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CHARACTERIZATION OF ENDOPHYTIC BACTERIA IN VIETNAMESE RICE SEEDS

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ABSTRACT

Rice is a staple food and is commonly used in daily life not only in Vietnam but worldwide. However, pests, diseases, and nutrient deficiencies are threatening Vietnam's rice production by causing significant damage. The endophytic bacteria (EB) which are isolated from plants, may help improve certain quality traits of rice seeds assist plants in coping with abiotic and biotic stresses from the environment. Therefore, this study aims to investigate some promising characteristics of EBs in rice seeds, including gelatinase production, starch hydrolysis, phosphate solubilizing, cellulase, and IAA synthesis. Rice seeds from various rice varieties were used to isolate the endophytic bacteria. The bacteria were grown in Petri dishes or glass test tubes in some selective media in a controlled environment to screen for investigated traits. A total of five EBs were isolated, and MALDI-TOF mass spectrometry with a log score of MALDI Biotypes greater than 2.0 was employed for bacterial identification. Interestingly, the results revealed that the bacteria in rice grains have a high ability to synthesize cellulase, hydrolyze starch and gelatin, and produce auxin. The highest cellulase activity was associated with *Staphylococcus caprae* while *Micrococcus luteus* exhibited maximum IAA hormon and gelatinase enzyme production. Starch hydrolysis was highest in *Bacillus*. However, these bacteria showed low phosphate solubilization ability. The promising bacteria identified in this study include *Bacillus cereus*, *M. luteus*, and *Bacillus atrophaeus*. The promising results from our study can be utilized for further *in vivo* studies in rice plants to develop biocontrol reagents and biofertilizers for agricultural applications.

Keywords: Auxin synthesis, cellulase, endophytic bacteria, gelatinase production, phosphate solubilizing, rice, starch hydrolysis.

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INTRODUCTION

Endophytic bacteria (EB) are microorganisms that infiltrate plant tissue without causing illness damage to their hosts. EBs have been identified in various plant components, including the roots, stems, leaves, seeds, fruits, tubers, ovules, and nodules of agricultural crops, meadow plants, wild plants, and perennial plants (Afzal et al., 2019). They affect a variety of plant species, including rice (Sen & Chandrasekhar, 2014; White et al., 2019). Numerous studies have demonstrated beneficial interactions between host plants and EBs (Inoue et al., 2007; Olanrewaju et al., 2017). These endophytes are especially attractive candidates for biocontrol agents due to their advantageous characteristics (Ahemad & Kibret, 2014). Certain EBs, such as *Azoarcus*, *Burkholderia*, *Gluconobacter*, *Klebsiella*, *Pantoea*, *Herbaspirillum*, *Rahnella*, and *Pseudomonas*, have been shown to stimulate plant growth (Shyam Kandel et al., 2017). Common characteristics of EBs include growth stimulation through phytohormone release, nitrogen fixation, nutrient solubilization, siderophore secretion, and phytopathogen inhibition via the production of antibiotics and/or cell wall-degrading enzymes (Rosenblueth & Martínez-Romero, 2007).

Moreover, rice is the staple food for more than half of the world's population (Fukagawa & Ziska, 2019), and Vietnam is one of the biggest rice producers in the world. Increasing rice production aimd global warming and rapid population growth is an urgent necessity. However, the overuse of fertilizer and the rapid depletion of available nutrient sources necessitate alternative solutions (Ashitha et al., 2021). If EB inoculants prove effective, they may enhance agronomic efficiency by reducing production costs and environmental damage, allowing for a decrease or elimination of chemical fertilizers (O'Callaghan et al., 2022). Several factors, including root exudation, bacterial colonization in the roots, and soil health, can influence the effectiveness of bacterial inoculants in promoting plant growth and yield. For example, *Serratia marcescens* AL2-16 can fix nitrogen in *Achyranthes aspera* by capturing atmospheric nitrogen and converting it into a form available to plants (Devi et al., 2016). Additionally, certain *Pseudomonas* isolates can solubilize precipitated phosphates to form soluble phosphate in *Pisum sativum* L. (Otieno et al., 2015).

In Vietnam, there are a number of studies about EBs isolated from different plant species including the weed plant (*Echinochloa colonum*) (Luu et al., 2021), and wild rice plants in the Mekong Delta region (Quach et al., 2024). These EBs have been investigated for their plant growth stimulation, microbial pathogen inhibition or *in vitro* antagonistic activity against *Alternaria alternate* fungal. However, EBs isolated from rice have not been widely investigated, even though Vietnam has a big collection of rice varieties.

Therefore, the objective of this study was to investigate the potential characteristics of EBs in Vietnamese rice seeds, including gelatinase production, starch hydrolysis, phosphate-degrading ability, cellulase activity, and auxin synthesis. The promising results of this research may contribute to the development of probiotics for rice cultivation in the future.

MATERIALS AND METHODS

Plant materials

Four rice accessions were obtained from the Plant Resources Center in Hanoi, Vietnam, to investigate the possible effect of accession characteristics on the seed endophytic community (Table 1). Their seeds were used to isolate endophytic bacteria.

Chemicals and reagents

Bacteria were cultured in Tryptic Soy Broth (TSB), and Tryptone Soya Agar (TSA) media (Himedia, India). Gelatine, starch, National Botanical Research Institute's phosphate (NBRIP), carboxymethylcellulose (CMC - $C_6H_9OCH_2COOH$) were from Sigma-Aldrich (Sigma Aldrich, St. Louis, MO, USA). Additionally, NaCl and sodium hypochlorite (NaClO) were supplied by Thermo Fisher Scientific (Waltham, USA).

Table 1. List of the four Vietnamese accessions used, including their province of origin, varietal type, population assignments based on DArT and GBS markers, and phenotypic characteristics

ID	Name	Province	Zone	Maturity class	Grain length	Grain width
G22	Trung Trang	Tuyen Quang	NE	na	Medium	Narrow
G26	Khau Cay Noi	Thanh Hoa	NW	Medium	Long	Large
G132	Padai Tlig Jug	Khanh Hoa	SCC	Late	Very long	Medium
G299	Blao Sinh Sai	Hoa Binh	NW	na	na	na

Notes: u = unknown; na = not analyzed; Zone: NE = Northeast; NW = Northwest; SCC = South Central Coast.

Surface sterilization of seeds

Rice seeds were surface-disinfected according to Van Nguyen et al. (2023). The effectiveness of sterilization was determined by plating 1 mL of the final rinse onto TSA plates and incubating them for 7 days at 28 ^{o}C.

Isolation of bacterial population

The sterilized seeds were ground using a sterilized mortar and pestle. A 0.7% NaCl solution was added to the homogenate, which was then diluted through a series of dilutions. For each dilution, 150 µL was spread onto TSA plates, which were incubated for several days at 28 ^{o}C.

Bacteria strain selection and identification

Isolated bacterial colonies from the TSA plates, which differed from one another, were collected. These colonies were then grown in liquid TSB medium overnight at 28 ^{o}C in the incubator. One part of these bacteria was mixed with liquid glycerol for storage at -80 ^{o}C, while the others were used for identification and functional characterization. The bacteria were grown overnight in a TSB medium and identified using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF) (Brucker, Germany) following the protocol of Nguyen et al. (2022). A log (score) of MALDI Biotypes greater than 2.0 indicated a valid identification.

Evaluation of phosphate solubilizing ability

To identify EB capable of dissolving phosphate, the National Botanical Research Institute's phosphate growth media (NBRIP) was prepared, consisting of 20 g of glucose, 5 g of calcium phosphate, 10 g of magnesium chloride, 0.25 g of magnesium sulfate, 0.2 g of potassium chloride, 0.1 g of ammonium sulfate, and 15 g of agar per liter (Nautiyal, 1999). The bacteria were grown in NBRIP-containing medium, and the plates were incubated at 28 ^{o}C for seven days. The halo-shaped zones surrounding the bacteria colonies on the NBRIP medium indicated phosphate solubilization. The Phosphate Solubilization Index (PSI) was calculated as follows:

$$\mathrm{PSI} = \left(\text{Colony diameter} + \text{Halo zone diameter}\right) / \text{Colony diameter}$$

Evaluation of the ability to synthesize IAA

The ability of bacteria to synthesize indole-3-acetic acid (IAA) is carried out following the guidelines of (Nguyen et al., 2022). The reaction utilized the Salkowski solution, which consists of 0.5 M iron chloride, 98% sulfuric acid, and distilled water. Twenty µL of the overnight bacterial suspension was added to the center of a TSA plate containing L-Tryptophan (12 g/L Tryptone Soya Broth, 0.408 g/L L-Tryptophan, 6.4 g/L agar). Sterile blotting paper was then placed on top of the bacterial suspension in the Petri dish. The plates were incubated at 28 ^{o}C for three days. After incubation, the paper was removed, and 100 µL of Salkowski solution was added to it and incubated for an additional 30 minutes. Pink color on the paper indicated positive IAA production by the endophytic bacteria.

Evaluation of cellulase synthesis

The cellulase activity of bacteria was determined as follows: First, 20 µL of overnight-bacteria suspension was added to the center of the CMC plate (12 g Tryptone Soya Broth (TSB), 0.8 g carboxymethyl cellulose (CMC), 6.4g Agar). The plates were incubated at 28 ^{o}C for three days. After incubation, 2 ml Lugol's solution (100 mg/mL KI, 50 mg/mL I_2) was added and the plates were incubated for an additional 10 minutes. Following this, the plate was rinsed with water, and the image of cellulose activity was recorded.

Evaluation of gelatinase production

The gelatinase activity was assessed according to the method described by Cappuccino and Welsh (2017) with some modifications. First, the gelatine medium was prepared. Overnight bacteria suspension was then inoculated in a test tube containing gelatine medium for two to seven days. Before evaluating the gelatine hydrolysis, the tubes were placed in the refrigerator at 8 ^{o}C for 30 minutes, or until the control tube without bacteria suspension had hardened. (Gelatin typically liquefies at temperatures of 28 ^{o}C or higher; thus, tubes are often stored in the refrigerator at 8 ^{o}C to ensure that the liquefaction is caused by gelatinase activity).

Evaluation of starch hydrolysis

The amylase production assay was adapted from Cappuccino and Welsh (2017). Five EB isolates were streaked on medium plates containing tryptic soy broth (TSB) ((5 g/L), starch (20 g/L), and agar (15 g/L)). After 5 days of incubation at 28 ^{o}C, the test plates were completely flooded with Lugol's solution for 10 minutes. The Lugol solution was then discarded. To determine the presence of starch in the medium, the test plates were air-dried and illuminated. Starch in the presence of iodine acquires a dark blue hue on the surface of the medium, indicating the lack of amylase and signalling a negative result. Conversely, a clear zone of hydrolysis surrounding the colonies suggests that starch has been hydrolyzed, indicating a positive result.

Statistical analysis

All the experiment was repeated at least three times. The differences between parameters were analyzed using the one-way ANOVA and Turkey post-hoc test with 95% confidence in GraphPad version 8.0.

RESULTS

The isolation of the different bacterial genera in the different rice varieties

The colony characteristics such as shape, color, and margins were thoroughly examined. Final isolates were selected based on typical dominant characteristics of the TSA medium. The identification of the bacteria was performed using the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) method, which allows for quick and accurate identification of bacterial isolates. Among the five isolates identified, the taxonomic composition indicated a predominance of the following genera *Micrococcus luteus, Bacillus cereus, Staphylococcus caprae, Bacillus atrophaeus* and *Bacillus ammyloliquefaciens*_ssp._*plantarum* (Table 2).

Table 2. Identification of tested isolates based on the MALDI-TOF method

Strain Code	Species (best match)	Score value
RSEB-22-1	*Staphylococcus caprae*	2.16
RSEB-26-1	*Bacillus atrophaeus*	2.01
RSEB-26-2	*Bacillus ammyloliquefaciens*_ssp._*plantarum*	2.19
RSEB-132-2	*Bacillus cereus*	2.19
RSEB-299-1	*Micrococcus luteus*	2.43

Note: RSEB: Rice seed Endophytic Bacteria.

Phosphate solubilizing ability test

Although our rice seed selection was based on the capacity of plants to be sensitive or resistant to low phosphate conditions, none of the five endophytic bacterial (EB) isolates were able to dissolve calcium phosphate, which is an un-soluble form of phosphate.

The ability to synthesize IAA

Among the five isolates, three displayed varying intensities of pink coloration on the paper discs after staining with the Salkowski reagent (Fig. 1, Table 3). Based on the intensity of colourization, IAA-producing isolates were qualitatively categorized into four groups: no production (2 isolates), low production (1 isolate), medium production (1 isolate), and high production (1 isolate) (Fig. 1, Table 3). Notably, the isolate with the highest levels of IAA production was *M. luteus* (RSEB-132-1) (Table 3).

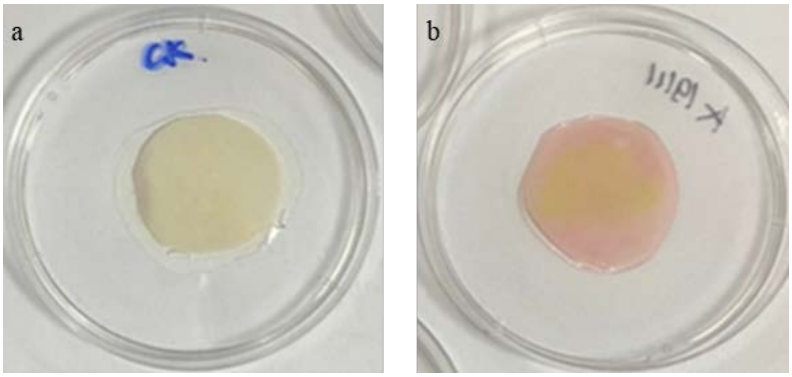

Figure 1. Qualitative assay for IAA production. (a) The negative control; (b) Strain RSEB-132-1 produced IAA with a high level indicated by pink color on the paper disc after staining by Salkowski reagent

Table 3. Enzyme production and antibacterial activities of rice root endophytic bacteria

Name of strain	IAA production	Cellulose hydrolysis	Gelatine hydrolysis			Starch hydrolysis
			2 dpi	5 dpi	7 dpi	
Staphylococcus caprae RSEB-22-1	-	+++	-	-	-	+
Bacillus atrophaeus RSEB-26-1	+	+	++	++	++	+++
Bacillus ammyloliquefaciens_ssp._*plant arum* RSEB-26-2	-	++	++	++	++	+++
Micrococcus luteus RSEB-132-1	+++	++	+++	+++	+++	+
Bacillus cereus RSEB-132-2	++	++	+	+	+	++

Notes: +, ++, +++ indicate the increased intensity of the activities, (-) indicates no activity

The ability to synthesize cellulase

All bacterial strains showed a decolourized zone around their growth on CMC agar (Fig. 2). The presence of a decolourization zone indicates that the bacteria secreted cellulase enzymes, which degrade cellulose in CMC. Among the isolates, *S. caprae* produced the largest decolourization zone, while *Bacillus atrophaeus* showed minimal reaction (Fig. 2).

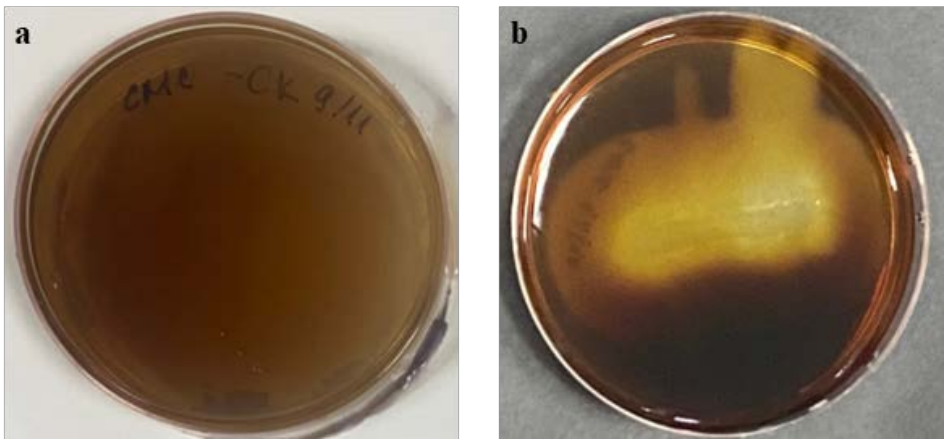

Figure 2. Qualitative assay for cellulase production. (a) The negative control; (b) Strain RSEB-22-1 produced cellulase with high level indicated by the efficiency of cellulase hydrolysis on CMC agar, halo zone surrounding the colonies

Gelatinase production

Seven days post-incubation, the gelatine liquefaction was observed with four isolates. Notably, the isolate producing the high level of gelatinase was *B. luteus* followed by *S. caprae* and *B. atrophaeus*. Lower gelatinase activity was observed in *M. luteus* and *B. ammyloliquefaciens*_ssp._*plantarum* (Table 2, Fig. 3).

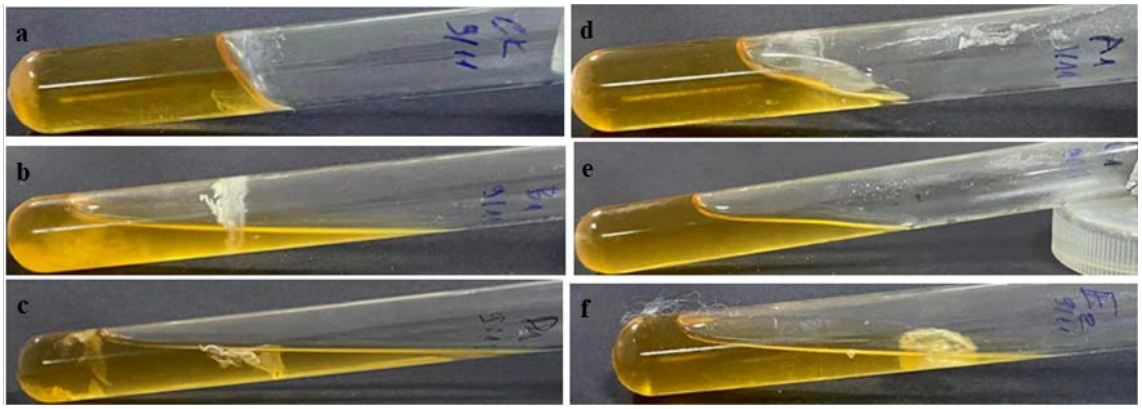

Figure 3. Qualitative assay for gelatinase production of endophytic bacteria. (a) The negative control, the gelatin medium without bacteria; (b-e) Different levels of gelatin liquefacien indicate the level of gelatinase production by *Micrococcus luteus*, *Staphylococcus caprae*, *Baccilus ammyloliquefaciens*_ssp._*plantarum*, *Bacillus cereus*, and *Bacillus atrophaeus*, respectively

The ability to hydrolyze starch

Five EB strains were screened for amylase production. The results showed that 3 EB isolates were able to hydrolyze starch: *B. ammyloliquefaciens*_ssp._*plantarum*, *B. cereus*, and *B. atrophaeus*. The remaining two isolates, *S. caprae* and *M. luteus* did not hydrolyze starch on the test plates (Table 3, Fig. 4). The highest starch hydrolysis capacity was observed in *B. atrophaeus* with a hydrolysis diameter of 3.53 ± 0.29 cm (Table 4).

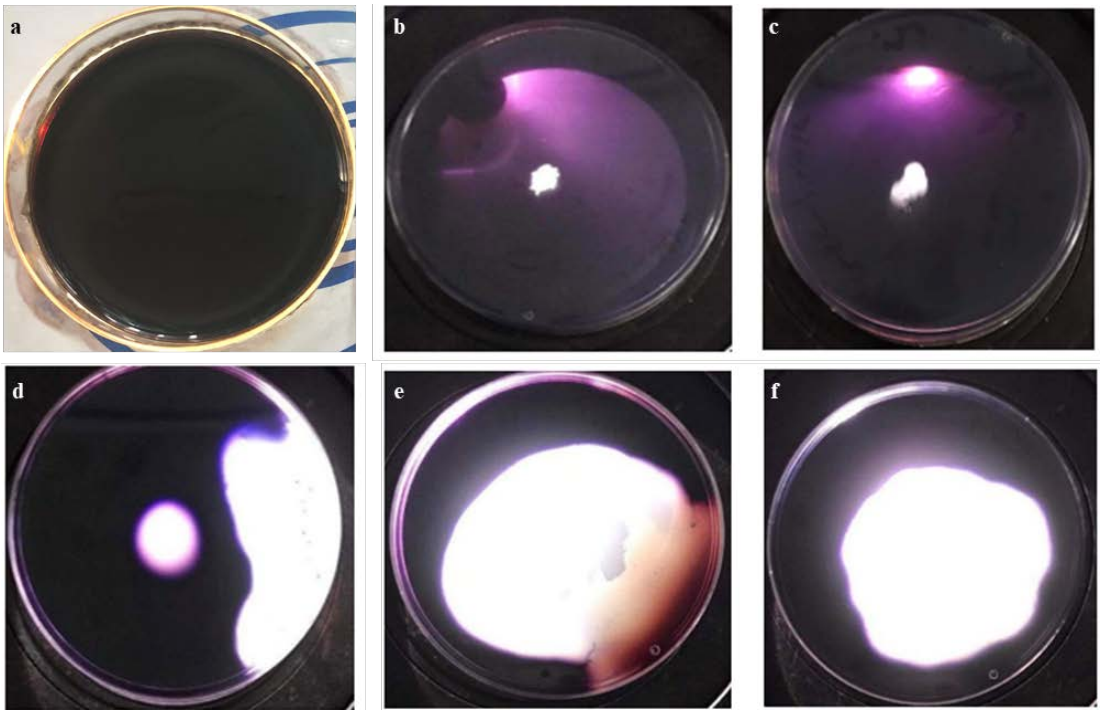

Figure 4. Qualitative assay for amylase production of endophytic bacteria. (a) The negative control, the starch medium without bacteria; (b-f) Different levels of gelatin liquefacien indicate the level of gelatinase production by *Micrococcus luteus*, *Staphylococcus caprae*, *Bacillus cereus*, *Bacillus ammyloliquefaciens*_ssp._*plantarum*, and *Bacillus atrophaeus*, respectively

Table 4. The ability of five EB bacteria to hydrolyze starch

Name of strains	Diameter of halo zone
Bacillus atrophaeus RSEB-26-1	3.53 ± 0.29^{a}
*Bacillus ammyloliquefaciens*_ssp._*plantarum* RSEB-26-2	3.2 ± 0.15^{a}
Bacillus cereus RSEB-132-2	2.43 ± 0.88^{b}
Staphylococcus caprae RSEB-G22-1	0.63 ± 0.06^{c}
Micrococcus luteus RSEB-132-1	0.43 ± 0.06^{c}

Note: Different letters indicate the significant difference between parameters, according to one-way ANOVA and Turkey post-hoc test ($p < 0.05$).

DISCUSSION

In our study, most isolated bacterial strains were found to be round, spherical, or straight rods, with colors ranging from milky white to light yellow and dark yellow. All bacteria were identified as gram-positive using MALDI-TOF.

Moreover, none of the EBs investigated were able to solubilize phosphate. It has been reported that the clear zone surrounding bacterial colonies in insoluble-Pi-containing media may result from phosphatase enzymes produced by phosphate-solubilizing bacteria that hydrolyze insoluble Pi (Pande et al., 2017). Furthermore, there are no studies in the literature demonstrating the ability of these five bacterial strains, isolated from rice grains, to dissolve phosphate. Our results also demonstrated that the Pi-solubility of plants is not due to the EBs isolated from the rice seeds, but may instead result from enzymes secreted by the plants themselves.

M. luteus (RSEB-132-1) was identified as the highest IAA-producing endophytic bacterial (EB) strain in our study. This strain was also found to produce a high concentration of IAA in other rice plants (Shahzad et al., 2017). Interestingly, the draft genome sequence of *M. luteus* (RSEB-132-1) also revealed several enzymes associated with salinity, oxidative stress tolerance as well as herbicide-resistance activity, indicating that this EB strain is promising for in vivo studies in plants. These findings support our results; however, further quantification is needed to determine the exact amount of IAA produced by these isolates.

Cellulase enzymes break down cellulose from plant cell walls into simple sugar. Through this cellulase activity, bacteria can penetrate the interior of plant roots by hydrolyzing wall-bound cellulose, potentially enhancing their ability to colonize plants (El-Deeb et al., 2011). Among our five endophytic bacterial (EB) isolates, *S. caprae* exhibited the highest cellulase activity. Additionally, *Bacillus* and *Paenibacillus* have also been reported to possess cellulase activity (El-Deeb et al., 2011; Chantarasiri, 2015). The variation in cellulase activity is influenced by the specific genes present in each strain (Meryandini et al., 2009).

The high gelatinase activity was obtained in the endophytic bacterial (EB) strains *B. atrophaeus* (RSEB-G26-1), *S. caprae* (RSEB-G22-1) and *B. ammyloliquefaciens_ssp._plantarum* (RSEB-G26-2) EB strains. In other studies, *B. cereus* (RSEB-G132-2) was found to exhibit gelatinase activity in *Limoniastrum monopetalum* and *Zea mays*, respectively (Abedinzadeh et al., 2019; Slama et al., 2019). Moreover, gelatinase can rapidly digest extracellular matrices produced by *Magnaporthe oryzae*. This activity detaches infection structures from membrane surfaces (Inoue et al., 2007). By hydrolyzing gelatine, EBs release amino acids, that are essential for their survival and enable them to compete with and suppress the growth of other pathogens (Inoue et al., 2007). Therefore, the selection of advantageous strains for the development of biocontrol agents heavily depends on the ability of rice seed EBs to hydrolyze gelatin.

By breaking down nutrient sources such as starch into smaller molecules, larger nutrient sources can be absorbed through the cell membrane via diffusion. The endophytic bacterial isolates with amylase activity that hydrolyzes starch are beneficial not only for the bacteria themselves but also for plants, as they enhance nutrient absorption from the environment. Furthermore, the amylase produced by EBs can be utilized in industrial applications, such as food and beverage production (Vijitra Luang-In et al., 2021). In our study, three EB isolates demonstrated high efficiency in hydrolyzing starch: *B. amyloliquefaciens* subsp. *plantarum* (RSEB-G26-2), *B. cereus* (RSEB-132-2), and *B. atrophaeus* (RSEB-26-1).

CONCLUSION

The findings are intriguing, as we discovered that B. cereus (RSEB-132-2) from the rice variety G132, B. atrophaeus (RSEB-G26-1), and B. amyloliquefaciens subsp. plantarum (RSEB-G26-2) from the G26 rice variety exhibit a high ability to synthesize cellulase and hydrolyze starch and gelatin. Additionally, the strain M. luteus (RSEB-132-1) from the G132 variety showed the highest IAA production capacity among the studied strains. In contrast, these rice varieties did not demonstrate the ability to dissolve phosphate, leading us to conclude that resistance to phosphate starvation in rice plants is not related to endogenous bacteria. Further studies should be conducted to isolate additional potential endophytic bacteria (EBs) from rice seeds. The promising activities of these EBs should also be further investigated *in vivo* for potential applications in rice cultivation.

Further studies should be performed to investigate other beneficial activities of these EBs. The *in vivo* test could also be carried out to study the potential application of these identified EBs in promoting plant growth or inhibiting pathogenic pathogens.

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