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RESEARCH ARTICLE

Bacillus subtilis (Bs-PLM), a bacterial bio-fungicide, effectively suppresses the chilli pathogen *Colletotrichum truncatum* by eliciting the host defense response

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Abstract

The present study was conducted to evaluate the efficacy of the bacterial bio-fungicide *Bacillus subtilis* against *Colletotrichum truncatum*, the causal agent of anthracnose and fruit rot in chilli (*Capsicum annuum* L.). A total of 10 *Bacillus* spp. isolates were obtained from the rhizosphere soil of major chilli-growing regions in Tamil Nadu, India. Characterisation through various methods, including molecular analysis, confirmed that these isolates belonged to the *Bacillus* genus. Additionally, molecular and *in vitro* screening identified *Bacillus subtilis* (Accession No. MZ618269) and *Bacillus cereus* (Accession No. MZ618270) as highly effective in inhibiting *C. truncatum*. Furthermore, PCR analysis revealed that *Bacillus* spp. isolates Bs-PLM and Bc-ADP possessed the genes responsible for the synthesis of the antibiotics iturin and surfactin. GC–MS analysis indicated that these isolates produced more than 30 antimicrobial compounds at different retention times, with the most notable antifungal compounds being *n*-Nonadecanol-1, 1-Hexadecanol, Behenic alcohol and Dibutyl phthalate. The volatile compounds released by Bs-PLM and Bc-ADP exhibited the highest mycelial growth inhibition. Under *in vivo* conditions, fruit lesion areas were significantly reduced when pathogen-inoculated fruits were treated with volatiles produced by these *Bacillus* strains compared to the untreated control fruits. Therefore, the *Bacillus* spp. could be used for the effective management of anthracnose and fruit rot disease in chillies.

Keywords: anthracnose; antibiotics; *Bacillus*; capsicum; GC-MS analysis; volatile compounds

Introduction

Chilli (*Capsicum* sp.) is a popular commercial spice and vegetable that comes from South and Central America and is known as a wonder spice crop (1). This species also has health benefits, including lipid-lowering, anti-diabetic, anti-hypertensive and anti-obesity (2). The biochemical compositions of chilli fruit allow consumers to use it as a bio-stimulator of blood circulation in treating various colds, relieve rhinitis, sore throats, treatment of gingivitis, regulate blood pressure, prevent gastric ulcers and intensify poor peripheral circulation (3). Capsaicin, a spicy alkaloid component found in chillies, has pharmacologically relevant biological effects and may have clinical use for pain relief, as well as reducing cancer risk by blocking carcinogens from binding to DNA and calorie intake by promoting thermogenesis (4). Chilli crops are frequently susceptible to a variety of biotic stressors, including diseases such as damping off, leaf curl and among them chilli anthracnose and fruit rot incited by *Colletotrichum truncatum* is one of the most economically significant diseases in Indias' chilli farming regions (5, 6).

Fungicides, resistance-inducing chemicals and biological agents are possible management practices for chilli anthracnose and fruit rot. Because of various ill effects with the use of fungicides, such as pathogen resistance, residual toxicity, pollution in the environment and high cost, fungicides are generally discouraged (7). Living organisms and their derivatives are used to control plant diseases, not only through direct antagonistic actions against

pathogens but also indirectly through the induction of resistance (8). Combinations of BCAs and fungicides could be used in the field to provide more reliable disease control. These types of combinations put the ideas of Integrated pest management (IPM) into effect by reducing the fungicide dose (under MRLs) or frequency of treatment while improving disease control (9).

Inducers of phytoalexins and/or elicitors of resistance in various plant species are known as resistance-inducing compounds (10). Salicylic acid has been found to produce resistance in a variety of crops (11, 12). Research indicates that no single treatment is especially effective in reducing chilli fruit rot, unlike the integrated disease management approach (13). IPM was introduced in response to growing awareness of the detrimental side effects of pesticide usage. IPM is described as the optimal combination of plant disease control measures, taking into account crop productivity, profit and safety profile (14). Therefore, the present studies were undertaken to investigate the effect of bio-protectants, fungicides and resistance-inducing chemicals for the successful and long-term management of chilli fruit-rot.

Materials and Methods

Isolation of the pathogen and pathogenicity studies

The pathogen was isolated from fruit-rot-infected chilli collected from various locations in Tamil Nadu using the tissue segment method on PDA (potato dextrose agar) medium (15). A pathogenicity test was conducted on 10 isolates of *Colletotrichum truncatum*, namely Ct-MLR, Ct-PKD, Ct-PLM, Ct-KVP, Ct-STR, Ct-ADP, Ct-KPV, Ct-SKK, Ct-SVP and Ct-BGU. Artificial inoculation was performed by applying a spore suspension of the pathogen onto chilli fruits (var. Bullet) under *in vivo* conditions using the pin-prick method. Additionally, a detached fruit assay was conducted on the same variety ('Bullet') to confirm pathogenicity. A control was maintained by injecting sterile water into the fruits, which were then placed in Petri dishes with moistened cotton to ensure high humidity. The fruits were monitored for infection for up to 11 days post-inoculation. It was observed that among the *Colletotrichum* isolates, Ct-PLM exhibited the highest fruit rot incidence (71.18 %) in both the pathogenicity tests, making it the most virulent isolate. Therefore, Ct-PLM was selected for further study. To establish the identity of the isolate, sequencing of the rDNA ITS region followed by homology searches using BLAST in the NCBI database indicated more than 90 % similarity with *C. truncatum* (16) (Accession No. MW929370).

Isolation of rhizospheric bacteria

Soil samples were collected from the rhizosphere of chilli plants grown in various regions of Tamil Nadu, India. Bacterial isolation was performed using the serial dilution method (SDM). Briefly, 5 g of soil was taken in a conical flask and mixed with 45 mL of sterile distilled water and placed on a rotary shaker at 120 rpm for 10 min. The soil mixture was then serially diluted at a 1:10 ratio with distilled water up to a 10^{-7} dilution. An aliquot of 100 µL from the 10^{-4} to 10^{-7} dilutions was spread onto nutrient agar (NA) plates. The plates were incubated at 28 ± 2 °C for three days, after which 10 morphologically distinct bacterial colonies were sub-cultured and purified. These bacterial isolates were stored as 20 % glycerol stock at -80 °C. The 10 isolates, designated as Bs-MLR, Bs-PKD, Bs-PLM, Bs-KVP, Bs-STR, Bs-ADP, Bs-KPV, Bs-SKK, Bs-SVP and Bs-BGU, were identified as *Bacillus* species based on colony and cultural morphology (data not shown) (Table 1). Biochemical analysis revealed that these *Bacillus* spp. Isolates tested positive for Gram staining, amylase activity, catalase test, Voges-Proskauer test, urease test, citrate utilisation and growth at 45 °C. However, they tested negative for growth at 4 °C.

Biochemical characterization

All the isolates of *Bacillus* were categorised based on Gram staining and biochemical analysis, motility, starch hydrolysis, gelatin liquefaction, glucose and sucrose utilisation, KOH test, methyl red test, H_2S test, maltose, lactose utilisation and urease activity, catalase activity tests carried out as per the standard procedure (17). The above biochemical parameter results, compared with Bergey's manual of determinative bacteriology and the antagonistic isolates were recognised as *Bacillus.*

Molecular characterisation of bacterial antagonists

DNA extraction

Total DNA was isolated from bacterial strains using standard protocols (18). Bacterial cultures were grown on NA broth for 48 hr at 25 °C. After that, bacterial colonies (2 mm diameter) were suspended in 100 µL of lysis solution (0.05 M NaOH, 0.25 % sodium dodecyl sulphate (SDS) and incubated for 15 min at 100 °C. The suspension was centrifuged for one min at 12000 rpm and diluted 50-fold with sterile distilled water. 5 µL of the diluted suspension was used for PCR analysis.

PCR amplification and sequencing of 16S rRNA

For the identification of rhizospheric bacterial isolates, 16S rRNA intervening sequence-specific primers 27F (5'-AGAGTTTGATCCTGGTCAG-3') and 1492R (5'-TACGGCTACCTTGTACGACTT-3') were used to amplify a 1500 bp

Table 1. Details of biocontrol agents isolated from the rhizosphere regions of chilli

S. No	Location	District	Isolates code
			***Bacillus* spp.**
1	Melur	Madurai	Bs-MLR
2	Paramakudi	Ramanathapuram	Bs-PKD
3	Palamedu	Madurai	Bs-PLM
4	Kovilpatti	Tuticorin	Bs KVP
5	Sathur	Virudhunagar	Bs-STR
6	Andipatti	Theni	Bc-ADP
7	Keelapavoor	Thenkasi	Bs-KPV
8	Sankarankovil	Thenkasi	Bs-SSK
9	Sivapuri	Cuddalore	Bs -SVP
10	Batlagundu	Dindugal	Bs -BGU

fragment (19). PCR reactions were performed in a 20 µl reaction volume containing the following components: 2 µL of 10X buffer (with 2.5 mM $MgCl_2$), 2 µL of 2 mM dNTP mixture, 5 µL of 2 µM primers, 3 U of Taq DNA polymerase, 8 µL of H_2O and 50 ng of template DNA. Amplification of the 16S rRNA gene was carried out in a thermal cycler (Eppendorf Master Cycler Gradient, Westbury, New York) under the following conditions: initial denaturation at 94 °C for 4 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 30 sec and extension at 72 °C for 1 min. A final extension was performed at 72 °C for 10 min. The PCR products were resolved on a 1.5 % agarose gel at 50 V, stained with ethidium bromide (0.5 µg/mL) and visualised using a gel documentation system (Alpha Innotech Corporation, San Leandro, California). The amplified products were then sequenced and the obtained sequences were aligned and compared with those available in the GenBank database to determine sequence homology. A phylogenetic tree was constructed using the Neighbour-Joining method.

In vitro assay of bacterial antagonists against the growth of *C. truncatum*

Efficacy of bacterial antagonists against *C. truncatum*

Antagonistic potential of ten different isolates of *Bacillus* spp was screened against the radial mycelial growth of *Colletotrichum truncatum* - Ct-PLM by dual culture technique (20). Briefly, the bacterial antagonists were streaked at one side of the Petri plate (1cm away from the periphery) on PDA medium and a 9 mm disc of 72 hr old actively growing *C. truncatum* culture was placed perpendicular to the bacterial streak on the opposite side. Control plates were maintained without bacterial streak. Three replications were maintained for each isolate. The plates were incubated at 28 ± 2 ^{0}C for 4 days. After ensuring the complete mycelial growth of the pathogen in the control plate, the percent inhibition (PI) was calculated with the following formula (21).

$$PI = \frac{Dc - Dt}{Dc} \times 100 \qquad \text{(Eqn. 1)}$$

Where, PI = percent inhibition

Dc = Average diameter of the pathogens' mycelial growth (cm) in control

Dt = Average diameter of the pathogens' mycelial growth (cm) in treatment

Mass multiplication of *Bacillus* spp

A loopful of *Colletotrichum truncatum*-effective *Bacillus subtilis* (Bs-PLM) and *Bacillus cereus* (Bc-ADP) was inoculated into nutrient broth and incubated in a rotary shaker at 150 rpm for 48 hr at room temperature. After incubation, the bacterial suspension was adjusted to a concentration of 9×10^8 colony-forming units (CFU) per mL and used for the preparation of a talc-based formulation. For this formulation, 400 mL of the two-day-old bacterial culture was mixed with 1 kg of talc powder, 15 g of calcium carbonate ($CaCO_3$) and 10 g of carboxymethyl cellulose (CMC). The mixture was shade-dried to reduce the moisture content to 20 % and then packed in polythene bags. At the time of packaging, the bacterial population in the talc powder was confirmed to be 2.5-3 × 10^8 CFU/g and was used for further *in vivo* experiments.

Efficacy of volatile compounds produced by bacterial antagonists against *C. truncatum*

The antifungal volatile compounds produced by *Bacillus* spp. were assessed *in vitro* against *C.truncatum*. For this study, the antagonistic isolates that showed effectiveness against the *C. truncatum* in the dual plate assay were used. Briefly, the *Bacillus* isolates at different concentrations, such as 1 %, 2 %, 3 % and 5 % were poured into four agar wells. Subsequently, a plug (9 mm) from the agar of each of the fungi, which were incubated for seven days, was punched and placed at the centre of a fresh PDA plate, sealed with parafilm and incubated at 28°C. Antifungal volatile compounds were identified after a week.

Extraction and identification of antifungal compounds from *Bacillus* sp.

Extraction of crude volatile compounds

The crude volatile compounds produced by the 10 selected isolates of *Bacillus* spp. were extracted separately following a modified version of the protocol (22). Briefly, a loopful culture of each isolate was inoculated into nutrient broth and incubated at 28 °C for three days. After 72 hr (stationary phase), the supernatant was collected by centrifugation at 8000 rpm for 30 min. The pH of each supernatant was then adjusted to 2.0 using diluted HCl, followed by stirring at 100 rpm in an orbital shaker for 8 hr. Antifungal compounds were extracted by adding an equal volume of ethyl acetate to the culture supernatant and shaking vigorously for 1–2 hr. To ensure complete extraction, each culture broth was extracted twice with ethyl acetate. The solvent fractions containing antifungal compounds were concentrated using a rotary evaporator at 60 °C and 80 rpm. The resulting crude extracts of extracellular antifungal compounds were dissolved separately in 1 mL of a methanol: chloroform (1:1) mixture for *in vitro* antifungal activity assays and GC-MS analysis.

Antifungal assay of volatile compounds against C.truncatum

In this study, *Bacillus* sp. was cultured in nutrient broth at 28 ± 2 °C. After three days of incubation, 10 mL of the culture was centrifuged at 10000 rpm for 20 min. The collected supernatant was first filtered through Whatman No.1 filter paper and then passed through a 0.34 µM Millipore filter. Different concentrations of the culture filtrate (0.1, 0.15, 0.2 and 0.25 mg/µL of mycelial extract) were introduced into four wells, each placed 2 cm apart from the centrally positioned *C. truncatum* virulent isolate Bs-PLM (5 mm disc) inoculated on a PDA plate. A control plate without the culture filtrate was maintained. All plates were incubated at 28 ± 2 °C for 7 days. The assay was performed twice, with four replications. PI of radial mycelial growth of the pathogen was calculated using the formula (21).

$$PI\,(\%) = C\text{-}T/C \times 100 \qquad \text{(Eqn. 2)}$$

where C represents the mycelial growth of *C. truncatum* in the control plate and T represents the mycelial growth in the treated plate.

Efficacy of volatiles on the fruit rot of chillies

Freshly harvested, mature and healthy chillies were used in this experiment. The chillies were washed with water, surface-sterilised with 2 % sodium hypochlorite for 10 sec, rinsed under sterile running water for 1 min and air-dried. Each fruit was wounded by pinpricking. A 20 µL bacterial suspension of *Bacillus* sp. (1×10^6 CFU/mL), obtained by culturing on NA for 24 hr at 27 ± 3 °C, was sprayed over the wounded fruits. Wounded fruits sprayed with an equal volume of sterile water served as the control. Each treatment was

performed in triplicate, with 18 fruits sprayed with *Bacillus* sp. considered as one replicate. The experiment was repeated three times. After eight days of spraying, the diameter of decayed spots and lesion areas was measured and compared to evaluate the treatment effects (23).

Detection of antibiotics- Iturin and Surfactin

PCR amplifications were carried out in a 20 µL reaction mixture. (10 µL of 2x PCR Master Mix (containing buffer, dNTPs, polymerase and $MgCl_2$) + 1 µL of each primer + 1 µL of template DNA +7 µl of sterile water). Thermal cycling conditions followed were: initial denaturation at 95 ºC for 15 min; 35 cycles of 95 ˚C for 1 min; 55 ˚C or 52 ˚C for 1 min and 72 ˚C extension for 1.5 min; and a final extension at 72 ˚C for 7 min. A total of 5 µL of each amplification reaction was analysed by electrophoresis using a 1.5 % agarose gel, followed by ethidium bromide staining and ultraviolet visualisation. The primers of Iturin (*Itu*D), Surfactin (*SRFAB*) and all the reagents were obtained from Bangalore Genei Pvt. Ltd., Bangalore, India (Table 2) (24).

GC-MS analysis of crude antibiotics produced by bacterial antagonists

Detection of active bio-molecules present in the volatile antimicrobial compounds of *Bacillus* spp. (Bs-PLM and Bc-ADP) responsible for the suppression of *C.truncatum* were carried out through GC-MS (GC Clarus 500 Perkin Elmer). Volatile compounds were identified by GC-MS using a column Elite-5MS (100 % Dimethyl poly siloxane), 30 × 0.25mm × 0.25µm df equipped with GC Clarus 500 Perkin Elmer. The turbo mass gold-perkin-Elmer detector was used. The carrier gas flow rate was 1 ml per min, split 10:1 and the injected volumes were 3µL. The column temperature was maintained initially at 110 ˚C at the rate of 10 ˚C /min - no hold followed by an increase up to 280 ˚C at the rate of 5 ˚C/min and 9 min (hold). The injector temperature was 250 ˚C and this temperature was held constant for 36 min. The electron impact energy was 70 eV, Julet line temperature was set at 2000 ˚C and the source temperature was set at 200 ˚C. Electron impact (EI) mass scan (m/z) was recorded in the 45-450 a MU range. Compounds present in the crude sample were identified by comparing the obtained spectra with the NIST Ver. 2005 MS data library (25).

Statistical analysis

S rRNA ITS sequence analysis of bacterial isolates

The rRNA homology searches were performed using the BLAST program of the National Center for Biotechnology Information (NCBI), USA. Sequences and accession numbers to be compared with our sequences were retrieved from the GenBank database (16). Average linkage cluster analysis of aligned sequences for the construction of a phylogenetic tree was performed with the Mega 7 software. Clustering was determined by UPGMA analysis of pairwise genetic distance values. Nucleotide sequences were aligned by using CLUSTAL W 1.81.

In the present study, all the *in-vitro* experiments were conducted in a Completely Randomised Block Design (CRD). The data obtained from all the experiments were statistically analysed following standard methods (26). To find out the significant difference between the treatments Least Significant Difference (LSD) at 5 % was computed in the AGRES. The data showing percentages were transformed into arcsine values before statistical analysis.

Results and Discussion

Pathogenicity studies

It was observed that among the *Colletotrichum* isolates, Ct-PLM exhibited the highest fruit rot incidence (based on the screening trails) in both the pathogenicity tests making it the most virulent isolate. Therefore, Ct-PLM was selected for further study. To establish the identity of the isolate, sequencing of the 16S rRNA followed by homology searches using BLAST in the NCBI database performed indicated more than 90 % similarity with *C. truncatum* (NCBI Accession No. MW929370).

Isolation and efficacy of rhizospheric bacterial isolates against *C. truncatum*

In vitro efficacy of different isolates of *Bacillus* spp. against the mycelial growth of *C. truncatum*.

Among the 10 isolates (Bs -MLR, Bs -PKD, Bs -PLM, Bs -KVP, Bs -STR, Bs -ADP, Bs -KPV, Bs -SKK, Bs -SVP and Bs -BGU) of *Bacillus* spp. evaluated for their antagonistic potential over the virulent isolates of *C.truncatum* Ct-PLM by dual culture technique the minimum mycelial growth (3.82 cm) with the mycelial reduction of 57.55 % over control was noticed in the isolate Bs- PLM followed by Bc-ADP with mycelial reduction of 54.56 % against the pathogen whereas the isolate Bs-SVP showed the least mycelial growth reduction (30.77 %) (Table 3).

In the present investigations, *Bacillus* spp. (Bs-PLM and Bc-ADP) showed 57.55 % and 54.56 % mycelial reduction against isolate of *C. truncatum*. The present results were in conformity with the previous findings, reported the bacterial antagonist on growth of *C.capsici* isolate *in vitro* and found that *B.subtilis* (Bs-3) isolate was found to record maximum growth reduction of *C.capsici* by 62.22 % over the control followed by *B.subtilis* (Bs-4) which recorded growth reduction of 55.55 % (27). Research indicates that bacterial bioagents i.e. *Bacillus cereus* showed 52.04 % mycelial growth inhibition of *C. gloeosporioides* (28). Marine bacteria strains *B. subtilis* RBM02 strain and *B. subtilis* RBM01 strain were found to inhibit *C. gloeosporioides* at high inhibition rate of 97 % and 93 % (29).

Biochemical characterization of *Bacillus*

The isolates of *Bacillus* were categorized based on the structure, Gram staining and biochemical analysis mentioned in the Table 4. All the *Bacillus* isolates shown positive results for Gram staining, motility, starch hydrolysis, gelatin liquefaction, glucose and sucrose utilization and negative results for KOH test, methyl red test, H_2S test,

Table 2. Primers of antimicrobial peptide (AMP) genes of *Bacillus* spp.

Lipopeptide antibiotics	Gene	Primer sequence (5'-3')	Product size	References
Iturin D	ItuD	ITUD-F1(5'-TTGAAYGTCAGYGCSCCTTT-3') ITUD-R1(5'-TGGCMAAATGGSGTCGT-3')	482 bp	(24)
Surfactin	SRFAB	SRFA-F1(5'- AGAGCACATTGAGCGTTACAAA-3') SRFA-R1(5'-CAGCATCTCGTTCAACTTTCAC -3')	626 bp	(24)

Table 3. *In-vitro* efficacy of *Bacillus* spp. isolates against the mycelial growth of *Colletotrichum truncatum*

Sl. No.	Isolates	Mycelial growth (cm)*	Percent reduction over control (%)
1	Bs-MLR	4.90[b]	45.54
2	Bs-PKD	4.18[bc]	53.56 (47.04)
3	Bs-PLM	3.82[a]	57.55 (49.34)
4	Bs KVP	4.37[c]	51.43 (45.82)
5	Bs-STR	5.13[de]	42.75 (40.83)
6	Bc-ADP	4.09[b]	54.56 (47.62)
7	Bs-KPV	5.49[e]	39.01 (38.65)
8	Bs-SSK	5.02[d]	44.21 (41.68)
9	Bs –SVP	6.23[g]	30.77 (33.69)
10	Bs –BGU	5.76[f]	36.00 (36.87)
C	Control	9.00	-
CD (*P* = 0.05)		**0.24**	-

*Mean of three replications. Data in parentheses are arc sine transformed values. Means in a column followed by the same superscript are not significantly different (*P* = 0.05).

Table 4. Biochemical parameters of *Bacillus* isolates

Characters	Bs-MLR	Bs-PKD	Bs-PLM	Bs KVP	Bs-STR	Bc-ADP	Bs-KPV	Bs-SSK	Bs –SVP	Bs -BGU
Gram-strain reaction	+	+	+	+	+	+	+	+	+	+
Motility test	+	+	+	+	+	+	+	+	+	+
KOH test	-	-	-	-	-	-	-	-	-	-
Starch hydrolysis	+	+	+	+	+	+	+	+	+	+
Gelatin liquefaction	+	+	+	+	+	+	+	+	+	+
Methyl Red test	-	-	-	-	-	-	-	-	-	-
H_2S test	-	-	-	-	-	-	-	-	-	-
Catalase test	+	+	+	+	+	+	+	+	+	+
Glucose utilization	+	+	+	+	+	+	+	+	+	+
Sucrose utilization	+	+	+	+	+	+	+	+	+	+
Maltose utilization	-	-	-	-	-	-	-	-	-	-
Lactose utilization	-	-	-	-	-	-	-	-	-	-
Urease activity test	-	-	-	-	-	-	-	-	-	-

Whereas + indicates Positive and - indicates negative.

maltose, lactose utilization and urease activity test. All the antagonistic *Bacillus* isolates were found to produce catalase activity. The above biochemical parameter results compared with Bergey's manual of determinative bacteriology and the antagonistic isolates were recognized as *Bacillus*.

Molecular characterization of *Bacillus* sp. isolates

rRNA sequence analysis

The PCR amplification of *Colletotrichum truncatum*-effective *Bacillus* sp. isolates, Bs-PLM and Bc-ADP, using universal primers (forward 27F and reverse 2492R) resulted in the generation of a 1500 bp product specific to the primers used (Fig. 1). Further sequence analysis of the 16S rRNA gene of *Bacillus* sp. isolates Bs-PLM and Bc-ADP revealed 99.22 % homology with *Bacillus subtilis* and *Bacillus cereus* sequences in the NCBI database. The assigned accession numbers are MZ618269 and MZ618270, respectively. These findings align with previous study results, identified aerobic Gram-positive bacteria through PCR amplification of the 16S rDNA gene sequence as *Bacillus subtilis* (30). Similarly, research characterized, screened and selected a soil bacterium, *Bacillus subtilis* isolate UASP17, from the rhizosphere region due to its high biocontrol activity (31). Molecular characterization revealed that amplification and sequencing of the 16S rRNA gene (1300 bp PCR product) exhibited 99 % similarity with reference strains of *B. subtilis* in the NCBI database. Since the 16S rRNA gene, located on the 30S ribosomal subunit, is species-specific and amplifies at 1500 bp, sequencing confirmed the *Bacillus* status of the strain (PCR 48).

Phylogenetic analysis of *Bacillus* spp.

The sequences and accession numbers of other *Bacillus* spp. retrieved from from Gene bank database for the construction of phylogenetic tree are provided in Table 5 and Fig 2. Large subunit ribosomal RNA gene sequences based various *Bacillus* species are identified and deposited in NCBI database (MZ618269, MZ618270, MN782244, MZ965049, KT986188, MN704495, LC512758, MF138127, MK116582, MK177189, KT986156). Further sequence analysis of the 16S rRNA gene of *Bacillus* sp. isolates Bs-PLM and Bc-ADP revealed 99.22 % homology with *Bacillus subtilis* and *Bacillus cereus* sequences in the NCBI database. The assigned accession numbers are MZ618269 and MZ618270, respectively. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the

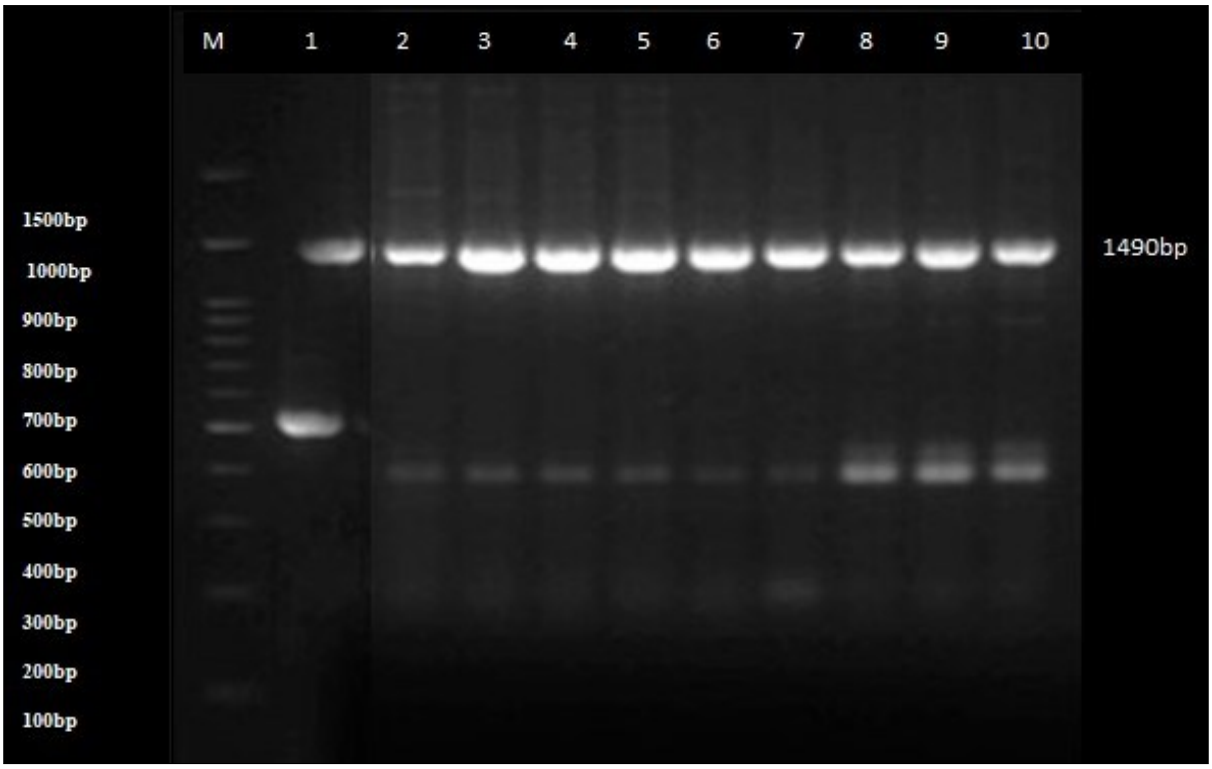


Fig. 1. PCR amplified product from *Bacillus subtilis* (Bs-PLM) and *Bacillus cereus* (Bc-ADP) isolates for the 16s rRNA gene, along with the marker.

Table 5. Large subunit ribosomal RNA gene sequences used for the construction of phylogenetic tree

S. No	Large subunit ribosomal RNA gene sequences	Accession number
1	*Bacillus subtilis* strain Bs PLM	MZ618269
2	*Bacillus cereus* strain Bc ADP	MZ618270
3	*Bacillus subtilis* subsp. *spizizenii* strain RKTK 4	MN782244
4	*Bacillus tequilensis* strain EP-05	MZ965049
5	*Bacillus sonorensis* strain Xmb063	KT986188
6	*Bacillus subtilis* subsp. *stercoris* strain EGI275	MN704495
7	*Bacillus licheniformis* KR7-12	LC512758
8	*Bacillus amyloliquefaciens* strain RB32	MF138127
9	*Bacillus mojavensis* strain	MK116582
10	*Bacillus vallismortis* strain DU12	MK177189
11	*Bacillus axarquiensis* strain Xmb027	KT986156
12	*Bacillus paramycoides* strain K5.2	MT299703
13	*Trichoderma asperellum* clone SF_94	MT529370

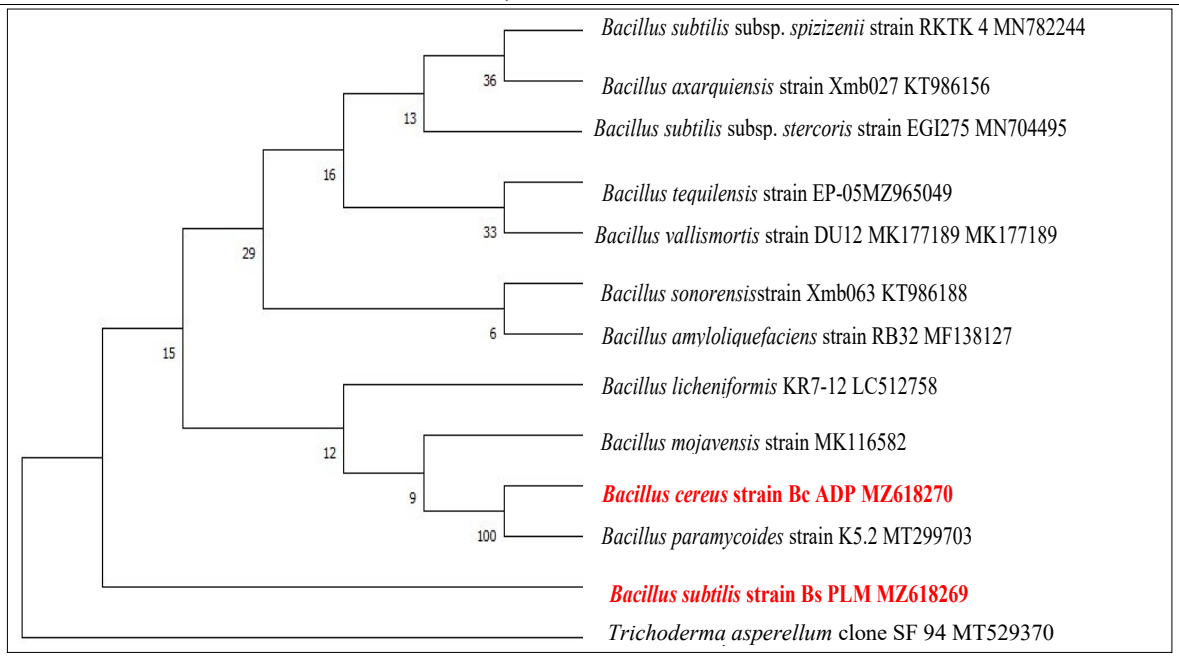


Fig. 2. Phylogenetic tree of *Bacillus* species based on 16S rRNA gene sequences. The isolates of *B. cereus* strain Bc ADP MZ618270 and *B. subtilis* Bs PLM MZ618269 were grouped as separate subclusters.

bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 20 nucleotide sequences. Codon positions included were 1st+ 2nd+ 3rd+ Noncoding. All positions containing gaps and missing data were eliminated (complete deletion option). There was a total of 626 positions in the final dataset. Evolutionary analyses were conducted in MEGA X. Phylogenetic analysis of 16 SrRNA gene of the study isolates and other *Bacillus* sp. retrieved from NCBI revealed the formation of three major clusters. The study isolates of *B. cereus* strain Bc ADP MZ618270 and *B. subtilis* Bs PLM MZ618269 were grouped as separate subclusters. On observing the sequences and evolutionary distance among the species, it was clear that all the *Bacillus* isolates had minimum evolutionary distance with closely related species and shared a common ancestor, indicating that the present isolate belonging to the same genotype of *Bacillus* sp (Fig. 2).

GC-MS analysis of antimicrobial compounds produced by *Bacillus* sp.

GC-MS analysis of *Bacillus* sp. detected more than 30 compounds at different retention time. At a particular retention time, components are separated out according to their mass/charge ratio. The analyses of extracellular antifungal compounds from *Bacillus* spp. (Bs-PLM and Bc-ADP) through GC-MS, resulted in the identification of a total of 50 compounds which included a range of alcohols, aldehydes, ketones, aliphatic alkanes, organic acids, etc. Major compounds detected in the extract and their molecular weight, retention time and antimicrobial property etc. are given in Table 6-7 and Fig. 3.

Antifungal compounds detected from the *B. subtilis* isolate Bs-PLM

The crude metabolites of *B. subtilis* strain Bs-PLM were analysed using GC-MS and the identified compounds were compared against the NIST 2005 library. The analysis revealed that the crude antifungal metabolite contained various compounds, including n-Nonadecanol-1, 1-Hexadecanol, 1-Pentadecene, Behenic alcohol, Dibutyl phthalate, 1-Dodecanol, 1-Heptacosanol, 1,2-Benzenedicarboxylic acid, bis(2-methyl), 1-Hexacosanol, Phthalic acid butyl 2-pentyl ester, Tetradecane, Hexadecane and Heneicosane (Table 6). The peak area of each compound was directly proportional to its quantity in the extract (32). Among the identified compounds, n-Nonadecanol-1, 1-Hexadecanol and 1-Pentadecene were the predominant metabolites produced by Bs-PLM. Behenic alcohol and Dibutyl phthalate were the secondary predominant compounds, while 1-hexacosanol and Heneicosane were the least abundant. n-Nonadecanol-1 exhibited the highest peak area of 13.43 % with a retention time of 27.66, whereas Heneicosane had the lowest peak area of 1.79 % with a retention time of 27.78.

Antifungal compounds detected from the *B. cereus* isolate Bc-ADP

The GC-MS analyses of crude antifungal metabolite revealed the presence of different compounds such as 1-Hexadecanol, 1-Nonadecene, 1-Tetradecene, Behenic alcohol, Dibutyl phthalate, 1-Dodecanol, 1-Heptacosanol, 1,2-Benzenedicarboxylic acid, bis (2-methyl, Butanoic acid, 2-methyl-, Oxirane-2-carboxylic acid, ethyl ester, Butanoic acid, 2-methyl-, 1-Hexacosanol, Heneicosane and Tetradecane. The peak area of the compound was directly proportional to its quantity in the extract. The highest area percentage of 12.88 % was observed in 1-Hexadecanol with a retention time of 22.93, followed by 1-Nonadecene, which had an area percentage of 12.23 % and a retention time of 27.63. The lowest area percentage of 2.18 % was recorded for Tetradecane (Table 7).

Recent research has shown that the primary and secondary metabolites produced by *B. subtilis* exhibit strong antifungal properties and have significant application potential (22). The findings of our study align closely with previous research on biocontrol agents against various fungal pathogens. Research indicates that extracted effective antifungal volatile organic compounds (VOCs) using headspace solid-phase microextraction and identified them through GC-MS (32). Eight *Bacillus* strains were found to produce ketones, including 3-methyl-2-pentanone, 2-heptanone, 2-octanone, 2-decanone, 5-methyl-2-hexanone, 2-nonanone, 2-dodecanone, 2-undecanone, 5-methyl-2-heptanone and 2-pentanone. These bioactive volatiles serve as rich resources and have been shown to effectively inhibit the growth of *Fusarium solani*. GC-MS analysis of VOCs produced by *B. subtilis* CF-3 revealed the presence of 74 compounds. Among these, 2,4-di-tert-butylphenol, 1-octanol and benzothiazole exhibited inhibitory effects against *M. fructicola* and *C. gloeosporioides*. Notably, benzothiazole demonstrated the strongest inhibitory effect on the mycelial growth of both pathogens under *in vivo* conditions, leading to a higher percentage of healthy fruit (33). Research indicates that 2,4-di-tert-butylthiophenol and benzothiazole, identified in the present study, are key inhibitory VOCs produced by *B. subtilis* CF-3.

Effect of volatiles produced by *Bacillus* spp. against *C. truncatum in vivo*

The *Bacillus* spp. isolates Bs-MLR, Bs-PKD, Bs-PLM, Bs-KVP and Bc-ADP which showed potential antagonistic activity against *C. truncatum* in dual culture assay, were further evaluated by testing the effect of their volatile production against *C. truncatum.* The results indicated in general, volatiles produced by *Bacillus* spp. Significantly inhibited the mycelial growth of the pathogen. However, the highest growth inhibition was observed in isolates Bs-PLM (55.22 %) and Bc-ADP (53.44 %) compared to the control. In contrast, Bs-MLR exhibited the lowest mycelial growth inhibition (42.88 %) against *C. truncatum* (Table 8).

Further *in vivo* evaluation of volatiles produced by *Bacillus* spp. Isolates-Bs-MLR, Bs-PKD, Bs-PLM, Bs-KVP and Bc-ADP-demonstrated their effectiveness in inhibiting the occurrence and progression of symptoms caused by the fruit-rot pathogen *C. truncatum*. In the inoculated control fruit, the lesion area expanded to 8.16 mm^2 after seven days of incubation at room temperature. However, in fruits exposed to volatiles from Bs-MLR, Bs -PKD, Bs-PLM, Bs-KVP and Bc-ADP, lesion areas were restricted to 3.18, 3.32, 2.33, 3.57 and 3.05 mm^2, respectively. Isolates Bs-MLR and Bc-ADP, as well as Bs-MLR and Bs-PKD, showed statistically similar effects (Table 9). Antifungal volatiles produced by antagonistic microbes have been extensively studied against various plant pathogens. Research indicates that screened four effective *Bacillus* spp. strains with antagonistic activity against the mango anthracnose pathogen. Among them, *B. pumilus* (TB09) and *B. thuringiensis* (TB72) exhibited 88.87 % and 80.07 % mycelial growth inhibition against *Colletotrichum* sp. under *in vitro* conditions (23). *In vivo* trials, disease inhibition by TB09 and TB72 against the anthracnose pathogen reached 94.28 % and 87.06 %, respectively. Similarly, research indicates that the antifungal effects of VOCs produced by *B. amyloliquefaciens* CPA-8 against *Monilinia laxa*,

Table 6. GC-MS analyses of the volatile profile of *Bacillus subtilis*- Bs-PLM

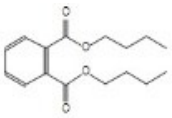
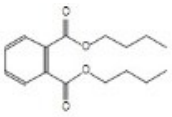
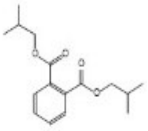
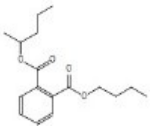
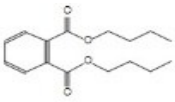
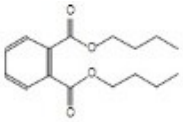
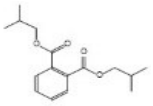
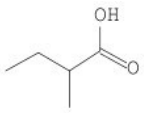
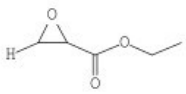
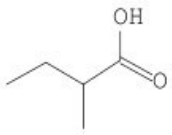
S.No.	Compound Name	RT	Area %	Molecular weight	Antagonist activities	Properties	Structure	Reference
1.	n-Nonadecanol-1	27.66	13.43	284	*Staphylococcus aureus*	Anti bacterial		(32)
2.	1-Hexadecanol	22.95	14.14	242	*Podosphaera pannosa*	Anti fungal		(35)
3.	1-Pentadecene	17.75	11.70	210	*Salmonella typhimurium*	Antibacterial		(40)
4.	Behenic alcohol	31.92	10.36	326	*Herpes simplex virus*	Antiviral		(27)
5.	Dibutyl phthalate	30.17	8.78	278	*Streptomyces albidoflavus*	Antibacterial		(32)
6.	1-Dodecanol	12.13	7.54	186	*S. aureus*	Antibacterial		(32)
7.	Dibutyl phthalate	31.17	7.10	278	*S.albidoflavus*	Antibacterial		(32)
8.	1-Heptacosanol	36.58	6.83	396	*Staphylococcus epidermidis*	Antibacterial		(27)
9.	1,2-Benzenedicarboxylic acid, bis(2-methyl	29.13	5.12	278	*A.alternata*	Antifungal		(35)
10.	1-Hexacosanol	41.09	3.74	382	*S. aureus*	Antibacterial		(40)
11.	Phthalic acid, butyl 2-pentyl ester	31.52	2.63	306	*F.oxysporum* f. sp.*cubense*	Antifungal		(29)
12.	Tetradecane	17.94	2.32	198	*Ceratocystis fimbriata*	Antifungal		(32)
13.	Hexadecane	23.11	2.28	226	*P.aeruginosa*	Antibacterial		(35)
14.	1-Hexacosanol	44.95	2.24	382	*Staphylococcus aureus*	Antibacterial		(35)
15.	Heneicosane	27.78	1.79	296	*Streptococcus pneumoniae*	Antimicrobial		(40)

Table 7. GC-MS volatile profile of Bc-ADP isolate *Bacillus cereus*

S. No	Compound Name	RT	Area %	Molecular weight	Antagonist activities against	Properties	Structure	Reference
1.	1-Hexadecanol	22.93	12.88	242	*Pestalotiopsis theae*	Antifungal		(32)
2.	1-Nonadecene	27.63	12.23	266	*Staphylococcus aureus*	Antibacterial		(35)
3.	1-Tetradecene	17.73	10.51	196	*Penicillium chrysogenum*	Antifungal		(40)
4.	Behenic alcohol	31.90	10.06	326	*Herpes simplex virus*	Antiviral		(27)
5.	Dibutyl phthalate	30.14	8.18	278	*S.albidoflavus*	Antibacterial		(32)
6.	Dibutyl phthalate	31.14	6.65	278	*S.albidoflavus*	Antibacterial		(32)
7.	1-Dodecanol	12.12	6.32	186	*S. aureus*	Antibacterial		(27)
8.	1-Heptacosanol	36.55	6.05	396	*Staphylococcus epidermidis*	Antibacterial		(35)
9.	1,2-Benzenedicarboxylic acid, bis(2-methyl	29.12	5.54	278	*A.alternata*	Antifungal		(32)
10.	Butanoic acid, 2-methyl-	5.02	4.85	102	*Ralstonia solanacearum*	Antifungal		(32)
11.	Oxirane-2-carboxylic acid, ethyl ester	6.34	4.77	88	*Fusarium oxysporum*	Antifungal		(27)
12.	Butanoic acid, 2-methyl-	5.06	4.17	102	*Ralstonia solanacearum*	Antifungal		(29)
13.	1-Hexacosanol	41.07	3.23	382	*Staphylococcus aureus*	Antibacterial		(32)
14.	Heneicosane	23.10	2.38	296	*Streptococcus pneumoniae*	Antimicrobial		(35)
15.	Tetradecane	17.93	2.18	198	*Ceratocystis fimbriata*	Antifungal		(35)

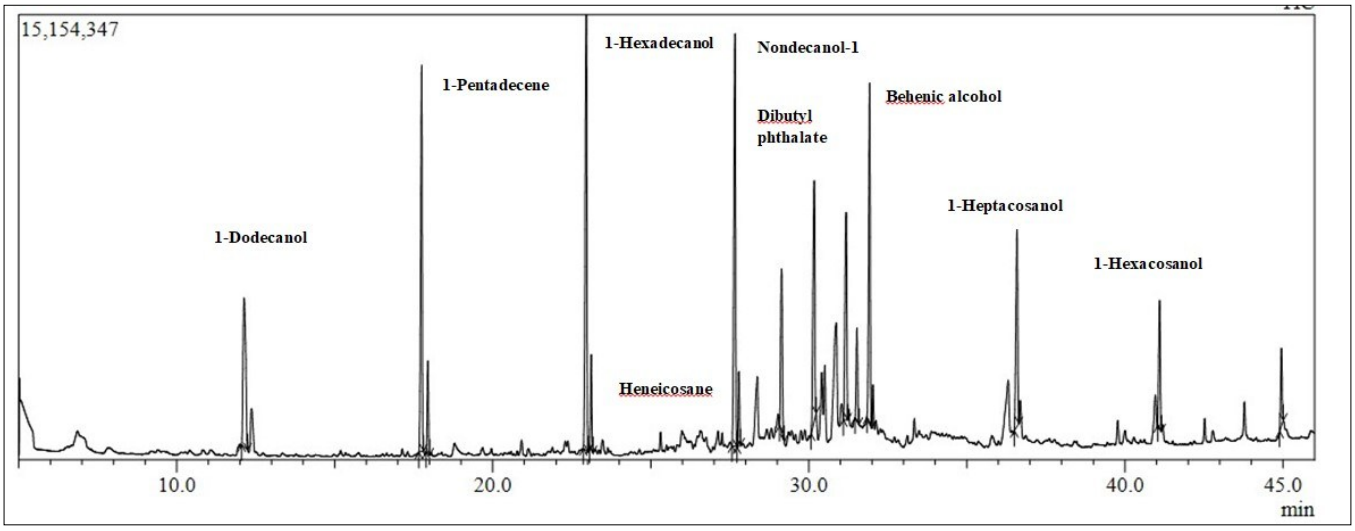


Fig. 3. Gas chromatogram of compounds identified from *B. subtilis* (Bs-PLM).

Table 8. *In vitro* efficacy of volatiles produced by *Bacillus* spp. against a virulent *Colletotrichum truncatum* isolate

S. No.	*Bacillus* sp. Isolates	Diameter of mycelial growth (cm)*	PROC
1	Bs-MLR	5.14^{d}	42.88 (40.91)
2	Bs-PKD	4.47^{c}	50.33 (45.19)
3	Bs-PLM	4.03^{a}	55.22 (48.00)
4	Bs-KVP	4.73^{b}	47.43 (43.53)
5	Bc-ADP	4.19^{a}	53.44 (46.97)
6	Control	9.00^{e}	-
	CD (*P* = 0.05)	0.19	

*Mean of three replications. Data in parentheses are arc sine transformed values. Means in a column followed by the same superscript are not significantly different (*P* = 0.05).

Table 9. *In vivo* efficacy of volatiles produced by *Bacillus* spp. against a virulent *Colletotrichum truncatum* isolate in chilli fruit

S. No.	*Bacillus* sp. Isolates	Diameter of Mycelial growth (mm²)*	PROC
1	Bs-MLR	3.18^{bc}	61.02
2	Bs-PKD	3.32^{c}	59.31 (50.37)
3	Bs-PLM	2.33^{a}	71.44 (57.70)
4	Bs-KVP	3.57^{d}	56.23 (48.58)
5	Bc-ADP	3.05^{b}	62.62 (52.31)
6	Control	8.16^{e}	-
	CD (*P* = 0.05)	0.24	

*Mean of three replications. Data in parentheses are arc sine transformed values. Means in a column followed by the same superscript are not significantly different (*P*=0.05).

M. fructicola and *Botrytis cinerea*, the three major postharvest pathogens of sweet cherry fruit (34). Their findings revealed approximately 82 % suppression of mycelial growth at the highest volatile concentration (1.35 mL/mL) under *in vitro* conditions, while in *in vivo* experiments, disease sporulation was completely inhibited, with no spores observed on decayed fruits.

Detection of antibiotic biosynthetic genes of *Bacillus* spp. by PCR amplification

Detection of iturin and surfactin

In general detection of antibiotic production by a particular bacterium is important in determining its capacity to be a good biocontrol agent for plant diseases. The majority of *Bacillus* species have the capacity to produce two to four antimicrobial peptides (AMPs) in the culture filtrates (35). Lipopeptide antibiotics, including the families surfactin, iturin and fengycin produced by *B. subtilis*, are known to have strong antagonistic activity and stability (36). Iturins, bacillomycin and fengycin have strong antifungal activity while bacilysin has antibacterial activity (37). Iturin family (iturin, mycosubtilin and bacillomycin) can disrupt surface tension of fungal cell membranes, leading to the formation of micropores and leakage of important ions, finally causing cell death (38, 39). Thus, antibiotic-producing *Bacillus* species antagonistic to fungal and bacterial pathogens are good candidates for suppressing plant diseases. In the present study, among the five isolates of *Bacillus* spp., the isolates Bs-PLM and Bc-ADP showed positive reaction for the presence of antibiotic gene *ItuD* and *srfAB* with amplicon size of 482bp and 626bp, respectively and thus have the potential to

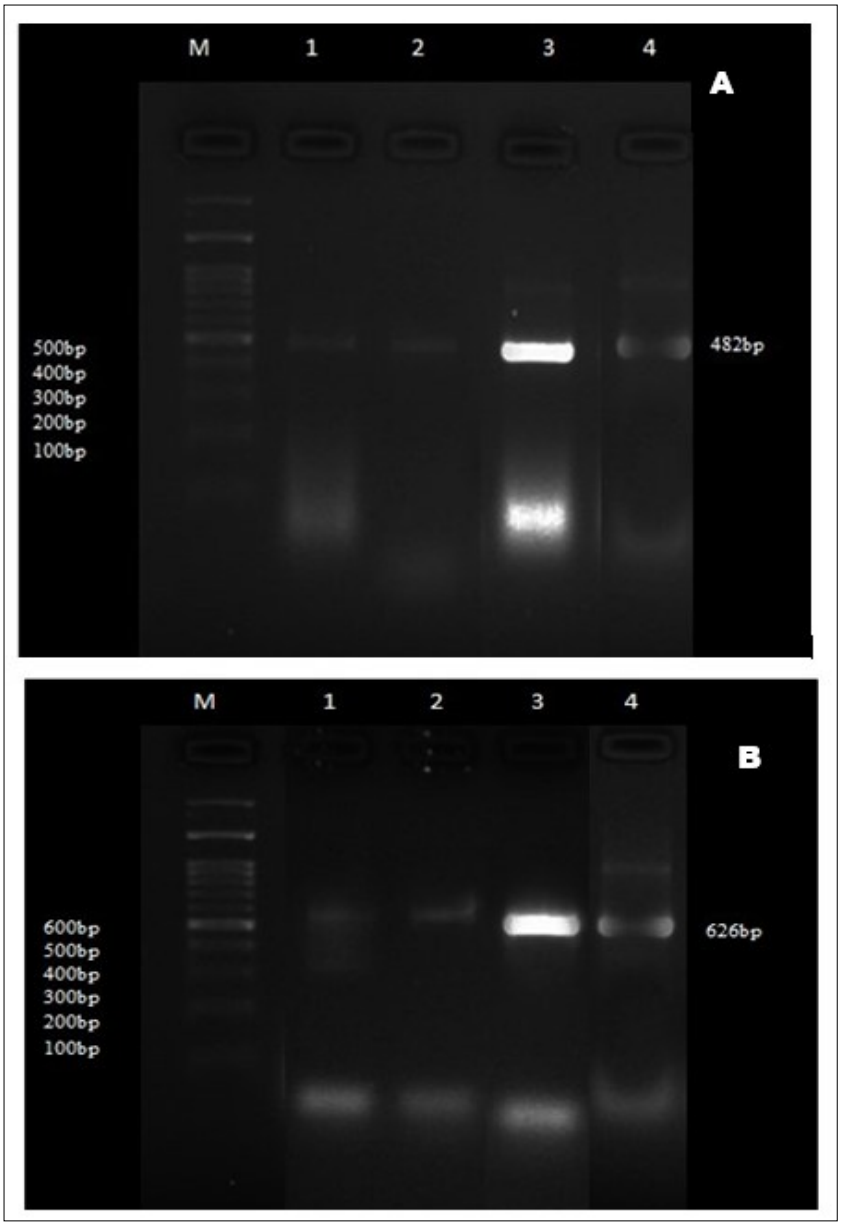


Fig. 4. PCR amplification of antibiotic biosynthetic genes A**)** ItuD (Iturin) of *Bacillus* sp. B**)** SRFAB (Surfactin) of *Bacillus* sp.

synthesise the antibiotics like iturin and surfactin (Fig. 4a-4b). It is very important to mention here that the *Bacillus* spp. Isolates Bs-PLM and Bc-ADP are also found to inhibit the fungal pathogen *C. truncatum* effectively and the reason might be due to the presence of different AMP genes, including Iturin (*Itu D)* and Surfactin (*srfAB)*. These AMP genes might have complemented the synergistic activity of the antagonists against the chilli fruit-rot pathogen. Similarly, research indicates that antagonistic activity of *B. subtilis* AKP against *C. capsici* and further analysed for the presence of antimicrobial peptide biosynthesis genes. *B. subtilis* AKP showed the presence of srfAA (201bp), fenD (670bp) and ituC (423bp) gene clusters, which were principally involved in antimicrobial activity against *C. capsici* (40).

Conclusion

In the present study, *Bacillus* isolates Bs-PLM (*B. subtilis* Palamedu) and Bc-ADP (*B. cereus* Andipatti) demonstrated strong potential as bio-fungicides, exhibiting the highest inhibition of *C. truncatum*, the causative agent of anthracnose in chilli. These isolates were also capable of synthesising antibiotics such as iturin and surfactin and producing effective VOCs. These combined factors contributed to significant pathogen growth inhibition and a substantial reduction in fruit lesion area compared to the untreated control. Therefore, the above-mentioned *Bacillus* isolates could be effectively utilised for the management of fruit rot disease in chilli plants.

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Authors' contributions

DSV conceptualized the study and wrote the original draft of the manuscript. TM contributed to revising the draft and improving the structure of the manuscript. TS assisted in the inclusion of tables and figures. JHS contributed to the revision and proofreading of the manuscript. JV reviewed the content and assisted in the arrangement of figures. JPB participated in proofreading and refinement of the manuscript. KPA contributed to the revision and final editing of the document. All authors read and approved the final version of the manuscript.

Compliance with ethical standards

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

Ethical issues: None

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