cured database of genomic bacteria strains EzBioCloud, in which 16S rRNA sequences of "type" strains are used, which are mostly based on complete genome sequences. In addition, every nucleotide difference (relative to the closest type strain) in the Sanger chromatogram was verified. Besides this, considering the phylogenetic tree it was corroborated the consistency of the proposed identification by using the genetic database described above. Furthermore, the quality of the 16S rRNA sequences for the studied isolates was very good in terms of quality and full-length sequence data. Considering this, from the analysis, the strain UTMR4 (*Atlantibacter* sp.) was identified only to the genus level, since the 16S only shared 98.5% sequence identity with the closest type strain.

Atlantibacter sp. UTMR4 and *Acinetobacter calcoaceticus* UTMR2 had greater capacity of phosphorus solubilization than the other isolates. Particularly, *Atlantibacter* sp. UTMR4 had a P solubilization index of 4.9, which was higher than the values for *Acinetobacter calcoaceticus* and A*cinetobacter* sp. in Pikovskaya's (PVK) agar, reported by Moreno-Ramirez et al. (2015) and *Pseudomonas aeruginosa*, *P. baetica*, and *P. jessenii* in NBRIP medium, reported by Ogata-Gutiérrez et al. (2017). In the quantitative assay, *Atlantibacter* sp. UTMR4 and *A. calcoaceticus* UTMR2 presented the highest solubilization capacity with 4.96 and 1.05 mg L^{-1} of solubilized phosphorus. These results were higher than the values reported by Ji et al. (2014) (0.3 and 3.3 mg L^{-1}) for *Paenibacillus kribbensis*, *Bacillus* (*aryabhattai*, *megaterium*, and *subtilis*), *Klebsiella pneumonia*, and *Microbacterium trichotecenolyticum* isolated from rice, but were lower than those reported by Li et al. (2020) (131.26 and 438.35 mg mL^{-1}) for *Trifolium pretense* and *Polygonum viviparum* (*Providencia rettgeri*, *Advenella incenata Acinetobacter calcoaceticus*, and *Serratia plymuthica).*

IAA is the most abundant and important auxin in plants (Simon and Petrášek, 2011) which can be secreted by bacteria and promote root growth by directly stimulating cell division

(Raddadi et al., 2008). In the *in vitro* tests, the strain *Atlantibacter* sp. UTMR4 produced the highest amount of IAA with 34.8 µg mL^{-1}, while *A. calcoaceticus* UTMR2 and *P. megaterium* UTMR3 produced 4.5 and 3.1 µg mL^{-1}. The IAA production by *Atlantibacter* sp. UTMR4 was greater than the values reported for endophytic-bacteria from rice cultivars *Paenibacillus kribbensis, Microbacterium binotii, M. trichotecenolyticum, Priestia aryabhattai, P. megaterium*, and *Klebsiella pneumoniae* with IAA production in the range from 3.1 to 24.6 µg mL^{-1} (Ji et al., 2014). Likewise, IAA production was greater than Chandra et al. (2018) reported IAA production of 7.2 to 18.2 µg mL^{-1} for bacterial isolates from sugarcane rhizosphere (*Bacillus subtilis* and *P. megaterium*). However, these results are lower than those reported by Zhang et al. (2017) for three strains of *Priestia megaterium* isolated from waste mushroom with IAA production ranging from 50.9 to 62.4 µg mL^{-1}. All of this indicates *Atlantibacter* sp. UTMR4 has the potential to promote plant growth through the secretion of phytohormones.

The phosphorus solubilizing activity and ammonia and IAA production presented by *Atlantibacter* sp. UTMR4, *A. calcoaceticus* UTMR2, and *P. megaterium* UTMR3 make them candidates as potential biofertilizing inoculants. These results are in agreement with other studies, such as that of Li et al. (2020) who reported that the strain *A. calcoaceticus*, isolated from the rhizospheric soil of red clover (*Trifolium pretense*)**,** increased the available nutrient content by phosphorus solubilization and nitrogen fixation, thus favoring the growth of *Avena sativa*, *Medicago sativa* and *Cucumis sativus* seedlings. In addition, Khanghahi et al. (2021) reported that bacterial strains of the *Acinetobacter* genus were capable of solubilizing inorganic P, K, and Zn, as well as fixing nitrogen. Foughalia et al. (2022) reported that an *A. calcoaceticus* strain, isolated from the rhizospheric soil of tomato plants (*Solanum lycopersicum*), provided high levels of protection (72.1%) against *Botrytis cinerea* in tomato plants and increased the length and fresh weight of shoots and roots, as well as stem diameter, and leaf number in comparison to untreated plants. The evaluation of the biofertilizing effect of *A. calcoaceticus* at the plant and fruit-production level is required to be able to propose its use as an efficient biofertilizer.

Atlantibacter strains have been isolated from alfalfa crop associated with different parts of the plant, establishing beneficial interactions that allow plant survival and development in saline conditions (Muñoz et al., 2020). In addition, strains of this genus have been

considered as endophytic bacteria which have shown growth-promotion potential as indole compound-producers (Belincanta et al., 2021). Likewise, the potential biofertilizing *P. megaterium* has been reported by Wang et al. (2021) capable of induced auxin biosynthesis while regulating auxin redistribution in *Arabidopsis* shoots and roots, thus stimulating plant growth and lateral root initiation. In a similar study, Chu et al. (2018) reported that *P. megaterium* colonized the meristematic areas and intervened in the elongation of the root tip and the middle segment of the root of maize seedlings, which resulted in a significant improvement in plant growth.

3.4.2. Growth of tomatillo seedlings

In general, in the *in vivo* tests, the inoculated tomatillo seedlings presented a significantly greater growth in terms of height, weight, and root length with respect to the control. It is probable that some of the mechanisms studied in the *in vitro* tests, alone or in combination, induced seedling growth promotion with IAA bacterial synthesis possibly playing a key factor. Agarwal et al. (2019) demonstrated the expression of gene OsASR6, induced by bacterial inoculants in roots of rice, has a multifaceted role where it improves root growth (branching and length), alters xylem structure, and physiological responses in transgenic plants. OsASR6 is activated by auxin and increases auxin responses and root auxin sensitivity. In a similar work, Ngo et al. (2019) reported an increase in the plant height and the shoot fresh weight of rice seedlings caused by *B. pumilus*. This was caused by an increase in nutrient and water uptake by the plant, therefore inducing acceleration of shoot development during the plant's early growth. In this regard, we observed that the rhizobacterial inoculants induced greater formation of secondary roots and root hairs while also enhancing the growth of the main root (Figure 4). The improvement in plant root development provides the roots with a greater contact surface with the rhizosphere for enhanced absorption of water and nutrients, thus enabling the plant to produce greater aerial biomass.

3.4.3 Mineral content

In the present study, the P content in the tomatillo seedlings treated with the rhizobacterial inoculants did not show significant differences as compared to the control. These results

can be explained by considering P is probably not a limiting nutrient for tomatillo. In this regard, Wu et al. (2019) reported *Priestia aryabhattai* and *Pseudomonas auricularis* increased the P content in the seedlings leaves of *Camellia oleifera* in the presence of low and medium P levels, but not in the presence of high P concentrations. Zhang et al. (2021) reported the strain *Burkholderia* sp. improved the growth of tomato seedlings under cadmium stress and the contents of N and K in inoculated tomato roots were higher than those of the control. However, the P content of the inoculated treatments was similar to the latter. In a similar study, Mendes and Rigobelo (2021) reported sugarcane plants inoculated with *Staphylococcus saprophyticus* and *Enterobacter* sp. did not show differences of P content in the shoot and the root compared to the control.

This study discovered the inoculation with *P. megaterium* UTMR3, *A. calcoaceticus* UTMR2, *C. cellulans* UTMR1, and *Atlantibacter* sp. UTMR4 rhizobacterial strains increased the K content in the aerial part of the tomatillo seedlings by 54, 37, 34.5, and 24.7% respectively, compared to the control. These results appear to be related to a greater bioavailability of this element by the action of rhizobacterial inoculants which caused greater K absorption by the plant roots. Qin et al. (2017) reported *Bacillus amyloliquefaciens* favored the presence of species of the genus *Agrobacterium* which helped to increase K availability in cucumber seedlings. The authors also reported the biofertilization treatment resulted in an increase in the leaf area and shoot height of the plants.

The Ca contents in the tomatillo seedlings of all treatments with *P. megaterium* UTMR3, *A. calcoaceticus* UTMR2, *Atlantibacter* sp. UTMR4, and *C. cellulans* UTMR1 were higher than the control at 88, 80, 76 and 52%. It is possible Ca improved assimilation and contributed to the higher growth of the inoculated plants. This element contributes to the formation and growth of the plant root system because this nutrient is essential in the synthesis of new cells of the root meristematic region (Galindo et al., 2020). In addition, our study showed the Mg content in all inoculated treatments was higher than the control seedlings. Guo et al. (2016) reported Mg is an essential nutrient in plants for the conformational stabilization of macromolecules such as nucleic acids, proteins, as well as wall, and cell membrane. In addition, Mg is involved in protein synthesis and associated with the chlorophyll by acting as a cofactor in the photosynthetic process, hence it is an

essential micronutrient for plant growth. Our results also showed higher Mn content observed in the tomatillo seedlings in all the treatments compared to the control. In accordance with this, Bhatt and Maheshwari (2020) reported that *Priestia megaterium* increased the contents of Ca, Mg, P, K, Fe and Zn in chilli (*Capsicum annum*) as compared to the control. Similarly, Ijaz et al. (2021) reported that a strain of *Bacillus* sp. (a genus closely related to *Priestia*) increased Mn uptake in the roots and shoots of maize seedlings through bacterial solubilization.

Finally, regarding the absence of Zn, Fe and Cu in the samples, it is known that these are essential elements for plant growth; however, the concentrations required by the crop are very low: Fe, 12.2-13.4; Zn, 4.4-4.9; and Cu, 0.9-1.0 µmol L^{-1} (Neocleous et al., 2020). The substrate used in the experiment was a mixture of peat moss, perlite and vermiculite that contain very low or no concentrations of these microelements. On the other hand, since the study was conducted at the seedling stage, the nutritional requirements were most probably lower. In addition to this, the detection limits of the ICP-OES equipment ranged from 0.1 to 20 ppm, so it is possible that the concentration of the elements tested could not be detected by the instrument.

3.5. Conclusion

30 bacterial strains was isolated from rhizospheric soil of wild *Physalis* sp. plants, which six bacteria were characterized for their potential use as biofertilizing inoculants in tomatillo. The strain *Atlantibacter* sp. presented activity in the four *in vitro* biofertilization mechanisms evaluated: ammonia production, phosphorus and potassium solubilization, and IAA synthesis. All four rhizospheric strains (*Cellulosimicrobium cellulans* UTMR1, *P. megaterium* UTMR3, *A. calcoaceticus* UTMR2, and *Atlantibacter* sp. UTMR4) evaluated *in vivo* had the ability to improve the plant growth parameters of tomatillo seedlings as well as mineral concentration (K, Ca, Mg, and Mn) in the leaves. These results indicate that all 4 rhizobacteria possess high potential as biofertilizers, with the strains *P. megaterium* and *A. calcoaceticus* presenting a superior performance in the seedling experiments. Additionally, this research constitutes one of the first studies reporting the biofertilizing potential on seedlings of the strain *Atlantibacter* sp.

The results obtained in this study were published in Scientia Horticulture 308 (2023) 111567.

3.6. Perspectives

Evaluation of the effect of rhizobacteria in the plant growth, quality and mineral content in tomatillo fruits under greenhouse conditions is a study that could be of great interest.

CHAPTER IV

4. Combined effect of the potassium dose and of plant biofertilization by *Acinetobacter calcoaceticus* on the growth, mineral content and nutritional quality of tomatillo fruits (*Physalis ixocarpa* Brot).

4.1. Introduction

Tomatillo, husk tomato or green tomato (*Physalis ixocarpa* Brot.) is one of the most cultivated vegetables in the American Continent due to its highly appreciated sensorial properties and its high nutritional value, containing 8.24 °Brix, 0.75-1.06 % protein, 1.12-2.10 % fat and 0.77-1.42 % ash (Shenstone et al., 2020). Tomatillo fruits are mainly rich in potassium; and they are also a rich source of Mg, Ca, Na, P and bioactive compounds (González-Pérez and Guerrero-Beltrán, 2021). In 2021, the cultivated area of tomatillo crop in Mexico was about 42,673 ha which produced about 824,977 tons (SIAP, 2022).

To increase tomatillo plant´s growth and productivity it is necessary to improve soil quality by the addition of essential nutrients to the plants (Ali et al., 2021). For this reason, it is known that a large number of chemical fertilizers have been used intensively despite the fact that the extensive use of these chemicals has negative effects on the environment and on soil's sustainability. The use of rhizobacterial biofertilizers, constitutes an alternative to reduce the use of chemical fertilizers aiming at developing sustainable crop cultivation systems.

The beneficial effects of rhizobacteria towards crop production have been attributed mainly to a number of mechanisms for nutrient assimilation and chemical signaling. Direct biofertilization mechanisms improve nutrient´s availability and uptake by the plant, which have a direct effect on plant´s growth. The main direct mechanisms include the following: N_2 fixation; P, K and Zn solubilization; and production of siderophores and phytohormones. On the other hand, indirect mechanisms (ISR, ACC deaminase) improve the tolerance of the plants against stress factors, which can be both biotic (pathogens) and abiotic (drought, salinity and heavy metal toxicity) (Shah et al., 2021).

The improvements in plant´s growth, crop yield and quality of a wide range of fruits crops with the use of rhizobacterial inoculants have been widely documented in the literature (Shah et al., 2021). A few studies in the literature have reported the effect of rhizobacterial application in tomatillo crop cultivation (*Physalis ixocarpa* Brot) on the growth parameters

of seedlings and plants. Rojas-Solis et al. (2016) reported that the application of *Pseudomonas fluorescens* strains alone and in combination with *Bacillus thuringiensis* resulted in significant beneficial effects on development of tomatillo seedlings, in comparison to the non-inoculated control (fresh weight, hypocotyl, and root length). In a similar study, Hernández-Pacheco et al. (2021) reported that the rhizobacterial consortium comprised by *Microbacterium oxydans*, *Stenotrophomonas maltophilia*, *Bacillus toyonensis*, *Microbacterium foliorum*, *Leifsonia shinshuensis*, and *Neobacillus drentensis* increased the length of the primary and lateral roots, the fresh weight of root and stem, and the total weight of tomatillo plants (*Physalis ixocarpa*) in comparison to the non-inoculated treatment. Similarly, in our previous work on tomatillo biofertilization (2023), we reported that the putative biofertilizer strains *Atlantibacter* sp., *Priestia megaterium* and *Acinetobacter calcoaceticus* increased the leaf dry weight (>349%), root length (>11%), root dry weight (>479%) and plant height (>140%) of tomatillo seedlings in comparison to the non-inoculated control. In addition, it was reported that the strain *A. calcoaceticus* increased the concentration of 3 minerals: K (37%), Ca (80%) and Mg (81%), in comparison to the non-inoculated seedlings. Considering these results, in the present work it was decided to continue the study of the biofertilization effect of the *A. calcoaceticus* strain on tomatillo fruit.

The strain *A. calcoaceticus* is a species found in natural places such as soil, fresh water, sediments, and contaminated areas (Pirog et al., 2021). This species has been reported as a phosphorus, potassium, and zinc solubilizer as well as nitrogen fixer (khanghahi et al. 2021). Yamakawa et al., (2018) reported that *Acinetobacter calcoaceticus* increased growth of duckweed (*Lema minor*) from 1.5 to 2-fold compared to the non-inoculated control. In addition, the co-inoculation of this species with *Pseudomonas* sp. increased 2.3-fold the growth of the plant. Similarly, Sadiq and Ali (2013) observed that *A. calcoaceticus* significantly increased the shoot and root length, and the roots/plant in wheat (*Triticum sativum*) in comparison to the non-inoculated treatment, whereas no effect was observed on the growth and yield of *T. aestivum* at full maturity. In another study, Foughalia et al., (2022) reported that the root and shoot length (99 and 43%, respectively), the root and shoot fresh weight (69 and 102%, respectively), and the number of leaves of tomato seedlings were significantly enhanced by the application of *A. calcoaceticus* compared to

the untreated. In similar study, Li et al. (2020) reported that the application of a rhizobacterial consortium comprised by *Providencia rettgeri*, *Advenella incenata*, *A. calcoaceticus* and *Serratia plymuthica* significantly increased the dry weight, plant height, root length, and root surface area of oat (*Avena sativa*), alfalfa (*Medicago sativa*), and cucumber (*Cucumis sativus*) seedlings.

To date, there are not reports on the effect of rhizobacteria over the yield and the nutritional quality of tomatillo fruits. In contrast, due to the worldwide utilization of tomato crop, several biofertilization studies of this vegetable have been reported in the literature. In a study, Yagmur and Gunes (2021) reported that the inoculants *Bacillus megaterium*, *Paenibacillus polymxa*, *Azospirillum* sp., and *Burkholderia cepacia* increased tomato crop yield: *Azospirillum* sp. increased 17% in root application, *Paenibacillus polymxa* increased 10% in soil application, and *Bacillus megaterium* increased 28% in leaf application in comparison to the control group. In another work, Lee et al. (2022) reported that the application of *Rhodopseudomonas palustris* increased the vitamin C (3x), lycopene (19%) and total phenolic compounds (16%) compared to the non-inoculated control. In a similar study, Katsenios et al (2021) evaluated the biofertilization effect of 9 rhizobacterial strains on the cultivation of industrial tomato. The application of *B. subtilis*, *B. amyloliquefaciens*, *Priestia megaterium*, and *B. licheniformis* increased the mean fruit weight per plant; with the latter also causing an increase in yield per plant. In terms of the nutritional quality of the fruits, *B. pumilus* increased the total soluble solids and *P. megaterium* improved the contents of lycopene and total carotenoids, with most of the bacterial strains causing an increase in the antioxidant activity of the fruit. Ochoa et al. (2016) evaluated the effect of the rhizobacterial inoculant *B. paralicheniformis* on the nutritional quality of tomato fruits at different doses of N fertilization. The authors reported that the bacterial inoculant had a positive effect on the synthesis of flavonoids by the plant at a 75% of the recommended nitrogen dose. The biofertilizer mainly contributed to the vitamin C and lycopene contents in the fruit, while the content of antioxidants compounds was affected by the interaction between the N fertilizing dose and biofertilizer inoculation. Similarly, Filiz et al. (2022) studied the effect of three phosphorus doses compared with different commercial biofertilizers in bean plants. The biofertilizer (*Bacillus subtilis*, *Bacillus megaterium* and

Loctococcus spp.) positively affected the grain yield, but the increment in P doses showed the best results in the yield parameters.

Considering the above, the objective of this work was to evaluate the effect of the application of the rhizobacteria *Acinetobacter calcoaceticuos* at different doses of potassium on the plant agronomic parameters, as well as on the fruit quality traits and mineral content on tomatillo plants (*Physalis ixocarpa* Brot).

4.2. Materials and methods

4.2.1. Experimental site and design

To perform the agronomical experiment, a macro tunnel was established at the village of San José de La Pradera located in the municipality of Santa Cruz Tacache de Mina, Oaxaca, México (17°47'51" North and 98°9'2" West at 1112 m.a.s.l.). In this study, certified tomatillo seeds of *Physalis ixocarpa* Brot cv. "cascara morada" were used as the crop cultivar. Tomatillo plants were transplanted on 15 August 2021, and harvested two times 60 and 70 days after transplanting (DAT). The mean, maximum, and minimum daily temperatures and relative humidities were measured with a HOBO Pro v2 temp/RH meter. The experiment followed a completely randomized design with 8 treatments (Table 11). Four potassium doses (0, 50, 75, 100%) were used as related to the recommended fertilization dose using Steiner's universal nutrient solution (Meneses-Lazo et al. 2020), which is a universal nutrient solution used for a wide variety of greenhouse crops. The macronutrients doses recommended are the following (meq L^{-1}): K^{+}, 7.0; Ca^{+2}, 9.0 Mg^{+2}, 4.0; NO_3^{-}, 12.0; $H_2PO_4^{-}$, 1.0; SO_4^{-2}, 7, and micronutrients, 20 (mg L^{-1}) (Meneses-Lazo et al. 2020). KNO_3, H_3PO_4, H_2SO_4, HNO_3, $CaNO_3$, and the microelements Fe, Mn, Cu, Zn, B, and Mo were used as fertilizer sources. The compound potassium nitrate was the only fertilizer used as a source of potassium, with and without bacterial inoculation. The rhizobacteria *Acinetobacter calcoaceticus* UTMR2, was used as the microbial inoculant in this study. The bacterial strain was isolated and characterized in a previous work for its biofertilizing traits on *Physalis* seedlings (Ramírez-Cariño et al., 2023). Plants were grown in plastic bags of 18 kg with a mix of site soil + river sand (60:40 v/v). The space between rows and between plants in the row was 70 cm. The analysis of the water and soil used in this experiment was determined. The soil texture was classified as medium textured

(loamy-clay-sandy) with an organic matter content of 2.0%, moderately alkaline pH (8.0), electrical conductivity of 1.4 dSm^{-1}, very high supply of available phosphorus (202 ppm), considerable supply of inorganic nitrogen (58.1 ppm), medium supply of potassium available (261 ppm), medium available Mg (332 ppm), low available Fe and Mn (4.67 and 3.32 ppm respectively), and cation-exchange capacity of (23.1 me/100 gr). On the other hand, the pH of the water was of medium salinity (7.4), high electrical conductivity (1.7 dSm^{-1}), medium K content, moderately high in Ca and Mg content, and hardness of 41.6 °f, which is considered high-hard. Solution nutritive was prepared according amounts recommended by Steiner. To prepare the nutrient solution for the irrigation of the treatments, the amounts suggested in the Steiner universal nutrient solution were discounted by the existing amount of mineral elements in the water and in the soil. Then the required amount of the nutritive minerals were formulated with different compounds and fertilizers used in agriculture. In the formulation, the amount of K was modified, which is one of the variables to be evaluated in this study. A concentrated solution (5 L), ajusted to a pH from 5.5 to 6.5, was used to prepare 250 L of nutrient solution for irrigation. The application of irrigation to the plants was based on the requirements according to their stage of development. Every day 3 irrigations were made: at transplantation, 100 mL were applied, whereas in the fruiting stage, 300 mL of nutrient solution were applied. Three replicates were used per treatment, and every experimental replicate consisted of 7 tomatillo plants. Two independent experiments were carried out.

Table 11. Experimental treatments to evaluate the biofertilizing effect of *Acinetobacter calcoaceticus* UTMR2 in tomatillo plants (*Physalis ixocarpa* Brot cv. "cascara morada") at different doses of potassium.

Number	Bacterial inoculant	K dose in nutritive solution (NS)	Treatment
1	No	100%	KF100
2		75%	KF75
3		50%	KF50
4		0%	KF0
5	Yes	100%	KB100
6		75%	KB75
7		50%	KB50
8		0%	KB0

The concentration of the bacterial suspension was adjusted to 1 x 10^9 UFC/ml, and then 10 ml of the inoculant was added to each of the tomatillo plant. The inoculants were applied twice at 10 and 25 DAT.

The weather parameters were monitored during the experimental period. The average relative humidity was 71%, with maximum and minimum of 99 and 19%, respectively; and mean temperature of 25 °C, with maximum and minimum average temperatures of 46 and 12 °C, respectively. The photosynthetically active radiation (PAR) was measured with a Light Scout® Mod. 3415FSE quantum light meter (Spectrum Technology Inc. USA) between 10:00 and 16:00 h, with an average PAR measurement of 997 mmol m^{-2} s^{-1}.

4.2.2. Cultivation of rhizobacteria

The inoculant strain *Acinetobacter calcoaceticus* UTMR2 was identified by 16S rRNA gene sequencing and was reported by Ramirez-Cariño et al. (2023). *A. calcoaceticus* strain was first tested for optimal growth. After that, the strain was cultured in LB broth and incubated at 37 °C for 72 h in an orbital shaker at 150 rpm, to obtain an optical density (600 nm) of 0.2. The bacterial suspension was adjusted to 10^9 cfu mL^{-1}.

4.2.3. Agronomic parameters

Physiological measurements (photosynthesis, and chlorophyll) of the plant were conducted two times at 30 and 60 DAT: photosynthesis was measured with a photosynthesis-fluorescens-meter LI-6400XT (LI-COR, USA) (µmol CO_2 $m^{-2}s^{-1}$) on fully expanded leaves when the sky was clear. The chlorophyll measurements were performed with a FieldScout CM 1000 Chlorophyll meter (Spectrum, USA). Six plants of each treatment were selected randomly, after which two leaves per plant were measured three times.

Stem thickness and plant height were measured using digital caliper (TTC, USA) at 70 DAT, when the experiment ended. The dry weight (g $plant^{-1}$) was weighed using an analytical scale (Ohaus, USA) after the samples were oven-dried at 70 °C for 72 h. The yield tomatillo fruits (g) per plant was obtained. All tomatillo fruits were counted and

weighted. The radial and equatorial diameter of the fruits were measured using a digital caliper.

4.2.4. Evaluation of quality parameters of tomatillo fruits

The fruits were harvested when it was observed that the fruit completely filled the calyx. At 60 and 70 DAT. Then, the fruits were removed from the calyx. The CIELab color parameters of tomatillo fruits were determined using an UltraScan Vis Spectrophotometer (HunterLab, USA). For phytochemical analyses, fruits of all samples were cut in halves and subsequently were blended in a home blender for 60 s. The samples were stored in high-density plastic bottles at -40 °C for further analysis. Total soluble solids (°Brix) was determined using a MA871 digital refractometer (Milwaukee, Europe). In addition, the pH and electrical conductivity (EC) of the fruit juice of tomatillo fruits were measured using a digital meter (Hanna, USA).

A proximal analysis of the fresh samples was performed according to the AOAC (1997) methods with the following determinations: moisture, protein, ash, total fiber, and titratable acidity (TA). For fat and total fiber determinations, the samples were first oven-dried at 70 °C for 48 h.

The fiber content was determined as follow: 1 g sample of defatted tomatillo fruit was weighed and added to the 500 ml balloon flask of the distillation equipment. 200 ml of 1.25% sulfuric acid was added and boiled for 30 min. The contents of the flask were filtered through normal wet filter paper. The sample was drawn back into the original flask using 200 ml of 1.25% sodium hydroxide and boiled for 30 minutes. subsequently, it was filtered through ash-free filter paper. all insoluble material was washed down with boiling water and then washed with 1% hydrochloric acid. The pH was adjusted between 6 and 7 and subsequently washed with alcohol, and then with acetone. The sample on the filter paper was transferred to a crucible and weighed. Subsequently, it was dried at 70 °C for 2 hours, weighed again and incinerated in a muffle at 600 °C for 2 h. After cooling in a desiccator, the crucible with the sample was weighed again and the corresponding calculations were made.

Sample weight(g) = W_1

Weight (g) of insoluble matter = W_2

Weight (g) of ashes = W_3

Then:

Fiber content (%) =[$(W_2 - W_3) / W_1$] x 100

Regarding the moisture, the porcelain capsules were weighed in constant weight. Subsequently, 5 g of the sample were transferred to the capsule. The capsule with its contents was quickly weighed. Subsequently, the capsule was placed in an oven at 100°C for 4 h until it had a constant weight. After cooling in a desiccator, it was weighed again and the weight loss corresponded to the loss of moisture.

For ashes determination, 2 g of sample was weighed and placed in a crucible weighed at constant weight, and then incinerated. Subsequently, the crucible with the sample was placed in a flask for 5 h and weigthed again.

Regarding the titratable acidity determination, the sample was filtered and 10 ml of liquid sample was obtained. Subsequently, 6 drops of phenoltalein were added as indicator. then, the titration with 0.1 N sodium hydroxide was done until a color change, the ml used were quantified.

In the proteins determination, a digestion was first done using 0.15 g of sample in a micro-Kjeldahl flask, adding 2.5 ml of sulfuric acid and 2 boiling beads, 0.04 g of copper sulfate pentahydrate and 1.3 g of potassium sulfate. Sample digestion was done in a micro-Kjeldahl for 90 min, when the sample showed a light blue-green coloration. 7 ml of distilled water was added and cooled. After distillation, the sample was added to the flask by removing the boiling beads and rinsed with 5 ml of distilled water. A bottle with 10 ml of boric acid and 2 drops of indicator was placed at the outlet of the distillation. 10 ml of saturated sodium hydroxide was added to the boiling chamber until a blue-gray or dark brown color. Approximately 20 ml of the distillate were collected and subsequently titrated with a 0.01 N HCl solution.

4.2.5. Determination of essential minerals

The mineral content (P, K, Ca, Na, Mg, and Mn) of fruits and plants samples was quantified using an inductive coupled plasma with optical emission spectroscopy (ICP-OES) Optima 7000 equipment (Perkin Elmer, USA). For the ICP-OES analysis, the samples were oven-dried at 60 °C for 48 h. Subsequently, the samples were subjected to acid digestion with reflux and later analyzed in a Spectroblue instrument equipped with Spectro Smart Analyzer software according to Ramírez-Cariño et al. (2023) Calibration curves for minerals (P, K, Ca, Na, Mg, and Mn) were performed using individual standard solutions (Perkin Elmer, USA). The linear correlation coefficient was 0.9999 for each mineral. Measurement results are presented as mean value ± standard deviation and are expressed as mg/kg dry weight.

4.2.6. Statistical analysis

One-way analysis of variance (ANOVA) was performed to determine the effect of *Acinetobacter calcoaceticus* application at different doses of potassium. For the experimental data analysis a statistical software (SAS Institute Inc., USA) was used. The comparisons of means were made using Tukey's honestly significant difference test at the 5% level of significance ($p < 0.05$). SAS v.9.00 (SAS 2002). To check the normality of data, the Shapiro-Wilk and Kolmogorov-Smirnov normality tests were performed considering a 95% confidence level.

4.3. Results

4.3.1. Agronomic and physiological parameters of growth and mineral content in tomatillo plants.

The mineral content in tomatillo plants was determined. It was observed that the treatments with the inoculant *Acinetobacter calcoaceticus* combined with low doses (50 and 0%) of K fertilizer (25.8 and 25.48, respectively) showed similar results than the treatment with chemical fertilizer only at 100% doses (27.11). This results can be attributed as an effect of the inoculant in the uptake of K for the plants. Similar results were observed for the P content when the inoculant *A. calcoaceticus* was combined with K fertilizer at 50% doses.

Regarding of the Na content, the inoculant showed effect in the treatment without K chemical fertilizer (K 0%). Similarly, the inoculant increased the Mg content in the treatment without K fertilizer (K 0%) (Table 12).

Table 12. Mineral content in tomatillo plants (g mineral kg^{-1} sample).

Treatment	K	P	Ca	Na	Mg	Mn
KF100	27.11 ± 1.25 ^{a}	0.93 ± 0.12 ^{c}	13.29 ± 1.36 ^{ab}	5.23 ± 0.14 ^{c}	6.38± 0.13 ^{c}	0.083 ± 0.003 ^{b}
KF75	21.93 ± 3.01 ^{bc}	0.68 ± 0.08 ^{d}	12.92 ± 0.87 ^{ab}	5.81 ± 0.41 ^{b}	7.20 ± 0.17 ^{b}	0.088 ± 0.004 ^{ab}
KF50	24.77 ± 0.51 ^{bc}	1.35 ± 0.05 ^{b}	11.90 ± 0.50 ^{c}	3.49 ± 0.25 ^{f}	7.45 ± 0.12 ^{ab}	0.068 ± 0.001 ^{c}
KF0	20.56 ± 2.47 ^{c}	0.63 ± 0.01 ^{d}	12.80 ± 0.22 ^{b}	4.73 ± 0.06 ^{d}	7.28 ± 0.04 ^{b}	0.092 ± 0.001 ^{a}
KB100	22.16 ± 0.75 ^{bc}	0.37 ± 0.06 ^{e}	10.52 ± 0.58 ^{c}	6.34 ± 0.23 ^{a}	6.32 ± 0.33 ^{c}	0.068 ± 0.004 ^{c}
KB75	21.06 ± 0.13 ^{c}	0.70 ± 0.05 ^{d}	10.58 ± 0.41^{c}	5.55 ± 0.09 ^{b}	6.68 ± 0.06 ^{c}	0.063 ± 0.003 ^{c}
KB50	25.80 ± 0.96 ^{ab}	1.63 ± 0.05 ^{a}	12.6 ± 0.47 ^{b}	3.91 ± 0.08 ^{f}	6.46 ± 0.20 ^{c}	0.082 ± 0.003 ^{b}
KB0	25.48 ± 0.28 ^{ab}	1.10 ± 0.01 ^{c}	14.58 ± 0.31 ^{a}	4.25 ± 0.37 ^{e}	7.89 ± 0.07 ^{a}	0.087 ± 0.001 ^{ab}

KF mean treatments with chemical fertilization only.
KB mean treatments with chemical fertilization combined with *Acinetobacter calcoaceticus* as bacterial inoculant.
Potassium fertilization dose based on the recommended dose for tomatillo (Meneses-Lazo et al. 2020).

Regarding the measurement of the photosynthesis, no significant differences were found between the treatments in the two measurements carried out (30 and 60 DAT). Therefore, photosynthesis was not affected neither by the different doses of K applied nor the *A. calcoaceticus* inoculation. The values of photosynthesis were similars between the flowering and fruiting stages (Table 13).

Table 13. Photosynthetic activity (µmol CO_2 m^{-2} s^{-1}) of tomatillo plants after 30 and 60 days after transplant (DAT).

Treatment	Photosynthesis 30 DAT	Photosynthesis 60 DAT
KF100	12.5 ± 0.72 [a]	16.1 ± 4.03 [a]
KF75	15.8 ± 1.93 [a]	12.4 ± 3.3 [a]
KF50	12.7 ± 2.28 [a]	13.2 ± 2.59 [a]
KF0	13.3 ± 0.73 [a]	12.3 ± 2.74 [a]
KB100	13.1 ± 1.14 [a]	14.4 ± 2.39 [a]
KB75	13.1 ± 0.65 [a]	11.8 ± 0.40 [a]
KB50	13.5 ± 1.05 [a]	13.1 ± 2.18 [a]
KB0	12.8 ± 1.85 [a]	12.8 ± 2.54 [a]

KF mean treatments with chemical fertilization only.
KB mean treatments with chemical fertilization combined with *Acinetobacter calcoaceticus* as bacterial inoculant.
Potassium fertilization dose based on the recommended dose for tomatillo (Meneses-Lazo et al. 2020).

The chlorophyll rate of plants was affected by the treatment *A. calcoaceticus* combined with doses of K (75 and 50%) at 30 DAT. Values of 200.2 and 200.5 mmol m^{-2}, were 15 and 22% higher in comparison with the similar treatments but without inoculant (173.8 and 164.5 mmol m^{-2}), respectively. Similarly the inoculant increased 22, 24 and 36% the chlorophyll content at 60 DAT when was combined with K 75, 50 and 0% doses, respectively. It was also observed that chlorophyll content in tomatillo plants decreased in the fruiting stage in comparison with the flowering stage (Table 14).

Table 14. Chlorophyll content (mmol m^{-2}) of tomatillo plants after 30 and 60 days after transplant (DAT).

Treatment	Chlorophyll 30 DAT	Chlorophyll 60 DAT
KF100	167.8 ± 8.94 ^{b}	134.9 ± 3.43 ^{bc}
KF75	173.8 ± 7.32 ^{b}	125.1 ± 13.82 ^{c}
KF50	164.5 ± 11.53 ^{b}	133.1 ± 6.24 ^{bc}
KF0	163.8 ± 7.05 ^{b}	129.9 ± 11.61 ^{bc}
KB100	170.2 ± 6.63 ^{b}	133.3 ± 13.58 ^{bc}
KB75	200.2 ± 3.26 ^{a}	152.8 ± 7.22 ^{ab}
KB50	200.5 ± 2.38 ^{a}	164.6 ± 9.81^{a}
KB0	180.9 ± 2.75 ^{ab}	176.9 ± 4.88 ^{a}

KF mean treatments with chemical fertilization only.
KB mean treatments with chemical fertilization combined with *Acinetobacter calcoaceticus* as bacterial inoculant.
Potassium fertilization dose based on the recommended dose for tomatillo (Meneses-Lazo et al. 2020).

Regarding the dry weight results, the inoculant *A. calcoaceticus* increased from 18 to 44 % the dry weight when was combined with the K chemical fertilizer at 50% doses in comparison with the treatments with chemical fertilizer only. Similarly, the inoculant increased the height of tomatillo plant (128.1 cm) when was combined with K fertilizer at 50% in comparison with the other treatments, which the plant height was ranged from 98.4 to 111.8 cm. Finally, in the plant stem thickness was not effected neither by the K fertilizing doses applied nor by the inoculant application (Table 15).

Table 15. Agronomic parameters in tomatillo plants.

Treatment	Dry weight (g)	Height (cm)	Stem thickness (mm)
KF100	53.3± 0.9 ^{cd}	109.1 ± 4.0 ^{b}	11.1 ± 0.3 ^{a}
KF75	44.7 ± 1.2 ^{e}	107.4 ± 7.4 ^{b}	10.8 ± 0.2 ^{a}
KF50	53.7 ± 1.3 ^{c}	111.7 ± 7.9 ^{b}	11.9 ± 0.4 ^{a}
KF0	54.6 ± 0.5 ^{c}	98.4 ± 4.1 ^{b}	10.8 ± 0.7 ^{a}
KB100	51.3 ± 0.6 ^{d}	102.4 ± 4.3 ^{b}	10.7 ± 0.7 ^{a}
KB75	52.9 ± 0.5 ^{cd}	109.6 ± 6.6 ^{b}	11.3 ± 1.0 ^{a}
KB50	64.2 ± 0.7 ^{a}	128.1 ± 4.0 ^{a}	11.0 ± 0.6 ^{a}
KB0	58.5 ± 0.1^{b}	111.8 ± 4.4 ^{b}	11.5 ± 0.9 ^{a}

KF mean treatments with chemical fertilization only.
KB mean treatments with chemical fertilization combined with *Acinetobacter calcoaceticus* as bacterial inoculant.
Potassium fertilization dose based on the recommended dose for tomatillo (Meneses-Lazo et al. 2020).

4.3.3. Yield production of tomatillo plants

The diameter fruit (radial and equatorial) was not affected neither by inoculant nor by the K fertilizing doses applied. The *A. calcoaceticus* inoculant increased the yield per tomatillo plant when was combined with K fertilizer 0% doses (272.7 ± 7.0) in comparison with the treatments with K chemical fertilizer only at 0 and 100% doses (238.4 and 262.0 g, respectively). Regarding to the fruits number, was observed that the treatment of the inoculant combined with K fertilizer at 100% was lower in comparison to the treatments with K fertilizer only at 100 and 75% doses and the inoculant combined with K fertilizer 0% doses. The fruit weight results showed that the inoculant application increased the weight of tomatillo fruits when was combined with K fertilizer at 75% doses compared to the treatments with the inoculant at low doses of K (50 and 0%) and the treatment with the K fertilizer only (100%)(Table 16).

Table 16. Yield parameters in tomatillo plants.

Treatment	Yield (g plant^{-1})	Fruits number	Weight fruit^{-1} (g)	Polar diameter (cm)	Equatorial diameter (cm)
KF100	262.0 ± 16.3 ^{cd}	14.0 ± 1.0 ^{a}	18.7 ± 0.3 ^{bc}	3.4 ± 0.2 ^{a}	4.2 ± 0.3 ^{a}
KF75	277.6 ± 7.4 ^{ab}	14.5 ± 0.5 ^{a}	19.4 ± 0.6 ^{ab}	3.4 ± 0.2 ^{a}	4.2 ± 0.1 ^{a}
KF50	267.6 ± 7.5 ^{bc}	13.6 ± 0.8 ^{ab}	19.8 ± 0.6 ^{ab}	3.4 ± 0.2 ^{a}	4.3 ± 0.1 ^{a}
KF0	238.4 ± 4.9 ^{d}	13.8 ± 0.4 ^{ab}	17.4 ± 0.8 ^{c}	3.4 ± 0.0 ^{a}	4.1 ± 0.1 ^{a}
KB100	239.8 ± 4.5 ^{d}	11.8 ± 0.3 ^{b}	20.4 ± 0.7 ^{ab}	3.4 ± 0.0 ^{a}	4.3 ± 0.2 ^{a}
KB75	287.6 ± 8.0 ^{a}	13.8 ± 1.0 ^{ab}	20.9 ± 1.0 ^{a}	3.4 ± 0.1 ^{a}	4.2 ± 0.1 ^{a}
KB50	245.0 ± 9.9 ^{cd}	13.0 ± 1.1 ^{ab}	19.0 ± 1.0 ^{bc}	3.4 ± 0.2 ^{a}	4.2 ± 0.2 ^{a}
KB0	272.7 ± 7.0 ^{ab}	14.7 ± 0.5 ^{a}	18.6 ± 0.3 ^{c}	3.5 ± 0.2 ^{a}	4.3 ± 0.3 ^{a}

KF mean treatments with chemical fertilization only.
KB mean treatments with chemical fertilization combined with *Acinetobacter calcoaceticus* as bacterial inoculant.
Potassium fertilization dose based on the recommended dose for tomatillo (Meneses-Lazo et al. 2020).

4.3.4. Quality characteristics of the harvested tomatillo fruits

The color indicates the freshness of the fruits, samples of tomatillo fruits were measured 15 days after the harvest. In this color parameter, no significant differences were obtained between the treatments in the three coordinates L*, a*, and b*, which ranged from 51.1 to 56.3, -5.6 to -7.7, and 24.1 to 27.8, respectively (Figure 12).

Regarding the physicochemical parameters: in the moisture and electrical conductivity parameters, no effect was observed neither by chemical fertilizer (K) nor by the inoculant. In the °Brix, the combination of the inoculant with K 100% doses and the treatment with the chemical fertilizer only at 75% doses had higher values compared to the other treatments. In the pH meditions, the treatment with chemical fertilizer only at 50% doses showed the best results followed by the combination of the inoculant with low doses of K (0%) in comparison with the other treatments. Regarding the proximal analysis of the tomatillo fruit, in the fats, ash and fiber content was not affected neither by fertilizer nor by the biofertilizer. In the protein content, it was observed that the best results were observed in the treatments with high doses of potassium (100 and 75%), both in treatment only with the fertilizer as well as in the combination with the inoculant. In this work, in the TA

parameter, the inoculant showed the best results when was combined with the chemical fertilizer at 75% doses (0.9 ± 0.01 %) compared to the other treatments (Table 17 and 18).

Figure 12. Tomatillo fruits evaluated 15 days after harvest to homogenize physiological maturity.

Table 17. Physicochemical parameters of the tomatillo fruits.

Treatment	Moisture (%)	°Brix (%)	pH	EC
KF100	91.7 ± 0.5 [a]	5.85 ± 0.00 [b]	3.80 ± 0.01 [d]	2.70 ± 0.14 [a]
KF75	91.7 ± 0.5 [a]	6.17 ± 0.03 [a]	3.83 ± 0.00 [c]	2.94 ± 0.36 [a]
KF50	92.2 ± 0.3 [a]	5.85 ± 0.05 [b]	3.96 ± 0.01 [a]	3.06 ± 0.11 [a]
KF0	92.3 ± 0.2 [a]	5.73 ± 0.03 [b]	3.79 ± 0.00 [d]	2.57 ± 0.08 [a]
KB100	91.5 ± 0.2 [a]	6.07 ± 0.08 [a]	3.79 ± 0.00 [d]	2.64 ± 0.19 [a]
KB75	92.1 ± 0.2 [a]	5.82 ± 0.03 [b]	3.78 ± 0.01 [d]	2.85 ± 0.13 [a]
KB50	91.9 ± 0.1 [a]	5.38 ± 0.08 [c]	3.79 ± 0.00 [d]	3.03 ± 0.31 [a]
KB0	91.6 ± 0.1 [a]	5.68 ± 0.12 [b]	3.86 ± 0.00 [b]	2.64 ± 0.17 [a]

KF mean treatments with chemical fertilization only.
KB mean treatments with chemical fertilization combined with *Acinetobacter calcoaceticus* as bacterial inoculant.
Potassium fertilization dose based on the recommended dose for tomatillo (Meneses-Lazo et al. 2020).

Table 18. Proximal analysis of the tomatillo fruits.

Treatment	Fat (%)	TA (%)	Ash (%)	Protein (%)	Fiber (%)
KF100	0.60 ± 0.01 [a]	0.85 ± 0.01 [b]	0.59 ± 0.05 [a]	1.4 ± 0.0 [bc]	22.0 ± 6.4 [a]
KF75	0.53 ± 0.09 [a]	0.70 ± 0.01 [c]	0.54 ± 0.05 [a]	1.8 ± 0.1[ab]	17.3 ± 7.3 [a]
KF50	0.45 ± 0.06 [a]	0.68 ± 0.00 [d]	0.57 ± 0.03 [a]	1.3 ± 0.4 [bc]	15.5 ± 1.3 [a]
KF0	0.64 ± 0.27 [a]	0.85 ± 0.00 [b]	0.55 ± 0.04 [a]	1.2 ± 0.3 [cd]	21.3 ± 0.7 [a]
KB100	0.59 ± 0.24 [a]	0.68 ± 0.00 [d]	0.65 ± 0.02 [a]	2.3 ± 0.2 [a]	18.4 ± 1.5 [a]
KB75	0.56 ± 0.30 [a]	0.90 ± 0.01 [a]	0.56 ± 0.10 [a]	1.7 ± 0.2 [ab]	15.8 ± 1.3 [a]

KB50	0.53 ± 0.12 [a]	0.69 ± 0.00 [cd]	0.61 ± 0.06 [a]	0.8 ± 0.2 [d]	21.0 ± 1.6 [a]
KB0	0.68 ± 0.08 [a]	0.66 ± 0.00 [e]	0.58 ± 0.05 [a]	0.6 ± 0.2 [d]	20.8 ± 0.6 [a]

KF mean treatments with chemical fertilization only.
KB mean treatments with chemical fertilization combined with *Acinetobacter calcoaceticus* as bacterial inoculant.
Potassium fertilization dose based on the recommended dose for tomatillo (Meneses-Lazo et al. 2020).

4.3.5. Mineral content in fruit

The mineral content in fruits and plant of tomatillo crop was determined. It was observed that the K, P, Na, Mg and Mn content in tomatillo fruits was not affected neither by the fertilizer doses applied nor by the biofertilzer *A. calcoaceticus*, only in the Mn content, the treatment with the chemical fertilizer at 0% doses was higher than the combination of the inoculant with the K fertilizer at 75% doses, the other treatments showed similar results (Table 19).

Table 19. Mineral content in tomatillo fruits (g mineral kg^{-1} sample).

Treatment	**K**	**P**	**Ca**	**Na**	**Mg**	**Mn**
KF100	20.61 ± 3.59 [a]	1.46 ± 0.13 [a]	0.04 ± 0.02 [b]	1.15 ± 0.24 [a]	2.65 ± 0.15 [a]	0.017 ± 0.002 [ab]
KF75	19.43 ± 2.42 [a]	1.57 ± 0.05 [a]	0.05 ± 0.05 [b]	1.15 ± 0.19 [a]	2.78 ± 0.05 [a]	0.018 ± 0.002 [ab]
KF50	22.46 ± 2.58 [a]	1.44 ± 0.11 [a]	0.09 ± 0.04 [ab]	2.43 ± 1.27 [a]	2.67 ± 0.05 [a]	0.017 ± 0.001 [ab]
KF0	20.62 ± 2.78 [a]	1.57 ± 0.11 [a]	0.23 ± 0.09 [a]	1.70 ± 82 [a]	3.10 ± 0.26 [a]	0.019 ± 0.003 [a]
KB100	20.33 ± 1.96 [a]	1.53 ± 0.18 [a]	0.05 ± 0.08 [b]	1.10 ± 0.31 [a]	2.83 ± 0.25 [a]	0.019 ± 0.002 [ab]
KB75	20.60 ± 1.37 [a]	1.38 ± 0.10 [a]	0.18 ± 0.05 [ab]	0.86 ± 0.10 [a]	2.54 ± 0.16 [a]	0.011 ± 0.005 [b]
KB50	19.59 ± 2.20 [a]	1.59 ± 0.08 [a]	0.15 ± 0.02 [ab]	1.42 ± 0.12 [a]	2.92 ± 0.10 [a]	0.019 ± 0.001 [ab]
KB0	18.53 ± 1.31 [a]	1.61 ± 0.32 [a]	0.09 ± 0.03 [ab]	0.91 ± 0.09 [a]	3.01 ± 0.36 [a]	0.018 ± 0.004 [ab]

KF mean treatments with chemical fertilization only.
KB mean treatments with chemical fertilization combined with *Acinetobacter calcoaceticus* as bacterial inoculant.
Potassium fertilization dose based on the recommended dose for tomatillo (Meneses-Lazo et al. 2020).

4.4. Discussion

4.4.1. Agronomic and physiological parameters of growth and mineral content in tomatillo plants.

Regarding the mineral content in tomatillo plants, our results ranged as follows (g kg^{-1}): K, from 20.56 to 27.11; P, from 0.37 to 1.63; Ca, from 10.58 to 14.58; Na, from 3.49 to 6.34; Mg, from 6.32 to 7.89; and Mn, from 0.063 to 092. It was observed that the inoculant increased the K content in tomatillo plants, when was combined with low doses of K chemical fertilizer (0%) and in the combination of biofertilizer with K fertilizer (50% doses) had similar results in comparison with K fertilizer only at high doses (100%). Similarly, *A. calcoaceticus* increased the P content in tomatillo plants, when was combined with low doses of K fertilizer (50%). The inoculant application increased also the Ca content when was combined with low doses of K fertilizer (0 and 50%) which were similar results compared with the high doses of K chemical fertilizer only (75 and 100%). Finally, the inoculant increased the Mg content in tomatillo plants in combination with low doses of K fertilizer (0%). In a study, Ali et al. (2021) reported that the concentrations of N, P, and K in the potato leaves were affected by the *Bacillus cereus* inoculation and concentrations were ranged between 22.7 to 26.5 g kg^{-1}, 6.05 to 6.72 g kg^{-1}, and 35.8 to 50.2, respectively. The inoculant increased the concentration of N, P and K in 34, 32 and 62%, respectively in comparison to the non-inoculated plants. In contrast to, Cisternas-Jamet et al. (2019) reported that *B. amyloliquefaciens* increased the Ca content in green bell pepper (*Capsicum annuum*) but an increase in the K content was not observed. In another study, (Nazir et al., 2017) noticed that the 50% urea-N combined with 50% BGS-N and *Bacillus* sp. presented the higher concentrations of K and P in shoot of okra plants.

In our study, the amount of K and other minerals contained in the water and in the soil was similar for all treatments. However, in the treatments with doses of K fertilizer, this was added in the form of KNO_3 to the soil through the nutrient solution. It is possible that potassium dissolved in the nutrient solution in ionic form, a part of that K was absorbed by mineral part of the soil to form unavailable compounds (Liu et al., 2016) and increased the soil pH due to the accumulation of the K^+ cation. On the other hand, the N content added to the soil through KNO_3 also was increased. Therefore, it is possible that the biofertilizer

activity decreased due to the increase in pH and the increase in N in the soil. In this sense, Reyes and Varela (2007) reported that the presence of beneficial microorganisms around the root depends of favorable condition environmental. Therefore, in treatments with high doses of K, possibly the activity of *A. calcoaceticus* was reduced and therefore, it was reflected in the low amount of minerals absorbed. In this sense, Xekarfotakis et al. (2021) observed that when KNO_3 was incorporated in the soil, resulted a stressful environment for the PGPR which affected its activity in the N fixation and uptake by the *Asparagus officinalis* plants as well as the application of N inorganic fertilizers inhibited the nitrogenase activity of free-living diazotrophs. In addition, the authors reported that the treatment NK fertilizers combined with the inoculant increased the K content in the leaf in comparison with the others treatments. In contrast, in the treatment with high K fertilizer input, the lowest foliar K was observed. In our study, is possible that the accumulation of K in the soil, decreased the uptake of the others minerals, and the biofertilizer also decreased its activity because to the unfavorable environmental conditions. For example, Khanghahi et al. (2018) reported that there are competition of potassium with calcium and magnesium for sites on the cation exchange in clay and acidic soils. This may explain that in the treatments with low doses of K, the inoculant is capable of solubilizing the K added by the water and in the nutrient solution, because it finds the amount of K and the appropriate environmental conditions, causing availability of other minerals.

On the other hand, the leaves represent one of the largest consumers of mineral nutrients extracted from the soil. A proportion of minerals remain in the foliar tissues and another proportion are remobilized during the senescence of the leaves. While the stems constitute the route for the traffic of minerals within the plant and represent an important consumer of minerals to sustain the production of vascular tissues and the storage of reserves (Gutiérrez, 1997). On the other hand, Fernandez-Escobar et al (1999) showed that the mineral nutrients which are most important for the plant are transferred faster to the roots than the others nutrients, and they would be expected to increase when root development is at a vital point. In addition, the authors reported that fluctuations in the nutrient content of the leaves during the plant growth cycle are attributed to the necessities of the crop depending on the development phase. In a study with *Sparagus officinalis* plants, Xekarfotakis et al. (2021) reported that the root P content was similar statistically in comparison with the other

treatments, which is a indication that P could be of the lower importance for *Sparagus officinales* growth compared to other macronutrients. Similarly, Krug and Kailuweit (1999) also found no differences in the N content of plants under different N applications rates. The authors attributed this recording to the fact that the plants do not absorb higher amounts of N, as it is not necessary for further growth.

Regarding the results obtained for photosynthesis in plants at 30 and 60 DAT, it was observed that the values of photosynthesis were higher in the the growth and flowering stage than the flowering and fructification stage. However, no effect was observed neither for the different doses of potassium nor for the application of the rhizobacteria. Regarding to the chlorophyll rate, it was observed an increment in the inoculated plants in the treaments at the K doses of 75, 50%, and 0% in comparison with their similar treatments non-inoculated. We considering that the increase of chlorophyll values was relationed with the availability and uptake of minerals, such as K, P, Ca, Mn, and mainly Mg, which is main component of the chlorophyll molecule, caused by the effect of the inoculation of *Acinetobacter calcoaceticus* UTMR2 strain. In line with our results, the *Acinetobacter* genus have been reported by increasing the chlorophyll content in plants. Susuki et al. (2014) revealed the capacity of *Acinetobacter calcoaceticus* to increasing the chlorophyll content in *Lactuca sativa* plants under poor nutrient conditions. Besides, according to Liu et al. (2019), *A. calcoaceticus* decrease the loss of clorophyll under drought stress and could keep the chlorophyll level in the plants similar to plants under normal conditions. Similarly, Foughalia et al. (2022) observed that *Acinetobacter calcoaceticus* strains and *Bacillus safensis* exercised protection against *Botrytis cinerea* in tomato plants through improve in the growth parameters incluided the increase of chlorophyll content. Works similars showed the effect of rhizobacteria in the chlorophyll content. Oliveira Reis et al. (2022) reported that *Paenibacillus alvei* and *Bacillus cereus* increased the chlorophyll index in the *Glycine max* plants due to the increased uptake of N, Fe, and Mg, Kalaji et al. (2018) reported that chlorophyll synthesis is influenced by the content of minerales in the plant, and that there is a correlation between the element availability in the soil (N, Mg, Cu, Mn Zn and Fe) and their content in the plant leaves, and Ali et al. (2022) observed that *Bacillus thuringiensis* increased the total chlorophyll in 42% in maize plants under salinity conditions compared with non-inoculated plants.

The results in our study, confirm that the lowest values of the chlorophyll content was in the treatments non-inoculated, therefore, we consider that the increment in the chlorophyll content in the tomatillo plants at 30 and 60 DAT was due to the *A. calcoaceticus* application. In contrast, in a study about of the use combined of different nitrogen levels with a cyanobacteria and/or a yeast in wheat plants, Hamed et al. (2022) observed that the fertilizer alone N level of 100% was higher in the chlorophyll content than the treatments at 75 and 50% N level combined with the cyanobacteria.

Regarding growth parameters, our results showed that the treatment of K 50% and 0% doses combinated with the inoculant increased the height as well as the dry weight of tomatillo plants, respectively, in comparison with it similar treatments of non-inoculated plants. This results can be attributed to the mineral content in the tomatillo plants because the highest mineral content of observed also in the treatments with low dose of K. On the other hand, the results in the stem thickness of tomatillo plants a significative difference was not observed between the treatments. The results in this study, suggests that *A. calcoaceticus* UTMR2 exert more effect in low amount of K, therefore, we consider that the increment observed in the growth attributes of tomatillo plants, were in response to inoculation with *A. calcoaceticus* UTMR2 and that chlorophyll content could be relationed with these enhances. In this sense, Helaly et al. (2022) considered that the enhance the growth parameters in kale plants inoculated with *Pseudomonas koreensis*, *Ralstonia pickettii*, and *Bacillus cereus* could be due to improved photosynthetic activity as a result of the higher contents of leaf chlorophyll. In other study, Zapata-Sifuentes et al. (2022) showed that *Acinetobacter radioresistens* and *Sinorhizobium meliloti* increased the root length and the number of secondary roots in cucumber (*Cucumis sativus*) plants compared with control plants non-inoculated, which improves water and nutrients uptake, besides that these rhizobacteria posses the mechanism to fix nitrogen and solubilize phosphate. Similarly, Pérez-Rodriguez et al. (2020) reported that the inoculation with *Pseudomonas fluorescens* RtM10 and *Azospirillum brasilense* Az39 in alone inoculation increased the root dry weight (62 and 41%, respectively) and the shoot dry weight (29 and 23%, respectively) in tomato plants. However, Hamed et al. (2022) reported that application of the fertilizer alone N level 100% significantly induced wheat growth, and was higher compared with N 75 and 50% level combined with cyanobacteria.

4.4.2. Yield parameters in tomatillo plants

Regarding the yield per tomatillo plant, it was observed a effect of the *Acinetobacter calcoaceticus* UTMR2 strain in the treatment combined with K 0% which showed a significative difference. In the weight per fruit it was observed an effect of the inoculant when was combined with K 75% doses. In line with our results, Filiz et al. (2022) reported that a consortium of biofertilizers (*Bacillus subtilis*, *Bacillus megaterium* and *Loctococcus* spp.) increased the seed germination, biological yield per plant, number of seeds per pod, number of seed per plant and grain yield per plant in bean plants. Similarly, Zapata-Sifuentes et al. (2022) reported that *A. radioresistens* increased the dry biomass, fruit length fruit diameter and yield per plant in comparison with non-inoculated plants and Perez-Rodriguez et al. (2020) showed that *Azospirillum brasilense* increased fruit number (35%) fruit weight (38%) in tomato plants in comparison with the non-inoculated plants. The non effect of biofertilizers in the agronomic parameters in plants have been reported. Toscano-Verduzco et al. (2020) did not find any effect by the biofertilization with *Beauveria brongniartii* in plants of *Capsicum chinense,* which did not show an increase in the fruit yield per plant and per hectare.

The increase in the size of the fruit is a way to observe the effect of rhizobacteria on the quality of the fruit of the crop. In our study, fruit size (polar and equatorial diameter) was not enhanced by the *A. calcoaceticus* inoculation, although previous works have reported distinct outcomes. For example, Perez-Rodriguez (2020) reported that *P. fluorescens* and *A. brasilense* alone or in combination, increased the fruit size in two varieties of tomato plants under field conditions. Similarly, Zapata-Sifuentes (2022) reported that *P. paralactis*, *S. meliloti* and *A. radioresistens* increased the fruit size of cucumber (*Cucumis sativus*) under greenhouse conditions, in comparison with the control treatment non-inoculated. In another work, Toscano-Verduzco et al. (2020) reported that the *B. brongniartii* inoculation increased the longitudinal and equatorial fruit diameter of the *C. chinense* plants.

4.4.3. Quality characteristics of tomatillo fruits

In our study, an effect was not observed by the inoculant application in the moisture content and EC parameters, °Brix in the treatments with the K doses (75%) combinated with the inoculant were higher compared to the other treatments. Regarding the titratable acidity (AT) the treatment K 100% doses combined with the biofertilizer and the treatment K 75% doses showed the highest values. Besides, all other treatments decreased the AT with the biofertilizer application. Inoculation of tomatillo plants with *A. calcoaceticus* had no effect on the ash and fiber content, because there was no significant difference between treatments. Our results of the physicochemical parameters ranged as follows: moisture content, from 91.6 to 92.36%; °Brix, from 5.38 to 6.17; pH, from 3.78 to 3.96; and EC, from 2.57 to 3.06. Regarding the proximal analysis parameters ranged as follow: fat, from 0.45 to 0.68%; TA, from 0.66 to 0.90%; ash, from 0.54 to 0.65%, protein content, from 0.6 to 2.3%; and fiber from 15.5 to 22.0%. Quality characteristics for the tomatillo fruit have been reported (USDA, 2019): proteins 0.96%, fat, 1.02%, and fiber 1.90%. Similarly, Shenstone et al. (2020) reported the chemical properties of tomatillo fruit: pH, 3.76; °Brix, 8.24; moisture content, 91.76; protein (% FW) 0.75-1.06; fat (% FW) 1.12-2.1, and ash (% FW) 0.77-1.42. However, no reports were found in the literature about the effect of rhizobacteria on the quality of the tomatillo fruit. Then, studies of the effect of rhizobacteria on quality characteristics in tomato fruit (which is similar to tomatillo fruit) were analyzed. In a study, Yagmur and Gunes (2021) reported that *Bacillus. megaterium*, *Paenibacillus polymxa*, *Burkholderia cepacia* and *Azospirillum*. sp, showed results on TA and pH ranged from 0.52 to 0.73, and from 3.75 to 4.07, respectively. In similar study, Ochoa-Velasco et al. (2016) reported the effect of *B. paralicheniformis* on tomato fruits: moisture ranged from 92.46 to 93.64; protein 0.35 to 0.95; total fiber, 0.76 to 1.09; ash, 0.51 to 0.59; TA 0.45 to 0.58, and °Brix 4.17 to 5.83. In addition, the authors reported that moisture and ash were not affected neither by the *B. paralicheniformis* nor the nitrogen applied on fresh tomatoes cultivated under different fertilization conditions. Similarly, Katsenios et al. (2021) reported that the moisture, pH value, and ash parameters in tomato fruits were not significantly affected from the single soil application of inoculants of *Bacillus* genus (*B. amyloliquefaciens*, *B. licheniformis*, *B. mojavensis*, *B. pumilus*, *B. subtilis*, *B. velezensis*) and *P. megaterium*. Interestingly, regarding the protein content, in our study was observed

that the inoculant showed better results when was combined with high K doses (100 and 75%) and therefore, decreased with low K doses (50 and 0%). Our results were in contrast to other reports in the literature, Ocho-Velasco et al. (2016) reported that the tomato fruits showed lower protein content in the plant inoculated with the biofertilizer in comparison with the non-inoculated plants. In other work, Cisternas-jamet and Salvatierra-martínez (2019) reported that an effect in the moisture and ash content by the inoculation of *Bacillus amyloliquefaciens* in pepper plants was not observed. In the same study, the protein content was increased by the inoculant when was inoculated in the seedbed before transplant, and the protein increment could be associated with the N fixation capacity by the rizhobacteria in which the N in the form of proteins could be increased. Similarly, the protein content can be increased when is inoculated with 50% urea-N + 50% biogas slurry (BGS) + *Bacillus* sp. compared with plants receiving only urea-N 100% (Nazir et al., 2017). The effect of rhizobacteria seems to be very specific, and deeper analyses are necessary to asses their use in determined crops (de la Osa et al., 2021). The benefics effects in plants by rhizobacteria also is in fuction of number of bacteria, plant-bacteria combination, plant genotype, soil type, soil organic matter and enviromental conditions (Yagmur and Gunes, 2021).

4.4.4. Mineral content in tomatillo fruit

The mineral content is an important characteristic in the nutritional and functional quality of the fruits and plants. In our study, we noticed that neither the biofertilizer nor the different doses of K had an effect on the content of K, P, Na, Mg, and Mn in the tomatillo fruit, since there was no difference between the treatments. Only in the Ca content was observed an effect by the K fertilizer doses, which low doses (0%) was higher that the high doses (75 and 100%). The values obtained in our study for the mineral content were ranged as follows (g mineral kg^{-1} sample) : K, from 18.53 to 22.46; P, from 1.38 to 1.61; Ca, from 0.04 to 0.23; Na, from 0.86 to 2.43; Mg, from 2.54 to 3.10; and Mn, from 0.011 to 0.019.

The values reported in the mineral content of tomatillo fruit has been reported by Shenstone et al. (2020) (g kg^{-1}): K, 2.68; P, 0.39; Ca, 0.07; Na, 0.01 and Mg, 0.2. However, studies of the mineral content in tomatillo fruit due to the effect of rhizobacteria, were not found. In a study, Nazir et al. (2017) reported that *Bacillus pumilus* and *Pseudomonas putida* were evaluated single and in combination on tomato plants. In the single application to *B.*

pumilus, the mineral content in tomato fruit were (g kg^{-1}): K, 15,3; P, 4.8; Ca, 1.25; Na, 1.0; Mg, 2.2, and Mn 0.04. In addition the single application of *P. putida* the mineral content in tomato fruit were (g kg^{-1}): K, 20; P, 5.0; Ca, 1.40; Na, 0.12; Mg, 2.1, and Mn 0.06.

Our results showed that there was no variation between the contents of K, P, and Mn in tomatillo fruit. It was observed also that the minerals content in fruits (K, Ca, Na, Mg, and Mn) was lower than in the tomatillo plants, only the P content was higher in the tomatillo fruits than in the tomatillo plants.

4.5. Conclusions

It is very important decreasing the use of the chemical fertilizers instead of the avoid. The use of rhizobacteria is a sustenaible option because are more the benefic traits than the negative effects. Rhizobacteria improve the agronomic parameters, yield, and fruit quality. Based in our results, it was noticed that *A. calcoaceticus* combined with low K doses (75 and 0%) increased the crop yield and fruit quality similar, even higher at K 100% recommended doses. In special the K fertilizer 0 and 50% doses combined with the inoculant, achieves a balance between a decrease in the fertilization dose and the improvement of yield and fruit quality. This study showed also that in the yield or tomatillo fruit quality parameters is involved biotic and abiotic factors, such as chemical fertilization, the microorganisms application, plant physiology, and the chlorophyll content, which interact between they giving as result the improvement of the tomatillo crop.

CHAPTER V.

5. General conclusions

5.1. Conclusions and final remarks

The use of rhizospheric bacterial strains as biofertilizers emerges as a promising strategy to produce food crops in a sustainable manner. A variety of rhizobacterial microorganisms have desirable characteristics that can foster plant growth and nutrimental enhancement of the harvested agro-products. Different biofertilization mechanisms are used by microorganisms in their interaction with the plant, such as mineral solubilization, production of phytohormones, siderophore, and other secondary metabolites and enzymes which favor the growth of the plant.

In our study, it was noticed that rhizobacteria isolated from rhizospheric soil of wild *Physalis* sp. plants, showed benefic traits *in vitro* tests of 4 biofertilization mechanisms evaluated (ammonia production, phosphorus and potassium solubilization, and IAA synthesis). Four bacterial strains (*Cellulosimicrobium cellulans* UTMR1, *P. megaterium* UTMR3, *A. calcoaceticus* UTMR2, and *Atlantibacter* sp. UTMR4) had the capacity of improve the growth of tomatillo seedlings and increase the length and weight of roots. On the other hand, the single application of bacterial strains increased the mineral content (K, Ca, Mg, and Mn) in leaves. The results in this study indicate that it is possible the use of rhizobacteria as an alternative to decrease the use of the chemical fertilizers in the production of tomatillo seedlings. On the other hand, the use of *A. calcoaceticus* as a biofertilizer in combination with low K doses (75, 50, and 0%) increased the chlorophyll content, agronomic parameters (dry weight, height, and yield), and mineral content in plants (K, P, Ca, and Mg). Regarding the quality of tomatillo fruits, only the titratable acidity was increased with the inoculant application, which indicates that the effect of *A. calcoaceticus* in the fruit quality parameters was no observed. Our results indicate that is possible decreasing the use of chemical fertilizer when the rhizobacteria are applicated in combined way with potassium fertilizers, without decreasing the production of tomatillo crop.

5.2. Areas for future research

The constant growth of the human population is demanding a greater amount of food production. The intensive agricultural activity is causing the overexploitation of non-renewable natural resources, mainly soil and water, which in turn damages the environment and poses serious health-risk to humans. Therefore, current agriculture should implement sustainable agronomical practices that consider the wellness of the agroecosystem as a whole. However, agricultural systems are very complex since they are affected by several factors, which include: crop product, botanical and biochemical traits of the plant, plant microbiome, soil properties, fertilization and irrigation characteristics, rhizobacterial inoculants, and agronomical factors. This complexity makes the fundamental study of crop biofertilization difficult to perform in terms of the biochemical mechanism that derive in plant development, crop productivity and nutritional quality of the harvested product.

To maintain a fertile and healthy soil, it is necessary to adopt strategies that include the use of the rhizospheric microbiome. In this sense, various research works have been carried out focusing on the utilization of rhizobacteria as efficient biofertilizers. Despite the high experimental and analytical load required for these studies and technologies, that seems to be the research direction in the future for the comprehensive biochemical study of the biofertilization of agricultural systems taking into account the nutritional quality and synthesis of bioactive compounds of the crop product. In addition, it has been well documented the effect of rhizobacterial inoculants on the enhancement of the nutritional quality and the concentration of compounds with biological activity in the crop product. Finally, it is required more research on the use of beneficial rhizobacteria in crop cultivation at field production scale, which require product formulation and packaging that extend the shelf life of biofertilizers and suits the local agricultural practices.

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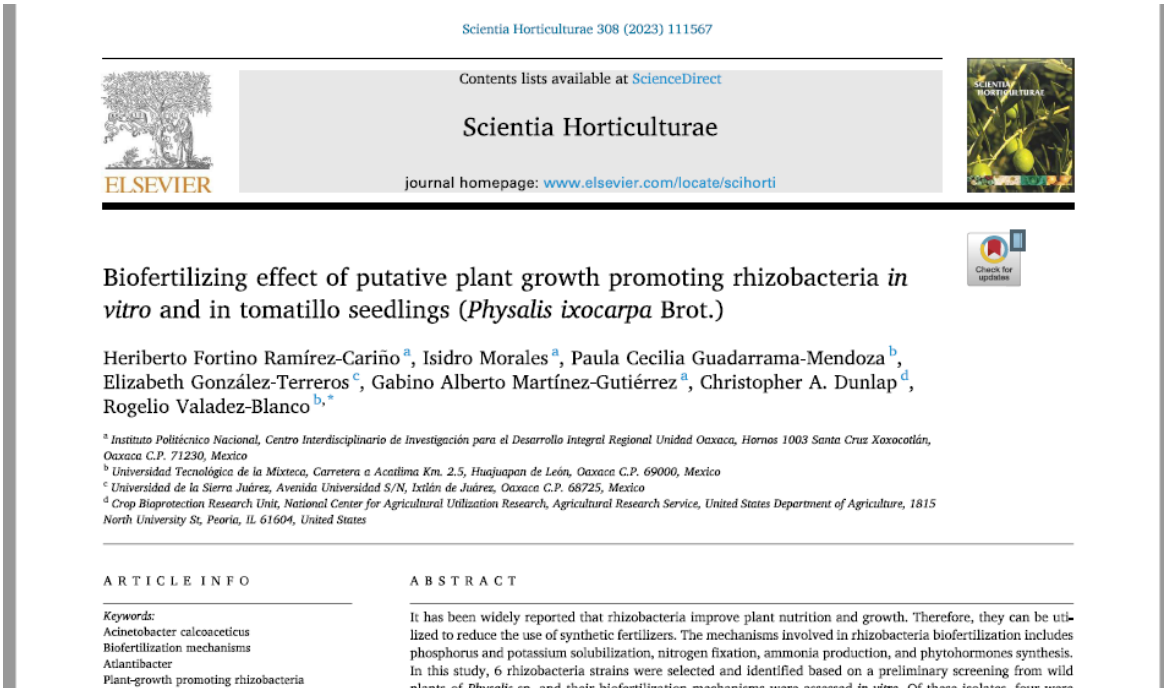
Annexes

Annex 1. Cover of the published article of the results obtained in chapter III



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Biofertilizing effect of putative plant growth promoting rhizobacteria *in vitro* and in tomatillo seedlings (*Physalis ixocarpa* Brot.)

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ABSTRACT

It has been widely reported that rhizobacteria improve plant nutrition and growth. Therefore, they can be utilized to reduce the use of synthetic fertilizers. The mechanisms involved in rhizobacteria biofertilization includes phosphorus and potassium solubilization, nitrogen fixation, ammonia production, and phytohormones synthesis. In this study, 6 rhizobacteria strains were selected and identified based on a preliminary screening from wild