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Unveiling the rhizosphere microbial community of castor plant (*Ricinus communis* L.) grown in textile effluent contaminated ecosystem and assessing its phylogenetic traits for plant growth promotion

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Abstract

Various factors influence the microbial community in the plant rhizosphere, including the variety, activity, and population structure. Plant species and soil type are critical determinants of the composition and diversity of the microbial communities linked to plant roots. Understanding these interactions is crucial for enhancing plant development and soil vitality, especially under adverse conditions such as salt-affected soils. This study evaluated the structure and composition of rhizosphere microbial communities in response to castor plant (*Ricinus communis* L.), a crop known for its resilience to various soil conditions. The bacterial community in the castor rhizosphere was examined by 16S rDNA sequencing in conjunction with an assessment of various plant growth-promoting (PGP) characteristics of the isolated bacterial strains. These findings indicated that the rhizosphere bacterial community was primarily comprised of *Bacillus* species, which are essential plant growth-promoting rhizobacteria (PGPR). These bacteria exhibit considerable potential to augment nutrient absorption, strengthen stress resilience, and promot overall plant vitality. Their supremacy underscores their adaptability and functional significance in castor root systems, particularly under diverse soil conditions. This study elucidates the microbial dynamics of the castor rhizosphere, highlighting the crucial role of *Bacillus* species in enhancing plant growth and soil fertility. These findings indicate that *Bacillus*-dominated microbial communities can be efficiently utilized for sustainable agriculture and soil remediation initiatives.

Keywords

bacterial community; castor; PGPR; rhizosphere; textile effluent

Introduction

The rhizosphere is a localized soil area immediately affected by the root system (1). In contrast to the bulk soil, the rhizosphere exhibits a higher nutritional concentration owing to the accumulation of diverse plant exudations, including sugars and amino acids, which provide a substantial source of energy and nutrients for bacteria (2). This leads to bacterial populations near plant roots being 10 to 100 times greater than those in the bulk soil (3). The rhizosphere functions as a locale for numerous microorganisms, and the bacteria inhabiting this area are designated as rhizobacteria (4).

Bacteria that inhabit plant roots and enhance plant growth are referred to as rhizobacteria that promote plant growth (PGPR). Bacteria can be classified into three categories according to their effect on plant growth: beneficial, harmful (deleterious), and neutral (1). Beneficial PGPR, free-living soil bacteria, enhance plant growth via both direct and indirect methods (5). Symbiotic nitrogen fixers include *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Allorhizobium*, *Sinorhizobium*, and *Mesorhizobium*, as well as free-living and associative nitrogen-fixing bacteria such as *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Citrobacter freundii*, and *Pseudomonas putida*. Phosphate-solu-bilizing bacteria, including *Bacillus* and *Paenibacillus*. Siderophore producers such as *Burkholderia vietna-miensis*, *Grimontella* and *Stenotrophomonas and Herbaspirillum* (6). Harmful plant pathogenic microbes include *Xanthomonas, Ralstonia solanacearum, Pseudomonas viridiflava, Erwinia amylovora, and Vibrio vulnificus* (7,8). *Alphaproteobacteria, Betaproteobacteria* and *Methylobacterium* are present in the rhizosphere and phyllosphere region of the plant found neutral in their role (9).

PGPR inhabit the vicinity of plant roots, significantly contributing to plant growth enhancement. They directly promote development by supplying critical nutrients, including nitrogen and phosphorus, or by synthesizing phytohormones, and indirectly by inhibiting plant pathogens or augmenting stress resilience. PGPR directly enhance plant growth through mechanisms including biological nitrogen fixation, production of plant growth promoting phytohormones, and increase in uptake of phosphorus nutrients by mineralization and solubilization. These bacteria also synthesis enzymes that modulate plant growth (e.g. ACC deaminase), vitamin production, mineralized organic nitrogen, sulfur and phosphorus, polysaccharide production, and improvement of soil aggregation, biogeochemical cycling of nutrients, production of metal chelators (e.g., siderophores), production of organic volatile compounds, increase in root surface area (by mycorrhizae), and help in water uptake. Various indirect plant growth promotion mechanisms include enhanced stress resistance (against salt, drought, metals, and xenobiotic compounds) to plants, biocontrol and antibiosis, induced systemic resistance, increase in biomass of roots adhering to the soil, and the potential to degrade synthetic pesticides and contaminants (1).

The ability of plant growth-promoting rhizobacterial (PGPR) strains to promote plant development through a variety of pathways has been extensively studied. *Bacillus* spp. PSB10 has been demonstrated to enhance the growth of chickpeas (*Cicer arietinum* L.) by producting IAA, ammonia, siderophores, and HCN (10). *Bacillus subtilis* DR2 promotes the development of coriander (*Coriandrum sativum*) via siderophore synthesis (11). *Burkholderia* strains in *Calendula officinalis* can create siderophores, solubilize heavy metals and phosphates, and synthesis ACC deaminase and IAA (12). *Klebsiella oxytoca* facilitates development in maize (*Zea mays*) via solubilizing phosphate, synthesizing IAA, and augmenting nitrogenase activity (13). *Pseudomonas aeruginosa* enhances maize development by producing exopolysaccharides, ammonia, siderophores, hydrogen cyanide, indole-3-acetic acid, and phosphate solubilization (14). *Bradyrhizobium* sp. 750 assists in alleviating heavy metal stress in rice (*Oryza sativa* L.) (15). *Flavobacterium* promotes growth in the common bean (*Phaseolus vulgaris* L.) by producing IAA and siderophores (16). *Pseudomonas* sp. enhances the growth of onions (*Allium cepa* L.) by solubilizing phosphate (17). In wheat (*Triticum aestivum*), *Rhizobium leguminosarum* Thy2 enhances growth by producing siderophores, facilitating nitrogen fixation, and alleviating cadmium stress (18). Finally, in sunflowers (*Helianthus annuus*), *Serratia marcescens* enhances growth through the production of IAA, siderophores, and HCN (19).

Castor plants are vital for ecological and economic purposes. Castor plants are rapidly growing, resilient crop well-suited for many environments, and requiring minimal inputs yet yielding high biomass in a short period. Castor plants facilitate environmental cleanup and carbon sequestration. The vast root system of castor plants aids in preventing soil erosion in deteriorated areas. Castor oil derived from castor seeds has multiple applications in the industrial, medical, and cosmetic industries. Consequently, all of these factors provide a desirable crop for sustainable agriculture, economic development, and environmental conservation. The studies conducted on the castor rhizosphere and their microbial associations were grouped into three types: (i) assessing castor rhizosphere microbial diversity (20, 21), (ii) screening for PGPR from castor and their inoculation effects as phytoremediation agents (22, 23), and (iii) isolating PGPR associated with castor and documenting their inoculation effects as plant growth promoting agents in castor crops (5, 24-26).

Evaluating microbial diversity in rhizosphere regions has become a main focus area for exploring the metabolic potential of several microbial communities fostered by root exudates. A varied rhizosphere microbiome enhances plant health by improving nutrient availability, synthesizing growth-promoting compounds, and providing protection against pathogens. Manipulating this diversity through rhizosphere engineering can result in more robust and productive agricultural systems (27). Such studies contribute significantly to the field of rhizosphere engineering, enabling the manipulation of microbial populations to enhance crop production. The present study aimed to isolate rhizosphere microorganisms from castor plants, focusing on their potential roles in nutrient availability and enabling phytoremediation in metal-contaminated soils.

Materials and Methods

Assessing the rhizosphere bacterial diversity

One gram of rhizosphere soil was taken and processed using the standard serial dilution method (28). For cultivating the organisms from the textile effluent contaminated soil, Reasoner's 2A agar, Modified (R2A agar, Modified) [Composition (g L^{-1}): Casein Enzymic hydrolysate - 0.250; Peptic digest of animal tissue-0.250; Casein Acid hydroly-

sate-0.500; Yeast extract-0.500; Glucose-0.500; Starch soluble-0.500; Dipotassium phosphate – 0.030; Magnesium sulphate, heptahydrate – 0.500; Sodium pyruvate – 0.030; Agar – 15; Final pH (at 25°C) – 7.2 ± 0.2] was used as a medium. This medium favours the growth of stressed slow growing organisms at longer incubation periods as compared to the use of high nutrient medium. R2A medium was prepared and the cultures were plated from the appropriate dilutions (the dilution ranged from 10^{-2} to 10^{-8}), and incubated for up to 48 hr. Morphologically unique and distinct colonies were identified, sub cultured, and maintained for further analyses.

Screening the bacterial isolates for PGPR traits

Bacterial isolates obtained from the castor rhizosphere were evaluated for their ability to produce essential plant growth-promoting features, such as indole acetic acid (IAA) synthesis, phosphate solubilization (PS), and siderophore synthesis.

Assay for quantifying IAA production

IAA synthesis by the bacterial isolates was quantified using the method outlined previously (29). Bacterial isolates were introduced into 5 mL of LB broth enriched with 0.2% L-tryptophan (pH 7.0) and incubated the culture in a shaking incubator at 28˚C with shaking at 125 rpm for 7 days. After incubation, the cultures were centrifuged at 11,000 rpm for 15 min. To ascertain IAA generation, 2 mL of the Salkowski reagent was added to 1 mL of the supernatant. The emergence of a pinkish hue signified the presence of IAA, and optical density (OD) was measured at 530 nm. A standard curve for IAA was used to quantify the quantity of IAA generated.

Assessment of phosphate solubilization efficacy

Pikovskaya's agar medium was used to assess the phosphate solubilization efficiency of the bacterial isolates. After spot-inoculation onto the medium, bacterial cultures were cultured for 48 hr at 30℃. Following incubation, the diameter of the halo (clear) zone that developed surrounding the bacterial colonies was measured, signifying phosphate solubilization. The following equation (Eq. 1) was used to determine the phosphate solubilization efficiency (PSE) (30). This method facilitated the quantitative evaluation of the phosphate solubilizing capacity of the isolates.

$$PSE\ (\%) = \frac{\text{Diameter of halo zone - Diameter of colony}}{\text{Diameter of colony}} \times 100 \quad \text{.....(Eqn.1)}$$

Assay on testing the production of siderophore

Both Chrome azurol sulphate (CAS) 60.5 g and 0.27 g of $FeCl_3$ were dissolved in 50 mL of distilled water with thorough mixing, the CAS solution was prepared. Subsequently, 364.6 µL of concentrated HCl was added and stirred thoroughly. This mixture was gradually added to an acetyltrimethylammonium bromide (CTAB) solution (prepared by dissolving 2.9 g CTAB in 40 mL of distilled water) under constant mixing, resulting in a dark blue solution (100 mL total), which was then autoclaved for 15 min at 121℃.

After increasing the volume of 50 mL of CAS solution to 1L (pH-7.0), agar was added and the mixture was autoclaved. Following autoclaving, it was allowed to cool before being transferred into sterile petri dishes, with approximately 25 mL of blue agar per plate. All isolates were spot injected into these plates after 24 hr (to check for contamination) and incubated for 3-4 days at the optimal growth temperature. Siderophore-producing isolates were defined as those that produced an orange halo zone against a dark medium background (31).

Determining the evolutionary relationship of the selected isolates

Preparation for subculturing and 16S rDNA sequencing

Culturing specimens were sub cultured to isolate and evaluate bacterial isolates. The selected isolates were prepared for 16S rDNA sequencing, with meticulous attention paid to the production of template DNA to guarantee the identification of pure bacterial cultures. Colonies were obtained with a sterile toothpick and suspended in sterile saline (0.5 mL) in a 1.5 mL centrifuge tube. The suspension was then centrifuged at 10,000 rpm for 10 min. Subsequent to the removal of the supernatant, the bacterial pellet was reconstituted in 0.5 mL of Insta Gene Matrix (Bio-Rad, USA). The suspension was incubated at 56℃ for 30 min and then heated to 100℃ for 10 min. The resultant supernatant served as the template DNA for PCR. For PCR amplification, 1 µL of template DNA was included into a 20 µL PCR reaction mixture. The bacterial 16S rDNA region was amplified using the primers 518F (5' CCAGCAGCCGCGGTAATACG 3') and 800R (5' TACCAGGGTATCTAATCC 3'). cA positive control (*E. coli* genomic DNA) and negative control was used to validate the PCR procedure.

Purification and sequencing of PCR Products

The PCR products were purified to exclude unincorporated primers and dNTPs using a Montage PCR Clean-up Kit (Millipore). The purified PCR products, approximately 1,400 base pairs in length, were sequenced using the primers 785F 5' GGA TTA GAT ACC CTG GTA 3' and 907R 5' CCG TCA ATT CCT TTR AGT TT 3' (32). Sequencing was conducted using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, USA), and the resultant sequencing products were evaluated using an Applied Biosystems 3730XL, an automated DNA sequencing machine (Applied Biosystems, USA).

Phylogenetic analysis

The acquired culture sequences were subjected to BLAST analysis to identify phylogenetically analogous strains and associated sequences from GenBank. The sequences were aligned using multiple sequence alignment, standardized to uniform nucleotide lengths, and used to construct a phylogenetic tree using the neighbour-joining approach in MEGA 6 (33). The bootstrap values at the tree nodes signify the level of statistical support, with elevated values (approximately 100%) indicating strong clustering.

The bootstrap technique utilizes 1,000 resampled datasets, excluding values below 50%. Scale bar indicates 0.005 replacements per site.

Results and Discussion

Evaluating bacterial isolates for PGP

An evaluation was conducted to document the culturable plant growth-promoting rhizobacteria prevalent in the rhizosphere of castor and to examine their plant growth-promoting capabilities. R2A medium was used to isolate the culturable bacteria present in the textile effluent-contaminated soils. Among the isolated 20 bacterial strains very few strains (35% of the isolated strains) showed common morphologies of *Bacillus* strains, such as large, spreading, and irregularly shaped, as per the descriptive information given in Bergey's Manual of Systematic Bacteriology (34). When screened for PGP traits (Table 1), 7 strains produced the phytohormone IAA. Among the 7 bacterial strains, 3 were identified as siderophore producers and phosphate solubilizers. Based on the uniqueness of the colony morphology and the presence of PGP ability, 7 strains were further subjected to phylogenetic identification and characterization.

Phylogenetic identification of bacterial isolates

For further identification, all cultures were subjected to 16 rRNA gene analyses, and the contig files generated through sequencing were identified using the comparative data obtained by the blast search in the ExTaxon server and based on the phylogenetic trees constructed with evolutionary neighbours. All the strains were identified as *Bacillus* genus, with two strains identified as *Bacillus altitudinis* (MH538126 and MH538127) and two as *Bacillus tequilensis* (MH538128 and MH538123), one belongs to *Bacillus paralicheniformis* (MH538129), another belong to *Bacillus paramycoides* (MH538124) and final one grouped under *Bacillus aryabhattai* (MH538125) (Fig.1.) All the 7 isolates were submitted to Genbank and assigned accession

Table 1. Plant growth promoting traits of rhizobacterial strains from castor plant

S. No		Bacterial strains	IAA production	Siderophore production	Phosphate solubilization
1	SPK1	*Bacillus aryabhattai* (MH538125)	+	+	+
2	SPK2	*Bacillus tequilensis* (MH538123)	+	-	+
3	SPK3	*Bacillus paramycoides* (MH538124)	+	-	-
4	SPK4	*Bacillus altitudinis* (MH538126)	+	+	-
5	SPK5	*Bacillus altitudinis* (MH538127)	+	+	-
6	SPK6	*Bacillus tequilensis* (MH538128)	+	-	+
7	SPK7	*Bacillus paralicheniformis* (MH538129)	+	-	-

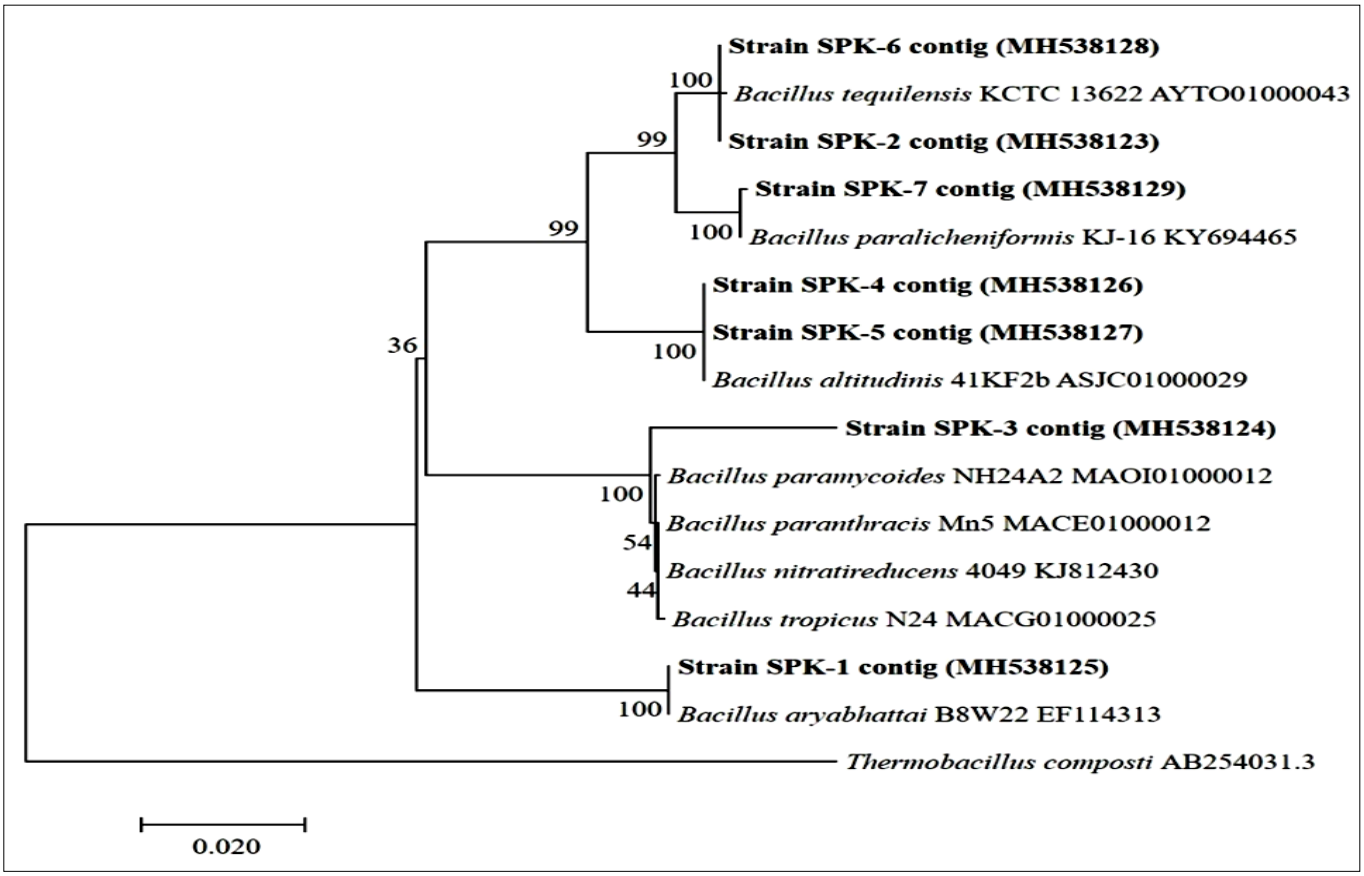

Fig. 1. Evolutionary relationship among the *Bacillus* strains revealed by sequencing the 16S rRNA genes. *Thermobacillus composti* was used as an outgroup to root the tree. Bacillus strains recovered in the present study were provided in bold. Scale bar, 0.020 nucleotide substitutions per nucleotide position.

numbers. *Bacillus* spp. is the foremost plant growth-promoting rhizobacteria because of their ability to generate durable stress-resistant spores. Interactions between *Bacillus* spp, and plants are mediated by chemical signals comprising several metabolites, leading to greater plant development and improved protection. *Bacillus* spp. invoke both direct (e.g., siderophore synthesis, nitrogen fixation, phytohormone synthesis, and nutrient solubilization) and indirect mechanisms (such as exopolysaccharide production, biofilm development, hydrogen cyanide synthesis, and lytic enzyme activity) to enhance plant growth and yield under diverse environmental conditions (35).

Bacillus species, classified as PGPR, are recognized for their ability to produce siderophores, solubilize phosphate, and synthesize IAA in the rhizosphere of castor plants, significantly enhancing plant health and development under stress conditions. To completely understand functions and potential of these activities in agricultural applications, it is necessary to critically evaluate their efficacy and ramifications. Among the 7 bacterial strains isolated *Bacillus aryabhattai* (SPK1) had all PGP features (Table 1). The bacterial strain *Bacillus paramycoides* (SPK 3) produced the highest quantity of IAA 78.0 mg ml^{-1} (Table 2). Recent metabolomic and molecular studies have enabled the identification of genes involved in IAA biosynthesis in *Bacillus amyloliquefaciens* (19). Furthermore, the application of IAA-producing *Bacillus altitudinis* alleviated iron stress in *Triticum aestivum* L. seedlings by both bioleaching of iron and upregulation of genes encoding ferritins (36). In a related investigation, seven bacterial isolates from the rhizosphere of common bean plants in the Uttarakhand Himalaya exhibited notable plant growth-promoting (PGP) and antagonistic characteristics (24). The isolate BPR7 was classified as *Bacillus* sp. BPR7 using 16S rRNA gene sequencing. Strain BPR7 demonstrated many plants growth-promoting (PGP) characteristics, including production of indole-3-acetic acid (IAA), siderophores, phytase, organic acids, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, cyanogens, lytic enzymes, and oxalate oxidase.

Table 2. Quantification of IAA production in rhizobacterial strains from castor plant

S. No		Bacterial strains	IAA production (mg mL^{-1})
1	SPK1	*Bacillus aryabhattai* (MH538125)	68.9
2	SPK2	*Bacillus tequilensis* (MH538123)	18.5
3	SPK3	*Bacillus paramycoides* (MH538124)	78.0
4	SPK4	*Bacillus altitudinis* (MH538126)	23.5
5	SPK5	*Bacillus altitudinis* (MH538127)	31.8
6	SPK6	*Bacillus tequilensis* (MH538128)	46.2
7	SPK7	*Bacillus paralicheniformis* (MH538129)	16.7

One of the main ways by which *Bacillus* species promote plant growth is through IAA synthesis. IAA is a phytohormone that is essential for cell division and root growth. *Bacillus*-induced IAA can result in longer roots with more surface area, which improves the capacity of plants to absorb nutrients and water in the castor rhizosphere. However, the amount of IAA produced by *Bacillus* varies and is affected by several environmental variables, including temperature, pH, and nutrients availability. Furthermore, overproduction of IAA can result in overly vigorous root growth, which could have a negative impact on the capacity of the plant to allocate resources and maintain its structural stability. Consequently, although *Bacillus* species can synthesize IAA and enhance castor root growth, the advantages must be judiciously evaluated against possible disadvantages.

Another essential PGPR characteristic is the formation of siderophores by *Bacillus* species, which serve as iron-chelating agents to promote iron uptake by plants. Owing to its limited solubility, iron is frequently a limiting component in soils and is necessary for the production of chlorophyll. Siderophores can sequester iron from the soil environment, increasing their availability for plant uptake and photosynthesis. 4 salt-tolerant plant growth-promoting rhizobacteria were isolated from the rhizosphere of salt-affected rice fields in Bangladesh and assessed for their producing siderophore capacity. *Bacillus aryabhattai* MS3 exhibited the highest yield of 60% and 43% under non-saline and saline conditions, respectively (37). Siderophore producing *Bacillus* species, however, make iron more available to castor plants. However, by competing with other microorganisms, both pathogens and helpful bacteria, they may also change the dynamics of the microbial population in the rhizosphere (38). Furthermore, siderophore synthesis has ecological ramifications that may have unanticipated negative effects owing to incomplete understanding, such as effects on the cycling of nutrients in the soil and microbial diversity.

Furthermore, *Bacillus* species solubilized multiple sources of organic and inorganic phosphates, together with potassium and zinc, which was substantiated by multiple investigations. An *in vitro* study was conducted to evaluate the zinc solubilization potential of bacterial isolates obtained from wheat rhizosphere, utilizing insoluble zinc carbonate ($ZnCO_3$), zinc oxide (ZnO), and zinc phosphate ($Zn_3(PO_4)_2$). 6 isolates, *Bacillus altitudinis* AJW-3, *B. subtilis* ABW-30, *B. megaterium* CHW-22, *B. licheniformis* MJW-38, *Brevibacillus borstelensis* CHW-2, and *B. xiamenensis* BLW-7, demonstrated the ability to solubilize these zinc compounds. This confirmation was further established in pot and field tests *via* inoculation with wheat crops (39). The potassium-solubilizing properties of *Bacillus aryabhattai* SK1-7 and its growth-promoting effects on plants were assessed in a study that demonstrated

B. aryabhattai SK1-7 can convert insoluble potassium in soil into bioavailable potassium. The SK1-7 strain dissolved 10.8 µg/mL of potassium, resulting in a potassium-solubilizing rate of 32.6%. The organic acids produced by this strain during growth and metabolism lower soil pH, solubilize insoluble potassium, and enhance chlorophyll content and total potassium levels in plants (40). The capacity to solubilize potassium and zinc underscores its potential as a biofertilizer and biocontrol agent for sustainable agriculture.

Earlier studies documented the diversity of *Bacillus* spp. occurring in the wild and cultivated castor plants. On total, 18 *Bacillus* isolates were recovered and they were tested for their ability to colonize the plants and biocontrol the pathogenic fungi *Macrophomina phaseolina* (21). Another study that attempted to characterize bacteria present inside the castor fruit waste, identified twelve species of bacteria, with *Bacillus* as common bacteria (41). A similar study on characterizing the proliferation of plants promoting endophytic bacteria from castor plants (*Ricinus communis* L.) revealed that the majority of the 15 isolates were identified as *Bacillus* species (29). The bacterial isolate Erc7 (*Bacillus cereus*) exhibited superior plant growth-promoting activities, including ammonia and IAA production, phosphate solubilization, nitrogen fixation, HCN production, and antagonistic activity against *Fusarium oxysporum* f. sp. *ricini* in *in vitro* conditions, which induces wilt in castor plants.

Conclusion

The bacterial strains isolated from the castor rhizosphere were predominantly of the genus *Bacillus,* with all seven strains exhibiting plant growth-promoting characteristics. Among these, *Bacillus aryabhattai* (SPK 1) exhibited all plant growth-promoting properties, including IAA biosynthesis, siderophore synthesis, and phosphate solubilization. Notably, *Bacillus paramycoides* (SPK3) produced the highest concentration of IAA, reaching 78.0 mg mL^{-1}. These findings suggest that the plant growth-promoting rhizobacterial strains derived from the castor rhizosphere may function as bioinoculants to augment the growth of the castor crop even under stressful environmental conditions.

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Authors' contributions

PK designed the concept of study, performed the field work, molecular sequencing and writing of manuscript, SPS worked on data analysis and writing of manuscript. VD worked on data analysis and manuscript writing. EP carried out the field work and data analysis, KS participated in data analysis, TI performed statistical analysis. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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