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A snapshot of bacterial endophytes isolated from the roots of Vetiver grass (*Chrysopogon zizanioides*) grown at Bien Hoa airbase, Dong Nai province

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Abstract. Vetiver grass *(Chrysopogon zizanioides)* is used for phytoremediation of contaminated soil, but the role of its associated endophytic bacteria remains unexplored. This study investigated endophytic bacteria from Vetiver grass grown in dioxin-contaminated soil at Bien Hoa airbase, Vietnam. Sixteen bacterial strains were isolated and identified using 16S rRNA gene sequencing, revealing nine distinct species. Notably, four strains (*Klebsiella variicola* B1, *Enterobacter cloacae* B4, B6, and *Enterobacter asburiae* B11) exhibited high indole acetic acid (IAA) production, a plant growth hormone. Strains *K. variicola* B1 and *E. cloacae* B4 also displayed phytase and phosphatase activity, potentially enhancing plant nutrient availability. These IAA-producing strains were further evaluated for their ability to promote tomato plant growth. After 28 days of root inoculation, tomato plants exhibited a 19 - 22 % increase in growth compared to controls. This study highlights the first isolation of endophytic

bacteria from Vetiver grass in dioxin-contaminated soil and identifies potential bacterial candidates for plant growth promotion in such environments.

Keywords: endophytic bacteria, dioxin-contaminated soil, indole acetic acid, plant growth promotion, Vetiver grass.

Classification numbers: 3.1.1, 3.1.2, 3.4.3.

1. INTRODUCTION

Vetiver grass (*Chrysopogon zizanioides*) is well-known for various applications, such as producing essential oils, making crafts, preventing soil erosion and landslides, and environmental pollution treatment. This plant can grow well in extreme conditions or low-nutrient soils. Vetiver grass is remarkably exploited for phytoremediation to remove toxic substances, including persistent organic pollutants, heavy metals, herbicides, etc. [1 - 5]. Bien Hoa airbase is a hotspot of dioxin contamination in Viet Nam [6]. In previous work, Vetiver grass was applied to mitigate dioxin pollution at Bien Hoa airbase. The results showed a significant decrease in dioxin content in the contaminated soil. Notably, Vetiver grass grew very well in the soil of this location [7]. The expanded root system promotes the growth of this grass. Plant growth may benefit from soilborne and endophytic microorganisms [3, 8, 9]. Endophytic bacteria living inside plant root tissues have been reported to play vital roles in the growth and development of plant hosts. They help plants absorb nutrients and produce biostimulants to promote the plant growth. These microbial endophytes can also assist crops in elevating their tolerance to salinity and drought stress [10 - 15].

In this study, we report for the first time sixteen endophytic bacterial strains isolated from the root tissues of Vetiver grass grown in dioxin-contaminated soil at Bien Hoa airbase, Dong Nai province. These strains could grow on the basic medium in the laboratory. Phenotypic analyses and 16S rRNA gene sequencing revealed that the isolated strains belong to different bacterial species. Notably, some of them could produce large amounts of indole acetic acid as a potential plant growth stimulant. The assays using tomato seedlings inoculated with two selected strains indicated that these endophytes were able to promote the growth of the tested plants.

2. MATERIALS AND METHODS

2.1. Materials

Materials: Root tissue samples were collected from Vetiver plants grown at Bien Hoa airbase, Dong Nai province. *Bacillus subtilis* VS1 used for reference control was provided by the Department of Microbiology, VNU University of Science, Ha Noi.

Cultivation media: Luria-Bertani (LB) medium contains 10 g of peptone, 5 g of yeast extract, 5 g of NaCl, 20 g of agar, and distilled H_2O to 1000 mL. LB-Tryptophan medium includes 2 % peptone, 0.15 % K_2HPO_4, 1.5 % $MgSO_4.7H_2O$, 1.5 % glycerol, 0.05 % tryptophan, and distilled H_2O to 1000 mL. Phytase screening medium (PSM) comprises 1 % glucose, 0.4 % sodium phytate, 0.2 % $CaCl_2$, 0.5 % $(NH_4)_2SO_4$, 0.05 % KCl, 0.05 % $MgSO_4.7H_2O$, 0.001 % $FeSO_4$, 2 % agar, and distilled H_2O to 1000 mL. Pikovaskya medium contains 1 % glucose, 0.5 % $Ca_3(PO_4)_2$, 0.05 % $(NH_4)_2SO_4$, 0.01 % $MgSO_4.7H_2O$, 0.02 % KCl, 0.05 % yeast extract, 0.0002 % $MnSO_2.H_2O$, 2 % agar, and distilled H_2O to 1000 mL.

2.2. Methods

Collecting Vetiver root samples: Root samples were taken at the root system of the Vetiver grass grown at Bien Hoa airbase, Dong Nai province. Firstly, the soil surface of the sampling locations was cleaned by removing all gravel and rocks. Subsequently, pickaxes, shovels, and scissors were used to dig and collect root samples (50 g/sample) at the selected locations. Root samples were rinsed with tap water to eliminate soil and sand. In the following step, the samples were washed sequentially with distilled water, hexane, and acetone to eliminate contaminated soil and toxic chemicals. The samples were air-dried and divided into different zip-bags with specific sample labels. The samples were stored at 4 °C and transported to the laboratory for further analysis.

Isolation of endophytic bacteria: The root samples were washed under running water for 3 min and then cut into small pieces of 5 cm. The surface of root samples was sterilized with 96 % ethanol (3 min), 3 % hydrogen peroxide (3 min), and then 1 % sodium hypochlorite (3 min). Finally, the samples were rinsed with sterile distilled water four times. To examine the effectiveness of the surface sterilization, 100 µL of the water of the last wash was spread on an LB plate, and the plate was incubated at 37 °C for 24 h. If there is no bacterial growth, surface disinfection is successful. The sterilized root samples were ground well using a mortar and a pestle with 1 mL of sterile distilled water. A volume of 100 µL of the liquid was spread on an LB plate, and the plate was kept at 30 - 37 °C to collect single bacterial colonies.

Genomic DNA extraction from bacterial strains: *Genomic DNA extraction from bacterial strains*: Bacterial strains were grown in the LB liquid medium for 24 h at 30 °C using an orbital shaking incubator. Bacterial cells were collected by centrifugation. The protocol for genomic DNA extraction was conducted as previously reported [16]. DNA samples were stored at 4 °C for further experiments.

DNA sequencing and identification of bacterial strains: 16S rRNA gene was amplified by PCR using the universal primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT). The thermal cycle for the PCR amplification includes 95 °C (3 min); 35 cycles of 94 °C (45 s), 53 °C (45 s), 72 °C (1 min); and 72 °C (7 min). PCR products were checked on an agarose gel by electrophoresis and purified using a MEGAquick-spin™ Plus Total Fragment DNA Purification Kit (iNtRON Biotechnology, South Korea). The purified DNA samples were sequenced from the forward direction by the 1st Base company (Singapore). DNA sequences were checked for quality using BioEdit 7.2 software and comparative analysis with the GenBank database using the BLAST tool.

Assay for the production of IAA: A bacterial strain was freshly grown in the LB medium at 37 °C for 24 h. Subsequently, 0.4 mL of the culture was added to a flask containing 40 mL of the LB-Tryptophan medium. Negative control flasks were not inoculated with bacterial strains. Flasks were shaken in an incubator at 37 °C, 150 rpm for 72 h in darkness. A volume of 1.5 mL of the culture was pipetted into an Eppendorf tube, and the bacterial cells were removed by centrifugation at 6000 rpm for 10 min. 1 mL of the supernatant was transferred to a new Eppendorf tube, and 4 mL of the Salkowski reagent (0.5 M $FeCl_3$ dissolved in 35 % H_2SO_4 at a ratio of 1:50) was added. The sample was well mixed and kept at room temperature for 25 min. The sample was then quantified for IAA concentration at 530 nm, as previously described [17, 18].

Assay for phytase activity: 50 µL of bacterial culture was added to a well of a PSM agar plate. After 24 h of incubation at 30 ^{o}C, phytate degradation was confirmed by a clear halo around the colony. For phytase activity quantification, a bacterial strain was grown in the PSM

medium at 37 °C, 200 rpm, for 72 h. The culture was centrifuged at 20 °C, 6000 rpm for 30 min. The supernatant as a crude enzyme solution was transferred to a new tube. The reaction mixture contained 125 µL of the crude enzyme solution, 125 µL of 15 mM sodium phytate (Sigma-Aldrich, USA), and 125 µL of 200 mM sodium acetate buffer (pH 5.5). The mixture was then incubated at 55 °C for 30 min, and the reaction was stopped by adding 0.5 mL of 15 % TCA. The development of yellow color began when 125 µL of the mixture was mixed with 1 mL of AAM solution (10 mM ammonium molybdate, 100 % acetone, 5 N sulfuric acid, at a ratio of 1:2:1). After 2 min of incubation at room temperature, 100 µL of 1 M citric acid was added. The sample was measured at 355 nm for the phytase activity quantification [19].

Detection of insoluble phosphate degradation [20]: Bacterial strains were grown on the Pikovaskya medium at 37 °C for 72 h. The ability of phosphate degradation was detected via clear halos around the bacterial colonies.

Assay for the tomato growth promotion by the selected bacterial strains: Tomato seeds were transplanted into nursery soil for 15 days. Afterwards, 30 plants with the same length of stem, number of leaves, and root length were selected for the next experiment. The root tips of all the plants were injured by cutting. For bacterial inoculation, 10 plants were soaked in a bacterial culture (10^6 CFU/mL) containing one of two selected strains for 20 min. As a control, 10 plants were grown without bacterial inoculation. Plant height was measured at 7, 14, 21, and 28 days. The experiment was repeated twice.

Statistical analysis: Data were presented as means ± standard deviations. Statistical analyses were performed with GraphPad Prism 8.0 using one-way ANOVA and Tukey's test. A statistical difference was considered when $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Successful isolation of culturable endophytic bacteria from Vetiver root samples

After surface sterilization of the Vetiver root samples, endophytic bacteria from the root tissues were isolated. Bacterial colonies appearing on the agar plates were separated and purified to obtain pure single strains. Sixteen bacterial isolates with diversified colony morphologies and colors were selected. These endophytic strains form mainly round colonies. The results from Gram staining showed that there are 10 Gram-negative and 6 Gram-positive bacterial strains. Further, cell morphological analysis under microscopy revealed that all 16 strains are rod-shaped bacteria or bacilli (Figure 1).

The isolated endophytic bacteria were identified based on morphological characteristics, Gram staining, and partial sequencing of the 16S rRNA gene. Genomic DNA samples were prepared from these strains for PCR amplification of the 16S rRNA gene using universal primer pair 27F/1492R. The analysis of PCR products on an agarose gel showed a single DNA band of approximately 1500 bp for all the strains (Figure 2A). Purified PCR products were sequenced from the forward direction, and the DNA sequences were compared with the GenBank database using the BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). All the 16S rRNA sequences of the endophytic bacterial strains were deposited in GenBank under accession numbers (OR921912-OR921927). Based on the results of 16S rRNA sequence analysis, 16 endophytic strains were preliminarily classified into 9 different species, including *Klebsiella variicola*, *Priestia megaterium* (previously known as *Bacillus megaterium*), *Citrobacter freundii*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Enterobacter hormaechei*, *Bacillus cereus*, *Enterobacter asburiae*, and *Serratia marcescens* (Figure 2B).

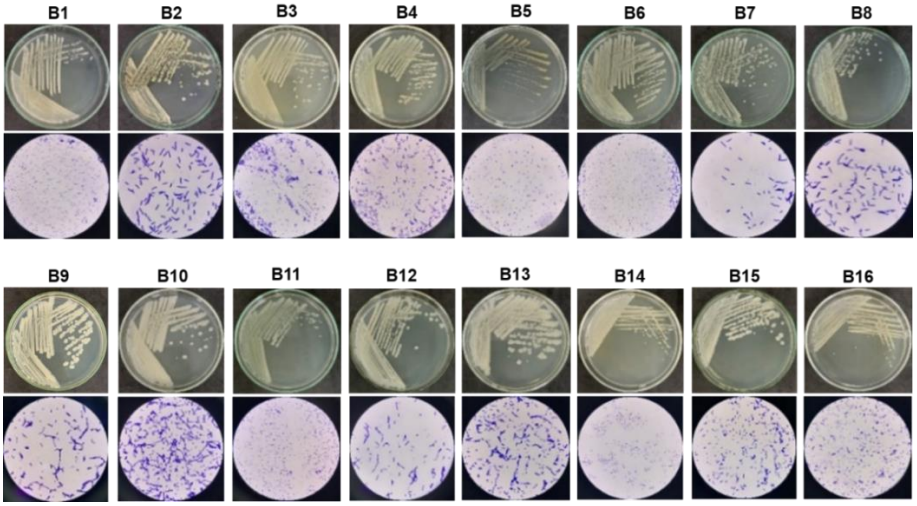
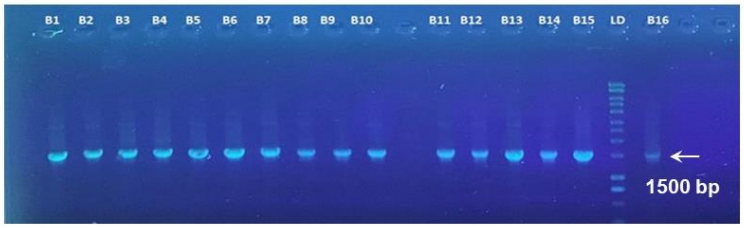


Figure 1. Phenotypic characteristics of sixteen endophytic bacterial strains. The strains were cultivated on the LB agar medium to examine colony morphology. Cell morphologies of these bacteria were observed under microscopy.

A



B

Bacterial strain (Accession number)	Nucleotide identity (%) to GenBank database	GenBank database (Accession numbers)	Suggested bacterial species
B1 (OR921912)	100	MT509531	*Klebsiella variicola*
B2 (OR921913)	100	MH559568	*Priestia megaterium*
B3 (OR921914)	100	MN420979	*Citrobacter freundii*
B4 (OR921915)	99.87	MZ676567	*Enterobacter cloacae*
B5 (OR921916)	100	CP133753	*Pseudomonas aeruginosa*
B6 (OR921917)	100	MT613371	*Enterobacter cloacae*
B7 (OR921918)	100	CP042551	*Enterobacter hormaechei*
B8 (OR921919)	100	OR793900	*Priestia megaterium*
B9 (OR921920)	100	OR793125	*Bacillus cereus*
B10 (OR921921)	99.76	MT111594	*Enterobacter cloacae*
B11 (OR921922)	100	MT613375	*Enterobacter asburiae*
B12 (OR921923)	100	CP138336	*Bacillus cereus*
B13 (OR921924)	100	CP138336	*Bacillus cereus*
B14 (OR921925)	100	MN069331	*Serratia marcescens*
B15 (OR921926)	100	CP138336	*Bacillus cereus*
B16 (OR921927)	100	MG461554	*Serratia marcescens*

Figure 2. Molecular identification of the endophytic bacterial strains. (A) The 16S rRNA gene was amplified from genomic DNA of 16 strains by PCR. Purified PCR products of the 16S rRNA gene were partially sequenced from the forward direction. (B) Comparative analysis of the 16S rRNA sequences using the GenBank database and the BLAST tool.

However, a major limitation of the 16S rDNA sequence is that it is unreliable for discriminating among closely related bacterial species within the same genus. To solve this problem, sequencing of other bacterial target genes, such as *rpoB* (β-subunit of RNA polymerase), *tuf* (elongation factor Tu), *gyrA* or *gyrB* (gyrase A or B), or *sodA* (manganese-dependent superoxide dismutase) may provide better separation of closely related species [21].

Therefore, some endophytic strains of the same genus from this study still need to be further confirmed for accurate species classification. All the endophytic bacterial strains reported in this study were already described as endophytes from different plants, such as *Klebsiella variicola* from *Saccharum officinarum* (sugarcane) [22], *Priestia megaterium* from *Bolboschoenus planiculmis* [23], *Citrobacter freundii* from *Zea mays* (maize) [24], *Enterobacter cloacae* from *Ocimum sanctum* [25], *Pseudomonas aeruginosa* from *Phragmites australis* [26], *Enterobacter hormaechei* from *Stevia rebaudiana* [27], *Bacillus cereus* from *Clitoria ternatea* [28], *Enterobacter asburiae* from *Phoenix dactylifera* [29], and *Serratia marcescens* from *Oryza sativa* (rice) [30]. However, this is the first report on culturable endophytes isolated from the root tissues of Vetiver grass cultivated in dioxin-contaminated soil at Bien Hoa airbase.

3.2. Production of large amounts of indole acetic acid by endophytic bacteria isolated from Vetiver grass

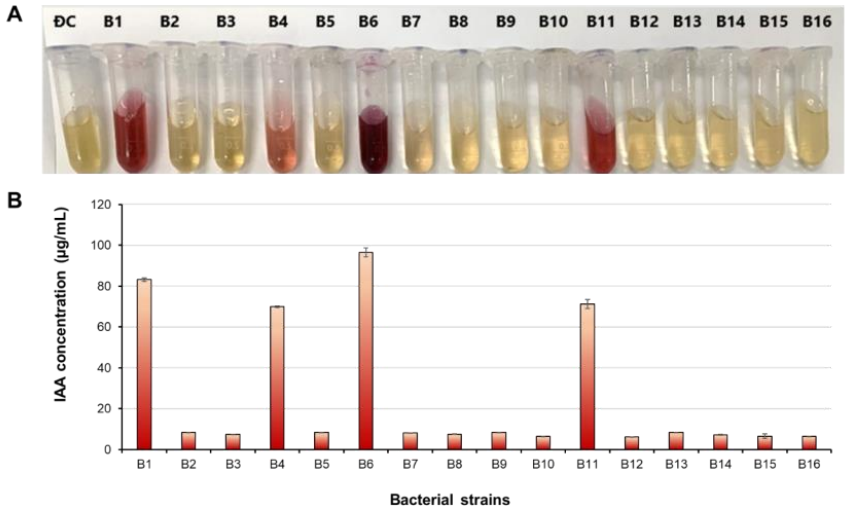


Figure 3. IAA biosynthesis in the endophytic bacterial strains. (A) Detection of IAA by the color changes of the reactions. (B) Quantification of IAA amounts produced by the bacterial strains.

Indole acetic acid (IAA) is a phytohormone of the auxin class, which plays a vital role in promoting the growth and development processes in plants, such as cell division, elongation, and differentiation [31]. This compound is also produced by numerous microorganisms, including bacterial endophytes [12, 13, 32]. The assays for IAA production showed that all the endophytic bacterial strains in this study exhibited the ability to produce IAA with concentrations ranging from 6.2 ± 0.02 to 96.5 ± 2.24 µg/mL (Figure 3).

Kim *et al*. (2017) indicated that *K. variicola* AY13 could synthesize IAA at a concentration of 84.27 ± 3.55 µg/mL [33]. An endophytic bacterium *E. cloacae* MG00145, isolated from the stem of *Ocimum sanctum*, produced IAA at a concentration of 17.807 µg/mL [25]. Our study showed that 4 endophytic bacterial strains isolated from Vetiver grass had large amounts of IAA in the liquid cultures after 72 h of cultivation (Figure 3). These strains include *K. variicola* B1 (83.2 ± 0.89 µg/mL), *E. cloacae* B4 (69.9 ± 0.45 µg/mL), *E. cloacae* B6 (96.5 ± 2.24 µg/mL), and *E. asburiae* B11 (71.2 ± 2.23 µg/mL).

3.3. Phosphate solubilizing ability of endophytic bacterial strains

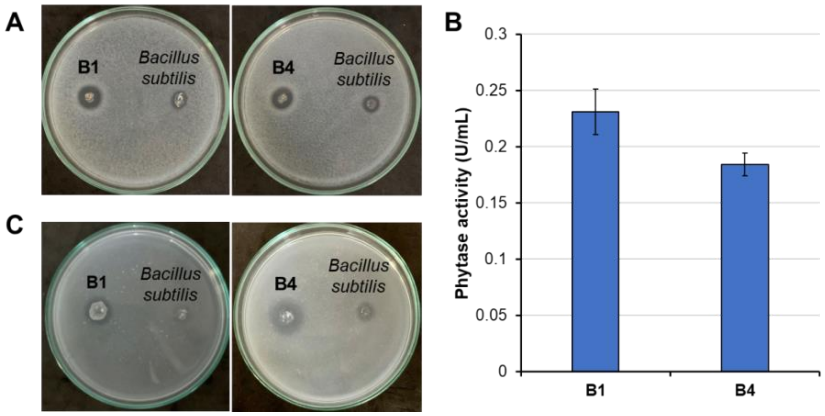


Figure 4. Enzyme production ability of two selected endophytic bacteria. (A) Phytate degradation by phytase activity of B1 and B4. *Bacillus subtilis* was used as a reference. (B) Quantification of phytase activity. (C) Phosphate degradation by B1 and B4.

Phosphate solubilization in plant rhizosphere by bacteria is a key driving force enabling higher P use efficiency and crop productivity [34]. Microbial degradation processes of phytate and insoluble phosphate in soil are usually involved in phytases and phosphatases [19, 34, 35]. Seven endophytic strains (B1, B2, B3, B4, B5, B6, and B8) exhibited phytate degradation, while only three (B1, B4, B5) degraded inorganic phosphate. Notably, *K. variicola* B1 and *E. cloacae* B4 could degrade both phytate and phosphate (Figure 4).

Phytate is the major form of organic phosphorus in soil but is not readily available to plants. Phosphate-solubilizing microorganisms are ubiquitous in soil and are vital in supplying P to plants [35]. Some bacterial genera, including *Pseudomonas*, *Enterobacter*, and *Pantoea*, were proven to produce phosphatases leading to high performance of inorganic phosphate release from insoluble phosphate [36]. Therefore, bacterial strains capable of degrading both phosphate and phytate are promising candidates for promoting plant growth.

3.4. Promotion of tomato plant growth by *K. variicola* B1 and *E. cloacae* B4

Endophytic strain *K. variicola* DX120E, isolated from roots of sugarcane, was reported to be able to colonize sugarcane roots and shoots and promote plant host growth [22]. Kim *et al*. (2017) reported that *K. variicola* AY13 could synthesize IAA at a concentration of 84.27 ± 3.55 µg/mL, promoting soybean growth and root development [33]. Endophytic bacterial strain *E. cloacae* MG00145, isolated from the stem of *Ocimum sanctum*, produced IAA at a concentration of 17.807 µg/mL. Plant assays with the inoculation of strain MG00145 proved that this strain significantly enhanced the growth of four crops [25].

In this study, strains *K. variicola* B1 and *E. cloacae* B4 could produce large amounts of IAA (Figure 3). They could also digest phytate and insoluble phosphate (Figure 4). Therefore, these strains were selected to evaluate their ability to stimulate the growth of tomato plants. After 28 days of bacterial inoculation, the plants inoculated with B1 or B4 increased their height by 19 - 22 % compared to the uninoculated control plants (Figure 5). Conclusively, strains B1 and B4 could promote the growth of tomato plants in vitro. These strains may also contribute to the growth simulation of Vetiver grass cultivated in dioxin-contaminated soil at Bien Hoa airbase.

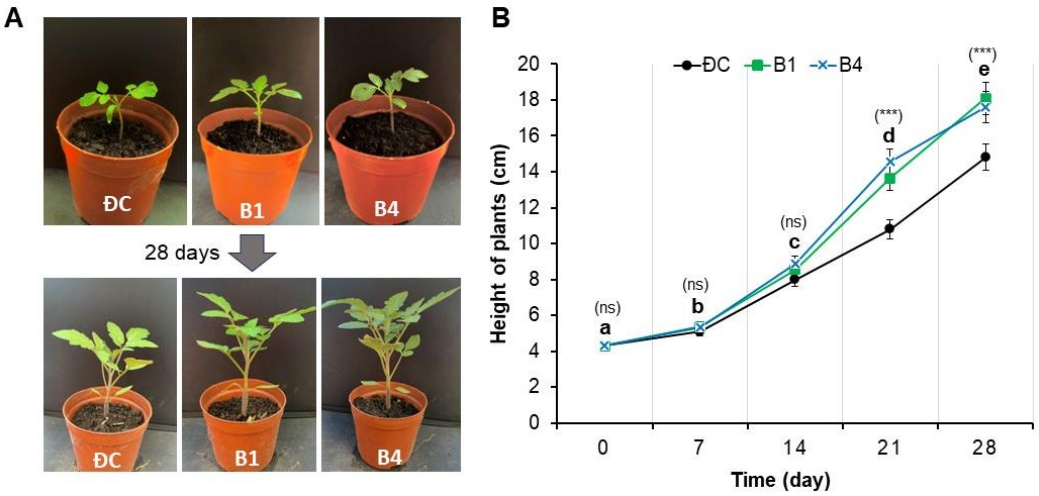


Figure 5. In vitro assays for promoting the growth of tomato plants by two endophytic bacterial strains (B1, B4). (A) The plants are at 0 day and 28 days of cultivation. (B) Quantification of the plant height through periods of cultivation. ĐC: control plant growth, B1: The growth of tomato plants inoculated with *K. Variicola* B1, B4: The growth of tomato plants inoculated with *E. cloacae* B4. Data were expressed as means ± standard deviations. One-way ANOVA analysis with Tukey's test was used for multiple comparisons. The lowercase letters indicate significant differences when the mean values of plants at different time points were compared. Asterisks (***) indicate a statistically significant difference ($p < 0.001$) between the means of tested plants and the means of control plants, and (ns) shows a non-significant difference.

4. CONCLUSIONS

Our study successfully isolated 16 endophytic bacterial strains from Vetiver grass roots cultivated in dioxin-contaminated soil at Bien Hoa airbase. 16S rRNA gene sequencing revealed these isolates belong to nine distinct species, including *Klebsiella variicola* (1 strain), *Priestia megaterium* (2 strains), *Citrobacter freundii* (1 strain), *Enterobacter cloacae* (3 strains), *Pseudomonas aeruginosa* (1 strain), *Enterobacter hormaechei* (1 strain), *Bacillus cereus* (4 strains), *Enterobacter asburiae* (1 strain), and *Serratia marcescens* (2 strains). Furthermore, four strains (*K. variicola* B1, *E. cloacae* B4, *E. cloacae* B6, and *E. asburiae* B11) exhibited significant IAA production, while strains B1 and B4 additionally displayed phosphate solubilization. In vitro assays indicated that strains B1 and B4 can promote the growth of tomato plants. These findings suggest endophytic bacteria associated with Vetiver grass hold promise for developing plant growth-promoting biofertilizers in contaminated environments.

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CRediT authorship contribution statement. Vu Thi Lan Anh: Methodology, Formal analysis, Data Visualization, Writing original draft. Dang Thi Ha Thu and Nguyen Thi Nhan: Formal analysis. Ngo Thi Thuy Huong, Nguyen Quoc Dinh: Methodology, Writing-Review and Editing. Tran Van Tuan: Conceptualization, Supervision, Writing-Review and Editing, Funding acquisition.

Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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