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Isolation and characterization of Vietnamese medicinal plant (Nhân trần tía, *Adenosma bracteosum* Bonati) bacterial endophytes displaying *in vitro* antibacterial activities

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ABSTRACT

In both traditional and modern Vietnamese medicine, *Adenosma bracteosum* Bonati is employed for the treatment of hepatitis, lung ailments, and liver disorders. Bacteria that reside within the cells of medicinal plants, use unique strategies to enhance the growth and survival of their host plants, often through distinctive secondary metabolites, are known as symbiotic or endophytic bacteria. In this study, the objective was to find bacterial endophytes with antibacterial properties. Fifty-eight endophytic isolates were obtained from the wild medicinal plant *A. bracteosum*. They were assessed for their *in vitro* antibacterial activities against common pathogenic bacteria, including *Escherichia coli*, *Staphylococcus aureus*, *Aeromonas hydrophila*, *Vibrio parahaemolyticus*, and *Dickeya dadantii*. Twelve isolates with broad antibacterial activity produced siderophores and lytic enzymes, with SB1R13.2 showing the greatest resistance against all five pathogenic bacterial strains, producing siderophores and synthesizing digestive enzymes. According to the 16S rDNA sequences, the SB1R13.2, SB4R5, and SB5T2 isolates demonstrated the most similar genetic affinity to *Bacillus velezensis*. Meanwhile, the SB4R2 isolate exhibits genetic similarity to *Burkholderia* sp. These findings suggest that this specific species, with its broad-spectrum antibacterial properties, holds significant potential as a promising agent for biological control and the treatment of diseases in humans and other organisms.

1. INTRODUCTION

The rapidly growing world population, coupled with climate change, is contributing to the widespread occurrence of diseases in humans, animals, and plants (Leal Filho et al., 2022). In response to this challenge, individuals have increasingly utilized antibiotics and plant protection chemicals. Human health is directly threatened by the residues of these

substances found in food, soil, water, and air (Ahmad et al., 2024).

Synthetic drug therapy is frequently limited by the twin problems of adverse side effects and emerging resistance. To date, researchers have identified roughly 20,000 antibiotic resistance genes (Saha & Sarkar, 2021). Despite notable achievements in decreasing infectious disease-related death and illness, the continuous appearance of new microbial

resistance mechanisms poses a substantial threat to established methods of prevention and cure (Saha & Sarkar, 2021). Inadequate dosing, the use of poor-quality drugs, and the genetic adaptability of microorganisms are the main factors contributing to this threat (Bisi-Johnson et al., 2017). Consequently, there is an urgent necessity to investigate untapped sources of bioactive compounds that hold pharmaceutical significance. Traditional medicinal plants are well-known for generating various compounds with diverse biological activities (Cariño-Cortés et al., 2007). Medicinal plants are the primary sources of bioactive compounds driving modern-day drug discovery and development (Newman, 2018). A significant majority—more than 80%—of drugs currently on the market derive from plant sources (Gouda et al., 2016). Because of unstable environmental conditions and accelerating climatic changes, the production of their essential plant metabolites faces challenges related to both productivity and quality control (Monika et al., 2017). An intensive search for newer and more effective agents to address these problems is underway, and endophytes are emerging as a novel source of potentially useful medicinal compounds. Among these, biological approaches involving the utilization of endogenous bacteria have emerged as effective solutions (Abd-Elgawad & Askary, 2020). Both endophytes and their host plant can produce similar bioactive compounds (Zhang et al., 2006). Endophytic bacteria colonize plant tissues asymptotically, causing no disturbance or harm to their hosts. Certain strains are known to synthesize secondary metabolites that provide beneficial effects, including promoting plant growth and inducing protection against both infections and environmental stresses (Eljounaidi et al., 2016). These endophytic bacteria generate a vast array of metabolites with unique structures and significant biological activities, such as biomass, alkaloids, flavonoids, phenolic acids, quinones, steroids, terpenoids, and essential enzymes. Although these compounds initially benefited the host plant, they are now broadly applied in industry, being harvested and utilized as agrochemicals, antibiotics, immunosuppressants, and antioxidants (Bhoonobong et al., 2012).

Bacterial endophytes are widely investigated for their potential in biocontrol against numerous pathogens (Dilfuza et al., 2017). This antagonistic activity is achieved through several methods: inhibiting pathogen growth via the secretion of antibiotics and toxins, releasing surface-active

compounds (antibiosis), and deploying extracellular digestive enzymes like chitinases, cellulases, and proteases (Chernin & Chet, 2002; De Souza et al., 2003). Recent research often spotlights bacterial endophytes from diverse crops, emphasizing their value as a source of antimicrobial metabolites and their role in biological control. However, there's been limited exploration into the antagonistic activity against plant pathogens, specifically by bacteria associated with medicinal plants (Dilfuza et al., 2017). Therefore, the search for bioactive metabolites from microorganisms (bioprospecting) has emerged as a promising alternative path for discovering new drugs (Buatong et al., 2011).

Adenosma bracteosum Bonati is a traditional Vietnamese medicine recognized for its multifaceted pharmacological properties, including antibacterial and antiviral effects, anti-inflammatory and detoxifying properties, as well as antioxidant and antitumor activities (Landa et al., 2009). Currently, there are no published studies examining the endophytes of this species or their potential application as biocontrol agents. Therefore, the objective of this study is to isolate endophytic bacteria from *A. bracteosum*, identify them, and investigate their antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, *Aeromonas hydrophila*, *Vibrio parahaemolyticus*, and *Dickeya dadantii*.

2. MATERIALS AND METHOD

2.1. Materials

Adenosma bracteosum Bonati samples were collected from Lo Go Xa Mat National Park, Tay Ninh Province, Viet Nam.

2.2. Method

2.2.1. Plant sampling and surface sterilization

After being randomly divided into five groups and sealed in separate plastic bags, all the samples were transported to the laboratory while being kept at 4°C. The samples were labeled based on the different sampling locations as SB1 (11°38'06.7"N 105°50'52.5"E), SB2 (11°38'00.9"N 105°50'51.0"E), SB3 (11°38'02.2"N 105°50'46.8"E), SB4 (11°38'06.7"N 105°50'48.1"E), and SB5 (11°38'05.1"N 105°50'50.0"E).

To prevent microbial contamination on the surfaces of *A. bracteosum* samples, the roots, stems, and leaves were surface-sterilized using the following method: the samples were washed with tap water,

then sterilized with 3% H₂O₂ for 1 minute and 30 seconds, followed by 70% ethanol for 1 minute and 30 seconds. Afterward, the samples were rinsed with distilled water five times to eliminate any remaining disinfectant chemicals. To verify the success of sterilization, 50 µL of the final rinse water was pipetted and spread onto a nutrient agar (NA) medium. The results were observed for 24 to 48 hours, and if no colonies appeared, the disinfection was deemed successful (Nxumalo et al., 2020; Nghia et al., 2024).

2.2.2. Isolation and characterization of endophytic bacteria

The isolation of endophytic bacteria was conducted using a modified method based on Nxumalo et al. (2020). Nutrient agar (NA) medium was employed for the isolation process. Under aseptic conditions, the disinfected samples were ground with a sterile mortar and pestle. After the tissue extract was serially diluted in a sterile aqueous solution, undiluted samples along with the 10⁻¹ and 10⁻² dilutions were pipetted onto NA plates. The volume used for each plating was about 100 µL. Daily isolation and monitoring of bacteria were performed, with the plates sealed and incubated at 28°C. Bacterial isolates were subsequently purified and characterized, including assessments of morphological characteristics, Gram properties, catalase tests, and motility tests (Mugiastuti et al., 2020; Nghia et al., 2024). After confirming the purity of the bacteria under a microscope, they were transferred to Nutrient Broth medium test tubes containing glycerol and stored in a refrigerator at -80°C until the continuation of subsequent experiments in the study.

2.2.3. Antibacterial activity test

Preparation of the endogenous bacterial culture solution involved culturing endogenous bacterial strains in liquid YEM (Yeast Extract Mannitol) medium (composed of mannitol 1%, yeast extract 0.05%, K₂HPO₄ 0.05%, MgSO₄·7H₂O 0.02%, NaCl 0.01%, pH 6.8-7.0) following 24 hours of shaking at 150 rpm at room temperature, the antibacterial activity of the endogenous bacterial isolates was tested against *E. coli*, *S. aureus*, *A. hydrophila*, *V. parahaemolyticus*, and *D. dadantii* using the agar disk diffusion method (Nghia et al., 2024). The antibacterial activity data of strain SB1R13.2 against *A. hydrophila* was previously reported in Nghia et al. (2024) and is reused here as reference data for further comparative analysis.

Preparation of *E. coli*, *S. aureus*, *A. hydrophila*, *V. parahaemolyticus*, and *D. dadantii*: Bacterial colonies, applied via a loopful, were introduced into five test tubes, each of which contained 5 ml of sterile 0.85% NaCl physiological saline solution. Subsequently, the bacterial suspension was spread on YEMA (Yeast Extract Mannitol Agar). The bacterial density used in the study was 10⁷ CFU/mL. Wells were subsequently formed on the agar surface using 6 mm sterile cotton tips. Into each well, 50 µl of the 24-hour endogenous bacterial culture solution was pipetted. The positive control contained the antibiotic tetracycline at 10 mg/mL, and the negative control contained 0.85% physiological saline. Following incubation at 30°C, the antibacterial zone diameter was measured after 24 hours. A randomized experimental design was employed, and the study was run in triplicate. The antibacterial zone diameter was calculated using the formula:

$$\text{Antibacterial ring diameter (mm)} = D - d$$

In this context, D measures the diameter of the bacterial inhibition zone (mm), while d is the well diameter, which is 6 mm (Nghia et al., 2024).

2.2.4. Production of siderophores

The capacity of isolates to generate siderophores was assessed (Senthil & Kumar, 2020). Preparation of the reagent involved two main components: 121 mg of CAS dissolved in 100 mL of distilled water, and 20 mL of a 1 mM FeCl₃ solution (prepared in 10 mM HCl). These two solutions were then combined. The mixture was then carefully added to 20 mL of HDTMA solution (73 mg combined with 40 mL of distilled water) and thoroughly stirred. Before being used, the resultant solution was autoclaved at 121°C.

The test bacterial strains were cultivated in Falcon tubes containing 10 ml of liquid LB medium and incubated for 48 hours at 30°C on a shaker. Then, 2 ml of bacterial fluid was centrifuged for ten minutes at 4°C at 10,000 rpm to collect supernatant.

After thoroughly combining 1 mL of centrifuge with 1 mL of CAS reagent, the mixture was left to incubate for 20 minutes at room temperature in the dark. The mixture's optical absorbance was measured at 630 nm. Siderophore activity was calculated using the following formula:

$$\text{Siderophore production capacity (\%)} = ((Ar - As) \times 100) / Ar$$

where Ar is the optical absorbance of the control (CAS reagent + LB medium) and As is the optical absorbance of the sample (CAS reagent + supernatant).

2.2.5. Production of extracellular hydrolytic enzymes

Isolated endophytes were screened for their ability to produce digestive enzymes (Mohamad et al., 2018).

The test medium for cellulase activity used the DSMZ1 composition (excluding CaCO_3), but carboxymethyl cellulose (5 g/L; Sigma-Aldrich) replaced the glucose. The plates were inoculated with 3 μL of bacterial suspension. After three to four days of incubation at 30°C, they were first stained with a Congo red solution and subsequently destained using a NaCl solution (Li et al., 2018). The presence of a clear or lightly colored halo around the colonies signified a positive cellulase reaction.

Protease activity: YEM agar medium with 5% (v/v) skim milk was used to measure the protease activity. A transparent halo surrounding the bacterial colonies as a result of milk hydrolysis showed a positive reaction during three to four days of incubation at 30°C.

Lipase enzyme activity: By adding ferrous citrate (0.2 g/L) and beef extract (3 g/L) to modified Sierra lipolysis agar, the activity of the lipase enzyme was measured. Following autoclaving, the medium was supplemented with 50 mL of Victoria Blue B solution (0.1 g/150 mL) and 10 mL of Tween-80. After 5–6 days of incubation at 30°C, white calcium precipitates around the bacterial colonies, indicating a positive reaction (Li et al., 2018).

The medium used to detect chitinase activity contained (per liter): 0.3 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3.0 g of $(\text{NH}_4)_2\text{SO}_4$, 2.0 g of KH_2PO_4 , 1.0 g of citric acid monohydrate, 15 g of agar, 200 mL of Tween-80, 4.5 g of colloidal chitin, and 0.15 g of bromocresol purple. This entire mixture was then autoclaved at 121°C for 15 minutes. Chitinase activity was subsequently assessed by incubating the inoculated plates at 30°C and observing them for the formation of a colored zone.

The amylase activity was assayed with starch-agar media (starch 20 g/L, peptone 5 g/L, beef extract 3 g/L, and NaCl 3%) and incubated for 7 days at 30°C. The appearance of a clear zone around the colonies after flooding the plates with an iodine solution

indicated the presence of amylase-positive isolates (Elmansy et al., 2018). In each of the aforementioned assays, sterile nutrient agar served as the control for the growth of bacteria. For every isolate, three replicates were used in each experiment.

2.2.6. Identification of endophytic bacteria

The endogenous bacterial strain demonstrating the greatest resistance to the pathogens (*E. coli*, *S. aureus*, *A. hydrophila*, *V. parahaemolyticus*, and *D. dandantii*) after isolation was selected for Polymerase Chain Reaction (PCR) amplification of its 16S rDNA gene. The amplification utilized the primers 16S-27F: 5'AGAGTTTGATCMTGGCT CAG-3' and 16S-1492R: 5'-CGGTTACCTTGTTA CGACTT-3' along with DreamTaq™ DNA polymerase. The total PCR reaction volume was 25 μL , containing: 2.5 μL of DreamTaq Buffer (10X), 2 μL of dNTPs (2.5 mM), 1.25 μL of each primer (10 μL stock concentration), 0.2 μL of DreamTaq DNA Polymerase (5 μL , Thermo Fisher Scientific), and 17.8 μL of sterile double-distilled water. The thermal cycling program included: an initial denaturation at 95°C for 10 min; 30 cycles of denaturation (95°C for 1 min), annealing (60°C for 1 min), and elongation (72°C for 2 min); and a final elongation step at 72°C for 10 min (Duong et al., 2021). PCR products were gel-extracted and sequenced in the forward and reverse directions using the BigDye™ Terminator v3.1 Cycle Sequencing Kit on the ABI 3500 Genetic Analyzer.

To determine the nearest bacterial species, the purified sequencing results were uploaded for a BLAST search via the NCBI database (Nxumalo et al., 2020). The phylogenetic tree was constructed using MEGA 11 (Molecular Evolutionary Genetics Analysis) software based on the neighbor-joining algorithm, with a bootstrap value of 1,000 replications (Tamura et al., 2013).

2.2.7. Statistical analysis

All data were stored, calculated, and visualized using Microsoft Excel 2016. Every experiment was conducted in triplicate, and results are reported as the mean $\pm$ standard deviation (SD). Statistical analysis was performed with Analysis of Variance (ANOVA) via Minitab 16 software. Differences between means were deemed statistically significant at the 5% level ($P < 0.05$). Finally, the phylogenetic tree was built using MEGA 11 software.

3. RESULTS AND DISCUSSION

3.1. Isolation of endophytic bacteria

Results from five different samples revealed the isolation and characterization of fifty-eight endophytic bacteria. All the endophytic isolates exhibited motility. Of these, fifty-one bacterial isolates tested positive for catalase (87.9%), while seven tested negative (12.1%). The colonies were mostly white; some were yellow, brown, or even purple. The morphological characteristics of the isolates are detailed in Table 1.

From the samples of roots, stems, and leaves of *A. bracteosum* after sterilization, 58 bacterial strains

were isolated on the NA medium. Of these, 31.03% were isolated from root samples, 44.8% were isolated from stem samples, and 24.14% were isolated from leaf samples. The strains of bacterial strains isolated are denoted as follows: SB, followed by the sample collection plot number denoted as 1, 2, 3, 4, and 5 respectively; followed by the symbols R (root), T (stem), L (leaf) and followed by the number of the isolated bacterial strain. Figure 1 shows the morphology of endophytic bacterial colonies growing on NA medium. Figure 1A was adapted from Nghia et al. (2014) for comparison and validation of the colony morphology of the potential bacterial strain previously described by us.

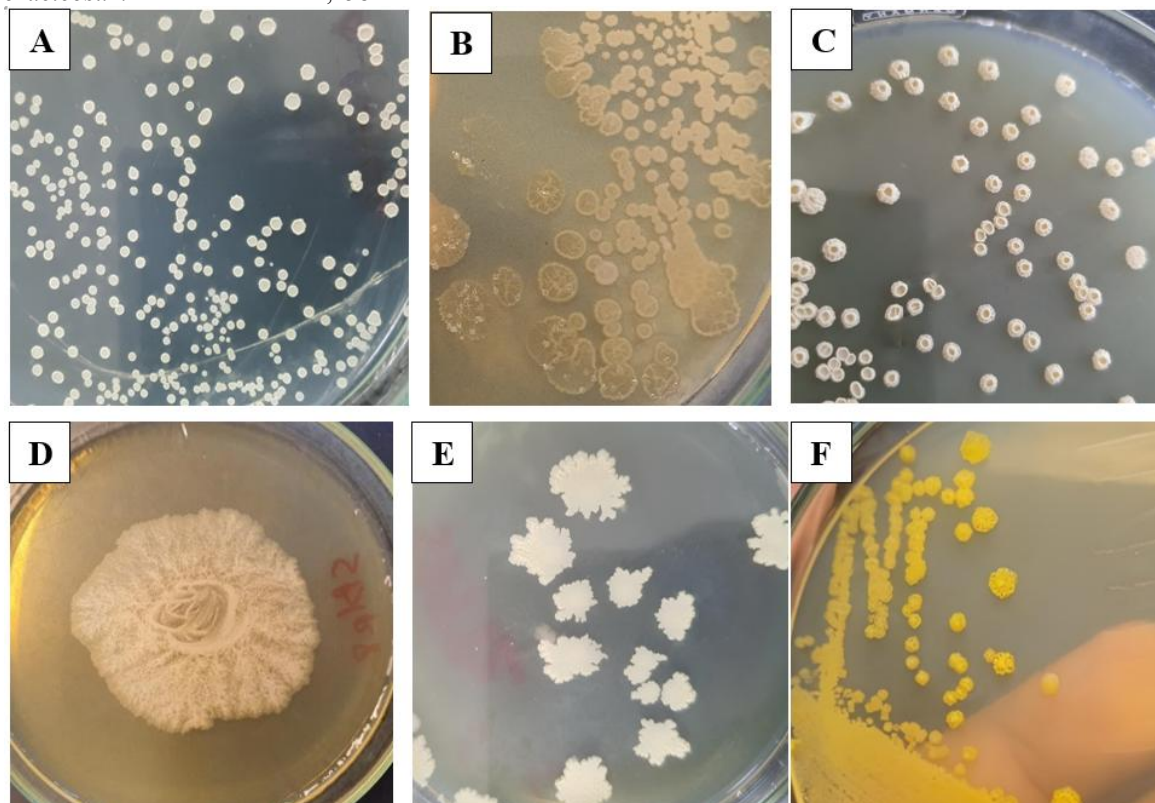


Figure 1. Morphology of endophytic bacterial colonies growing on NA medium

A: SB1R13.2 (Adapted from Nghia et al. (2024)); B: SB4T10; C: SB4R5; D: SB1R8; E: SB5T2; F: SB2T3

3.2. Antibacterial activity

Endophytic bacteria recovered from wild *A. bracteosum* populations were tested for their antagonistic activity against a panel of five common bacterial pathogens, encompassing both Gram-positive and Gram-negative types. Of the 58 bacterial strains isolated, 22 showed resistance to at least one pathogenic bacterium, with inhibition zones ranging from 1.33 to 20.67 mm (Table 2).

Specifically, 8 isolates inhibited *E. coli*, 6 isolates inhibited *S. aureus*, 8 isolates inhibited *A. hydrophila*, 10 isolates inhibited *V. parahaemolyticus*, and 10 isolates inhibited *D. dadantii*. Notably, the SB1R13.2 isolate exhibited the highest antibacterial activity against the selected Gram-positive and Gram-negative bacteria (Table 2).

After 24 hours of incubation, SB1R13.2 had the largest antibacterial zones of 15.33 ± 0.57 mm for *E. coli*, 20.67 ± 0.57 mm for *S. aureus*, and 17.33 ± 0.57 mm (Nghia et al., 2024) for *A. hydrophila* (Figure 2), which were statistically significantly different from the other strains. This result is also similar to some domestic studies on the isolation of endophytic bacteria with the ability to resist *E. coli*, *S. aureus*, and *A. hydrophila* such as *Mimosa pudica* (Hieu & Hiep, 2016), *Moringa oleifera* (Vy & Hiep, 2019). In addition, there are some studies abroad, such as endophytic bacteria from *Andrographis paniculata* Nees (Truong et al., 2023), *Hoya multiflora* Blume (Alvionita et al., 2020), *Bacillus velezensis* Ea73 isolated from *Ageratina adenophora* (Ren et al., 2022) also can resist *E. coli*.

Moreover, the porin protein of *Streptomyces coelicolor* AZRA 37 isolated from the roots of *Azadirachta indica* A. Juss., commonly known as Neem tree in India, also can resist *S. aureus* ATCC 25923 (Kumar et al., 2016). The crude ethyl acetate extract from the endophytic actinomycete *Streptomyces griseorubens* MPT42 isolated from *Litsea cubeba* can also work against *S. aureus* (Nguyen et al., 2019). Besides, the ethyl acetate extract from the endophytic actinomycete *S. griseorubens* MPT42 isolated from *L. cubeba* can also resist *A. hydrophila* (Nguyen et al., 2019). The endophytic bacteria strain of the *Enterobacter* genus isolated from *Coscinium fenestratum* can inhibit the formation of *A. hydrophila* biofilm (Shastry et al., 2019).

Table 1. Morphological characteristics of endophytic bacteria on NA medium

Morphology of bacteria	Features	Number of isolates	Rate (%)
Form of colonies	Circular	36	62.1
	Irregular	22	37.9
Color of colonies	White	41	70.7
	Yellow	8	13.8
	Brown	8	13.8
	Violet	1	1.7
Elevation of colonies	Convex	37	63.8
	Flat	20	34.5
	Raised	1	1.7
Margin of colonies	Entire	29	50
	Undulate	23	39.7
	Lobate	5	8.6
	Filiform	1	1.7
Surface of colonies	Smooth	36	62.1
	wrinkled	22	37.9
Size of colonies	Small (< 1mm)	3	5.2
	Medium (= 1mm)	4	6.9
	Large (> 1mm)	51	87.9
Shape of bacterial cells	Rods	52	89.7
	Spheres	6	10.3
Gram stain	Positive Gram	13	22.4
	Negative Gram	45	77.6

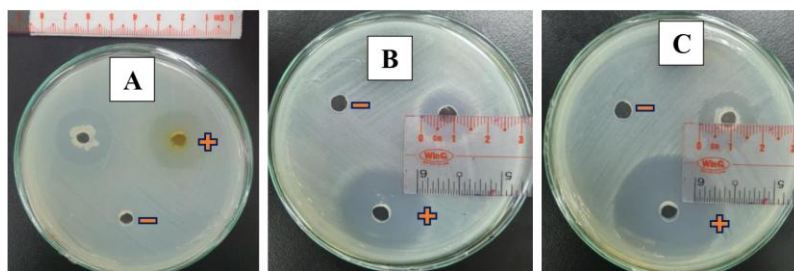


Figure 2. Antibacterial ability of some endogenous bacterial strains after 24h inoculation

A: SB1R13.2 strain on YEMA medium spread with *A. hydrophila*; B: SB4R5 strain on YEMA medium spread with *E. coli*; C: SB5T2 strain on YEMA medium spread with *S. aureus*; (+): Positive control, (-): Negative control.

Table 2. The clear zone indicates the antibacterial effectiveness of the endophytic bacterial isolates

No	Endophytes	Pathogenic bacteria				
		<i>E. coli</i>	<i>S. aureus</i>	<i>A. hydrophila</i> *	<i>V. parahaemolyticus</i>	<i>D. dadantii</i>
1	SB1R11	5.33±0.57 ^{qrs}	-	10.3±0.57 ^{ijkl}	4.67±0.57 ^{rs}	5.33±0.57 ^{qrs}
2	SB1R13.2	15.3±0.57 ^c	20.7±0.57 ^c	17.3±0.57 ^d	11.3±0.57 ^{hij}	9.67±0.57 ^{ijklm}
3	SB1T7.1	-	-	-	8.0±0.00 ^{mnop}	7.33±0.57 ^{op}
4	SB1T7.2	-	-	-	-	9.33±0.57 ^{klmn}
5	SB1T13	-	-	11.7±0.57 ^{ghi}	-	-
6	SB1T14	-	-	4.0±0.00 ^s	-	-
7	SB1L4	-	3.67±0.57 st	-	-	8.67±0.57 ^{lmno}
8	SB1L6	-	-	-	8.0±0.00 ^{mnop}	8.0±1.00 ^{mnop}
9	SB1L7	3.67±0.57 st	-	-	-	-
10	SB2R14	-	-	13.3±0.57 ^{fg}	-	-
11	SB2T3	-	-	-	9.67±0.57 ^{ijklm}	11.7±0.57 ^{ghi}
12	SB2T7	-	-	-	-	7.67±0.57 ^{nop}
13	SB2L2	-	-	-	5.33±0.57 ^{qrs}	-
14	SB3T7.2	9.67±0.57 ^{ijklm}	13.3±0.57 ^{fg}	-	-	-
15	SB4R2	1.33±0.57 ^{fg}	-	8.0±1.00 ^{mnop}	-	-
16	SB4R4	-	-	-	7.0±0.00 ^{opq}	-
17	SB4R5	10.7±0.57 ^{ijk}	14.3±0.57 ^{ef}	-	-	-
18	SB4T6	7.67±0.57 ^{nop}	9.33±0.57 ^{klmn}	10.7±0.57 ^{ijk}	4.67±0.57 ^{rs}	7.0±0.00 ^{opq}
19	SB4L4.1	-	-	2.0±0.00 ^{tu}	-	-
20	SB4L4.2	-	-	-	8.0±1.00 ^{mnop}	6.33±0.57 ^{pqr}
21	SB4L5	-	-	-	3.67±0.57 st	-
22	SB5T2	10.3±0.57 ^{ijkl}	12.7±0.57 ^{fgh}	-	-	-
23	PC	23.7±0.57 ^b	31.0±0.00 ^a	17.5±0.86 ^d	31.3±0.57 ^a	30.3±0.57 ^a

(-): denotes no formation of a clean zone; PC: Positive control; At the 5% significance level (based on Tukey's test), mean values that share the same letter in any column or row are considered statistically insignificant different

* Data from Nghia et al. (2024).

Medicinal endophytic bacteria are prolific producers of secondary metabolites that exhibit strong antibacterial potential. They reside and complete their life cycles entirely within plant tissues without inducing infection or disease symptoms (Bacon & White, 2000; Saikkonen et al., 2004). Evidence shows that these endophytes can generate similar metabolites both in laboratory conditions (in vitro) and inside their host plants (Dos Santos et al., 2016; Kusari et al., 2013). In this study, the antibacterial activities against various pathogen bacteria of diverse endophytic bacteria isolated from wild populations of *A. bracteosum* were discovered. This research aims to pave the way for the development of antibacterial bioactive compounds that could be applied in the pharmaceutical and veterinary industries.

The isolated endophytic bacteria presented their antibacterial activities. Previous research has shown that *Bacillus* strains from medicinal plants can inhibit pathogenic bacteria (Akinsanya et al., 2015; Gao et al., 2017; Jiang et al., 2015). Many studies, however, have documented that *Bacillus* strains found in medicinal plants possess the ability to inhibit common pathogens. Specifically, this activity has been shown against *S. aureus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, and *E. coli* (Mohamad et al., 2018; Nejatizadeh-Barandozi, 2013).

Of the 22 endophytic bacterial isolates with antibacterial abilities shown in Table 2, twelve were found to be inhibitory to at least 2 or more pathogenic bacteria. These 12 isolates were further examined for their ability to produce siderophores and extracellular enzymes.

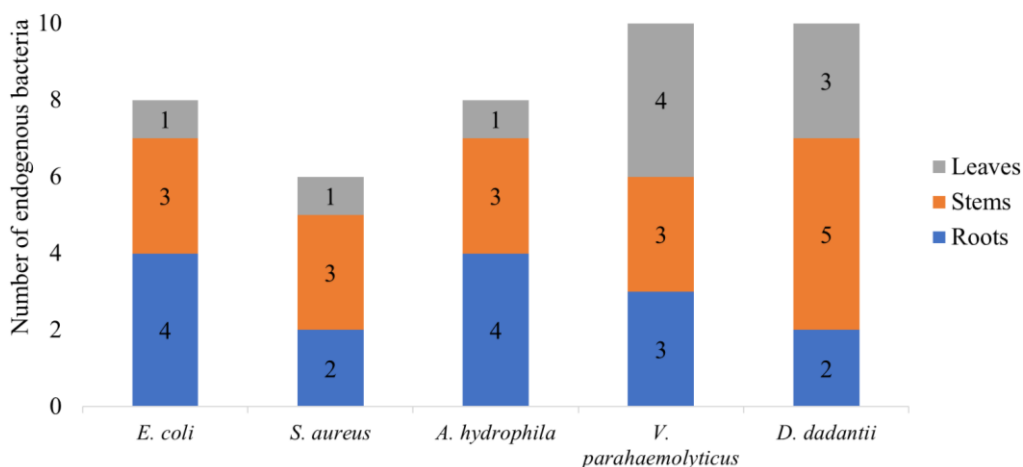


Figure 3. The number of endophytic bacteria with antibacterial ability distributed in *Adenosma bracteosum* Bonati

Figure 3 shows that endophytic bacteria strains can resist *E. coli*, *S. aureus*, *A. hydrophila*, *V. parahaemolyticus*, and *D. dadantii* which are present in the plant parts. The number of endophytic bacteria in roots and stems was higher than in leaves, accounting for 76.2%, and in the leaves, accounting for 23.8%. This finding is consistent with findings from other studies on endophytic bacteria in *Moringa oleifera* (Vy & Hiep, 2019), maize (Prihatiningsih & Soesanto, 2020).

3.3. Digestive enzyme and siderophores activities

The same 12 endophytic bacterial isolates were assayed for the production of siderophores and various lytic enzymes, specifically protease, cellulase, lipase, amylase, and chitinase. These activities are crucial for the breakdown of phytopathogens or the regulation of virulence factors. The successful production of one or more lytic enzymes by several tested strains was demonstrated using specific indicators, including a pH change (observed with soluble chitin and bromocresol purple) that confirmed chitinase activity. The diameter of the clear zone on specific media confirmed the presence of the other enzymes: protease (on skim milk agar), cellulase (on carboxymethyl cellulose agar), lipase (on Sierra lipolysis agar), and amylase (on starch-agar media) (see Table 3).

From this study, the 12 most antibacterial strains produced siderophores, and at least one digestive enzyme was collected (refer to Table 3). The protective effect of these endophytic bacteria on plants is likely achieved through their ability to

break down fungal cell walls and membranes, to degrade membrane proteins or extracellular virulence factors, or to trigger systemic resistance in the plants (Frankowski et al., 2001). These can cleave polymeric compounds, such as chitin, proteins, cellulose, hemicellulose, and even DNA (Heydari & Pessarakli, 2010), and they also can interfere with pathogen metabolic activity (Nicot et al., 2016), inhibit conidia germination, and lyse germ tubes (Elad et al., 2004). Studies on endophytic bacteria from medicinal plants, including *Ferula songorica*, *Hypericum perforatum*, and *Ferula sinkiangensis*, have reported the production of similar enzymes, which supports this current finding (Liu et al., 2016; Y. Liu et al., 2017). Most bacteria generate lytic enzymes capable of breaking down complex polymers like proteins, chitin, glucans, lipids, and cellulose, which form the main structure of phytopathogenic fungal cell walls (Lee et al., 2013; Villarreal-Delgado MF et al., 2018). These lytic enzymes are natural byproducts of endophytic bacteria, helping them colonize plant roots (H. Liu et al., 2017). Furthermore, endophytic *Bacillus* species have been specifically noted for producing these enzymes that target and break down pathogen cell walls (Kalai-Grami et al., 2014). It has been demonstrated that the *B. subtilis* J9 strain protected strawberry plants against *B. cinerea* in field conditions and produced extracellular chitinase and protease (Essghaier et al., 2012). Certain lactobacilli could inhibit the hyphae formation of fungi *in vitro* by producing bifunctional enzymes with chitinase/peptidoglycan hydrolase activity (Allonsius et al., 2019). Among the most extensively researched lytic enzymes

produced by rhizobacteria are cellulases, β -1,3-glucanases, chitinases, and proteases (Mota et al., 2017). Of these, chitinases represent the largest group of enzymes associated with plant defense

(Jalil et al., 2015). They function by hydrolyzing the glycosidic bonds within the chitin components of fungal cell walls, thereby inhibiting phytopathogenic fungi (Lopes et al., 2017).

Table 3. Enzymatic and siderophore activities of bacterial strains showing the highest antibacterial efficacy

Endophytes	Siderophore	Protease	Cellulase	Lipase	Chitinase	Amylase
SB1R11	+	+	+	+	+	-
SB1R13.2	+	+	+	+	+	+
SB1T7.1	+	-	+	-	+	-
SB1L4	+	-	+	-	+	+
SB1L6	+	+	+	+	+	+
SB2T3	+	+	-	+	+	+
SB3T7.2	+	+	+	+	-	+
SB4R2	+	+	-	+	-	+
SB4R5	+	+	-	-	-	+
SB4T6	+	+	+	+	-	+
SB4L4.2	+	+	-	-	-	+
SB5T2	+	+	+	-	+	+

(+): Denotes siderophore/enzyme production; (-): Denotes no siderophore/enzyme production

Siderophore production is common among endophytic bacteria. Siderophores are small organic molecules that bind competitively with the ferric ion (Fe^{3+}) when iron is scarce (Goswami et al., 2016; Verma et al., 2019). By scavenging iron in the rhizosphere, siderophores effectively starve phytopathogens of iron, thereby preventing their growth (Aloo et al., 2022). Furthermore, some siderophores, such as phenazines, possess a secondary antifungal action: they catalyze the creation of hydroxyl radicals. These radicals damage the lipids and other macromolecules in the pathogen's cell membranes, leading to inhibition (Britigan et al., 1989). For instance, analysis of the secondary metabolites produced by the endophytic *P. aeruginosa* BRp3 in rice, against *X. oryzae*, identified the presence of the phenazine siderophores pyochelin and pyocyanin via mass spectrometry (Yasmin et al., 2017). Siderophore production by the endophytic bacterium *P. fluorescens* ENPF1 (isolated from *Phyllanthus amarus*) was identified as a mechanism against the blight pathogen *Corynespora casicola*, specifically through the secretion of hydroxamate and carboxylate siderophores (Mathiyazhagan et al., 2004). Similarly, siderophores produced by *Rhizobia* have been shown to improve the inhibition of fungal pathogens under both laboratory (*in vitro*) and field (*in vivo*) conditions (Singh et al., 2018; Srinivasan, 2017).

Endophytic bacteria utilize siderophore production as a key antagonistic mechanism against various plant pathogens. For example, strains like *B. niabensis*, *B. subtilis*, and *B. mojavensis* exhibit antagonistic effects against banana wilt pathogens in dual cultures by secreting these compounds. A positive correlation has been established between siderophore output from endophytes such as *Pseudomonas*, *Acinetobacter*, *Enterobacter*, and *Bacillus*, and the inhibition of pathogens like *P. sojae* (Zhao et al., 2018). Furthermore, in bean plants, endophytes including *B. amyloliquefaciens*, *B. halotolerans*, *B. velezensis*, *Agrobacterium fabrum*, and *P. lini* produce siderophores that show antifungal activity against root rot pathogens like *Fusarium* sp., *Macrophomina* sp., and *Alternaria* sp. (Sendi et al., 2020). In a similar vein, endophytes from *C. longa* (specifically *Acinetobacter* sp., *P. aeruginosa*, and *Enterobacter* sp.) produce siderophores that demonstrate antagonistic activities both *in vitro* and *in planta* against *P. aphanidermatum* and *R. solani* (Vinayarani & Prakash, 2018).

The results of assessing siderophore and extracellular enzyme production activities in 12 endophytic bacterial strains indicated that strains of SB1R13.2 produced siderophores and all five types of enzymes, its broad antibacterial spectrum, stability, and superior antibacterial activity, so SB1R13.2 was selected for identification.

3.4. Identification of interest strains

In developing biocontrol agents, ensuring biosafety is essential. Therefore, we selected bacterial strains with notable colony morphological characteristics to determine preliminarily if they belonged to the genus *Bacillus*. Sequencing results of the 16S rDNA gene of four bacterial strains (SB1R13.2, SB4R2, SB4R5, and SB5T2) showed that three of the isolates (SB1R13.2, SB4R5, and SB5T2) belonged to the genus *Bacillus* with a similarity of 99.3% or higher. The isolate SB4R2 was identified as belonging to the genus *Burkholderia* with a similarity of 99.71%. A phylogenetic tree was constructed using MEGA 11 (Molecular Evolutionary Genetics Analysis) software based on the neighbor-joining algorithm (related to Figure 4). Based on these results, we proceeded to evaluate the antibacterial activity of the isolated bacterial strains.

Bacillus velezensis is highly valued for its bioactive compounds with applications in medicine, biocontrol, and the environment. This Gram-positive, endospore-forming bacterium is widespread (ubiquitous) and non-pathogenic, commonly isolated from sources like soil, water, plant roots, and fermented foods. As a host-adapted strain, *B. velezensis* holds significant economic importance due to its proven capability to enhance plant growth across diverse stress environments, both biotic and abiotic. In addition, this species suppresses many plant diseases, including those

caused by bacteria, oomycetes, and fungi (Alenezi et al., 2021; Rabbee et al., 2023).

Bacillus velezensis Ea73 isolated from *Ageratina adenophora* has antagonistic activity against *Staphylococcus aureus*, and the metabolites cyclo (L-Pro- L-Val) and cyclo (L-Leu- L-Pro) were found (Ren et al., 2022). Lipopeptides exhibiting strong antibacterial and antifungal potential were extracted from *B. velezensis* YA215 isolated from the mangrove forest area in the Gulf of Tonkin, Guangxi, China (Yu et al., 2022). The strain *Bacillus velezensis* LDO2 strongly inhibited the growth of both pathogenic fungi and bacteria affecting peanuts. Of particular note, it caused marked inhibition and hyphal deformation of *Aspergillus flavus* mycelia. The gene clusters responsible for the synthesis of antifungal and antibacterial metabolites were successfully identified by the researchers. Beyond its antimicrobial action, the LDO2 strain also demonstrated several features that promote plant growth, including phosphorus solubilization, siderophore production, and root growth promotion. Genes associated with these plant growth promotion features, along with protein-related genes, were also identified and analyzed. These results indicate that the peanut-derived strain, *B. velezensis* LDO2, is a promising candidate for use as a biocontrol agent, a peanut growth promoter, and a source of antimicrobial compounds (Chen et al., 2019).

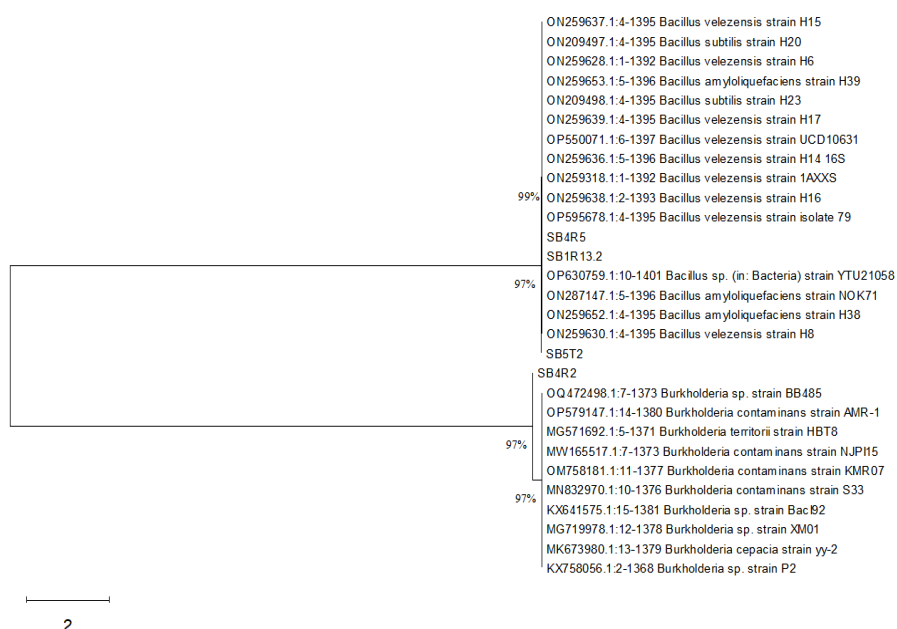


Figure 4. Phylogenetic tree illustrating the relationship between endophytic bacteria in *Adenosma bracteosum* Bonati and closely related bacterial strains on NCBI (MEGA 11)

Of the 22 strains that exhibited diverse antibacterial properties *in vitro*, the SB1R13.2 strain showed the highest against all tested strains of these 5 pathogenic bacteria and concurrently produced siderophores along with all mentioned digestive enzymes. Molecular and phylogenetic analysis using MEGA 11 software indicated that SB1R13.2 is most closely related to *Bacillus velezensis*. The *Bacillus* genus, particularly *B. velezensis*, has emerged as a potent antibacterial agent with broad-spectrum activity. To the best of our knowledge, this work is the first to isolate *B. velezensis* from *A. bracteosum* and produce bioactive substances with antibacterial qualities. Future research should focus on field-based evaluations to confirm the efficacy, stability, and practical applicability of these strains under real-world conditions. If validated, *B. velezensis* SB1R13.2 and similar

isolates could serve as sustainable alternatives to agrochemicals, offering a natural, eco-friendly approach to biocontrol in agriculture and potentially beyond.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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