








Native Bacterial Consortium as a Biofertilizer Strategy in the Cultivation of the Fedearroz 2020 Rice Variety

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ABSTRACT

The use of plant growth-promoting bacteria is a sustainable strategy for reducing dependence on chemical fertilizers in agricultural production. This study evaluated the biofertilizer potential of native bacteria in rice plants (*Oryza sativa* L.) var. Fedearroz 2020, isolated from rhizospheric soils at the La Victoria experimental center (Monteria, Colombia). Ten samples were collected from soil at a depth of 15cm, which were mixed and used for the isolation of microorganisms in Burk's medium. Biological nitrogen fixation, phosphate solubilization, and indole-3-acetic acid production were evaluated. Five treatments were arranged in a completely randomized block design: inoculation of the bacterial consortium at concentrations of 10^{-6} , 10^{-7} , and 10^{-8} CFU mL⁻¹, chemical fertilization (urea, DAP, KCl), and a control without fertilization or inoculation. Isolate C1 showed the highest nitrogen fixation (3.831mg L⁻¹), while C2 showed the highest phosphate solubilization (3171.2mg L⁻¹) and indole-3-acetic acid production (35.99mg L⁻¹). Molecular characterization identified C1 as *Herbaspirillum* sp. and C2 as *Klebsiella* sp., with no evidence of antibiosis. Inoculation of greenhouse plants with the bacterial consortium promoted significant increases in height, root length, number of tillers, fresh and dry weight, number of panicles, number of grains, grain yield, and nitrogen content, with no statistical differences compared to chemical fertilization. These results demonstrate the potential of the *Herbaspirillum-Klebsiella* consortium as a biofertilizer alternative in rice under controlled conditions.

Keywords: *Herbaspirillum*; *Klebsiella*; Biofertilizers; *Oryza sativa*; Plant Growth-Promoting Bacteria.

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INTRODUCTION

Sustainable agricultural production requires new strategies to reduce the use of agrochemicals, especially synthetic fertilizers, whose production and application pose a threat to the environment (de Andrade et al., 2023). In recent decades, the intensive use of these inputs has been a common practice in agriculture, without considering their negative effects on human health, ecosystems, and production costs (Bora, 2022). This problem particularly affects rice cultivation, one of the

main crops in South America, not only because of its nutritional value, providing 11.5% of daily caloric intake with an average consumption of 37kg person⁻¹ year⁻¹, but also because of its economic and social importance, as it is the main source of income for nearly one million farmers in the region (Barrios-Perez et al., 2021).

Rice is currently the cereal with the largest planted area worldwide, first cultivated more than 5000 years ago (Álvarez et al., 2023). However, its high dependence on chemical fertilizers, particularly nitrogen and phosphorus, has led to the indiscriminate use of fertilizers in tropical

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soils with low availability of these nutrients (Bora, 2022). In fact, only an estimated 50% of nitrogen fertilizer is absorbed by plants, with the rest being lost through leaching or microbial denitrification (Bora, 2022). These significant losses have led to higher production costs and a range of environmental issues, including water pollution, alterations in the nitrogen cycle and risks of poisoning. A critical consequence is the emission of nitrous oxide (Ríos-Ruiz et al., 2020), a greenhouse gas with a Global Warming Potential (GWP100) that is 273 times greater than that of carbon dioxide (CO₂) on a mass-equivalent basis (de Haas & Andrews, 2022).

Following the 2010 Hague Conference on agriculture, food security, and climate change, the Food and Agriculture Organization of the United Nations (FAO) officially endorsed the concept of climate-smart agriculture (CSA). Since then, this approach has gained significant international interest and support, as its core objectives are to sustainably enhance agricultural productivity and incomes, foster adaptation to climate change, and develop strategies that effectively reduce greenhouse gas (GHG) emissions (Kavadia et al., 2020).

Given this situation, it is necessary to adopt sustainable technologies that improve crop nutrition efficiency and reduce dependence on chemical fertilizers. Among these alternatives is the use of plant growth-promoting bacteria (PGPB), beneficial soil microorganisms with the ability to improve plant development through mechanisms such as biological nitrogen fixation, phosphorus solubilization, and the production of phytohormones such as indole-3-acetic acid (Ríos-Ruiz et al., 2020). In addition, these microorganisms can act indirectly in plant protection through competition for ecological niches, the production of siderophores, lytic enzymes, and the induction of systemic resistance (Harahap et al., 2023). Through these functions, microbes assist crops in adapting to abiotic stress (water limitations, salinity, nutrient depletion, etc.) which are conditions primarily driven by climate change (Kavadia et al., 2020).

Biological nitrogen fixation is a key natural mechanism for counteracting nitrogen deficiency in soils, a condition that may intensify as an indirect consequence of climate change. The microbial communities responsible for this process are classified into two main groups: those that establish symbiotic relationships with plants and those that are free-living (Zilli et al., 2020). Symbiotic nitrogen-fixing bacteria, such as genera like *Rhizobium*, *Mesorhizobium*, *Sinorhizobium*, and *Bradyrhizobium*, are recognized as PGPB because they form associations with leguminous plants. In contrast, species of the genus *Frankia* establish mutualistic relationships with non-leguminous plants (Zilli et al., 2020). Free-living nitrogen-fixing bacteria comprise genera such as *Azotobacter*, *Azospirillum*, *Pseudomonas*, *Bacillus*, *Agrobacterium*, *Enterobacter*, *Erwinia*, and *Burkholderia* (Zilli et al., 2020). Collectively, these microorganisms enhance nitrogen availability in soils, thereby increasing crop yields.

Reducing the use of nitrogen fertilizers in agricultural systems is an essential strategy to mitigate climate change, as it lowers both direct and indirect greenhouse gas emissions. Direct nitrous oxide (N₂O) emissions from soils

are expected to decline when efficient nitrogen-fixing bacteria are applied, since they reduce the need for nitrogen fertilization. However, additional research is required to confirm this effect (Zilli et al., 2020). Evidence from soybean cultivation has shown decreased N₂O emissions after inoculation with *Bradyrhizobium* strains (Kavadia et al., 2020). Furthermore, several studies have reported positive effects of co-inoculating free-living nitrogen-fixing bacteria with symbiotic fixers, as these free-living species can facilitate nodulation and further enhance plant growth (Zilli et al., 2020).

Phosphorus (P) is the third most important nutrient for plant development, after nitrogen (N) and potassium (K). It plays a central role in almost all metabolic pathways in plant cells, including energy conversion, respiration, molecular synthesis, and photosynthesis. However, phosphorus in soils is often present in forms that plants cannot assimilate (Kavadia et al., 2020). The intensive use of phosphate fertilizers increases P deposits in the soil, which poses a significant risk of eutrophication in surface waters. Consequently, relying on increased phosphorus fertilization is not a sustainable practice. Instead, specific microbial communities, such as phosphorus-solubilizing bacteria, offer a viable alternative by solubilizing and mineralizing phosphorus, thereby improving its availability and promoting its absorption by plants (Kavadia et al., 2020).

Studies have consistently shown that plant growth-promoting bacteria (PGPB) exert significant positive effects on plant development when applied under controlled laboratory or greenhouse conditions (Chandra et al., 2020). However, their effectiveness often decreases under real field conditions, which makes their exclusive use in greenhouses a limitation. This reduction in performance may be due to their limited capacity to colonize the roots of cultivated plants or to their low competitiveness against native microbial communities already established in the rhizosphere. These factors restrict their persistence and functional activity in the natural environment of agricultural soils. Recent evidence suggests that the combined application of microbial inoculants may be more effective than the use of individual strains in improving crop growth and yield (Berg et al., 2020). The use of microbial consortia, rather than single inoculants, is thought to provide a significant advantage by exploiting the complementary properties of diverse microbial strains (Kavadia et al., 2020).

The study of native bacterial strains with biofertilizer potential represents a promising strategy for optimizing rice yields, reducing the use of agrochemicals, and promoting cleaner and more efficient agriculture, particularly in tropical regions with high dependence on external inputs. The objective of this study was to evaluate the biofertilizer potential of native bacteria in rice plants (*Oryza sativa* L.) var. Fedearroz 2020.

MATERIALS & METHODS

Sampling

Soil samples were collected from rice crops established at the Fedearroz (National Rice Fund - FNA) experimental center "La Victoria," located at kilometer 8 of

the municipality of Montería, Córdoba, Colombia, at coordinates 9°29' N and 75°49' W. Sampling was carried out using a zigzag pattern, collecting ten subsamples at a depth of 15cm. The subsamples were homogenized to form a composite sample, from which one kilogram of soil was taken for analysis. This sample was transported to the Biotechnology Laboratory of the University of Córdoba for the isolation of microorganisms and *in vitro* evaluation of growth-promoting capabilities. At the same time, a portion was sent to the Soil and Water Laboratory of the same institution for the physicochemical characterization of the soil (Fig. 1).

Isolation of Microorganisms

Ten grams of the composite soil sample were incubated in liquid Burk's medium under constant agitation for 48 hours (Hernández-Forte & Nápoles, 2017). Serial dilutions (10^{-1} - 10^{-8} CFU mL⁻¹) were then performed, and aliquots were seeded in solid Burk's medium in Petri

dishes. These were incubated at 28°C for 48h. The colonies obtained were transferred to new dishes with the same medium for purification. Verification was performed by considering the morphology of the colony (size, edge, consistency, color, and elevation) and by Gram staining (Alcarraz Curi et al., 2019).

In vitro Evaluation of Plant Growth-promoting Capabilities Nitrogen-fixing Activity

The biological nitrogen fixation capacity was evaluated qualitatively by directly inoculating each bacterial morphotype into Burk's medium. To quantitatively estimate the fixed nitrogen, an indirect measurement based on the detection of the ammonium ion produced was used, employing the Berthelot colorimetric technique (Zavala et al., 2020). Absorbance was measured at 632.9nm, using a standardized standard curve from an ammonium chloride (NH₄Cl) solution, with a concentration range of 0.8-5.6mg L⁻¹ (R² = 0.999).

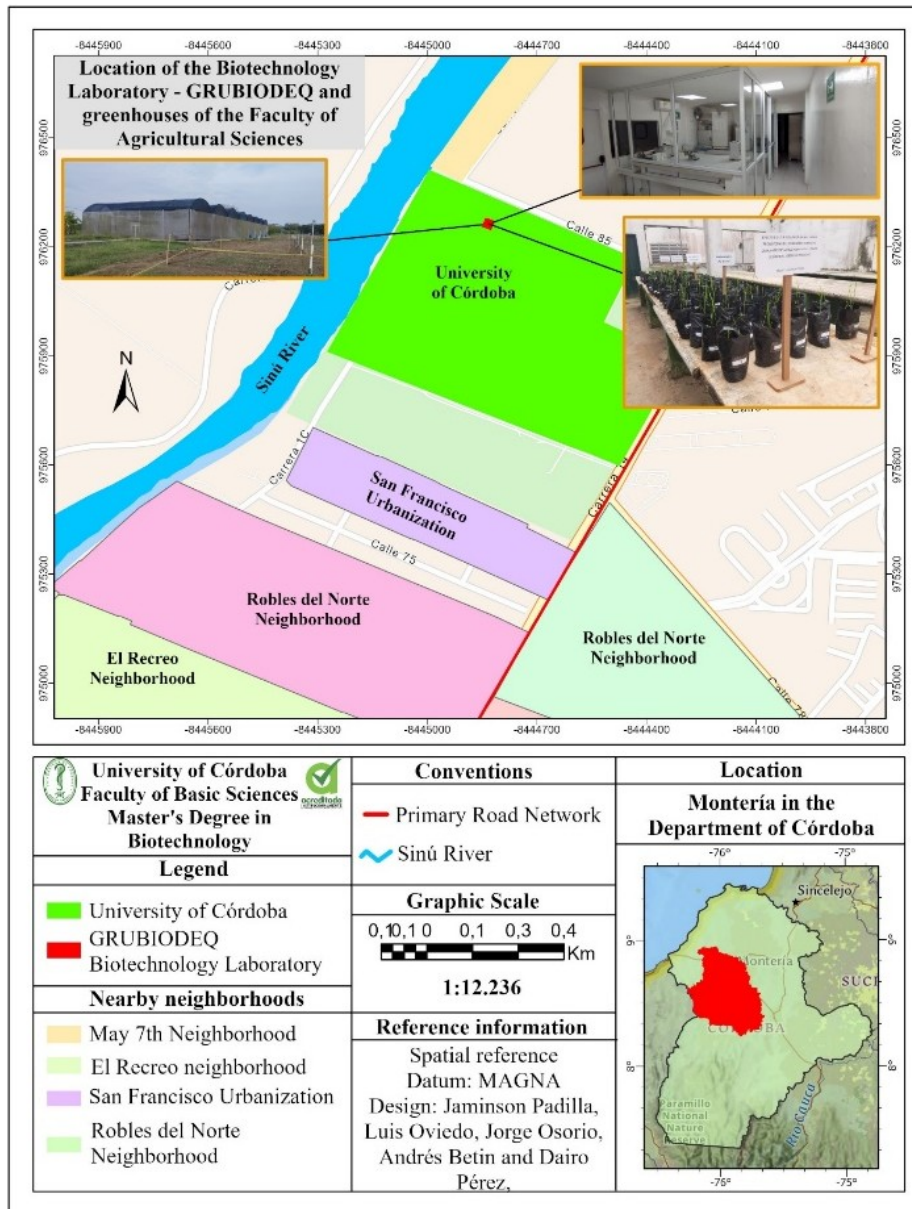


Fig. 1: Geographic location of the study area.

Phosphate Solubilization Activity

Phosphate solubilization was initially evaluated qualitatively by direct inoculation of each bacterial morphotype into NBRIP solid medium. Isolates that grew in this medium were selected for quantitative evaluation using the vanadomolybdophosphoric method (Almenares-Casanova et al., 2020). Absorbance was measured at 440nm and the results were interpreted using a standardized calibration curve based on a potassium phosphate (KH_2PO_4) solution, with a concentration range of 40-240mg L^{-1} ($R^2= 0.999$).

Production of Indole-3-acetic Acid

The microorganisms were inoculated in liquid Burk's medium supplemented with 1% tryptophan (a precursor of indole acetic acid). For the quantitative determination of the phytohormone, the Salkowski reagent was used, a colorimetric method prepared from ferric chloride and concentrated sulfuric acid (Zavala et al., 2020). Absorbance was measured at 527nm, and the estimate was made by comparison with a standardized calibration curve based on standard solutions of IAA, with concentrations of 3.2-45mg L^{-1} ($R^2= 0.999$).

Evaluation of Antagonistic Activity

The strains with the best performance in growth-promoting activities were isolated separately in Burk's liquid medium. Subsequently, the bacterial suspensions were adjusted to the 0.5 McFarland standard ($1.5 \times 10^8 \text{CFU mL}^{-1}$) and applied onto sterile filter paper discs with a 6mm diameter, each impregnated with 10 μL of the corresponding suspension. For the compatibility test, a lawn culture of each bacterium was performed in Petri dishes with Mueller-Hinton agar (4mm depth, equivalent to 25mL plate^{-1} ; pH 7.3 at 25°C), whose standard formulation includes 17.5g L^{-1} of hydrolyzed casein, 2g L^{-1} of meat extract, 1.5g L^{-1} of starch, and 17g L^{-1} of agar. Next, the discs impregnated with the other microorganism were placed equidistantly ($\geq 24\text{mm}$ center to center and $\geq 15\text{mm}$ from the edge) on the surface of the culture, allowing them to absorb for 5–10min before incubation. The plates were incubated at $35 \pm 2^\circ\text{C}$ for 48h and then the presence or absence of zones of inhibition between the isolates was evaluated, measuring their diameter in millimeters where appropriate. Discs impregnated only with sterile medium were used as a negative control, while a strain with known antagonistic activity was used as a positive control (Rojas Badía et al., 2021).

Molecular Identification of Bacteria with Greater Plant Growth-promoting Capacity

DNA extraction was performed using the commercial Wizard Genomic kit (Promega). The 16S rRNA gene region was amplified by PCR in a Biorad T100 thermocycler using universal primers 27F and 1492R. The reaction was prepared in a volume of 50 μL , as described in the protocol for the use of DreamTaq Polymerase (Thermo Fisher Scientific Inc, Waltham, United States). The amplified products were purified and sequenced at the Institute of Genetics of the National University of Colombia, and the raw sequences were visualized and manually trimmed in

Chromas v.2.6.6 to remove low-quality bases. Subsequently high-quality consensus sequences were used for subsequent analyses. Taxonomic identification was performed by alignment with reference sequences, using the BLAST algorithm against the NCBI GenBank database.

Effect of Bacterial Concentration (CFU mL^{-1}) on Rice Growth and Yield

The strains with the highest plant growth-promoting activity were cultured in Burk's liquid medium under constant agitation at room temperature for 48 hours. From these cultures, bacterial suspensions with concentrations of 10^{-6} , 10^{-7} , and $10^{-8} \text{CFU mL}^{-1}$ were prepared using the serial dilution method and colony-forming unit count (Zavala et al., 2020).

Rice seeds of the Fedearroz 2020 variety, a short-cycle variety with high tillering potential, were used. These seeds were disinfected by successive washing in 5% sodium hypochlorite, 70% ethanol, and sterile distilled water, for 4 minutes each, modifying the protocol described previously (Hernandez et al., 2021). The physiological quality of the seeds was evaluated through a germination test, using 50 seeds incubated on moistened paper for four days; the germination percentage was then calculated. This test was performed in triplicate.

Establishment of the Greenhouse Experiment

The rice seeds were inoculated for 2h with the evaluated concentrations (Sultana et al., 2024) and sown 2 cm deep in plastic bags containing soil with low organic matter content. As controls, uninoculated seeds (negative control) and seeds treated with chemical fertilization (positive control) were included, which was adjusted to the nutritional requirements of the Fedearroz 2020 variety, considering the soil analysis: 92kg ha^{-1} of nitrogen, 23kg ha^{-1} of phosphorus, and 90kg ha^{-1} of potassium. The results of the physicochemical analysis of the soil are presented in Table 1.

The experiment was carried out in the phytopathology shade house of the Faculty of Agricultural Sciences at the University of Córdoba. Growth parameters (plant height, root length, number of tillers, fresh weight, and dry weight) were evaluated at 30, 60, and 90 days after sowing. After 90 days, yield components were recorded, including the number of panicles per plant, number of grains per plant, and grain yield (g plant^{-1}). The percentage of nitrogen was also determined by the Kjeldahl method, including reagent blanks and replicates in each batch of analysis.

Statistical Analysis

A completely randomized block design was used with five treatments: T1, $10^{-6} \text{CFU mL}^{-1}$; T2, $10^{-7} \text{CFU mL}^{-1}$; T3, $10^{-8} \text{CFU mL}^{-1}$; PC, positive control (chemical fertilization); and NC, negative control (plants without inoculation or fertilization). Each block included five replicates per treatment were established in, for a total of 25 pots per block. Each pot contained three plants as a subsample. Destructive sampling was performed 30, 60, and 90 days after planting. On each sampling date, one replicate (one pot) per treatment was evaluated in each block, for a total of five replicates per treatment per date. The three plants

Table 1: Results of the physicochemical analysis of the soil used as a basis for fertilization.

Parameters	Results	Units	Uncertainty	Technique
pH	4.9	Unidades de pH	NA	Potentiometry
Total organic carbon	1.00	%	NA	Titrimetric
Available sulfur	2.2		NA	Turbidimetric
Available phosphorus	1.8	mg kg ⁻¹	NA	Colorimetric
Exchangeable calcium	0.97		NA	Atomic absorption spectrophotometry
Exchangeable magnesium	0.740		NA	
Exchangeable potassium	0.068		NA	Emission spectrometry
Exchangeable sodium	< LOQ		NA	
ECEC	2.2	Cmol(+) kg ⁻¹	NA	Sum of cations
Exchangeable acidity	0.40		NA	KCl 1.0 M
Available copper	0.62		NA	
Available iron	65.9		NA	Atomic absorption spectrophotometry
Available zinc	0.71		NA	
Available manganese	4.7	mg kg ⁻¹	NA	
Available boron	0.01		NA	Hot water- oven
Textural class	Sandy-loamy			Hydrometer

Abbreviations: ECEC, effective cation exchange capacity.

in each pot were measured and their values were averaged to obtain a single data point per experimental unit. To ensure independence between time points, different pots were used for each destructive sampling. The response variables were subjected to Kolmogorov-Smirnov normality tests, analysis of variance using Fisher/Kruskall-Wallis ANOVA, and Tukey and Dunn post hoc tests.

Statistical analyses were performed using the Graph Pad Prism statistical package for Windows version 5.01. The significance criterion was set at $P < 0.05$.

RESULTS

Burk's selective medium allowed the growth of seven different bacterial morphotypes, from which three strains (C1, C2, and C3) with morphological characteristics compatible with diazotrophic bacteria were purified. These strains showed visible colonies after 24h of incubation in solid medium, with varying sizes. Strain C1 showed small (1mm), circular, opaque, whitish, raised, smooth colonies with entire edges and a mucoid consistency; it corresponded to Gram-negative bacilli. C2 presented large (>3mm), transparent, circular, smooth, convex, and mucoid colonies, also with Gram-negative bacillary morphology. C3 developed large, shiny, circular, smooth, convex, and creamy colonies, with Gram-positive bacillary forms in Gram staining.

In vitro Evaluation of Plant Growth-promoting Capabilities

The quantitative results of these functional activities of bacterial isolates C1, C2, and C3 are presented in Table 2. These demonstrated their ability to fix atmospheric nitrogen, solubilize inorganic phosphates, and produce indoleacetic acid (IAA). The nitrogen fixation test revealed that isolate C1 had the highest ammonium production (3.831mg L⁻¹). In terms of phosphorus solubilization and IAA biosynthetic capacity, C2 recorded the highest values with 3171.2mg L⁻¹ and 35.99mg L⁻¹, respectively. Meanwhile, C3 showed the lowest concentrations in the analyses.

Since isolates C1 and C2 showed the highest levels of plant growth-promoting activity, their compatibility was evaluated using an antagonism test. After the incubation period, no inhibition halos or restricted growth zones were observed, indicating the absence of antibiosis and confirming the compatibility between the two isolates.

These results allowed the formation of a bacterial consortium between the two isolates.

Table 2: Plant growth-promoting activity of bacterial isolates expressed as ammonium (Berthelot method), soluble phosphate (vanadomolybdophosphoric method), and IAA (Salkowski method). Calibration curves showed $R^2 > 0.99$. Data represent the mean of three replicates

Isolate	Ammonium concentration (mg L ⁻¹)	Phosphate concentration (mg L ⁻¹)	IAA concentration (mg L ⁻¹)
C1	3.83±0.30	1636.83±0.15	16.20±1.23
C2	2.50±0.50	3171.23±0.15	35.99±1.50
C3	1.46±0.17	1599.20±1.20	14.91±0.73

Molecular Identification of Bacteria with Greater Plant Growth-promoting Capacity

Partial sequences of the 16S rRNA gene, with sizes of 485 and 775bp, were amplified for isolates C1 and C2, respectively. Isolate C1 was identified as *Herbaspirillum* (98.76% identity, 100% coverage), while C2 corresponded to the genus *Klebsiella* (98.97% identity, 100% coverage). Phylogenetic analyses, based on the dendrograms generated, showed a close relationship with other bacteria of the genera *Herbaspirillum* and *Klebsiella* (Fig. 2). The chromatograms obtained revealed peaks corresponding to the nucleotides of the DNA fragments of both isolates. The sequences were reported in GenBank with the code PP935261 for C1 and PP935262 for C2.

Although some species of *Klebsiella* are recognized as opportunistic human pathogens, the strains used in this study were isolated from agricultural soils and characterized as plant growth-promoting bacteria (PGPB). All experimental procedures were classified as biological risk type 1 and were carried out under appropriate biosafety conditions, following institutional regulations.

Effect of Bacterial Concentration on Rice Growth and Yield

Serial dilutions were used to obtain concentrations of 10⁻⁶, 10⁻⁷, and 10⁻⁸CFU mL⁻¹ of the bacterial consortium, which were used to inoculate rice seeds to evaluate their effect on growth and yield parameters. The seed germination rate was 90%, indicating adequate physiological quality for establishing the trial under greenhouse conditions. The seeds were sown in early December 2023, with emergence evident on the fifth day in all treatments, with uniform germination and adequate initial development.

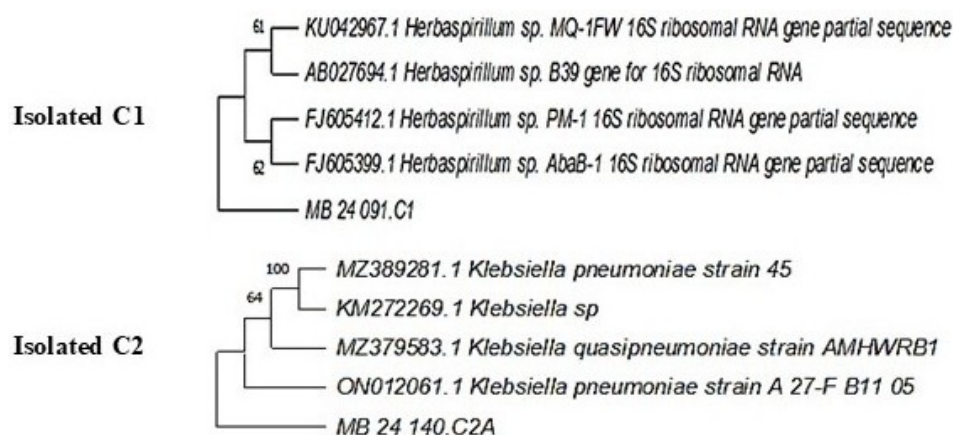


Fig. 2: Phylogenetic tree of isolates C1 and C2 and their relationships with bacterial species of the genera *Herbaspirillum* and *Klebsiella*.

Growth Parameters

The results for height, root length, number of tillers, fresh weight, and dry weight of rice plants are shown in Fig. 3. During the first 30 days, the average height of the plants showed significant differences between the treatments evaluated ($P=0.004$). Treatment T1 showed higher values compared to T2, PC, and NC. At 60 days, significant differences were observed ($P=0.001$), with treatments T1, T2, and T3 showing greater heights than PC and NC. At 90 days, the data did not follow a normal distribution, but significant differences between treatments were still identified ($P=0.005$). In this period, treatments T1, T2, T3, and PC did not differ from each other, but all significantly exceeded NC, which showed the lowest average height (Fig. 3A).

The average root length at 30 days showed significant differences between the treatments evaluated ($P=0.048$), with treatment T1 standing out with higher values than NC. At 60 days, there were significant differences ($P=0.018$), with all treatments with bacterial inoculation and chemical fertilization significantly outperforming the negative control, with no differences between them. At 90 days, the significant differences between treatments remained ($P<0.001$); treatments T1, T2, T3, and PC showed similar lengths to each other and higher than NC, which had the lowest root growth (Fig. 3B).

No tillering was observed in any of the treatments evaluated in the first 30 days. After 60 and 90 days, there were significant differences between treatments in both periods ($P=0.002$ and $P<0.001$, respectively). In both cases, treatments T2 and PC had the highest average tillers per plant, being statistically superior to NC, which did not produce tillering throughout the crop cycle (Fig. 3C).

The average fresh weight of plants during the first 30 days did not show significant differences between treatments ($P=0.106$). At 60 days, significant differences ($P=0.015$) were observed between treatments T2 and PC and NC. At 90 days, no significant differences were recorded between treatments ($P=0.084$); however, treatments with bacterial inoculation and chemical fertilization maintained higher weights compared to the negative control (Fig. 3D).

The average dry weight of the plants at 30 days showed significant differences between treatments ($P=0.008$), with treatment T1 being significantly higher

than NC. At 60 and 90 days, significant differences ($P=0.002$ and $P=0.014$, respectively) were observed between the inoculated treatments and PC with respect to NC (Fig. 3E).

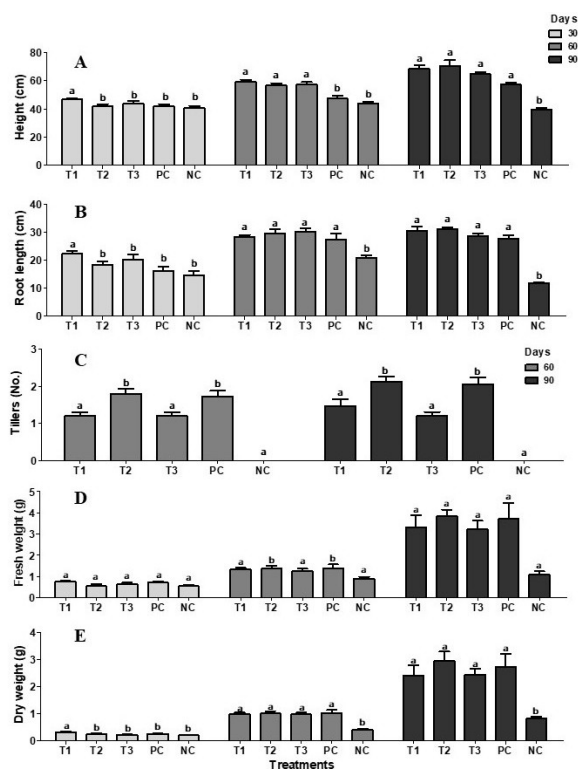


Fig. 3: The effect of bacterial concentration (CFU mL^{-1}) on rice growth. T1, 10^6CFU mL^{-1} , T2, 10^7CFU mL^{-1} , T3, 10^8CFU mL^{-1} ; PC, Positive control; NC, Negative control. a,b Statistical differences with respect to the negative control. $n=5$ replicates per treatment. Error bars indicate the standard deviation. ANOVA + Tukey's test: Root length (cm) and fresh weight (g). Kruskal-Wallis + Dunn's test: Height (cm), tillers (No.), and dry weight (g).

Performance Parameters

The results of the performance parameters evaluated 90 days after sowing are presented in Fig. 4. The number of panicles per plant showed that T2 and PC presented significant differences with respect to the other treatments ($P=0.001$) (Fig. 4A). Meanwhile, T1, T2, T3, and PC showed differences from NC for the variable number of grains per plant ($P<0.007$). Negative control recorded the lowest

average, while the treatments with bacterial inoculations and chemical fertilization showed higher values that were statistically like each other (Fig. 4B).

Grain yield (g plant^{-1}) showed significant differences between treatments ($P < 0.001$). The treatments with bacterial inoculation and chemical fertilization did not show statistical differences between them, but all recorded significantly higher weights than the treatment without inoculation or fertilization (Fig. 4C). Similarly, the results for nitrogen content showed that T1, T2, T3, and PC were significantly higher than NC ($P < 0.001$) (Fig. 4D). This suggests that the nitrogen provided by the bacteria is comparable to that provided by fertilization.

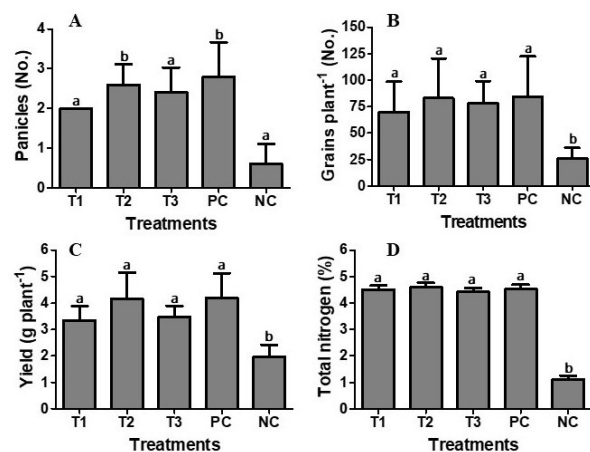


Fig. 4: Effect of bacterial concentration (CFU mL^{-1}) on rice yield. T1, 10^6CFU mL^{-1} ; T2, 10^7CFU mL^{-1} ; T3, 10^8CFU mL^{-1} ; PC, Positive control; NC, Negative control. a, b Statistical differences with respect to the negative control. $n=5$ replicates per treatment. Error bars indicate the standard deviation. ANOVA + Tukey's test: grains plant^{-1} (No.), yield (g plant^{-1}), and total nitrogen (%). Kruskal-Wallis + Dunn's test: panicles (No.).

DISCUSSION

Several studies have documented bacteria with biofertilizer potential in the rice rhizosphere. For example, isolates from the Cuban rice cultivar "INCA LP-7" included 14 Gram-negative non-spore-forming *coccobacilli* (Santa Cruz-Suárez et al., 2021). A separate study in different regions of Jammu and Kashmir (India) characterized 15 diazotrophic bacteria from rice rhizospheres, five of which exhibited plant growth-promoting traits. Gram-negative strains predominated, a characteristic that reflects their adaptability, efficiency in rhizosphere colonization, and diverse mechanisms for promoting plant development (Avsar, 2024).

In vitro Evaluation of Plant Growth-promoting Capabilities

Ammonium concentrations in the bacterial isolates from in this study were higher than those reported for rhizospheric actinobacteria by Condori-Pacsi et al. (2019), and similarly surpassed the concentrations reported by Pérez-Cordero et al. (2018) for endophytic bacteria from colosoana grass. The concentrations reported in these studies ranged from $0.03\text{--}0.63 \text{mg L}^{-1}$ for actinobacteria and $0.23\text{--}1.17 \text{mg L}^{-1}$ for endophytic bacteria. These results

demonstrate the ability of the microorganisms evaluated to fix atmospheric nitrogen through the action of the nitrogenase enzyme complex, responsible for catalyzing the conversion of molecular nitrogen into ammonium (Fadiji & Babalola, 2020).

Phosphate levels observed in the bacterial isolates in this study were higher for rhizospheric bacteria in rice, whose maximum value was 10.22mg L^{-1} , than those reported by Gupta et al. (2022) similarly, they exceeded the concentrations of endophytic bacteria reported by Pérez-Cordero et al. (2018), which ranged from $1112\text{--}1530 \text{mg L}^{-1}$. Phosphate solubilization is a key process for plant nutrition, facilitated by bacteria that mobilize phosphorus through the release of low molecular weight organic acids and by the action of phosphatase enzymes that degrade organic phosphate compounds (Khatoun et al., 2020).

The resulting indoleacetic acid (IAA) concentrations were higher than those reported in bacteria isolated from colosoana grass, with values between $1.15\text{--}12.30 \text{mg L}^{-1}$, indicating that even low concentrations of this phytohormone can stimulate plant growth (Pérez-Cordero et al., 2018). However, other studies have reported higher levels than those achieved by isolate C2 in this research. Native bacterial strains isolated from the rhizosphere of *Coffea* had concentrations of up to 72.35mg L^{-1} (Alcarraz Curi et al., 2019). It should be noted that excessive levels of IAA can have negative effects on growth, such as inhibiting stem and root elongation (Lam et al., 2020).

Characteristics of *Herbaspirillum* and *Klebsiella* as PGPB

Herbaspirillum is a metabolically versatile microorganism with multiple mechanisms associated with promoting plant growth, including biological nitrogen fixation and phytohormone production. In addition, this genus has been shown to stimulate H^+ vacuolar pumps, which contributes to improving photosynthetic efficiency in rice plants (Ramos et al., 2020). All *Herbaspirillum* species are diazotrophs and establish close associations with plants, especially those of the Poaceae family, where they have been used as biofertilizers with promising results (Ramos et al., 2020).

Klebsiella is a bacterial genus widely reported as a plant growth promoter due to its ability to fix nitrogen, solubilize phosphates, and produce indole-3-acetic acid (Schmidt-Durán & Chacón-Cerdas, 2021). In some studies, it has shown higher nitrogenase activity than genera such as *Azospirillum*, highlighting its potential as a nitrogen fixer in grasses. It has been proven to be effective in improving nitrogen nutrition in barley crops (Sharma et al., 2021), as well as in promoting rice growth under adverse conditions, such as salt stress and pollution, increasing parameters such as germination, biomass, root and stem length, and chlorophyll content (Mitra et al., 2018; Girma et al., 2022).

Effect of Bacterial Concentration (CFU mL^{-1}) on Rice Growth and Yield

The behavior observed in plant height suggests that plant growth-promoting bacteria (PGPB) have a positive effect from the early stages of development, possibly

associated with their ability to rapidly colonize the rhizosphere and stimulate cell elongation. This effect may be mediated by the production of phytohormones such as indole-3-acetic acid (IAA), which has been widely recognized for its role in modulating plant growth, especially in relation to stem elongation (Ramos et al., 2020).

The fact that bacterial inoculations have induced growth comparable to that of plants treated with chemical fertilization reinforces the hypothesis of the biofertilizer potential of these microorganisms. This ability to replace or complement conventional nutrition is related to the increase in height of plants inoculated with PGPB, processes such as biological nitrogen fixation and the production of phytohormones, especially IAA (Corrales-Lozada et al., 2020). This result aligns with the findings of Li et al. (2020), who demonstrated inoculating rice plants with two strains of *Klebsiella* increased their height under adverse conditions, highlighting the adaptability and effectiveness of these bacteria in stressful environments. In forage systems, a positive response in the height of ryegrass (*Lolium spp*) has also been observed when inoculating seeds with a consortium of *Herbaspirillum* and *Pseudomonas*, reaffirming the potential of these microbial associations as a biotechnological strategy to promote sustainable plant development (Cortés-Patiño et al., 2022).

The similarity in root system response between plants treated with PGPB and those receiving chemical fertilization has been pointed out as evidence of the potential of these microorganisms to act as functional biofertilizers. This effect has been widely documented; PGPB activity has been linked to longer root length, attributed to the production of phytostimulant compounds such as IAA. This type of auxin promotes both root formation and elongation, which has a positive impact on nutrient absorption and plant development (Corrales-Lozada et al., 2020).

The pattern observed is consistent with previous reports where inoculation with bacterial consortia favors root system expansion. Results showing longer root lengths in rice plants inoculated with two PGPB bacteria were attributed to the hormonal stimulation exerted by auxins (Hernández et al., 2023). However, the effects may vary depending on the inoculum or plant species, such as the decrease in main root length in ryegrass inoculated with *Herbaspirillum* and *Pseudomonas*, possibly due to an overproduction of indole compounds (Cortés-Patiño et al., 2022). Likewise, the effectiveness of certain strains may be linked to complementary functions, such as phosphate solubilization. In this sense, *Herbaspirillum* in combination with insoluble sources of phosphorus promoted more pronounced root development than *Pseudomonas*, due to its ability to release nutrients and generate hormonal compounds that promote growth (Almenares-Casanova et al., 2020).

The absence of tillering in the early stages contrasts with other studies that have reported early responses to the use of growth-promoting bacteria in different rice varieties. This discrepancy could be related to the specific interaction between the variety used and the bacterial consortium evaluated, as well as to physiological factors

inherent to the phenological cycle (Barzan et al., 2023). The presence of tillering in some inoculated treatments, as well as in the treatment with chemical fertilization, coincides with what has been described by various authors who associate this characteristic with adequate nitrogen supply and the hormonal stimulus exerted by PGPB (Barzan et al., 2023; Harahap et al., 2023). In particular, the microbial consortium could favor physiological processes related to the development of tillers, provided that an effective interaction with the host plant is established. The fact that some treatments failed to induce noticeable tillering suggests that factors such as inoculum concentration or the adaptability of the strains used could influence the expression of this agronomic trait (Barzan et al., 2023).

The fresh weight of the plants appeared to be influenced by the number of tillers rather than by height and root development. In treatments where greater tillering was observed, higher fresh weight values were also recorded, suggesting that this morphological component could favor biomass accumulation by increasing the number of shoots and the amount of photosynthetically active tissue, which would facilitate greater uptake and utilization of resources (Tian et al., 2022). In advanced stages of cultivation, the absence of significant differences between treatments could be associated with the transition of plants to reproductive phases, in which vegetative growth stabilizes or even decreases due to processes such as leaf senescence or the maturation of reproductive organs. This pattern has been noted in previous studies as a natural factor that limits the continuous increase in fresh weight, regardless of the treatment applied (Barzan et al., 2023).

Bacterial inoculation seems to favor greater accumulation of dry biomass compared to plants that did not receive any type of fertilization, even from early stages of development. These results may be due to the consortium's ability to dissolve soil nutrients, increasing dry matter production (Ríos-Ruiz et al., 2020), and to mechanisms such as the production of IAA, which regulates cell enlargement and can stimulate the development of lateral roots and root hairs, thus increasing the dry weight of the plant (Harahap et al., 2023). This agrees with Hernandez et al. (2021), who observed the increasing dry weight of plants inoculated with species of the genus *Klebsiella*, reaching up to 20.52g, in contrast with uninoculated treatments, which was attributed to the enhanced availability of nitrogen, phosphorus, and potassium in the soil.

The number of panicles per plant was favored in treatments with bacterial inoculations, especially in those where greater tillering was also observed, which is consistent with the role of this morphological characteristic as a key factor in determining the number of fertile stems (Arias-Badilla et al., 2020). This is consistent with the findings of Harahap et al. (2023), who reported that inoculation of rice with *Delftia tsuruhatensis* D9, *Delftia sp.* strain MS2As2, and *Bacillus sp.* increased the number of panicles by 163.63% compared to the control. This highlights the positive effect of growth-promoting bacteria on the reproductive performance of the plant. This

increase is mainly attributed to biological nitrogen fixation and phytohormone production, which favor the development of reproductive structures.

The number of grains per plant showed a favorable response to bacterial inoculation, with values like those achieved with chemical fertilization. This trend suggests that the application of the *Herbaspirillum-Klebsiella* consortium could contribute to nutrient supply and reproductive development of the crop. Previous research supports this observation, demonstrating that the application of a liquid biofertilizer formulated with *Delftia tsuruhatensis* D9, *Delftia sp.* strain MS2As2, and *Bacillus sp.* significantly increased the number of grains per mound, with a 115.93% increase in yield compared to the treatment without biofertilizer, under the same dose of nitrogen fertilizer (Harahap et al., 2023). This effect is attributed to the ability of microorganisms to fix atmospheric nitrogen and improve nutrient absorption, promoting flower formation and grain filling. Likewise, it was shown that inoculation with a consortium of *Rhizobium* and *Methylobacterium* promoted an increase in the number of grains per panicle in rice, an effect associated with improvements in plant physiology and morphology (Priya et al., 2019). Similarly, increases in the number of seeds per ear were reported after inoculation with species of the genus *Methylobacterium* (Nysanth et al., 2019).

Grain weight in rice is closely related to the availability of nutrients during filling, particularly nitrogen, which is essential for protein synthesis and biomass accumulation in the seed (Barzan et al., 2023). In this study, treatments with bacterial inoculations showed a positive effect on this variable, significantly outperforming the treatment without input. This result is associated with the action of the *Herbaspirillum-Klebsiella* bacterial consortium, whose ability to fix nitrogen and solubilize nutrients favors root absorption and subsequent translocation to reproductive organs, thus contributing to higher grain weight (Bertham et al., 2021; Harahap et al., 2023). Several studies have supported the potential of these plant growth-promoting bacteria to improve grain yield, reporting that the application of a liquid biofertilizer formulated with nitrogen-fixing bacteria increased grain yield by 102% compared to the treatment without biofertilizer, under the same nitrogen dose (Harahap et al., 2023). This effect is attributed to the biological activity of microorganisms, which promote nitrogen fixation and improve the absorption and availability of nutrients in the soil, thus enhancing yield components when combined with nitrogen fertilization. Similarly, inoculation with *Streptomyces* strains was shown to improve rice yield, reinforcing the effectiveness of plant growth-promoting bacteria under control conditions (Ngalimat et al., 2022). Similar findings have been documented, reporting 20-30% increases in grain yield after the application of microbial consortia that included genera such as *Azotobacter*, *Azospirillum*, *Pseudomonas*, and *Bacillus*, compared to treatments without inoculation (Tualar et al., 2018).

The improvement in nitrogen content can be attributed to the action of the microorganisms in the

bacterial consortium, which can fix atmospheric nitrogen and facilitating its assimilation during the vegetative stage of the crop (Domínguez et al., 2020). In fact, it has been pointed out that an adequate range of nitrogen in rice is between 2.6-3.2%, so the values obtained in the inoculated treatments reflect an even higher nutritional level (IPek, 2019). Similar results reported an increase in nitrogen content to 5.7% after inoculating *Herbaspirillum seropedicae* in rice, in contrast to 2.7% in the treatment without inoculation, which showed visual symptoms of deficiency (Ramos et al., 2020). These results coincide with performance in variables such as number and weight of grains, reinforcing the key role of biological nitrogen fixation in crop productivity, especially in scenarios with reduced or absent chemical fertilization (Ramos et al., 2020).

Conclusion

Isolated native strains of *Herbaspirillum* and *Klebsiella*, exhibit key functional capabilities such as biological nitrogen fixation, phosphate solubilization, and indole-3-acetic acid production, fundamental attributes of plant growth-promoting bacteria. Inoculation with different concentrations of the *Herbaspirillum-Klebsiella* bacterial consortium promotes significant improvements in growth and yield parameters of Fedearroz 2020 rice variety under greenhouse conditions, comparable to those obtained with conventional chemical fertilization. These results suggest that the application of biofertilizers based on these bacteria could reduce dependence on synthetic fertilizers, contributing to more sustainable and efficient agricultural practices.

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Author's Contribution: JPC and LOZ conceived and designed the experiment. JPC carried out field sample collection and laboratory analyses. LOZ and DPS supervised and coordinated the experimental development. JOM performed data processing and statistical analysis. The manuscript was written by JPC,

JOM, and ABR. All authors participated in critical reviews of the content and approved the final version of the document.

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