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In vitro antagonistic potential of bacterial endophytes against chilli anthracnose pathogen *Colletotrichum acutatum*

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The escalating threat of chilli anthracnose and the adverse effects of chemical pesticides underscore the urgency for sustainable management of chilli anthracnose. This study aims to identify the potential endophytic bacteria as eco-friendly solutions to combat chilli anthracnose pathogen. Through evaluating their antagonistic effects, we aim to provide effective biological control strategies for this destructive plant disease. Among the 74 bacterial endophytes isolated from healthy chilli plants, *Bacillus amyloliquefaciens* (ENLB 4) exhibited the most pronounced antagonistic effect against the chilli anthracnose pathogen *Colletotrichum acutatum*. *In vitro* tests revealed that ENLB 4 inhibited the pathogen's growth by 56.43%. Additionally, the cell-free culture filtrate of ENLB 4 exhibited substantial inhibitory effects on pathogen growth at various concentrations (46.74% at 5%, 54.99% at 10%, and 63.55% at 15%, respectively). Similarly, volatile organic compounds produced by ENLB 4 resulted in a higher (37.78%) growth inhibition of the pathogen. Analysis of the culture filtrate using GC-MS identified 17 compounds, some of which possess antimicrobial properties (hexanoic acid, 3-Isobutylhexahydropyrrolo[1,2-a] pyrazine-1,4-dione, isovaleric acid), while others are known to induce systemic resistance in treated plants (acetic acid). These findings underscore the potential of endophytic bacteria, exemplified by *B. amyloliquefaciens*, as effective and environmentally friendly agents for disease management in agriculture.

Keywords: *Bacillus amyloliquefaciens*, Biocontrol, GC-MS, Sustainable disease management

Chilli (*Capsicum annuum*) is one of the important spices cum vegetable crops, first introduced into India by Portuguese traders in the year 1584[1]. The pungency of chilli plants results from the presence of an alkaloid called capsaicin. Capsaicin possesses antioxidant, antimutagenic, and anticarcinogenic properties[2]. Chilli is rich in essential vitamins such as vitamin A, vitamin C, thiamine, and riboflavin, besides it also provides a wealth of nutrients including carbohydrates, sugar, protein, omega 3 and omega 6 fatty acids, volatile oils, and numerous minerals[3]. India is one of the largest chilli producing countries, with an annual production of 20.49 lakh tons and a productivity of 2918 kg/ha[4]. There are various constraints in chilli production, among which anthracnose, caused by *Colletotrichum* spp., results in losses in both pre-harvest and post-harvest conditions[5]. Anthracnose disease, caused by *Colletotrichum* spp., is a seed, soil, and airborne disease that affects the crop at all stages of growth, and even after harvest, during transit and storage[6]. *Colletotrichum spp.* are designated as one of the ten most destructive plant pathogens in the world[7]. A total of three *Colletotrichum* species were reported to be pathogenic on chilli plants in India[8]. The combination of high humidity, rainfall and extended periods of leaf wetness increases the incidence of anthracnose disease[9]. Symptoms of the disease appear as sunken necrotic lesions, with concentric rings of acervuli containing masses of conidia under severe conditions[10]. Anthracnose disease prevails in all the chilli growing areas of the country[11] causing loses ranging from 40 – 80%[12], and also resulting in latent infections[13]. Currently, this disease is controlled through the widespread application of various chemical fungicides such as carbendazim, propiconazole, difenoconazole, azoxystrobin, trifloxystrobin, maneb, and mancozeb[14,15]. Biocontrol agents are environmentally friendly, economically efficient, self-sustaining, and enhance crop productivity[16] while protecting plants from invading pathogens, avoiding any detrimental, long lasting consequences[17]. The primary means by which antagonistic microorganisms inhibit pathogens include competing for both space and nutrients, releasing cell wall degrading enzymes, antibiotics, solubilising inorganic soil nutrients, and producing various other antimicrobial compounds[18].

Bacterial endophytes exhibit a broad spectrum of functional activities within their host plants, encompassing the synthesis of antimicrobial secondary metabolites, root colonisation to safeguard plants from a variety of biotic and abiotic stresses, solubilisation of inorganic soil nutrients, and the regulation of plant growth through the production of various plant

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growth promoting hormones, among other functions[19,20]. They play a pivotal role in suppressing plant pathogens through various mechanisms, including direct antagonism via competitive interactions and hyperparasitism, as well as the release of diverse cell wall degrading enzymes, antibiotics, antimicrobial volatile secondary metabolites[21], and indirectly by activating plant defense mechanisms[22].

The objective of the present investigation is to identify potential antagonistic endophytes from healthy chili plants and assess their antagonistic activity *in vitro* against the pathogen responsible for chili anthracnose. Exploring the beneficial properties of plant endophytes holds promise in identifying novel endophytes for promoting plant growth and serving as potential biocontrol agents.

Materials and Methods

Isolation of chilli anthracnose pathogen and evaluating the virulence of all the pathogen isolates on the K 2 chilli variety

Chilli plants exhibiting anthracnose symptoms were sampled from Andhra Pradesh and Tamil Nadu. After rinsing to remove debris, small sections (2×2 mm) of infected and healthy tissue were excised. These tissue fragments underwent surface sterilisation with 70% alcohol for 20 seconds and 1% sodium hypochlorite solution for 1 min, followed by rinsing with sterile distilled water three times. After drying in a controlled laminar airflow chamber, the fragments were aseptically transferred onto Petri plates containing potato dextrose agar (PDA), with four fragments periphery per plate. The plates were then incubated in a BOD incubator at 28 ± 1°C for 7 days to observe fungal growth.

The standard method of Koch's postulates was followed to evaluate the virulence of pathogen isolates with some modifications[23]. The topsoil underwent tyndallisation for 3 days before being filled into cement pots with farmyard manure. Healthy K 2 chilli seedlings were transplanted and maintained with 25% soil moisture. At flowering, plants were sprayed with spores of pathogen isolates. The experiment was replicated thrice. After anthracnose symptoms appeared[24], the pathogen was re-isolated on PDA medium, confirming its pathogenicity by comparing the cultural and morphological characteristics with the standard manual for the identification of *Colletotrichum* spp.[25].

Isolation and *in vitro* screening of endophytic bacteria against *Colletotrichum acutatum*

The endophytic bacteria from healthy chilli plants were isolated following the methodology outlined[26]. Each sample (leaf, fruit, and root), weighing one gram, was surface sterilised using 15% H_2O_2 (v/v) for 1 min and rinsed four times with 0.02 M potassium phosphate buffer (*p*H 7.0). Following this, the samples were individually macerated in a clean mortar and pestle with 9 mL of 0.02 M phosphate buffer. Serial dilutions of 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} were prepared. From each dilution, 0.1 mL was pipetted onto Petri plates containing tryptic soy agar (TSA) and spread using sterile L-shaped spreaders. The plates were then incubated in a BOD incubator at 27 ± 1°C for 4 days.

The antagonistic activity of endophytic bacteria was studied using the dual-culture technique on PDA plates as described[27]. A 6 mm disc containing the pathogen was carefully positioned in a sterile manner at one corner of a Petri plate. After 48 h of pathogen inoculation, 24 h old endophytic bacterial cultures were inoculated onto the plate in a zigzag pattern on the opposite side. Control plates were solely inoculated with the pathogen. The entire experiment was replicated thrice, and the plates were then incubated at 27 ± 1°C for seven days. Following the incubation period, the radial growth of the pathogen was measured and percent growth inhibition was calculated using Formula [28] which is

$$\text{Percent growth inhibition (I)} = \frac{C - T}{C} \text{ X } 100$$

C – Radial growth of pathogen in control plates; T – Radial growth of pathogen in treated plates

Assessing different concentrations of antagonistic bacterial culture filtrate on pathogen growth

The antagonistic activity of cell free culture filtrate from endophytic bacterial isolates was assessed using the poisoned food technique[29]. A loop containing a culture of antagonistic bacteria was introduced into 100 mL of NB broth and incubated in a shaker incubator at 150 rpm for 2 days at a temperature of 37 ± 1°C. The broth was then filtered through Whatman No. 1 filter paper and centrifuged for 12 min at 6000 rpm. The resulting supernatant (cell free filtrate) was collected and passed through a Millipore membrane filter with a pore size of 0.2 µm to eliminate any bacterial cells. This cell free filtrate was evaluated for its impact on the mycelial growth of the pathogen

at three different concentrations: 5%, 10%, and 15%. The culture filtrate was added to PDA (Potato Dextrose Agar) media, and the mixture was poured into Petri plates. In the center of each plate, an 8 mm disc containing a 7day old culture of the pathogen was inoculated. These plates were then incubated at a temperature of 28 ± 1°C for a period of 15 days. In parallel, control plates were inoculated without the antagonist culture filtrate, and this procedure was repeated three times for consistency. The extent of mycelial growth was documented, and the percentage of inhibition in comparison to the control was calculated using Formula 1[28] which was mentioned earlier. The percent inhibition of pathogen growth was further compared with a standard fungicide (Hexaconazole 5% SC @ 100 ppm).

Evaluation of antimicrobial properties of volatile organic compounds of selected antagonistic bacterial isolates against pathogen

The antagonistic activity of volatile organic compounds from selected endophytic isolates was assessed using the double petri dish assay, as described[30]. In this experiment, a 6 mm disc containing actively growing 7-day old pathogen was introduced into a Petri plate filled with PDA (Potato Dextrose Agar). The cover of this plate was substituted with another base plate, which had been streaked with antagonistic endophytic bacteria on a nutrient agar medium. These two base plates were sealed using Saran wrap and subsequently placed in an incubator at a temperature of 27 ± 1°C for a duration of seven days. Notably, the base plate containing the pathogen was positioned facing upward during this incubation period. In the control plates, inoculation was carried out in a manner where only the pathogen was introduced into the upper base plate. The lower base plate contained only a nutrient agar medium without any antagonist. This process was repeated three times for consistency. At the conclusion of the incubation period, the percentage of growth inhibition was calculated using the prescribed formula[29].

$$\text{Percent growth inhibition (I)} = \frac{C - T}{C} \text{ X } 100$$

C – Radial growth of pathogen in control plates;
T – Radial growth of pathogen in treated plates

Cultural and molecular characterisation of promising endophytic bacterial isolate by partial sequencing of 16S rRNA

Cultural characters like colony colour, cell shape, pigmentation, elevation and margin were determined. In order to study the shape of cells, a smear of bacterial isolate was prepared in a clear slide and observed at 100× magnification by mounting on Euromex- iscope Trinocular Fluorescence Microscope (Model IS.3152 PLi-3). The DNA from the ENLB 4 isolate was extracted using standard procedures[31]. PCR was employed to preferentially amplify the 16S rRNA gene from genomic products using the universal forward primer 27F (5′-AGAG-TTTGATCCTGGC TCAG-3′) and reverse primer 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) (Fig. 1). Subsequently, the 16S rRNA gene sequences of the bacterial isolates were aligned with standard reference sequences from GenBank, NCBI, using BLAST. Using MEGA 6.0, a phylogenetic tree was constructed[32] (Fig. 2). Finally, the sequence was submitted to NCBI GenBank, resulting in the acquisition of an accession number.

Partial characterisation of secondary metabolites from promising endophytic antagonistic bacteria

The most effective endophytic bacterial strain, specifically *Bacillus amyloliquefaciens*, was selected for characterisation of its secondary metabolites using gas chromatography and mass spectrometry (GC-MS), following the procedure described[33]. Bacterial cultures were grown in nutrient broth and incubated at 37°C for 7 days. After the incubation period, the broth was filtered, and metabolites were extracted using a

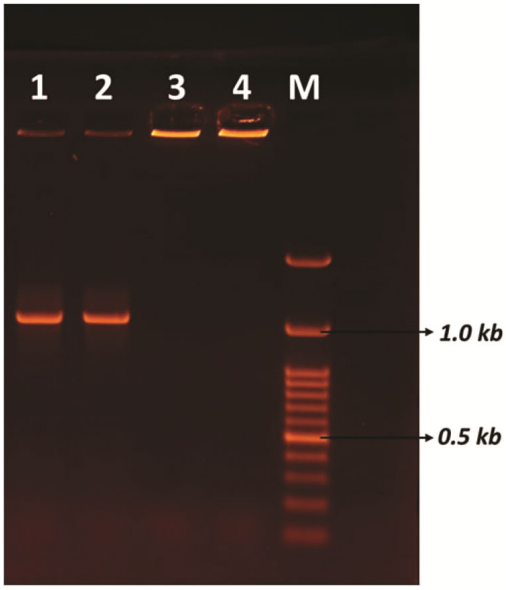

Fig. 1 — PCR amplification of 16S rRNA gene of ENLB 4 (*Bacillus amyloliquefaciens*) using 27F and 1492R primers. [M, Molecular weight marker – 100bp; Lane 1, ENLB 4 bacterial sample; Lane 2, PCR positive control; Lane 3, PCR genomic DNA as negative control; Lane 4, PCR water as negative control]

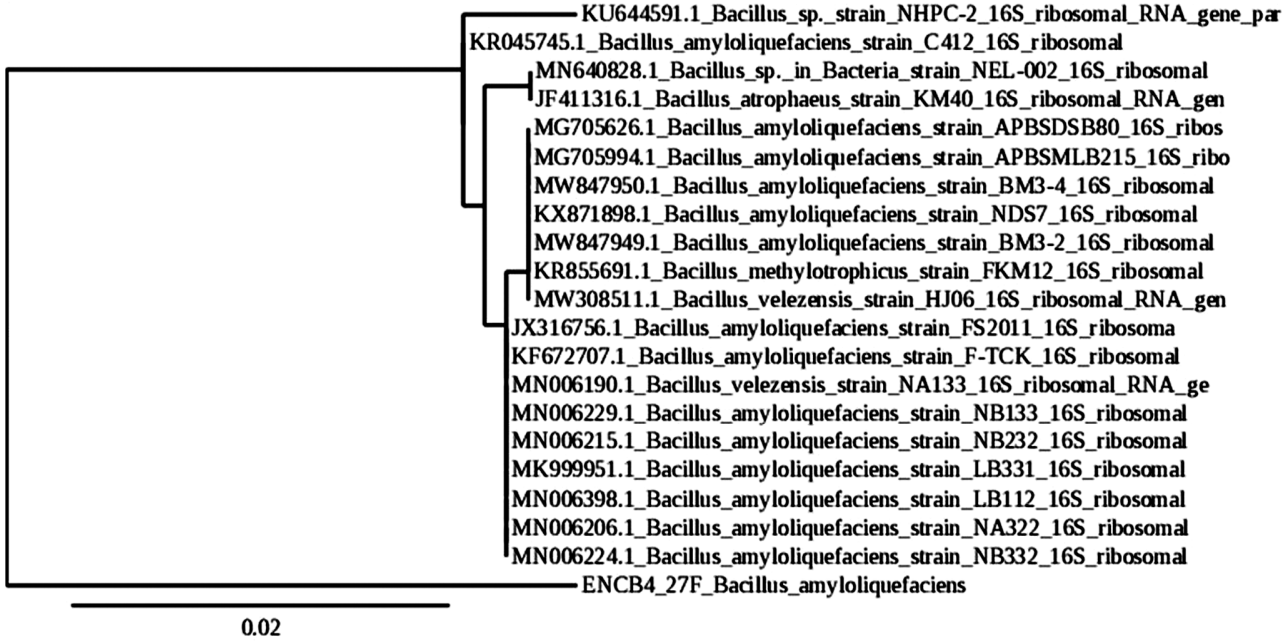

Fig. 2 — Phylogenetic tree of ENLB 4 (*Bacillus amyloliquefaciens*) using maximum likelihood method.

mixture of hexane and acetone in a 1:1 (v/v) ratio. The organic phase was separated and concentrated using a rotary evaporator. The resulting residue was dissolved in 1 mL of high-performance liquid chromatography (HPLC) grade methanol and sent to the SRM Institute of Science and Technology in Chennai for GC-MS analysis.

Results and Discussion

A total of 20 pathogenic isolates were obtained from infected samples collected from different chili-growing areas in Andhra Pradesh and Tamil Nadu. The responsible pathogens (*Colletotrichum* spp.) were isolated on Potato Dextrose Agar (PDA) medium. The fungal cultures underwent additional purification using the hyphal tip technique[34] and were preserved on PDA slants at 4°C in a refrigerator. They were labelled from C1 to C20. When tested under pot culture conditions for their pathogenicity, C7 recorded the highest virulence among all the pathogen isolates. Hence, it was selected for further *in vitro* studies. C7 exhibited whitish grey mycelium with fluffy growth, with a colony diameter of 85.2 mm, producing clavate spores measuring 14.8 µm in length and 4.4 µm in width, alongside dark brown acervuli consisting of 3–5 segments. Based on cultural and morphological characteristics, as well as sequence similarities of the ITS region of 5.8S rDNA, the isolate C7 was identified as *Colletotrichum acutatum* (OL348327).

Isolation and *in vitro* screening of endophytic bacteria against *Colletotrichum acutatum*

A total of 74 bacterial endophytes were collectively isolated, with 34 originating from the leaves, 21 from the fruits, and 19 from the roots. These isolates were evaluated for their effectiveness against the chili anthracnose pathogen, *C. acutatum* (C7), using a dual culture technique. The findings indicated that among the endophytic isolates, ENLB 4 displayed the smallest radial growth at 31.8 mm, followed by ENRB 17 at 34.6 mm, exhibiting high inhibition rates of 56.43% and 52.60%, respectively (Table 1). Conversely, the least inhibitory effect on the pathogen's radial growth, at a mere 0.27%, was observed with ENLB 29. In another study, a total of 87 endophytic bacteria were isolated from both chili and tomato plants. It was reported that isolates BETS11 and BETL9 displayed the highest inhibition, recording a remarkable 46.7% inhibition against *C. capsici*[35]. In their study, 58 endophytic bacteria were isolated from *Capsicum annum* and assessed for their antagonistic effects against *C. scovillei*. It was reported that 8 endophytic bacteria exhibited inhibition levels ranging from 70% to 80%, while only 2 endophytic bacteria managed to inhibit the pathogen's growth by more than 80%[36]. Antagonistic bacteria like *Bacillus* possess a wide variety of

Table 1 — *In vitro* evaluation of antagonistic activity of bacterial endophytes against *Colletotrichum acutatum* (Dual Culture)

Isolate No.	Source	Radial Growth (mm)	Percent inhibition
ENLB 1	Leaf	44.1	39.58
ENLB 2	Leaf	53.31	26.97
ENLB 3	Leaf	56.21	23
ENLB 4	Leaf	31.8	56.43
ENLB 5	Leaf	49.9	31.64
ENLB 6	Leaf	71.4	2.19
ENLB 7	Leaf	59.3	18.76
ENLB 8	Leaf	63.2	13.42
ENLB 9	Leaf	61.4	15.89
ENLB 10	Leaf	54.3	25.61
ENLB 11	Leaf	50.2	31.23
ENLB 12	Leaf	54	26.02
ENLB 13	Leaf	56.3	22.87
ENLB 14	Leaf	64.1	12.19
ENLB 15	Leaf	66.8	8.49
ENLB 16	Leaf	43.1	40.95
ENLB 17	Leaf	69.3	5.06
ENLB 18	Leaf	56.3	22.87
ENLB 19	Leaf	52.1	28.63
ENLB 20	Leaf	54.8	24.93
ENLB 21	Leaf	42.6	41.64
ENLB 22	Leaf	67.3	7.80
ENLB 23	Leaf	66.4	9.04
ENLB 24	Leaf	59.3	18.76
ENLB 25	Leaf	56.4	22.73
ENLB 26	Leaf	62.1	14.93
ENLB 27	Leaf	51.4	29.58
ENLB 28	Leaf	72.1	1.23
ENLB 29	Leaf	72.8	0.27
ENLB 30	Leaf	67.2	7.94
ENLB 31	Leaf	52.1	28.63
ENLB 32	Leaf	61.4	15.89
ENLB 33	Leaf	56.4	22.73
ENLB 34	Leaf	51.3	29.72
ENFB 1	Fruit	49.9	31.64
ENFB 2	Fruit	53.3	26.98
ENFB 3	Fruit	71.4	2.19
ENFB 4	Fruit	56.1	23.15
ENFB 5	Fruit	72.1	1.23
ENFB 6	Fruit	70.3	3.69
ENFB 7	Fruit	49	32.87
ENFB 8	Fruit	50.6	30.68
ENFB 9	Fruit	67.4	7.67
ENFB 10	Fruit	60.4	17.26
ENFB 11	Fruit	37.1	49.17
ENFB 12	Fruit	67.4	7.67
ENFB 13	Fruit	56.6	22.46
ENFB 14	Fruit	61.4	15.89
ENFB 15	Fruit	65.6	10.13
ENFB 16	Fruit	59.6	18.35
ENFB 17	Fruit	54.2	25.75
ENFB 18	Fruit	36.9	49.45
ENFB 19	Fruit	67.4	7.67
ENFB 20	Fruit	71.2	2.46
ENFB 21	Fruit	50.4	30.95
ENRB 1	Root	56.3	22.87
ENRB 2	Root	40.3	44.79
ENRB 3	Root	55.1	24.52
ENRB 4	Root	50.4	30.95
ENRB 5	Root	67.4	7.67
ENRB 6	Root	61.5	15.75
ENRB 7	Root	65.4	10.41
ENRB 8	Root	69.3	5.06
ENRB 9	Root	53.4	26.84
ENRB 10	Root	56.2	23.01
ENRB 11	Root	61.4	15.89
ENRB 12	Root	57.4	21.36
ENRB 13	Root	47.2	35.34
ENRB 14	Root	50.4	30.95
ENRB 15	Root	61.8	15.34
ENRB 16	Root	63.9	12.46
ENRB 17	Root	34.6	52.6
ENRB 18	Root	53.8	26.30
ENRB 19	Root	65.4	10.41
Control	-	73	-
C.D.		2.702	-
SE(m)		0.915	-
SE(d)		1.294	-
C.V.		3.635	-

(*Contd.*)

biological functions such as secretion of antimicrobial compounds, lytic enzymes such as amylase[37], siderophores, antibiotics[38] and other cell wall degrading enzymes such as extracellular chitinases, mycolytic enzymes, hydrolytic enzymes, etc. which leads to the suppression of plant pathogens[39,40].

Efficacy of cell-free culture filtrate of selected antagonistic bacterial isolates at different concentrations on the growth of *Colletotrichum acutatum*

Ten bacterial antagonists were selected based on their *in vitro* antagonistic ability to study the effect of their cell-free cultural filtrate at three different concentrations on the mycelial growth of *Colletotrichum acutatum* (C7), which showed the highest incidence of chili anthracnose disease under pot culture conditions. Results revealed that the cultural filtrate of the chosen antagonistic bacterial endophytes consistently and significantly suppressed the mycelial growth of the pathogen across all tested concentrations. At all the examined concentrations (5%, 10%, and 15%), ENLB 4 demonstrated notably greater inhibition in the mycelial growth of the pathogen, with respective percentages of 46.74%, 54.99%, and 63.55% (Table 2). This was followed by ENRB 17 and ENFB 18. ENFB 7 consistently exhibited the lowest inhibition of mycelial growth of the pathogen at all

Table 2 — Efficacy of culture filtrate of antagonistic bacterial isolates at different concentrations on the mycelial growth of *Colletotrichum acutatum* (Poisoned food technique)

Isolates	Radial growth (mm)			Percent inhibition		
	5%	10%	15%	5%	10%	15%
ENLB-1	56.4	52.3	47.3	25.09	30.35	36.84
ENLB-4	40.1	33.8	27.3	46.74	54.99	63.55
ENLB-16	54.3	50.6	46.1	27.88	32.62	38.45
ENLB-21	50.4	47.6	43.1	33.06	36.61	42.45
ENFB-7	63.1	59.1	54.2	16.20	21.30	27.63
ENFB-11	46.4	40.9	36.9	38.37	45.53	50.73
ENFB-18	42.1	38.1	33.4	44.09	49.26	55.40
ENRB-2	47.4	44.1	40.6	37.05	41.27	45.79
ENRB-13	61.3	56.2	51.4	18.59	25.16	31.37
ENRB-17	41.2	35.1	29.8	45.28	53.26	60.21
Hexaconazole 5% SC (100 ppm)	0	0	0	100	100	100
Control	75.3	75.1	74.9	-	-	-
C.D.	1.492	1.528	1.143	-	-	-
SE(m)	0.505	0.518	0.387	-	-	-
SE(d)	0.715	0.732	0.548	-	-	-
C.V.	1.666	1.851	1.522	-	-	-

tested concentrations. In contrast, the fungicide Hexaconazole 5% SC, at 100 ppm, achieved complete (100%) inhibition of the pathogen's mycelial growth. Our results align with those reported[41], where *Bacillus subtilis* and *Pseudomonas fluorescens* were isolated, and their cultural filtrate tested on six plant pathogens. The findings showed significant inhibition, with both bacteria's cultural filtrate exhibiting strong effects (*B. subtilis* 74.44% and *P. fluorescens* 76.67%) against *C. gloeosporioides* at a 20% concentration. In a comparable experiment, the cultural filtrate of *B. amyloliquefaciens* Y1 isolate at a concentration of 5% recorded a significant inhibition of growth of *C. gloeosporioides* by 69.79%[42]. Similarly, *Bacillus* spp. are also known to produce cyclic tetrapeptides such as cyclo-(prolyl-valyl-alanyl-isoleucyl) which show excellent antifungal activity against *Colletotrichum gloeosporioides*[43]. The presence of surfactins, iturin, and fengycin in the culture filtrates of *B. subtilis* and *B. amyloliquefaciens* which were previously reported to inhibit spore germination of certain plant pathogens[44] may be responsible for the strong antagonistic activity against *Colletotrichum acutatum*

Evaluation of antimicrobial properties of volatile organic compounds of selected antagonistic bacterial isolates against *Colletotrichum acutatum*

The inhibitory effects of volatile organic compounds from five selected antagonistic bacterial endophytes specifically ENLB 4, ENFB 11, ENFB 18, ENRB 2, and ENRB 17 were assessed against the mycelial growth of the chilli anthracnose pathogen (C7) using a double petri dish assay. Among the tested isolates, ENLB 4 exhibited the lowest mycelial growth of the pathogen at 44.3 mm, resulting in a percent inhibition of 37.78% (Table 3). Following closely was ENRB 17 with a mycelial growth of 49.2 mm and a percent inhibition of 30.89%. In contrast, ENRB 2 recorded the highest mycelial growth of the pathogen at 59.3 mm, accompanied by a percent inhibition of 16.71%. Previously, the impact of volatile substances from nine isolates of *Bacillus amyloliquefaciens* was examined on *Colletotrichum acutatum*, and it was reported that the Bc2 isolate exhibited the highest growth inhibition of the pathogen, reaching 72%[45]. Similar findings were reported by[46] who observed that volatile compounds produced by *B. tequilensis* GYUN-300 isolate reduced the growth of *C. acutatum* KACC42403 by 20%. Similar results were reported earlier[47]. These volatile organic compounds produced by antagonistic *Bacillus* spp. encompass a range of chemical components, including organic acids, S-containing compounds, hydrocarbons, N-containing compounds, phenols, aldehydes, amines, esters, alcohols, ketones, and other hydrocarbons. These compounds are recognised for their inhibitory effects on the growth of plant pathogens[48].

Table 3 — Evaluation of antimicrobial properties of volatile organic compounds of the selected antagonistic bacterial isolates against *Colletotrichum acutatum* (Double petri dish assay)

Isolate	Radial growth (mm)	Percent inhibition
ENLB 4	44.3	37.78
ENFB 11	56.1	21.20
ENFB 18	54.1	24
ENRB 2	59.3	16.71
ENRB 17	49.2	30.89
Control	71.2	-
C.D.	1.98	-
SE(m)	0.636	-
SE(d)	0.899	-
C.V.	1.976	-

Cultural and molecular characterisation of promising endophytic bacterial isolate by partial sequencing of 16S rRNA

The colony of ENLB 4 displayed a creamy white hue with a flat elevation and rough margin, devoid of any pigmentation, while the bacterial cells exhibited a rod shape. After running the extracted rRNA gene on 0.8% agarose gel electrophoresis, a band of high molecular weight intact clear genomic DNA was observed. Upon resolving the amplicon on 1%

Table 4 — Compounds detected in culture filtrate of *Bacillus amyloliquefaciens* (ENLB 4)

Retention time	Compound	Molecular Formula	Composition (%)
3.162	N-Acetylisoxazolidine	$C_5H_9NO_2$	9.887
3.283	Isovaleric acid	$C_5H_{10}O_2$	3.531
3.474	Acetic acid	$C_2H_4O_2$	3.835
3.665	Propanoic acid, 2-methyl-	$C_4H_8O_2$	1.058
3.760	Tetraacetyl-d-xylonic nitrile	$C_{14}H_{17}NO_9$	0.995
3.951	Isovaleric acid	$C_5H_{10}O_2$	1.691
6.458	Butanoic acid	$C_4H_8O_2$	5.482
8.008	Heptanoic acid	$C_7H_{14}O_2$	9.545
15.052	Hexanoic acid, 1-cyclopentylethyl ester	$C_{13}H_{24}O_2$	11.711
19.983	3-Methyl-1,4-diazabicyclo[4.3.0]nonan-2,5-dione, N-acetyl-	$C_{10}H_{14}N_2O_3$	4.121
20.422	Glycyl-L-proline	$C_7H_{12}N_2O_3$	10.784
21.383	3-Isobutylhexahydropyrrolo [1,2-a]pyrazine-1,4-dione	$C_{11}H_{18}N_2O_2$	7.174
22.051	trans-13-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	3.036
22.267	Methyl stearate	$C_{19}H_{38}O_2$	3.286
22.574	Nonanoic acid, 9-(o-propylphenyl)-, methyl ester	$C_{19}H_{30}O_2$	0.287
23.457	L-Proline, N-valeryl-, pentyl ester	$C_{15}H_{27}NO_3$	3.295
25.493	l-Leucyl-d-leucine	$C_{12}H_{24}N_2O_3$	20.281

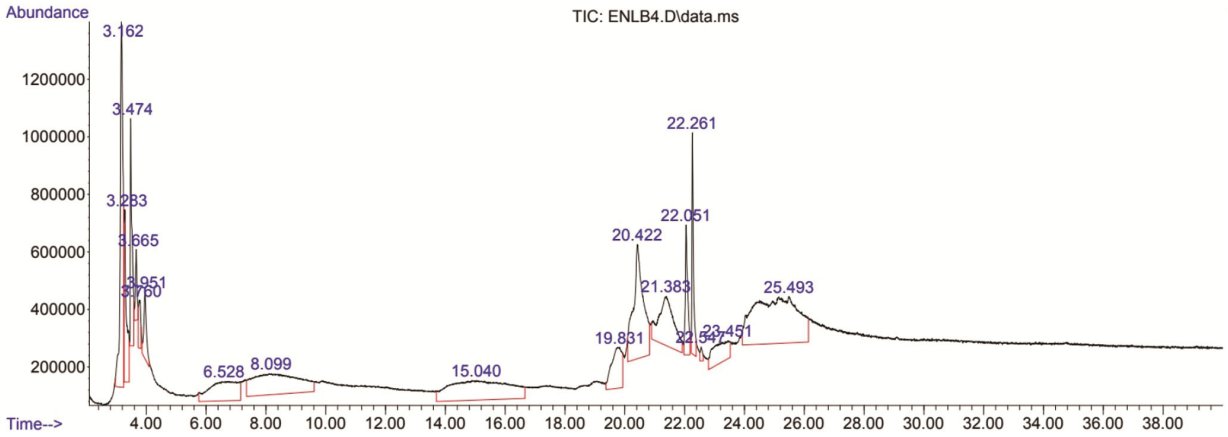

Fig. 3 — GC-MS chromatogram of the culture filtrate of ENLB 4 (*Bacillus amyloliquefaciens*).

agarose gel, a band of 938 bp was detected (Fig. 1). The consensus sequence of the 16S rRNA was used to conduct a BLAST search in NCBI GenBank, revealing a 96.25% match with *Bacillus amyloliquefaciens* for the ENLB 4 sequence. Using the Neighbor joining method and BioNJ algorithms, a phylogenetic tree was constructed, demonstrating the close clustering of the ENLB 4 isolate with previously reported *B. amyloliquefaciens* isolates (Fig. 2). Based on cultural characters, sequence similarities and phylogenetic analysis, the endophytic bacterial isolate ENLB 4 was identified as *B. amyloliquefaciens*. The sequence was deposited in NCBI GenBank under accession number OM842970.

Partial characterisation of secondary metabolites from promising endophytic antagonistic bacteria

The database search unveiled that the culture filtrates of *Bacillus amyloliquefaciens* contained a total of 17 compounds (Fig. 3). The identified compounds in the culture filtrates of *B. amyloliquefaciens* were listed in Table 4. Earlier 3 compounds were detected from the cell free culture filtrate of *B. amyloliquefaciens* sub sp. *amyloliquefaciens* RLS19 namely 2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-; acetamide, N-methyl-N- [4-(3- hydroxypyrrolidinyl)-2-butynyl]-, and pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro- among which 2,5-Piperazinedione, 3,6-bis(2-methylpropyl)- was reported to possess antifungal activity against *Aspergillus flavus*[49].

Compounds such as Albuterol and 1,3-propanediol, identified from the culture of B. subtilis strain SYST2, along with 2-pentylfuran identified from *B. megaterium* XTBG34, were reported to have a positive impact on plant growth and yield in treated plants[50]. Similarly, cultures of *B.valenzensis* expressed genes responsible for synthesising metabolites,

specifically lipopeptides and polyketides. These metabolites were found to have the ability to induce systemic resistance in treated plants[51].

Conclusion

The results of the present study clearly indicate that among the 74 endophytic bacterial isolates, ENLB 4 (*Bacillus amyloliquefaciens*) consistently exhibited higher inhibition of the growth of the pathogen *Colletotrichum acutatum* (C7). In the dual-culture technique, where endophytic antagonistic bacteria reduce pathogen growth through antibiosis, ENLB 4 achieved a 56.43% growth reduction. Additionally, in the poisoned food technique, the culture filtrate of ENLB 4 resulted in a 63.55% reduction in pathogen growth, attributed to the action of antimicrobial secondary metabolites. Furthermore, in the double petri dish assay, ENLB 4 demonstrated a 37.78% reduction in pathogen growth due to the presence of diffusible volatile organic compounds with antimicrobial properties. Analysis of the culture filtrate of ENLB 4 revealed the presence of 17 compounds with antimicrobial activities, alongside beneficial effects on plant growth and induction of systemic resistance in treated plants. *Bacillus amyloliquefaciens* (ENLB 4) holds potential as a sustainable alternative to synthetic fungicides for anthracnose management in chili plants, reducing environmental impact. However, field validation is needed, as in vitro results may not directly translate to field conditions. Further research on specificity, long-term stability, and regulatory considerations is crucial. Monitoring for unintended effects and resistance development is essential to ensure sustainable use.

Conflict of interest

The authors have no conflicts of interest to declare.

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