



Biofertilizer and Biostimulant Potential of Pigeonpea (*Cajanus scarabaeoides* L.) Bacterial Endophytes *In vitro* and in Planta Study

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Authors' contributions

This work was carried out in collaboration among all authors. Author KBS designed the study and performed the research and wrote the first draft of the manuscript. Authors Arati and DC performed statistical analysis, wrote the protocol. Author PB managed the analyses of the study and literature searches. Author NN reviewed the manuscript and supervised the research. All authors read and approved the final manuscript.

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ABSTRACT

Endophytes are microorganisms that establish intimate, non-pathogenic associations with host plants, were isolated from wild pigeonpea. The study aimed to determine their potential as biofertilizers and biostimulants for enhancing the cultivated pigeonpea variety ICP 8863 growth. The

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experiment was laid out in completely randomized design with three replications at the Department of Plant Pathology, UAS, Bangalore, between January 2023 and February 2024. Endophytic bacteria were isolated from leaf and stem tissues of *Cajanus scarabaeoides*. These isolates were subjected to a qualitative screening assay to evaluate their potential for biofertilization, assessing phosphate, potassium, and zinc solubilization, siderophore production, and atmospheric nitrogen fixation. *In planta* experiments were conducted to determine the biostimulatory effects of these endophytes on plant growth. High-performance liquid chromatography (HPLC) was employed to profile phytohormone production by a selected, effective endophyte. Subsequent molecular characterization was performed for taxonomic identification. Twenty-five bacterial endophytes were isolated from wild pigeonpea, with nine displaying biofertilization potential. *In planta* evaluation identified SB3 as the most effective, significantly improving germination (91.11%), plant height (83.21 cm), branching (15.66), leaf production (49.66), and dry matter accumulation (139.84 g/plant). SB3 was shown to produce indole acetic acid, cytokinin, gibberellic acid, and abscisic acid via HPLC, and was identified as *Bacillus subtilis* through 16S rRNA sequencing. This study reveals the potential of wild pigeonpea endophytes as a bioresource for sustainable agriculture due to their ability to enhance plant growth through nutrient solubilization and phytohormone production, making them ideal for eco-friendly biofertilizers and biostimulants.

Keywords: Pigeonpea; endophytic bacteria; biofertilization; biostimulation; phytohormone production.

1. INTRODUCTION

The term "endophyte," derived from Greek, refers to microorganisms (fungi, bacteria, protozoa, viruses, or algae) that reside within plant tissues without causing disease (De Bary, 1866; Saikkonen et al., 2004). These organisms are widespread, inhabiting nearly all plant tissues, and a single plant species can harbor a remarkable diversity, sometimes exceeding 100 different endophyte species. This highlights the intricate ecological relationships between plants and their endophytes, shaped by both environmental pressures and evolutionary history (Liu et al., 2017).

Cajanus cajan (L.) Millspaugh, commonly known as pigeonpea, redgram, arhar, or tur, is a diploid ($2n=2x=22$) legume species within the *Fabaceae* family. This significant *kharif* crop ranks as the second-most-important pulse crop in India, after chickpea. While India dominates global pigeonpea cultivation and production, its productivity lags behind the global average, likely due to various biotic and abiotic stresses. In contrast, wild pigeonpea varieties, exposed to diverse environmental pressures, may harbor unique endophytes with beneficial plant growth-promoting and stress-resistant traits. These endophytes represent a valuable resource for enhancing crop resilience and productivity (Kuzniar et al., 2019; Lacava et al., 2022).

Plant growth-promoting endophytes (PGPE) enhance plant development through three primary mechanisms: biostimulation

(phytohormone modulation), biofertilization (nutrient availability), and biocontrol (Santos et al., 2018). They also contribute to bioremediation (Afzal et al., 2019). The long-standing symbiotic relationship between plants and endophytes, evident in fossil records, underscores their crucial role in plant evolution and adaptation (Clay and Scharl, 2002). In previous cases, the native endophytes have improved the performance of pigeonpea (Rajendran et al., 2008) and other legume crops (Bhutani et al., 2018; Alok et al., 2020). But the role of endophytes from wild relatives in enhancing the growth traits of pigeonpea is untouched. Hence, it is hypothesized that endophytes from resilient plants, such as wild pigeonpea, offer opportunities to improve agricultural sustainability and environmental management through their beneficial traits.

This study assesses the effectiveness of wild pigeonpea-derived bacterial endophytes in improving the growth performance of the cultivated pigeonpea variety.

2. MATERIALS AND METHODS

2.1 Isolation of Bacterial Endophytes

Isolation of endophytic bacteria from leaf and stem tissues of *Cajanus scarabaeoides* (L.) Thouars (wild pigeonpea) was done using a standard protocol (Kumar et al., 2016). This research was conducted in the Department of Plant Pathology at the University of Agricultural Sciences, GKVK, Bengaluru, Karnataka, India,

between 2023 and 2024. Specifically, 1 cm segments of leaf and stem tissue were surface-sterilized by sequential washes: 70% ethanol (1 min), 1% sodium hypochlorite (30 sec), and a final 70% ethanol wash (1 min), to eliminate surface contaminants without excessively damaging the plant tissue or killing endophytes located just beneath the surface, followed by rinsing with sterile distilled water and air-drying. These segments were then aseptically placed onto nutrient agar (NA) and King's B media and incubated for 48-72 hours to observe bacterial growth from the cut ends. Imprints of the surface-sterilized tissues were also made on the media to confirm the absence of epiphytic contamination. Endophyte isolates were coded using a system that denoted the tissue of origin (L for leaf, S for stem) and the type of microorganism (B for bacteria).

2.2 Colonization Frequency of Endophytes

For each stem and leaf tissue, four segments were placed onto separate media, with three replicates per treatment. In total, 24 segments from each tissue type were assessed for bacterial colonization. The colonization frequency (Cf) of endophytic bacteria was determined by the number of segments colonized by each endophyte to the total number of segments observed (Suryanarayanan et al., 2003).

2.3 Phenotypic Differentiation of Bacterial Endophytes

The isolated bacterial endophytes were characterized phenotypically by macroscopic studies. All the isolates were streaked on agar plates and incubated at 30°C for 24 h. Thereafter colony morphological traits viz., colour, form, elevation and margin were then noted. Microscopic observations were performed to investigate the Gram reaction as per standard methods (Cappuccino and Sherman, 2008).

2.4 Evaluation of Bacterial Endophytic Biofertilization Potential

Qualitative assessment of the potentiality of isolated bacterial endophytes for biofertilization traits, viz., phosphorus, potassium, and zinc solubilization, siderophores production, and atmospheric nitrogen fixation was done following established methods and specific media: Pikovskaya medium for phosphorus solubilization

(Jasim et al., 2013), Aleksandrow medium for potassium (Hu et al., 2006), a minimal medium with ZnO for zinc (Fomina et al., 2005), Norris glucose nitrogen-free medium for free-living nitrogen fixation (Pathak and Kalekar, 2012) and Chrome Azurol S (CAS) solid medium for siderophore production (Schwyn and Neilands, 1987). For *in vitro* assays, bacterial isolates were pre-cultured in tryptic soy broth at 30°C and 120 rpm for 24 hours. A 5 µL aliquot of each culture was then spot-inoculated onto the respective media and incubated at 30°C for three days. Solubilization of phosphorus, potassium, and zinc was indicated by clear halo zones around bacterial colonies. Siderophore production was identified by an orange halo around the colonies. For nitrogen fixation, isolates were streaked onto Norris glucose nitrogen-free medium and incubated at 28°C for seven days. Growth on this nitrogen-free medium indicated positive nitrogen fixation.

2.5 Assessing Endophytic Biostimulant Activity in Pigeonpea

In planta studies were done by priming the pigeonpea (ICP 8863) seeds with the respective endophytic bacterial suspension (1×10^8 CFU/mL). Germination percentage was determined after 2-3 days of germination on blotter paper. Primed seeds were then sown in 2 kg pots and grown under glasshouse conditions. After 12 weeks, plant height (cm), number of branches, number of leaves, and dry matter accumulation (g/plant) were measured.

2.6 High-Performance Liquid Chromatography (HPLC) Based Phytohormone Analysis from Effective Bacterial Endophyte

Endophytic bacteria, previously identified as possessing biostimulant and biofertilizer properties, underwent High-Performance Liquid Chromatography (HPLC) to quantify phytohormones, specifically indole-3-acetic acid (IAA), gibberellic acid (GA), cytokinins, and abscisic acid (ABA). Bacterial cultures were grown in 50 mL Tryptic soy broth (TSB) supplemented with 5 mg/mL tryptophan for IAA quantification, following Tien et al. (1979), and incubated at 37°C for 7 days. Uninoculated TSB served as a control. After incubation, cultures were centrifuged at 6000 rpm for 15 minutes. The supernatant's pH was adjusted to 2.5-3.0 with 0.1 N HCl, and then extracted with an equal volume of diethyl ether overnight at 4°C. The

aqueous phase was discarded, and the organic phase was evaporated using a rotary vacuum evaporator at 40°C and 10000 rpm. The resulting residue was dissolved in HPLC-grade acetonitrile/methanol, filtered through a 0.22 µm filter, and immediately analyzed via HPLC. C18 reverse phase HPLC column was fitted with the column temperature of 30°C was maintained for all samples.

2.7 Characterization of Efficient Bacterial Endophyte

Molecular characterization of efficient endophyte was done by DNA isolation by the by the alkaline lysis method (Ramu et al., 2010). The quality of the isolated DNA was verified by 0.8% agarose gel electrophoresis. Further, amplification of the 16S rRNA gene, by using universal primers 27F: 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R: 5'-TACGGYTACCTTGTTACGACTT. PCR mixture prepared contained 1x Taq buffer (2 µL), dNTP mix (2µL), forward and reverse primer (0.5 µL each) template DNA (1 µL) and Taq polymerase (0.3 µL) and final volume is adjusted to 20 µL of sterile water. The PCR was conducted using a Proflex PCR thermal cycler (Carlsbad, California, United States). The amplification protocol included an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 sec, and extension at 72°C for 60 sec, with a final extension at 72°C for 10 min. The amplified products were verified *via* electrophoresis on a 1% (w/w) agarose gel to confirm the specific region's amplification.

Sequence analysis of the PCR products was performed by Eurofins Genomics. The resulting sequence was then subjected to a BLASTn search against the GenBank database to determine sequence similarity. Using BioEdit (v5.0.9), sequence identity matrices were created to compare the target endophyte to closely related sequences. To further understand the evolutionary context, a phylogenetic tree was generated using MEGA X.

2.8 Statistical Analysis

The experiment followed a completely randomized design (CRD). Data collected from both laboratory and pot experiments were statistically analyzed using the WASP: 2.0 software (available at www.icargoa.res.in/wasp2/index.php), and mean

comparisons were performed using Duncan's Multiple Range Test (DMRT).

3. RESULTS AND DISCUSSION

3.1 Endophytic Bacterial Community: Isolation and Colonization Analysis

From 48 tissue segments (24 leaf and 24 stem) of wild pigeonpea, 25 bacterial endophytes were successfully isolated of which 9 from leaf tissue (Fig. 1) and 16 from stem tissue (Fig. 2). Colonization frequencies were 58.33% for leaf segments and 87.50% for stem segments, indicating a higher prevalence of endophytes in stems (Table 1). The isolated endophytes were then characterized based on their morphology, as detailed in Table 2.

A diverse bacterial endophyte community was isolated and phenotypically characterized from wild pigeonpea (*Cajanus scaraboides*) leaf and stem tissues, revealing insights into their ecological roles. The viability and successful recovery of these endophytes were confirmed by high colonization frequencies and bacterial colony formation within 48-72 hours, indicating an effective isolation protocol.

Twenty-five endophytes were isolated from 48 tissue segments of *Cajanus scaraboides*, with a higher isolation frequency from stems (16 isolates) compared to leaves (9 isolates). Colonization frequencies, ranging from 58.33% to 87.50%, indicated a significant presence of endophytic bacteria. Notably, stem segments exhibited a higher colonization frequency (87.50%), suggesting a tissue-specific distribution of endophytes. This higher bacterial colonization in stems aligns with previous studies indicating stems as favorable microbial habitats (Rai et al., 2007; Yang et al., 2011). The vascular system within stems may facilitate endophyte movement and colonization, contributing to this increased presence (McCully, 2001), consistent with our findings. Recent studies on tea and sweet potato also reported higher bacterial diversity in stems compared to leaves, potentially due to the more stable internal environment of stems (Lin et al., 2022; Wang et al., 2023; Kandel et al., 2017). However, significant colonization was also observed in leaf tissues, highlighting both stems and leaves as important habitats for endophytic bacterial communities in *C. scaraboides*.

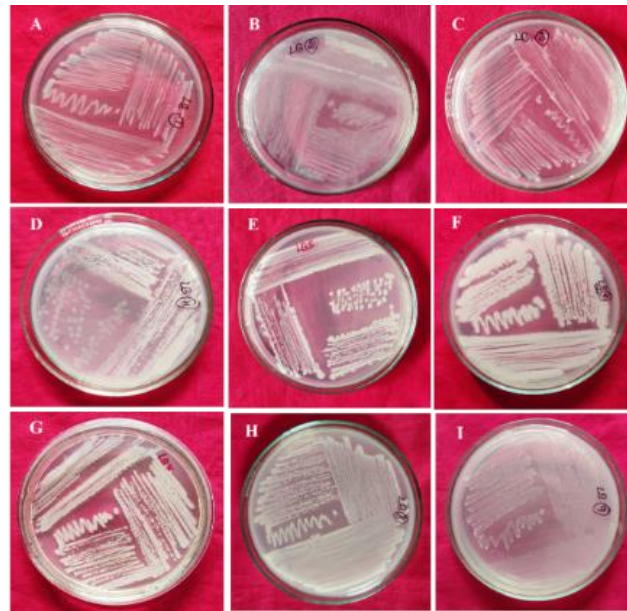


Fig. 1. Leaf bacterial endophytes: A-LB1, B-LB2, C-LB3, D-LB4, E-LB5, F-LB6, G-LB7, H-LB8, I-LB9

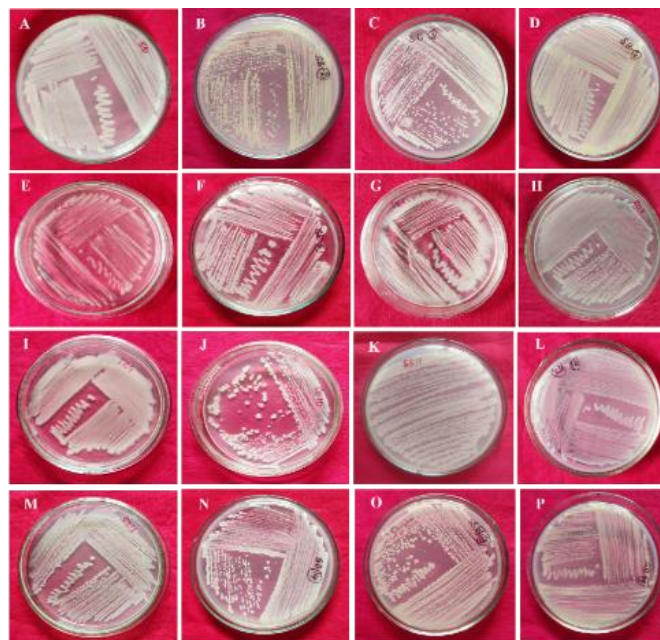


Fig. 2. Stem bacterial endophytes: A-SB1, B-SB2, C-SB3, D-SB4, E-SB5, F-SB6, G-SB7, H-SB8, I-SB9, J-SB10, K-SB11, L-SB12, M-SB13, N-SB14, O-SB15, P-SB16

Table 1. Endophytic colonization frequency from diverse tissues of pigeonpea

Plant part	Number of segments placed on media	Number of segments colonized by endophytes	Number of endophytes emerged	Colonization frequency (%)
Leaf	24	14	9	58.33
Stem	24	21	16	87.50

3.2 Endophyte Biofertilization Potential: A Qualitative Assessment

The plant growth-promoting traits of 25 bacterial endophytes were assessed, focusing on siderophore production, nitrogen fixation, and the solubilization of phosphate, potassium, and zinc. Qualitative analysis revealed universal phosphate solubilization, evidenced by clear zone formation. However, only nine isolates (LB5, LB7, LB8, SB1, SB3, SB4, SB5, SB8, and SB13) displayed positive results for all tested parameters. These isolates demonstrated clear zones for mineral solubilization, orange zones for siderophore production, and bacterial growth on nitrogen-free media, indicating nitrogen fixation. The variability in these traits among the isolates is summarized in a binary category plot (Fig. 3).

The consistent phosphate solubilization observed across all isolates suggests a potential role in enhancing soil phosphorus availability, a crucial

factor for plant growth (Singh et al., 2020). This observation aligns with previous reports demonstrating improved phosphorus acquisition following endophytic bacterial inoculation (Chauhan et al., 2013; Lovecka et al., 2023). While phosphate solubilization was universal, potassium and zinc solubilization was selective. These micronutrients are essential for plant physiological processes, including enzyme activation and protein synthesis (Ehsanullah et al., 2015; Perelman et al., 2022). The nine isolates capable of solubilizing phosphate, potassium, and zinc display a broad spectrum of nutrient-mobilizing properties, as previously reported in rice, maize, and potato (Walitang et al., 2017; Marag et al., 2018; Singh et al., 2022). This suggests their potential efficacy in biofertilization, enhancing nutrient uptake and plant health. Furthermore, siderophore production by some isolates indicates their ability to improve iron availability in crop systems (Han-Song et al., 2011), thereby supporting plant growth in iron-deficient environments.

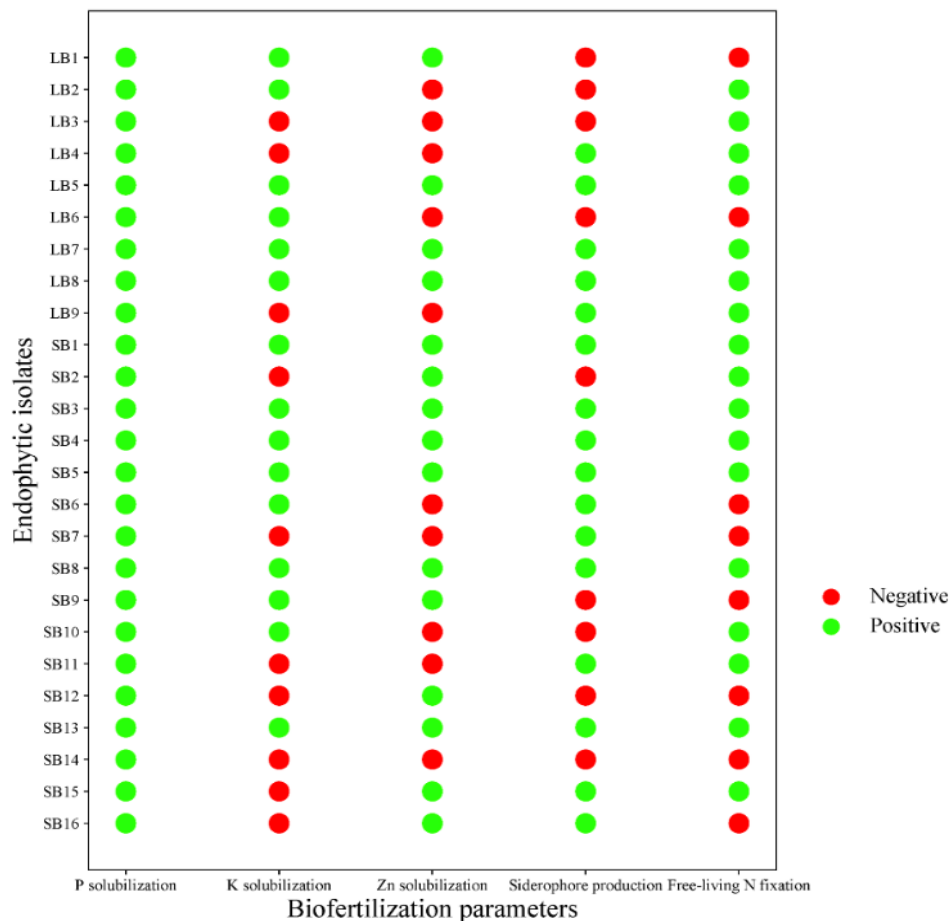


Fig. 3. Binary category plot depicting endophyte biofertilization activities

Table 2. Morphological traits of bacterial endophytes of pigoenpea

SL.No.	Isolate	Colony characteristics					Gram reaction	Cell shape
		Color	Form	Elevation	Margin	Surface		
1.	LB1	Creamy white	Circular	Flat	Entire	Smooth	Gram -ve	Cocci
2.	LB2	Creamy White	Circular	Flat	Entire	Smooth	Gram +ve	Rod
3.	LB3	White	Circular	Flat	Entire	Smooth	Gram -ve	Cocci
4.	LB4	Creamy White	Circular	Flat	Entire	Smooth	Gram -ve	Coccobacilli
5.	LB5	Pale Yellow	Circular	Raised	Entire	Smooth	Gram +ve	Rod
6.	LB6	Creamy White	Circular	Convex	Entire	Smooth	Gram -ve	Coccobacilli
7.	LB7	Creamy White	Circular	Raised	Entire	Smooth	Gram +ve	Coccobacilli
8.	LB8	Pale Yellow	Circular	Flat	Entire	Smooth	Gram +ve	Rod
9.	LB9	White	Circular	Flat	Entire	Smooth	Gram -ve	Cocci
10.	SB1	White	Circular	Raised	Entire	Smooth	Gram +ve	Rod
11.	SB2	Pale Yellow	Circular	Convex	Entire	Smooth	Gram +ve	Rod
12.	SB3	White	Circular	Convex	Entire	Smooth	Gram +ve	Rod
13.	SB4	Pale Yellow	Circular	Raised	Entire	Smooth	Gram +ve	Coccobacilli
14.	SB5	White	Circular	Convex	Entire	Rough	Gram -ve	Cocci
15.	SB6	Creamy White	Irregular	Raised	Undulate	Smooth	Gram +ve	Rod
16.	SB7	White	Irregular	Raised	Undulate	Smooth	Gram -ve	Cocci
17.	SB8	Creamy White	Circular	Raised	Entire	Smooth	Gram -ve	Cocci
18.	SB9	White	Circular	Raised	Entire	Smooth	Gram -ve	Cocci
19.	SB10	Pale Yellow	Circular	Flat	Entire	Smooth	Gram +ve	Coccobacilli
20.	SB11	White	Irregular	Raised	Entire	Smooth	Gram +ve	Rod
21.	SB12	Creamy White	Irregular	Raised	Undulate	Smooth	Gram -ve	Coccobacilli
22.	SB13	Pale Yellow	Circular	Raised	Entire	Rough	Gram +ve	Rod
23.	SB14	Creamy White	Circular	Flat	Undulate	Smooth	Gram -ve	Cocci
24.	SB15	Pale Yellow	Circular	Flat	Entire	Smooth	Gram +ve	Coccobacilli
25.	SB16	Pale Yellow	Circular	Flat	Entire	Smooth	Gram +ve	Rod

The observed universal phosphate solubilization suggests that these bacterial endophytes can effectively increase soil phosphorus availability, a critical factor for plant growth. These microorganisms can convert unavailable elements into plant-accessible forms through root exudates, thereby enhancing plant growth. The nine isolates (LB5, LB7, LB8, SB1, SB3, SB4, SB5, SB8, and SB13) demonstrating phosphate, potassium, and zinc solubilization, along with siderophore production, are particularly promising candidates for biofertilizer development. Their multifunctional properties enable simultaneous mitigation of multiple nutrient limitations, leading to enhanced plant growth.

3.3 Biostimulant Activity of Selected Endophytes

Nine endophytes, previously identified for their biofertilizer activity, were evaluated for in planta growth promotion. Seed priming with these endophytes resulted in enhanced germination rates compared to the control (62.22%). Notably, seeds treated with SB3 exhibited the highest germination rate (91.11%), followed by SB13 (86.66%), with a significant difference observed between these two treatments (Table 3). Consistent with germination results, significant improvements were also observed in plant height, branching, leaf number, and dry matter accumulation in endophyte-treated plants. Specifically, SB3-treated plants showed significant increases in plant height (83.21 cm),

branch number (15.66), leaf number (49.66), and dry matter accumulation (139.84 g/plant) compared to the control (69.26 cm, 7.33, 30.33, and 97.78 g/plant, respectively). These results highlight the potential of SB3 as an effective biostimulant.

In planta growth promotion analysis demonstrated the potential of these endophytes to enhance plant growth and development through various mechanisms. The increased germination percentages observed in endophyte-treated seeds, compared to the control, suggest a positive impact on seed viability and early growth stages (Verma et al., 2019). This aligns with prior research on seed endophytic *Bacillus*, which accelerated quinoa germination and indicated improved seed health (Pitzschke, 2016). The significant increase in germination rates, particularly with the SB3 isolate (91.11%), suggests the production of growth-promoting substances, such as phytohormones, which likely contribute to enhanced seed germination and vigor. For example, indole acetic acid (IAA) produced by *Bacillus amyloliquefaciens* has been shown to enhance seed germination and seedling vigor in capsicum (Gowtham et al., 2018). Furthermore, gibberellin biosynthesis intermediates, which are transported between cells to produce active gibberellins, play a crucial role in seed germination by activating the embryo, weakening the endosperm, mobilizing food reserves, and counteracting abscisic acid (Kucera et al., 2005).

Table 3. Biostimulatory effects of endophytes on pigeonpea growth

Treatments	Isolates	Plant height (cm)	Number of branches	Number of leaves	Dry matter accumulation (g/plant)	Germination percent (%)
T ₁	LB5	75.54 ^e	12.33 ^c	44.33 ^c	132.01 ^c	82.22 ^{bc}
T ₂	LB7	74.61 ^f	12.00 ^{cd}	41.33 ^d	128.83 ^d	77.77 ^{cde}
T ₃	LB8	78.40 ^c	11.33 ^{cde}	39.33 ^e	125.03 ^e	68.88 ^{fg}
T ₄	SB1	75.93 ^{de}	10.33 ^{ef}	35.33 ^g	121.89 ^f	66.66 ^{fg}
T ₅	SB3	83.21 ^a	15.66 ^a	49.66 ^a	139.84 ^a	91.11 ^a
T ₆	SB4	76.32 ^d	9.33 ^f	33.66 ^h	112.80 ^h	80.00 ^{bcd}
T ₇	SB5	71.22 ^h	10.66 ^e	36.33 ^g	118.72 ^g	73.33 ^{def}
T ₈	SB8	73.45 ^g	11.00 ^{de}	37.66 ^f	124.85 ^e	71.11 ^{ef}
T ₉	SB13	80.89 ^b	13.66 ^b	46.66 ^b	136.77 ^b	86.66 ^{ab}
T ₁₀	control	69.26 ⁱ	7.33 ^g	30.33 ⁱ	97.78 ⁱ	62.22 ^g

Values are mean (\pm SE) (n=3) and values followed by the same letter in each column are not significantly different from each other as determined by DMRT (P= .05)

Analysis at 12 weeks post-sowing revealed that endophyte treatment not only enhanced germination but also significantly improved other growth parameters. Plants treated with the SB3 isolate exhibited the greatest increase in plant height compared to the control, suggesting enhanced cell elongation and division, and improved vertical growth. This could be attributed to the endophyte's ability to increase nutrient availability and potentially produce phytohormones (Sandhya et al., 2017). Furthermore, SB3-treated plants showed a notable increase in branch and leaf number, indicating stimulated lateral growth and foliage development, possibly through improved nutrient solubilization and availability (de Melo Pereira et al., 2012). Increased branching and leaf production are associated with enhanced photosynthetic capacity and resource acquisition, ultimately leading to greater biomass production.

The observed increase in dry matter accumulation in endophyte-treated plants indicates enhanced biomass production, a key indicator of overall plant health and productivity. This enhancement is likely attributed to improved nutrient availability, increased photosynthetic efficiency resulting from a larger leaf area, and the overall stimulation of plant metabolic processes by the endophytes (Rana et al., 2020, Li et al., 2021, Lin et al., 2022). These results highlight the potential of these endophytes, particularly SB3, as effective biostimulants for improving plant growth and productivity. Our findings corroborate previous reports on bacterial endophyte-mediated growth promotion (Kabir et al., 2023; Tspinana et al., 2024).

3.4 Quantification of Phytohormones Produced by Efficient Endophyte

The endophytic bacterium SB3, known for its biofertilizing and biostimulating properties, was analyzed using HPLC to quantify the production of key phytohormones: IAA, gibberellin (GA), cytokinin, and abscisic acid (ABA). The resulting data, visualized in a balloon plot (Fig. 4), demonstrates the levels of these hormones synthesized by SB3.

While some microorganisms can produce small amounts of auxins without L-tryptophan (TRP), *in vitro* studies consistently show a significant increase in auxin production when TRP is present ((Zahir et al., 2010; Kabir et al., 2023). This was confirmed in our study, where endophyte SB3 produced substantially higher

levels of IAA (2.83 to 8.28 µg/mL) in a medium supplemented with L-TRP compared to a standard medium. This highlights the importance of root exudates, which include TRP, in facilitating IAA synthesis by plant growth-promoting microorganisms under natural conditions (Mano and Nemoto, 2012). SB3 also demonstrated significant production of gibberellin GA3 (92.52 µg/mL), the most prevalent and biologically active form among gibberellins. These hormones are known to influence stem elongation, seed dormancy, leaf and fruit senescence, and flowering, often through interactions with other phytohormones (Yamaguchi, 2008; Gusmiaty et al., 2019). Furthermore, SB3 produced cytokinin (41.79 µg/mL), a level comparable to IAA production with TRP supplementation. Cytokinins play a vital role in cell division, tissue growth, and delaying leaf senescence, thereby enhancing plant productivity (Kiba and Sakakibara, 2010; Li et al., 2021). In contrast, SB3 produced a low concentration of abscisic acid (ABA, 1.38 µg/mL), aligning with its known growth-inhibitory effects. ABA acts antagonistically to auxins, gibberellins, and cytokinins, promoting stress responses and limiting growth under unfavorable conditions (Brookbank et al., 2021).

In summary, SB3's ability to synthesize a range of plant growth and stress-related phytohormones confirms its potential as a versatile biofertilizer and biostimulant, capable of enhancing plant performance across diverse environmental conditions.

3.5 Molecular Characterization of SB3 (Efficient Endophyte)

Molecular characterization of endophytic bacteria SB3 was performed to ascertain its genetic identity. Genomic DNA was extracted via alkaline lysis, and the 16S rRNA gene was amplified using PCR, producing a fragment of approximately 1500 base pairs (Fig. 5a). Subsequent sequencing and BLASTn analysis against the NCBI GenBank database facilitated the identification of SB3 as *Bacillus subtilis* with accession number PQ569343 (Fig. 5b).

Endophytic microorganisms within the *Bacillus* genus, notably *Bacillus subtilis*, are extensively studied for their plant growth-promoting and biological control attributes (Khan et al., 2018; Kushwaha et al., 2020). Endophytic *B. subtilis* enhances plant development through

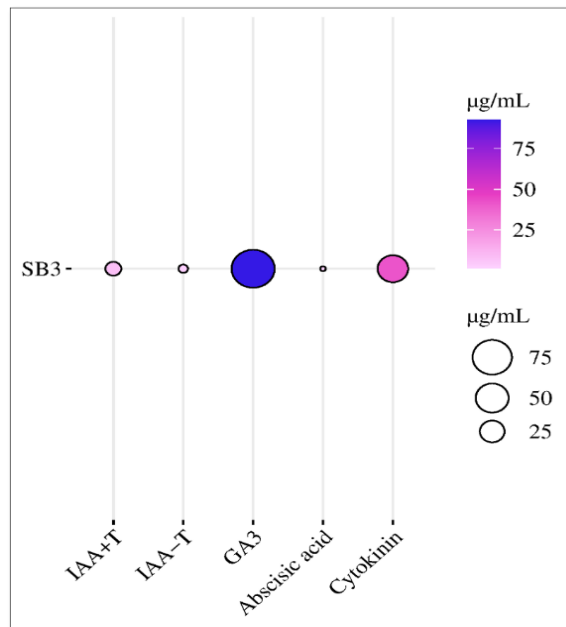


Fig. 4. Ballon plot displaying the phytohormone quantification in efficient endophyte SB3

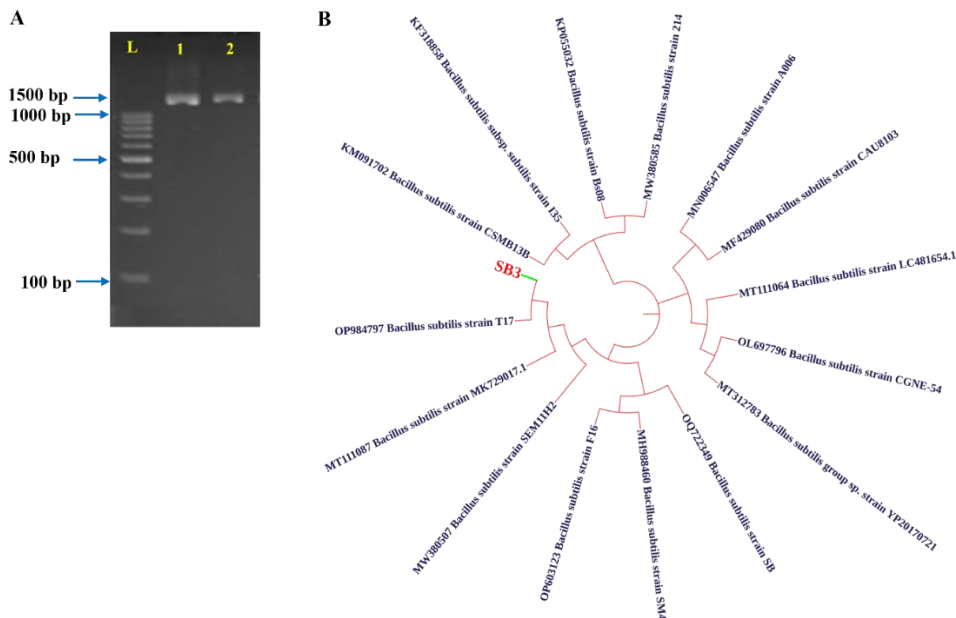


Fig. 5. Identification of efficient endophyte, SB3: A- Gel picture showing 1500 bp amplification (L: Ladder; 1 and 2: DNA samples), B- Phylogeny tree constructed by a neighbor-joining method

phytohormone synthesis and nutrient acquisition (Sorokan et al., 2021; Di et al., 2023) and confers biotic and abiotic stress resistance via secondary metabolite production (Lastochkina et al., 2020; Mageshwaran et al., 2022). The identification of isolate SB3 as *Bacillus subtilis* underscores its potential utility in agricultural biotechnology.

4. CONCLUSION

The study underscores the potential of wild pigeonpea derived endophytic bacteria as biofertilizers and biostimulants for enhancing the cultivated pigeonpea variety ICP 8863 growth. Thus establishes the significant plant growth-

promoting potential of endophytic bacteria from wild pigeonpea, particularly *Bacillus subtilis* isolate SB3 which serves as a valuable bioresource for sustainable agriculture. To fully capitalize on this potential, future research should prioritize: (1) large-scale field trials to validate efficacy across diverse agro-climatic conditions; (2) mechanistic studies to elucidate the underlying biostimulation and biofertilization processes; and (3) exploration of biocontrol applications against plant pathogens. These investigations will facilitate the development of sustainable agricultural practices through the utilization of these beneficial endophytes.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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