International Journal of Agriculture and Biosciences 2025 xx(x): xx-xx.
https://doi.org/10.47278/journal.ijab/2025.072

RESEARCH ARTICLE
eISSN: 2306-3599; pISSN: 2305-6622

Effect of an *Enterobacter cloacae*-based Biofertilizer on the Growth and Performance of Cassava Plants (*Manihot esculenta* Crantz) MCol 2066 Variety (Chirosa)

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ABSTRACT

Biofertilizers are valuable resources that supply indispensable nutrients for plants, improving the physiological development and agronomic quality of crops, producing vegetable hormones, supporting and promoting a sustainable agricultural production. This study focused on evaluating the impact of an *Enterobacter cloacae*-based biofertilizer on the growth and performance of cassava plants (*Manihot esculenta* Crantz), MCOL 2066 variety (Chirosa). The traits associated with plant growth promotion were evaluated employing a biotest under controlled greenhouse conditions, using a completely randomized experimental design. The 16S rRNA gene sequencing reveals the confirmation of *Enterobacter cloacae* isolates (strain S105E PP405613.1 and FB105B PP761660.1). Under *in vitro* conditions, it was found that the isolates produced indole acetic acid, solubilized P and fixed nitrogen. The phosphorus (P) solubilizing activity was associated with a simultaneous decrease in the medium's pH (pH 7.0-<4.5). It was found that the biofertilizer induced a significant increase in plant height, root development, and biomass accumulation. As a result, these *Enterobacter cloacae* isolates could be further formulated for field applications.

Keywords: Indole acetic acid, Phosphate-solubilizing bacteria, Nitrogen-fixing bacteria, Colombia, Cordoba department.

Article History
Article # 25-065
Received: 12-Feb-25
Revised: 04-Apr-25
Accepted: 14-Apr-25
Online First: 13-May-25

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a starchy root that constitutes an essential staple food source for over 800 million people worldwide (Li et al., 2024). It has an extensive application in the food, medical, chemical, and light manufacturing industries, where it can be transformed into starch, alcohol, and other chemical compounds, as well as being used in food production (Li et al., 2024). Cassava plays a crucial role in food security and global agricultural development (Garreto et al., 2023). This crop is vital in more than 100 countries, mainly in tropical and subtropical regions, providing not only a significant nutritional value through its roots but also offering substantial economic value through various by-products (Olasanmi et al., 2021). In 2021, global production reached 304 million tons, with Africa leading at 192 million, followed by Asia and the Americas (Boukhers et al., 2022). Within this context, Colombia is worthy of note as a significant producer in Latin America, following Brazil and Paraguay (Garcia & Alzate, 2024), thereby underscoring the importance of cassava to its agricultural economy. Nevertheless, the conventional reliance on chemical fertilizers in cassava farming states a number of challenges, including environmental contamination, increased production costs and a negative impact on agricultural sustainability (Canellas et al., 2022).

Nitrogen (N) and phosphorus (P) are pivotal to agriculture, playing a significant role in plant growth and vital metabolic processes (Qin et al., 2023). The P present in soil is classified into three main forms: dissolved P, adsorbed or exchangeable P, and mineral P. Dissolved P is predominantly found as orthophosphate, which is directly assimilable by plants; however, its concentration in soil is often quite limited (Dong et al., 2025). Mineralized phosphorus is composed of primary and secondary

Cite this Article as: Ruiz AJB, Guevara MEC, Zumaqué LEO and Plaza YJP, 2025. Effect of an *Enterobacter cloacae*-based biofertilizer on the growth and performance of cassava plants (*Manihot esculenta* Crantz) MCol 2066 variety (Chirosa). International Journal of Agriculture and Biosciences xx(x): xx-xx. https://doi.org/10.47278/journal.ijab/2025.072

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phosphate minerals, which are less effective in terms of availability for plants. Adsorbed P, on the other hand, refers to phosphorus retained on the surface of clay minerals or associated with metal oxides and hydroxides, such as iron (Fe), aluminum (Al), or calcium (Ca), through mechanisms such as anion exchange or ligand displacement, like -OH or -COOH groups (Gao et al., 2020). The application of phosphate fertilizers has been a common response to this limitation, but with very low efficiency, since plants only use between 5% and 25% of these inputs, leaving non-assimilable residues in the soil (Chen et al., 2025). On the other hand, nitrogen is a vital nutrient that frequently constitutes the primary limiting factor in crop growth. In the absence of nitrogen fertilizers, agricultural production would decrease drastically, reaching insufficient levels to satisfy the food needs of more than half of the global population (He et al., 2021). However, the application of large amounts of nitrogen fertilizers to the soil often exceeds the actual demand of crops, surpassing their capacity for absorption and assimilation in plant tissues (Buisset et al., 2025). In cropping systems, between 60% and 70% of nitrogen applied through chemical fertilizers is lost in the form of nitrous oxide (N_2O) and molecular nitrogen (N_2) (Congreves et al., 2021). As a result of these high losses, the efficiency of nitrogen utilization from fertilizers remains low, at approximately 30% to 35% (Anh et al., 2025).

In numerous agricultural regions worldwide, intensive practices over decades or even centuries have resulted in soils exhibiting either depletion or excessive accumulation of essential minerals required for soil fertility and plant development. This phenomenon is particularly evident in the case of N and P, whose balance has been significantly disrupted in many soils dedicated to agricultural production (Buisset et al., 2025). While the utilization of chemical fertilizers has been demonstrated to enhance crop yields, it concomitantly poses a range of hazards to human health and the environment, due to the frequent use of chemical fertilizers, which has been shown to lead to the accumulation of active N and P species in the soil, with these subsequently leaching into nearby water bodies (Rehman et al., 2021). The extensive use of chemical fertilizers, coupled with apprehensions regarding their environmental sustainability, has prompted to explore alternative solutions to address crop nutritional requirements. Confronted with these challenges, it is imperative to adopt more sustainable and innovative approaches in agriculture, such as the utilization of alternative inputs of microbial origin that ensure environmental safety and agricultural sustainability (Maitra et al., 2021). In this context, microbial biotechnology offers innovative solutions through the use of multifunctional biofertilizers containing plant growth-promoting rhizobacteria (PGPR) as sustainable alternatives to enhance the availability of essential nutrients and reduce dependency on chemical inputs (Khourchi et al., 2022; De Lima et al., 2024). A sustainable alternative consists of the application of agricultural inputs, such as bioinoculants formulated from soil microorganisms, capable of stimulating plant development and optimizing the health of both plants and the soil ecosystem. A variety of studies have demonstrated the direct and indirect effects of rhizosphere bacteria, promoting the growth of the root system and shoot formation in plants (Espinosa et al., 2025). Bacterial inoculants provide numerous advantages, including the supplementation of nutrients, the promotion of plant growth, the improvement of soil health, and the suppression of diseases, which allows them the enhancement of resilience in adverse environmental conditions. Moreover, in contrast to chemical fertilizers, biological fertilizers do not have negative effects on the ecosystem (Maitra et al., 2021). This approach offers the potential for significant environmental benefits, as well as substantial improvements in crop growth and yields, thereby representing a promising advancement for green and sustainable agriculture (Tang et al., 2020).

The use of microorganisms in soil management and plant health has emerged as a key approach to ensure long-term agricultural sustainability, and represents a promising paradigm for green agriculture, progressively reducing dependency on mineral fertilizers while minimizing environmental impact (Wang et al., 2022). The integration of diverse strains of plant growth-promoting rhizobacteria (PGPR), such as *Enterobacter cloacae*, with complementary functions, can enhance plant development by acting as multifunctional providers of essential nutrients and improving the stability and effectiveness of the inoculum under biotic and abiotic conditions (Zhou et al., 2021). The fundamental role of microorganisms in supplying minerals to plants is well-documented, either through nutrient mobilization in the soil or via specialized interactions in the rhizosphere, which collectively promote plant growth (Habibi et al., 2023). These microorganisms facilitate the availability of nitrogen and phosphorus in forms assimilable to plants through chelation, ion exchange, the production of organic acids such as gluconic acid, ketogluconic acid and lactic acid, and other mechanisms that prevent the immobilization of these nutrients in the soil (Elhaissoufi et al., 2022). Furthermore, they provide plant hormones such as indole-acetic acid (IAA), abscisic acid (ABA), gibberellic acid (GA), 1-aminocyclopropane-1-carboxylate (ACC) deaminase, and salicylic acid (Shameem et al., 2023). *E cloacae* has been identified as a rhizospheric bacterium characterized by its ability to promote plant development under a wide range of conditions, both optimal and adverse (Devi et al., 2022). It contributes to an increase in the production of auxins, ethylene, cytokines, and siderophores, thereby enhancing the availability of essential nutrients such as nitrogen and phosphorus (Kumar et al., 2022). It has been recognized as a bacterium that boosts plant growth due to its ability to produce various growth promoters and has played a pivotal role in soil fertility, increasing the growth and yield of various agricultural crops (Ali et al., 2022). Moreover, this strain has been shown to produce indole-acetic acid, a key factor in its plant growth-promoting effects (Panigrahi et al., 2020).

In Colombia, the increasing use of synthetic fertilizers has had significant negative environmental impacts, including issues such as salinization and eutrophication of water bodies (Qin et al., 2023), highlighting the urgent need to adopt more sustainable agricultural practices, such

as the use of microbial inoculants, which are gaining recognition as a key strategy for future agricultural management practices. Numerous studies have explored the potential of bacteria, both individually and in consortia, with the aim of their eventual commercialization in global agricultural markets. In particular, the application of microorganisms capable of supplying nutrients to plants could reduce dependency on chemical fertilizers, enhancing soil health and maintaining the balance of its microbiome (Bloch et al., 2020). This underscores the necessity to broaden and deepen studies focused on the identification and characterization of native strains with high biofertilizer potential. The objective of this research was to evaluate the effect of a biofertilizer based on *E. cloacae* on the development and yield of cassava (*Manihot esculenta* Crantz) plants of the MCOL 2066 (Chirosa) variety under greenhouse conditions.

MATERIALS & METHODS

Study Area

The research was conducted at the GRUBIODEQ biotechnology laboratory and in the greenhouses of the Agronomic Engineering program at the University of Córdoba (8°47′037″ N; 75°50′51″ W, 15 masl) (Fig.1), located in Montería, Córdoba- Colombia (Pompelli et al., 2019).

Characterization of Native Phosphorus Solubilizing Bacteria (PSB), Free-living Nitrogen-fixing (FLNF) and Indole Acetic Acid-producing Bacteria (IAAPB)

Soil Sampling

The samples were collected from rhizospheric soil cultivated with Chirosa cassava at Farm 13 Rojo (8°51'45.0 "N 75°35'40.2 "W) located in the municipality of Cienaga de Oro, Córdoba-Colombia. Five subsamples were combined, taken in a zigzag pattern at depths of 1-5cm (Cezar et al., 2023) to obtain representative samples. Samples were stored in Ziploc plastic bags at room temperature during transportation and subsequently refrigerated at 4°C, until their processing at the GRUBIODEQ biotechnology laboratory and the University of Córdoba soil laboratory.

Isolation

Ten g of the previously homogenized rhizospheric soil sample were dispersed in 90mL of a sterile 0.85% NaCl saline solution and subjected to orbital shaking at 150rpm for 45min, the upper turbid phase was serially diluted, then seeded on NBRIP and Burk's agar plates which were incubated at 28°C for 72h (Hii et al., 2020). Independent colonies from each plate were picked for PSB detection in petri dishes with NBRIP (National Botanical Research Institute's Phosphate) culture medium (Aliyat et al., 2022). All inoculated plates were incubated at 30°C for 7 days (Dey et al., 2024). In the same way, to isolate FLNF and IAAPB independent, colonies were seeded by depletion in petri dishes with modified Burk's Nitrogen-free culture medium comprising: 1.3g/L Burk's salt (5g $MgSO_4.7H_2O$, 20g KH_2PO_4, 5g K_2HPO_4, 3.25g $CaSO_4$), 1.0mL L^{-1} Fe-Mo mixture (1.45g $FeCl_3$, 0.253 g $NaMoO_4$ and 1000mL distilled water), 20g/L sucrose and 15g/L agar-agar (Pérez & Oviedo, 2019) before autoclaving (15 psi, 121°C for 15 min) and incubated at 27°C for 7 days (Wang et al., 2024).

Molecular Identification and Phylogenetic Analysis

The extraction of genomic DNA was carried out using the PureLink™ Genomic DNA Minikit kit (Pbact) (Thermo Fisher Scientific, USA), in strict accordance with the manufacturer's instructions. The amplification of the samples was performed using the universal primers 27F (5′–AGAGTTTGATCCTGGCTCAG3′) and 1492R (5′–CGGTTACCTTGTTACGACTT3′) (Sigma-Aldrich, Darmstadt, Germany), which amplify the 16S ribosomal RNA (rRNA) gene (Sano et al., 2021). The PCR mixture consisted of 25µL of DreamTaq Hot Start PCR Master Mix (2X) (Thermo Fisher Scientific, USA), 1µL of each 10mM primer, 2µL of DNA (~157ng μL^{-1}), and molecular-grade water, yielding a total volume of 50µL. The amplification process was conducted in a thermocycler (Bio-Rad T100™ thermocycler, USA). The amplification protocol comprised an initial denaturation step at 95°C for 5min, followed by 30 cycles of denaturation at 95°C for 30s, annealing at 54°C for 40s, and an extension step at 72°C for 1min. Subsequently, a final extension step was carried out at 72°C for 10min. The amplicons generated by PCR were subjected to analysis using 2% agarose gel electrophoresis, performed. The visualization of the bands was then carried out using a UV transilluminator (Enduro GDS Labnet, USA). The estimation of the size of the amplified fragments was performed by comparison with a 100 bp molecular weight marker (Sigma-Aldrich, Germany).

The determination of gene sequences was carried out by bioinformatic analysis with the BLAST tool, performing a homology search in the GenBank database of NCBI (National Center for Biotechnology Information) to find the sequences with the highest similarity. Phylogenetic inference of the isolates with the highest activity was carried out using the Neighbor-Joining method, allowing the reconstruction of their evolutionary history. The phylogenetic relationships among the analyzed taxa were represented by a bootstrap consensus tree, constructed from 1000 replicates to evaluate the robustness of the nodes (Briega et al., 2025). Statistical support for the grouping of similar taxa was expressed as the percentage of trees in which these groups were recovered during a process of bootstrapping, or repeatedly sampling a data set to generate a distribution of estimates. Evolutionary distances were computed using the composite maximum likelihood method and expressed in terms of base substitutions per site (Bhardwaj et al., 2023). The study considered the analysis of a set of 10 nucleotide sequences. Evolutionary analyses were performed in MEGA11 (Tamura et al., 2021).

In vitro Evaluation of Phosphorus Solubilizing Activity, Nitrogen Fixing and Indole Acetic Acid Producer from the Isolated Bacterial Populations

In vitro Detection of Phosphorus Solubilizing Activity

The isolated bacteria were inoculated in 10mL of liquid NBRIP medium and maintained at 28°C under incubation for 9 days, under constant agitation at 150rpm

in an orbital shaker (Gao et al., 2024). Samples were collected on days 3, 5, 7 and 9. Soluble phosphorus was quantified using the Molybdovanadate Method (Amri et al., 2023). These samples underwent centrifugation at 10,000rpm for a duration of 15min to obtain a supernatant devoid of cells (Dey et al., 2024). Seven mL of the obtained supernatant (with previously measured pH) was collected, followed by the addition of 2mL of Molybdovanadate reagent, and subsequently diluted to a final volume of 10mL using distilled water. They were left to stand for 10min, after which the absorbance was measured at 440nm (Han et al., 2024). A calibration curve was established using potassium acid phosphate (K_2HPO_4) to calculate the concentration of soluble phosphorus in the form of phosphate.

***In vitro* Detection of Nitrogen-fixing Activity**

The nitrogen-fixing capacity of the isolated populations was evaluated by the Berthelot colorimetric technique (phenol-hypochlorite), using ammonium ion titration method (Pérez & Oviedo, 2019). The FLNF were inoculated in 10% soil medium and incubated at 28 to 29°C for 48 hours with constant agitation at 150rpm. After incubation, 25mL of 2M potassium chloride (KCl) was added, and the system was agitated for an additional hour. It was then left undisturbed to allow soil precipitation; 10mL of the supernatant were collected, centrifuged at 2000rpm for 20min and 0.4mL of 10% phenol alcohol solution, 0.4mL of 0.5% sodium nitroprusside and 1mL of oxidizing solution (20 g of sodium citrate, 1 g of sodium hydroxide and 1mL of 1.5N sodium hypochlorite in 100mL of water) were added. The mixture was allowed to stand for 1 hour and afterwards the absorbance was measured at 632.9nm (Pérez & Oviedo, 2019). A calibration curve was constructed using ammonium chloride (NH_4Cl) as a standard with the aim of determining the concentration of nitrogen fixed in the form of ammonium.

***In vitro* Quantification of Indole Acetic Acid (IAA) Production**

Each IAAPB colony was inoculated in liquid Burk's Korea medium supplemented with tryptophan (Pérez & Oviedo, 2019) in containers previously sterilized and kept under constant agitation at 150rpm for 48h at 28°C (Abo Elsoud et al., 2023). Subsequently, the microbial culture underwent centrifugation at 3,000rpm for a duration of 10min (Aliyat et al., 2022). A 4mL aliquot of the supernatant was transferred into a test tube, and mixed with 2mL of Salkowski reagent ($FeCl_3$. $6H_2O$ 0.01M and H_2SO_4 1M) was added (Husseiny et al., 2021; Dey et al., 2024). The development of a pink to red color indicated the formation of IAA, and the color intensity was proportional to the production of IAA. After that, the absorbance reading was taken by UV spectrophotometer at 530nm (Abo Elsoud et al., 2023). To calculate IAA production, a calibration curve was established using pure indole acetic acid.

Evaluation of the Effect of Inoculation with PSB, FLNF and IAAPB on the Development and Performance of a Chirosa Cassava Crop under Greenhouse Conditions 120 days after Planting

Antagonism Test

The antagonistic effect was determined in vitro by agar diffusion method (Palladini et al., 2023). The strains were inoculated on liquid medium (NBRIP and Burk's) and on Mueller Hinton agar (Dehydrated beef infusion 2.0g/L, Casein hydrolysate 17.5g/L, Starch 1.5g/L and Agar 15.0g/L) (Haile et al., 2022). Antimicrobial discs of 7 mm diameter impregnated with a bacterial suspension were placed on the Mueller Hinton medium. The culture plates were maintained at 28°C under incubation for a period of 72 hours (Liu et al., 2024). At the end, the inhibition diameters zone around each disk was measured in millimeters.

Experimental Design

The greenhouse experiment followed a Completely Randomized Design (CRD) with a single factor, five treatments, and fifteen replicates, totaling 75 experimental units. The details of the treatments are presented in Table 1.

Sowing of Planting Material under Greenhouse Conditions

Seventy-five 20 ft x 24 ft C4 plastic bags were used, each filled with 20kg of sandy loam soil per bag from the 13 Rojo farm, located in the municipality of Cienaga de Oro-Córdoba. The soil was transported to the University of Córdoba, where 75 cassava cuttings, each approximately 20cm long, were prepared for planting. For treatment T2 (chemical fertilization), according to the soil analysis and the nutritional requirements of the Chirosa cassava variety. The soil was amended based on the recommended nutrient requirements for cassava cultivation, that is: KCl (181mg kg^{-1} soil), urea (550mg kg^{-1} soil), and Diammonium phosphate (DAP; 311mg kg^{-1} soil). For the treatments (T3-T5) the cuttings were immersed (60min) in the biofertilizer, using the concentrations established in Table 1. Then, all the cuttings were planted in the plastic bags previously filled with the soil described above and distributed at a distance of one meter between rows by one meter between plants (1m x 1m), following the experimental design proposed for this trial. In treatments T3-T5 (Table 1), 25mL of the biofertilizer was applied per bag every 15 days for 120 days after planting (Ferreira et al., 2021).

Table 1: Summary of the experimental treatments

Treatments	Description
T1 (absolute control)	Cuttings without any type of treatment (water)
T2 (chemical control)	Edaphic fertilization according to soil analysis
T3	Cuttings treated with biofertilizer at the concentration of 10^6CFU mL^{-1}
T4	Cuttings treated with biofertilizer at the concentration of 10^7CFU mL^{-1}
T5	Cuttings treated with biofertilizer at the concentration of 10^8CFU mL^{-1}

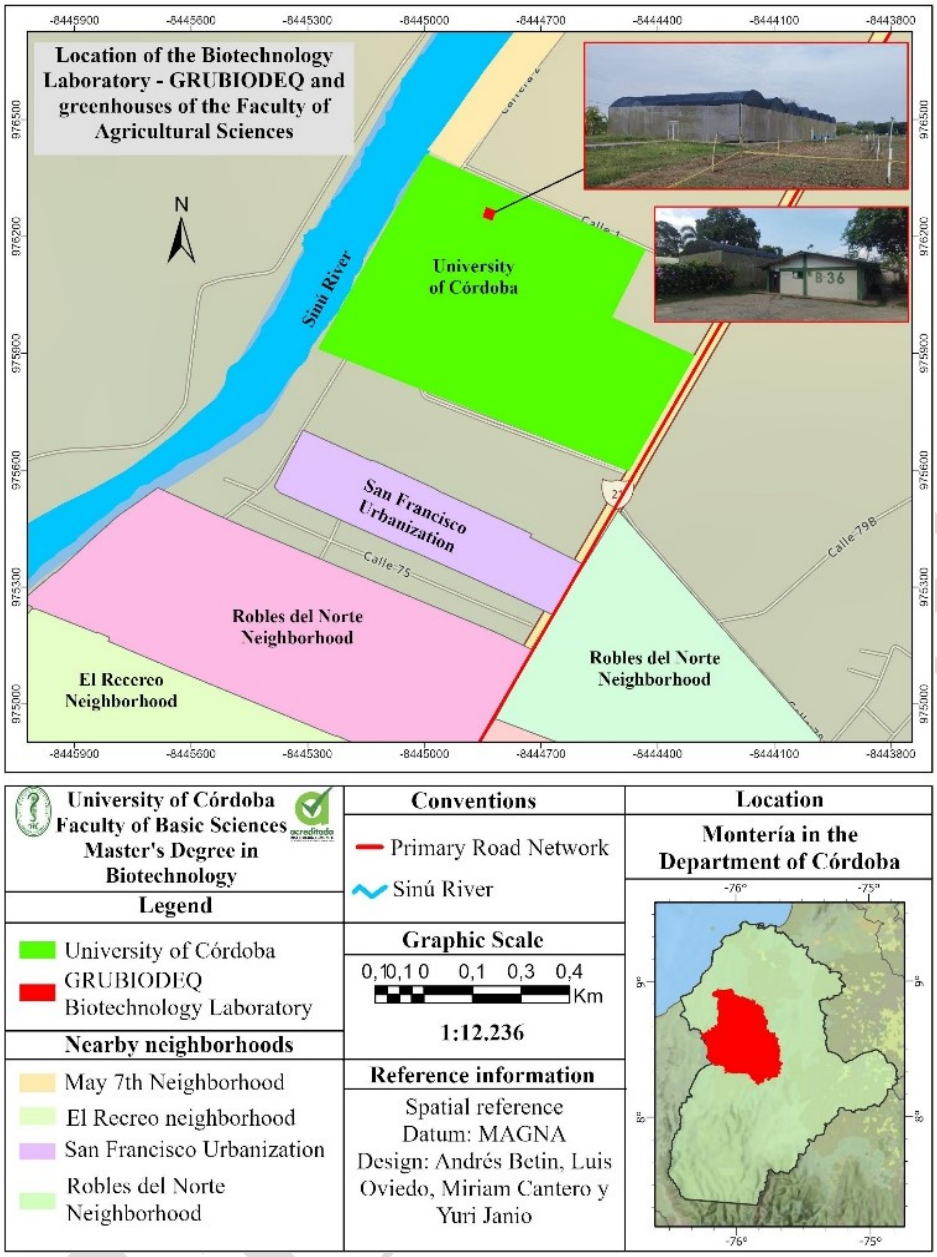

Fig. 1: Location of the study area.

Evaluation of Biometric Parameters of Plants

The following biometric parameters were evaluated: plant height (cm), stem diameter (cm), number of leaves, dry weight of the aerial part (g), number of roots, root length (cm), root diameter (cm), dry weight of the root (g), and fresh weight of the root (g).

Determination of the Percentage of Protein in Leaves and Starch in Cassava Roots

After 120 DAP, Three plants per treatment were randomly selected and their dried samples were ground to a particle size of less than 0.5 mm (Du et al., 2024). The Kjeldahl method was used to determine the N concentration in the leaves (Jakienė et al., 2025). For starch determination in roots a powder of crushed roots was obtained as described above. Then the starch percentage was analyzed as described in detail (Negi et al., 2022; Shameem et al., 2023).

Statistical Analysis

A one-way analysis of variance (ANOVA) was performed using Tukey's post-hoc test ($p < 0.05$). R version 4.3.1 was employed for statistical analysis and model fitting, while SigmaPlot version 12.0 was used for graphical visualization. For the construction of the heatmap, the means of each treatment were divided by the mean of the control, obtaining a ratio between these two values. Then, the values were log-transformed ($\log 2x$), where x represents the ratio of treatment means to control means. With a color scale the values were presented where the green ones are the values with $\log 2x$ greater than or equal to 0.05 and the values in red are those with $\log_{2x}$ less than -0.05.

RESULTS

Isolation

Eight strains of PSB, FLNF, and IAAPB, each exhibiting

distinct morphologies, coming from the rhizospheric region of agricultural soils. The PSB were distinguished by forming remarkable colonies on NBRIP plates, characterized by prominent solubilization halos around $Ca_3(PO_4)_2$ and FLNF and IAAPB formed remarkable colonies on Burk's plates. These isolates were initially categorized as nitrogen-fixing bacteria because they were able to grow on Burk's medium, which is a selective medium for nitrogen fixers.

In vitro Evaluation of the Phosphorus Solubilizing Activity, Nitrogen Fixative and Indole Acetic Acid Producing of Isolated Bacterial Populations

In vitro Detection of Phosphorus Solubilizing Activity

The phosphorus solubilization capacity of the isolated strains, focused to the development of efficient and sustainable biofertilizers are presented in Fig. 2. The phosphorus solubilization indicated that strains S104B (1122.04 mg L^{-1}), S105E (1122.04 mg L^{-1}) and S106F (1050 mg L^{-1}) exhibited a high ability to solubilize phosphorus compared to strains S104A2 (283.33 mgL^{-1}), S104C (273.66 mgL^{-1}), S104B2 (239.25 mgL^{-1}), S104A (201.62 mgL^{-1}) and S104D (233.87 mgL^{-1}). It was shown that the strains S104B, S105E, and S106F exhibited 469%, 456%, and 427% greater phosphorus solubilization compared to the control strains (Fig. 2). Based on these results, the strains S104B, S105E and S106F were selected for P solubilization profiling and pH variation for nine continuous days.

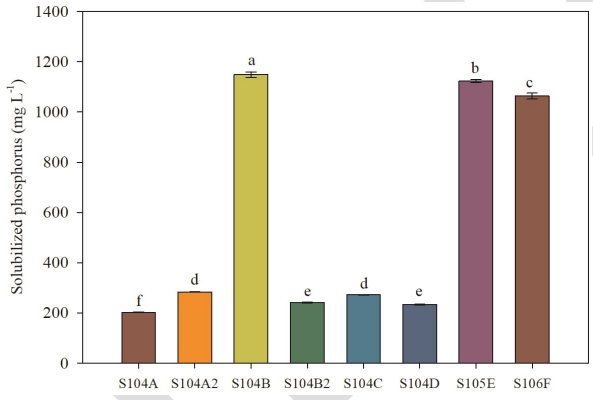

Fig. 2: Phosphorus solubilization by PSB cultured in NBRIP medium. This Figure provides a visual representation of the ability of different BSF bacterial isolates to solubilize phosphorus (expressed in mg L^{-1}). For each characteristic, the means followed by different letters denote statistical significance ($p < 0.05$). Means (±SE) (n = 3).

The water-soluble P concentration was concomitant with the acidification of the culture medium supernatants (Fig. 3A), meaning that phosphorus solubilization was associated with a significant decrease in pH (Fig. 3B). P solubilization by strains S104B, S106F and S105E at 9 days was 28%, 17% and 12% respectively, as compared to time zero (Fig. 3A). The strains S104B, S106F and S105E reduced the pH of the culture supernatant to values below 4.6 after three days (Fig. 3B) with average water-soluble P concentrations of 1744.62±76.93, 1746.77±6.72 and 1976.88±17.20mgL^{-1} respectively (Fig. 3A). After nine days the strain S104B, solubilized a mean phosphorus concentration of 2169.11±49.31mg L^{-1}, S106F (2028.62±44.03mg L^{-1}) and S105 E (2224.73±26.16mg L^{-1}) (Fig. 3A). By the ninth day, the pH significantly decreased to average values below 4.5, the strain S104B could decrease pH to 4.49±0.03, S106F (4.52±0.01) and S105E (4.42±0.01) (Fig. 3B). The above results indicate that strain S105E produced the highest concentration of soluble phosphorus at nine days.

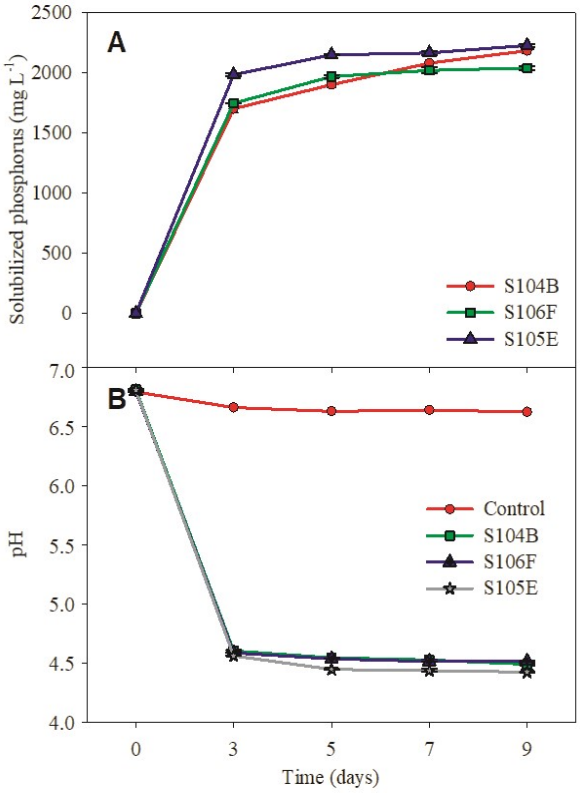

Fig. 3: Phosphorus solubilizing activities of bacterial strains cultured on NBRIP medium. PSB strains were inoculated on NBRIP medium and samples were collected aseptically at different time intervals; water-soluble phosphorus concentration (A) and pH of the medium (B) were determined in each sample. Means (±EE) (n = 3).

In vitro Quantification of Indole Acetic Acid (IAA) Production and Atmospheric Nitrogen Fixation

The Fig. 4A, strongly illustrates the preeminence of strain FB105B in atmospheric nitrogen fixation, standing out with an average concentration of 8.67±0.01mgL^{-1}. In an efficiency hierarchy at the conversion of atmospheric nitrogen to ammonium, strains FG104-1 and FB104B occupy the subsequent positions, exhibiting important concentrations of 7.82±0.01mgL^{-1} and 5.79±0.01mgL^{-1}, respectively. These strains are followed in a more distantly way by FG108B, with a concentration of 1.66±0.01mgL^{-1}. In the same way, Fig. 4B shows the superiority of FB105B strain in indole acetic acid (IAA) production, with an average concentration of 53.28±1.55mgL^{-1}, placing it as the top IAA producer among all the strains examined. In decreasing order are found the strains FG104-1 (23.44±0.20mg L^{-1}) and FB104B (23.18±0.21mg L^{-1}). On the other hand, the strain FG108B produced 4.68±0.30mg L^{-1} of AIA (Fig. 4B).

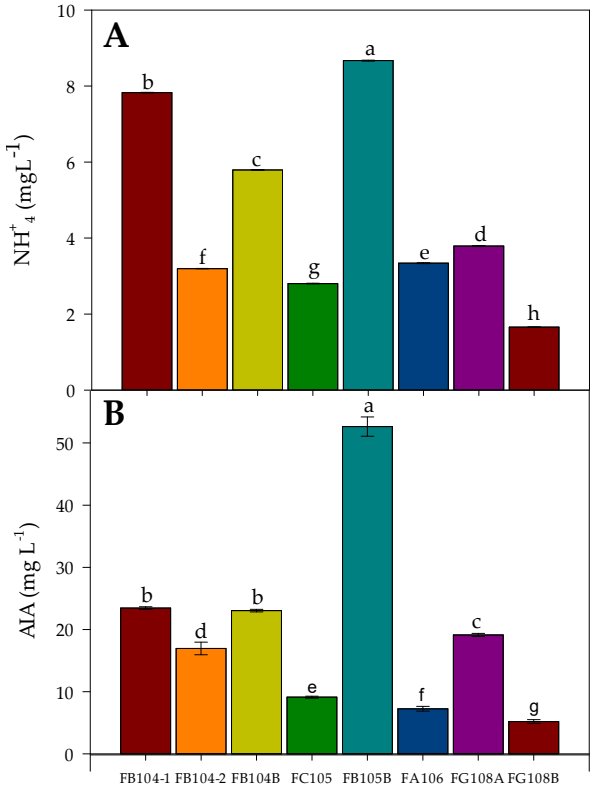

Fig. 4: (A) Atmospheric nitrogen fixation and indole acetic acid (IAA) production (B) in FLNF and IAAPB strains cultured on Burk's culture medium. Each bar represents mean (±EE) values (n = 3). Averages followed by different lowercase letters are statistically significant up to α = 0.05.

Molecular Identification

Four isolates that revealed the highest potential for P solubilization, N fixation, and IAA production were subjected to molecular identification (strains S105E, S104B, S106F, and FB105B) using 16S rRNA sequence analysis (Fig. 5). Using 16S rRNA gene sequence analysis, the isolate S105E demonstrated 96.79% identity with the Enterobacter sp. strain 71E (MH021685.1), isolate S104B was identified as similar to the *Enterobacter hormaechei* strain 0992-77 (NR042154.1), S106F as similar to the *Enterobacter cloacae* strain ATCC 13047 (NR118568.1), and FB105B as similar to the *Enterobacter cloacae* strain ATCC 13047 (NR118568.1). A phylogenetic tree of the identified isolates is shown in Fig. 6, demonstrating the relationship among these four isolates, indicating that strains S105E, S104B, and FB105B of *Enterobacter cloacae* are closely related, while the strain S104B of *Enterobacter hormaechei* is slightly separated.

Evaluation of the Effect of Inoculation with *Enterobacter cloacae* (strain S105E and FB105B) on the Development and Yield of a Chirosa cassava Crop under Greenhouse Conditions up to 120 days after Planting (DAP)

Evaluation of Biometric Parameters

Among the variety of microorganisms evaluated for their ability to solubilize phosphorus and produce IAA, specific isolates identified were inoculated by sequencing of the 16S rRNA region in cassava plants under greenhouse conditions. The strains used to prepare the biofertilizer and inoculate the cassava plants were: S105E and FB105B, both strains belonging to *E. cloacae*. There was no antagonism between the strains evaluated.

The number of leaves, plant height, dry weight of the aerial part and stem diameter under the effect of the control, chemical and biofertilizer treatments are shown in Fig. 7. The biofertilizer, at the lower concentration (10^6 CFU mL-1) did not present significant differences compared to the control (T1), however, it presented an increase of about 19.4%. The chemical treatment was similar to the biofertilizer treatment at the maximum concentration (10^8CFU mL^{-1}) with an increase of leaves in the order of 98% more. However, at the medium concentration (10^7CFU mL^{-1}) there was an increase in leaves of the 33.7% in relation to the control (Fig. 7A). Plant height exhibited a similar trend to chemical fertilization (T2) and the highest concentration of biofertilizer, where plants under 10^8CFU mL^{-1} (T5) had an average height of 93.5% and 88% greater than the control treatment, but between the treatment with 10^8CFU mL^{-1} (T5) and the treatment with chemical fertilization there was a non-significant manner increase of 3%. The treatments with medium and low concentrations of biofertilizer promoted plant growth by 10.2% and 2.4%, respectively, in contrast to the control (Fig. 7B). In addition, the treatment with 10^8CFU mL^{-1} promoted an increase of 115% and 116% in plant dry weight (Fig. 7C) and stem diameter (Fig. 7D). The medium and low treatment with 10^7CFU mL^{-1} and 10^6CFU mL^{-1} promoted an increase of 77.8% and 54.2% in aerial part dry weight (Fig. 7C), while the same treatments promoted an increase of 1.2% and 44.4% in stem diameter (Fig. 7D).

The application of T2 and T5 resulted in significant increases in root number by 38.5% and 57.7%, respectively, compared to the control treatment (Fig. 8A), the root diameter increased by 85.0% (T2) and 115.0% (T5) (Fig. 8C), and the root length grew by 58.9% (T2) and 65.37% (T1) (Fig. 8B), compared to untreated plants (control, T1). Statistically significant differences between treatments T2 and T5 versus the control are highlighted. There were no statistical differences between T2 and T5.

Statistical analysis revealed significant differences in root dry weight between T2, T5, and the control group ($p < 0.05$). T2 and T5 increased root dry weight by 170.60% and 167.51%, respectively (Fig. 9A). Similarly, in the analysis of root fresh weight, it was observed that all tested treatments showed superiority to the control treatment (T1; PFR = 93.91±1.11g) with 72.87% (162.34±0.47g in T2), 21.88 % (114.46±15.98g in treatment T3), 23.39% (115.87±0.21g in T4) and 77.54% (166.73±12.74g in T5) higher than the control treatment (Fig. 9B). T5 significantly outperformed both the control and treatments T3 and T4 in increasing root fresh weight. Statistically important differences ($P<0.05$) were found between treatments T2, T5 and T1. Regarding the percentage of starch and dry matter, a similar behavior was observed, presenting significant differences ($P<0.05$) between treatments T2, T4 and T5 compared to the control treatment. T2, T4 and T5 had increases of 56.42%, 46.92% and 55.42% respectively (Fig. 9C-D). There was no statistical difference between T2 and T5.

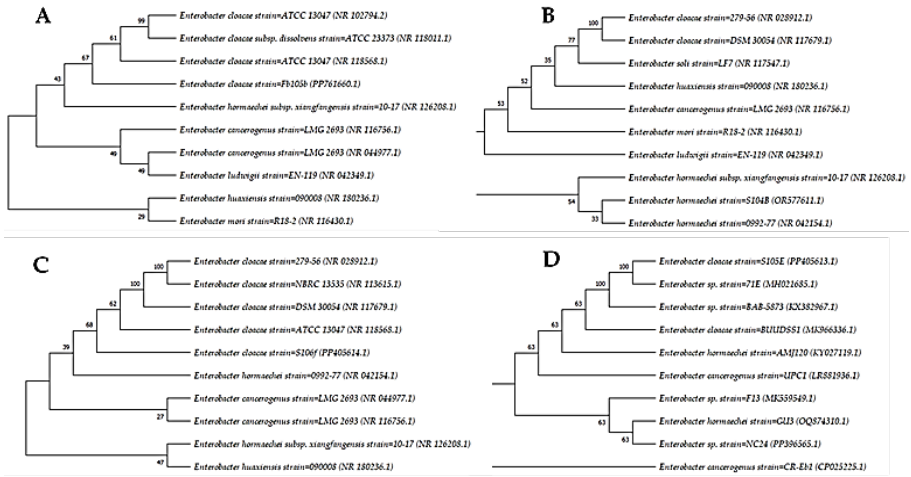

Fig. 5: Phylogenetic tree constructed using the maximum likelihood test of the bacterial isolates and homologous sequences available in the GenBank database obtained using the NCBI BLAST algorithm. (A) strain FB105B (PP761660.1); (B) strain S104B (OR577611.1); (C) strain S106F (PP405614.1); and (D) strain S105E (PP405613.1).

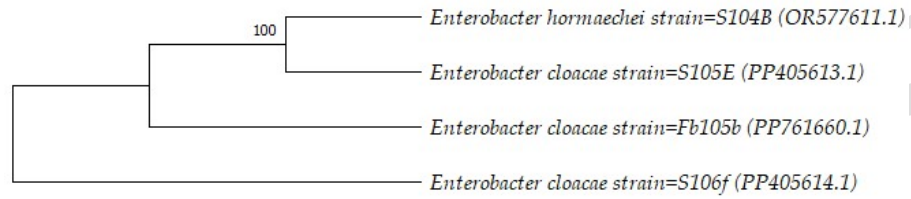

Fig. 6: Phylogenetic relationship of the 16S rRNA gene sequences of the four isolates, using the maximum likelihood test, performed with the software MEGA11.

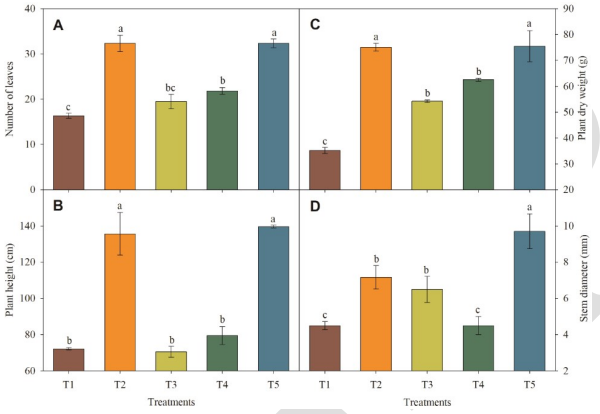

Fig. 7: Number of leaves (A); plant height (B); plant dry weight (C) and stem diameter (D) evaluated at different treatments; T1: control; T2: chemical fertilization; T3: concentration of 10^6CFU mL^{-1}; T4: concentration of 10^7CFU mL^{-1} and T5: concentration of 10^8CFU mL^{-1} under greenhouse conditions up to 120 DAP. For each characteristic, the means followed by different letters denote statistical significance ($p < 0.05$). Means (±EE) (n = 6).

Determination of Protein Percentage in Leaves of Chirosa Cassava Plants

Table 2 displays significant increases in nitrogen (N) concentrations, expressed as a percentage of protein, in the leaves of the cassava plants treated with different concentrations of biofertilizer and chemical fertilization, compared to the control treatment. The statistical analysis revealed significant differences between treatments as follows: between T2 and T3; T4 and T3, as well as between T4 and T1; and between T5, T1 and T3 ($p < 0.05$). increment in protein percentage was detected with the application of T2 and T5, being 128.55% and 218.57%, respectively.

Heatmap

The heatmap illustrates that T5 consistently yielded the best results across all evaluated characteristics.

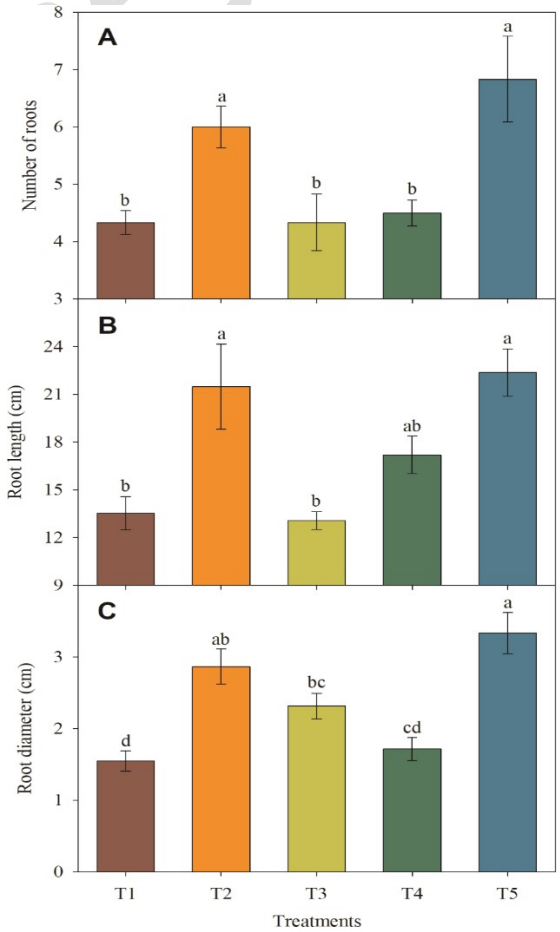

Fig. 8: (A) Root number; (B) root length; (C) root diameter; evaluated at different treatments; T1: control; T2: chemical fertilization; T3: concentration of 10^6CFU mL^{-1}; T4: concentration of 10^7CFU mL^{-1} and T5: concentration of 10^8CFU mL^{-1} under greenhouse conditions up to 120 DAP. For each characteristic, the means followed by different letters denote statistical significance ($P<0.05$). Means (±EE) (n = 6).

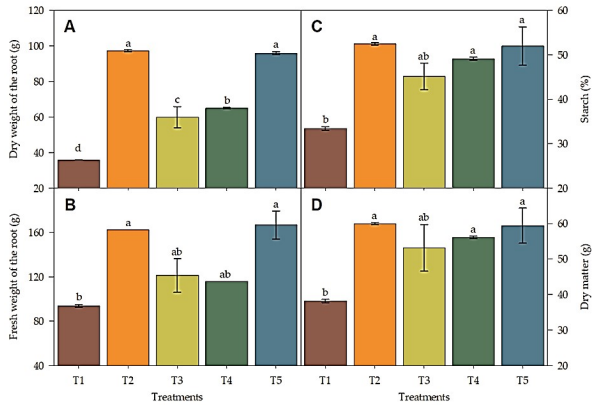

Fig. 9: (A) root dry weight; (B) root fresh weight; (C) starch percentage and (D) dry matter percentage, evaluated at different treatments; T1: control; T2: chemical fertilization; T3: concentration of 10^6CFU mL^{-1}; T4: concentration of 10^7CFU mL^{-1} and T5: concentration of 10^8CFU mL^{-1} under greenhouse conditions up to 120 DAP. For each characteristic, the means followed by different letters denote statistical significance ($p < 0.05$). Means (±EE) (n = 6).

Table 2: Percentage of protein evaluated in the leaves of Chirosa cassava plants. For each characteristic, means followed by different letters denote statistical significance ($p < 0.05$). Means (±EE) (n = 3)

Treatamient	Total Protein(%)
T1	6.78±1.07d
T2	17.64±0.86b
T3	9.34±1.67c
T4	19.81±1.06a
T5	21.61±0.61a

Values (Mean+SD) bearing different letters in a column indicate significant ($P<0.05$) differences

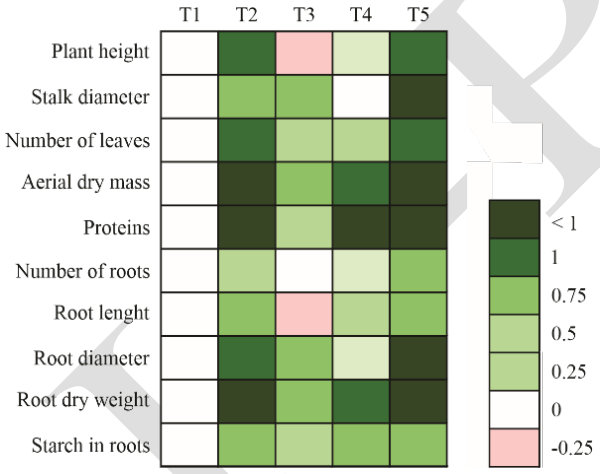

Fig. 10: Heatmap showing the variation in all evaluated characteristics in respect to non-treated plants. The color intensity denotes higher ratio between treated and non-treated plants in accordance color scale. T1, non-treated plants; T2, plants fertilized by chemical compounds, T3 to T5 denote plants treated with 10^6, 10^7, and 10^8UFC mL^{-1}, respectively.

Regarding aerial growth, T5 exhibited 2.7-, 1.6-, 1.6-, and 2.1-fold increases in plant height, stalk diameter, leaf count, and aerial dry mass, respectively, compared to non-treated plants. Additionally, T5 plants contained double the total protein of non-treated plants (Fig. 10). In root characteristics, T5 treatments resulted in 1.9-, 2.2-, 1.6-, and 2.2-fold increases in root number, length, diameter, and dry weight, respectively. Moreover, roots from T5 plants contained 3.2-fold more starch than those of non-treated plants (Fig. 10).

DISCUSSION

Global agriculture remains reliant on chemical fertilizers, whose widespread use poses significant risks to both the environment and human health. Additionally, the production of mineral fertilizers requires high energy input, accounting for approximately 2% of global greenhouse gas emissions (Beesigamukama et al., 2022). Although these inputs play a key role in modern agriculture by providing essential macronutrients such as nitrogen (N) and phosphorus (P), their excessive or inappropriate use has been linked to a wide range of negative environmental impacts. These include soil acidification, imbalances in ionic composition, reduction of beneficial microbial activity, accumulation of harmful compounds, salinisation, groundwater contamination by nutrient lixiviate and degradation of soil quality (Verardi et al., 2025). To address these challenges, biological fertilizers have emerged as a promising alternative to chemical fertilization and are increasingly valued as a fundamental strategy to promote agricultural sustainability (Zhai et al., 2022). The diversity of soil microorganisms, provided by nature, plays a crucial role in maintaining soil fertility and plant development. Within this diversity, bacteria are particularly important due to their ability to actively colonise the rhizosphere, which contributes significantly to improving plant growth and development (Cheng et al., 2024).

The majority of the phosphorus present in the soil is found in insoluble forms, with different levels of availability, which limits its uptake by plants. Furthermore, a significant portion of soluble inorganic phosphate from chemical fertilizers is quickly immobilized, limiting its uptake by crops (Aliyat et al., 2022). In this context, phosphate solubilizing bacteria (PSB) play a pivotal role by facilitating phosphorus solubilization in soil and enhancing its availability to plants. The phosphate solubilization process is critical for generating bioavailable phosphorus, which directly enhances plant growth and development. The presence of phosphate-solubilising bacteria in the soil represents a promising indicator for their application as biofertilizers in agricultural production, contributing to the sustainability of the sector. In this process, the release of organic phosphorus present in the soil is facilitated by acid phosphatases and phytases produced by rhizosphere microorganisms (Petkova et al., 2025).

In this study, *E. cloacae* strain S105E was isolated from rhizospheric soil cultivated with chirose cassava, thereby demonstrating its capacity to solubilize P. Notably, an inverse correlation was observed between P concentration and pH levels in the medium (Fig. 2). These findings are consistent with those reported by Dey et al. (2024), who observed an increase in the concentration of P solubilized by *E. cloacae* (JKD01) from the rhizospheric region of *Avicenna* sp. Similarly, Habibi et al. (2023) reported that *E. cloacae* (strains AF74 and AF42) isolated from salt-stressed alkaline soils of Afghanistan, solubilized phosphorus and were able to reduced in a significant way the pH of the medium. In a similar vein, Goyal et al. (2024)

isolated *E. cloacae* strain BHUJPVR02 from rhizospheric soil cultivated with rice, demonstrating the capacity to solubilize 943.51mg L^{-1} of P after a 48-hour incubation period. Concurrently, Ji et al. (2020) isolated *E. cloacae* (HG-1) from saline-alkali soil, exhibiting enhanced P solubilization after 72 hours of incubation. As demonstrated in previous studies, phosphorus solubilizing bacteria have been shown to reduce the pH of the medium (Liu et al., 2021; Hk et al., 2022; Maharana & Dhal, 2022). It is known the ability of phosphate-solubilizing bacteria (PSB) to dissolve phosphates from insoluble mineral sources in soil is primarily associated with the production of organic acids and chelating agents (Aliyat et al., 2022). Such production during their metabolism leads to a decrease in pH (Do Carmo et al., 2019; Chawngthu et al., 2020; Nacoon et al., 2020; Rfaki et al., 2020). New studies have reported that different PSB strains, including *E. cloacae*, excrete organic acids that convert tricalcium phosphate into mono- and dicalcium forms that are more accessible to plants, facilitating P uptake (Zúñiga et al., 2020; Arenas et al., 2022; Barin et al., 2022). In this sense, (Habibi et al., 2023) reported that *E. cloacae* (strains AF42 and AF74) produced high concentrations of shikimic acid, oxalic acid, malic acid and tartaric acid, and the secretion of these acids was associated with a significant solubilisation of P and a decrease in the pH of the culture medium. Organic acids facilitate the solubilisation of insoluble P because the carboxyl groups present in these acids play a key role in chelating cations bound to phosphate salts, such as Ca^{+2}, Fe^{+3} and Al^{+3}. This process, therefore, facilitates the release of phosphorus in a form more accessible to plants (Vasseur et al., 2021). The potential specific role of organic acids in P solubilisation observed in the present study requires further investigation, it means, it is necessary the determination of the type of organic acid and its quantification.

This study highlights the remarkable capacity of *Enterobacter cloacae* strain S105E to produce soluble phosphorus at high concentrations (2224.73±26.16mg L^{-1}). This phenomenon can be attributed to the role of phosphate-solubilising bacteria (PSB) in facilitating the dissolution and mineralization of mineral phosphates in soil through the generation of protons, the secretion of organic and inorganic acids, and the production of phosphatases (Vassileva et al., 2022). Also, they facilitate the release of phosphate ions through the chelation of metal cations by the action of organic acids (Rawat et al., 2022). The released phosphorus is adsorbed on colloidal sites in the soil, thereby reducing the adsorption capacity and intensity of this nutrient. The decrease in phosphorus retention capacity may be linked to a reduction in soil pH, as research has shown that the maximum phosphorus adsorption capacity decreases with lowering pH. In soil, a reduction in pH may decrease phosphorus fixation by colloids, favouring its availability and uptake by plants (Penn & Camberato, 2019). Furthermore, the composition of plant root exudates, which contain a wide variety of organic acids, phenolic compounds, hormones and other secondary metabolites, has been shown to favour the solubilisation of insoluble P present in the soil (Santoro et al., 2024).

Nitrogen is a vital nutrient for plant development, with biological nitrogen fixation (BNF) serving as a fundamental mechanism for its acquisition through the activity of beneficial microorganisms (Guerrieri et al., 2020). The ability of certain plant growth-promoting bacteria to produce ammonia is a beneficial trait that contributes to plant development (Prodhan et al., 2023). BNF facilitates early seedling establishment, improves soil fertility, and enhances phosphate solubilization, ensuring essential nutrient availability for plant growth (Prodhan et al., 2023). In this study, the FB105B strain of *E. cloacae* demonstrated robust growth in N-free Burk's culture medium, accompanied by a clear blue zone indicative of nitrogen fixation (Ferreira et al., 2021; Shameem et al., 2023). Strain FB105B (*E. cloacae*) demonstrated its capacity to fix atmospheric nitrogen and synthesize indole-acetic acid (IAA). These results align with Dey et al. (2024), who reported that *E. cloacae* (JKD01) produced 24.07mg L^{-1} of IAA and fixed nitrogen at a concentration of 120mg L^{-1}. Furthermore, Goyal et al. (2024) reported that *E. cloacae* (BHUJPVR02) produced 42.29mg L^{-1} of IAA and fixed 11.46 mg L^{-1} of N in ammonium form. In a similar vein, Ji et al. (2020) isolated *E. cloacae* (HG-1) from saline-alkali soil, which confirmed its capacity to produce IAA and fix N after a 72-hour incubation period. In a similar vein, Singh et al. (2022) isolated *E. cloacae* (ZNP-4) from the rhizosphere of *Ziziphus nullifera*, emphasising its capacity to produce IAA. In contrast, Panigrahi et al. (2020) underscored the potential of *E. cloacae* (MG001451) as a valuable agricultural resource due to its high IAA production and positive impact on plant growth.

The efficacy of *E. cloacae* (strains S0105E and FB105B) lies not only in their ability to solubilize inorganic phosphates and increase phosphorus availability but also in their capacity to produce growth regulators such as indole-acetic acid plays a critical role in plant development, facilitating processes such as cell division, root elongation, stress tolerance, stimulation of nitrogen fixation, and the biosynthesis of various metabolites (Meng et al., 2019). Consequently, the contribution of these bacteria to sustainable agricultural practices becomes increasingly significant. The incorporation of practices that promote the activity of PGPR (Plant Growth-Promoting Rhizobacteria) in the soil can be an effective strategy to increase phosphorus use efficiency, reduce dependence on synthetic phosphate fertilisers, and consequently mitigate the negative environmental impacts associated with their excessive use (Mitter et al., 2021).

Cassava seedlings (*Manihot esculenta*) treated with a biofertilizer composed of *E. cloacae* strains S105E and FB105B, at elevated concentrations (10^8CFU mL^{-1}), yielded a marked enhancement in the assessed biometric parameters. The enhanced growth of treated plants was further substantiated by the increase in biomass of inoculated plants. These observations are consistent with the results reported by Goyal et al. (2024), who

documented that the application *of E. cloacae* (BHUJPVR02) to rice seedlings resulted in a significant increase in root length, leaf number, and root count compared to the control. In contrast, Habibi et al. (2023) reported that inoculation trials in rice with *E. cloacae*, applied individually or in combination, promoted plant development as evidenced by increased root length and dry biomass of roots and shoots in all test plants compared to untreated control plants. In a similar vein, Ji et al. (2020) observed that inoculating wheat plants with the HG-1 strain of *E. cloacae* resulted in substantial increases in root length, plant height, fresh weight, and dry weight when compared to non-inoculated plants. In a similar study, Dey et al. (2024) reported that inoculation of *Brassica chinensis* seedlings with *E. cloacae* (JKD01) resulted in significantly increased root and shoot lengths compared to untreated seedlings. In a separate study, Al Dayel & El Sherif (2021) reported that the stem diameter and plant height of *Moringa oleifera* under saline stress increased in plants treated with *E. cloacae*. The enhancement in growth parameters, particularly root length, observed in this study can be attributed to indole-acetic acid (IAA). The promotion of cell division, elongation, and differentiation by IAA is achieved through the opening of calcium channels in the plasma membrane, thereby altering calcium homeostasis in the cytoplasm (Guerrieri et al., 2020).

Although the application of chemical fertilizers also resulted in a significant increase in nitrogen uptake, dry and fresh biomass of the plant and root compared to control plants, no significant differences were observed between biological treatments and chemical fertilization, suggesting similar effectiveness between chemical and biological fertilization. The findings of this research align with those reported by Tsegaye et al. (2022), who demonstrated that individual or consortium inoculation of *E. cloacae* significantly improved growth, yield, and nutrient uptake in grain of the two teff varieties compared to uninoculated plots. In a similar study, Khuong et al. (2024) reported that *E. cloacae* N-VT01 treatments applied in consortium resulted in higher nitrogen uptake in pineapple plants compared to controls. According to Franco et al. (2021), the application of bacterial isolates solubilizes P and also produce ammonia, which has been demonstrated to help in nitrogen accumulation that is finally delivered to the plant, thereby increasing plant biomass by enhancing root and shoot lengths, and facilitating nutrient metabolism in the soil, allowing better availability for plants (White et al., 2019). Other studies have reported that *E. cloacae* is associated with the growth and development of plants such as bell pepper (*Capsicum annuum* L. Nervin) (Nejati Sini et al., 2024), wheat (Singh et al., 2022), rice (Khumairah et al., 2022), *Oryza sativa* (rice), *Arachis hypogaea* (peanut), *Vigna mungo* (black gram), and *Brassica rapa* var. Toria (toria) (Panigrahi et al., 2020), and teff (Tsegaye et al., 2022). However, it is crucial to recognise the limited information available on the influence of *E. cloacae* on the growth and development of cassava plants; thus, this study becomes one of the first reports on this topic. In this research, it was verified that the S105E and FB105B strains of *E. cloacae* effectively promoted the growth and development of cassava plants. This finding support their potential safe as biofertilizers. Also, they stimulated plant growth at 120 DAS, showing a notable increase in root and whole-plant biomass. This effect may be attributed to the activity of ACC deaminase (ACCD), which could play a crucial role in promoting plant growth under controlled greenhouse conditions (Ortega et al., 2024). Future studies should focus on validating these results by investigating the enzymatic activity of ACC deaminase (ACCD) in these strains under varying environmental conditions.

Conclusion

The native *Enterobacter cloacae* strains S105E and FB105B demonstrated remarkable proficiency in phosphorus solubilization, nitrogen fixation, and indole-acetic acid (IAA) biosynthesis.

This study underscores the potential of *Enterobacter cloacae* strains S105E and FB105B as effective biofertilizers for enhancing growth, yield, and nutrient absorption in Chirosa cassava plants cultivated under greenhouse conditions.

Integrating *Enterobacter cloacae* strains S105E and FB105B into biofertilizers for agricultural management systems represents a substantial step toward sustainable agriculture, contributing not only to food security but also to the conservation of ecosystems and biodiversity.

These strains have demosntrated both direct and indirect abilites, to enhance plant growth offering the potential to reduce reliance on chemical fertilizers. Furthermore, with additional research, their application in real environments could be proposed as a promising strategy towards agricultural sustainability.

Financing: This research was financially supported by the Faculty of Basic Sciences, University of Córdoba, CO, Colombia, through the program: "Strategies for the sustainability of research groups, year 2019". Line of research: agricultural biotechnology. Grants: FCB-12-19.

Acknowledgment: The authors would like to thank the University of Córdoba, the Biotechnology group of the Department of Chemistry and Department of Biology-GRUBIODEQ, Professor Juana Robles, Professor Luis Alfonso Rodríguez and Professor Mauricio Begambre, to significantly contribute to the development of this manuscript.

Conflict of Interest: The authors declare that they have no competing financial interests or known personal relationships that could have influenced the work presented in this article.

Data Availability: Data can be provided on appropriate request.

Author's Contribution: First author (A.J.B.R): methodology, research, data analysis, conceptualization,

writing, revising and editing. Second author (M.E.C.G): research, conceptualization, data analysis, writing, review and editing. Third author (L.E.O.Z): visualization and revision. Fourth author (Y.J.P.P): review and editing. All authors have read and accepted the published version of the manuscript.

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