NOVEL *BACILLUS* SPP. AS PROMISING HALOTOLERANT GROWTH PROMOTING RHIZOBACTERIA FROM MANGROVE PLANT *SONNERATIA CASEOLARIS*

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Abstract. The use of salt-tolerant plant growth promoting rhizobacteria (ST-PGPR) for reducing environmental stress and enhancing quality of crops has recently emerged as a perspective for sustainable development of agriculture, especially in the context of climate change. Within the mining of potential bacterial candidates serving for such purposes, bacterial strains from rhizosphere of mangrove plants in Vietnam were isolated and characterized. Results revealed two microbial isolates from *Sonneratia caseolaris*, symbolized as S13 and S15, exhibited 1-aminocyclopropane-l-carboxylic acid (ACC) deaminase activity and salt tolerance up to 7% sodium chloride. These bacteria performed phosphate solubilizing property with 57.04-61.16% of degradation after 9 days in NBRIP medium. Besides, S13 and S15 showed antimicrobial activity against plant pathogens, including *F. oxysporum* and *A. niger*. By analyzing the ribosomal 16S sequences, bacterial strains S13 and S15 were identified to be *Bacillus* spp. with phylogenetically close relation to *B. velezensis* and *B. siamensis*, respectively. Additionally, sprouts encoated with S13 and S15 bacterial suspensions (~4*10^8 CFU/L each) exhibited enhanced shoot and root elongation in both normal and saline conditions. The bacterial strains were *in vitro* evaluated to be non-toxic to tested cells and animals. Achieved results brought out mangrove derived ST-PGPR strains with highly applicable potential for development of saline agriculture.
Keywords: *growth promoting, mangrove plants, rhizobacteria, salt-tolerant, Sonneratia caseolaris*

Introduction

Sonneratia spp. (Lythraceae, Myrtales) are perennial evergreen plants, distributed commonly in coastal swamp areas that are submerged or inundated with seawater (Kathiresan and Bingham, 2001). These were claimed to play important roles in coastal ecosystems and possess medicinal significance (Kathiresan and Bingham, 2001; Tian et al., 2009; Shefa et al., 2014). *S. caseolaris* is among the most common mangrove plant in northern Vietnam (Pham et al., 2017), particularly in the protection of coastal areas from erosion and salinization. Besides, the plant has been also known as a novel habitat for other species, especially halophile and halotolerant microbes (Subedi et al., 2022).

The interaction between microbies and extreme habitats has been claimed to be beneficial, offering strategies for host plants in enhancing stress tolerance and resistance (Rodriguez and Durán, 2020). Various salt-tolerant plant growth promoting rhizobacteria (ST-PGPR) have been shown to help plants in mitigation of damaging effects in unfavorable conditions by diverse mechanisms, including the production of

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1-aminocyclopropane-l-carboxylic acid (ACC) deaminase for reducing plant stress hormone ethylene, exopolysaccharides for rhizosphere sodium absorption, and volatile organic compounds for facilitating sodium transportation in saline soils (Shultana et al., 2022).

As a consequence of climate change, salinization is dramatically happening and directly threatening cropping areas, food productivity and security. Such effects are predicted to be extremely severe in coastal countries like Vietnam, where two low-lying deltas (Red River Delta and Mekong Delta) are pillars of the country's agriculture and economy (Anh, 2023). Scholars and researchers have conducted various studies to highlight effective management practices for the purpose of counteracting negative climate change's impacts on agricultural crops worldwide, including strategic initiatives, such as improved land and water resources management, as well as scientific approaches in agronomy and cultivation technologies. Of these, the introduction of microbial secondary metabolites and osmoprotective compounds to improve plant stress tolerance has been found in perspective (Majeed et al., 2015; Djumaniyazova et al., 2015; Chen et al., 2021). The application of poly-γ-glutamic acid bioproduct from glutamic acid producing bacteria *Brevibacterium flavum* and *Bacillus amyloliquefaciens* was confirmed to significantly decrease salinity and improve soil structure, offering an eco-friendly way to improve salinized soils (Chen et al., 2021). Above all, the application of different ST-PGPR based formulations in enhancement of agricultural plants' tolerance against salinity stress is being considered the most promising methodology owing its efficacy and broad-spectrum property. For instances, Sharma et al. (2016) found a statistically significant relationship between ST-PGPR inoculation and salt tolerance of peanut seedlings. In 2018, halotolerant *Enterobacter* sp. strain P23 was found to promote rice seedling growth under salt stress in India (Sarkar et al., 2018). Likewise, prior studies highlighted positive impacts of ST-PGPR inoculation on the vegetative growth and vigor of certain cereal grain crops, such as wheat and paddy plants, under saline conditions (Upadhyay et al., 2012; Bal et al., 2013; Jha and Subramanian, 2013). The isolation and selection of potential ST-PGPR strains from mangrove plant *S. caseolaris* for more effective withstand against climate change in local agricultural conditions are thus of great concern.

The present study aimed to investigate ST-PGPR isolates from the rhizosphere of *S. caseolaris* in Vietnam and characterize their biological properties. Results were expected to bring out appropriate bacterial candidates for raising plantations and crop cultivation in saline agriculture.

Materials and Methods

Isolation of salt tolerant and ACC deaminase producing bacteria from S. caseolaris rhizospheres

Soil samples were collected in May 2022 from rhizosphere of *S. caseolaris* at mangrove of Xuan-thuy National Park (20°12'22.5" N to 20°12'65.8" N, 106°32'00.7" E to 106°33'08.2" E). In sterilized box, the rhizomes were detached from the rhizosphere soils and transferred to flasks containing DM medium (Davis and Mingioli, 1950; Sarkar et al., 2018), which was composed of (in g/L) K_2HPO_4 5.34; KH_2PO_4 2; $(NH_4)_2SO_4$ 1; $MgSO_4.7H_2O$ 0.1; $Na_3C_6H_5O_7.2H_2O$ 0.5. The mixture was shaked for 15 h (220 rpm, 30°C) and then settled for 15 mins to separate soil supernatant from soil particles. In the next step, aliquots (10 mL each) of the supernatant were pipetted into

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flasks containing 90 mL of freshly prepared DM medium supplemented with 1% (w/v) glucose (DM-glucose medium), 1% (w/v) NaCl and 3 mM of ACC, and incubated (220 rpm, 30ºC, 10 days) for enrichment. Thereafter, 100 µL aliquots of enriched cultures were pipetted and spread on agar plates for isolation of halotolerant bacterial strains. The isolation was carried out on DM-glucose agar plates containing 1% (w/v) NaCl and 3 mM of ACC as the sole nitrogen source (instead of $(NH_4)_2SO_4$), incubated at 30°C for 3 days. The bacterial isolates were then repeatedly sub-cultured in ACC supplemented saline DM-glucose agar plates for pure straining.

PGP traits assays

Phosphate solubilizing

The phosphate solubilizing property of bacterial isolates were colorimetrically assessed by bromophenol blue containing NBRIP medium [composition (in g/L): glucose 10; $Ca_3(PO_4)_2$ 10; $MgCl_2.6H_2O$ 5; $MgSO_4.7H_2O$ 0.25; KCl 0.2; $(NH_4)_2SO_4$ 0.1; bromphenol blue 0.025] following Mehta and Nautiyal (2001). The method depended on the correlation between blue color intensity and pH changes resulting from the solubilization of phosphate in the medium. Briefly, bacterial isolates were seeded in LB (Luria-Bertani) liquid medium [composition (in g/L): Tryptone 10; NaCl 10; Yeast extract 5] (30ºC, 24 h), adjusted (OD_{600nm}=0.8) and transferred to NBRIP medium (1:500, v/v). Aliquots (5 mL) of culture supernatants were collected at 3 days interval, centrifuged for biomass removal (10.000 rpm, 15 mins) and diluted for optical density measurement at 600 nm by Tecan F150 microplate reader (Switzerland).

Phytohormone production

Bacterial isolates were inoculated in LB medium supplemented with 5 mM tryptophan (30°C, 200 rpm, 7 days). The production of bacterial indole acetic acid (IAA) was assayed by modified colorimetric method as described by Sarwar and Kremer (1995). Briefly, 150 µL of culture filtrate were dispensed into wells of 96-well microplates (Corning, USA) and incubated with 100 µL of Salkowski reagent (solution of $FeCl_3$ 0.5M in perchloric acid 35%, 1:50, v/v) (30°C, 30 min). The absorbance at 530 nm was recorded by Tecan's infinite spectrophotometer. Concentration of IAA produced by bacterial isolates was calculated by regression analysis (Excel, Microsoft Office, USA) using a pre-determined calibration curve established with IAA standards (Sigma-Aldrich, USA). Sterile LB medium served as negative control in the test.

Antimicrobial test

The antimicrobial property of bacterial isolates was assessed by agar plug diffusion method depending on the formation of inhibition zone after depositing an agar culture on test microorganism-inoculated agar plates (Jiménez-Esquilín et al., 2005). The assay employed five test microorganisms, including *Escherichia coli* ATCC 25922 (Gram negative), *Pseudomonas aeruginosa* ATCC 25923 (Gram negative), *Staphylococcus aureus* subsp. *aureus* ATCC 11632 (Gram positive), *Aspergillus niger* 439 (filamentous fungi) and *Fusarium oxysporum* M42 (filamentous fungi). While all the test bacteria were purchased from Oxoid (United Kingdom), test fungal strains were originated from Vietnam Type Culture Collection (VTCC, Vietnam).

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Bacterial growth kinetics with carbon sources

Bacteria exhibiting ability of salt tolerance and PGP traits were inoculated into 100 mL flasks containing DM broth supplemented with 1% (m/v) of tested carbon sources, including mannitol, glucose, sucrose and starch. The culture was orbitally shaken at 220 rpm (30 °C) and sampled every 24 hours during the incubation period. Bacterial densities in culture suspensions after washing by centrifugation (8000 *xg*, 10 min) and sterile phosphate-buffer saline (HiMedia, India) were measured at 620 nm using Tecan's infinite spectrophotometer (TECAN, Switzerland).

In vitro sprouting experiment

Isolated rhizosphere bacterial isolates exhibiting PGP traits and salt tolerance were selected for study of their influence on the growth of mung bean sprouts. For the experiment, they were incubated in DM-glucose medium supplemented with 0.65% (w/v) NaCl and 3 mM of ACC (30^{o}C, 48 h), harvested by centrifugating (3000 rpm, 10 min) and adjusted in phosphate-buffered saline (PBS) to OD_{600nm}=0.5.

Mung bean seeds (TX05, Viet Seed Ltd., Vietnam) were surface sterilized with 70% ethanol (1 min) and 0.02% (w/v) sodium hypochloride (2 min), rinsed throughly with sterile water and incubated (30^{o}C, 70% humidity, 48 h) for gemination. These were then selected to be engulfed in prepared bacterial suspensions (10^9 CFU/mL) (30^{o}C, 4 h) before being transferred to tubes containing semi-liquid Knop medium [composition (in g/L): KNO_3 0.61; $Ca(NO_3)_2$ 0.66; $MgSO_4$ 0.24; $(NH_4)H_2PO_4$ 0.12; $MnSO_4.H_2O$ 0.0023; H_3BO_3 0.0005; $MoO_3 \cdot H_2O$ 0.00025; $ZnSO_4.7H_2O$ 0.0005; $CuSO_4.5H_2O$ 0.000025; agar 8; pH ~ 7]. The experiment was lasted for 7 days at room temperature in aseptic condition. The shoot height and root length of sprouts in each treatment were estimated using ImageJ (Tajima and Kato, 2013) and statistically analyzed.

Identification of microorganism

The 16S rRNA gene sequences of bacterial isolates were amplified by PCR with universal primers 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-TAC GGT TAC CTT GTT ACG ACT T-3′) and sequenced on ABI PRISM® 3100 Avant Genetic Analyzer (Applied Biosystems). Using BlastN tool, obtained sequences were compared to available data at GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Physiological characteristics of bacterial isolates were determined by API kits 50CH and API20E (bioMerieux, France) following manufacturer's instructions. Bacterial characterization was accomplished in consideration to Bergey's Manual of Determinative Bacteriology (Bergey and Holt, 2000).

Toxic test

Cytotoxicity

Portions of bacterial culture was extracted twice with equal volumes of ethyl acetate, and concentrated microbial crude extract was obtained by vacuum evaporation (EYELA, Japan) with 40 rpm at 40°C. The extract was subsequently dissolved in dimethyl sulphoxide (DMSO) (Sigma Aldrich, Germany) for assessment of cytotoxicity on Vero cells (*Cercopithecus aethiops* kidney cells, ATCC CCL-81.4) by Sulforhodamine B (SRB) method as described by Likhiwitayawuid et al. (1993).

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Safety test in animals

Safety test of a bacterial isolates in BALB/c mice was carried out as the procedure mentioned earlier by Carter (1984). Accordingly, BALB/c mice were given either a 0.5 mL intraperitoneal (IP) or 0.2 mL intravenous (IV) injection of brain heart infusion (BHI) broth (Merck, Germany) containing 10^7 CFU/mL of bacterial biomass. Animals were observed daily for health evaluation up to 15 days postinfection.

Statistical analysis

Phylogenetic analysis of bacterial isolates was carried out using MEGA 7 software (Kimura, 1980; Kumar et al., 2016). Freeware ImageJ (Tajima and Kato, 2013) was applied for measurement of total root length.

Experiments were performed in triplicate and results are expressed as mean ± standard deviation (SD). Comparison of variances within groups was conducted by statistical software R 3.5.0 (R Core Team, 2014) using one-way analysis of variance (ANOVA). Post-hoc Tukey HSD (Honestly Significant Difference) analysis was employed for comparing multiple treatments after ANOVA. *p* values less than 0.05 were considered statistically significant.

Results

Isolation and taxonomic identification of bacterial strains

By subculturing on DM-glucose agar plates containing 3 mM of ACC as the sole nitrogen source (instead of $(NH_4)_2SO_4$) and 1% (w/v) NaCl, bacterial isolates, namely S13 and S15 were obtained. These were continuously subcultured three times in the medium to ensure the ability of ACC deaminase production. The morphological characteristics of these bacteria on agar plates are demonstrated in *Fig. 1*.

As shown in *Fig. 1 (A-1, B-1)*, on LB medium, both isolates S13 and S15 appeared as rough creamy white, circular, slightly raised bacterial colonies, 1.2-1.7 mm diameter. The optimal temperature range for growth of these was 25-40^{o}C, while their optimal pH range was 7.0-8.0. Under microscope, the cells were rod shaped, 0.5-0.8 x 1.6-2.5 μm in size (*Fig. 1 (A-3, B-3)*). Both S13 and S15 were found as motile, aerobic Gram-positive bacteria. The identification by API 50 CHB V4.1 revealed 97.9% identity of S13 to *Bacillus amyloliquefaciens/B. subtilis*, and 96.7% identity of S15 to *B. subtilis/B. licheniformis*. Biochemically, bacterial isolate S13 was positive in starch hydrolysis, catalase, gelatinase and Voges Proskauer tests, and negative in β-D-galactosidase, nitrate reduction, H_2S production, lysine decarboxylase, omithine decarboxylase tests. Meanwhile, isolate S15 was positive in starch hydrolysis, catalase, β-D-galactosidase, argninine dihydrolase, citrate, indole, nitrate reduction and Voges Proskauer tests, and negative in H_2S production, lysine decarboxylase, omithine decarboxylase and gelatinase tests. Interestingly, both isolates were able to grow in 7% sodium chloride selective media, indicating they are salt tolerant bacteria.

From extracted genomic DNAs, 16S rRNA genes of isolates S13 and S15 were amplified (primers 27F and 1492R), resulting in 1412 bp and 1413 bp long sequences, respectively. The 16S rRNA gene of S13 was found similar in sequence with high identity percentage of 100% to *Bacillus velezensis* strain EN01 (Sequence ID: CP053377.1), *B. amyloliquefaciens* strain WF02 (Sequence ID: CP053376.1), *B. siamensis* strain cqsM9 (Sequence ID: MN826567.1) and *B. velezensis* strain NN04

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(Sequence ID: MT114570.1). On the other hand, the 16S rRNA gene sequence of S15 showed 100% identity to *B. subtilis* strain BSFT-39 (Sequence ID: MN945444.1), *B. velezensis* strain 3618 (Sequence ID: MT538490.1), *B. siamensis* strain JBRI-MO-2019-0019 (Sequence ID: MN865931.1) and *B. amyloliquefaciens* strain CIAD-IB72 (Sequence ID: MK941881.1). The phylogenetic analysis conducted in MEGA7 suggested a close relationship of S13 and S15, whereby both of them belong to bacterial genus *Bacillus* (*Fig. 2*), therefore an association with determined characteristics were required for deepened taxonomic identification. In terms of aforementioned DNA barcoding, morphological, biochemical and physiological characteristics, S13 and S15 were identified as bacterial strains of *B. velezensis* and *B. siamensis*, respectively.

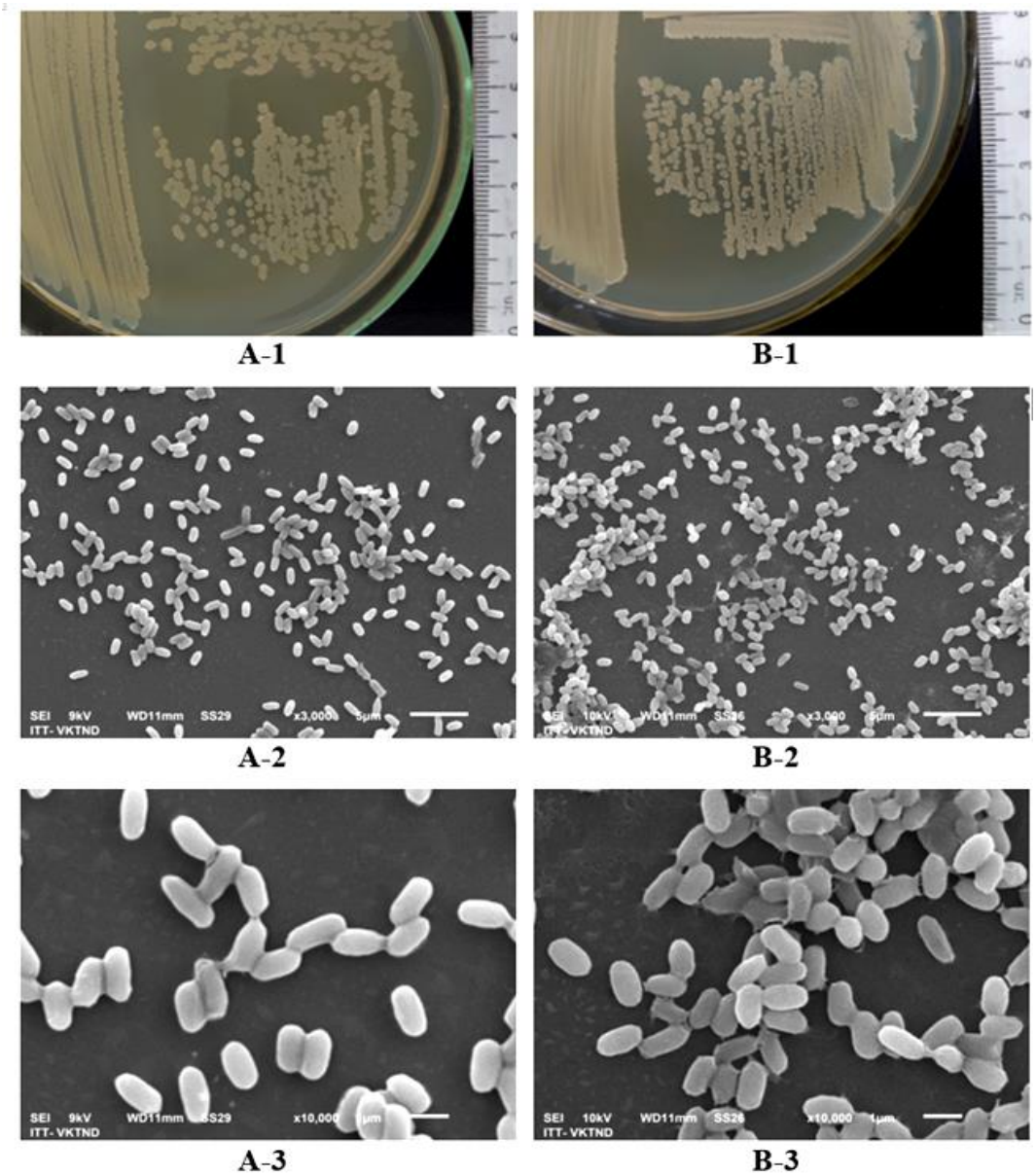

Figure 1. *Macro- and micromorphology of bacterial isolates S13 and S15. Colonies of S13 (A-1) and S15 (B-1) on LB agar plate (30°C, 3 days of incubation); SEM microscopic images of S13 (A-2, A-3) and S15 (B-2, B-3) at x3,000 and x10,000 magnifications, respectively (taken by Jeol SM-6510LV, Japan)*

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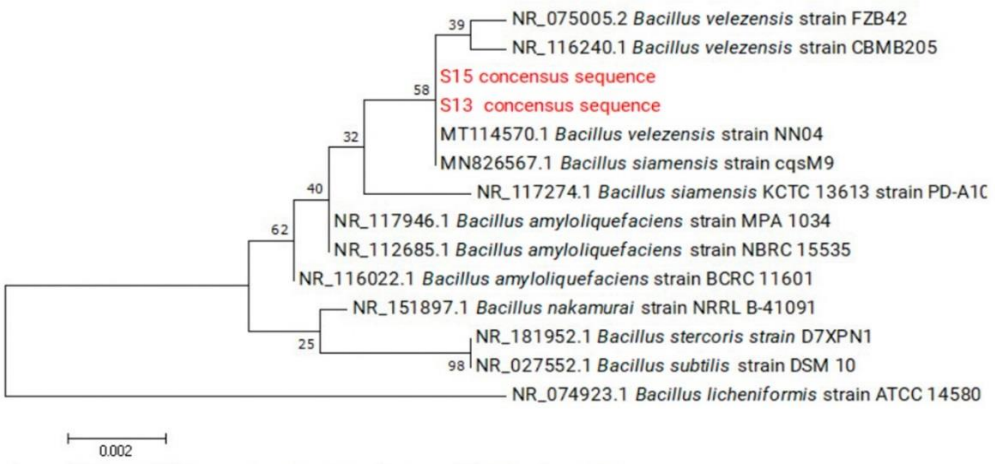

Figure 2. *Phylogenetic tree of bacterial strains S13, S15 and other published species in GenBank reconstructed from the 16S rRNA gene sequences. (Maximum likelihood method, 1000 bootstrap replicates, 1378 positions)*

It is worth noting that *B. velezensis* and *B. siamensis* have been referenced as plant-associated species of the operational group *B. amyloliquefaciens* (Fan et al., 2017), whose members known as important candidates with biotechnological potentials (Ngalimat et al., 2021). To determine plant growth promoting (PGP) traits of bacterial strains *B. velezensis* S13 and *B. siamensis* S15, their abilities to solubilize phosphate, produce phytohormone and antimicrobial compounds were assayed. Besides, their effects to sprouting process, *in vitro* cells and in animals were additionally considered.

PGP properties

Phosphate solubilizing and phytohormone production

Phosphate-solubilization is known as a basic growth promoting trait for improving nutrient availability in adjacent soils and rhizospheres to plants. The assessment of solubilized phosphate amounts in culture supernatants of bacterial strains S13 and S15 revealed a significant variation to negative control (*Fig. 3*). As shown in the figure, during nine days of experiment, no significant change was observed in optical density at 600 nm of uninoculated medium, while the trend of continuously reduced phosphate in bacterial infected media was obvious. In detail, the rate of solubilization by S13 and S15 varied from 15.83-25.29% at day 3 to 38.17-45.23% at day 6 and peaked 57.04-61.16% at the ninth day of incubation in comparison to vehicle.

Along with phosphate solubilizing, phytohormone producing property is an important trait of plant growth promoting microorganisms for increasing plant vigor. The spectrophotometrically measured amounts of *in vitro* produced IAA after inoculating bacterial strains S13 and S15 in L-Tryptophan supplemented media were elucidated by interpolating from standard IAA calibration. As depicted in *Table 1*, *B. velezensis* strain S13 exhibited higher rate of produced phytohormone than *B. siamensis* strain S15, and both of them were significantly higher than uninoculated negative control (*Table 1*).

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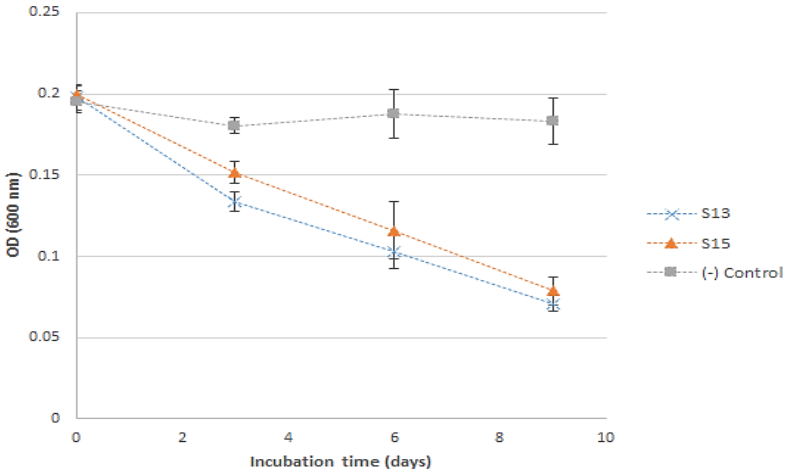
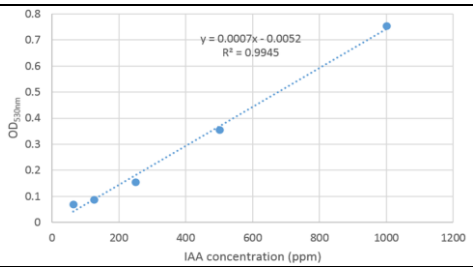


Figure 3. *Solubilized phosphate by inoculating with bacterial strains S13 and S15. Incubation in NBRIP medium (30°C, 9 days). Uninoculated NBRIP medium served as the negative control. Absorbance recorded at 600 nm. Error bars represent standard deviations of three replications*

Table 1. *Bacterial indole acetic acid production by strains S13 and S15. Data are expressed as mean ± SD of three replicates*

Test samples	IAA production (ppm)	Calibration of IAA concentration and optical density at 530 nm
Negative control	0	
Strain S13	34.53 ± 2.37	
Strain S15	31.42 ± 3.19	

Antimicrobial activity

Assessment of the antagonistic activity of *B. velezensis* strain S13 and *B. siamensis* strain S15 against five test microbes using agar plug method showed diverse inhibition by both bacteria (*Table 2*). Despite the difference in inhibiting spectra, both S13 and S15 exhibited antimicrobial effect against 4/5 test microorganisms on agar plates. Interestingly, they were found antagonistic to ubiquitous fungal pathogen *F. oxysporum* and saprophytic fungus *A. niger*. Fungal strains of *A. niger* were commonly reported as saprophytic fungi and opportunistic invaders of agricultural crops, and recently have been mentioned for plant pathogenicity, such as in ginger and peanut (Pawar et al., 2008; Xu et al., 2015). The present results indicate the possible use of *Bacillus* strains S13 and S15 in biocontrol of plant diseases.

Achieved results indicate potentially beneficial impacts to host plants and crops of bacterial strains isolated from mangrove plant *S. caseolaris*. Further studies were taken place to figure out prospects of applicating these microbes in saline agriculture.

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Table 2. *Antimicrobial activity of bacterial strains S13 and S15. Results were expressed as mean value ± SD of growth inhibition zones diameters obtained of two replicates*

Test microorganisms		Antagonistic area (D-d, mm)*		
		Negative control	S13	S15
(-) Gram bacteria	*Escherichia coli*	0	4.50±0.25	0
	Pseudomonas aeruginosa	0	0	6.25±0.13
(+) Gram bacteria	*Staphylococcus aureus*	0	17.25±0.13	9.25±0.25
Filamentous fungi	*Aspergillus niger*	0	4.50±0.25	4.00±0.50
	Fusarium oxysporum	0	5.50±0.25	4.25±0.13

* D: Inhibition zone diameter; d: Agar plug diameter. Uncultured agar plug served as negative control

Carbon sources utilization

Strains S13 and S15 were inoculated into carbon sources supplemented medium for quantitative observation of their substrate utilization. Results are summarized in *Fig. 4*. In general, results showed that both bacterial strains were able in assimilating a wide range of carbonhydrates, including mannitol, glucose, sucrose and starch. However, they showed different manners of utilizing these.

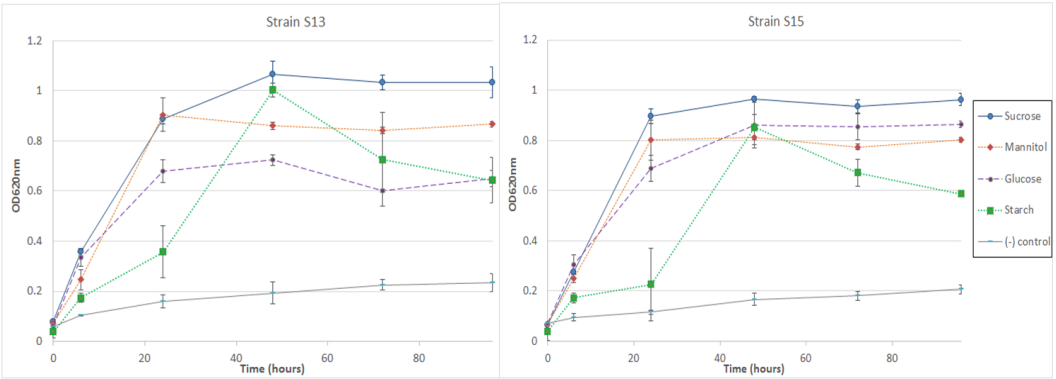

Figure 4. *Carbon sources utilization of bacterial strains S13 and S15. Incubation in DM medium (30°C, 96 hours). Uninoculated DM medium served as blank. Carbon source unsupplemented DM medium served as negative control. Error bars represent standard deviations of three replications*

As demonstrated, the bacterial growth was significantly increased in the presence of all investigated carbon sources, suggesting possibly miscellaneous metabolic pathways by *B. velezensis* S13 and *B. siamensis* S15. Another important similarity between the carbon utilization of S13 and S15 were the preference of sucrose for proliferation in all growth phases (*Fig. 4*). Additionally, growth kinetics of these bacteria with starch as the substrate reached the peak 48h after inoculation, before reducing without obvious stationary phase. The ability to metabolize glucose was divergent between two *Bacillus* strains, since it was found to be more preferable to S15 than S13. Besides, they had a similar tendency in taking up mannitol for growth.

Achieved results indicate capabilities of *B. velezensis* and *B. siamensis* to metabolize a wide range of carbohydrates, including monosaccharides (glucose), sugar alcohols

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(mannitol), disaccharides (sucrose), amylose/amylopectin (starch), under favourable conditions. These findings may play a role as prerequisites to continuing studies on metabolic pathways of *Bacillus* spp., as well as selection of suitable substrates for sustainable fermentation in the future.

In vitro sprouting experiment

The isolated *Bacillus* spp. bacteria were inoculated with germinated mung bean seeds and observed for their effects on sprouting development. Results demonstrated in *Table 3* and illustrated in *Fig. 5* revealed significant differences in growth parameters of experimental mung bean seedlings. Under both normal and saline (0.65% NaCl) conditions, the inoculation with strains S13 and S15 improved root length and shoot height in comparison to uninoculated controls. The seedlings' growth parameters indicated positive responses with the inoculation of *B. velezensis* S13 and *B. siamensis* S15, especially in saline medium. In detail, after 7 days, under saline condition, the maximum shoot growth was recorded by inoculation with S15, with 51.56% increased shoot height, followed by 26.6% of increase by S13. Furthermore, the roots were elongated by 114.16% and 111.94% after 7 days of inoculation with bacterial strains S13 and S15, respectively.

Table 3. *Effects of bacterial inoculation on mung bean seedlings' growth parameters. Data expressed as mean values ± SD of three replications*

Samples	Salt concentrations (%)	3 days		7 days		p
		Averaged total root length (cm) *	Averaged shoot height (cm) *	Averaged total root length (cm) *	Averaged shoot height (cm) *	
Control	0	3.35±1.10 [a]	6.54±0.75 [a]	6.82±1.22 [a]	10.50±0.76 [a]	
	0.65	1.50±0.75 [b]	3.56±0.55 [b]	2.26±0.85 [b]	5.45±0.81 [b]	<0.05
S13	0	5.80±0.61 [c]	10.50±1.43 [c]	8.19±1.81 [c]	13.48±1.72 [c]	
	0.65	3.08±0.83 [d]	5.35±0.68 [d]	4.84±2.32 [d]	6.90±1.46 [d]	<0.05
S15	0	6.29±1.28 [e]	11.44±0.83 [c]	10.06±1.29 [e]	14.21±0.72 [e]	
	0.65	2.93±1.11 [d]	5.59±1.27 [d]	4.79±0.70 [d]	8.26±1.49 [f]	<0.05

*Different superscript letters in the same column represent significant differences verified by Tukey's HSD test at $p < 0.05$. Uninoculated sprouts served as control

The presented results could serve as a basis for further effective infection tests and expandable studies before application in field condition.

Toxic test

A consideration to the database of safety-assessed microorganisms (https://zag.bvl.bund.de/, last accessed on 28th Jan 2024) revealed no information relating either *B. velezensis* or *B. siamensis*, notwithstanding that both species have been claimed as applicable PGPB in the literature (Dunlap et al., 2015; Ngalimat et al., 2021). Therefore, examination of toxin production by bacterial strains *B. velezensis* S13 and *B. siamensis* S15, both *in vitro* and *in vivo*, were required. According to OECD "Environment Health and Safety Publications Series on Testing and Assessment No. 46" and OECD Guideline 453, a combination of cytotoxicity on mammalian cells and rodent toxicity were evaluated.

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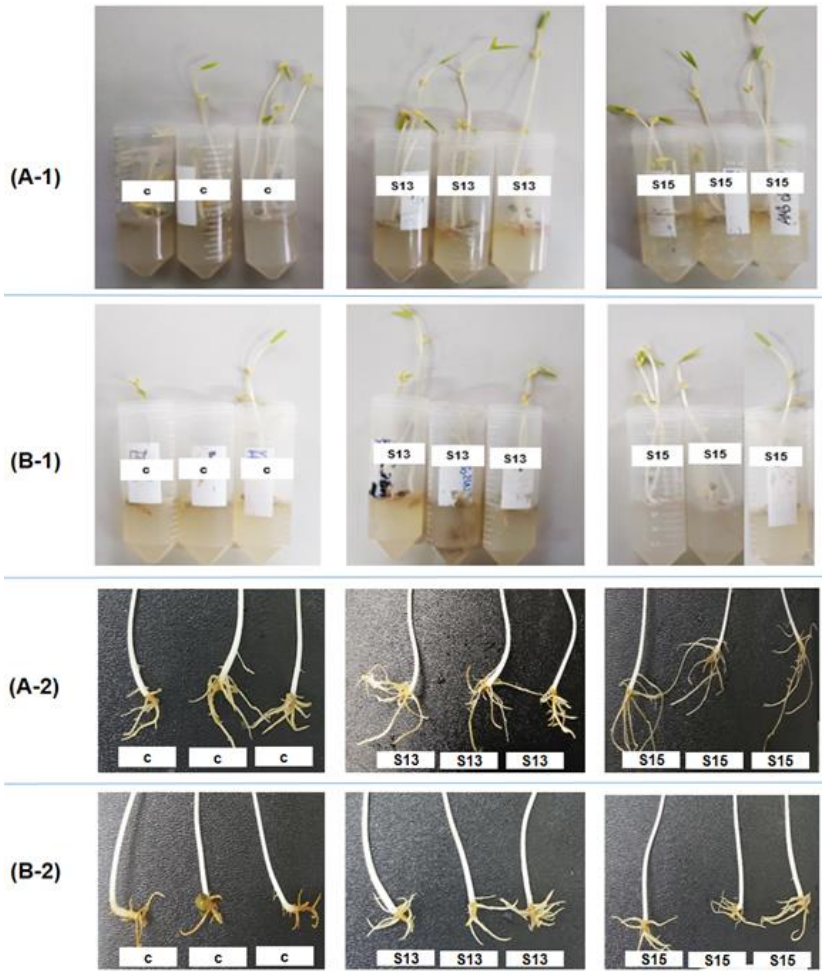

Figure 5. *Shoot and root morphological changes of sprouts under experimental inoculation with bacterial strains S13 and S15 after 3 days. Incubation at medium containing 0% NaCl (A-1, A-2). Incubation at medium containing 0.65% NaCl (B-1, B-2). Uninoculated sprouts served as control*

As a result of cytotoxicity on Vero cells, crude extracts of *B. velezensis* S13 and *B. siamensis* S15 exhibited no cytotoxic effects, with 99.45±0.82 and 98.09±1.15% of cell survival percentage at the highest test concentration of 100 µg.mL^{-1}, respectively.

As mentioned by "SANCO/12116/2012–rev.0" - Guidance of European Commission Health & Consumer Protection Directorate-General, intravenous/intraperitoneal (IV/IP) toxicity and pathogenicity (infectivity) test data on the pre-formulated product is a prerequisite for the safety of a microbial product on animal, therefore IV/IP administration was applied in the present assessment of S13 and S15. In BALB/c mice, no fatal case was recorded when injected with aliquots containing *B. velezensis* S13 and *B. siamensis* S15 cells, both intraperitoneally and intravenously. The mortality rates as well as animal's conditions 15 days after the injection were determined presented in *Table 4*.

For application as PGP preparations, bacterial inoculants must be validated to be safe to living organisms. The fact that both *B. velezensis* S13 and *B. siamensis* S15 exhibited no detectable hazard potential in cells and test animals could thus be considered an evidence to highlight scenario of applying these bacteria in saline agriculture.

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Table 4. *Presentations associated with bacterial biomass of S13 and S15 in BALB/c mice*

Sample	Number of test animals	Injection route*	Mortality rate (%)	Animal's condition 15 days post-injection	Test result
Control (BHI broth)	6	IV	0	Healthy	Safe
	6	IP	0	Healthy	Safe
BHI broth containing S13 biomass	6	IV	0	Healthy	Safe
	6	IP	0	Healthy	Safe
BHI broth containing S15 biomass	6	IV	0	Healthy	Safe
	6	IP	0	Healthy	Safe

* IP: intraperitoneal injection; IV: Intravenous injection

Discussion

Specificity and novelty of the bacterial isolates

The cooperation between plants and rhizobacteria has been well-documented and was claimed to be dependent on specific chemotaxis of microbes towards root exudates. While some bacteria exerted genotype specific colonization in plant rhizospheres, some were identified to be able in establishing association to multiple plant partners (Drogue et al., 2012). The host specificity was assumed as an obstacle for broad application of any PGPB in different agricultural plants, but also an advantage for selectively promoting target crops excluding invasive species. In our study, two halotolerant PGP bacterial strains of *Bacillus* spp. were found to exhibit phytostimulating effects in *in vitro* tests, especially on the growth of mung bean sprouts. These indicated an occasionally commensalism association between isolated *Bacillus* spp. strains and plant's rhizome, where stringent dependence of bacteria to host-genotype was not obligatory.

It was noted that both *B. velezensis* and *B. siamensis* were documented as beneficial bacteria inhabiting diverse environments and have been broadly applied in agriculture. They were reported as associated bacteria of different hosts, including terrestrial and mangrove plants (Subha et al., 2020; Alenezi et al., 2021; Kaleh et al., 2022). Nevertheless, isolates S13 and S15 showed a number of novel properties like discrete cells, lowered IAA production, multiple carbon sources utilization, suggesting discrepancies in characteristics among strains of similar taxon. To the best of our knowledge, it is the first time these bacterial species be isolated from *S. caseolaris*, therefore the result could serve as contributing data for the mangrove plant – bacteria association.

On the other hand, the distribution of *Bacillus* spp. in mangrove sediments has been mentioned in earlier studies. *Bacillus* bacteria were reported as the predominant cellulase-producing mangrove species in Eastern Thailand (Bamrungpanichtavorn et al., 2023), as well as the prevalent halophilic bacteria from mangrove forests of Qeshm Island in Iran (Javid et al., 2020). Recently, a *B. velezensis* strain exhibiting antibacterial activity was isolated from the mangrove area in Beibu Gulf, China (Yu et al., 2022). The species was also found as one of dominated cellulolytic *Bacillus* spp. in mangroves of Indonesia (Dewiyanti et al., 2024). These results raised an idea that the bacteria commonly appeared as a permanent inhabitant of mangrove areas rather than a specific plant symbiont. Given the fact that mangroves have been known as a habitat for diverse

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flora and fauna with plasticity, particularly those with salinity tolerance, our isolation of two *Bacillus* strains belonging to *B. velezensis* and *B. siamensis* with PGP traits may not be a coincidence. Furthermore, these bacteria could hypothetically play a role as indicators for such saline environments. Insights into the correlation between the existence of these microbial species and ecological conditions in mangroves could thus be a scientific prospect to be explored.

Potential use of the bacterial isolates

As proposed by Fan et al. (2017), the 'operational group *B. amyloliquefaciens*' (OGBa) consisting of four species, namely *B. amyloliquefaciens*, *B. siamensis*, *B. velezensis* and *B. nakamurai*, was sub-grouped from the *B. subtilis* species complex due to their sequence homology of *rpoB* gene, as well as similarities revealed by genome and digital analysis. Interestingly, *B. siamensis*, *B. velezensis* and other members of OGBa were introduced to present ability to colonize plant and be successfully used as agricultural applications (Fan et al., 2017). In the present study, *B. velezensis* strain S13 and *B. siamensis* strain S15 were identified to exhibit plant growth promoting traits, able in metabolizing a wide range of carbohydrates and non-toxic to animal cells, are thus considered supporting evidence for practical features of OGBa, prompting one of the most appropriated Bacilli for sustainable vegetation purpose. Furthermore, their halotolerant properties and growth enhancing effects in tested sprouts under saline stress have helped pointing out *B. velezensis* strain S13 and *B. siamensis* strain S15 as ST-PGPR candidates of desirability for bio-formulations of crops under saline conditions. In addition, a number of researchers have been focused on the production of antimicrobial compounds by bacteria members of OGBa, especially non-ribosomal antifungal peptides and antibacterial polyketides (Ngalimat et al., 2021). As revealed by the above-mentioned results, isolated strains S13 and S15 were found as antagonists against 4/5 tested microbial pathogens, indicating the biosynthesis of broad-spectrum secondary metabolites for possible use in pharmaceuticals. Most notably, both of them exhibited inhibition against fungal phytopathogens *A. niger* and *F. oxysporums*, suggesting the possible use as biocontrol agents for management of diseases in sustainable agriculture. Future research should include further tests on effects of microbial inoculation in varying environmental parameters, as well as an extensive optimization process for formulating biopreparations before application in field condition.

Conclusion

Minimizing plantation and cultivation areas of important crops due to salinization has led to increased demands on biologically effective products for renaturation and growth promotion. From rhizosphere of mangrove plant *S. caseolaris* in Vietnam, two potential ST-PGPR strains of *Bacillus* spp. were isolated and characterized. These bacteria were identified to belong to species *B. velezensis* and *B. siamensis* and possessed potentials to be a new microbial resource for enhancing crops' productivity and withstand against abiotic stresses in local condition. Our results suggest that the potential for mining effective ST-PGPR isolates from mangroves deserves further attention, also in order to identify microbes possessing potentials in bioremediation and renaturation of salinized areas.

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