South African Journal of Botany 174 (2024) 768–778

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Plant growth-promoting potential of bacterial endophytes isolated from *Lessertia frutescens*

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ARTICLE INFO

Article History:
Received 17 May 2024
Revised 2 September 2024
Accepted 19 September 2024
Available online 3 October 2024

Edited by: Dr I. Risenga

Keywords:
Lessertia frutescens
Endophytes
Indole acetic acid
Plant growth promoting
Seedling growth

ABSTRACT

Many endophytic bacteria have plant growth promoting attributes that could improve agricultural yield as well as resistance to various types of stress and disease. Detection of such attributes usually involves *in vitro* screening and the subsequent testing of the isolates on the plants for specific or combined effects on germination, seedling growth and yield. Despite many studies in this field, there is a knowledge gap on the influence of endophytes on early growth of certain economically important plants such as medicinal plants. In the present study, we evaluated the *in vitro* capabilities of bacterial endophytes isolated from *Lessertia frutescens* (L.) Goldblatt & J.C.Manning (syn. *Sutherlandia frutescens* (L.) W.T.Aiton) for plant growth promotion and their effect on initial seedling growth. Using a culture-dependent approach, plant samples of *Lessertia frutescens* were screened for bacterial endophytes. The isolated bacterial endophytes were subsequently evaluated for their plant growth-promoting attributes along with their ability to produce hydrolytic enzymes. Molecular identification of selected endophytic bacteria based on the 16S rRNA genes of strains that tested negative in the hemolysis test was conducted. The two most promising isolates were evaluated for their ability to promote *Lessertia frutescens* seedling growth in a two-month pot trial study. The obtained results revealed that many of the bacterial endophytes had potential to promote plant growth. Specifically, 86 % of the endophytes possessed nitrogen-fixing, phosphate solubilizing, and IAA-producing abilities, while approximately 71 % were able to exhibit siderophore-producing capabilities. The endophytes exhibited significant production of essential hydrolytic enzymes, including amylase (86 %), gelatinase (86 %), protease (29 %), lipase (43 %), and D-nase (57 %). The two best isolates were identified as relatives of *Bacillus* spp. (*Bacillus licheniformis* BaDB06 and *Bacillus velezensis* strain SM-95). Their plant growth-promoting properties such as their ability to enhance plant height and their ability to be used as bio-agent were further confirmed in the pot trial study as they enhanced the growth of *Lessertia frutescens* seedlings compared to the control. This study provides insights into the functional roles of endophytic bacteria of *Lessertia frutescens* in seedling growth and their potential plant growth enhancement, highlighting their potential for sustainable agriculture and ecosystem management.
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1. Introduction

Exploring interactions between plants and microbes presents significant potential for sustainable agriculture, providing innovative approaches to enhance crop yield while minimizing dependence on chemical interventions (Sudheer et al., 2020). Among these microorganisms, endophytes inhabiting plant tissues have emerged as key contributors to enhancing plant growth and bolstering resilience against environmental pressures (Anukool Vaishnav et al., 2019). Endophytes have gained a massive commercial and economic interest due to their significant role in improving plant health and development (Cardoso et al., 2019). Endophytes and their host plants have no competition for space and essential substances such as nutrients (Ghasemnezhad et al., 2021). Instead, endophytes play a vital role in plant health through various processes, such as phytostimulation, biological nitrogen fixation, phosphate solubilisation, siderophores production as well as protection of host plants against stress and diseases.

Endophytes from several crops, including maize, soybean, wheat, and some vegetables, have been exploited for their potential to promote plant growth (Rana et al., 2020). However, research interests on endophytes from medicinal plants as microbial bio-stimulants are currently low. Endophytes from medicinal plants have been reported to exhibit antifungal, antiviral and antibacterial and other biological properties, similar to those of their host plants (Palanichamy et al.,

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https://doi.org/10.1016/j.sajb.2024.09.043
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2018). The use of endophytes from medicinal plants with antimicrobial traits could play a big role in protecting plants from biotic stress while promoting plant growth.

Leguminous medicinal plants such as *Lessertia frutescens* L. R. Br. (= *Sutherlandia frutescens* L.) is a shrub belonging to the Fabaceae family and is native to the southern Africa: Namibia, Lesotho, Botswana, Zimbabwe, and South Africa (Aboyade et al., 2014; Zonyane et al., 2019). Even though it is available in the coastal and inland parts of South Africa, *Lessertia frutescens* is highly distributed in the Western Cape and Karoo regions of South Africa (Street and Prinsloo, 2013). *Lessertia frutescens* holds significant medicinal and ecological importance. Identified bioactive compounds within the *L. frutescens* exhibit pharmacological activities including anti-inflammatory, antioxidant, immunomodulatory, and anticancer properties, among others. Extracts and formulations from *L. frutescens* are currently being investigated for their potential therapeutic benefits in conditions such as diabetes, HIV/AIDS, cancer and immune −related disorders (Mudau et al., 2022). The ability of *L. frutescens* to thrive in different habitats including disturbed habitats helps to stabilize ecosystems and aid in rehabilitation of degraded environments (Zonyane et al., 2020). Understanding and harnessing the dual significance of *L. frutescens* can pave the way for holistic approaches to health and environmental sustainability.

Lessertia frutescens harbours a variety of endophytic microbes with different essential functions. Endophytes associated with *L. frutescens* have been implicated in various applications, including antimicrobial abilities (Sishuba, 2022) and the production of bioactive compounds (King, 2021), among others. However, the full extent and significance of their contributions to plant growth and development have largely remained unexplored. Exploring endophytes from *L. frutescens* can be helpful in promoting the cultivation of this medicinal plant, which is highly valued in the pharmaceutical industry.

Understanding the diversity and dynamics of the endophytic bacterial community within *L. frutescens* holds significant potential for exploration and application in agriculture and medicine. It is critical to ascertain the degree to which essential microbes can influence the growth of their host plant and to track their impacts separately from those of other endophytes. Thus, this study aims to evaluate the ability of plant-growth promoting endophytes isolated from *Lessertia frutescens* medicinal plant as potential gateways to sustainable agriculture by evaluating their plant growth-promoting attributes along with their ability to produce hydrolytic enzymes and their ability to enhance *Lessertia frutescens* seedling growth.

2. Methods

2.1. Isolation of bacterial isolates from Lessertia frutescens

Lessertia frutescens medicinal plants (18 plants) were obtained from Eden nursery in Klerksdorp, North-West Province of South Africa in April 2021. *Lessertia frutescens* cultivation practices included seed sowing and the use of chemical fertilizer (fertilizer 2:3:2 (N: P: K)) as a source of nutrients. The plants were watered daily or as needed during their growth period. The plant samples were collected and transported on ice to the laboratory at North-West University Potchefstroom campus, where they were surface sterilized. The plants were immediately rinsed with tap water and separated into segments for effective surface sterilization. Surface sterilization was carried out as described by Palanichamy et al. (2018). Plant parts were submerged in 95 % ethanol for 30 s, 2.5 % sodium hypochlorite solution for 5 min, sodium hypochlorite was washed off by 70 % ethanol for 1 min, and finally washed three times with double distilled water to wash the substances off. To test the efficiency of surface sterilization, small portions of each plant part was plated on nutrient agar and incubated at 37 °C for 24–48 h. The absence of microbial growth indicated the success of the sterilization process. The plant segments (roots, stem and leaves) were air-dried in a sterile environment and later crushed using a pestle and mortar. For each sample, 1 g of crushed plant material was mixed with 9 ml of sterile distilled water and shaken. The suspension was serially diluted (10^0–10^{-6}) and 100 μL aliquots of 10^{-6} dilution were plated in nutrient agar and incubated at 37 °C for 24 h for bacterial endophytes isolation. The endophytes were then sub-cultured until pure cultures were obtained.

2.2. Test for pathogenicity of endophytes: haemolytic test

All the pure culture isolates of the bacterial endophytes were subjected to hemolysis test as described by (Amaria et al., 2023). Bacterial endophytes were spot inoculated on sheep blood agar. The agar plates were then incubated for 48 h at 30 °C. The change in colony color and/or appearance of clear zone around the colony of the bacterial endophytes on the blood agar indicated positive hemolytic reaction of the strain. A change in the color of the strain to dark or greenish grey indicated partial lysis of the blood cells, which is known as alpha hemolysis, while the appearance of a clear zone around the strain indicates a complete lysis of the blood cells and the reaction is known as beta hemolysis. The absence of color change or a clear zone around the colony indicates a negative hemolytic reaction of blood cells to the bacterial endophytes and the reaction is known as the gamma hemolysis. The endophytes that tested negative for hemolysis were identified molecularly and then tested for their plant growth promoting properties by testing their ability to fix biological nitrogen, solubilize insoluble phosphate, and produce indole acetic acid and siderophore as well as their ability to produce hydrolytic enzymes.

2.3. Molecular identification of bacterial endophytes from Lessertia frutescens plants

Endophytes that tested negative for haemolytic test were identified using SANGER sequencing as described by (Palanichamy et al., 2018) with modifications. Bacterial genomic DNA from each pure endophytic strain was extracted using Fungal/Bacterial DNA mini-Prep™ kit (Zymo Research Corporation, USA) following manufacturer's instructions. Polymerase chain reaction (PCR) was used to amplify the V3-V4 region of bacteria's 16S rRNA gene using universal forward primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). To achieve the final volume of 25 μL, the PCR reaction included 12.5 μL of Taq 2x Master Mix with Standard Buffer (New England, Biolabs Inc.), 1 μL of each primer (10 μM), 5 μL of bacterial DNA, and nuclease-free water to bring to 25 μL. Following that, the mixture was amplified for 35 cycles at the following settings: 95 °C for 5 min for initial denaturation, 95 °C for 30 s for denaturation, 52 °C for 30 s for annealing, 72 °C for 1 min for extension, and 72 °C for 1 min for final extension in a T100 (BioRad) thermal cycler. The size of the amplicons were confirmed by electrophoresis on a 1 % agarose gel in TBE buffer, and the gel was visualized on a Gel DOC (BioRad Laboratories) after 45 min of running at $\geq$ 80 V. The amplicons were sequenced using SANGER sequencing at Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa) and the sequences were compared with available sequences in the National Center for Biotechnology Information (NCBI) database by the Basic Local Alignment Search Tool (BLAST) program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4. Determination of plant growth promoting attributes of endophytic bacteria

2.4.1. Dynamics of nitrogen fixation by endophytic bacteria

Two approaches were utilized to determine the nitrogen-fixing capacity of bacterial endophytes isolated from *Lessertia frutescens*. The ability of bacterial endophytes to fix biological nitrogen was

determined as described by (Hamza et al., 2017) on a nitrogen-free media (Burk's media) containing magnesium sulphate (0.2 g), dipotassium phosphate (0.8 g), monopotassium phosphate (0.2 g), calcium sulphate (0.13 g), ferric chloride (0.00145 g), sodium molybdate (0.000253 g), sucrose (20 g) and bacteriological agar (15 g) in 1 L. The endophytes that tested negative for hemolysis were grown in this N-free medium and incubated at 30 °C for 7 days. Bacterial endophytes developed into strong, elevated globular-like structures, demonstrating the dynamic nature of nitrogen-fixing activity on nitrogen-free media. The *NifH* gene, which regulates the microbial biological nitrogen fixation, was identified molecularly, providing additional evidence of those endophytes' capacity to fix nitrogen. The *NifH* gene profiles from the endophytes that demonstrated nitrogen fixation were identified using PoL-F GC (5′- TGCGAYCCSAARGCBGACTC-3′) and Pol-R (5′- ATSGCCATCATYTCRCCGGA −3′) primer set as described by (Poly et al., 2001). To achieve the final volume of 25 μL, the PCR reaction included 12.5 μL of Taq 2x Master Mix with Standard Buffer (New England, Biolabs Inc.), 1 μL of each primer (10 μM), 5 μL of bacterial DNA, and nuclease-free water to bring to 25 μL. Following that, the mixture was amplified for 30 cycles at the following settings: 95 °C for 5 min for initial denaturation, 95 °C for 1 min for denaturation, 52 °C for 1 min for annealing, 72 °C for 1 min for extension, and 72 °C for 5 min for final extension in a T100 (BioRad) thermal cycler. The size of the amplicons were confirmed by electrophoresis on a 1 % agarose gel in TBE buffer, and the gel was visualized on a Gel DOC (BioRad Laboratories) after 45 min of running at ≥ 80 V.

2.4.2. Phosphate solubilisation

P-solubilization of bacterial endophytes were evaluated qualitatively on Pikovskaya agar medium (PVK) enriched with 0.5 % of insoluble tricalcium phosphate (TCP) $Ca_3(PO4)^2$ as indicated by (Pikovskaya, 1948). Pure isolates were inoculated on nutrient broth and incubated at 37 °C for 24 h. Three wells (with equal distance from between each and close to the centre) were then made on Pikovskaya's agar plates and 100 μl overnight cultures were transferred to each hole, and the plates were incubated at 30 °C for 7 days. The diameter of the well and halo were measured. The same bacterial isolate was added to each of the three wells, and this was performed twice (two plates) to provide six replicates, with each well representing one replicate. The agar plates were examined for formation of a clear zone around the colonies for their capacity to solubilize TCP and their solubilizing index (SI) values were calculated as follows:

$$SI = \frac{\text{Solubilisation diameter} + \text{Colony diameter}}{\text{Colony diameter}}$$

The average SI from the wells were then computed, and the results were utilized to generate a pie chart that compared each bacteria's phosphate solubilizing capacity to others.

2.4.3. Siderophore production

To determine the ability of the endophytes to produce siderophores, the Chrome Azural S (CAS) agar plate method was used, as described by Mukherjee and Dutta (2019). Blue agar solution was prepared by dissolving 60.5 mg of CAS in 50 ml of double distilled water and was mixed with 10 ml of iron (III) solution containing 1 mM $FeCl_3{\cdot}6H_2O$ in 10 mM HCl. This solution was autoclaved after adding 72.9 mg of hexadecyltrimethylammonium bromide (HDTMA) diluted in 40 mL of double distilled water. In a separate 1 L bottle, a mixture of 15 g of bacteriological agar, 100 ml (10 mM9 salts), 30.24 g of piperazine (PIPES), 12.0 g of a 50 % (w/w) NaOH solution to raise the pH of PIPES (6.8), and 25 % NaCl, all dissolved in 750 ml of double distilled water, was autoclaved. When the agar and blue solution reached 50 °C, the two solutions were mixed with stirring, and the agar was poured in agar plates and allowed to solidify before use. The endophytic microbes were then spot inoculated in blue agar and incubated for 7 days at 30 °C and this was replicated 6 times for each endophyte. The presence of a clear zone around the strain demonstrated the endophyte's ability to produce siderophores.

2.4.4. Indole acetic acid (IAA) production

Indole acetic acid production by endophytes was determined as described by Putrie et al. (2017). In 6 replicates, pure isolates were incubated in 10 mL nutrient broth supplemented with 1% of l-tryptophan for 48 h at 30 °C on a shaker at 200 rpm. Two ml of the microbial suspension was centrifuged at 10,000-rpm speed for 10 min at 4 °C. Following the transfer of the supernatant into a test tube, 2 ml of Salkowski's reagent (150 mL concentrated sulfuric acid (H_2SO_4), 250 mL distilled water, and 7.5 mL of 0.5 M $FeCl_3$) was added. The mixture was then incubated for 30 min at room temperature in the dark. The absorbance was measured using a spectrophotometer at a wavelength of 540 nm with V-1100 D Spectrophotometer (Labex (pty), LTD). The standard IAA calibration curve was set up by determining the prepared different concentrations of IAA solution (0, 5, 10, 20, 50 and 100 μg/mL) as described by (Sarker and Al-Rashid, 2013). Two ml of each standard prepared was transferred to separate tubes, and 2 ml of Salkowski's Reagent was added to each standard. They were incubated at room temperature in the dark for 30 min, and IAA concentrations were measured at 540 nm. The results were plotted, and the equation from the standard curve was used to calculate the concentration of IAA for the samples.

2.5. Determination of the hydrolytic activity of extracellular enzymes

The plate method was used to detect enzymatic activity qualitatively, in which solid agar media was supplied with the basic components and appropriate substrates for the enzymes examined in this study. Proteolytic activity of bacterial endophytes was measured using the method described by (Olajuyigbe and Ajele, 2005) and (Khan et al., 2017) on skim milk agar containing 3 % (w/v) skim milk (Oxoid, UK) and 35 g/L Mueller-Hinton agar (Merck, Germany). After incubation, colonies with a clear zone of skimmed milk hydrolysis suggested the endophyte's protease activity. Amylase activity was detected on Tryptone Soya Agar (Merck, Germany) supplemented with 1 % w/v soluble starch, and 3 g of beef extract. Following incubation, the plates were flooded with 1 % lugol solution, and the presence of a clear zone indicated endophyte amylase activity (Khan et al., 2017). Gelatinase activity was determined on agar plates containing Tryptone Soya Agar (Merck, Germany), supplemented with 3 % gelatine powder (Merck, Germany). The endophytic isolates were spot inoculated, and the development of a turbid or clear zone around the bacteria following incubation indicated endophytic gelatinase activity. Lipase activity of endophytes was determined on agar plates containing Tryptone Soya Agar (Merck, Germany), supplemented with 1 % of tween 80 (Sigma Aldrich, German). The endophytic isolates were also spot inoculated, and the development of a turbid or clear zone around the bacteria following incubation indicated endophytic lipase activity. The Dnase activity of the endophytes was determined using Dnase agar (Oxoid, UK) following the manufacturer's instructions. After incubation, the plates were flooded with 1 M hydrochloric acid (HCl) and the appearance of clear zones around the colony indicated the endophytes' Dnase activity. All the plates for enzyme determination were incubated at 37 °C for 24–72 h.

2.6. Pot experiment

The pot seedling trial was conducted in a growth chamber using two strains (*Bacillus licheniformis* BaDB06 and *Bacillus velezensis* SM-95) that showed all the plant growth promotion capacity and highest IAA production. The best IAA producing strains were chosen because IAA is known to stimulate seedling growth. *Lessertia frutescens* seeds were obtained from Seed for Africa company (Cape Town, South

Africa). The experiment consisted of 4 treatments replicated 6 times. The treatments were Treat-C (control=0 inoculum), Treat-A (*A* = *B. licheniformis* BaDB06 inoculum), Treat-B (*B* = *B. velezensis* SM-95 inoculum) and Treat-AB (AB = *B. licheniformis* BaDB06 + *B. velezensis* SM-95 inoculum). Six seeds for each replicate were incubated overnight in a nutrient broth containing the following: (1) nutrient broth only for the control (Treat-C), (2) *B. licheniformis* BaDB06 for Treat-A, (3) *B. velezensis* SM-95 for Treat-B, and (4) *B. licheniformis* BaDB06 + *B. velezensis* SM-95 for Treat-AB. The seeds were then planted in pots of the same size (20 cm in diameter) containing sterile coconut peat and they were placed in a growth chamber for the duration of the experiment. Temperatures at the growth chamber were kept at 30 °C during the day and 18 °C at night with constant 70 % humidity. All treatments were watered every other day with 100 ml of double distilled water and fertilized with 100 ml of Hoagland solution on a weekly basis for nutritional supplementation right after emergence. The seedling growth was recorded after two months.

2.7. Statistical analysis

The influence of *Bacillus licheniformis* BaDB06 and *Bacillus velezensis* SM-95 on *Lessertia frutescens* seedling growth was determined statistically. Obtained data were subjected to one-way analysis of variance (ANOVA) to determine the differences among different treatment groups. The normality assumption was checked based on the Shapiro–Wilk Test (α=0.05). Tests were set at 5 % significance level ($P < 0.05$). Post hoc tests, Fisher's Least significant difference (LSD) and Tukey Honest Significance Difference (HSD) were used for pair-wise comparisons.

3. Results

3.1. Hemolytic test results

A total of 25 bacterial endophytes were isolated from *Lessertia frutescens* plant parts, all together. Only seven endophytes tested negative for hemolysis, 10 of the isolates had a beta hemolytic reaction on blood cells and eight had an alpha haemolytic reaction on blood cells.

3.2. Plant growth promotion attributes

Table 1 summarizes plant growth-promoting activities of the *Lessertia frutescens* endophytic bacteria that tested negative for hemolysis. These endophytes from *Lessertia frutescens* exhibited a variety of activities, with each demonstrating at least one plant growth-promoting attribute. In total, 86 % fixed biological nitrogen, 86 % solubilized insoluble phosphate, 86 % produced IAA, and 71 % produced siderophores.

Fig. 1 demonstrates bacterial endophytes that amplified for *NifH* gene

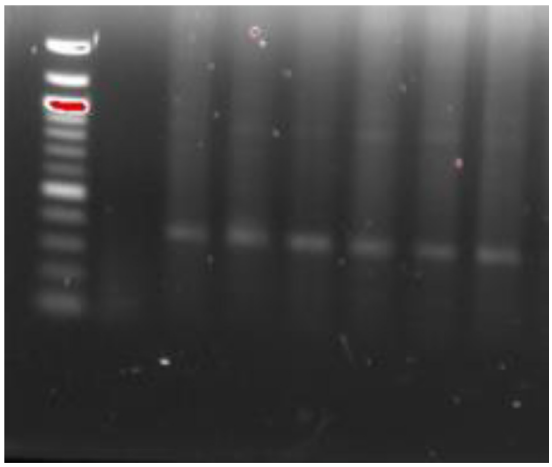

Fig. 1. *NifH* gene for endophytic bacteria isolated from *Lessertia frutescens*.

Fig. 2 exhibits the ability of each bacteria to solubilize phosphate in comparison to others. Each strain's percentage contribution to other strains has also been noted, with strains with high solubilizing index values having a higher percentage contribution. Six of the endophytes were capable of solubilizing insoluble tricalcium phosphate on agar plate with solubilizing index values between 1.29 and 1.36. In terms of the percentage contribution to the solubilization of insoluble phosphate, *Bacillus licheniformis* BaDB06, *Bacillus subtilis* LCDB-BP2, *Bacillus paralincheriformis* AJVR1, and *Kosakonia* sp. MBWS.8 were the equal phosphate solubilizers.

Fig. 3 exhibits the ability of each bacteria to produce siderophore in comparison to others.

The percentage contribution of each strain against other strains has also been noted, with the strains that had significant siderophore production having a high % contribution. Five of the endophytes were capable of producing siderophore with average siderophore production with halo zones ranging from 2.5 to 5.33 mm. *Bacillus subtilis* ZIM3 was the highest producer with 26 % contribution followed by *Bacillus licheniformis* BaDB06 at 24 % and the least producer was *Kosakonia* sp. MBWS2,8 at 12 % contribution.

Fig. 4 exhibits the ability of each bacteria to produce indole acetic acid in comparison to others. The percentage contribution of each strain against other strains has also been noted, with strains with high indole acetic acid synthesis having a high % contribution. Six of the endophytes demonstrated their ability to produce IAA with concentrations ranging from 0.15 to 1.60 μg/mL. *Bacillus velezensis* SM-95 was the highest IAA producer (1.60 μg/mL) with 49 % total contribution and followed by *Bacillus licheniformis* BaDB06 (0.64 μg/mL) with 20% total contribution while *Kosakonia* sp. MBWS.8 was the least IAA producer (0.15 μg/mL) with only 5 % contribution.

3.3. Hydrolytic enzyme activity

Table 2 illustrates hydrolytic enzyme activities of some *Lessertia frutescens* endophytic bacteria. Endophytes from *Lessertia frutescens*

Table 1
Plant growth-promoting activities of some isolated *Lessertia frutescens* endophytes.

Sample ID	Plant Part	Phylogenetic affiliation with similarity (16S rDNA sequencing)	Genebank Accession number	Similarity (%)	BNF	PS	SD	IAA
CBL1	Leaf	*Bacillus subtilis* LCDB-BP2	OP703608.1	97	+	+	–	+
CBL7	Leaf	*Bacillus licheniformis* BaDB06	JX237842.1	99	+	+	+	+
CBR4	Root	*Bacillus licheniformis* QT445	MT065669.1	97	–	+	–	+
CBR6	Root	*Kosakonia* sp. MBWS2.(8)	OP990041.1	99	+	+	+	+
CBR7	Root	*Bacillus velezensis* SM-95	MT377909.1	100	+	+	+	+
CBR11	Root	*Bacillus paralicheniformis* AJVR1	MT459810.1	97	+	+	+	+
CBR16	Root	*Bacillus subtilis* ZIM3	MT539995.1	99	+	–	+	–
Total					6/7 (86%)	6/7 (86%)	5/7 (71%)	6/7 (86%)

BNF=Biological Nitrogen Fixation, PS=Phosphate Solubilisation, SD=Siderophore, IAA=Indole Acetic Acid, AM=Amylase, PT=Protease, GT=Gelatinase, LP=Lipase and DN=*D*-nase.

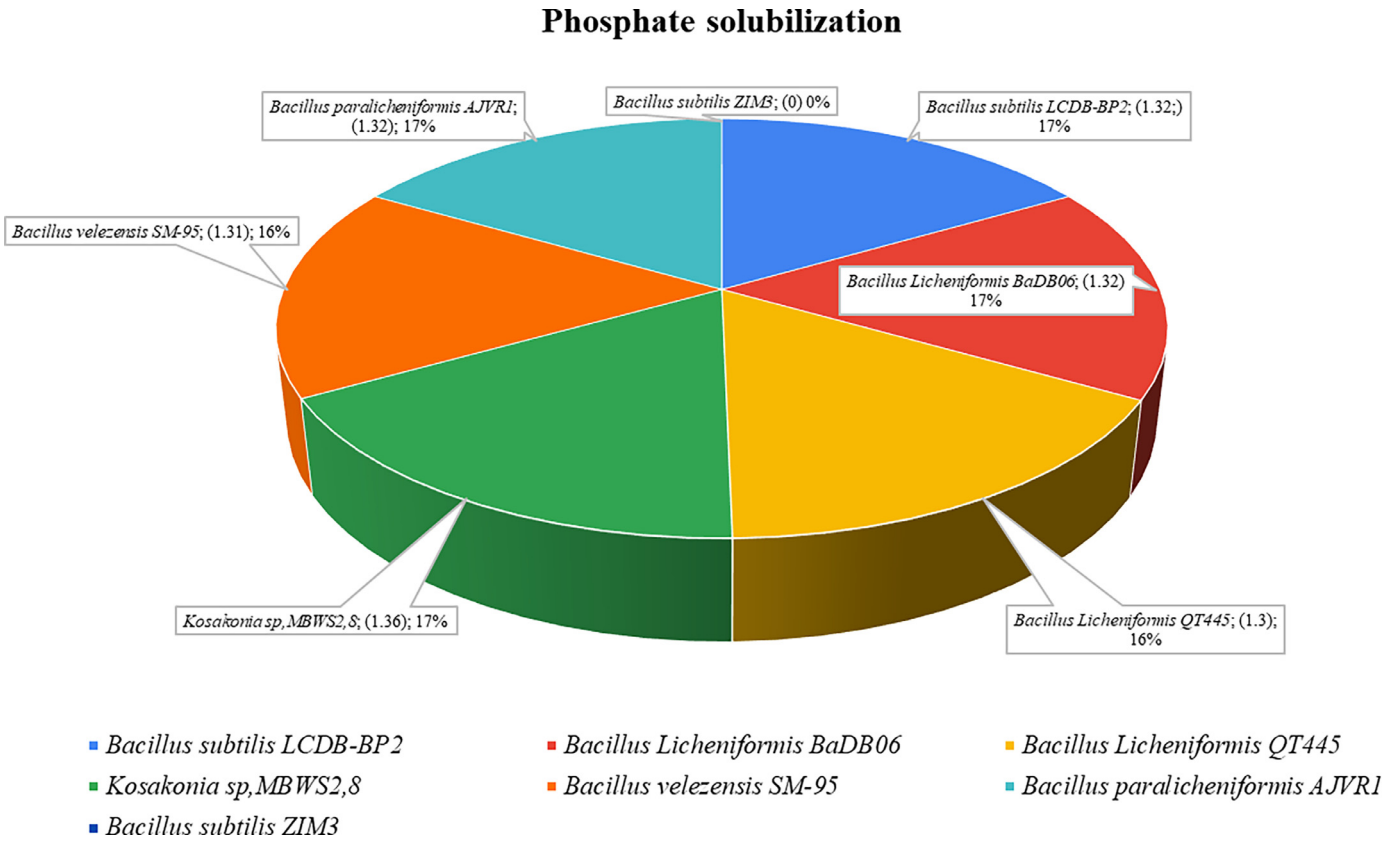

Fig. 2. Solubilization of tri-calcium phosphate by bacterial endophytes of *Lessertia frutescens*.

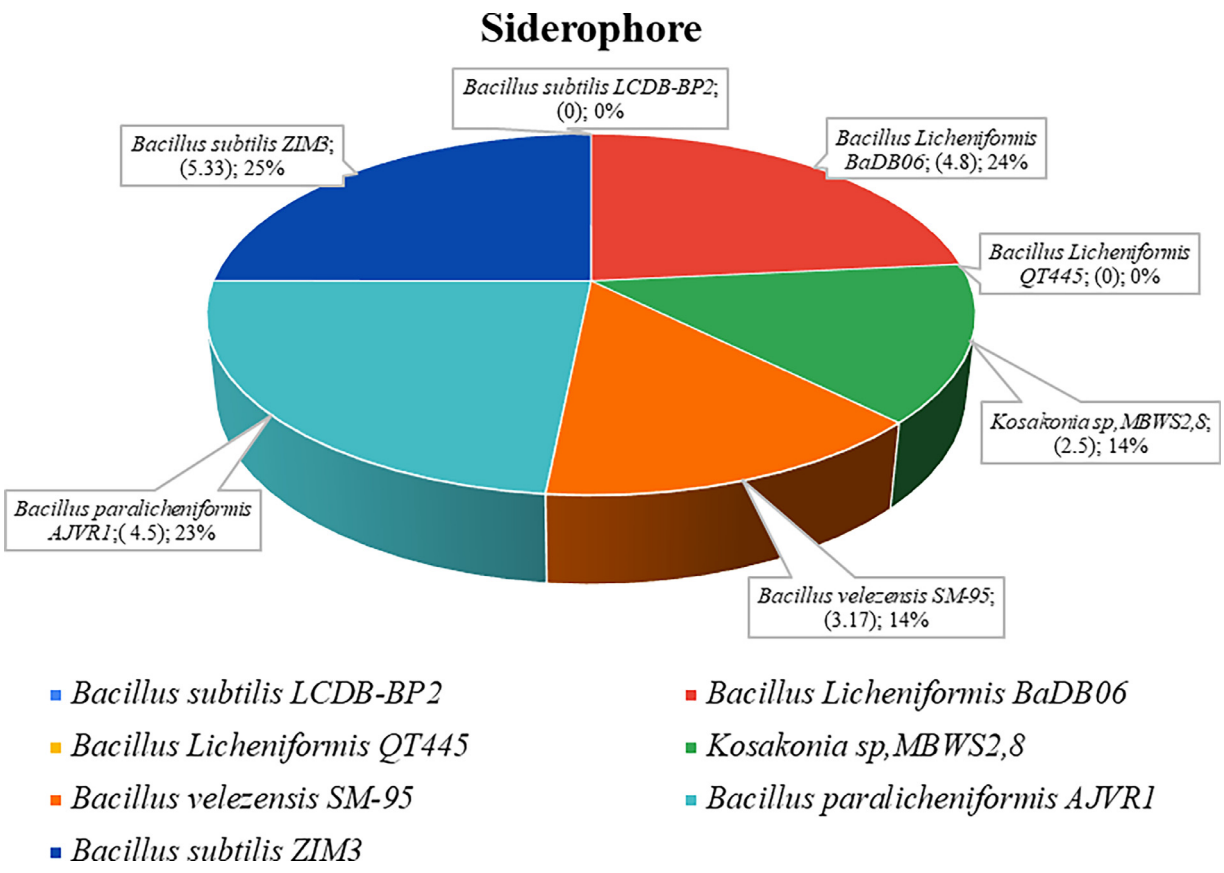

Fig. 3. Siderophore production by endophytes isolated from *Lessertia frutescens*.

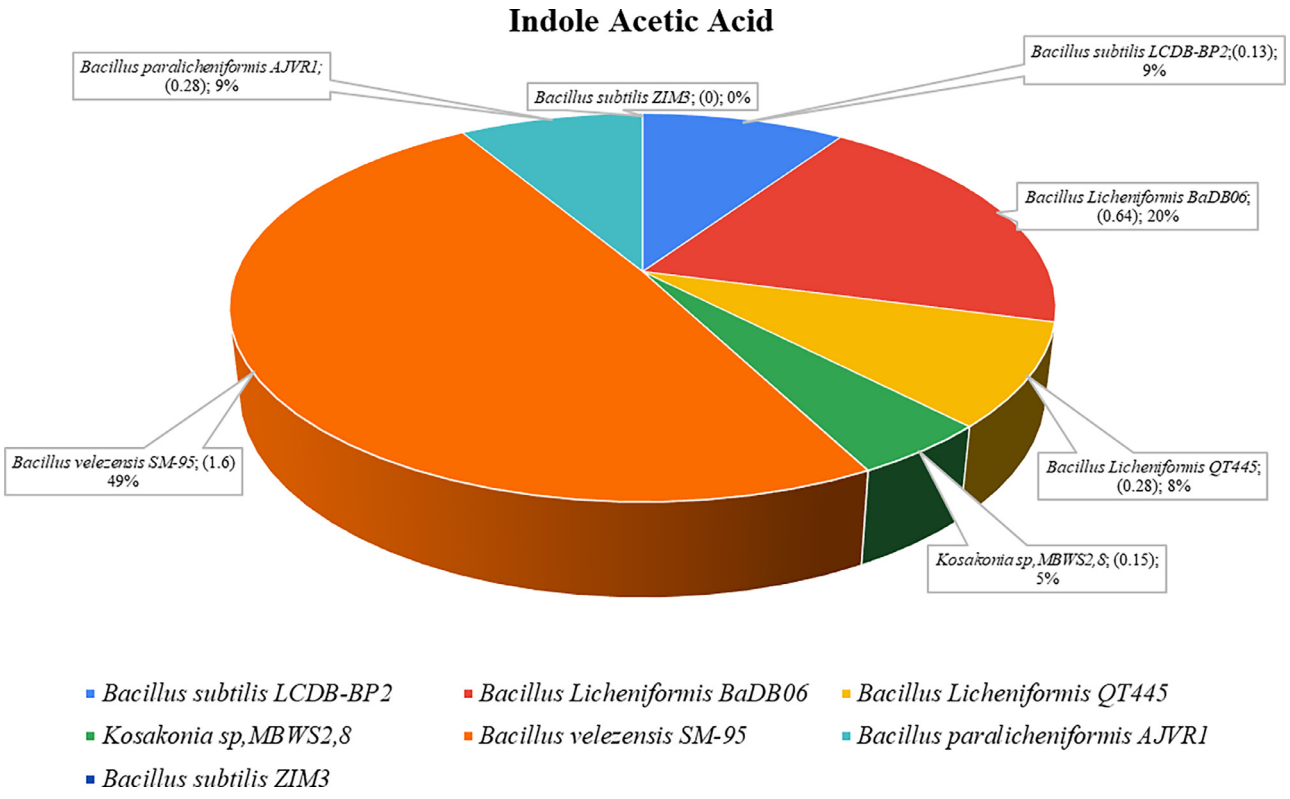

Fig. 4. Indole acetic acid production by isolated endophytes from *Lessertia frutescens*.

demonstrated ability to produce essential hydrolytic enzymes (amylase (86 %), gelatinase (86 %), protease (29 %), lipase (43 %), and D-nase (57 %). CBL7, CBR7, CBR4, and CBR11 were the only strains that showed favourable results for all of the plant growth-promoting properties investigated (IAA, siderophore, phosphate solubilization, and N-fixation). CBL1 (amylase, protease, gelatinase, and lipase), CBR4 (amylase, gelatinase, lipase, and D-nase), and CBR16 (amylase, protease, gelatinase, and D-nase) were the dominant strains in hydrolytic enzyme activity, producing four of the five enzymes tested. CBL7 (amylase and gelatinase) and CBR11 (lipase and gelatinase) were the least enzyme-producing strains, producing only two out of five enzymes.

Fig. 5 illustrates the hydrolytic enzyme activity of endophyte isolated from *Lessertia frutescens*. With a zone of hydrolytic activity ranging from 2 to 15 mm, gelatinase was the most abundantly produced enzyme. Six of the endophytes also produced amylase, which had a hydrolytic activity ranging from 1 to 7 mm. Protease and lipase were the least produced enzymes. CBR16 was a notable producer of these enzymes with the capacity of producing, gelatinase, amylase, protease and D-nase, followed by CBL1, which produced gelatinase, amylase, protease, lipase, and CBR4 which gelatinase, lipase, D-nase and amylase. The strains that produced the fewest enzymes were CR11, which could only create protease and gelatinase, while CBR6 could only make gelatinase and D-nase as well as CBL7 that produced gelatinase and amylase but had a better hydrolytic activity.

3.4. Lessertia frutescens *seedling growth*

Endophytic bacteria significantly affected *Lessertia frutescens* seedling growth after two months (Figs. 6, and 7). Compared to the control (without inoculum) with the lowest seedling height, observable leaf loss and yellowing, the combined treatment (Treat-AB) resulted in the highest seedling growth (Fig. 6). Similarly, the individual treatment with *B. licheniformis* BaDB06 and *B. velezensis* SM-95 significantly promoted seedling height.

4. Discussion

Besides offering raw materials and diverse bioactive compounds which are vital in agriculture and pharmaceuticals, medicinal plants host a variety of bacterial endophytes with diverse potentials including plant growth promotion (Guerriero et al., 2018). The current

Table 2
Hydrolytic enzyme activities of *Lessertia frutescens* endophytes.

Sample ID	Plant Part	Phylogenetic affiliation with similarity (16S rDNA sequencing)	AM	PT	GT	LP	DN
CBL1	Leaf	*Bacillus subtilis* LCDB-BP2	+	+	+	+	–
CBL7	Leaf	*Bacillus licheniformis* BaDB06	+	–	+	–	–
CBR4	Root	*Bacillus licheniformis* strain QT445	+	–	+	+	+
CBR6	Root	*Kosakonia* sp. strain MBWS2.(8)	+	–	–	–	+
CBR7	Root	*Bacillus velezensis* strain SM-95	+	–	+	–	+
CBR11	Root	*Bacillus paralicheniformis* strain AJVR1	–	–	+	+	–
CBR16	Root	*Bacillus subtilis* strain ZIM3	+	+	+	–	+
Total			6/7(86 %)	2/7 (29 %)	6/7 (86 %)	3/7(43 %)	4/7(57 %)

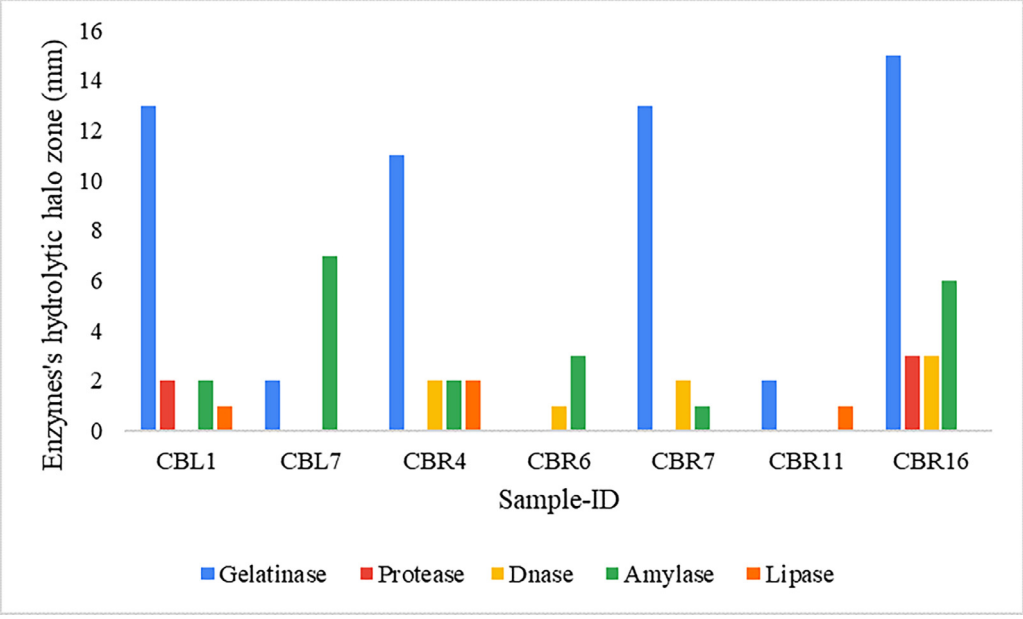

Fig. 5. Hydrolytic enzyme activity of endophytes from *Lessertia frutescens*.

investigation thus sought to isolate endophytes from the medicinal leguminous plant *Lessertia frutescens* and evaluate their capacity to promote plant growth. As indicated in Section 3.1, only seven endophytes showed no harm to blood cells and the majority of them belonged to *Bacillus*. This presents a significant advantage in agriculture as such organisms are suggested to be non-pathogenic. From the present study, as indicated in Table 1, a greater number of isolates were obtained from the roots compared to other plant components such as leaves, while none of the isolates from stems exhibited negative hemolysis. The higher abundance of endophytes in roots relative to other plant parts can be attributed to their natural affinity for soil habitats, with colonization predominantly occurring through the root system (Golinska et al., 2015). Limited bacterial entry into the xylem (Afzal et al., 2019; Arora and Ramawat, 2017) results in fewer bacterial endophytes being isolated from non-root tissues compared to roots. The study predominantly identified *Bacillus* species, with only one strain being non-*Bacillus*. *Bacillus* species are potential bio-fertilizer candidates because their spores allow for simple colonisation of either a seed or roots and they have a good survival rate in various soil types and conditions (Lopes et al., 2018).

Nutrients such as nitrogen and phosphorus, are primary macronutrients with high demand from plants, significantly impacting crop outcomes by determining success or failure. Understanding different avenues utilized by endophytes to stimulate plant growth is critical, especially when selecting the optimal strains for bio-fertilizer production. Endophytes play a crucial role in N acquisition and exchange. For instance, free-living and symbiotic endophytes can fix biological nitrogen by converting atmospheric nitrogen into soluble form, which is accessible to plants (Ek-Ramos et al., 2019; Hassan, 2017). It is believed that endophytes use carbon assimilates produced by plants as an energy source to transform insoluble nitrogen into soluble ammonia which plants can utilise (de Vilhena Araújo et al., 2020). It is not surprising that more than 80 % of endophytes isolated from *Lessertia frutescens* were able to fix nitrogen as *Lessertia frutescens* is a leguminous plant. Symbiotic nitrogen-fixing bacteria, known as alpha and beta rhizobia, perform BNF with legume species, enhancing ecosystem sustainability (Bomfeti et al., 2011). There is a tight relationship between *Nif* gene notation and nitrogen-fixing activity (Hurek et al., 2002). As shown in Fig. 1, all six endophytic bacteria that tested positive for nitrogen fixation possessed the *NifH* gene. *NifH* gene

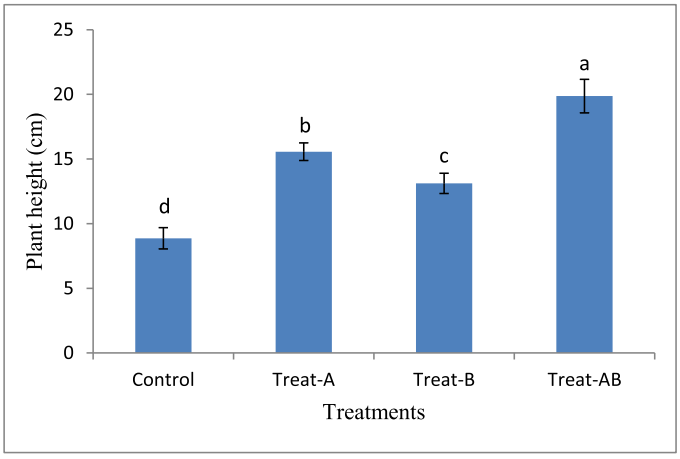

Fig. 6. Effect of bacterial endophytes on *Lessertia frutescens* seedling growth after 2 months. Error bars represent standard deviation ($n = 6$). Different letters on the upper portion of each bar indicates significant differences between treatments after 2 months ($P < 0.05$). *A* = *B. licheniformis* BaDB06, *B* = *B. velezensis* SM-95 and AB = *B. licheniformis* BaDB06 + *B. velezensis* SM-95.

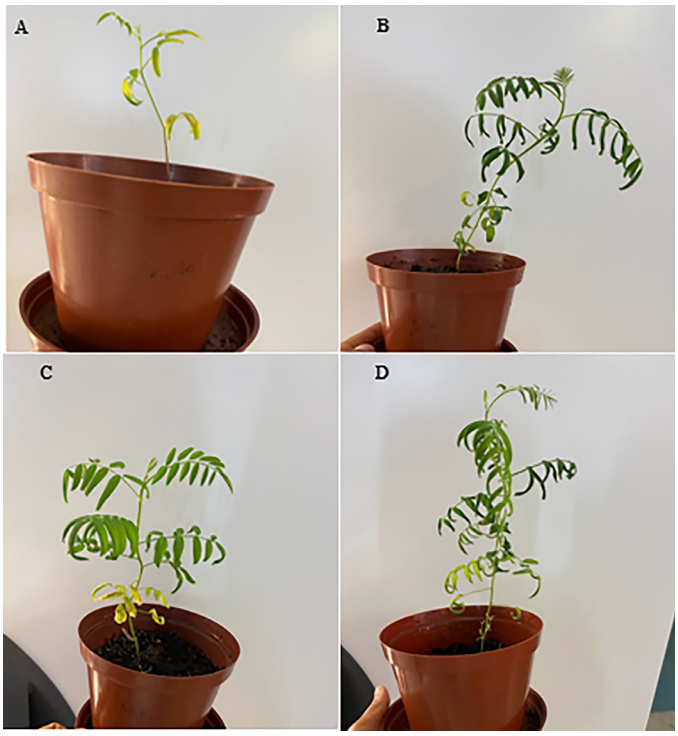

Fig. 7. Seedling growth of *Lessertia frutescens* in a pot experiment after two months, (**A**) Control (treatment without an inoculum), (**B**) Treat- A (treatment with *B. licheniformis* BaDB06 inoculum), (**C**) Treat- B (treatment with *B. velezensis* SM-95 inoculum) and (**D**) Treat- AB (treatment with *B. licheniformis* BaDB06 + *B. velezensis* SM-95 inoculum).

fragments have been effectively used to identify diazotrophic bacteria (n-fixing microorganisms) in the environment using the polymerase chain reaction (PCR) technique and the *NifH* gene's presence aids in clarifying the relationship between the associated n-fixing endophytes and their host (Terakado-Tonooka et al., 2013). *NifH* genes plays a vital role in plant nitrogen regulation. For example, biological N-fixation uses the nitrogenase enzyme to catalytically convert atmospheric nitrogen to ammonia (Terakado-Tonooka et al., 2013). Whereas, *NifH* encodes the Fe protein (dinitrogenase reductase) in nitrogen-fixing bacteria and controls the production of nitrogenase synthesis (Thaweenut et al., 2011). Plant development is mostly restricted by N, which is usually supplemented with chemical fertilizers, which are hazardous to the environment (Defez et al., 2017). Endophytes from *Lessertia frutescens* have the potential to be a better supply of nitrogen, reducing reliance on chemical fertilizers. Additionally, maximizing high crop yield without damaging the environment while conserving biodiversity and ecosystems is extremely important (Defez et al., 2017). The plant-endophyte interaction has been shown to be one of the most sustainable strategies for improving crop production (Sher et al., 2024).

Bacillus species, especially *B. subtilis*, not only fix nitrogen but also promote nodulation by other bacteria, facilitating symbiotic microbe colonization (Blake et al., 2021). Endophytes also employ processes like acidification and chelation to aid in P exchange by releasing immobile insoluble phosphate and make this available for plant use (Hassan, 2017; Jayakumar et al., 2020). About 86 % of the endophytes were found to solubilize insoluble tricalcium phosphate (TCP) on agar plate with solubilizing indices ranging from 1.29 to 1.36 as shown in Fig. 2. The disappearance of TCP indicates the high potential of those endophytes to solubilize inorganic bound phosphate (TCP) (Bekkar and Zaim, 2023). In addition, formation of halo zones around the colonies are in response to decrease in pH due do organic acid production, exchange reaction and ion chelation (Bekkar and Zaim, 2023). Endophytes have been suggested to solubilize different forms of insoluble phosphate differently, for instance, they solubilise inorganic phosphate through lowering the pH of the soil, by competing with P for absorption sites in the soil and through chelation reaction of cations bound to P (Ingle and Padole, 2017; Sharma et al., 2013). The hydroxyl and carboxyl group of acids form chelates with cations (Al, Fe and Ca) (Khan et al., 2009). The PSM results in chelation reactions, and this leads to the release of bound P in the soil (Ingle and Padole, 2017). Endophytic microbes produce certain enzymes such as phytase and phosphatase to mineralise the P-bound making it available for plant usage (Ghosh et al., 2021; Ingle and Padole, 2017).

Endophytes can also improve plant growth through production of siderophores. As shown in Table 1, 71 % of endophytes isolated from *Lessertia frutescens* were able to produce siderophores. Even though iron is only needed in minute amounts, it is essential for plant growth and it plays a part in enzymatic activities that are necessary for physiological processes (Eid et al., 2021; Ghosh et al., 2021). Iron exists in two oxidation states in the environment: ferric (Fe^{3+}), which is insoluble, and soluble ferrous (Fe^{2+}), which plants can only use (Ghosh et al., 2020). The insoluble ferric (Fe^{3+}) form exists as either oxides, hydroxides, carbonates and phosphates and it is not available for plant use (Eid et al., 2021). Iron deficiency in plants leads to leaf chlorosis and reduced metabolic activities (Chowdappa et al., 2020). Additionally, siderophore-producing endophytes can also act as biocontrol of phytopathogens by chelating iron and liming its availability for uptake by pathogens (Burragoni and Jeon, 2021; Ek-Ramos et al., 2019). For instance, when the hydroxamate iron binding is utilized, two oxygen molecules from the hydroxamate group operate as chelating agents, creating hexadentate octahedral with Fe3+ and rendering iron inaccessible to pathogens, leading to their ultimate death (Chowdappa et al., 2020). Siderophores degrade pathogen cell walls, which aids in the prevention of sporulation (Mohamad et al., 2022). Thus, siderophores offer dual benefits to plants as they shield plants from pathogens and also help them with iron acquisition and absorption (Iqbal et al., 2021). An investigation by Myo et al. (2019) reported that bacterial endophytes, *B. velezensis* produced siderophoes and IAA significantly improved tomato growth.

Endophytes also promote plant development through biosynthesis and regulation of phytohormones (Mercado-Blanco and Lugtenberg, 2014). As shown in Table 1, about 86 % of endophytes were able to produce IAA with concentrations ranging from 0.15 to 1.60 μg/mL. *Bacillus velezensis* SM-95 as the highest IAA producer (1.60 μg/mL) has also been triggering systemic resistance in plants besides production of auxins (Wang et al., 2022). The second best performing isolate, *Bacillus licheniformis* BaDB06 (0.64 μg/mL) has been associated with the ability to produce IAA and improve plant yield (Kwon et al., 2021). It is not surprising that most of the endophytes were able to produce IAA, in the present study l-tryptophan was used as an indicator and IAA is known to be produced through the help of amino acid, tryptophan which is a product of l-tryptophan metabolism (Ghosh et al., 2021). Auxins like indole acetic acid (IAA), helps in plant tissue differentiation, cell division and elongation, apical dominance, and plant pathogen protection (Mukherjee et al., 2022).

Not only does a lack of essential nutrients cause stagnation and lower crop yield, but it also makes plants vulnerable to pathogenic attack due to inadequate immunity (Ghosh et al., 2021, 2020). Endophytes not only provide essential nutrients such as N and P to crops, but they also indirectly stimulate plant growth by antagonizing pathogens and producing lysis enzymes (Ghosh et al., 2021). It has been established that more than 8% of the *Bacillus* genome is dedicated to producing inhibitory components like lytic enzymes (Márquez et al., 2020). For example, *B. velezensis*, which was present in this study (Table 1), forms stable relationships with plants, which encourages the promotion of plant growth and the inhibition of soilborne plant diseases. It does so through formation of biofilms, production of quorum-quenching enzymes such as phytase production which plays a role in phosphorus solubilisation (Iqbal et al., 2021;

Rabbee et al., 2019). Screening new potential endophytes that produce enzymes is essential to understand the role of endophytes in plant defense (Saran et al., 2007). Among these enzymes are hydrolytic enzymes such as gelatinase, lipase, protease, and amylase (Naik et al., 2019) and these enzymes were evaluated in the present study. Findings from the current study demonstrated that different endophytes from *Lessertia frutescens* have varying capacities for producing these enzymes as shown in Table 2. Mohamad et al. (2022), also screened bacteria endophytes for hydrolytic enzymes and found that a number of those bacteria could produce enzymes such as protease, lipase. Aydi Ben Abdallah et al. (2019) also confirmed the ability of endophytic bacteria to produce amylase, lipase, cellulase, and protease enzymes. Microbial enzymes indirectly enhance plant growth by assisting in the mobilization of certain activities, such as the production of pathogen-protecting chemicals and the catabolism of complex organic molecules that promote plant growth (Yan et al., 2018). The fact that the majority of these endophytes from the present study produced several enzymes is remarkable.

It has been indicated that having a single microorganism that can synthesis both lipase and protease is one of the rare cases (Suryia Prabha et al., 2015) and this was the case for *Bacillus subtilis* LCDB-BP2 isolated from the leaves as shown in Table 2. Furthermore, these microbial enzymes enable the colonization of plants by endophytes and the transformation of inaccessible forms of nutrients into plant-usable ones (Maggini et al., 2018). In a real experiment *B. paralicheniformis* isolated from *Enicostema axillare* improved the production of the tomato's defensive enzymes and thus the tomato plant was resistant to *F. oxysporum* infection and such defence was attributed to the ability of the strain to produce hydrolytic enzymes (Jinal et al., 2020).

Although medicinal plants like *Lessertia frutescens* are commercial plants, proper commercialization, which includes greater sustainable returns and consistency of generated raw materials, can only be achieved through the application of sustainable propagation cropping systems (Kleynhans et al., 2016). *Lessertia frutescens* is known to have multiple curative properties including, anti-cancer, anti-virus, anti-microbial, anti-stress, and anti-diabetic qualities and consequently its demand has surged dramatically (Kleynhans et al., 2016). Standard medicinal plant production can lead to increased plant biomass yield (Isah et al., 2018). Thus, the need for medicinal plants propagation cannot be emphasised any less as the supply sometimes becomes less than the demand adding to issues associated with inconsistent supply. As earlier mentioned, the present study evaluated the effect of native bacillus endophytes of *L. frutescens* on its seedling growth. As shown in Figs. 6 and 7, endophytes significantly improved *Lessertia frutescens* seedling growth after two months compared to control with the combined treatment (= *B. licheniformis* BaDB06 + *B. velezensis* SM-95 inoculum) showing the highest seedling growth. The mode of action of endophytes' influence on plant growth commences in seed where the receptors in the plasma membrane send a signal to the seed (Shasmita et al., 2018). Both the seed and microbial cellular activities are the determinant for cell responsiveness, survival and adaptability in changes in environment (Shasmita et al., 2018). It is only after endophytes have been established inside the seed's systems that they can alter the host's metabolic and growth pathways. They can then trigger seed germination, seedling development, and other plant growth stages while giving fitness and an improved growth rate (Finch-Savage and Bassel, 2016).

On the other hand, endophytes' capacity to enhance seedling growth has been attributed to their ability to produce plant-growth promoting attributes. IAA, for example, has been suggested to help in seed germination and the production of adventitious and lateral roots, as well as in improving shoot length (Jayakumar et al., 2020). It is not surprising that both of the endophytic strains employed in the present investigation showed a significant difference in seedling growth compared to control as shown in Fig. 6, during the seed germination trial test because they both produced IAA. Furthermore, IAA endophytic bacteria helps in improving nutrition absorption and exchange by plants due as they trigger root length growth and photosynthetic plant components (Fadiji and Babalola, 2020). This appears to be the first study that reports on the influence of *Bacillus licheniformis* and *Bacillus velezensis* on *L. fructensces* plant growth. However, the effect of these strains (*Bacillus licheniformis* and *Bacillus velezensis*) and their consortia on medicinal plant *T. vulgaris* growth and oil content at 65 and 140 days was investigated by (Abdel-Hamid et al., 2021). The control heights at 65 and 140 cm were 10 and 12.7 cm and *Bacillus licheniformis* inoculation resulted in the maximum T. vulgaris plant height of 42.3 and 55.9 cm at 65 and 140 days, respectively. This was followed by the combined treatment (*Bacillus licheniformis* and *Bacillus velezensis*) with 38.9 and 55.7 cm in the same order of days. These strains' capacity to improve plant development was linked to their ability to boost nutrient availability to plants through these plant growth promoting traits, IAA and siderophore synthesis, phosphate solubilization, and N-fixation. Due to the potential of microorganisms, particularly endophytes, to influence plant development and biomass accumulation, they have been specified as one of the factors for plant health and growth (Klaedtke et al., 2016). Lastly, endophytes are a sustainable source of nutrients and this study revealed that endophytes from *L. frutescens* can be used as a better alternative for nutrient nourishment to promote plant growth. This can ensure better supply of *Lessertia frutescens* as it has been indicated that the monthly demand of *L. frutescens* in South Africa is roughly 20 tons for production of commercial herbal dietary supplements and supplying this demand is becoming increasingly challenging as the population grows (Shaik et al., 2011). Furthermore, the market and economic value of medicinal plants in South Africa cannot be overlooked, as they constitute a significant contribution with annual turnovers of over US$ 60 million from over 20 000 tons of medicinal plant trades (Van Wyk, 2011).

5. Conclusions and recommendations

Endophytes are a sustainable source of nutrients and this study revealed that native *Lessertia frutescens* endophytes can be used as a better alternative for nutrient nourishment to promote plant growth. The capacity of most of the strains to possess a wide variety of hydrolytic activities as well as plant growth promoting abilities indicates their long-term benefits in agriculture. *Bacillus licheniformis* BaDB06 and *Bacillus velezensis* strain SM-95, promoted *Lessertia frutescens* seedling growth. This has demonstrated that *Bacillus* species are ideal candidates for bio-fertilization and biocontrol since they can promote plant growth and produce bio-control agents like siderophores and hydrolytic enzymes.

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.

CRediT authorship contribution statement

Sinawo Tsipinana: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Linda Obi:** Writing – review & editing. **Stephen Amoo:** Writing – review & editing, Validation, Supervision, Investigation, Funding acquisition, Data curation, Conceptualization. **Rasheed Adeleke:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Acknowledgments

The authors would like to extend their gratitude to the EIER research group at North-West University for the technical and moral support.

Ethical approval

Not applicable.

Consent to publish

Each author gave a written consent to publish the manuscript.

Funding

The National Research Foundation (NRF) of South Africa funded this study. The South African Department of Agriculture, Land Reform and Rural Development funded the project.

Availability of data and materials

Data and material are available for research and reference purpose.

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