

ISOLATION AND CHARACTERIZATION OF PLANT-GROWTH-PROMOTING RHIZOBACTERIA FROM ORGANIC COMPOST AND BENEFICIAL EFFECT ON *ORYZA SATIVA*

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Abstract

Plant-growth-promoting rhizobacteria (PGPR) are beneficial bacteria in the rhizosphere that enhance plant growth. This study aimed to isolate and characterize PGPR for biofertilizer development. Sixteen bacterial strains were isolated from organic compost at the On Nut organic composting plant in Bangkok, Thailand. These isolates were evaluated for plant-growth-promoting (PGP) properties, such as phosphate solubilization and the production of indole-3-acetic acid (IAA), ammonia, and chitinase. Isolates 10, 12, 15, and 18 exhibited PGP properties, with the highest phosphate solubilization. In particular, isolate 12 demonstrated the production of IAA at 0.0478 µg/mL and of gibberellin (GA3) at 0.3193 µg/mL, solubility of phosphate at 11.0119 µg/mL, and the highest percent inhibition of radial growth (PIRG) against *Colletotrichum gloeosporioides* C1060. In pot experiments, rice (*Oryza sativa* L. cv. RD47) grown with isolate 12 for 10 days showed statistically significantly increased root dry weight ($p \leq 0.05$) compared to the other treatments. 16S rRNA sequence analysis in EzBioCloud revealed that isolate 12 was closely related to *Bacillus licheniformis*. Therefore, this bacterium exhibits the potential for us as a biofertilizer for early-stage *Oryza sativa* L. cv. RD47.

Keywords: Biofertilizer; Plant-growth-promoting rhizobacteria; PGPR; Rice

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Introduction

Rice is a staple crop consumed by populations worldwide, especially on the Asian continent, and accounts for up to 90% of dietary intake. Global predictions for cereal production indicate an increase from 320 million metric tons to 3.1 billion metric tons by the year 2032, with rice as the major contributor to this growth (OECD, 2023). The pursuit of increased agricultural productivity has resulted in the adoption of chemical fertilizers. However, the excessive application of potassium-containing fertilizers such as potassium chloride can disrupt the balance of other essential nutrients in the soil. Overuse of potassium can interfere with the uptake of magnesium (Mg) and calcium (Ca), both vital for plant growth and development (Zörb *et al.*, 2014). For example, soybean cultivation in sandy soils often shows a limited response to potassium fertilization, as high rates of potassium application in these soils can significantly reduce the absorption of macro- and micronutrients, such as Ca and Mg, due to increased competition for exchange sites (Volf *et al.*, 2022). Additionally, nitrogen-based fertilizers can lead to the accumulation of nitrates in soil, resulting in high nitrate levels in groundwater, which can harm various living organisms (Savci, 2012).

Plant-growth-promoting rhizobacteria (PGPR) have the ability to form communities within the rhizosphere, enabling them to effectively compete with other microorganisms. They can directly enhance nutrient uptake through processes such as fixing nitrogen or solubilizing phosphorus into a form available for plants to utilize. Additionally, they can produce phytohormones such as auxins, which are primarily involved in root and shoot development, and gibberellins, which regulate seed germination, flowering, and fruit development (Grover *et al.*, 2021). Another notable characteristic is their ability to inhibit plant pathogens by producing biocidal compounds or inhibitory substances such as chitinase and oxidase enzymes.

The main objectives of this study were the isolation and identification of plant-growth-promoting (PGP) bacteria from organic compost at the On Nut organic composting plant, Bangkok, Thailand. The isolates were evaluated for their potential to promote plant growth through qualitative measurements. Subsequently, the selected isolates underwent quantitative assessments of PGP properties and their ability to inhibit phytopathogens. Finally, the selected isolates were assessed for their capacity to promote rice plant growth over a period of 10 days and were molecularly identified using 16S rRNA sequencing.

Materials and Methods

Bacterial Isolates

To isolate bacteria from organic compost at the On Nut organic composting plant, Bangkok, Thailand, samples that had undergone fermentation for 3, 5, and 7 weeks were collected from beneath the compost pile at a depth of approximately 50 cm. These samples were subsequently air-dried at room temperature for 24 h. 10-fold serial dilutions were prepared with sterile water, and 100 μ L of each dilution was plated on Nutrient Agar (NA) with the addition of cycloheximide at a final concentration of 25 μ g/mL. The plates were incubated at 37°C for 24 h. The isolates were purified and preserved in 20% (v/v) glycerol at -20°C.

Qualitative Characterization of PGP Traits of Bacterial Isolates

Indole acetic acid (IAA) production was evaluated using Salkowski's reagent method. The bacterial isolates were inoculated into 5 mL of Tryptic Soy Broth (TSB) with and without 1 μ g/mL of L-tryptophan and incubated at 37°C for 7 days in a shaking incubator at 170 rpm. Subsequently, the culture broth was centrifuged at 12,000 rpm for 10 min, and 1 mL of the supernatant was tested with Salkowski's reagent. The mixture was then placed in darkness at room temperature for 30 min. IAA production was indicated by the development of a pink-red color.

Ammonia production was evaluated using Nessler's reagent method. The bacterial isolates were inoculated in 10 mL of Peptone Water (1% peptone, 0.5% NaCl, 0.9% Na₂HPO₄ · 12H₂O, and 0.15% KH₂PO₄) and incubated at 37°C for 2 days in a shaking incubator at 170 rpm. Subsequently, the culture broth was centrifuged at 12,000 rpm for 10 min, and 1 mL of the supernatant was tested with 0.5 mL of Nessler's reagent. The presence of orange sediment indicated ammonia production.

Phosphate solubilization activity was evaluated on Pikovskaya's Agar (PVK agar) plates. Bacterial isolates were spotted on PVK agar. After incubation at 37°C for 3 days, we observed samples for the appearance of clear zones surrounding the colonies, which indicated phosphatase activity.

Chitinase activity was evaluated on Colloidal Chitin Agar (CC agar) plates (1% colloidal chitin, 0.6% Na₂HPO₄, 0.3% KH₂PO₄, 0.1% NH₄Cl, 0.05% NaCl, 0.005% yeast extract, and 1.5% agar). Bacterial isolates were spotted on CC agar. After incubation at 37°C for 7 days, the samples were observed for clear zones surrounding colonies, and the Hydrolysis Capacity (HC value) was calculated (Gupta *et al.*, 2012).

Quantitative Characterization of PGP Traits of Bacterial Isolates

To quantitatively measure IAA and GA3 production using the modified methods of Nonthakaew *et al.* (2022); Castillo and Martinez (1997), selected bacterial isolates were cultured in 100 mL of TSB supplemented with 1 µg/mL of L-tryptophan for IAA production and without L-tryptophan for GA3 production. The cultures were incubated at 37°C for 4 days in a shaking incubator at 170 rpm. Subsequently, the culture broth was centrifuged at 10,000 rpm for 15 min, and the supernatant was adjusted to a pH of 2.5. The supernatant was extracted twice using 50 mL of ethyl acetate. The organic phase was then evaporated at 40°C using a rotary evaporator to obtain a crude extract, which was subsequently weighed. For HPLC sample preparation, the entire crude extract from each sample was dissolved in 1 mL of HPLC-grade methanol. The quantity of IAA and GA3 were determined using reversed-phase HPLC with a Cadenza CD-C18 column (250×4.6 mm, 3-µm particle size). The mobile phase consisted of 20% CH₃CN and 0.1% HCOOH, with a flow rate of 1 mL/min, to measure IAA production; and 15% CH₃CN and 0.1% HCOOH, with a flow rate of 1 mL/min, to measure GA3 production. UV detection was performed at 280 nm for IAA and 204 nm for GA3. The amounts of IAA and GA3 were extrapolated from the standard curve using IAA and GA3 standards, respectively. Each experiment was performed in triplicate.

To quantitatively measure phosphate solubilization, selected bacterial isolates were cultured in 50 mL of PVK broth and incubated at 37°C for 4 days in a shaking incubator at 170 rpm. After incubation, the culture was centrifuged at 10,000 rpm for 15 min and filtered through Whatman No.1 filter paper. Next, 0.1 mL of the supernatant was tested with 0.25 mL of Barton's reagent, and the volume was adjusted to 5 mL with distilled water. After 10 min, the intensity of the yellow color was measured using a BioTek Fluorescence Microplate Reader at 430 nm, and the amount of phosphate solubilized was extrapolated from the standard curve using KH₂PO₄ (Pande *et al.*, 2017). The experiments were performed in triplicate.

Determination of Antibacterial Activity of Crude Extracts

Crude extracts obtained from bacterial cultures in 100 mL of TSB, dissolved in 1 mL of HPLC-grade methanol, were tested for antibacterial activity. *Xanthomonas campestris* pv. *campestris* and *Xanthomonas axonopodis* pv. *manihotis* were

cultured on Mueller–Hinton agar (MH agar) for 48 h at room temperature. Colonies of *Xanthomonas* spp. were suspended in 0.85% NaCl to achieve an approximate absorbance of 0.1 at 600 nm. Adjusted cultures were swabbed onto MH agar, and 20 µL/disc of crude extract was placed on the swabbed plates. Inhibition zones were measured after overnight incubation at room temperature (Rabbee and Baek, 2023). The experiments were performed in triplicate.

In Vitro Antagonistic Activity in Dual Culture

Colletotrichum gloeosporioides C1060 was cultured on Potato Dextrose Agar (PDA) at room temperature for 7 days. For the dual culture method, the bacterial isolate was streaked 20 mm from the edge of an NA plate, and an 8 mm mycelial agar disc of *C. gloeosporioides* C1060 (previously cultured) was placed on the opposite side, 30 mm from the edge. Cultures of *C. gloeosporioides* C1060 alone on NA plates were used as negative controls. The radial growth of the mycelium toward the bacterial isolate strip was measured after incubation at room temperature for 7 days (Rahman *et al.*, 2009). The experiments were performed in triplicate.

Plant-Growth-Promoting Ability on *Oryza sativa* L. cv. RD47

The *Oryza sativa* L. cv. RD47 seeds were soaked in a 15% (v/v) sodium hypochlorite (NaOCl) for 15 min, followed by five subsequent rinses with sterile water. The treated rice seeds were then immersed in a microbial inoculum with a final concentration of 10⁵ CFU/mL for a duration of 2 h. A control set was immersed in water. Following the soaking, the rice seeds were sown in seedling trays containing non-sterilized paddy soil, with 16 seeds planted in each tray. Upon reaching 10 days, 6 seedlings were randomly selected from each planting assessment for the assessment of growth parameters, including shoot dry weight and root dry weight.

16S rRNA Sequencing

Total genomic DNA was extracted using a heat treatment method (Dashti *et al.*, 2009). After DNA extraction, PCR reactions were performed in a T100 thermal cycler (BIO-RAD). The universal forward primers, 27F (5'-AGAGTTT GATCMTGGCTCAG-3'), and reverse primer, 1492R (5'TACGGYTACCTTGTTACGACTT-3') (Heuer *et al.*, 1997), were used for the amplification of the 16S rRNA gene fragment. PCR amplifications were carried out in a 20-µL PCR reaction mixture consisting of 7 µL of deionized

water, 10 μ L of 2 $\times$ PCR master mix solution (iNtRON Biotechnology, Korea), 1 μ L of template DNA, and 1 μ L of both forward and reverse primers. The amplification cycle consisted of an initial denaturation step of 5 min at 95°C, followed by 30 cycles of 30 sec at 95°C (denaturation), 30 sec at 52°C (annealing), and 30 sec at 72°C (extension), with a final extension step for 5 min at 72°C. PCR products were electrophoresed using 1.5% agarose stained with fluorescent staining dye (SMOBIO) and visualized under blue light. PCR products of isolates were purified using a PCR purification kit (Bioneer, Korea), and the amplified product was sequenced on a Sanger sequencing platform (U2bio, Korea). The sequences were submitted to the GenBank database under accession numbers OR743720, OR686313, OR743721, and OR743722 for isolates 10, 12, 15, and 18, respectively.

The sequences were trimmed and aligned using Vector NTI software. 16S rRNA gene sequences were identified using the EzBioCloud server (Yoon *et al.*, 2017).

Statistical Analysis

Quantitative characterization of PGP traits of the bacterial isolates, including IAA and GA3 production, and phosphate solubilization—along with the determination of antibacterial activity of crude extracts and *in vitro* antagonistic activity in dual culture—were performed in triplicate. The plant-growth-promoting ability in *Oryza sativa* L. cv. RD47 was evaluated in six replicates. Data from all experiments were analyzed by analysis of variance (ANOVA) and the means were compared using Duncan's test at $p < 0.05$. All analysis was conducted using SPSS version 26 software.

Results and Discussion

Selection of Bacteria through the Evaluation of PGP Characteristics

A total of 16 isolates (isolates 1, 2, 3, 4, 5, 8, 9, 10, 12, 13, 14, 15, 16, 18, 21, and 22) were obtained from organic compost at the On Nut organic composting plant, Bangkok, Thailand. Among these 16, 2, 4, and 10 isolates demonstrated IAA production, chitinase activity, phosphate solubilization, and ammonia production, respectively as illustrated in Figure 1. Due to the fact that isolates 10, 12, 15, and 18 exhibited remarkable plant-growth-enhancing characteristics, particularly their phosphate solubilization capability, they were selected for quantitative study.

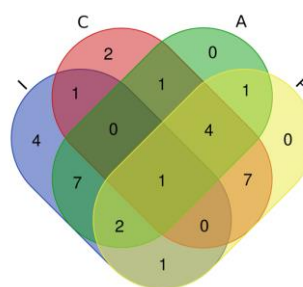


Figure 1. Number of isolates with qualitatively characterized PGP traits. I: IAA production, C: chitinase activity, A: ammonia production, and P: phosphate solubilization

In the current study, we focused on isolating bacteria from compost for potential use as biofertilizers. We specifically targeted compost as it is rich in beneficial plant nutrients, as well as useful microorganisms that promote plant growth and inhibit phytopathogens. The primary bacterial phyla commonly found in the composting process include *Bacillota*, *Actinomycetota*, and *Pseudomonadota*. These bacteria play key roles in organic matter decomposition and plant growth promotion. For instance, bacteria from the *Bacillota* have been reported to enhance plant growth by facilitating the absorption of essential resources such as nitrogen, iron, and phosphorus (Hashmi *et al.*, 2020). Additionally, bacteria from the *Actinomycetota*, particularly *Streptomyces* species, are known for their ability to inhibit phytopathogenic microorganisms by producing antibiotics that combat plant pathogens (Aguilar-Paredes *et al.*, 2023).

Evaluating Quantitative PGP Characteristics of Bacterial Isolates

The amounts of IAA, phosphate solubilization, and GA3 product were investigated. Isolate 18 exhibited the highest production of IAA (0.1119 μ g/mL) ($p \leq 0.05$), followed by isolates 15, 12, and 10 in descending order (Figure 2a). In terms of GA3 production, isolate 12 showed the highest production (0.3193 μ g/mL) but without statistically significant differences ($p > 0.05$) (Figure 2b). Regarding phosphate solubilization capability, isolate 18 had the highest value, at 58.6310 μ g/mL ($p \leq 0.05$), followed by isolates 12, 15, and 10 in decreasing order (Figure 2c).

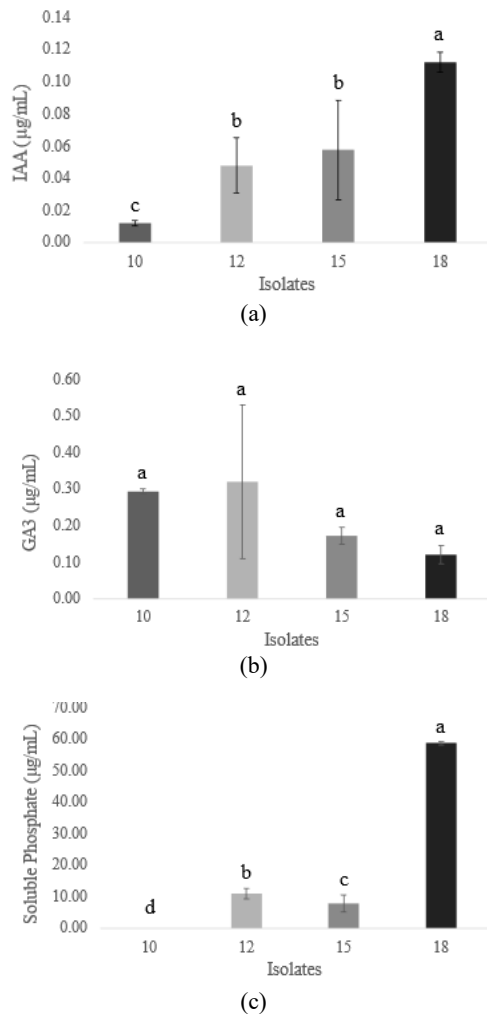


Figure 2. Quantitative analyses of various PGP characteristics of selected bacterial isolates. (a) IAA (b) GA3, and (c) phosphate solubilization. Different letters indicate statistically significant differences between isolates according to Duncan's test ($p < 0.05$)

The initial evaluation of the isolate's PGP properties focused on 4 characteristics: IAA production, ammonia production, phosphate solubilization, and chitinase production. Since all four PGP traits are essential for plants, they were selected as representatives in the screening process. IAA is a vital plant hormone that plays a role in root elongation and in the formation of lateral roots and root hairs, which aid in nutrient absorption from the surrounding area (Alotaibi *et al.*, 2022; Chandra *et al.*, 2018). Ammonia-producing bacteria help increase the nitrogen content in the soil, which is an important nutrient for plant growth, leading to

better root and leaf development (Wei *et al.*, 2024). Phosphate solubilization is another critical trait because phosphorus—a major nutrient required by plants—typically exists in inorganic forms in the soil, making it difficult for plants to absorb. Microorganisms that can solubilize phosphate play a role in converting phosphorus into a form that is easily absorbed by plants (Otieno *et al.*, 2015). Additionally, PGPR that produce chitinase can strengthen the plant's immune system, enabling plants to better combat various pathogens by limiting their invasion (Veliz *et al.*, 2017). In the present study, isolates 10, 12, 15, and 18 were selected due to their outstanding plant-growth-promoting properties, especially in phosphate solubilization. Pande *et al.* (2017) showed that phosphate-solubilizing bacteria (PSB) significantly ($p \leq 0.05$) influenced the length of roots, shoots, and leaves, as well as the fresh and dry weight of roots and shoots, when compared to control groups. PSB have the capability to produce organic acids such as gluconic or formic acid to convert insoluble phosphate into a form that plants can readily utilize.

Antibacterial Activity of Crude Extracts

The antibacterial potential of the bacterial isolates was evaluated based on their zone of inhibition against *X. campestris* pv. *campestris* and *X. axonopodis* pv. *manihotis*. Isolates 10 and 18 exhibited potential antibacterial activity against both strains of *Xanthomonas* spp., as shown in Table 1.

Table 1. Antibacterial activity of crude extracts from bacterial isolates. Different letters indicate statistically significant differences between isolates according to Duncan's test ($p < 0.05$)

Isolates	Average zone of inhibition (mm)	
	<i>X. campestris</i> pv. <i>campestris</i>	<i>X. axonopodis</i> pv. <i>manihotis</i>
10	8.0 ^b ± 0.0	8.3 ^b ± 0.58
12	0.0 ^c ± 0.0	0.0 ^c ± 0.0
15	0.0 ^c ± 0.0	0.0 ^c ± 0.0
18	12.0 ^a ± 1.0	11.3 ^a ± 0.58

Antagonistic Activity Against Phytopathogens in Dual Culture

The interaction between the bacterial isolates and *C. gloeosporioides* C1060 was investigated using a dual culture test (Figure 3 and Figure 4). Isolate 12 exhibited the most effective inhibition of the plant-pathogenic *C. gloeosporioides* C1060, with a PIRG value of 69.84%, as shown in Figure 3.

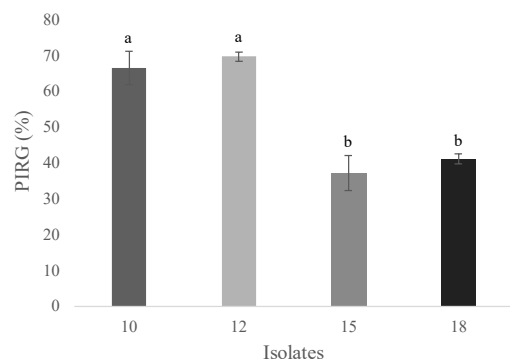


Figure 3. Bacterial isolates' antagonistic activity against the growth of *C. gloeosporioides* C1060 from bacterial isolate. Different letters indicate statistically significant differences between isolates according to Duncan's test ($p < 0.05$).

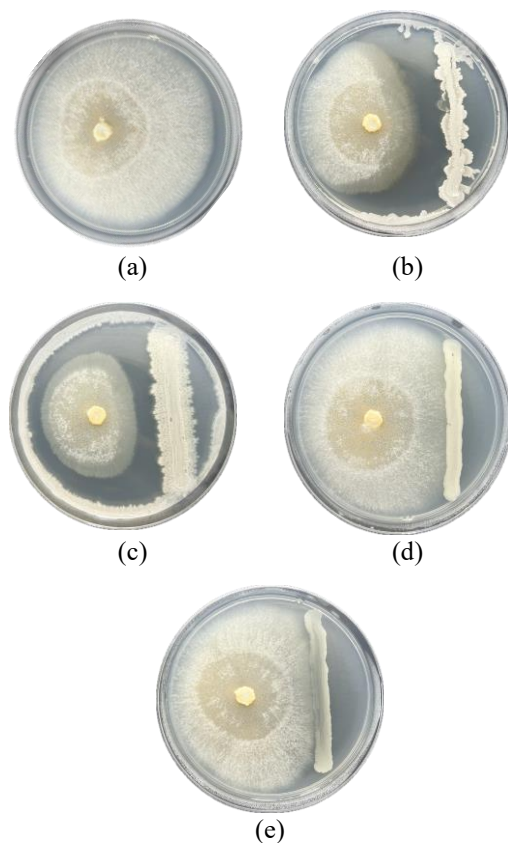


Figure 4. Antagonistic zone between *C. gloeosporioides* C1060 against (a) *C. gloeosporioides* C1060 (control), (b) isolate 10, (c) isolate 12, (d) isolate 15, and (e) isolate 18.

Phytopathogens have a significant negative impact on global agricultural production by affecting economically important crops. One well-known phytopathogen that affects plants is *X. campestris* pv. *campestris*, which causes black rot in the *Brassicaceae* family. Additionally, *X. axonopodis* pv. *manihotis* is the causative agent of bacterial leaf blight in cassava, and *C. gloeosporioides* C1060 is responsible for anthracnose in various fruit crops worldwide (Anon, 2022; Liu *et al.*, 2022; Peralta-Ruiz *et al.*, 2023). In our study, we observed that isolates 10 and 18 demonstrated inhibitory zones against both *X. campestris* pv. *campestris* and *X. axonopodis* pv. *manihotis*. These isolates can thus be classified as PGPR, and could be used as biocontrol agents to combat plant pathogens. For example, *Priestia megaterium* JR48, another PGPR, was previously confirmed to inhibit *X. campestris* pv. *campestris* by enhancing the accumulation of salicylic acid in plants, a biocontrol agent known to induce resistance (Li *et al.*, 2022). Furthermore, evaluating the inhibition of *C. gloeosporioides* C1060 by calculating the PIRG values, we found that all isolates were effective in inhibiting this plant pathogen. Notably, isolate 12 exhibited the highest PIRG value, 69.84%, signifying its exceptional inhibitory activity—a desirable trait for demonstrating PGPR properties.

Plant Growth Promotion in *Oryza sativa* L. cv. RD47

The *in vivo* effects of the PGP isolates were investigated in *Oryza sativa* L. cv. RD47 (Figure 5 and Figure 6). Bacterial isolate 12 gave the highest shoot dry weight, but without statistically significant differences ($p > 0.05$), while it significantly increased root dry weight compared to the other treatments ($p \leq 0.05$), as illustrated in Figure 5A and B respectively.

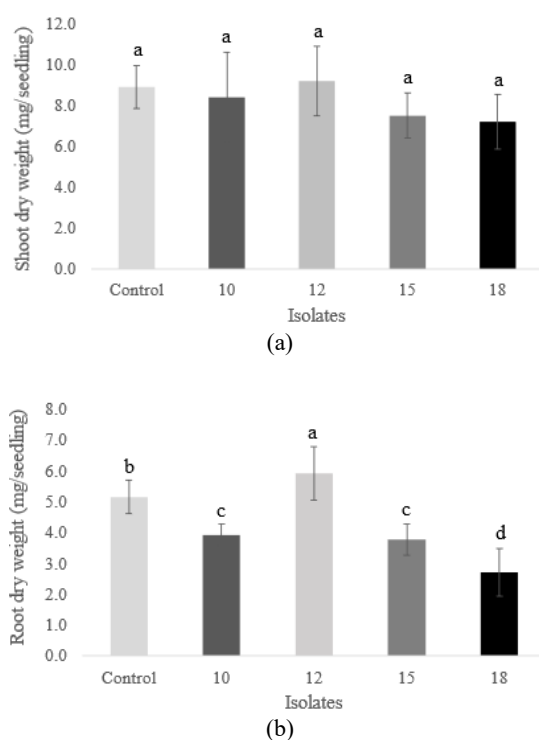


Figure 5. (a) Shoot dry weight and (b) root dry weight of 10-day-old RD47 seedlings germinated from seeds treated with bacterial solution. Different letters indicate statistically significant differences between isolates according to Duncan's test ($p < 0.05$)



Figure 6. 10-day-old RD47 seedlings germinated from seeds treated with (A) control, (B) isolate 10, (C) isolate 12, (D) isolate 15, and (E) isolate 18

The treatment of rice seeds with selected bacterial isolates significantly improved root dry weight, which is a key growth parameter for early plant growth. This positive effect can be attributed to several mechanisms employed by PGP bacteria.

For example, IAA, a crucial growth regulator for rice, is known to stimulate root development and nutrient uptake (Wang *et al.*, 2017). Additionally, the improved root dry weight can be attributed to another mechanism: the production of GA3, which enhances shoot and root elongation, leaf expansion, flowering, fruit ripening, and seed germination. Miceli *et al.* (2019) found that a GA3 concentration of 10^{-6} M was highly effective in promoting plant growth and increasing crop yield by enhancing various growth parameters, including biomass accumulation and leaf expansion. In the present study, isolate 12 significantly increased root dry weight compared to the other treatments ($p \leq 0.05$). One possible explanation for the observation that certain isolates with PGP properties did not enhance plant or root development compared to the control is the delayed response associated with the effects of PGP traits. These beneficial effects, including enhanced root development and improved nutrient uptake, may require a longer timeframe to manifest than the 10-day observation period employed in this study (Grover *et al.*, 2021). Jain and Patriquin (1985) emphasized that inoculation significantly increases the number and branching of root hairs and lateral roots during the initial three weeks post-germination. In contrast, root biomass often shows considerable increases at later growth stages (Okon and Kapulnik, 1986). As a result, any positive impact of the isolates on plant growth may not have been visible at the early stage observed herein, and a longer assessment duration could reveal the true extent of their influence.

16S rRNA Sequencing Analysis

From this study, the 16S rRNA gene sequences were taxonomically identified using the EzBioCloud server program. Isolate 10 was found to be closely related to *Bacillus tequilensis*, with 100% sequence similarity; isolate 12 to *Bacillus licheniformis*, with 99.86% sequence similarity; isolate 15 to *Weizmannia ginsengihumi*, with 100% sequence similarity; and isolate 18 to *Sphingomonas colocasiae*, with 97.17% sequence similarity.

Previous research has demonstrated that *B. licheniformis* FMCH001 acts as a PGPR, enhancing the fresh and dry masses of the shoot and root system of tomato plants when compared to the control (de O. Nunes *et al.*, 2023). The ability of *B. licheniformis* to inhibit the growth of plant pathogenic fungi can be attributed to various mechanisms, one of which is the production of antimicrobial substances. Shleeva *et al.* (2023) found that *B. licheniformis* strain BFP011 is capable of producing a complex with a lipid or carbohydrate moiety. This complex has

demonstrated the ability to combat microorganisms that contribute to plant and human diseases, such as *C. capsici* and *Escherichia coli*. This comprehensive study highlights the potential of isolate 12 as a beneficial microorganism for enhancing crop growth and effectively suppressing plant pathogens.

Conclusions

In this study, four bacterial isolates displayed exceptional qualitative traits in promoting plant growth, as well as the ability to inhibit phytopathogens such as *Xanthomonas* spp. and *C. gloeosporioides* C1060. Evidence from analyses conducted both *in vitro* and *in vivo* indicated that *B. licheniformis* 12 enhances the early (10-day) growth of rice by facilitating an increase in root dry weight. These findings highlight the potential of isolate 12 for further development as a biofertilizer for commercial use in early-stage rice growth. Future research should consider extending the experimentation period with crops to investigate this isolate's long-term effects.

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