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RESEARCH ARTICLE

Biological control of citrus canker by endophytic bacteria

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Abstract

Citrus is an important fruit crop in India, playing a significant role in the agricultural economy and experiencing high demand due to its rich nutritional content. Despite its economic importance, citrus canker, caused by the Gram-negative bacterium *Xanthomonas citri* subsp. *citri*, poses a significant threat to citrus production globally, including in India. This study focuses on using endophytic bacteria, specifically *Bacillus amyloliquefaciens* ESK-8 and *Bacillus subtilis* EPM-3, for the biological management of this pathogen. A roving survey in major citrus cultivating areas of Tamil Nadu revealed varying disease prevalence rates (21% - 65%). The citrus canker pathogen and endophytic *Bacillus* isolates were isolated from the surveyed locations and identified based on biochemical and molecular analyses using 16S rDNA sequences. *In vitro* assays through agar well diffusion method using culture filtrates of various *Bacillus* isolates revealed significant inhibition rates, with *Bacillus amyloliquefaciens* ESK-8 and *Bacillus subtilis* EPM-3 exhibiting inhibition diameters of 14.1 mm and 11.6 mm, respectively, compared to the control. GC-MS analysis of *Bacillus amyloliquefaciens* ESK-8 and *Bacillus subtilis* EPM-3 unraveled important antibacterial compounds such as bis(2-ethylhexyl) phthalate, n-hexadecanoic acid, D -erythro-pentose, 2-deoxy, hexadecanoic acid, octadecanoic acid and tridecane. Furthermore, the metabolic pathways related to these compounds include glycerolipid metabolism, glutamate metabolism and tryptophan metabolism, all of which play significant roles in plant growth and antagonism-promoting activities. Additionally, pot culture studies confirmed the antagonistic potential of *Bacillus amyloliquefaciens* ESK-8 and *Bacillus subtilis* EPM-3 against the citrus canker pathogen. This research highlights the potential of endophytic *Bacillus* isolates for the sustainable management of citrus canker disease.

Keywords

Bis(2-ethylhexyl) phthalate; biocontrol; *Bacillus amyloliquefaciens; Bacillus subtilis;* metabolic pathways; siderophore

Introduction

Citrus is one of the most important tropical and subtropical crops globally, including in India. It belongs to the *Rutaceae* family and is an excellent source of vitamin C. India ranks 6th in the world for citrus fruit production (1). In India, citrus cultivation covers 10.6 million hectares, yielding 125.10 lakh metric tons with a productivity of 10.3 metric tons per hectare (2). Various factors contribute to the decline in citrus production, in that citrus canker is one of the most serious problems which is caused by phytopathogenic bacterium *Xanthomonas citri* subsp. *citri*. It is a destructive and highly contagious disease that significantly impacts citrus plants, leading to considerable economic losses of up to 20 % to 50 % (3). The disease is prevalent in many citrus-growing regions, leading to a decline in production and marketability. The

symptoms are brown, corky necrotic lesions on leaves stems and fruits, often surrounded by yellow halos and exuding bacterial ooze. These lesions on fruits significantly reduce market value while causing premature fruit drop, twig dieback, defoliation and stunting. This multifaceted impact diminishes fruit yield and affects tree longevity, posing a major threat to the citrus-growing countries all over the world. Conventional management of citrus canker has primarily relied on chemical control measures, including the use of copper-based fungicides and antibiotics like streptomycin. It will reduce disease incidence with significant drawbacks in the development of resistance in the bacterial population (4). The use of excessive synthetic fungicides has led to soil and water contamination, and declining the soil beneficial microorganisms demands an alternative biocontrol strategy (5). Interestingly, endophytic bacteria specifically, *Bacillus* sp. pave a potential alternative for the biological management of this pathogen. Moreover, species specifically *Bacillus amyloliquefaciens* and *B. subtilis have been recognized for their efficacy in suppressing the pathogen growth of plant pathogens, particularly bacteria such as Xanthomonas spp., Ralstonia spp., and Erwinia spp. by employing a broad spectrum of antagonistic mechanisms, including the production of antibiosis, lytic enzymes, competition for nutrients and space and enhancement of plant defense mechanism through Induced Systemic Resistance (ISR) (6). Conversely, B. amyloliquefaciens* and *B. subtilis are renowned for their genetic capacity of synthesis of antibacterial compounds viz., octadecanoic acid, 2,3, Butanediol and Alpha bisabolol (AB) (7). In the past, many pot culture studies have demonstrated the antimicrobial activities of B. amyloliquefaciens* and *B. subtilis against citrus canker. For instance, applying a spore suspension of B. amyloliquefaciens reduced the disease incidence and enhanced plant growth in rice by producing novel antibacterial compounds like difficidin and bacilysin (*8). *In another study, (*9) *reported the antibacterial activity of B. amyloliquefaciens against Xanthomonas citri* subsp. *citri* by producing extracellular enzymes and lipopeptide compounds. In summary, all above-mentioned studies demonstrated the antibacterial role of various endophytic *Bacillus* isolates against citrus canker, paving a biocontrol tool for the management of this disease. In this study, potential endophytic *Bacillus* isolates were obtained from citrus leaves and evaluated for their antibacterial role through various *in vitro* studies. Furthermore, GC-MS analysis was performed to identify novel antibacterial compounds. In addition, a pot culture study was conducted to assess the efficacy of the potential endophytic *Bacillus* isolates for the sustainable management of bacterial canker in citrus.

Materials and Methods

Survey and isolation of bacterial pathogen of citrus canker

A roving survey was conducted to evaluate the disease incidence in the major citrus-growing regions of Tamil Nadu, which are Madurai, Virudhunagar, Dindigul, Thenkasi, Theni and Trippur. The symptoms show raised lesions on both the surface of leaves, often appearing crater-like with concentric rings, water-soaked margins and a corky texture. In the latter stage, lesions may develop yellow halos, which can turn into shot holes as the infected tissue falls away. On fruits, lesions can merge, forming irregular, scabby patches that begin as small, watery spots and darken at a later stage (10). The disease severity was calculated based on the severity index (11).

$$PDI = \frac{\text{Sum of all numerical ratings}}{\text{Total number of leaves graded}} \times \frac{100}{\text{Maximum grade}}$$

The pathogen was isolated from the infected plant tissues (leaves, fruits and stems) using the tissue segmentation method in a nutrient agar medium. The diseased samples were collected from 15 different locations. The infected tissues were surface sterilized serially with sterile water, 0.4 % sodium hypochlorite, then washed twice with sterile water separately. Then the tissues were directly placed onto a sterile Nutrient Agar (NA) plate. The plates were incubated at 28°C for 72 hours (12). The suspected bacterial colonies as mucoid with a typical yellow pigment were examined and preserved. A pathogenicity test was performed by using a detached leaf assay to confirm the pathogen (13). Healthy citrus leaves were inoculated with culture filtrates of *Xanthomonas* isolates. Lesion development was observed for 7 to 14 days, with the size and quantity of lesions recorded to evaluate the most virulent isolate (14).

Isolation of endophytic bacterial isolates

To isolate endophytic bacteria, healthy, disease-free citrus leaves were collected from 15 different locations in southern parts of Tamil Nadu. The serial dilution method was used to isolate the endophytic bacteria. After surface sterilization, the tissue was aseptically macerated using a pestle and mortar. It is incubated for 3 hours at 28°C to facilitate the release of endophytic bacteria, then homogenized and serially diluted up to 10^{-2} in sterilized NaCl solution. For each dilution, 50 µL of the suspension was spread onto 25 percent yeast extracts nutrient agar medium and the plates were incubated at 28 °C for 15 days (15). Colonies exhibiting diverse morphology and colours were selected and purified through sub-culturing under aseptic conditions (16). Totally 15 endophytic bacterial isolates were successfully isolated from different citrus growing regions.

Cultural and morphological characterization of Xanthomonas spp.

A loopful of 72-hour-old *Xanthomonas* culture was serially diluted, and 500 µl from the 10th dilution was spread onto NA plates to ensure the isolation of bacterial colonies. The plates were incubated at 27-28°C for 72 hours. The characteristics such as shape, size, pigmentation, margin, elevation and number of colonies were recorded for each isolate (17).

Biochemical characterization of Xanthomonas spp.

The bacterial isolates were biochemically characterized according to Bergey's manual of determinative bacteriology viz., Gram staining, KOH test, Catalase test, Starch hydrolysis and Simmons citrate utilization test (18).

Molecular characterization of Xanthomonas spp.

The bacterial DNA extraction was done by using a thermo-scientific genomic DNA purification kit in standard protocol. The DNA was suspended in TE buffer and quality was determined by 1.5 % agarose gel electrophoresis. To identify the pathogen the internal transcribed spacer (ITS) region was amplified by using the universal bacterial primers 799F (5'-AACMGGATTAGATACCCKG-3') and 1193R (5'-ACGTCATCCCCACCTTCC-3') (19).

Cultural and biochemical characterization of endophytic bacterial isolates

The cultural characterization of endophytic bacterial isolates was studied by taking a loopful of 72-hour-old bacterial endophytic culture that was serially diluted. From the 10^{th} dilution, 500 µl of bacterial suspension was plated onto a separate NA medium. The inoculated plates were then incubated at 27-28°C for 72 hours. The observations were made regarding colony shape, size, pigmentation, margin, elevation, appearance, diameter, number of colonies and characteristics of endophytic bacteria (20).

The biochemical tests were conducted for the characterization of 15 isolates of endophytic bacteria by following standard methods like Gram staining, ammonia production, gelatin liquefaction, phosphate solubilization and siderophore production (21).

Molecular characterization of potent endophytic bacteria

The effective endophytic bacterial isolates *viz*., EBPM-3, EBSK-8 and EBPK-10, which exhibited antibacterial activity against *Xanthomonas axonopodis* pv. *citri* strains were further characterized by 16S rRNA gene sequencing. The obtained sequences were compared using BLAST within the NCBI GenBank database with the accession numbers PQ128363, PQ128364 and PQ113705 (22). Phylogenetic analysis was performed using the neighbour-joining method implemented in the MEGA software. The phylogenetic tree was constructed based on type strains of species closely related to *Bacillus* spp. Bootstrap replication (1000 iterations) was utilized to statistically assess the robustness of the nodes within the phylogenetic tree (23).

In vitro efficacy of different isolates of endophytic bacteria against Xanthomonas spp.

The effect of different isolates of endophytic bacteria was tested against *Xanthomonas* spp. by following the agar well diffusion method (24). Overnight culture of the pathogenic isolates was prepared in nutrient broth and the prepared suspension was mixed in nutrient agar media. It was poured into the Petri plates. Three wells were created in the agar plate and 150 µl of the endophytic bacterial strains viz., ESK-8 and EPM-3 at 10^8 CFU/mL was poured in each well. It was incubated at 28°C for 24-48 hours, after that the inhibition zones were measured.

Extraction of antibacterial compounds from cell free supernatant

The crude metabolites were obtained by following the methodology by (25). The *Bacillus* strains were cultivated in nutrient broth and subjected to incubation at 28 ± 2 °C for 10 days and 3 days respectively. After 72 hours, corresponding to the stationary phase, the supernatant was harvested through centrifugation at 8000 rpm for 30 min, maintaining the temperature at 28 ± 2 °C. Subsequently, the supernatant's pH was adjusted to an acidic value of 2.0 using concentrated HCl, and this mixture was stirred at 100 rpm using an orbital shaker for 8 hours while being maintained at 28 ± 2 °C. To extract antibacterial compounds, present in the culture broth, an equal volume of ethyl acetate was added to the supernatant. The resulting mixture was then agitated for 2 h in an orbital shaker at a rate of 200 rpm. This extraction process using ethyl acetate was conducted twice to ensure comprehensive extraction. The solvent fraction encompassing the antibacterial compounds was pooled and subsequently concentrated through evaporation using a rotary flask evaporator at 60°C at 8000 rpm. The concentrated crude metabolites were dissolved in ethyl acetate. This resultant solution was then employed for both *in vitro* antibacterial assessment and GC-MS analysis (26).

GC-MS analysis of solvent extracts

The selected endophytic bacterial strains *viz*., ESK-8 and EPM-3 demonstrate the highest antibacterial activity. The bacterial cultures were grown in nutrient broth and the metabolites were extracted using ethyl acetate. These extracts were then analyzed using a GC-MS to identify the bioactive compounds showing antimicrobial properties.

The analysis of the crude ethyl acetate extracts derived from *Bacillus* spp. cell-free supernatant samples were conducted using the Shimadzu GC-MS QP-2020 gas chromatograph system. This system consisted of an autosampler gas chromatograph paired with a mass spectrometer, featuring a silica capillary column. Detection in the GC-MS utilized an electron ionization system set to 70 eV. The temperature program for the oven began at 80 °C for 2 minutes and then increased at a rate of 9 °C per minute until it reached 200 °C, where it was held for 4 minutes. The temperature was further raised by 10°C per minute until reaching 300 °C, which was maintained for an additional 5 minutes. Helium served as the constant carrier gas, with a flow rate of 1.5 ml/min and an injection volume of 1 µl. The injector temperature was set at 250°C, while the ion source temperature was maintained at 230 °C. Mass spectra were recorded at energy of 70 eV. The relative percentage of each component was calculated by comparing the average peak area of each component to the total area. This analysis facilitated the identification of specific compounds by correlating their characteristics with those found in the NIST libraries (27).

Formulation of ESK-8 and EPM-3

The potent endophytic bacterial strains *viz*., ESK-8 and EPM-3 were further formulated with stable and effective formulation. A loopful of *B. amyloliquefaciens* and *B. subtilis* was inoculated into the Nutrient broth and incubated in a rotary shaker at 150 rpm for 72 h at room temperature (28 ± 2°C) (28). After 72 h of incubation, the broth containing 9x 10^8CFU/ml was used for the preparation of talc-based formulation.

Evaluating the efficacy of Bacillus amyloliquefaciens ESK-8 and Bacillus subtilis EPM-3 against Xanthomonas spp. under pot culture condition

An experiment was conducted at the Department of Plant Pathology, Agricultural College and Research Institute, Madurai, to assess the effectiveness of *B. amyloliquefaciens* ESK-8 and *B. subtilis* EPM-3 against *X. axonopodis* pv. *citri* in pot culture condition. Virulent isolates of the pathogen were inoculated using the pinprick method. The foliar application of bioagents with treatment T_1- *B. amyloliquefaciens* ESK-8, T_2- *B. subtilis* EPM-3 and T_3- BPV at 100 ppm concentrations. Additionally, foliar spraying of T_4-Streptocycline at 100 ppm was applied 24 hours after pathogen inoculation, with a second spray seven days later. A T_5 -control treatment was without inoculation of the pathogen. Each treatment was replicated four times in a completely randomized design under glasshouse conditions (29). Disease intensity was recorded 30 days after inoculation and the percent disease index (PDI) was calculated using a standard formula (30).

$$PDI = \frac{\text{Sum of all numerical ratings}}{\text{Total number of leaves graded}} \times \frac{100}{\text{Maximum grade}}$$

Statistical Analysis

Data were analyzed statistically by using appropriate statistical methods, with significance determined at $p < 0.05$. ANOVA was performed to compare the efficacy of different endophytic bacterial strains across all assays.

Results

Survey and isolation of bacterial pathogen and characterization

A survey was carried out across citrus-growing regions in the districts of Madurai, Dindigul, Tenkasi, Tirupur, Virudhunagar, and Theni to evaluate the incidence of citrus canker. The percent disease index (PDI) ranged from 20.31% to 85.47 %, with the highest incidence recorded in Sankarankovil (85.47 %), followed by Palamedu (Madurai) with a PDI of 79.63 %. The lowest disease incidence, with a PDI of 20.31%, was observed in Ayyanarkulam (Madurai). Fifteen isolates of *Xanthomonas* spp. (designated CCMR-1 to CCPL-15) were isolated from infected plant tissues showing a typical symptom on leaves with raised corky lesions (Fig. 1), by leaf sap and leaf bit method.

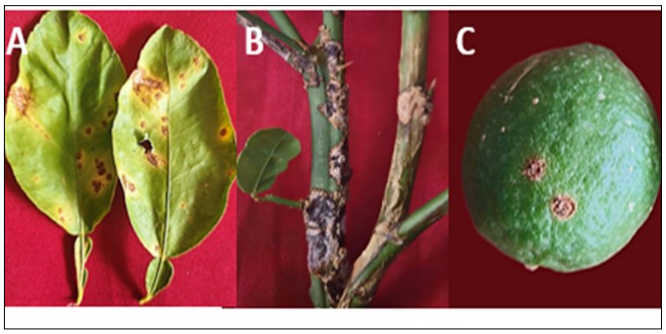

Fig. 1. Symptoms of citrus canker on leaves, stem and fruits. **A.** Small, raised, oily-looking spots appear on the surfaces of leaves, lesions surrounded by a yellow or chlorotic halo **B.** Infected stems may exhibit dieback, leading to reduced tree vigour.

A pathogenicity test was performed on three-month-old acid lime seedlings for all fifteen *Xanthomonas* spp. isolates, using clipping, pinprick and spray inoculation methods. Leaf wounds were created with sandpaper to enhance bacterial entry. The symptoms are observed as water-soaked, yellowish chlorotic lesions, which later progressed into raised necrotic spots (Fig. 2a). The most virulent isolate, CCSK-8 was identified, showing the highest PDI of 82.21 % by detached leaf assay (Fig. 2b).

The cultural and morphological characteristics of 15 isolates of *Xanthomonas spp.* were analyzed by various morphological traits of bacterial colonies. These include colony size, which denotes the overall dimensions of the growth medium; colony shape (circular to irregular), colony colour (straw yellow to deep yellow), colony elevation (convex or raised) and colony margin (smooth, wavy and lobate) were observed. All *Xanthomonas* spp. showed rod-shaped and motile morphology and identified as Gram-negative through Gram staining (Fig. 3). The biochemical characterization of all 15 isolates showed positive response for Gram staining,

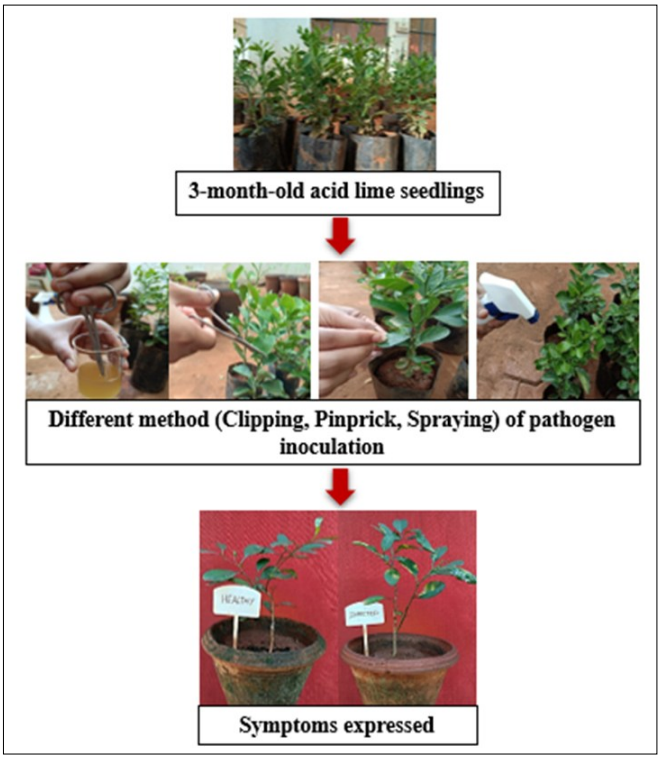

Fig. 2a. Pathogenicity test by different methods of pathogen inoculation (Clipping, Pinprick and Spraying).

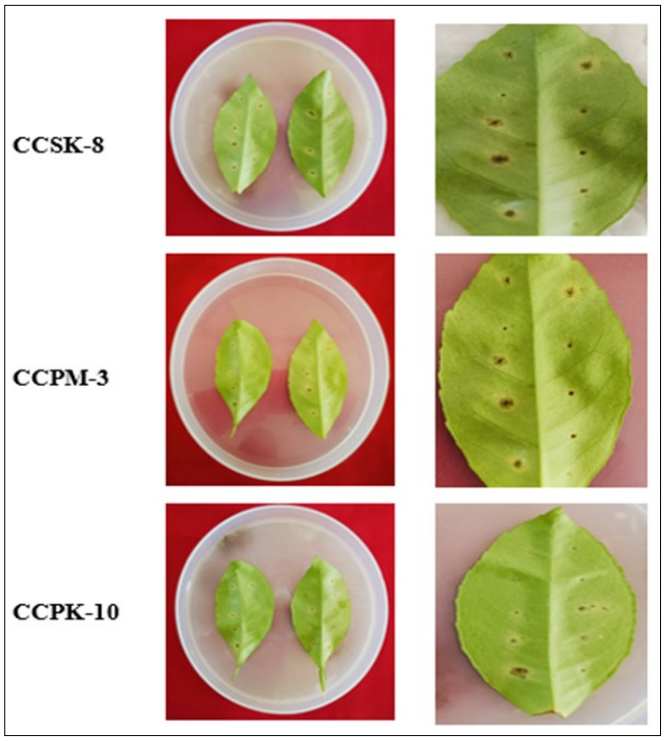

Fig. 2b. Pathogenicity test by detached leaf assay. *Xac* strains (CCSK-8, CCPM-3 and CCPK-10) infiltration. **B.** Leads to smaller infection lesions and water-soaked symptoms in citrus leaves.

Catalase test, starch hydrolysis, Simmons citrate utilization and KOH test (Table 1).

PCR amplification of genomic DNA from citrus canker isolates using universal and 16S rRNA primers confirmed the presence of the 16S rRNA gene in all fifteen isolates, with 400-1500 bp fragments. BLAST analysis of a virulent isolate 16S rRNA gene sequence showed 100% identity with *X. axonopodis* pv. *citri (Xac)* and the sequence were deposited in GenBank under accession number PQ124896.1 (Fig. 4).

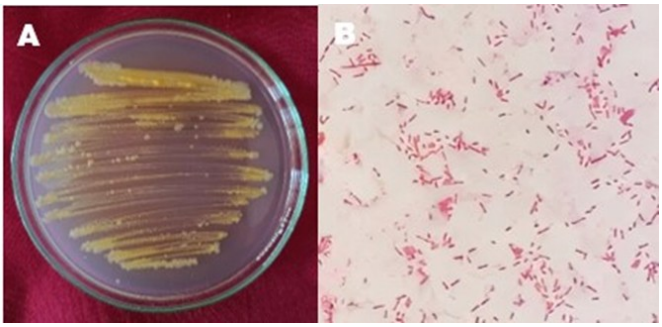

Fig. 3. Cultural and Morphological characteristics of *Xanthomonas* spp. **A**. Pure culture of *Xanthomonas* spp. **B.** Rod-shaped gram-negative bacteria through gram staining.

Isolation of endophytic bacterial isolates and their characterization

Endophytic bacteria were isolated from disease-free, healthy plant parts viz., leaf surface, root tissues and seeds in various citrus growing areas of different districts using standard isolation procedures.

The morphological traits of the isolated endophytic bacteria were observed, including colony colour (creamy white), shape (circular to irregular), elevation (convex, raised and spreading) and margin (smooth and wrinkled). The biochemical characteristics of Gram staining revealed that all strains were motile and rod-shaped Gram-positive bacteria (Fig. 5). Furthermore, all the isolates showed positive results in gelatin liquefaction, ammonia production, siderophore production and phosphate solubilization (Table 2).

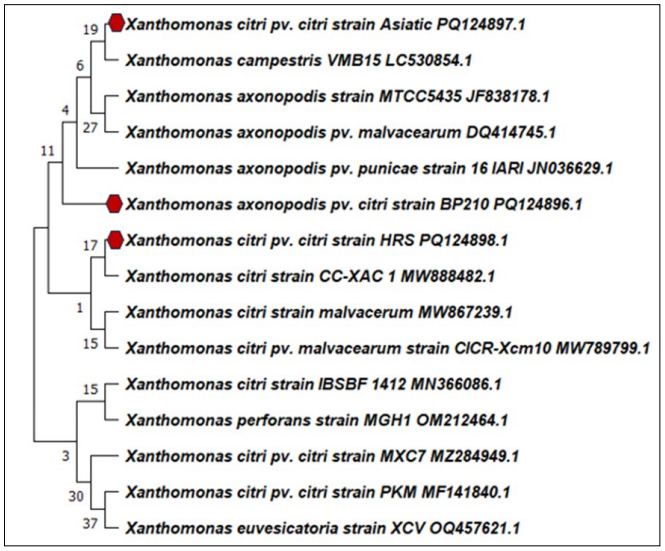

Fig. 4. Phylogenetic tree based on the 16S rRNA gene sequences that highlight the position of CCPM-3, CCSK-8 and CCPK-10 relative to the other strain types within the genus *Xanthomonas*. The highlighted sequence accession numbers were retrieved from the NCBI.

The BLAST homology analysis revealed that the 16S rRNA gene sequences of the three bacteria belong to the genus *Bacillus*. In addition, the phylogenetic tree inferred from the 16S rRNA gene sequences indicated that the isolates EBSK-8, EBPM-3 and EBPK-10 share a high degree of identity with *B. amyloliquefaciens* ESK-8, *B. subtilis* EPM-3 and *B. velezensis* EPK-10, respectively (Fig 6).

Table 1. Biochemical characterization of *Xanthomonas* spp

Isolate code	Gram staining	Potassium hydroxide test	Catalase test	Citrate utilization	Starch hydrolysis	Siderophore production
CCMR-1	Negative	Positive	Positive	Positive	Positive	Positive
CCNS-2	Negative	Positive	Positive	Positive	Positive	Positive
CCPM-3	Negative	Positive	Positive	Positive	Positive	Positive
CCAK-4	Negative	Positive	Positive	Positive	Positive	Positive
CCVS-5	Negative	Positive	Positive	Positive	Positive	Positive
CCOM-6	Negative	Positive	Positive	Positive	Positive	Positive
CCTP-7	Negative	Positive	Positive	Positive	Positive	Positive
CCSK-8	Negative	Positive	Positive	Positive	Positive	Positive
CCVP-9	Negative	Positive	Positive	Positive	Positive	Positive
CCPK-10	Negative	Positive	Positive	Positive	Positive	Positive
CCKT-11	Negative	Positive	Positive	Positive	Positive	Positive
CCPR-12	Negative	Positive	Positive	Positive	Positive	Positive
CCVN-13	Negative	Positive	Positive	Positive	Positive	Positive
CCVK-14	Negative	Positive	Positive	Positive	Positive	Positive
CCPL-15	Negative	Positive	Positive	Positive	Positive	Positive

Table 2. Biochemical characterization of endophytic bacteria

Isolate code	Gram staining	Ammonia production	Gelatin liquefication	Phosphate solubilization	Siderophore production
EBMR-1	Positive	Positive	Positive	Positive	Positive
EBNS-2	Positive	Positive	Positive	Positive	Positive
EBPM-3	Positive	Positive	Positive	Positive	Positive
EBAK-4	Positive	Positive	Positive	Positive	Positive
EBVS-5	Positive	Positive	Positive	Positive	Positive
EBOM-6	Positive	Positive	Positive	Positive	Positive
EBTP-7	Positive	Positive	Positive	Positive	Positive
EBSK-8	Positive	Positive	Positive	Positive	Positive
EBVP-9	Positive	Positive	Positive	Positive	Positive
EBPK-10	Positive	Positive	Positive	Positive	Positive
EBKT-11	Positive	Positive	Positive	Positive	Positive
EBPR-12	Positive	Positive	Positive	Positive	Positive
EBVN-13	Positive	Positive	Positive	Positive	Positive
EBVK-14	Positive	Positive	Positive	Positive	Positive
EBPL-15	Positive	Positive	Positive	Positive	Positive

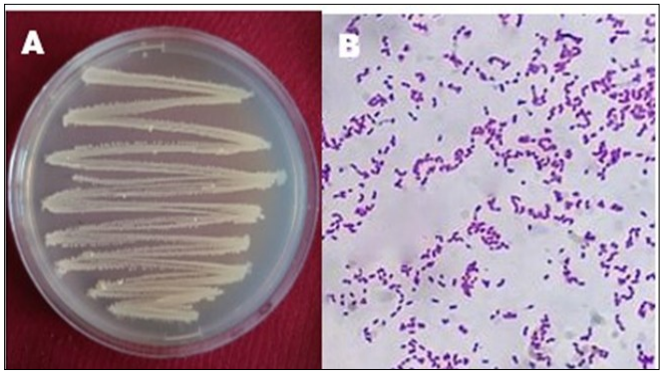

Fig. 5. Cultural and Morphological characteristics of endophytic bacteria. **A.** Pure culture of endophytic bacteria. **B.** Rod-shaped gram-positive bacteria through gram staining.

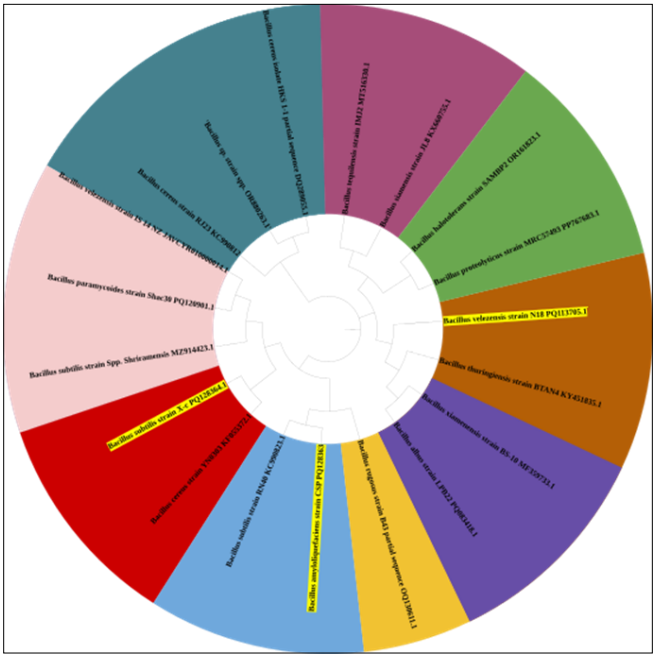

Fig. 6. Phylogenetic tree based on the 16S rRNA gene sequences that highlight the position of EPM-3, ESK-8, and EPK-10 relative to the other strain types within the genus *Bacillus*. The highlighted sequence accession numbers were retrieved from the NCBI.

Screening for antibacterial ability

Numerous endophytic bacterial strains (*Bacillus* spp.) were screened, with the agar well diffusion method for the significant inhibition of *Xanthomonas* spp. Notably, the effective endophytic bacteria (*Bacillus* spp.) EBPM-3, EBSK-8 and EBPK-10 exhibited the highest inhibition zones 15.3 mm, 14 mm and 11.6 mm (Table 3, Fig. 7).

GC-MS analysis of solvent extract of culture supernatant from endophytes

From the GC-MS analysis, fifty important compounds were identified in both the antagonist and enriched pathway constructed from the MetaboAnalyst software 6.0. The crude extracts of *B. amyloliquefaciens* ESK-8 and *B. subtilis* EPM-3 were reported to contain the following antibacterial bioactive compounds that were identified and quantified in the sample, as revealed by the chromatogram. These include Bis (2-ethylhexyl) phthalate with a retention time of 22.52 and a peak area percentage of 67.13 %, n-Hexadecenoic acid at 17.520 with 3.50 %, D-erythro-Pentose, 2- deoxy at 9.813 with 2.83 %, Hexadecenoic acid, 2-hydroxy-1-(hydroxymethyl) at 24.875 with 2.64 %, Octadecanoic acid, 2,3-dihydroxy propyl ester at 22.348 with 1.97%, Tridecane, 1-iodo- at 12.347 with 1.92 %, Octadecanoic acid at 19.414 with 1.66 %, 2(3H)-Furanone, dihydro-4,4-dimethyl- at 16.319 with 1.31 % and 3'-O-Acetylthymidine at 16.017 with 1.26 %. The metabolism linked to the *Bacillus* spp. are glycerolipid metabolism, glutamate metabolism and tryptophan metabolism (Table 4 and 5, Fig. 8).

Formulation of endophytic bacterial isolates

The formulation of ESK-8 and EPM-3 effectively manages citrus canker by outcompeting *X. axonopodis* pv. *citri* and inhibiting its growth. These bacteria promote plant growth and enhance disease resistance. Applications such as soil treatment, foliar sprays and seed coating improve plant health and control the disease.

Table 3. Screening the antibacterial activity of effective endophytic bacteria against *Xac by* agar well diffusion

Isolates code	Species	Strain	Zone of Inhibition (mm)
EBPM-3	*Bacillus subtilis*	EPM-3	15.3[a]
EBSK-8	*Bacillus amyloliquefaciens*	ESK-8	14.1[b]
EBPK-10	*Bacillus velezensis*	EPK-10	11.6[c]
	Control		0.0[d]
	CD (*P*=0.05)		**1.50**

*Mean of three replications

Data followed by the same letter in a column are not significantly different by DMRT at a 5 % level of significant

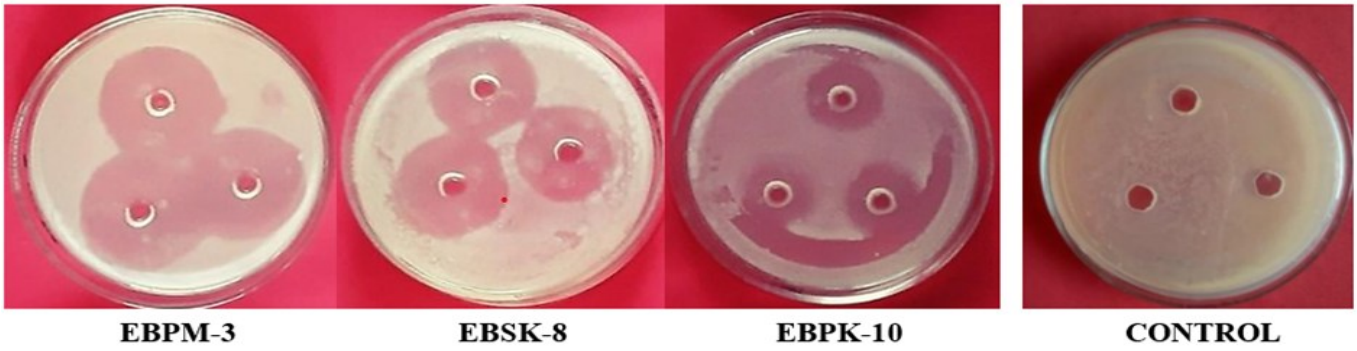

Fig. 7. Inhibitory effect on the growth of virulent *Xac* isolates CCSK-8, CCPM-3, and CCPK-10 by using agar well diffusion method.

Table 4. GC-MS analysis of secondary metabolites of *Bacillus amyloliquefaciens* ESK-8

S.NO	Compound Name	Peak Area %	R. Time	MW (g/mole)	Molecular formula	Specific role	Reference
1	Bis (2-ethylhexyl) phthalate	16.57	22.522	390.5561	$C_{24}H_{38}O_4$	Antibacterial activity	(50)
2	Octadecanoic acid,2,3-	11.58	23.928	358.5558	$C_{21}H_{42}O_4$	Antibacterial activity	(51)
3	Furan, tetrahydro-2,2-dimethyl-5-(1-methyleth	7.33	24.875	154.2493	$C_{10}H_{18}O$	Antibacterial activity	(52)
4	Hexanoicacid,2-propenylester	5.93	24.438	156.2221	$C_9H_{16}O_2$	Antibacterial activity	(53)
5	D-erythro-Pentose,2-deoxy-	4.83	9.883	134.1305	$C_5H_{10}O_4$	Antibacterial activity	(54)
6	Pentanoic acid,4-methyl-ethyl ester	4.25	25.565	144.2114	$C_8H_{16}O_2$	Antimicrobial activity	(55)
7	Carbonicacid,decylnonylester	3.05	25.940	328.5298	$C_{20}H_{40}O_3$	Antimicrobial activity	(56)
8	Pentane-1,2,3,4,5-pentaol	2.83	13.024	152.146	$C_5H_{12}O_5$	Antibacterial activity	(57)
9	1,4:3,6-Dianhydro-.alpha.-d-glucopyranose	2.80	21.541	144.1253	$C_6H_8O_4$	Antibacterial activity	(58)
10	Aceticacid,5-hydroxy-4-nitrotetrahydropyran	2.61	15.948	205.17	$C_7H_{11}NO_6$	Antibacterial activity	(59)

Table 5. GC-MS analysis of secondary metabolites of *Bacillus subtilis* EPM-3

S.NO	Compound Name	Peak Area %	R.Time	MW (g/mole)	Molecular formula	Specific role	Reference
1	Bis(2-ethylhexyl) phthalate	67.13	22.520	390.5561	$C_{24}H_{38}O_4$	Antibacterial activity	(50)
2	n-Hexadecanoic acid	3.50	17.520	256.43	$C_{16}H_{32}O_2$	Antibacterial activity	(60)
3	D-erythro-Pentose,2-deoxy-	2.83	9.813	134.1305	$C_5H_{10}O_4$	Antibacterial activity	(54)
4	Hexadecanoicacid,2-hydroxy-1-(hydroxymet	2.64	24.875	330.5026	$C_{19}H_{38}O_4$	Antibacterial activity	(60)
5	Octadecanoic acid, 2,3-dihydroxypropyl ester	1.97	22.348	358.5558	$C_{21}H_{42}O_4$	Antibacterial activity	(51)
6	Tridecane, 1-iodo-	1.92	12.347	310.2579	$C_{13}H_{27}I$	Antibacterial activity	(45)
7	Octadecanoic acid	1.66	19.414	284.48	$C_{18}H_{36}O_2$	Antibacterial activity	(61)
8	2(3H)-Furanone, dihydro-4,4-dimethyl-	1.31	16.319	154.2493	$C_{10}H_{18}O$	Antibacterial activity	(52)
9	3'-O-Acetylthymidine	1.26	16.017	364.24	$C_{12}H_{16}N_2O_6$	Antimicrobial activity	(62)
10	Hexadecane	1.12	14.839	226.445	$C_{16}H_{34}$	Antibacterial activity	(63)

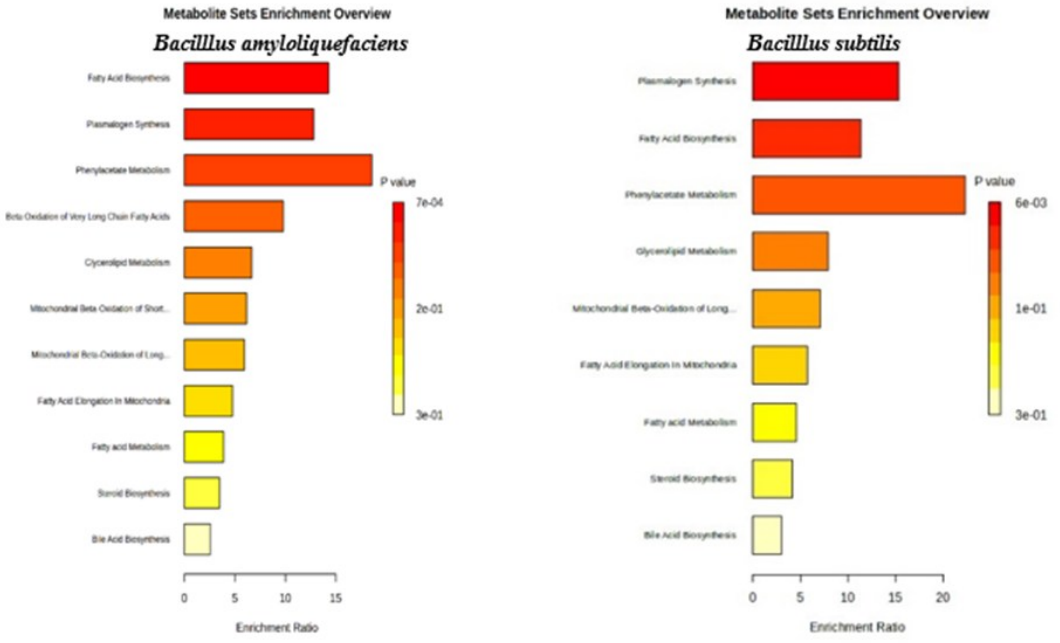

Fig. 8. *Bacillus amyloliquefaciens* ESK-8 and *Bacillus subtilis* EPM-3 and their metabolites enriched through Metabo Analyst software 6.0.

Evaluating the efficacy of promising B. amyloliquefaciens and B. subtilis against Xanthomonas spp. under pot culture condition

The study assessed various treatments for disease control and plant growth over 4 and 8 weeks, highlighting significant differences in efficacy. T_1(Streptocycline) at 100 ppm achieved moderate disease control, reducing PDI from 55.6 % to 50.0 % in 4 weeks and increasing plant height from 20.0 cm to 40.0 cm. After 8 weeks, disease severity increased to 40.0 %, but plant height continued to 27.5 cm. BPV at 100 ppm had minimal impact, with PDI slightly increasing and plant height improving from 19.0 cm to 28.0 cm by 8 weeks. T_2 (*B. amyloliquefaciens* ESK-8 strain) at 100 ppm provided moderate disease control and PDI range from 22.2 % to 50.0 % in 4 weeks and 20.0 % in 8 weeks, while plant height increased to 30.0 cm. Similarly, T_3 (*B. subtilis* EPM-3 strain) at 100 ppm effectively reduced PDI from 33.3 % to 50.0 % in 4 weeks and further to 25.0 % by 8 weeks, with plant height at 27.0 cm. The control treatment expressed disease severity increasing from 20.0% to 55.0% and plant height decreasing from 50.0 cm to 15.0 cm in 4 weeks. It was concluded that T_2 (*B. amyloliquefaciens*) was the most effective, followed by T_3 (*B. subtilis*).

Discussion

Gottwald *et al*. (2002) (10) and Wang *et al.* (2019) (31) reported that citrus canker, primarily caused by *X. axonopodis* pv. *citri* (*Xac*), poses a significant threat to citrus production globally, resulting in severe economic losses. The isolation of fifteen *X.* spp. isolates from infected tissues, exhibiting typical symptoms such as raised corky lesions, is consistent with established diagnostic methods (32). This discussion aims to elaborate on the findings from a survey conducted across several citrus-growing districts Madurai, Dindigul, Tenkasi, Tirupur, Virudhunagar and Theni to assess the incidence of citrus canker and explore the isolation and characterization of *Xanthomonas* isolates along with the potential biocontrol agents identified during the study. The isolation of fifteen *Xanthomonas spp.* isolates from infected plant tissues exhibiting typical symptoms of citrus canker reaffirms established diagnostic protocols. The pathogenicity tests conducted using various inoculation methods (clipping, pinprick and spray) confirmed that these isolates can cause disease in acid lime seedlings, which is crucial for understanding their virulence (33). Notably, isolate CCSK-8 demonstrated the highest PDI of 82.21%, emphasizing the variability in virulence among different strains (34). Francis and Graham (2010) (13) introduced a detached leaf protocol for screening germplasm for resistance to citrus canker and bacterial spots. This method involves injecting bacterial inocula into the underside of disinfested immature leaves and assessing symptoms after a specific duration. It is particularly effective for small amounts of leaf material and allows rapid evaluation of pathogen infectivity. In this study, the detached leaf assay revealed that *Xac* isolates CCPM-3, CCSK-8 and CCPK-10 significantly produced disease development in citrus leaves, as visible symptoms were seen compared to control leaves inoculated with *Xac* isolates. Kumar *et al. (*2020) (6) highlighted the importance of strain-specific responses in managing bacterial pathogens. Morphological characterization of the fifteen isolates revealed traits consistent with the *Xanthomonas* genus, such as rod-shaped, motile, Gram-negative bacteria (35). The biochemical tests, which showed positive results for Gram staining, catalase, starch hydrolysis, and Simmons citrate utilization, corroborate the classification of these isolates within the *Xanthomonas* group (36). The molecular characterization through PCR amplification of the 16S rRNA gene confirmed the identity of the isolates, revealing a 100% similarity to *X. axonopodis* pv. *citri*. González *et al.* (2020) (37) reported that molecular confirmation supports the use of PCR as an efficient diagnostic tool. Liu *et al*. (2022) (38) suggested that the isolation of endophytic bacteria from healthy citrus tissues provides a promising avenue for developing biological control strategies against citrus cankers. The *Bacillus* spp. identified, exhibiting beneficial traits such as ammonia production and phosphate solubilization, highlighting their potential role in promoting plant health and disease resistance. The antibacterial activity of strains EBPM-3, EBSK-8 and EBPK-10 against *Xanthomonas spp.* emphasizes the utility of these endophytes as biocontrol agents. Kloepper *et al*. (2004) (39) findings aligned with earlier studies that suggest endophytic bacteria can suppress pathogenic bacteria while promoting plant growth and stated that the agar well diffusion method demonstrated significant inhibition of *Xanthomonas spp.* by the endophytic *Bacillus* spp., reinforcing their potential as biocontrol agents. The antagonistic effects of these isolates were tested using the agar well diffusion method, which showed that the most effective strains-EBPM-3, EBSK-8 and EBPK-10 had significant antibacterial activity against the virulent strain CCSK-8, with EBPM-3 displaying the largest inhibition zone. Al-Saleh (2014) (40) noted that *Pseudomonas fluorescens* from Saudi Arabia demonstrated inhibitory activity against *X. campestris* pv. *citri (XCC)* within 24 hours of culture. *In vitro* assays of strains B1 and C8 exhibited strong inhibitory effects at 168 hours, with inhibition zone diameters of 8.42 mm and 7.52 mm, respectively. Similarly, Daungfu *et al.* (2019) (15) reported that endophytic *B. subtilis* LE024 and *B. amyloliquefaciens* LE109, isolated from *Citrus maxima* and *Citrus aurantifolia,* showed inhibition zones of 7.8 mm and 8.2 mm, respectively. BLAST analysis of the 16S rRNA gene sequences indicated that these strains belong to the genus *Bacillus*, showing high similarity to *B. amyloliquefaciens, B. subtilis* and *B. velezensis*. According to Singh *et al*. (2018) (41) and Zhang *et al*. (2019) (42), this similarity suggests that the endophytic strains may possess comparable biocontrol properties, making them promising candidates for managing citrus canker. Further investigation is needed to elucidate the specific mechanisms by which these isolates exert their antibacterial effects and to assess their effectiveness in field conditions. GC-MS analysis identified fifty significant compounds in the crude extracts of *B. amyloliquefaciens* and *B. subtilis*, which were analyzed using MetaboAnalyst software 6.0. These compounds are likely responsible for the antibacterial properties of these endophytic bacteria against *Xac.* The GC-MS analysis revealing the presence of bioactive compounds in the culture supernatants of *B. amyloliquefaciens* ESK-8 and *B. subtilis* EPM-3 further underscores the metabolic diversity of these bacteria and their possible mechanisms of action against pathogens (29). The identification of key compounds such as Bis (2-ethylhexyl) phthalate and n-Hexadecanoic acid may provide insights into the antimicrobial properties of these endophytes. Notably Bis (2-ethylhexyl) phthalate, which has been linked to antibacterial properties (43). The metabolic pathways associated with these compounds, including glycerolipid and tryptophan metabolism,

suggest a complex interaction between the endophytes and their environment, further enhancing their potential for application in disease management. This metabolic profiling is crucial for understanding the mechanisms through which these bacteria exert their beneficial effects on plants (29). For example, Zheng *et al*. (2024) (44) utilized GC-MS analysis to identify bioactive compounds in *B. amyloliquefaciens*, highlighting the presence of lipopeptides and other metabolites with potential antibacterial effects. Likewise, Ajilogba and Babalola (2019) (45) demonstrated that bacterial metabolites identified via GC-MS are critical for the biocontrol efficacy of *Bacillus* species. These findings emphasize the necessity of identifying and characterizing the bioactive compounds in endophytic bacteria to better understand their mechanisms of action and improve their use as biocontrol agents. The formulation of *B. amyloliquefaciens* ESK-8 and *B. subtilis* EPM-3 demonstrated effectiveness in managing citrus canker by outcompeting *X. axonopodis* pv. *citri* and promoting plant growth (46). The efficacy of these formulations in pot culture experiments, where significant reductions in PDI and increases in plant height were observed, reinforces the importance of utilizing biocontrol agents in integrated disease management strategies (47). T_2 (*B. amyloliquefaciens* ESK-8) was particularly effective, with results indicating potential for practical applications in citrus production (35). The formulation of *B. amyloliquefaciens* and *B. subtilis* has shown promising results in managing citrus canker by outcompeting *X. axonopodis* pv. *citri* and inhibiting its growth. These species produce antimicrobial compounds, such as lipopeptides, which compromise the integrity of the pathogen's cell membrane, thus inhibiting its growth and spread. Qian *et al.* (2020) (48) highlighted the biocontrol potential of *B. amyloliquefaciens* QC-Y against citrus canker, showing a strong inhibitory effect on *Xac*. Studies by Ke *et al*. (2023) (49) and Wang *et al*. (2022) (29) also confirmed the effectiveness of *B. amyloliquefaciens* and *B. subtilis* in managing citrus canker through their antimicrobial activities. Assessing the effectiveness of *B. amyloliquefaciens and B. subtilis* against *Xanthomonas* spp. in pot culture experiments has yielded promising outcomes. These results indicate that formulations of these endophytic bacteria could serve as an eco-friendly and sustainable alternative to chemical pesticides for controlling citrus canker.

Conclusion

In conclusion, the research findings from this study not only identify the promising endophytic bacterial strains *viz*., *B. amyloliquefaciens* ESK-8, *B. subtilis* EPM-3 and *B. velezensis* EPK-10 with antibacterial properties against *Xac* but also provide insight into their mechanisms of action and could act as a practical and powerful biocontrol agent (either bacterial spray or extract) and potential applications in biocontrol strategies for managing citrus diseases. Further research is warranted to elucidate the specific bioactive compounds involved and to evaluate their effectiveness in field conditions.

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Authors' contributions

SV as responsible for drafting the original manuscript, investigation, and analysis. MP contributed to supervision and project administration, reviewing and editing. RN played a role in the resources and data curation. YI carried out the data analysis. MK and MML provided the resources and data analysis. AM^1 did the formal analysis, review and editing, while AM^2 handled the methodology and analysis. (AM^1- Ananthan M & AM^2- Ayyandurai M)

Compliance with ethical standards

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Declaration of generative AI and AI-assisted technologies in the writing process

While preparing the manuscript, the authors used Grammarly to improve the language and readability. After using this tool/ service, the authors reviewed and edited the content as needed and took full responsibility for the publication's content.

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