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Research Article
Identification and Evaluation of Endophytic Bacteria Against Phytopathogenic Fungi *in vitro* Assay

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Abstract

Background and Objective: Plant health problems are a major cause of economic losses in crops worldwide. In recent years, it has been shown that endophytic microorganisms associated with many plant species produce secondary metabolites with antifungal action; among these are endophytic bacteria, which can promote plant growth and control the growth of phytopathogenic fungi. The objective of this study was to identify antagonistic bacteria against phytopathogenic fungi and evaluate their effectiveness in tomato seedlings. **Materials and Methods:** Strains of endophytic bacteria were isolated from pine roots and identified by amplifying the 16S gene. The isolated and identified strains were evaluated *in vitro* in antagonistic tests against fungi of the genera Alternaria and Fusarium. A greenhouse trial was conducted with tomato seedlings of the Floradade variety 15 days after transplant. The species *Bacillus atrophaeus*, *Bacillus subtilis* and *Brucella intermedia* were identified by molecular identification. **Results:** The strain that induced the highest percentage of inhibition was found to be a strain of *Brucella intermedia*, presenting values of up to 50% inhibition of the evaluated fungi. Highly significant differences were shown in the *in vitro* antagonism tests with the evaluated endophytic bacteria strains. *Bacillus atrophaeus* increased fresh root biomass by 46% and both *Brucella intermedia* and *Bacillus atrophaeus* increased fresh plant biomass by 34%. **Conclusions:** The data suggest that the strains shown here inhibit the growth of phytopathogenic fungi and promote plant development in greenhouses.

Key words: Antagonism, *Bacillus atrophaeus*, *Bacillus subtilis*, *Brucella intermedia*, plant growth-promoting bacteria

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Mexico has a wide variety of ecosystems and climatic conditions, which are favorable for horticultural production[1]. One of the most important regions for food production in Mexico is the Northwest, which includes the states of Baja California, Baja California Sur, Chihuahua, Sinaloa and Sonora[2]. In Mexico, phytosanitary control problems have arisen, where the symptoms of known pathogens are confused with those of "new" phytopathogens that are arriving in agricultural areas[3]. Phytosanitary problems are largely the cause of global economic losses in crops, which are mainly caused by fungi[4].

Phytopathogenic fungi, including *Alternaria* spp., *Fusarium* spp., *Rhizoctonia* spp. and *Colletotrichum* spp., are major biotic contributors to soilborne diseases in agriculture, causing significant damage to economically important crops worldwide[5-7]. With increasing regulatory restrictions on synthetic pesticides and a rising demand for organic farming[8], alternative disease management strategies such as beneficial microorganisms and their bioactive metabolites have gained attention as sustainable substitutes for conventional fungicides[9].

Currently, there is great interest in finding environmentally friendly solutions for controlling plant pathogens[10]; these include the use of antagonistic microorganisms which have been isolated from some economically important crops and some have even been commercialized[11,12]. The use of microorganisms, in addition to providing biological control of some pathogens, also stimulates the plant, reduces the use of chemicals such as fungicides and reduces the ecological impact they cause[13]. On the other hand, it has been shown that endophytic bacteria associated with many plant species produce secondary metabolites with antifungal action[14,15]. It has been reported that both beneficial microorganisms and pathogenic species coexist in the same botanical species, causing an antagonistic role and competition for the niche where they live[16], they can reside in plant tissues, carrying out mutualism processes that generate the production of low molecular weight organic compounds that, in many cases, are responsible for providing protection and resistance to the plant[17-19], the effectiveness of these organisms depends on factors such as: host specificity, the ability to move within plant tissues and the induction of systemic resistance[20,21]. Based on these considerations, this study aimed to isolate and identify endophytic bacteria capable of antagonizing phytopathogenic fungi from the genera *Fusarium* and *Alternaria* through *in vitro* assays.

MATERIALS AND METHODS

Study area: This study was conducted in the Horticulture Department at the Agrarian Antonio Narro University, located in Saltillo, Coahuila, Mexico. Field root samples were collected between February and March 2023, while endophytic bioassays and greenhouse experiments were performed from September to December 2023.

Isolation of antagonistic strains: Roots were collected from 20 pine trees (*Pinus cembroides* Zucc.) in the Cañón de Caballos locality, Saltillo, Coahuila (25°14'47.63", 100°53'07.84"). This area has an arid, semi-warm climate, with temperatures ranging from 18 to 22°C, with the coldest month below 18°C and the hottest month above 22°C.

Fig. 1: Bacterial strain with potential antagonist activity

Summer rainfall occurs and winter rainfall accounts for 5 to 10.2% of the annual total. The roots were cut into 1 cm fragments with a scalpel and washed with tap water in a colander to preserve plant material. They were disinfected in a series of steps that consisted of 5 min in 3% hydrogen peroxide, washing with sterile distilled water, 1 min in 70% ethanol, washing with sterile water, 1 min in 6% chlorinated solution and two washes with sterile distilled water. Roots were surface dried with sterile forceps and blotting paper, then plated on potato dextrose agar (PDA) to isolate endophytic microorganisms. Following 72 hrs incubation, bacterial growth adhering to root tissues was subcultured onto fresh PDA for purification. The zones of inhibition (Fig. 1) indicated the presence of antagonistic bacteria.

Identification of endophytic bacterial strains by molecular biology methods: Strain identification was carried out by amplification of the 16S rDNA gene[22]. Strains were propagated in Czapek-Dox medium[23] with 30% glucose for 4 days to obtain greater biomass production. Biomass was centrifuged at 10,000 rpm. The supernatant was discarded and DNA was extracted from the pellet.

The bacteria pellet was placed in a 1.5 mL Eppendorf tube, with 850 µL of extraction buffer was added (20 Mm EDTA, 0.1 M Tris-HCl, pH 8.0, 1.4 M NaCl, CTAB (Hexadecyltrimethylammonium bromide) 2%, 1% β-mercaptoethanol and 1% P VP (polyvinylpyrrolidone) plus 20 µL of proteinase K with 5 min of zoning, then, the Dumolin *et al.*[24]. Protocol was followed. For spectrophotometric analysis, DNA samples were diluted 1:100 by mixing 2 µL of extracted DNA with 198 µL of sterile molecular-grade water, followed by gentle pipette mixing to ensure homogenization. A blank reference was prepared using TE buffer. Absorbance measurements were taken at 260 nm and 280 nm to assess DNA concentration and purity. DNA quality was assessed by visualizing the samples on a transilluminator following agarose gel electrophoresis, with well-defined, high-molecular-weight bands indicating successful extraction. For bacterial identification, partial sequencing of the 16S rRNA gene was performed using universal primers 27F (5 -AGAGTTTGATCCTGGCTCAG-3) and 1492R (5 -CTACGGCTACCTTGTTACGA-3). PCR amplification was performed in 50 µL reaction volumes using Bioline® Taq DNA Polymerase 2X Master Mix, containing 20 mM each of forward and reverse primers and 2 ng/µL of template DNA. PCR conditions were as follows: 10 min at 95°C, 30 cycles (30 sec at 95°C, 30 sec of primer annealing at 55°C and primer extension 1 min at 72°C) with a final extension of 10 min at 72°C.

The obtained PCR amplicons were sent to the National Laboratory of Agricultural, Medical and Environmental Biotechnology (LANBAMA) in San Luis Potosí, Mexico, for sequencing. Sequence homology was searched using the BLAST tool (www.ncbi.nlm.nih.gov/BLAST/) from the NCBI (National Center for Biotechnology Information) database.

Antagonistic effectiveness test against phytopathogenic fungi: The tested strains were *Alternaria* sp., *Alternaria alternata*, *Fusarium* sp., *Fusarium oxysporum* and *Fusarium verticillioides*. These strains were donated by the Department of Parasitology of the Universidad Autónoma Agraria Antonio Narro and the Department of Microbiology of the Faculty of Chemical Sciences of the Universidad Autónoma de Coahuila. The isolated strains were selected for subsequent evaluation against four phytopathogenic fungi: *Alternaria alternata*, *Fusarium* sp., *Fusarium oxysporum* and *Fusarium verticillioides.* The antagonism assay was performed by placing a 5-mm fungal plug at the center of a PDA plate, with four equidistant bacterial inoculation points (3 cm from the center, 0.5 cm from the plate edge) marked using a sterile bacteriological loop. Quadruplicate plates for each bacterial-fungal combination were incubated at 25°C for 7 days. Fungal growth inhibition was quantified by measuring radial expansion (cm) from the colony edge to the plate periphery, comparing test plates to uninoculated controls.

Endophytic bacteria inoculation test on tomato seedlings assay: Tomato seeds (*Solanum lycopersicum* 'Floradade') were used for this test. A growth substrate consisting of 50% peat moss and 50% perlite (v/v) was sterilized by autoclaving at 121°C (1.05 kg cm^{-2}) for 120 min. 2-3 seeds were sown per well in germination trays containing the sterile substrate. After 8 days of growth, uniform seedlings were selected and transplanted into 500 mL sterile containers (10 seedlings per treatment, including controls).

Endophytic bacterial inoculation was performed for 15 days post-transplantation to allow for seedling establishment before treatment application. Three antagonistic bacterial strains (*Bacillus atrophaeus* J1-2, *Brucella intermedia* (*Ochrobactrum intermedium* J2-1 and *Bacillus atrophaeus* J4-3) demonstrating optimal fungal inhibition were selected for inoculation. From fresh cultures, bacterial biomass was harvested by gently scraping plate surfaces with a sterile scalpel after adding 3 mL of sterile nutrient broth to each Petri dish. For each strain, 1 mL of the resulting suspension was transferred to three replicate flasks (n = 3 per strain). Control flasks contained sterile nutrient broth only. All cultures were incubated at

28°C with constant agitation (150 rpm) for 48 hrs. McFarland turbidity standards (0.5-5.0) were prepared by combining barium chloride (1% w/v $BaCl_2$) with 1% sulfuric acid (v/v H_2SO_4) as described by Mahesh *et al.*[25], with each standard corresponding to specific bacterial concentrations (1-10×10 CFU/mL). For optical density calibration, each standard was measured spectrophotometrically at 540 nm using triplicate readings, with sterile distilled water serving as a blank. Based on McFarland standard calibration curves, all bacterial suspensions were adjusted to a standardized concentration of 2.0×10 CFU/mL (equivalent to McFarland 4.0).

Tomato seedlings were maintained under controlled greenhouse conditions with a 16:8 hr (light:dark) photoperiod and constant temperature of 28±2°C. Plants were inoculated weekly for three consecutive weeks following transplantation. The experiment was terminated 30 days post-transplantation, with 10 biological replicates maintained per treatment. The seedlings were watered every third day with Steiner nutrient solution® for hydroponics. Upon completion of the 30-day greenhouse trial, the following growth parameters were quantified for each treatment: Root length (RL), plant height (PH), stem diameter (SD) and fresh biomass (FB).

Statistical analysis: All quantitative data, including fungal radial growth measurements and plant agronomic parameters, were analyzed using One-way Analysis of Variance (ANOVA) in R software (version 4.2.3) with the agricola package. A *post hoc* mean separation was performed using Tukey's honest significant difference (HSD) test at $\alpha = 0.05$ significance level. Treatment effects were considered statistically significant when $p<0.05$.

RESULTS

Molecular identification of bacterial isolates: The 16S rRNA gene sequencing identified the isolated strains as belonging to the genera *Bacillus* and *Brucella* (Table 1). Strains J3-4 and J5-1 showed 100% sequence identity with *Bacillus atrophaeus* (GenBank MT434773.1 and KU836511.1, respectively), while strain J4-2 exhibited 99.86% similarity to *B. atrophaeus* (MN826517.1). Strain J4-1 shared 95.86% identity with *B. atrophaeus* (KJ469797.1), suggesting potential intraspecific variation. Additionally, strain J2-1 was closely related to *Brucella intermedia* (95.56% identity; MK249656.1) and strain J3-6 aligned with *Bacillus subtilis* (94.93% identity; HQ256520.1). The lower sequence identities (94.93-95.56%) for these latter strains indicate possible novel phylogenetic lineages, necessitating further multi-locus or whole-genome analysis for definitive taxonomic classification.

Antagonistic effectiveness test against phytopathogenic fungi: The evaluation of fungal inhibition by the isolated bacterial strains revealed distinct patterns of antagonism against the phytopathogenic fungi after 7 days of inoculation (Table 2, Fig. 2a-d).

Table 1: Identification of obtained strains in the GenBank database

Strain	Identified organism	Percentage of identity	Access number
J2-1	*Brucella intermedia* (*Ochrobactrum intermedium*)	95.56	MK249656.1
J3-6	*Bacillus subtilis*	94.93	HQ256520.1
J1-2	*Bacillus atrophaeus*	99.87	MN826517.1
J3-4	*Bacillus atrophaeus*	100	MT434773.1
J5-1	*Bacillus atrophaeus*	100	KU836511.1
J4-1	*Bacillus atrophaeus*	95.86	KJ469797.1
J4-3	*Bacillus atrophaeus*	99.86	MN826517.1

Table 2: Radial growth of phytopathogenic fungi evaluated with the selected endophytic strains

	Evaluated strain	*Alternaria alternata*	*Fusarium* sp.	*Fusarium oxysporum*	*Fusarium verticillioides*
J1-2	*Bacillus atrophaeus*	3.08±0.14^{b}	3.16±0.38^{b}	4.28±0.10^{b}	3.63±0.05^{d}
J2-1	*Brucella intermedia (basonym: Ochrobactrum intermedium)*	2.83±0.28^{b}	3.41±0.38^{b}	4.56 ±0.40^{b}	5.51±0.44^{c}
J3-4	*Bacillus atrophaeus*	2.75±0.50^{b}	2.91±0.38^{b}	6.25±0.00^{a}	6.46±0.05^{ab}
J3-6	*Bacillus subtilis*	3.23±0.20^{b}	3.58±0.38^{ab}	6.00±0.25^{a}	5.70±0.69^{bc}
J4-1	*Bacillus atrophaeus*	3.36±0.12^{b}	3.50±0.43^{ab}	5.83±0.62^{a}	6.03±0.16^{bc}
J4-3	*Bacillus atrophaeus*	3.08±0.28^{b}	2.91±0.14^{b}	4.78±0.20^{b}	4.41±0.07^{d}
J5-1	*Bacillus atrophaeus*	3.73±0.27^{b}	2.58±0.38^{b}	6.41±0.14^{a}	5.76±0.20^{bc}
Control		5.00±0.75^{a}	4.65±0.72^{a}	6.25±0.25^{a}	7.18±0.07^{a}
Pr(>F)		4.44-05^{e***}	0.0009***	2.87-07^{e***}	6.97-09^{e**}

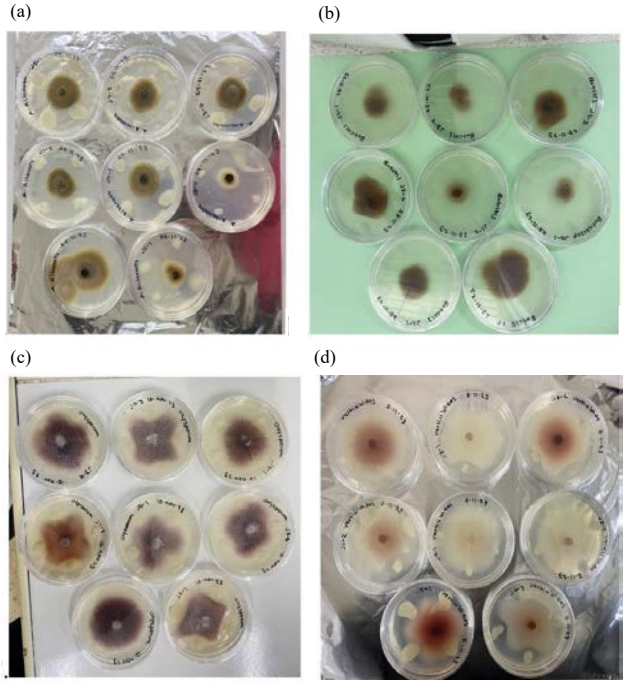

Fig. 2(a-d): Phytopathogenic fungi against bacterial strains (J1-2, J4-1, J5-1, J2-1, J3-4, J3-6, J4-3) 7 days after incubation, (a) *Alternaria alternata*, (b) *Fusarium* sp., (c) *Fusarium oxysporum* and (d) *Fusarium verticillioides*

Fig. 3: Inoculated seedlings with strains of endophytic bacteria, from left to right, Control, J2-1: *Brucella intermedia*, J4-3: *Bacillus atrophaeus* and J1-2: *Bacillus atrophaeus*

Table 3: Morphological variables evaluated in tomato seedlings inoculated with endophytic bacterial strains

Endophytic strain	RL	HP	SD	FBS	FBR
Control	22.71±4.57^{a}	15.57±2.76^{a}	0.44±0.05^{a}	2.35±0.97^{b}	4.65±1.37^{b}
Ochrobactrum intermedium J2-1	28.28±6.18^{a}	18.57±4.46^{a}	0.50±0.05^{a}	4.42±1.33^{a}	7.15±1.92^{a}
Bacillus atrophaeus J4-3	24.42±4.19^{a}	18.2±4.46^{a}	0.50±0.05^{a}	4.01±1.21^{ab}	7.13±1.47^{a}
Bacillus atrophaeus J1-2	26.71±6.55^{a}	18.85±4.01^{a}	0.50±0.00^{a}	4.39±1.39^{a}	6.72±1.46^{ab}
p<F	0.263	0.424	0.178	0.0138*	0.019*

RL : Root length, HP: Plant height, SD: Stem diameter, FBS ; Stem fresh biomass, FBR: Root fresh biomass, Values are expressed as mean±standard deviation. Different letters in the same column indicate statistically significant differences (p<0.05) based on One-way ANOVA, p<F: Probability value from ANOVA and *p < 0.05 indicates significant differences among groups

All tested bacterial strains significantly reduced fungal growth compared to the control, with highly significant p-values ($p<0.001$) for each pathogen. The control group showed the highest radial growth across all pathogens, with *F. verticillioides* reaching 7.18±0.07 cm. Among the treatments, *Bacillus atrophaeus* J3-4 showed the strongest inhibitory effect against *A. alternata* (2.75±0.50 cm) and matched the control in suppressing *F. oxysporum* (6.25±0.00 cm), while *B. atrophaeus* J5-1 showed the highest inhibition against *Fusarium*. sp. (2.58±0.38 cm). *Brucella intermedia* J2-1 was notably effective against *F. verticillioides* (5.51±0.44 cm) compared to the control. These results confirm the antifungal potential of multiple *Bacillus* strains and *Brucella intermedia* in biocontrol strategies against major phytopathogens.

All strains showed significant inhibition compared to the control, *Fusarium* sp., strains J1-2, J2-1, J4-3, J3-4 and J5-1 exhibited significant growth reduction (17-45%), though inter-strain variation was non-significant. The most pronounced antifungal activity occurred against *Fusarium oxysporum*, with strain J2-1 (*Brucella intermedia*) showing 50% inhibition; significantly outperforming other treatments ($p<0.05$). Similar efficacy was observed against *F. verticillioides*, where J1-2 achieved 50% inhibition, while J3-4 showed no significant effect. Among all tested strains, *B. intermedia* (J2-1) emerged as the most consistent antagonist across multiple pathogens. The *Bacillus* strains displayed variable efficacy, with inhibition ranges of 25-50% (*A. alternata*), 23-44.5% (*Fusarium* sp.), 23-31% (*F. oxysporum*) and 17-50% *(F. verticillioides*). These results highlight the strain-specific nature of fungal antagonism and the potential of select endophytes for biological control applications.

Endophytic bacteria inoculation test on tomato seedlings assay: The Table 3 presents the morphological responses of tomato seedlings inoculated with different endophytic bacterial strains. Although no statistically significant differences were observed in root length (RL), hypocotyl height (HP) and stem diameter (SD) among treatments, seedlings inoculated with *Ochrobactrum intermedium* J2-1 exhibited the highest RL (28.28±6.18 cm) and HP (18.85±4.01 cm). Similarly, both *Bacillus atrophaeus* strains (J4-3 and J1-2) showed moderate improvements in these traits compared to the control. Notably, significant differences were observed in fresh biomass of shoot (FBS) and root (FBR). *O. intermedium* J2-1 significantly enhanced FBS (4.42±1.33 g) and FBR (7.15±1.92 g) compared to the control (2.35±0.97 g and 4.65±1.37 g, respectively), indicating its potential as a growth-promoting endophyte. The p-values for FBS (0.0138) and FBR (0.019) confirm statistical significance at the 5% level.

These results demonstrate the strain-specific growth promotion capabilities of these endophytes, with B. intermedia J2-1 showing particularly consistent performance across multiple biomass parameters (Fig. 3).

DISCUSSION

Brucella intermedia (*Ochrobactrum intermedium)* has been reported to secrete hydrolytic enzymes and antibiotic metabolites that induce permanent abnormalities in soil-borne pathogens[26]. Sipahutar and Vangnai[27] and Sun *et al.*[28] demonstrated that *Ochrobactrum* spp. enhance soybean and mung bean yields while exhibiting significant rhizoremediation potential in triclocarban-contaminated agricultural soils. These microorganisms are known to synthesize phytohormones, produce siderophores and exhibit antibiotic activity. Similarly, *Bacillus atrophaeus* represents another important plant growth-promoting rhizobacterium (PGPR) that enhances plant development and controls pathogenic microorganisms[29]. Xue *et al.*[30] reported that *B. atrophaeus* exhibits strong antifungal activity against *Fusarium oxysporum* through secretion, which inhibits the spore germination and induces reactive oxygen species production.

Bacillus subtilis also demonstrates antifungal activity through lipopeptide production, including mycosubtilin and surfactin, which inhibit mycotoxin biosynthesis (e.g., deoxynivalenol and fumonisins)[31]. These strains show significant biocontrol potential via multiple mechanisms against diverse pathogens[32]. Their efficacy stems from both direct antimicrobial action and induction of plant defense responses. Additionally, *Bacillus* spp. can form endospores, enhancing their suitability for formulation and long-term storage as biocontrol agents[32-34].

Ochrobactrum intermedium exhibited 50% inhibition against the tested phytopathogenic fungi, similar results were observed for *Ochrobactrum ciceri*, which demonstrated 70% antifungal activity, outperforming other bacterial candidates[35]. The antagonistic assays revealed distinct inhibition patterns among the bacterial strains using *Bacillus subtilis* strains; these values were lower than those reported by researcher[32], who found that *B. subtilis* effectively inhibited *F. oxysporum* and *F. solani* by 54.7-85.3% compared to untreated controls. Similarly, Baard *et al.*[36] documented 60% inhibition of *F. verticillioides* by a *B. subtilis* strain, while Cavaglieri *et al.*[37] reported variable antibiosis levels (28-78%) for *B. subtilis.* Strains of *Bacillus* spp. displayed multiple modes of action, including siderophore production and secretion of hydrolytic enzymes (e.g., chitinase and β-1,3-glucanase), likely contributing to their direct antifungal effects[38].

Although the trial did not result in increased tomato fruit yield, a positive trend was observed in the fresh weight of roots and stems following inoculation with endophytic bacteria. This aligns with findings by Albayrak[39], who highlighted *Bacillus* species as particularly effective due to their resilience under adverse environmental conditions and broad-spectrum pathogen control. *Bacillus* spp. antagonize fungal pathogens through multiple mechanisms, including nutrient competition, antifungal compound production, systemic resistance induction and plant growth promotion via siderophores and other metabolites[38].

Greenhouse experiments by Renu *et al.*[40] further support these observations, demonstrating that *Ochrobactrum intermedium* inoculation enhanced early germination, chlorophyll content and fresh weight in *Spinacia oleracea* (28.33% increase in shoots, 72.60% in roots). Similar results were reported by Naz *et al.*[41] for *Zea mays*, with improved seed germination, shoot length and auxin-mediated growth.

Bacillus atrophaeus emerges as a promising biocontrol agent for tomato diseases, exhibiting both localized and systemic effects. Hou *et al.*[42] documented its role in enhancing maize growth, biomass yield and antioxidant activity under salt stress. Plant Growth-Promoting Rhizobacteria (PGPR) like *B. atrophaeus* optimize crop nutrition through direct mechanisms (e.g., nitrogen fixation, phosphorus solubilization, IAA and siderophore production) and indirect pathways (e.g., antioxidant defense, exopolysaccharide synthesis)[43].

CONCLUSION

The inoculation of *Bacillus atrophaeus* and *Ochrobactrum intermedium* demonstrates significant potential as a strategy for biocontrol of phytopathogenic fungi *in vitro* assays and enhancement of plant growth in greenhouse conditions. *B. atrophaeus* effectively suppresses fungal pathogens while concurrently promoting root and shoot biomass. Although fruit yield improvements were not observed in this trial, the consistent biomass augmentation and pathogen inhibition underscore their value as sustainable alternatives to chemical inputs. Future studies should optimize strain formulations and application timing to maximize synergistic effects under field conditions. These findings align with broader evidence of PGPR efficacy, reinforcing *B. atrophaeus* and *O. intermedium* as promising candidates for integrated pest and growth management systems.

SIGNIFICANCE STATEMENT

This study highlights the potential of endophytic bacteria, particularly *Brucella intermedia*, *Bacillus atrophaeus* and *Bacillus subtilis*, as biocontrol agents against phytopathogenic fungi (*Alternaria* and *Fusarium*) and as plant growth promoters in tomato crops. The findings demonstrate that these strains significantly inhibit fungal growth (up to 50%) and enhance plant biomass, with *B. atrophaeus* increasing fresh root biomass by 46%. These results suggest that endophytic bacteria could offer a sustainable alternative to chemical fungicides, improving crop health and productivity while reducing economic losses caused by plant pathogens.

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