Isolation and Functional Characterization of Plant Growth-Promoting Bacteria Associated with Peanut (Arachis hypogaea L.) Cultivation, Revealed by 16S rRNA Sequencing and BOX-PCR

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Isolation and Functional Characterization of Plant Growth-Promoting Bacteria Associated with Peanut (Arachis hypogaea L.) Cultivation, Revealed by 16S rRNA Sequencing and BOX-PCR

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ABSTRACT:
Soil is a dynamic ecosystem rich in microorganisms that play essential roles in plant health, nutrient cycling, and organic matter decomposition. In Brazil, peanut (*Arachis hypogaea* L.) cultivation is concentrated in the state of São Paulo, where crop rotation systems contribute to soil conservation, increased fertility, and erosion control. Through symbiosis with microorganisms such as *Rhizobium*, peanut enhances nutrient acquisition and plant development, thereby reducing dependence on chemical fertilizers. Despite its agronomic relevance, information on the taxonomic and functional diversity of microorganisms associated with peanut cultivation remains limited. This study aimed to isolate, characterize, and select microorganisms with potential to promote peanut growth and nutrition. Samples of nodules, roots, and soil were collected during the dry and rainy seasons. A total of 92 bacterial isolates were obtained and qualitatively evaluated for indole-3-acetic acid (IAA) production, phosphate solubilization, and siderophore production. Selected strains were subjected to IAA quantification and molecular identification by 16S rRNA gene sequencing. Genomic diversity was analyzed using BOX-PCR. High variability in plant growth-promoting traits was observed. The best-performing isolates were selected to establish a collection of promising strains for future in planta and field studies. This integrated approach improves understanding of the soil microbiota associated with peanut and supports the development of more sustainable agricultural strategies.

KEYWORDS: Soil health, Arachis hypogaea, Microorganisms, taxonomy

1. INTRODUCTION

Peanut (*Arachis hypogaea* L.) is a species native to South America and belongs to the genus *Arachis*, which comprises approximately 80 species distributed across diverse environments (Bertioli et al., 2011; Nascimento et al., 2018). In Brazil, the crop shows broad edaphoclimatic adaptability and high economic importance. More than 90% of national production is concentrated in the state of São Paulo, especially in the Ribeirão Preto region, where peanut is frequently included in crop rotation systems, particularly during sugarcane field renovation(Peixoto et al., 2008; Barbosa et al., 2014; CONAB, 2022).

As a legume, peanut establishes symbiotic associations with soil microorganisms that play a fundamental role in plant nutrition and growth promotion. Biological nitrogen fixation, carried out in association with bacteria of the genus *Rhizobium*, contributes significantly to reducing dependence on nitrogen fertilizers and enhances the sustainability of agricultural systems (Nannipieri et al., 2017; Wang et al., 2017; Camargo et al., 2019). In addition to nitrogen fixation, plant growth-promoting microorganisms can contribute to nutrient solubilization,

phytohormone production, siderophore production, phytopathogen suppression, and increased plant tolerance to abiotic stresses such as drought and salinity (Gomes et al., 2016; Wang et al., 2017; Da Silva et al., 2022).

Advances in metataxonomic approaches have expanded knowledge of the diversity of prokaryotes associated with plants. However, for peanut cultivation, information on the microbial communities presents in cultivation areas and the biotechnological potential of native strains remains limited. Currently, only one strain is registered for commercial use in Brazil (SEMIA 6144), highlighting the need for studies focused on the isolation, characterization, and selection of microorganisms adapted to regional cultivation conditions.

Given this context, the present study aimed to isolate and identify microorganisms associated with peanut cultivation that have the potential to promote plant growth and nutrition, with the goal of reducing dependence on chemical fertilizers.

2. MATERIAL AND METHODS

2.1 Sample Collection and Processing

Soil and plant samples were collected in a peanut cultivation area at Universidade Estadual Paulista "Júlio de Mesquita Filho" (UNESP), Jaboticabal Campus, SP, Brazil (21°08'38.3"S; 48°10'32.5"W; 606 m above sea level). Sampling was conducted at two distinct time points, representing the rainy season (March 2025) and dry season (September 2024).

Three sampling areas were established, each separated by at least 1 km, to capture greater spatial variability. Cultural practices followed those adopted in the experimental area and were recorded in the field history.

Approximately 100 g of soil was collected at each point from a depth of up to 20 cm using a sterilized aluminum spatula and stored in 50 mL Falcon tubes.

Plants were collected manually, with the aerial part discarded. Roots were washed under running water, and secondary roots bearing nodules were separated. Plant material was surface-disinfected with 95% ethanol for 1 min, followed by 2% sodium hypochlorite for 5 min and five washes with autoclaved ultrapure water.

Nodules and roots were macerated separately in Yeast Mannitol medium (YML) following Vincent (1970). Bacterial isolation was performed on modified Yeast Mannitol Agar (YMA) from serial dilutions of soil, root, and nodule samples, with the addition of cycloheximide (100 mg L^{-1}).Plates were incubated at 30 °C until colonies appeared.

Finally, isolates were stored in 20% glycerol and kept at −80 °C. A total of 92 bacterial isolates were obtained from soil, roots, and nodules and were classified as shown in Table 1.

Table 1: Classification of isolates according to origin and climatic condition at the time of collection

Isolation Code	**Characteristic**
NS	Isolates from nodules/roots, dry season (September/2024)

Isolation Code	Characteristic
SS	Isolates from soil, dry season (September/2024)
NC	Isolates from nodules/roots, rainy season (March/2025)
SC	Isolates from soil, rainy season (March/2025)

2.2 Cell Density Standardization

To standardize the cell density of the isolates at 10^8 CFU mL^{-1}, different initial OD_{600} values were used for each isolate after 48 h of cultivation.

2.3 Indole-3-Acetic Acid (IAA) Production

Isolates were cultivated in YML medium supplemented with L-tryptophan (1.0 g L^{-1}) at 30 °C for 96 h under agitation at 150 rpm. After centrifugation, the supernatant was used to evaluate IAA production using a colorimetric method with modified Salkowski reagent (Gordon & Weber, 1951).

Initial screening was performed by visually assessing color intensity, which was classified as low, high, or very high production. Analyses were conducted in triplicate and included a positive control and a blank.

Selected isolates were subjected to spectrophotometric quantification using an IAA standard curve (5–125 µg mL^{-1}).

2.4 Statistical Analysis

Data obtained from indole-3-acetic acid (IAA) quantification were analyzed by analysis of variance (ANOVA) to determine whether significant differences existed among the evaluated isolates. When significant effects were detected, means were compared using Tukey's test at a 5% significance level. These statistical methods improve the reliability of experimental interpretation and are widely used in microbiological and agronomic studies involving treatment comparisons. (GOMES, 2009; FERREIRA, 2018).

2.5 Phosphate Solubilization

Phosphate-solubilizing ability was evaluated in modified NBRIP medium (Nautiyal, 1999). Isolates were incubated at 30 °C for 10 days, and halo formation around the colonies indicated solubilizing activity.

2.6 Siderophore Production

Siderophore production was evaluated after culturing the isolates in King B medium (King et al., 1954) at 30 °C for 48 h under agitation. Supernatants were obtained by centrifugation and used in assays with Chrome Azurol S (CAS) reagent prepared according to Schwyn and Neilands (1987).

Detection was performed in microplates, and a color change from blue to yellow or orange indicated siderophore production.

2.7 DNA Extraction, Sequencing, 16S rRNA Gene Analysis, and Phylogenetic Analysis

Obtained sequences were compared with GenBank database through BLAST. Multiple alignment was performed in MAFFT, and phylogenetic reconstruction conducted in IQ-TREE, with SH-aLRT support and ultrafast bootstrap. Trees were visualized in iTOL v.6.

The best-performing isolates were selected for molecular characterization. Cultures were grown in LB medium (Bertani, 1951), and genomic DNA was extracted using a commercial Extracta® DNA and RNA from Pathogens kit according to the manufacturer's instructions.

The 16S rRNA gene was amplified by PCR, purified, and sequenced using the BigDye™ Terminator v3.1 kit on a SeqStudio™ genetic analyzer (Thermo Fisher Scientific).

The obtained sequences were compared with the GenBank database using BLAST. Multiple sequence alignment was performed in MAFFT, and phylogenetic reconstruction was conducted in IQ-TREE with SH-aLRT support and ultrafast bootstrap analysis.

The trees were visualized in iTOL v.6. Sequences were processed using bcl2fastq software and analyzed with the USEARCH package for quality control. Amplicon sequence variant (ASV) inference was performed using the DADA2 package, with taxonomic assignment based on reference databases. Spurious sequences and potential contaminants were removed before comparative analyses were performed.

2.8 BOX-PCR

Given the limited discriminatory power of the 16S rRNA gene for closely related species, BOX-repeat element PCR (BOX-PCR) was employed as a complementary approach to assess genomic variability among the isolates.

Amplification was performed using the BOXA1R primer (5'-CTACGGCAAGGCGACGCTGACG-3'), as described by Versalovic et al. (1994) and Koeuth et al. (1995), with modifications proposed by Fernandes et al. (2003) and Kaschuk et al. (2006).

The combination of 16S rRNA gene sequencing and BOX-PCR allowed integrated analysis of taxonomic identity and genomic diversity among the genetically distinct isolates, enabling subsequent functional characterization.

2.9 Antagonistic Potential

The antagonistic potential of the selected bacterial isolates was evaluated in vitro using a dual-culture assay in Petri plates with phytopathogens previously cultivated on potato dextrose agar (PDA) medium.

Antifungal activity was determined by measuring the inhibition zones formed between fungal mycelial growth and the bacterial inoculation points. In addition, the in vitro mycelial growth inhibition rate (R) was calculated using the following equation:

$$R = \frac{C - T}{C} \times 100$$

where:

- **R** corresponds to the percentage of mycelial growth inhibition;
- **C** represents the mycelial diameter of the fungus in the control treatment (mm);
- **T** corresponds to the mycelial diameter of the fungus in the treatment with bacterial isolates, including the initial fungal plug (mm)

Each treatment was performed in triplicate, and the experiment was repeated independently three times.

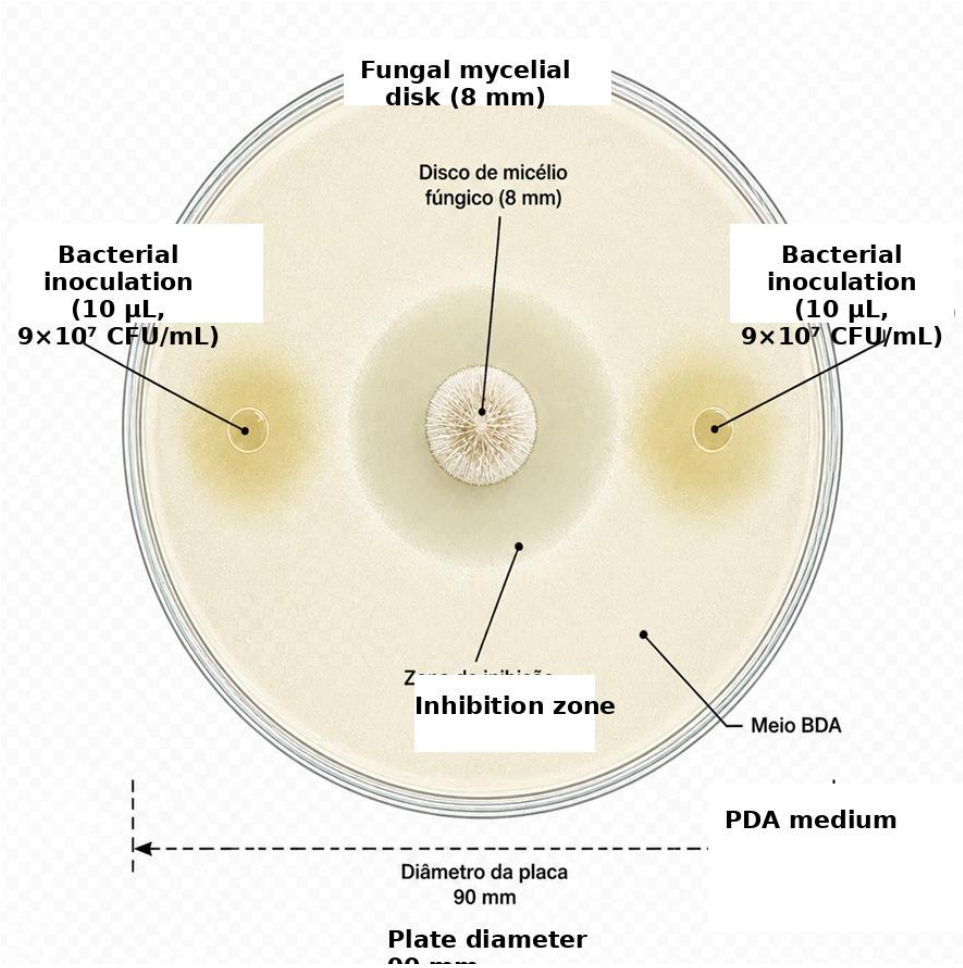

Figure 1. Representation of a Petri plate with double culture plate technique for antagonistic potential evaluation. Source: prepared by the author.

3. RESULTS AND DISCUSSION

3.1 Bacterial Isolation

Bacterial isolations were performed on YMA medium (Vincent, 1970) supplemented with cycloheximide (100 mg L^{-1}), a condition that proved efficient in inhibiting fungal growth and facilitated the visualization and selection of bacterial colonies. Morphological screening was performed using a stereomicroscope (MZ-8, Leica), allowing the identification of different colonial morphology patterns.

A total of 92 bacterial isolates were obtained, 43 from nodules/roots and 49 from soil, collected during the dry season (September 2024) and rainy season (March 2025) (Table 2). The distinction between exclusively nodular and root isolates was not possible in some cases due to the strong adherence of nodules to the main roots. All isolates were preserved at −80°C.

Table 2: Results of the quantity of isolates obtained on YMA culture medium containing cycloheximide (100 µg/mL), from soils, nodules/roots collected at different times of the year.

Collection Period	Nodules/Roots Isolates	Soil Isolates	Total
September/2024 (dry period)	27 (NS)	29 (SS)	56
March/2025 (rainy period)	16 (NC)	20 (SC)	36
Total	**43**	**49**	**92**

3.2 Indole-3-Acetic Acid (IAA) Production

Qualitative evaluation of IAA production in vitro demonstrated wide variability among the isolates. Color intensity after reaction with Salkowski reagent was proportional to the concentration of the phytohormone produced, as described by Gilbert et al. (2018).

After 72 h of cultivation, YML medium containing L-tryptophan used as a control showed a yellowish coloration after mixing with Salkowski reagent, while the supernatants from bacterial cultures showed wide variability in IAA production, expressed by different color intensities ranging from pink to orange, wine, and purple.

Of the 92 isolates analyzed, 20 showed very high IAA production (++++) , 13 showed high production (+++), 12 showed moderate production (++), 12 showed low production (+), and the remaining isolates practically did not produce IAA (Table 3; Figure 2).

Table 3. Qualitative classification of IAA production by the 92 bacterial isolates.

IAA Production	Symbol	Number of Isolates	(%)
Very high	++++	20	21.7
High	+++	13	14.1
Moderate	++	12	13
Low	+	12	13

IAA Production	Symbol	Number of Isolates	(%)
Non-detectable	–	35	38
Total	--	**92**	**100**

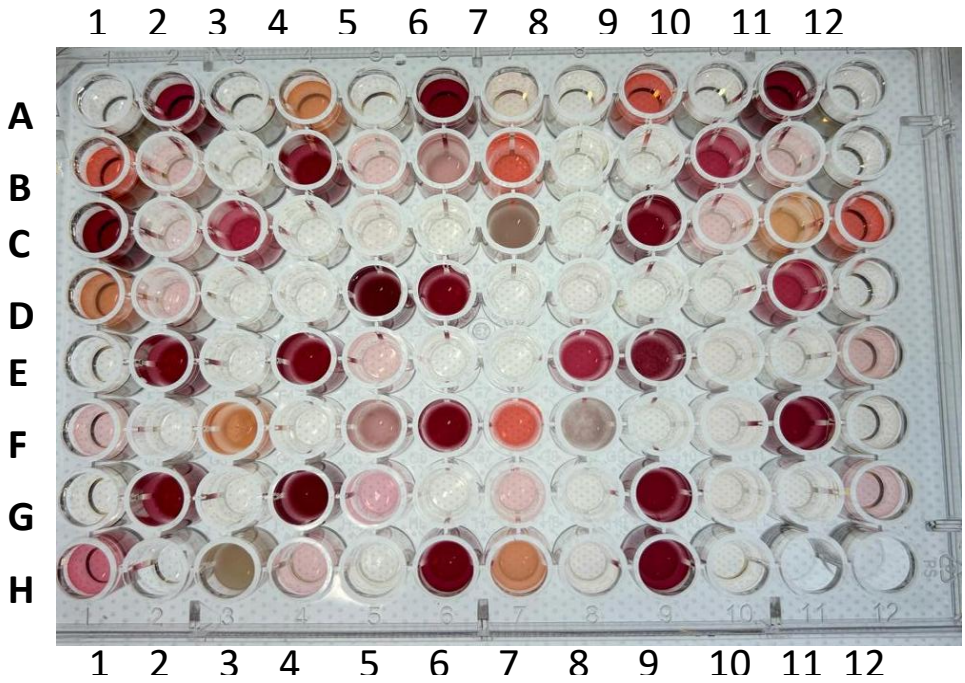

Figure 2. Representative test of IAA production by isolates in YML supplemented with cycloheximide 100 (µg/mL), in dry (September) and rainy (March) seasons, being A1: negative control (YML); A2: positive control (SEMIA 587); A3 – C5: dry season nodules/roots (NS); C6 - E10: dry season soil (SS); E11 – G12: rainy season nodules/roots (NC) and G3 – H10: rainy season soils (SC).

Quantification of indole-3-acetic acid (IAA) in selected isolates was performed using a standard curve (5–125 µg mL^{-1}) by spectrophotometry (Table 4).

Table 4. Quantification of indole-3-acetic acid of isolates. Means followed by the same letter in the column do not differ statistically from each other by Tukey's test at 5% probability ($p < 0.05$).

Group	Isolate	IAA Production (µg/mL)
NC	NC 3	151.31 ± 2.96 c
	NC 2	123.28 ± 0.91 e

Group	Isolate	IAA Production (µg/mL)
	NC 4	73.02 ± 7.49 h
	NC 5	49.03 ± 0.40 i
	NC 6	48.28 ± 1.04 i
	NC 1	8.40 ± 0.07 k
NS	NS 2	162.46 ± 1.04 c
	NS 4	161.83 ± 1.74 c
	NS 1	152.91 ± 1.01 c
	NS 3	42.14 ± 0.28 e
	NS 5	15.46 ± 0.50 i
SC	SC A	144.48 ± 1.15 d
	SC C	92.35 ± 2.20 g
	SC D	54.38 ± 0.69 f
	SC E	51.94 ± 0.70 i
	SC B	13.36 ± 0.15 j
SS	SS D	117.39 ± 7.18 e
	SS B	109.10 ± 3.41 a
	SS A	101.42 ± 5.11 b
	SS C	51.31 ± 0.68 i

Quantitative analysis revealed wide variation in IAA production among the evaluated isolates, with values ranging from 8.40 to 162.46 µg mL^{-1} (Table 4). The results showed statistically significant differences among isolates in IAA production capacity (ANOVA; $F = 594.53$; $p < 0.0001$), demonstrating high physiological variability among the evaluated microorganisms.

Isolates NS 2 (162.46 ± 1.04 µg mL^{-1}), NS 4 (161.83 ± 1.74 µg mL^{-1}), and NC 3 (151.31 ± 2.96 µg mL^{-1}) stood out as the highest IAA producers. The lack of a significant difference among these isolates, indicated by the same Tukey grouping, reinforces the high biotechnological potential of these strains for plant growth promotion.

In contrast, isolates NC 1 (8.40 ± 0.07 µg mL^{-1}) and SC B (13.36 ± 0.15 µg mL^{-1}) showed the lowest values, revealing marked metabolic diversity in the evaluated microbial community.

The presence of high-producing strains in both dry and rainy seasons suggests that IAA synthesis is more closely related to intrinsic genetic characteristics than to environmental seasonality.

Isolates obtained from nodules and roots under water stress conditions, corresponding to the dry season, showed expressive auxin production, as observed for NS 2, NS 4, and NS 1. These results may indicate an adaptive mechanism associated with promoting root development and optimizing water and nutrient uptake by the host plants.

Similarly, the high production observed in soil isolates, such as SS D and SC A, reinforces the importance of this ecological niche as a reservoir of microorganisms with strong bioestimulant potential for plant growth promotion and agricultural inoculant development.

Overall, the results indicate that isolates with high IAA production potential are distributed across different areas and seasons. However, although high in vitro production is a promising indicator of bioestimulant potential, additional assays under controlled and field conditions are necessary to confirm the effectiveness of these isolates in promoting plant growth

3.3 Integration of 16S rRNA and BOX-PCR Techniques in Isolate Identification

3.3.1 Phylogenetic Analysis

Based on the sequences obtained from the 16S rRNA gene, phylogenetic trees were constructed for the different bacterial groups identified among the isolates obtained from peanut cultivation.

The analyses allowed grouping of the isolates according to their genetic proximity to sequences deposited in public databases, revealing high taxonomic diversity among the evaluated microorganisms. The results showed isolates belonging to the genera *Bacillus*, *Klebsiella*, *Rhizobium*, *Priestia*, *Microbacterium*, *Burkholderia*, *Mycolicibacterium*, *Lysinibacillus*, and *Kosakonia*.

The clusters observed in the phylogenetic trees indicated high genetic similarity between the isolates and bacterial species previously described in the literature, reinforcing the usefulness of 16S rRNA gene sequencing for taxonomic identification.

Among the evaluated isolates, isolates 01, 02, 03, 05, and 10 clustered with species of the genus *Bacillus*, while isolates 04, 07, and 16 showed phylogenetic proximity to species of the genus *Rhizobium*. Isolate 09 clustered with species of the genus *Klebsiella*, whereas isolates 13, 14, and 15 showed similarity to species of the genus *Priestia*. Isolates related to the

genera *Microbacterium* (isolate 12), *Burkholderia* (isolate 17), *Mycolicibacterium* (isolate 18), *Lysinibacillus* (isolate 19), and *Kosakonia* (isolate 20) were also identified.

The diversity observed among bacterial groups evidence wide microbial variability associated with peanut culture, especially in rhizospheric and endophytic environments, which represent important reservoirs of microorganisms with biotechnological potential for plant growth promotion and biological control of phytopathogens (Figure 3).

Figure 3. Phylogenetic trees inferred by maximum likelihood method showing taxonomic positioning of bacterial isolates recovered in this study relative to type lineages and phylogenetically close reference lineages. Analysis was organized in eight panels at genus level: (A) Bacillus, (B) Burkholderia, (C) Klebsiella, (D) Microbacterium, (E) Mycolicibacterium, (F) Priestia, (G) Rhizobium and (H) Lysinibacillus. Isolates from this study are indicated as Isolate 01–19, while reference taxa were included to resolve their phylogenetic positioning within recognized species complexes and closely related clades. Node support was evaluated by bootstrap resampling and is represented by a color scale ranging from 50 to 100, where higher values indicate higher topological support.

3.3.2 Genetic Diversity of Isolates: 16S rRNA Gene Sequencing and BOX-PCR Techniques

The combination of 16S rRNA gene analysis with fingerprinting techniques such as BOX-PCR is considered a robust approach for studying bacterial diversity. While 16S rRNA sequencing provides taxonomic identification and phylogenetic positioning of the isolates, BOX-PCR allows genetic discrimination at the strain level, providing a more detailed view of the diversity existing within the same taxonomic group. Thus, the combination of these two techniques was used for the identification of 20 previously selected isolates: five from nodules/roots in the dry season (NS 1, 2, 3, 4, 5), four from dry-season soil (SS A, B, C, D), six from nodules/roots in the rainy season (NC 1, 2, 3, 4, 5, 6), and five from rainy-season soil (SC A, B, C, D, F).

DNA extraction was performed using a commercial kit, and the DNA was quantified using a NanoDrop® 1000, which yielded good quantity and quality, as shown in Figure 4.

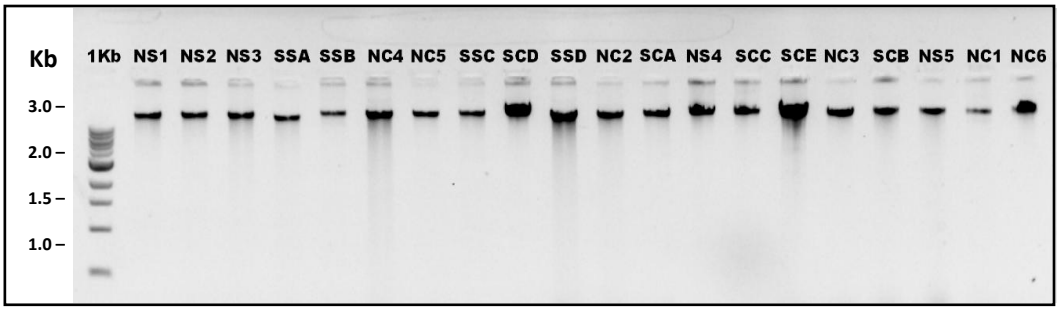

Figure 4. Profile of DNAs from bacterial isolates from soils and nodules/roots of peanut in 0.8% agarose gel. Cassettes: (1) 1 Kb DNA ladder standard; (2 to 5) NS 1,2,3,4,5; (6 to 9) SS A, B, C, D; (10 to 15) NC 1,2,3,4,5,6; (16 to 20) SC A, B, C, D, E.

PCR was performed using DNA and oligonucleotides specific for the 16S rRNA gene, resulting in the amplification of fragments of approximately 1,500 bp, suitable for sequencing. The sequences obtained were submitted to nucleotide similarity analysis by comparison with the GenBank and RDB databases (Table 5).

Additionally, phylogenetic analysis was performed based on reference sequences that presented similarity with analyzed sequences (Figure 3). It was observed that, when comparing 16S rRNA analysis results with indole-3-acetic acid (IAA) production, there is a notable discrepancy in IAA production levels between isolates classified as identical based on sequencing analyses.

Additionally, phylogenetic analysis was performed based on reference sequences that showed similarity to the analyzed sequences (Figure 3). When the 16S rRNA analysis results were compared with indole-3-acetic acid (IAA) production, a notable discrepancy was observed in IAA production levels among isolates classified as identical based on sequencing analyses.

Table 5. Identification of bacterial isolates based on 16S rRNA gene. Taxonomic identification was inferred from the closest matches obtained through BLASTn searches against the NCBI GenBank database.

Nomenclature	Bacterial Species	Coverage (%)	Identity (%)
NS 1	Bacillus velezensis	85	90.49
NS 2	Bacillus velezensis	100	99.12
NS3	Bacillus velezensis	100	97.82
NS4	Rhizobium viscosus	100	96.41
NS5	Mycobacterium cosmeticum	99	100
SS A	Bacillus velezensis	100	99.85

Nomenclature	Bacterial Species	Coverage (%)	Identity (%)
SS B	Bacillus velezensis	100	97.40
SS C	Bacillus paranthais	99	99.12
SS D	Bacillus zanthoxyli	99	95.69
NC 1	Burkholderia cepacia	100	99.87
NC 2	Rhizobium dioscoreae	100	95.10
NC 3	Klebsiella pneumonice	100	92.79
NC 4	Bacillus velezensis	100	97.44
NC 5	Bacillus velezensis	100	94.51
NC 6	Kosakonia coubnii	100	97.21
SC A	Rhizobium dioscoreae	99	96.53
SC B	Microbacterium zeae	99	74.02
SC C	Priestia aryabhattai	100	97.53
SC D	Lisinibacillus sp.	100	99.74
SC E	Priestia aryabhattai	100	96.78

To investigate why isolates genetically identical at the genus and species level, according to 16S rRNA gene sequencing, showed different results in certain analyses, PCR was performed using the BOX-PCR technique. This approach allowed differentiation and comparison of genetically close bacterial isolates, enabling discrimination among bacteria of the same species.

Gel analysis (Figure 4) showed that, among the seven *Bacillus velezensis* isolates identified by 16S rRNA sequencing, only isolates SS-A, SS-B, and NC-4 showed identical banding profiles in BOX-PCR analysis, whereas isolates NS-3 and NC-5 showed similar profiles distinct from the three previously described isolates; however, they shared a band of approximately 1.1 kb.

In contrast, isolates NS-1 and NS-2 showed distinct banding patterns, sharing only one common band at approximately 2.0 kb. Despite their marked differences from the other isolates, NS-1 also showed a band at approximately 1.1 kb, which appears to correspond to a conserved region of *Bacillus velezensis*. This band was absent in NS-2, raising questions about the identification of this isolate.

Isolates NC-2 and SC-A (*Rhizobium dioscoreae*) showed similar profiles despite being isolated from different niches, whereas the soil isolates SC-C and SC-E (*Priestia aryabhattai*) showed completely different BOX-PCR profiles, except for a single band of approximately 1.25 kb.

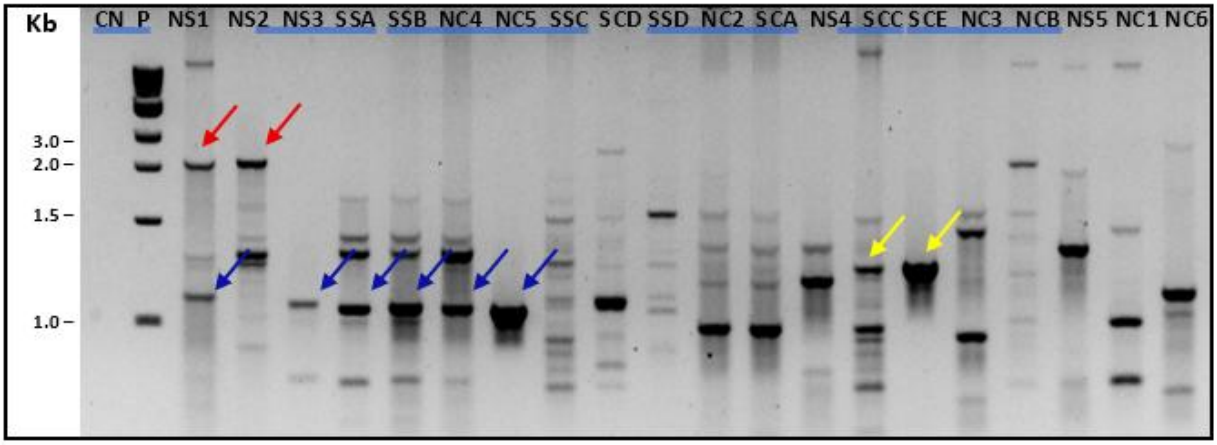

Figure 5. Profile of DNA fragments after amplification with BOXA 1R in 1.5% (w/v) agarose gel in TBE containing Ethidium Bromide (0.5 µl/mL). Red arrows 2.0 kb, blue arrows ≈ 1.1 kb and yellow arrows ≈ 1.25 kb.

The combination of 16S rRNA gene sequencing and BOX-PCR enabled not only the taxonomic identification of the isolates, but also the construction of comparative genomic profiles with greater discriminatory power at the intraspecific level. This integrated approach contributed to the interpretation of functional differences observed among taxonomically similar isolates and allowed the establishment of a reference genetic marker for each strain.

Defining these profiles is particularly relevant for maintaining the genetic identity of isolates through successive subcultures in the laboratory, since contamination or genetic variation may occur during repeated subculturing. Thus, the banding pattern obtained by BOX-PCR can be used as a tool to monitor the purity and stability of isolates belonging to the strain bank.

Overall, similarities and divergences between isolates belonging to the same genus or species were evident when comparing 16S rRNA sequencing results with BOX-PCR profiles. These findings indicate that, especially for the genus *Bacillus*, identification based exclusively on partial 16S rRNA gene sequencing may not be sufficient for accurate discrimination among phylogenetically close species.

Species such as *Bacillus subtilis*, *Bacillus velezensis*, and *Bacillus amyloliquefaciens* show high similarity in the 16S rRNA region, often with practically identical sequences, which limits taxonomic resolution (Glaeser & Kämpfer, 2015). In such cases, complementary approaches are recommended, such as housekeeping gene sequencing (for example, gyrB, rpoB, or recA) or more comprehensive genomic analyses, to ensure more robust and reliable taxonomic identification.

3.4 Siderophore Production

Siderophore production by plant growth-promoting bacteria (PGPR) is recognized as one of the main mechanisms involved in iron acquisition by plants and also plays an important role in microbial competition and the biocontrol of phytopathogens, thereby contributing to more sustainable agricultural systems. (Timofeeva et al., 2022; Timofeeva, 2023).

Sid The siderophore-producing capacity of the isolates was evaluated qualitatively using the Chrome Azurol S (CAS) assay. The transition of the blue CAS–Fe^{3+} complex to greenish or yellowish tones indicated the presence of iron-chelating compounds. Color intensity was used as a semiquantitative criterion and classified as low (+), moderate (++), or high (+++).

Among the 20 evaluated isolates, 10 showed high siderophore production (+++) (Table 6), indicating relevant functional potential for agricultural applications.

The genera *Burkholderia*, *Bacillus*, and *Rhizobium* stood out among the best producers, with particular emphasis on isolates NC-1 (*Burkholderia cepacia*), NC-4, and NS-4 (*Bacillus velezensis* and *Rhizobium viscosus*, respectively), which showed intense yellow-orange coloration after 72 h of cultivation in King B medium.

Table 6. Qualitative production of siderophores by bacterial isolates evaluated in King B medium using CAS assay.

Bacterial Species	**Isolate Code**	**Siderophores King B**
Bacillus velezensis	NS 1	+++
Bacillus velezensis	NS 2	+++
Bacillus velezensis	NS3	++
Rhizobium viscosus	NS4	+++
Mycobacterium cosm	NS5	-
Bacillus velezensis	SS A	+++
Bacillus velezensis	SS B	+++
Bacillus paranthais	SS C	++
Bacillus zanthoxyli	SS D	++
Burkholderia cepacia	NC 1	+++
Rhizobium dioscoreae	NC 2	+++

Bacterial Species	Isolate Code	Siderophores King B
Klebsiella pneumonice	NC 3	-
Bacillus velezensis	NC 4	+++
Bacillus velezensis	NC 5	+++
Kosakonia coubnii	NC 6	++
Rhizobium dioscoreae	SC A	+++
Microbacterium zeae	SC B	+
Priestia aryabhattai	SC C	++
Lisinibacillus sp.	SC D	++
Priestia aryabhattai	SC E	++

Similar results were reported by Batista et al. (2018), who observed high siderophore production by rhizospheric and endophytic isolates belonging to the genera *Bacillus*, *Burkholderia*, and *Pantoea* in maize roots.

Isolate NC-1 (*Burkholderia cepacia*) showed high siderophore activity, corroborating the findings of Zheng et al. (2025), who demonstrated high siderophore production by *Burkholderia cenocepacia* P3 after optimization of cultivation conditions. Members of the cepacia complex are known to produce ornibactin and pyochelin, siderophores with high affinity for Fe^{3+}, which are associated with both iron acquisition and biocontrol mechanisms.

Lalitha and Nithyapriya (2021) demonstrated that *Bacillus subtilis* LSBS2 produces a bacillibactin-type siderophore and that inoculation increases biomass, iron content, and growth in peanut.

Within the genus *Bacillus*, Lalitha and Nithyapriya (2021) demonstrated that *Bacillus subtilis* LSBS2 produces bacillibactin, resulting in increased biomass, iron content, and growth in peanut. Similarly, several studies indicate that *Bacillus velezensis* synthesizes bacillibactin-type siderophores, often associated with the concomitant production of secondary metabolites with antimicrobial activity. Chen et al. (2025) reported that *B. velezensis* SS-20 carries genes involved in the biosynthesis of bacillibactin, surfactin, and other bioactive compounds, reinforcing the multifunctional potential of this species.

Although siderophore production is frequently associated with *Bacillus* and *Burkholderia*, the presence of siderophore-producing rhizobia is also relevant. Arcas et al. (2024) demonstrated

that *Rhizobium radiobacter* produces siderophores capable of being used in an efficient natural iron fertilizer for soybean cultivation.

Siderophore production by rhizobia is particularly important because iron is an essential element for leghemoglobin synthesis and nitrogenase function in root nodules, directly affecting biological nitrogen fixation (BNF). Thus, isolates with high siderophore-producing capacity can simultaneously contribute to mineral nutrition and symbiotic efficiency.

3.5 Phosphate Solubilization

Phosphorus (P) is an essential macronutrient for plant development, participating in fundamental processes such as photosynthesis, respiration, energy transfer (ATP), cell division, and nucleic acid synthesis. Despite its high total concentration in soil, a large proportion is present in insoluble forms and is therefore unavailable for plant uptake. In this context, phosphate-solubilizing bacteria (PSB) play a strategic role in mobilizing this nutrient and supporting the sustainability of agricultural systems.

Phosphate-solubilizing capacity was evaluated in NBRIP medium according to the methodology described by Nautiyal (1999), with readings taken after 10 days of incubation. Of the 20 tested isolates, 19 showed growth and halo formation, indicating positive solubilizing activity. Only isolate SC-B (*Microbacterium zeae*) showed neither growth nor halo formation. (Table 7) and (Figure 6).

The solubilization index (SI) ranged from 1.11 to 2.35. The highest value was observed for NC-1 (*Burkholderia cepacia*), with SI = 2.35, followed by NC-3 (*Klebsiella pneumoniae*, SI = 2.08) and NC-2 (*Rhizobium dioscoreae*, SI = 2.00). Isolates belonging to the genus *Bacillus* also showed high indices, ranging from 1.50 to 2.00.

These results indicate high functional diversity among the isolates, with particular emphasis on *B. cepacia*, which also demonstrated superior performance in other evaluated traits, such as siderophore and IAA production, suggesting multifunctional potential.

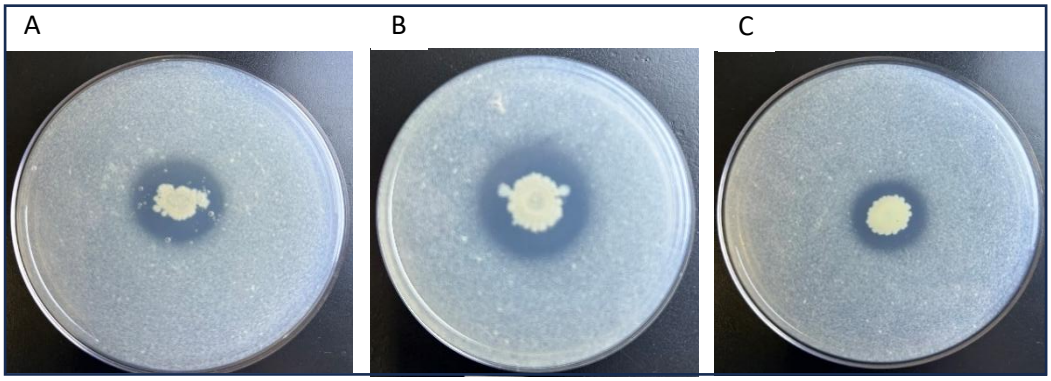

Figure 6. Phosphate solubilization halos observed in NBRIP medium after 10 days of cultivation. A – NC-1; B – NC-3 and C – NC-2.

Table 7. Phosphate solubilization index (SI) of bacterial isolates evaluated in NBRIP medium after 10 days of incubation. *(+) Bacteria that grew and (-) bacteria that did not grow.*

Bacterial Species	Isolate Code	Phosphate Solubilization	SI	(+) / (-)
Bacillus velezensis	NS 1	1.93	+	
Bacillus velezensis	NS 2	2.0	+	
Bacillus velezensis	NS3	1.5	+	
Rhizobium viscosus	NS4	1.5	+	
Mycobacterium cosm	NS5	-	+	
Bacillus velezensis	SS A	1.92	+	
Bacillus velezensis	SS B	1.94	+	
Bacillus paranthais	SS C	1.11	+	
Bacillus zanthoxyli	SS D	1.22	+	
Burkholderia cepacia	NC 1	2.35	+	
Rhizobium dioscoreae	NC 2	2.00	+	
Klebsiella pneumonice	NC 3	2.08	+	
Bacillus velezensis	NC 4	1.80	+	
Bacillus velezensis	NC 5	1.94	+	
Kosakonia coubnii	NC 6	1.91	+	
Rhizobium dioscoreae	SC A	1.98	+	
Microbacterium zeae	SC B	-	-	
Priestia aryabhattai	SC C	1.60	+	

Bacterial Species	Isolate Code	Phosphate Solubilization	SI	(+) / (-)
Lisinibacillus sp.	SC D	1.50	+	
Priestia aryabhattai	SC E	1.63	+	

NBRIP medium proved suitable for in vitro screening, since solubilization of insoluble phosphates is mainly associated with the production of organic acids derived from glucose metabolism, which promote medium acidification and chelation of cations bound to phosphate. In addition, phosphatase production can contribute to the mineralization of organic phosphorus forms (Alori et al., 2017).

Traditionally, the genera *Pseudomonas* and *Bacillus* are described as the main phosphate solubilizers in agricultural soils. However, a wide diversity of PSB has been reported in the literature. Santos et al. (2025) highlight that genera such as *Pseudomonas*, *Enterobacter*, and *Pantoea* can solubilize phosphorus under different pH conditions, reinforcing the central role of local acidification in the process.

The results obtained in this study are consistent with these reports, since isolates from *Burkholderia*, *Bacillus*, and *Rhizobium* demonstrated high solubilizing capacity. It should be noted that *Burkholderia cepacia* was previously classified as *Pseudomonas cepacia*, reflecting the historical phylogenetic proximity between these groups.

Hossain et al. (2023), when evaluating peanut-derived isolates with multiple growth-promoting traits, including IAA production, siderophore production, and phosphate solubilization, observed a significant increase in biomass after reinoculation, especially for *Burkholderia*, *Pseudomonas*, *Rhizobium*, *Herbaspirillum*, and *Pantoea* strains. These findings reinforce the potential of PSB to improve nutrient acquisition and tolerance to environmental stresses.

Although soybean cultivation has concentrated on most of the established inoculation technologies for legumes, there is growing interest in exploring the microbiome associated with peanuts. The identification of isolates with high phosphate-solubilizing capacity, especially when combined with other growth-promoting traits, expands the prospects for developing bioinputs aimed at reducing dependence on phosphate fertilizers and promoting more resilient and environmentally sustainable agricultural systems.

3.6 Antifungal Activity

After 16S rRNA gene sequencing and evaluation of enzymatic activities, the selected isolates NS-3, SS-A, SS-B, NC4, and NC-5 were tested against phytopathogens using a dual-culture plate technique.

Mycelial plugs of each phytopathogen (approximately 8 mm in diameter), previously cultivated on solid PDA medium, were removed and placed at the center of PDA plates. The bacterial isolates were inoculated at points opposite the plugs.

The antifungal screening results demonstrated that some bacterial isolates have high potential as biocontrol agents.

Among the evaluated isolates, some stood out for the breadth of their activity, inhibiting multiple pathogenic fungi. Table 8.

Table 8. Percentage of mycelial growth inhibition of phytopathogens by bacterial isolate.

Nomenclature	Bacterial Species	Phytopathogen	Inhibition (%)
NS3	Bacillus velezensis	Fusarium oxysporum	35.83%
NS3	Bacillus velezensis	Rhizoctonia solani	60.83%
SS A	Bacillus velezensis	Fusarium oxysporum	37.50%
SS A	Bacillus velezensis	Rhizoctonia solani	56.67%
SS B	Bacillus velezensis	Fusarium oxysporum	43.33%
SS B	Bacillus velezensis	Rhizoctonia solani	65.00%
SS B	Bacillus velezensis	Aspergillus flavus	24.17%
NC 4	Bacillus velezensis	Fusarium oxysporum	45.83%
NC 4	Bacillus velezensis	Rhizoctonia solani	60.00%
NC 5	Bacillus velezensis	Fusarium oxysporum	0.00%
NC 5	Bacillus velezensis	Rhizoctonia solani	40.00%
NS 1	Bacillus velezensis	Fusarium oxysporum	0.00%
NS 1	Bacillus velezensis	Rhizoctonia solani	0.00%
NS 1	Bacillus velezensis	Aspergillus flavus	0.00%
NS 2	Bacillus velezensis	Fusarium oxysporum	0.00%
NS 2	Bacillus velezensis	Rhizoctonia solani	0.00%
NS 2	Bacillus velezensis	Aspergillus flavus	0.00%

Nomenclature	Bacterial Species	Phytopathogen	Inhibition (%)
SS C	Bacillus paranthais	Fusarium oxysporum	0.00%
SS C	Bacillus paranthais	Rhizoctonia solani	0.00%
SS C	Bacillus paranthais	Aspergillus flavus	0.00%
SS D	Bacillus zanthoxyli	Fusarium oxysporum	0.00%
SS D	Bacillus zanthoxyli	Rhizoctonia solani	0.00%
SS D	Bacillus zanthoxyli	Aspergillus flavus	0.00%

Among the evaluated isolates, *Bacillus velezensis* SS-B showed the highest inhibition percentages, with 65.00% against *Rhizoctonia solani*, 43.33% against *Fusarium oxysporum*, and 24.17% against *Aspergillus flavus*.

Although the highest antagonistic activity was observed against *Rhizoctonia solani*, inhibition of *Aspergillus flavus* by isolate SS-B deserves emphasis, since this fungus is of major agricultural and sanitary importance because it can cause grain losses and produce metabolites of toxicological concern. In this context, even a moderate inhibition percentage indicates relevant biotechnological potential, especially given the complexity of controlling this pathogen.

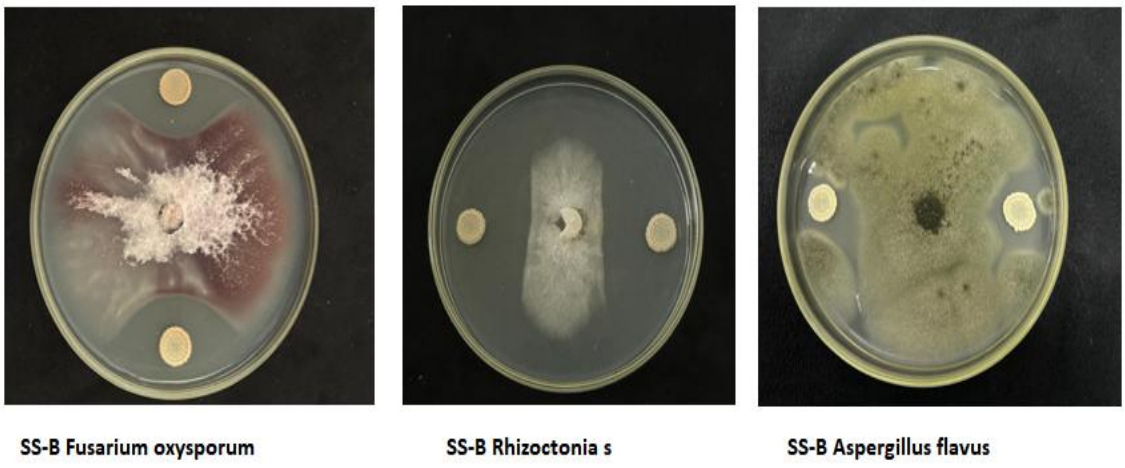

Figure 7. In vitro antagonistic activity of bacterial isolate SS-B against the phytopathogens *Fusarium oxysporum*, *Rhizoctonia solani*, and *Aspergillus flavus*.

Isolate NC4 also showed strong performance, with 60.00% inhibition against *R. solani* and 45.83% against *F. oxysporum*.

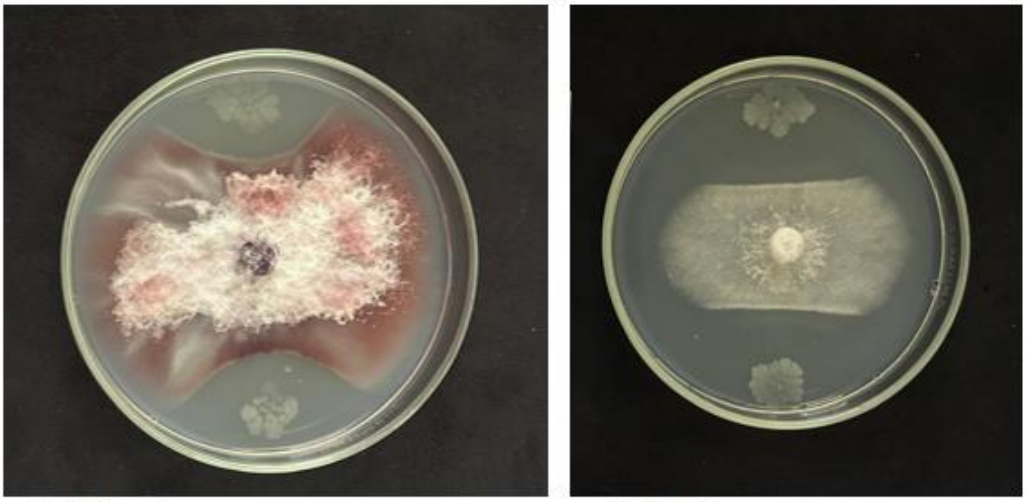

Figure 8. n vitro antagonistic activity of bacterial isolate NC-4 against the phytopathogens *Fusarium oxysporum* and *Rhizoctonia* sp.

Isolate NS-3 inhibited 60.83% of *R. solani* growth and 35.83% of *F. oxysporum* growth, while SS-A showed 56.67% and 37.50% inhibition against these same pathogens, respectively.

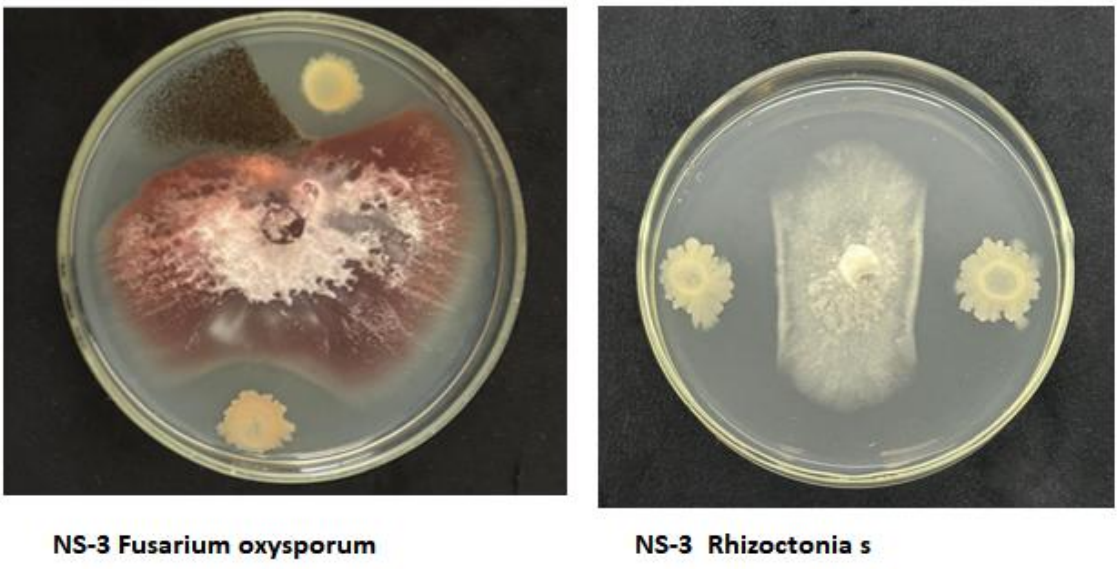

Figure 9. In vitro antagonistic activity of bacterial isolate NS-3 against the phytopathogens *Fusarium oxysporum* and *Rhizoctonia* sp.

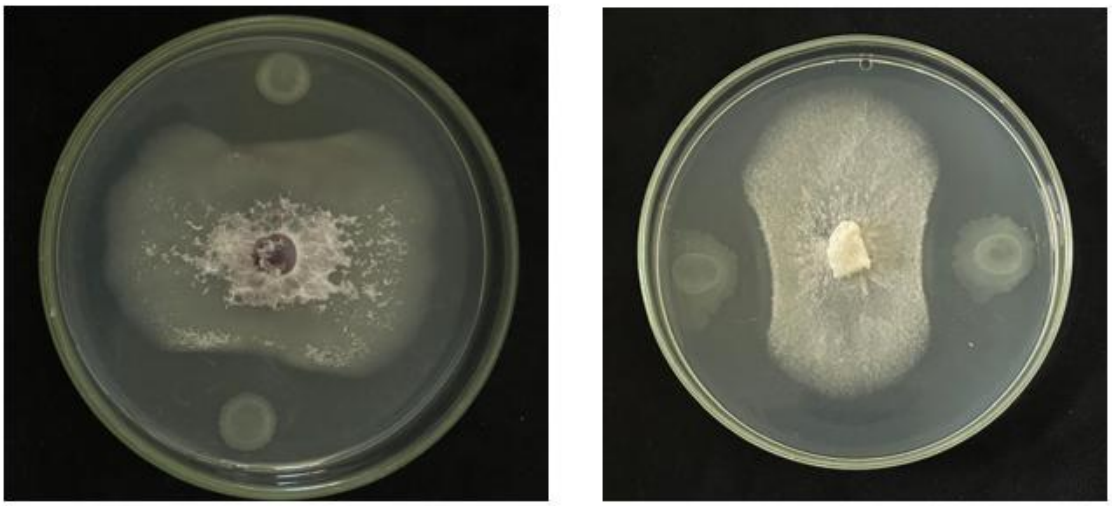

Figure 10. In vitro antagonistic activity of bacterial isolate SS-A against the phytopathogens *Fusarium oxysporum* and *Rhizoctonia* sp.

Isolate NC-5 showed a moderate effect against *R. solani* (40.00%) but showed no inhibition against *F. oxysporum*.

Figure 11. In vitro antagonistic activity of bacterial isolate NC-5 against the phytopathogen *Rhizoctonia* sp.

This result suggests that antagonistic activity among isolates of the same bacterial species may vary according to the specificity of the target pathogen and, possibly, differential production of bioactive metabolites.

Overall, the results indicate that the evaluated isolates, especially SS-B, NC4, NS-3, and SS-A, show promising potential for the biological control of agriculturally relevant phytopathogens.

The high inhibition observed against *R. solani* reinforces the potential of these microorganisms as candidates for further greenhouse and field studies.

In addition, the taxonomic identification of the most promising isolates as *Bacillus velezensis* is consistent with the history of this taxon as a biocontrol agent and plant growth promoter.

Considering the context of peanut cultivation and the search for more sustainable management strategies, these results strengthen the prospect of applying these isolates in the development of agricultural bioinputs.

4. CONCLUSIONS

The present dissertation demonstrated that the microbiome associated with peanut cultivation, encompassing both endophytic and rhizospheric niches, constitutes a diverse reservoir of microorganisms with biotechnological potential for plant growth promotion and biocontrol.

One of the distinguishing features of this research was the strategic application of the BOX-PCR technique as a genetic fingerprinting tool. While 16S rRNA gene sequencing provided taxonomic support, BOX-PCR proved to be an innovative and high-resolution method for intraspecific discrimination, allowing the identification of subtle differences between strains of the same taxon, as observed, for example, in the genus *Bacillus*. This highly discriminatory approach complemented conventional taxonomic analyses by providing strain-level resolution. The integration of sequencing with the genetic banding profile therefore establishes a robust identity marker, essential for ensuring the purity, stability, and functional quality of isolates in future biotechnological applications and in the large-scale production of agricultural inoculants.

Among the 92 isolates obtained, 20 stood out for high indole-3-acetic acid (IAA) production, with values reaching 162.46 µg mL^{-1}, showing that metabolic variability is not necessarily restricted to phylogenetic classification based solely on 16S rRNA.

Isolates belonging to *Burkholderia cepacia*, *Bacillus velezensis*, and *Rhizobium dioscoreae* simultaneously showed high IAA production and phosphate-solubilizing capacity, as well as consistent siderophore production. The convergence of these traits suggests relevant multifunctional potential for application as bioinputs, especially in systems seeking to reduce dependence on chemical fertilizers.

The results also reinforce a limitation already described in the literature: high similarity within the *Bacillus subtilis* complex can hinder taxonomic discrimination when based exclusively on partial regions of the 16S rRNA gene. In this context, complementary approaches are recommended to improve the robustness of identification.

Although the in vitro assays indicate promising performance, validation under controlled conditions and subsequently in the field will be essential to confirm the agronomic effectiveness of the selected strains and support their incorporation into more sustainable management strategies.

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