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RESEARCH ARTICLE

MALDI-TOF MS identification of *Bacillus subtilis* SOE 7 against red rot pathogen *Colletotrichum falcatum* in sugarcane

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Abstract

The present research focuses to isolate, identify, characterize, screen and application of bacterial endophyte with antifungal potential against red rot causing pathogen *Colletotrichum falcatum* in sugarcane. Endophytic bacteria and *C. falcatum* were isolated from healthy roots and infected sugarcane plants respectively. MALDI-TOF MS was used to identify endophytic bacterial isolates from sugarcane roots and were screened for the antagonistic activity, hydrogen cyanide and siderophore production. The quantity of lipopeptides produced by different isolate was evaluated using HPLC and the efficient strain was selected, screened and employed for biocontrol of red rot in sugarcane under field conditions. Endophytes such as *Bacillus*, *Enterobacter* and *Klebsiella* were the predominant genera. Notably, *Bacillus* sp. SOE 7 inhibited *C. falcatum* mycelial growth by 62.25 %, showing high hydrogen cyanide production (15.68 µg mg^{-1}) and lower siderophore production compared to *Bacillus* sp. SOE 1 and SOE 3. HPLC analysis revealed *Bacillus* sp. SOE 7 produced lipopeptides surfactin (2.05 mg L^{-1}) and iturin (1.52 mg L^{-1}), likely contributing to its antagonistic activity. Field application of this isolate significantly reduced disease incidence and increased cane yield compared to the control. The bacterial endophyte *Bacillus subtilis* SOE 7 isolated from sugarcane, exhibits maximum antifungal activity against the red rot pathogen *C. falcatum* and effectively controls the disease in the field.

Keywords: climate change; drought; intercropping; salinity; sustainability; tolerance

Introduction

Sugarcane (*Saccharum officinarum*), the primary source of commercial sugar, is affected by over 50 diseases, primarily caused by viruses, bacteria, fungi, phytoplasma and nematodes. Among these, red rot, caused by *Colletotrichum falcatum*, is the most severe, significantly reducing yields across India's sugarcane regions (1). Red rot manifests as red patches on leaf sheaths, elongated lesions on midribs and distinct symptoms on stalk tissues (2). The pathogen spreads rapidly through soil, facilitated by root borers and irrigation water have elaborately discussed the biocontrol of red rot, wilt and Pokkah Boeng diseases in sugarcane by documenting the antagonistic potential of rhizobacterial species *Bacillus subtilis* that produces indole acetic acid, chitinase and glucanases (3).

The excessive use of chemical fungicides in pest management has led to environmental degradation and resistance in pathogens. Despite various control methods, including chemical treatments, red rot remains a persistent problem due to the fungus's ability to evolve with the conditions (4). Researchers worldwide have attempted to identify eco-friendly fungicides as substitutes for toxic chemicals (5,6). Endophytic bacteria, which produce antimicrobial compounds, solubilize nutrients and promote plant growth, offer a promising approach (7). These bacteria can induce systemic resistance, particularly in vegetative propagated crops like sugarcane, reducing the need for frequent applications of chemical fungicides (8,9). This dynamic interaction highlights the complexity of plant-microbe relationships and underscores the importance of studying the plant microbiome in diverse organs and developmental stages for agricultural applications (10).

Identifying and screening native antagonistic bacteria is crucial for effective disease management. Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) has proven to be a rapid and reliable method for identifying bacterial endophytes through protein profiling (11). This technique allows for accurate identification and classification, even with limited cell numbers or mixed flora (12). Rapid analysis of biomolecules/ microorganisms based on the protein's patterns present in any live sample can be carried out through MALDI-TOF MS. It a high throughput and rapid tool for species classification because of its, minimal pre-sample preparation and displays high dynamic ranges for the organism identified. Most importantly, MS allows for the detection of a wide microbial protein spectrum without the use of

standard specific DNA primers or antibodies. These protein patterns serve as fingerprint region that could be constructed as yeast/ bacteria/fungi or mold for genus and species classification of microorganisms. Intact cell mass spectrometry was also developed to discriminate bacteria without any sample pre-treatment. Currently, most published studies of the direct mass spectrometric analysis of microorganisms are based on MALDI techniques because of its speed and simplicity. There is only handful of studies for identifying endophytic bacteria in sugarcane by MALDI-TOF MS.

The development of an effective biological control agent against *C. falcatum* requires screening of the identified isolates capable of reducing red rot under *in vitro* conditions. In this study, sugarcane fields were surveyed to isolate *C. falcatum* from infected plants and endophytic bacteria from different growth stages of healthy plants. The bacterial endophytes were identified using MALDI-TOF MS and screened for antagonistic activity, siderophore and hydrogen cyanide production. The most effective isolates underwent lipopeptide detection via HPLC, followed by genomic identification. The potential of these endophytes to suppress red rot under field conditions was also evaluated. While MALDI-TOF MS has been recognized as a rapid and reliable tool for microbial identification in various contexts, its application in identifying and screening endophytic bacteria within sugarcane plants particularly for biocontrol against *C. falcatum* remains limited. Most existing studies have focused on bacterial identification in other systems, with few or no reports applying MALDI-TOF MS to sugarcane microbiomes. This gap underscores the novelty of our approach, which combines MALDI-TOF MS-based identification with functional screening of endophytes for antagonistic activity, advancing sustainable disease management strategies in sugarcane cultivation.

Materials and Methods

Red rot pathogen isolation

A field survey was conducted in 15 sugarcane-growing areas in the Cuddalore district of Tamil Nadu to identify diseased fields. A stratified random sampling approach was used across the 15 sugarcane fields, with field history of red rot disease, contributing 15 symptomatic plants, one from each field, selected at random. Infected sugarcane samples were longitudinally sliced open with a sterilized blade, revealing transverse white spots and reddish tissues typical of red rot (13). The symptomatic tissues were cut into 3 × 3 mm pieces, surface-sterilized with 5 % sodium hypochlorite for 30-60 sec (14), rinsed with sterilized water, blotted dry and placed on Potato Dextrose Agar (PDA) plates. The plates were incubated at 28-30 °C for fungal growth. After five days, the growing fungal hyphae were transferred to fresh PDA. Colonies initially appeared white and cottony, gradually turning greyish to pink with age and exhibited a concentric ring pattern a characteristic cultural feature of *C. falcatum*. The reverse side of the colony typically showed pale brown to pink pigmentation. Microscopic examination of lactophenol cotton blue-stained mounts revealed fusiform, hyaline, single-celled conidia measuring approximately 18-24 µm in length and 4-6 µm in width. Conidiophores were septate and branched, bearing slimy masses of conidia. These morphological and cultural characteristics were consistent with the description of *C. falcatum* reported in earlier studies, confirming its identity at the genus and species level. Spore masses were picked with a sterilized wire loop and streaked onto water agar. The hyphal tips of single germinated conidia were then transferred to PDA slants for maintaining pure colonies, which were kept viable with periodic subcultures at 4 ± 1 °C.

Isolation of bacterial endophytes from sugarcane

Bacterial endophytes were isolated from root samples of healthy sugarcane plants using Tryptic Soy Agar (TSA) and PDA media, both supplemented with 0.1 mg mL^{-1} cycloheximide (15,16). Soil particles adhering to the roots were thoroughly washed away with tap water. The roots were then cut into 0.5 cm pieces and surface-sterilized by soaking for 30 sec in 70 % ethanol, followed by 3 min in 2 % sodium hypochlorite and another 30 sec in 70 % ethanol. The roots were rinsed three times with distilled water to remove any residues. To confirm successful surface sterilization, 100 µL of the final rinse water was plated onto the media. The sterilized root segments were then ground using a sterile mortar and pestle with 2 mL of sterile phosphate-buffered saline. 1 mL of the root extract was serially diluted to the fifth dilution and 1 mL from this dilution was incubated at 28 ± 2 °C for 48 hr to monitor bacterial colony growth. In total, twenty endophytic bacterial isolates were obtained and stored at -80 °C in nutrient broth containing 50 % glycerol.

Identification of the bacterial endophytes by MALDI-TOF MS

Endophytic isolates were identified using MALDI-TOF MS and the MALDI BioTyper system (Bruker Daltonics GmbH, Leipzig, Germany) (17). Briefly, 24 hr old bacterial isolates grown on Petri dishes were used. A small amount of bacterial cells was washed with 300 µL of deionized water, followed by the addition of 900 µL of absolute ethanol. The suspension was centrifuged for 3 min at 14000 rpm. The pellets were then resuspended in 50 µL of 70 % formic acid and 50 µL of 100 % acetonitrile. After another 3 min of centrifugation at 14000 rpm, the supernatant containing the extracted proteins was collected. For MALDI-TOF MS analysis, 1 µL of 0.5 % (w/v) α-cyano-4-hydroxycinnamic acid in a 50:48:2 acetonitrile: water: trifluoroacetic acid solution was added to the supernatant and the mixture was dried. All dried extracts were analysed in triplicate. The analysis was performed using a laser frequency of 20 Hz, with two ion sources at 20 kV and 18.4 kV, a lens at 9.1 kV and a mass range of 2 to 20 kDa (18). The logarithmic value of the final score was used for identification, with a score of 2.3 to 3.0 indicating species level identification, 2.0 to 2.3 indicating genus level identification, 1.7 to 2.0 representing less reliable genus level identification and scores below 1.7 indicating poor identification.

In vitro assay of bacterial antagonists against *C. falcatum*

The dual culture technique was employed to assess the antagonistic activity of the bacterial strains against the fungal pathogen *Colletotrichum falcatum* (19). For reference, *Bacillus amyloliquefaciens* strain VB7 (20), known for its biocontrol potential and maintained at the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore was used. In each Petri dish, bacterial isolates were streaked on three diagonals, maintaining equal distance between the bacterial streaks and a central disc of fungal mycelium. A control PDA plate with only the pathogen was also maintained. After 72 to 120 hr of incubation at 28 ± 2 °C, the antagonistic activity of each bacterial strain was evaluated by measuring the inhibition of fungal growth. The Percent Inhibition (PI) value was calculated using the following formula:

$$PI = \frac{(Dc - Dt)}{Dc} X100$$

Where, Dc= average diameter (cm) of fungal growth in the control, Dt= average diameter (cm) of fungal growth in treatment

Siderophore production of the endophytic bacterial isolates

Siderophores strongly attract ferric iron, leading to a change in the color of the medium from blue to yellow. Using a ternary compound of chrome azurol S (CAS)/Fe^{3+}/hexadecyl trimethyl ammonium bromide as the indicator, qualitative analysis was performed (21). A zone of bright yellowish fluorescence was observed on CAS agar plates, indicating the occurrence of positive siderophore production in the dark medium. Quantitative estimation was performed by growing the isolates in nutrient broth for three days, followed by centrifugation. The supernatant of each bacterial culture (0.5 mL) was mixed with 0.5 mL of CAS reagent for 20 min. The extracted siderophores were dissolved in ethanol and the absorbance was measured at 560 nm for salicylate type siderophores and 700 nm for catechol type siderophores using a UV-VIS spectrophotometer. The quantity of the extracted siderophores was determined and expressed as µg mg^{-1} of a microbial cell.

Hydrogen cyanide (HCN) production by endophytic bacterial isolates

The production of HCN was assessed using a modified version (22). Briefly, 48 hr old bacterial isolates were individually streaked on nutrient agar supplemented with 4.4 g L^{-1} glycine. The lids of the Petri dishes were lined with filter papers soaked in a picric acid solution (composed of 2.5 g picric acid and 12.5 g Na_2CO_3). These sealed plates were then incubated at 32 ± 2 °C for 48 hr. The filter paper discs changed colour, ranging from reddish-brown (strong), brown (moderate), to yellow or light brown (weak), indicating varying levels of HCN production. Quantitative estimation of HCN production involved incubating the bacterial isolates in 250 mL flasks containing nutrient broth at 32 ± 2 °C. Filter paper strips saturated with alkaline picrate solution were suspended inside the conical flasks. After 48 hr of incubation at 30 ± 2 °C, the colour change in the sodium picrate filter paper was measured at 625 nm using a spectrophotometer and the HCN concentration was recorded as µg mg^{-1} of microbial cells (23).

Identification of antifungal lipopeptides from the endophytic *Bacillus* sp. SOE 7

The isolates *Bacillus* sp. SOE 7, *Bacillus* sp. SOE 1 and *Bacillus* sp. SOE 3, which demonstrated the highest growth inhibition of *C. falcatum* and the greatest production of HCN, salicylate type siderophores and catechol type siderophores, respectively, were individually cultured in a seed culture medium. Each isolate was grown in a 500 mL shaker flask containing 200 mL of Luria Bertani broth and incubated at 30 °C with agitation at 150 rpm for 60 hr. After incubation, the cultures were centrifuged at 8000 rpm for 10 min and the cell-free supernatants were collected. The pH of the supernatants was adjusted to 2.0 using HCl and stored overnight at 4 °C (24). The precipitate formed was collected by centrifugation at 8000 rpm for 10 min at 4 °C, then dried completely using a rotary evaporator. The resulting metabolites were suspended in HPLC grade methanol (Sigma) and filtered through a 0.2 µm membrane filter (Sartorius).

For lipopeptide identification, High-Performance Liquid Chromatography (HPLC) was employed using a C18 column (4.6 x 150 mm, Nacalai Tesque, Inc., Japan). A 20 µL sample was injected into the column, which utilized a mobile phase of 90 % methanol and 10 % water, along with 0.1 % trifluoroacetic acid (TFA). The column was maintained at 28 ± 1 °C, with a flow rate of 1 mL min^{-1}. UV absorbance at 210 nm was used for detection. The presence of surfactin and iturin lipopeptides in the sample was confirmed by comparing the retention times and absorption spectra with those of known surfactin and iturin standards (HPLC grade, Sigma). The quantity of lipopeptides produced was calculated using the following formula.

$$\text{Concentration of the sample} = \frac{\text{Concentration of the standard} \times \text{Area of the sample}}{\text{Area of the standard}}$$

Evaluation of the endophytic bacterial culture *Bacillus subtilis* SOE 7 in sugarcane under field conditions

A field experiment was conducted to assess the impact of the endophytic bacterial culture *Bacillus subtilis* SOE 7 from August to June in a farmer's field in Erumanur village, Virudhachalam taluk, Cuddalore district. The experimental field, previously used for a ratoon crop severely affected by red rot, was cleared and ploughed thoroughly before fresh planting. The trial was laid out in a Randomized Block Design (RBD) with four replications. Each plot measured 6 m x 5 m, accommodating five rows of sugarcane with row-to-row spacing of 90 cm and plant-to-plant spacing of 30 cm. Seedlings grown from single-node chip buds in pro-trays were subjected to three different treatments. In the first treatment, chip buds were soaked in a cell suspension of *Bacillus subtilis* SOE 7 in nutrient broth (10 log CFU mL^{-1}) for 1 hr before sowing in pro-trays. At 30 Days After Sowing (DAS), the plants were drenched with the bacterial cell suspension for 24 hr prior to transplanting. A second treatment followed the same procedure but used a standard culture of *Bacillus amyloliquefaciens* VB7. The third treatment involved dipping seedlings grown from untreated chip buds in a solution containing 50 g of carbendazim, 200 mL of malathion and 1 kg of urea in 100 L of water for 15 min before planting in the main field. Seedlings treated with bacterial cell suspension were exempted from carbendazim treatment before planting in the main field. An untreated control plot, with no microbial agent or carbendazim application, was also maintained. The main field had uniform agronomic practices for all plots, including irrigation, weeding and fertilizer application according to local recommendations. Environmental conditions during the trial were typical of the region, with an average temperature range of 28 °C, relative humidity between 60-85 % and total seasonal rainfall of approximately 900-1100 mm, predominantly during the northeast monsoon. Disease incidence was naturally monitored under field conditions with prior red rot inoculum likely persisting in the soil due to the history of infected ratoon crops.

Red rot disease incidence in sugarcane plants

The reduction in red rot disease incidence due to the application of *Bacillus subtilis* SOE 7 was studied and expressed as a percentage of disease incidences.

$$\text{Percent disease incidence} = 100 \times \frac{\text{Number of plants with symptoms}}{\text{Total number of plants}}$$

Yield parameters of sugarcane

The experimental crop was harvested approximately 10 months

after planting. After removing the tops and trash, the harvested canes were weighed and the cane yield and number of millable canes were recorded for each plot, expressed in t ha^{-1} and '000 ha^{-1}, respectively. For juice quality analysis, 15-25 canes were randomly selected from each plot to create a composite juice sample. The juice was analysed for brix and sucrose percent using a hand refractometer and polarimeter respectively.

Statistical analysis

All the experiments were performed in triplicate and the data were analysed using one-way ANOVA with the statistical software IRRISTAT (version 3/93, Biometrics Unit, International Rice Research Institute) to determine the mean differences between treatments with a critical difference (P = 0.05) via Duncan's Multiple Range Test (DMRT). For MALDI-TOF MS identification, the mass spectra generated were compared by using MALDI BioTyper software regarding the spectrum peak frequency, position and intensity.

Result and Discussions

MALDI-TOF MS-based identification of endophytic bacteria isolates

Pathogens isolated from red rot-infected sugarcane were morphologically confirmed as *C. falcatum*. Among 20 endophytic bacterial isolates collected from various sugarcane growth phases and about 15 strains were identified using MALDI-TOF-MS and MALDI BioTyper, predominantly as *Bacillus* sp., *Enterobacter* sp. and *Klebsiella* sp. These genera were found to colonize sugarcane irrespective of location and growth stage (Table 1). Of the isolates, 10.90 % had highly probable species identification, 28.62 % secure genus identification, 35.86 % probable genus identification and 24.62 % were unidentified.

Numerous studies have investigated the utilization of the rhizospheric bacterial community in combating plant diseases (25). A previous study investigated 226 rhizospheric bacterial isolates that are effective against the red rot pathogen (5). They emphasized on the need for isolating a variety of potential microorganisms since the pathogen *C. falcatum* is available with many pathotypes and is compatible with the host variety. Endophytes, known for Inducing Systemic Resistance (ISR) and preventing pathogen colonization, are crucial in managing red rot (7). Moreover, biopriming of seed materials with antagonistic endophytic microbial biocontrol agents provides protection against soil-borne pathogens (26).

The application of fungal and bacterial biocontrol agents such as *Chaetomium globosum*, *Trichoderma* sp., *Pseudomonas* sp. and *Paenibacillus* sp. and their efficacy in red rot control under sugarcane field conditions have been reported (27). Studies on the antagonistic effects of endophytic *Bacillus* sp. on *C. falcatum* under *in vitro* conditions have revealed the fungistatic properties of volatile organic compounds produced by bacterial endophytes.

The whole-cell MALDI-TOF MS technique determines the molecular weight of proteins and peptides, using mass spectra as unique identifiers for specific organisms. This method is highly effective for screening and identification due to its minimal sample preparation, rapid data acquisition, high throughput and automated processing capabilities (28). Using MALDI-TOF MS, isolates were identified, primarily from three genera: *Bacillus* sp., *Enterobacter* sp. and *Klebsiella* sp., which were found to be endophytes in sugarcane. The impact of these bacterial endophytes on inhibiting *C. falcatum* mycelial growth was assessed to evaluate their antagonistic potential. Previous research has highlighted *Bacillus* sp. for its production of various compounds such as polyhydroxyalkanoates, isobutanol, macrolactin, bacillaene, difficidin, surfactin, iturin A, fengycin and the iron-siderophore bacillibactin, which are effective against fungal pathogens in diseased sugarcane leaves (29). *Bacillus* sp. is frequently used in phytopathogen management due to their lipopeptides, which hydrolyse fungal hyphal membranes. Additionally, *Bacillus* sp. can sporulate to survive under various stressful conditions, germinate when conditions become favourable and inhibit pathogen colonization in the field.

In vitro assay of endophytic bacteria against *C. falcatum*

In this study, the bacterial endophyte *Bacillus* sp. SOE 7, isolated from the roots of sugarcane plants in the formative stage, exhibited the highest inhibitory effect on *C. falcatum* (62.25 %) (Fig. 1). This inhibition rate was notably 18.54 % higher than that of the standard *Bacillus amyloliquefaciens* strain (50.58 %). In contrast, bacterial isolates with the least or no antifungal activity included *Escherichia* sp. SOE 4, *Sphingomonas* sp. SOE 8 and *Arthrobacter* sp. SOE 14, all derived from sugarcane root samples. Additionally, siderophore and HCN production was assessed among the endophytic bacterial isolates (Fig. 2) and their levels were quantified. *Bacillus* sp. SOE 1 produced the highest amount of salicylate-type siderophores (21.70 µg mg^{-1} of cells), while *Bacillus* sp. SOE 3 produced the highest catechol-type siderophores (13.01 µg $mg^{-1}$of cells). Interestingly, *Bacillus* sp. SOE 7 produced the most HCN (15.68 µg mg^{-1} of cells) but lower amounts of salicylate-type siderophores (12.43 µg/mg of cells) and catechol-type siderophores (11.67 µg mg^{-1} of cells), compared to the standard *B. amyloliquefaciens* strain, which

Table 1. Identification of endophytic bacterial isolates using MALDI-TOF MS

S. No.	Isolate code	Identification using MALDI-TOF MS	Score value
1	SOE1	*Bacillus amyloliquefaciens* (CIP 103265T)	1.700
2	SOE2	*Bacillus subtilis* ssp*subtilis* (DSM 10T)	2.081
3	SOE3	*Bacillus mojavensis* (DSM 9205T)	1.723
4	SOE4	*Escherichia coli* (MB11464-1 CHB)	1.516
5	SOE5	*Bacillus pumilus* (DSM 1794)	1.754
6	SOE6	*Bacillus subtilis* ssp*subtilis* (DSM 5660)	1.940
7	SOE7	*B. subtilis*ssp*subtilis* (DSM 5660)	2.149
8	SOE8	*Sphingomonas*sp (B605 UFL)	1.300
9	SOE9	*E. cloacae*ssp*dissolvens* (DSM 16657T)	2.047
10	SOE10	*Acidovoraxtemperans* (DSM 7270T HAM)	1.408
11	SOE11	*Bacillus altitudinis* (CS 809_1 BRB)	1.738
12	SOE12	*Bacillus megaterium* (DSM 32T)	2.301
13	SOE13	*Bacillus mojavensis* (DSM 9205T)	1.712
14	SOE14	*Arthrobacter ramosus* (IMET 10685T HKJ)	1.310
15	SOE15	*Klebsiella pneumoniae* ssp *pneumoniae* (9295-1 CHB)	2.300

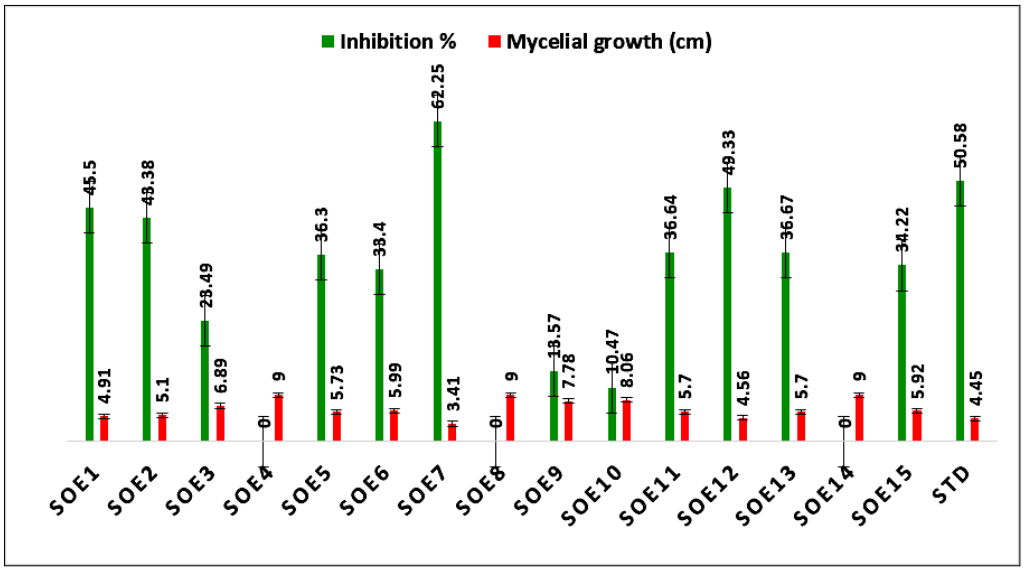

Fig. 1. Antagonism of bacterial endophytes against *Colletotrichum falcatum* (dual culture assay). The data are presented as percent inhibition and cm of mycelial growth, error bars indicate the standard deviation obtained from three replicates per treatment

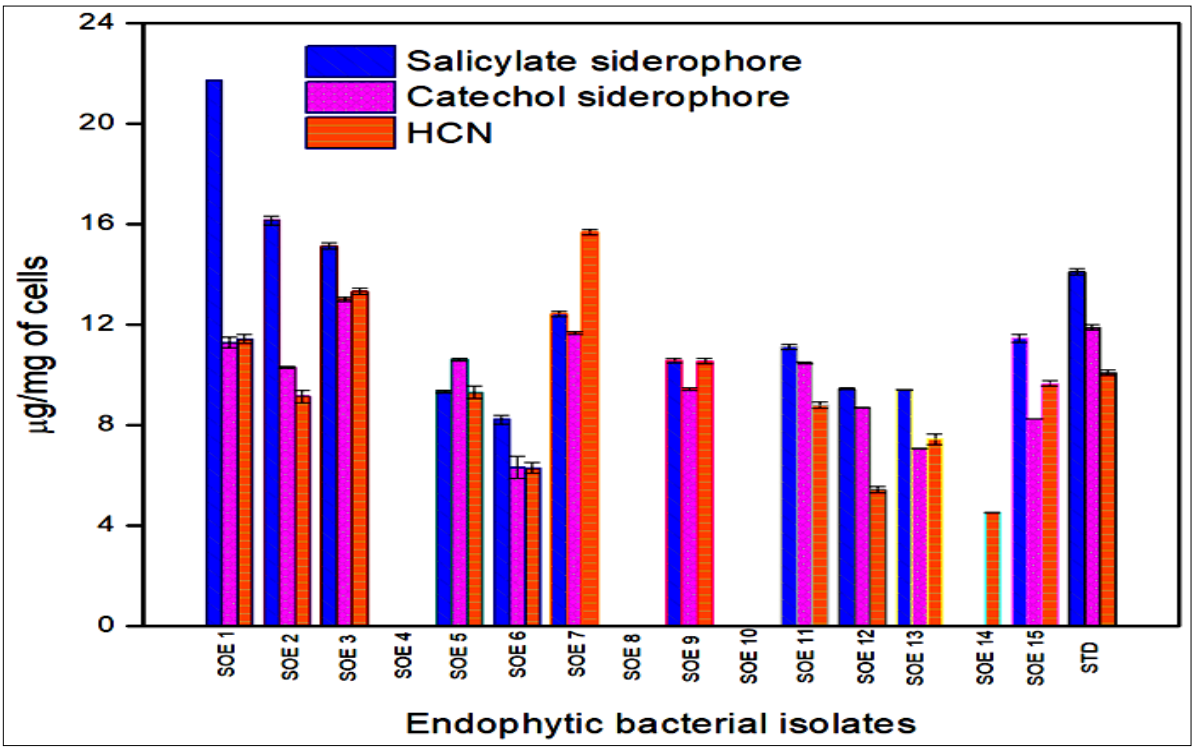

Fig. 2. Quantitative estimation of salicylate-type (blue) and catechol-type (pink) siderophores and HCN (orange) production by the endophytic bacterial isolates expressed in µg/mg of cells.

The SOE4, SOE8 and SOE10 isolates showed no production and the SOE14 isolate showed only HCN production. Error bars indicate the standard deviation obtained from three replicates per treatment

produced 14.1 µg mg^{-1} of cells and 11.89 µg mg^{-1} of cells, respectively. Overall, *Bacillus* sp. SOE 7 demonstrated the highest antifungal activity against *C. falcatum* (30) have corroborated the anti-oomycetic mode of activity for disease inhibition against foot rot disease caused by *Phytophthora capsici* in black pepper using *Bacillus amyloliquefaciens, B. pumilis* and *B. velezensis* which are endospore forming rhizosphere bacteria with more relative abundance percentage that witnessed the disease suppression of soil borne fungi. The results obtained in the present investigation are in consensus with above findings where the native *Bacillus subtilis* SOE 7 were evidentially suppresses the red rot causing *C. falcatum.*

Identification of antifungal lipopeptides of *Bacillus* sp. SOE 7 by using HPLC

Based on these findings, HPLC analysis of the antifungal lipopeptides produced by the endophytic isolate *Bacillus* sp. SOE 7 revealed the presence of two major classes of lipopeptides: iturins and surfactins. Specifically, surfactin homologs were eluted at 3.80 min (peak 1), 4.0833 min (peak 2) and 4.2833 min (peak 3), while iturin homologs were detected at 1.7833 min (peak 4) and 2.7833 min (peak 5). These retention times matched those of standard reference iturin and surfactin lipopeptides (Fig. 3). Quantitative analysis of the chromatogram indicated that SOE 7 produced 2.05 mg L^{-1} surfactin and 1.52 mg L^{-1} iturin. In contrast, other *Bacillus* sp. isolates (SOE 1 and SOE 3) did not produce detectable levels of lipopeptides (data not shown). These HPLC results suggest that the potent antifungal activity of *Bacillus* sp. SOE 7 against the pathogen is likely due to its

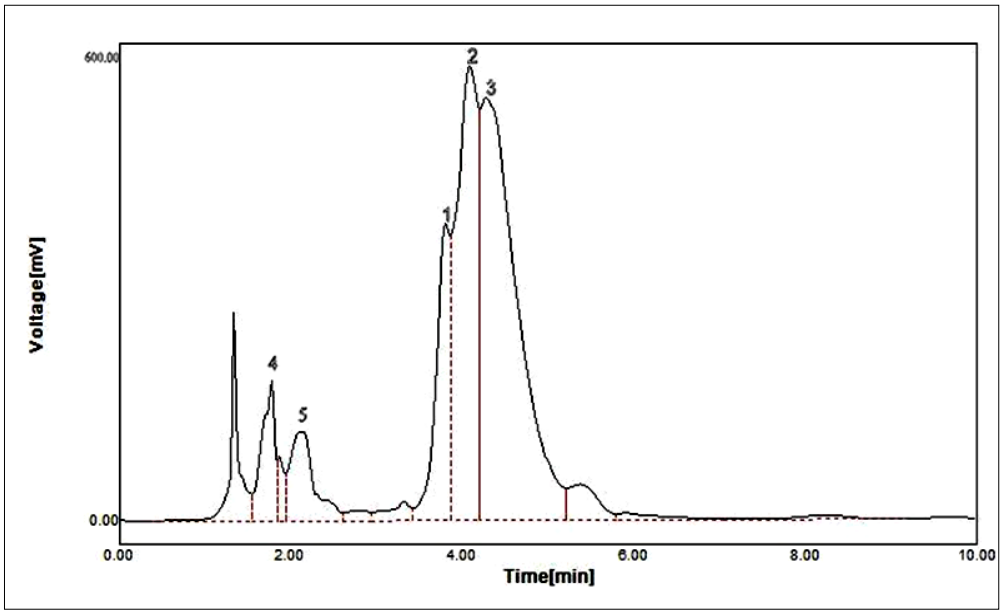

Fig. 3. HPLC chromatograms of the lipopeptide surfactin and iturin compounds extracted from the endophytic bacterial isolate *Bacillus* sp. SOE 7. Peaks 1, 2 and 3 represent surfactin homologs; peaks 4 and 5 represent Iturin homologs

production of surfactin and iturin lipopeptides. In the control of sugarcane red rot, earlier researches using pot culture tests in green house conditions (31) have highlighted that *Bacillus velezensis* YC89 possess circular chromosomes containing gene clusters which encodes secondary metabolites that are offering resistance inducer codon that produces plant defence enzymes such as beta-1-3-glucanases, chitinase, peroxidases, polyphenol oxidases, superoxide dismutase etc., that are antagonistic against *Colletotrichum* sp. The present study also envisages the concurrence of the above cited findings.

In the present study, the *Bacillus subtilis* SOE 7 isolate showed maximum inhibition percentage, less siderophore production and maximum HCN production than the other endophytic isolates. This led to the opinion that the production of lipopeptides could be the possible reason for the inhibition of *C. falcatum*. The role of lipopeptides synthesized by *Bacillus* sp. against plant disease-causing pathogens has been described (32,33). Biocontrol of fungal pathogens causing plant disease by lipopeptide production and their mechanism of action by *Bacillus subtilis* strains have been reported in sugarcane (29), against the leaf spot disease pathogen *Alternaria alternata* (34) and against *Plasmopara viticola*, which causes downy mildew in grape plants (35). Our findings indicates that lipopeptides, namely, surfactin and iturin from the native endophyte *Bacillus subtilis* SOE 7, are effective in controlling the isolated pathogen *C. falcatum* in sugarcane. The role of any potential antagonist and the compounds derived from them against a plant pathogen is likely to be specific and their mode of action should be demonstrated clearly prior to field application of the biocontrol agent. Molecular identification of the selected endophyte *Bacillus subtilis* SOE 7 was performed to study its taxonomic relatedness with *B. subtilis* since our MALDI-TOF MS results showed a match with *B. subtilis* subsp. *subtilis* DSM 5660, with a score of 2.149. Using the 16S rRNA gene within the *Bacillus subtilis* lineage pinpointed effective strains (36). This exploration of *Bacillus* species extending to endophytic regions was even delved which showed that three distinct *Bacillus* species namely, *B. amyloliquefaciens*, *B. subtilis* and *B. tequilensis*, thrive within plant endophytic environments (37).

Effect of the endophytic bacterial culture *Bacillus subtilis* SOE 7 in sugarcane under field conditions

The influence of the application of *Bacillus subtilis* SOE 7 on the reduction in red rot disease incidence was studied under field conditions. The seedlings treated with *Bacillus subtilis* SOE 7 had the lowest red rot incidence (12.0 ± 3.6 %), which was significantly lower than that in the standard culture of *Bacillus amyloliquefaciens* VB7 treatment (26.5 ± 4.1 %) and the untreated control (47.5 ± 3.8 %). However, the red rot incidence in the *Bacillus subtilis* SOE 7 treatment was slightly higher than that in the carbendazim treatment, which recorded an incidence of 7.5 ± 2.1 %. The yield attributes of the harvested canes, including cane yield, number of millable canes, brix and sucrose percentage, were assessed. The application of the endophytic bacterial culture *Bacillus subtilis* SOE 7 resulted in a significantly higher cane yield (100.4 ± 4.4 t ha^{-1}) and millable cane yield (98.3 ± 2.6 t ha^{-1}), followed by *Bacillus amyloliquefaciens* VB7 (95.3 ± 4.7 t ha^{-1}; 92.3 ± 1.8 t ha^{-1}). In contrast, the untreated control showed significantly lower cane yield and millable canes (81.7 ± 1.1 t ha^{-1}; 73.5 ± 1.9 t ha^{-1}). The brix and sucrose percentages were also notably higher following the application of *Bacillus subtilis* SOE 7 (18.6 ± 1.1 %; 16.2 ± 1.2 %, respectively), which were comparable to those achieved with *Bacillus amyloliquefaciens* VB7 (18.1 ± 1.3 %; 15.9 ± 1.2 %). The lowest brix percentage (15.2 ± 0.06 %) was observed in the untreated control. The lowest sucrose percentage (10.1 ± 0.1 %) was also observed in the untreated control.

In the present study, a distinctly greater percentage reduction of 54.72 % in disease incidence and a percentage increase (5.4 %) in cane yield were observed in response to the application of *Bacillus subtilis* SOE 7 than in response to the application of standard *Bacillus amyloliquefaciens* VB7. Additionally, a significantly greater percentage reduction of 74.74 % in disease incidence and a percentage increase of 26.8 % in cane yield were observed in comparison with those in the untreated control. In the case of carbendazim treatment, even though an increase in disease percentage (37.5 %) was observed in comparison with that of *Bacillus subtilis* SOE 7, the yield outcomes were found to be significantly lower (Table 2). Similar studies on the effectiveness of native *Bacillus* sp. in suppressing red rot disease in sugarcane under

Table 2. Red rot disease incidence and yield parameters observed in sugarcane under field conditions

Treatments	Red rot incidence (%)	Cane yield (t ha^{-1})	Millable cane yield (t ha^{-1})	Brix (%)	Sucrose (%)
Bacillus subtilis SOE 7	12.0 ± 3.6^{b}	100.4 ± 4.4^{d}	98.3 ± 2.6^{d}	18.6 ± 1.1^{c}	16.2 ± 1.2^{d}
B. amyloliquefaciens VB7	26.5 ± 4.1^{c}	95.3 ± 4.7^{c}	92.3 ± 1.8^{c}	18.1 ± 1.3^{c}	15.9 ± 1.2^{c}
Carbendazim	7.5 ±2.1^{a}	90.1 ± 5.6^{b}	88.5 ± 4.9^{b}	17.3 ± 1.2^{b}	13.2 ± 1.1^{b}
Untreated control	47.5 ± 3.8^{d}	81.7 ± 1.1^{a}	73.5 ± 1.9^{a}	15.2 ± 0.06^{a}	10.1 ± 0.1^{a}

Data presented are mean values ± standard deviation (n=10). Values in the same column with different superscript letters are statistically significant from each other (p >0.05) by DMRT.

field conditions have been reported (38). A reduction in red rot incidence was observed in a previous study following the application of *B. amyloliquefaciens* RB 19 as a seedling treatment (39). The potential mechanisms by which *Bacillus* sp. controls red rot disease include the induction of Induced Systemic Resistance (ISR) through surfactin production and enhancements in yield attributes such as weight, number of millable canes and sucrose percentage (40,41). *Bacillus subtilis* SOE 7, being an endophyte, possesses the ability to induce resistance and produce unique secondary metabolites (surfactin, iturin A, fengycin) through horizontal gene transfer and coevolution with its host. This balanced antagonism provides it with a distinct advantage over conventional microorganisms used as biocontrol agents (42).

Conclusion

In this study, endophytic bacterial species were isolated, identified and screened from sugarcane root samples to evaluate their antagonistic activity against the red rot pathogen *C. falcatum*. Using MALDI-TOF MS, the identified strains were predominantly from the genus *Bacillus*. Among these, *Bacillus subtilis* SOE 7 exhibited the strongest inhibition of the pathogen, employing multifaceted mechanisms to combat plant diseases. These mechanisms include the production of siderophores, HCN and antimicrobial lipopeptides such as surfactin and iturin compounds with significant antifungal potential that could be extracted, validated and formulated for targeted action against specific *C. falcatum* pathotypes for commercial use. The study also demonstrated the ability of *Bacillus subtilis* SOE 7 to suppress red rot disease under field conditions, functioning as an endophytic plant colonizer. Despite the slightly higher disease incidence under the SOE 7 treatment, the yield and quality parameters (cane yield, millable canes, brix and sucrose content) were significantly higher than those of both the untreated control and even the carbendazim-treated plots. These results suggest that SOE 7 not only provides disease suppression but may also promote plant vigour and productivity through mechanisms such as ISR induction and lipopeptide-mediated antagonism. Given increasing concerns about fungicide overuse, pathogen resistance and environmental residues, a valuable next step would be to explore the combined application of *B. subtilis* SOE 7 with reduced doses of carbendazim, aiming to retain high levels of disease control while minimizing chemical inputs. Such integrated biocontrol-chemical strategies align with sustainable agricultural goals highlighting the role of endophytic bacteria as eco-friendly bioagents in crop disease management, offering a sustainable alternative to chemical fungicides.

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Authors' contributions

All authors contributed equally and approved the final manuscript.

Compliance with ethical standards

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

Ethical issues: None

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