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RESEARCH ARTICLE

Rhizospheric and phylloplane bacteria from *Capsicum annuum*: Uncovering candidates for biocontrol of *Ralstonia solanacearum*

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Abstract

Ralstonia solanacearum, the causative agent of bacterial wilt, is a major plant pathogen affecting many economically significant crops, including pepper (*Capsicum annuum*). This pathogen causes severe yield losses due to the limited effectiveness of current control measures. This study aimed to evaluate potential biocontrol agents for managing *Ralstonia solanacearum* by isolating and testing microorganisms from the rhizosphere and phylloplane of pepper plants. A total of 32 bacterial isolates were screened, and four strains showed the most pronounced antagonistic activity *in vitro*, producing inhibition zones ranging from 4.0 to 6.12 cm. The most effective isolates included three rhizospheric strains identified as *Bacillus* sp., *Serratia* sp., and *Pseudomonas* sp., and one phylloplane strain identified as *Pseudomonas aeruginosa*. These microorganisms effectively suppressed *Ralstonia solanacearum* growth under laboratory conditions and show strong potential as biocontrol agents for bacterial wilt in pepper crops.

Keywords: *Ralstonia solanacearum*; biocontrol; *Pseudomonas*; halo; inhibition.

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1. Introduction

Bell pepper (*Capsicum annuum*) is an economically important crop in the *Solanaceae* family, recognized as one of the fastest-growing and most in-demand vegetables. Currently, both sweet and hot pepper varieties are widely cultivated due to their culinary, nutritional, and economic importance. Global production continues to increase, driven by increased consumption and health awareness, as peppers are rich in pigments (chlorophyll, anthocyanins, lutein) and bioactive compounds such as vitamins, flavonoids, and capsaicinoids (González-Mendoza et al., 2023; Rehman et al., 2022). In recent years, the pepper industry has also faced challenges related to climate variability, emerging diseases, and market fluctuations, highlighting the need for sustainable production strategies (Mahmood et al., 2023).

Chemical-based management of *Ralstonia* is often ineffective and poses risks to both the environment and human health. In response, recent research has emphasized the use of plant growth-promoting bacteria (PGPB) as sustainable alternatives for disease management and productivity enhancement. These beneficial microorganisms not only enhance soil fertility and stimulate plant growth but also exhibit direct antagonistic effects against plant pathogens through various mechanisms, including antibiotic production, enzyme secretion, and the induction of systemic resistance (Kumar et al., 2024; Zhang et al., 2023a). Understanding the composition and functional potential of native bacterial populations is crucial for developing targeted biocontrol strategies adapted to local agroecosystems (Abdelsalam et al., 2023; Sánchez-Montesinos et al., 2023).

Based on the description above, it was proposed to isolate and study microorganisms from pepper plants with antagonistic potential against *R. solanacearum*, aiming to identify sustainable alternatives for managing this pathogen, as most current products are only marginally effective in controlling this harmful bacterium.

2. Methodology

Recovery and pathogenicity confirmation of *Ralstonia solanacearum*

The *Ralstonia solanacearum* strain used in this study was an isolate from the Phytopathogenic Bacteria Laboratory collection at the Faculty of Agronomy, Central University of Venezuela. Bacterial suspensions were prepared in nutrient agar (NA) to confirm their pathogenicity and adjusted to a concentration of 10^8 cells/mL. These suspensions were inoculated into the stems of healthy, homogeneous, and vigorous bell pepper plants through stem infiltration. The plants were then placed in a shade house and monitored daily for symptom development. Once symptoms appeared, bacterial re-isolation was conducted.

Isolation of antagonistic bacteria from rhizosphere and phylloplane

For bacterial isolation, pepper plants were collected from vegetable production areas. Isolates from the rhizosphere were obtained by macerating 10 g of root tissue and suspending it in 90 mL of saline solution to prepare the stock solution. Serial dilutions were then performed up to 10^{-4}. From each dilution, 0.1 mL was transferred with a pipette and plated onto Petri dishes containing NA and KB media using the spread plate method.

Bacterial isolation from the phyllosphere was performed using the method described by Rose (1975) with some modifications. Healthy leaves were selected, thoroughly washed with sterile distilled water, and a portion of each sample was excised. The leaf samples were then placed in beakers containing 50 mL and 100 mL of sterile saline solution, as well as in others containing a 5% Tween 80 solution. Both solutions were gently shaken for 10 minutes and then placed on a shaker for 24 hours. After the incubation period, the resulting suspensions were plated onto NA and KB media for bacterial growth. After 48 hours of incubation, isolates were selected based on their ability to form inhibition halos, their high colony count, and their fluorescence under UV light when cultured on BK medium. Colonies exhibiting these characteristics were further isolated to obtain pure cultures, which were subsequently used for further testing.

In vitro effect of bacterial isolates on *Ralstonia solanacearum*

To measure the *in vitro* effectiveness of the selected bacteria on *R. solanacearum*, the filter paper method was used (Lorian, 1980; Balouiri et al., 2016). The procedure was as follows: in Petri dishes containing NA and KB culture medium, a suspension of *R. solanacearum*, adjusted to 10^8 CFU/mL with Mc Farland scale tube number 3, was surface seeded at a rate of 100 µL (Gayathiri et al., 2018), then sterile filter paper discs of 5 mm diameter, impregnated with 10 µL of the treatment (bacteria isolated from rhizosphere and phyllosphere) were placed equidistantly; 5 discs per plate were used. The design was completely randomised with 5 replicates. The control consisted of discs to which 10 µL of sterile distilled water was added. The evaluations were carried out after 48 hours, measuring with a graduated ruler the size of the halo of the zone of inhibition of the growth of *Ralstonia solanacearum*.

Data analysis

Data were initially tested for normality using the Shapiro–Wilk test and for homogeneity of variances using Levene's test. Since the inhibition zone diameters obtained from the rhizospheric isolates did not meet the assumptions of normality and homoscedasticity, comparisons among treatments were performed using the non-parametric Kruskal–Wallis test. In contrast, data from the phylloplane isolates satisfied both assumptions and were analyzed using one-way ANOVA, followed by Tukey's Honestly Significant Difference (HSD) post hoc test at a significance level of $\alpha = 0.05$. All statistical analyses were performed using Statistix version 9.0 (Analytical Software, Tallahassee, FL, USA).

Extraction of nucleic acids

The three bacterial isolates from the rhizosphere and one from the phylloplane, which exhibited the highest *in vitro* antagonistic activity against *R. solanacearum*, were selected for molecular identification. Genomic DNA from selected antagonistic strains was extracted using a modified protocol from Sambrook and Russell (2001). Bacterial pellets were obtained by centrifugation (Universal 320R, Hettich, Germany) and resuspended in Tris-HCl buffer. Cell lysis was achieved with lysozyme, RNase A, and proteinase K, followed by thermal shock treatments. DNA was precipitated with cold isopropanol and stored at −20 °C. DNA quantification was performed using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, USA).

PCR amplification and visualization

PCR was performed in a thermal cycler (Mastercycler EP Gradient S, Eppendorf AG, Germany), with primers fD1 and rP2 targeting the 16S rDNA gene. Each 50 µL reaction included Platinum Taq polymerase, dNTPs, $MgCl_2$, and DNA template. Following amplification, 5 µL of the PCR products were electrophoresed on a 1% agarose gel. The expected amplicon size was approximately 1500 bp, which was compared against a molecular weight marker. The 16S rDNA PCR products were subsequently purified using the AccuPrep Kit (Bioneer®), following the manufacturer's protocol. Purified PCR products were submitted to Macrogen (Korea; www.macrogen.com) for sequencing, employing a modified Sanger chain termination method. Sequence data were analyzed and edited using BioEdit sequence alignment software.

Phenotypic characteristics of the most promising bacteria *in vitro*

Cultural, physiological and biochemical tests were carried out to identify the most promising bacteria: oxygen requirement, Gram staining, catalase, oxidase, fluorescence, gelatin liquefaction, starch hydrolysis, typical colony formation in Tween 80 medium, tobacco hypersensitivity, levan production, growth at 38 °C, arginine dihydrolase, nitrite reduction to nitrite, hydrogen sulphide production, growth at different NaCl concentrations. 5 °C, arginine dihydrolase, nitrate reduction to nitrite, hydrogen sulphide production, growth at different NaCl concentrations, phenol red dextrose agar, acid production from arabinose, galactose, ethanol, sorbitol, geraniol and polyethylene glycol (Moore et al., 2001; Holt et al, 1994).

3. Results and discussion

Morphological characterization of *Ralstonia solanacearum*

Colonies of *Ralstonia solanacearum* grown on Triphenyl tetrazolium chloride (TZC) medium exhibited typical characteristics, including a fluid (mucoid) consistency due to the production of extracellular polysaccharides (EPS). The colonies displayed a shiny, smooth surface with irregular edges and a distinct white color with a red center (Figure 1A). On nutrient agar, the colonies were milky white, fluid, and mucoid in appearance (Figure 1B).

These observations align with Yang et al. (2020) and Zhang et al. (2023b), who identified mucoid, red-centered colonies on tetrazolium chloride (TZC) medium as characteristic of virulent *Ralstonia solanacearum* strains. The abundant production of exopolysaccharide (EPS) correlates strongly with the pathogen's capacity to occlude xylem vessels, precipitating wilting in susceptible hosts (Musa et al., 2024). Consequently, colony morphology on selective media such as TZC provides a reliable preliminary diagnosis of bacterial wilt when used alongside pathogenicity assays and molecular confirmation.

Isolation of bacteria from the rhizosphere and phylloplane

During the isolation process, a high density of microorganisms associated with the root and phylloplane of pepper plants was observed. A total of 32 bacterial isolates with potential plant growth-promoting (PGPB) properties were selected: 17 isolates were obtained from the rhizosphere and identified with the letter "R" followed by a number, while 15 isolates were derived from the phylloplane and identified with the letter "F" and a number.

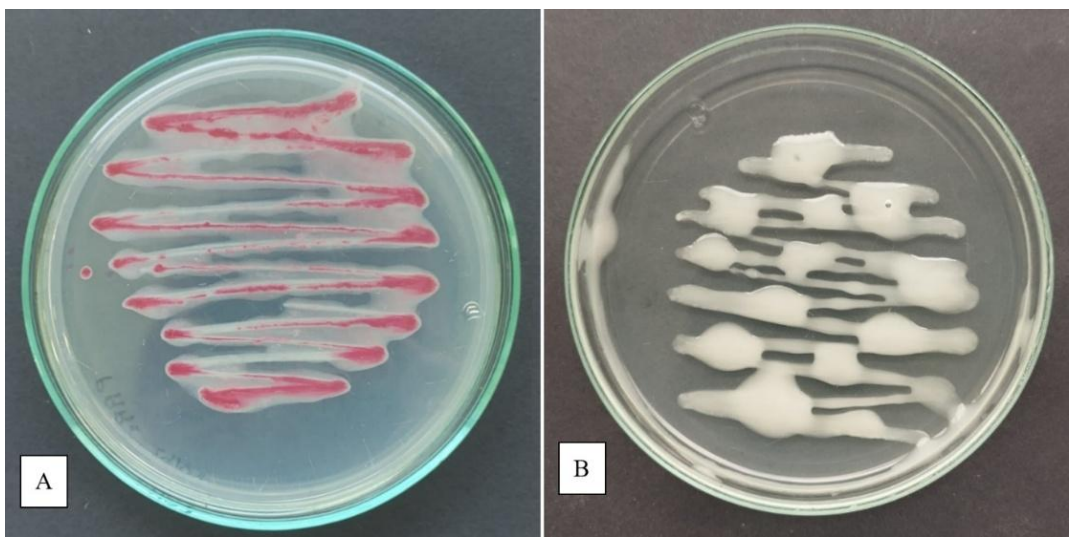

Figure 1. A) *R. solanacearum* colonies on TZC medium. 1. B) Colonies of the pathogenic bacterium on King's medium B.

Among these isolates, several strains exhibited antagonistic activity against *Ralstonia solanacearum*. The large number of isolates reflects the high microbial diversity present in both the rhizosphere and phylloplane. Similar findings were reported by Gavande et al. (2024), who isolated 43 bacteria with antagonistic potential from the rhizosphere of maize (*Zea mays*), soybean (*Glycine max*), and pepper (*Capsicum annuum*) crops. Furthermore, Narasimhan and Banerjee (2021) identified bacteria in the phylloplane of *Carica papaya* plants, which were subsequently selected as biocontrol agents following their successful antagonistic performance.

In vitro comparison of bacterial isolates with *Ralstonia solanacearum*

All rhizosphere isolates exhibited *in vitro* antagonism against *Ralstonia solanacearum*. Statistical differences between treatments and the control were observed using the non-parametric Kruskal-Wallis test (Table 1).

Table 1
Comparison of inhibition halos of *R. solanacearum* produced by bacteria isolated from the rhizosphere

Rhizospheric isolates	Group	Arithmetic mean (cm)
R7	A	6.12
R1	AB	5.1
R23	AB	4.96
R20	AB	4.86
R8	ABC	4.64
R22	ABCD	3.84
R9	ABCD	3.24
R3	ABCD	2.8
R13	ABCD	2.74
R10	ABCD	2.5
R26	ABCD	2.46
R6	ABCD	2.1
R14	ABCD	1.54
R27	BCD	1
R17	CD	0.74
R25	D	0.5
R19	D	0.4

P (0.05) Kruskal-Wallis Non-Parametric Test. Each value followed by a letter refers to a specific group.

The largest inhibition halos were produced by the isolates R7, followed by R1 and R23, while the smallest inhibition halos were observed with isolates R25 and R19.

Regarding the bacteria isolated from the phylloplane, differences were also observed (Table 2). The inhibition halos obtained in the confrontation assays ranged from 0.7 cm to 2.7 cm, with statistically significant differences compared to the control. All isolates from the phylloplane were able to grow on *Ralstonia solanacearum*, inhibiting its development, although to a lesser extent when compared to the bacteria isolated from the rhizosphere.

Strains R1, R23, R7, and R8 exhibited the highest biocontrol activity against *Ralstonia solanacearum*, with growth inhibition radii ranging from 4 cm to 6.12 cm on the agar medium (Figure 2). The varying degrees of antagonistic activity observed among the isolates can be attributed to their capacity to produce antimicrobial compounds that inhibit pathogen growth, as well as their high growth rates and metabolic versatility.

Table 2
Comparison of inhibition halos of *R. solanacearum* produced by bacteria isolated from the phylloplane

Phylloplane isolates	Group	Arithmetic mean (cm)
F1	A	2.72
F2	AB	2.354
F21	AB	2.3
F15	AB	1.98
F17	AB	1.68
F19	AB	1.76
F4	AB	2.06
F18	AB	1.56
F7	AB	1.58
F20	AB	1.2
F3	AB	1.3
F30	AB	1.12
F31	AB	1.14
F8	AB	0.78
F5	B	0.72

P (0.05) Kruskal-Wallis Non-Parametric Test. Each value followed by a letter refers to a specific group.

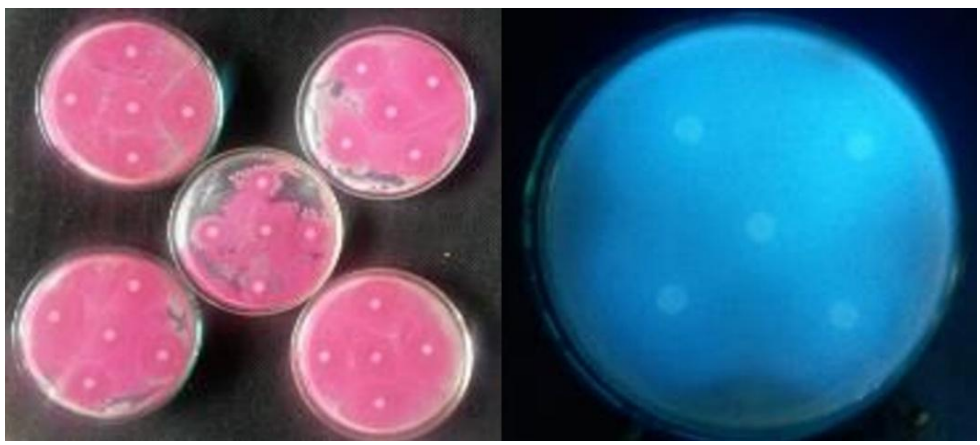

Figure 2. Biocontrol test: Inhibition of R. solanacearum growth by strains R23 and R7.

In the laboratory tests, microorganisms with antagonistic potential isolated from the rhizosphere exhibited a restrictive effect on the growth of the pathogenic bacterium. Two distinctive characteristics were particularly noted in the development of *R. solanacearum*: direct inhibition of the pathogen and growth on the medium. Specifically, the R1 strain, identified as *Bacillus sp.*, produced an inhibition halo, while the remaining bacterial isolates grew on the medium, effectively preventing the pathogen's development (Figure 3).

In contrast, as shown in Figure 4, the bacteria isolated from the phylloplane produced smaller inhibition zones. A plausible hypothesis to explain this behavior may be related to the characteristics of the bacteria associated with the site of isolation. In this regard, ABD Alamer et al. (2020) isolated 245 microorganisms from the rhizosphere of eggplant (*Solanum melongena*), of which 10 strains produced inhibition zones greater than 15.0 mm. Similarly, Kurabachew & Wydra (2013) evaluated the *in vitro* potential of 150 rhizobacteria isolated from tomato (*Solanum lycopersicum*) plants, finding that 13 isolates reduced pathogen growth with inhibition zones ranging from 5.4 to 21.5 mm, with *Bacillus* spp. and *Pseudomonas* spp. strains producing the largest halos. Additionally, Tahir et al. (2017) demonstrated the *in vitro* effects of volatile compounds produced by microorganisms, highlighting beneficial interactions between plant growth-promoting rhizobacteria (PGPRs) and plants, which resulted in the induction of systemic resistance against biotic and abiotic stressors, growth promotion, and inhibition of fungal and bacterial pathogens.

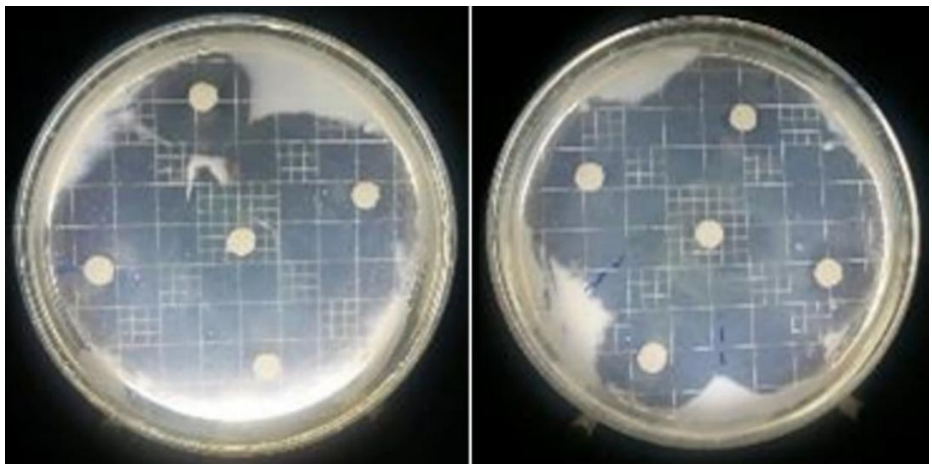

Figure 3. In vitro antibiosis effect of strain R1, identified as *Bacillus* sp., on *Ralstonia solanacearum*.

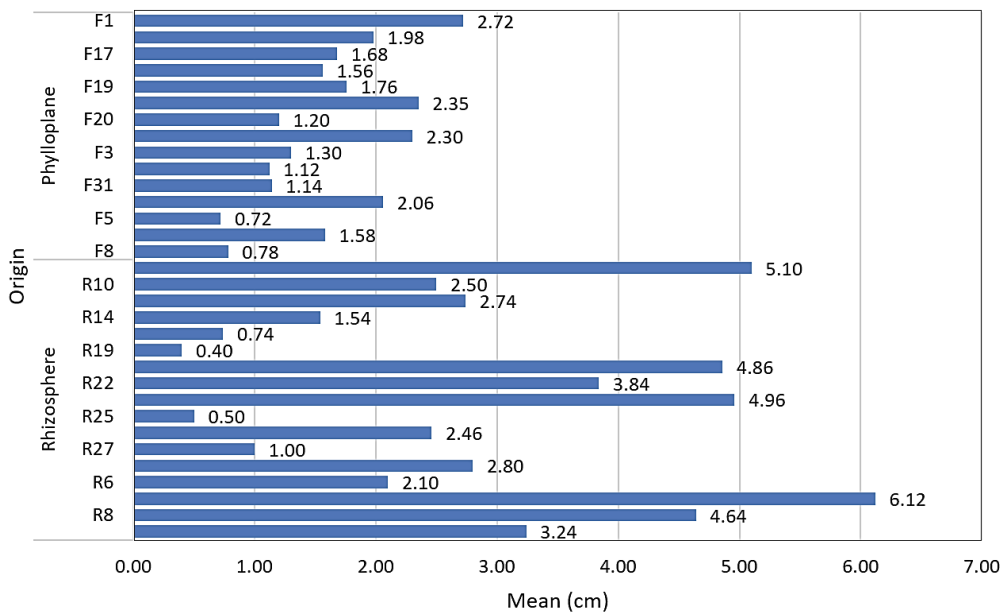

Figure 4. Distribution of the average growth (cm) of bacterial strains isolated from the phylloplane and rhizosphere.

Molecular identification of microorganisms

The results of the molecular analysis revealed that strains R1 and R23 were identified as *Bacillus* and *Serratia*, respectively, with a similarity percentage of 99% when compared to the GenBank database. Sequence analysis of strains R7 and F1 indicated that these microorganisms belong to the genus *Pseudomonas*, with 99% similarity to sequences of this genus. Furthermore, strain F1 was specifically identified as *Pseudomonas aeruginosa*. Electrophoresis of the PCR product resulted in a single band of approximately 1.5 kb, which corresponds to the expected fragment size (Figure 5).

The inability to assign species-level identity to strains R1, R23, and R7 may be due to the high genetic similarity within each genus, particularly among closely related species, which limits the discriminatory power of 16S rDNA sequencing alone.

The identified bacterial genera, including *Bacillus*, *Pseudomonas*, and *Serratia*, have been recognized for their Plant Growth-Promoting Rhizobacteria (PGPR) characteristics (El-Sersawy et al., 2021; Goswami et al., 2016; Kundan et al., 2015). *Bacillus* strains, in particular, are known to produce a diverse range of bioactive compounds that contribute to plant pathogen biocontrol and promote plant growth, making them promising candidates for various agricultural and biotechnological applications. The antagonistic activity of *Bacillus* species is attributed to the secretion of extracellular metabolites, such as antibiotics, cell wall hydrolases, and siderophores, which inhibit pathogen growth. Furthermore, *Bacillus* species are known to enhance plant defense mechanisms against pathogen attacks by activating induced systemic resistance (ISR) (Etesami et al., 2023; Miljaković et al., 2020).

Two of the bacteria identified molecularly belong to the genus *Pseudomonas*. This group of bacteria is widely distributed in agricultural soils, and their properties have been extensively discussed, with *Pseudomonas* being one of the most promising Plant Growth-Promoting Bacteria (PGPB) due to their ability to exhibit various mechanisms of action. The benefits of using microorganisms of this genus are numerous, with some of the most well-known mechanisms including atmospheric nitrogen fixation, phosphorus solubilization, production of phytohormones, lytic enzymes, volatile organic compounds, antibiotics, and secondary metabolites under stress conditions (Mehmood et al., 2023).

The R23 strain was identified as *Serratia* sp., a bacterium that has been attributed to biocontrol potential, mainly due to its production of chitinases (Zhao et al., 2024). Additionally, *Serratia marcescens* has been recognized for their growth-promoting capabilities, primarily through the production of ammonium, indole-3-acetic acid, phosphate solubilization, and other mechanisms (Rico-Jiménez et al., 2023).

Biochemical and physiological characterization of bacterial strains

Biochemical and physiological tests confirmed the molecular identification of the bacteria. For the R7 and F1 strains, identified as *Pseudomonas* sp. and *Pseudomonas aeruginosa*, the results were positive for the following tests: fluorescence on King's B medium, KOH, oxidase, gelatin liquefaction, growth at 7% NaCl, and growth at 38.5 °C. Regarding starch hydrolysis, the result was negative, and no acid production was observed from carbohydrates such as sorbitol, sucrose, arabinose, trehalose, and cellobiose (Table 3).

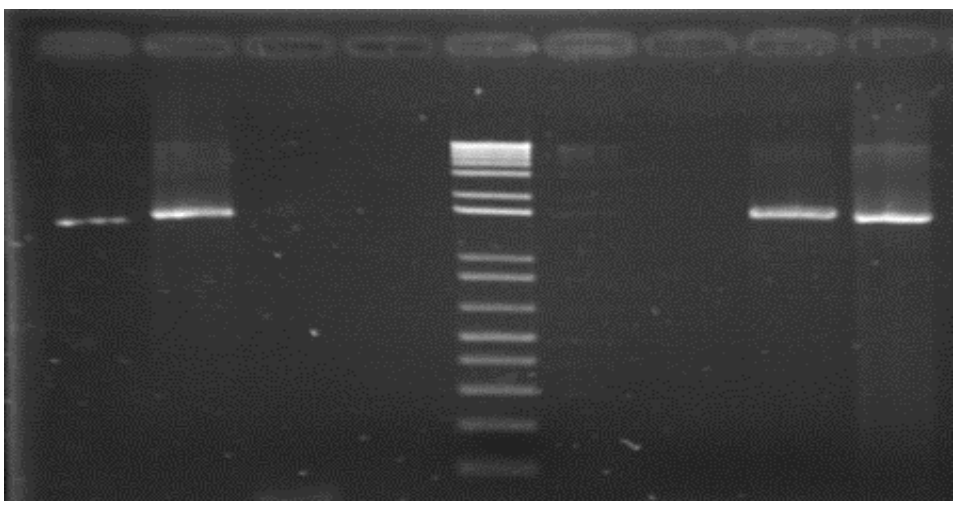

Figure 5. Amplification of the 16S rDNA gene. Line 1: Strain R1. Line 2: Strain R23, Line 5: 100bp molecular weight marker, Line 8: Strain R7, Line 9: Strain F1.

Table 3
Physiological and biochemical characteristics of bacterial strains antagonistic to *Ralstonia solanacearum* Mill

Cultural, physiological, and biochemical tests	*Serratia* sp	*Pseudomonas* sp	*Pseudomonas aeruginosa*	*Bacillus* sp
3% KOH test	+	+	+	-
Oxygen requirement	Facultative anaerobic	Aerobic	Aerobic	Facultative
Oxidase	-	+	+	-
Catalase	+	+	+	+
Fluorescence on King's B medium	-	+	+	-
Tween 80 utilization	+	+	+	+
Levan production	-	-	-	-
Starch hydrolysis	-	-	-	+
Gelatin liquefaction	+	+	+	+
Hydrogen sulfide (H_2S) production	+	-	-	-
Growth at 10% NaCl	+	+	+	+
Acid production from:				
Lactose	-			+
Sorbitol	+	-	-	-
Xylose	+	+	+	+
Sucrose	+	-	-	+
Arabinose	+	-	-	+
Trehalosa	+	-	-	+

The bacterium identified as *Bacillus* was positive for the differential tests of starch hydrolysis, growth in 10% NaCl, and acid production from arabinose and xylose (Table 3), which aligns with the descriptions by Holt (1994) and Moore et al. (2001) for the *Bacillus* genus. However, the results obtained were not sufficiently precise to determine the species. Regarding the bacterium R23, identified as *Serratia* sp., it exhibited physiological and biochemical characteristics consistent with the genus, such as growth in Tween 80, in 7% and 10% NaCl, and acid production from sucrose, lactose, xylose, arabinose, and sorbitol.

4. Conclusions

A total of 32 bacterial strains with potential as plant growth-promoting bacteria (PGPB) were isolated, 17 from the rhizosphere and 15 from the phylloplane of banana crop fields. The rhizosphere microorganisms exhibited superior biocontrol activity, with inhibition of halo diameters ranging from 0.4 cm to 6 cm, while the phylloplane strains showed halos between 0.72 cm and 2.72 cm. Molecular identification of the three most antagonistic bacteria from the rhizosphere and one from the phylloplane revealed they belonged to the genera *Serratia*, *Pseudomonas*, and *Bacillus*.
Given their significant antagonistic potential, further field studies are essential to assess the effectiveness of these bacteria as biocontrol agents under real agroecological conditions.

Author contribution

P. Rodulfo-Acuña: Conceptualization, Methodology, Formal Analysis, Visualization, Writing - Review & Editing Original Draft. **Y. Hernández:** Supervision, conceptualization, Methodology, Review original draft. **P. Terrero-Yepez:** Conceptualization, Methodology, Formal Analysis, Writing Review & Editing. **B. Paiva:** Conceptualization, Writing - Review & Editing Original Draft. **E. Marys-Sarabia:** Supervision, Conceptualization. **R. E. Mejias-Herrera**: Conceptualization, Methodology, Formal Analysis, Visualization, Writing original draft.

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