



RESEARCH ARTICLE

Deciphering the antibacterial metabolome of endophytic *Bacillus velezensis* ER4 against *Xanthomonas citri* pv. *malvacearum* through integrated metabolomics

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Abstract

Bacterial blight of cotton, caused by *Xanthomonas citri* pv. *malvacearum*, remains a major constraint to global cotton productivity. In the quest for sustainable and eco-friendly disease management strategies, endophytic *Bacillus* species were isolated, identified and evaluated for their antagonistic potential against this pathogen. A total of five endophytic *Bacillus* isolates namely ER4, EI3, ER5, EP5 and EI4 were obtained from internal root tissue of cotton and the molecular characterisation confirmed these isolates as *Bacillus velezensis*, *Bacillus subtilis*, *Priestia megaterium*, *Priestia endophyticus* and *Bacillus tequilensis* respectively. A cross-streak assay was performed to evaluate the efficacy of the above endophytes against *X. citri* pv. *malvacearum*. Among these, *B. velezensis* ER4 exhibited the highest inhibitory activity, producing a pronounced inhibition zone of 17 mm, indicative of potent diffusible antimicrobial metabolite production. To further assess volatile-mediated inhibition, an agar well diffusion assay employing volatile extracts of *B. velezensis* ER4 was conducted, demonstrating substantial suppression of pathogen growth. The strong antagonistic activity warranted an in-depth metabolomic investigation. Comprehensive metabolite profiling using gas chromatography–mass spectrometry (GC-MS) and liquid chromatography–mass spectrometry (LC-MS) revealed a total of 147 metabolites, grouped into 42 chemical classes and associated with 57 metabolic pathways, as determined through MetaboAnalyst 5.0, KEGG and HMDB analyses. Functional characterisation highlighted key roles in antimicrobial, antioxidant and plant growth-promoting activities. Notably, phenolic compounds, fatty acid derivatives, phthalates and pyrrolo[1,2-a] pyrazine analogues were identified as major bioactive components. These findings demonstrate the potential of *B. velezensis* ER4 as a promising biocontrol agent and provide a metabolomic foundation for the development of sustainable bioformulations for the effective management of bacterial blight in cotton cultivation.

Keywords: antagonistic activity; bacterial endophytes; biocontrol; cotton bacterial blight; metabolomic profiling; pathway enrichment analysis

Introduction

Cotton (*Gossypium* spp.) is one of the world's most crucial fibre crops and serves as the cornerstone of the global textile industry and a significant economic pillar for many agrarian economies. However, its cultivation is continuously threatened by numerous pathogens, among which bacterial blight caused by *Xanthomonas citri* pv. *malvacearum* (*Xcm*) stands out as a historically devastating disease (1). The disease manifests through characteristic symptoms including angular leaf spots, stem lesions known as black arm and boll rot, collectively leading to catastrophic yield losses that can exceed 60 % under epidemic conditions (1, 2). The pathogen's persistence and adaptability make it a formidable challenge in major cotton-growing regions worldwide, including India, Pakistan and the United States, where its management is further complicated by

climate change-induced fluctuations that favour disease outbreaks (3, 4).

Conventional management of bacterial blight has heavily relied on an integrated approach combining copper-based bactericides and the deployment of resistant cotton cultivars (5). While these methods have provided a measure of control, their long-term efficacy is increasingly compromised. The emergence of copper-resistant *Xcm* strains and the continuous breakdown of major resistance genes, such as the B genes, render cultivars susceptible over time, thereby creating a cycle of dependency on chemical inputs that are environmentally detrimental (3, 4, 6). The environmental toxicity of persistent copper accumulation in soils, coupled with the growing need for sustainable agricultural practices, has catalysed the search for alternative, biologically based control strategies (7).

In this context, microbial biocontrol agents (BCAs), particularly plant-associated endophytic bacteria, offer a promising and sustainable alternative to plant disease management (8). These endophytes confer protection through multiple mechanisms, including antimicrobial metabolite production, niche competition and induction of systemic resistance (ISR) in the host plant (9, 10). The genus *Bacillus* has proven to be a prolific source of such BCAs, with *Bacillus velezensis* emerging as a paradigm species due to its genomic richness and metabolic versatility (11, 12).

The biocontrol efficacy of *B. velezensis* is largely attributed to its extensive repertoire of secondary metabolites. Genomic analyses reveal that 5–10 % of a typical *B. velezensis* genome is dedicated to biosynthetic gene clusters (BGCs) encoding a diverse array of bioactive compounds (13, 14). These include cyclic lipopeptides namely surfactin, fengycin and iturin, which disrupt pathogen membrane integrity; polyketides such as diffidin, macrolactin and bacillaene, which inhibit protein synthesis and bacteriocins (11). Notably, specific metabolites like oxydiffidin have demonstrated potent and specific antibacterial activity against various *Xanthomonas* species, positioning *B. velezensis* as a viable alternative to copper sprays (15). Preliminary studies in cotton systems have shown that seed treatment or foliar application of *B. velezensis* strains can significantly reduce the bacterial blight severity under both controlled and field conditions, underscoring their practical potential (16).

Despite these advances, current studies are largely limited to genomic predictions or targeted metabolite identification, with relatively few reports integrating comprehensive metabolomic profiling with functional validation against *Xcm*. Moreover, the specific metabolic signatures associated with endophytic *B. velezensis* strains inhabiting cotton tissues and their mechanistic linkage to antibiosis and host interaction remain insufficiently characterized. Modern metabolomics provides a powerful, high-resolution platform for such investigations. The integration of analytical techniques like gas chromatography–mass spectrometry (GC–MS) for primary metabolism and liquid chromatography–mass spectrometry (LC–MS) for secondary metabolism, coupled with sophisticated bioinformatics tools (e.g., MetaboAnalyst, KEGG, HMDB), enables the comprehensive profiling, annotation and pathway mapping of the entire metabolite suite produced by a biocontrol strain (17). Such an approach provides an opportunity to link metabolic signatures with biocontrol mechanisms, thereby addressing the existing knowledge gap in understanding metabolite-mediated pathogen suppression. Unlike previous studies, this integrative multi-platform approach facilitates the simultaneous characterisation of both primary and secondary metabolic networks and their correlation with biocontrol efficacy.

Therefore, this study focuses on *B. velezensis* strain ER4, an endophytic isolate recovered from the internal root tissues of healthy cotton plants. We hypothesise that the endophytic lifestyle of *B. velezensis* ER4 is associated with a unique metabolome rich in antimicrobial and plant-defense-priming compounds that confer robust protection against *Xcm*. The objectives of this research are to systematically characterise the metabolome of *B. velezensis* ER4 using integrated GC–MS and LC–MS platforms, to employ computational pathway analysis to identify key metabolic pathways and bioactive metabolites implicated in antibiosis, host interaction and to correlate these metabolic signatures with *in vitro* and *in planta* biocontrol efficacy against bacterial blight in cotton. The

insights garnered from this metabolomic dissection are anticipated to not only validate *B. velezensis* ER4 as a leading candidate for developing a sustainable bio-pesticide but also to uncover a reservoir of novel metabolites with potential applications in both agriculture and pharmacology.

Materials and Methods

Isolation of endophytic bacterium

The bacterial endophytes were isolated from internal root tissues of apparently healthy cotton plants (Cultivars RCH 659 and MCU 5) collected from Erode and Dindigul. The isolation procedure followed a rigorous surface sterilisation protocol to exclude epiphytic microorganisms (18). Samples were surface-sterilised using 70 % ethanol (2 min), 2 % sodium hypochlorite (3 min), followed by three rinses with sterile distilled water. To confirm the efficacy of the surface sterilisation, 100 µL of the final rinse water was plated onto Nutrient Agar (NA; HiMedia, India) and monitored for microbial growth after 48–72 hr of incubation at 28 °C. No microbial growth was observed, confirming the successful elimination of epiphytic contaminants and validating the effectiveness of the surface sterilisation procedure. The surface-sterilised root segments were aseptically macerated in 1 mL of sterile 0.85 % saline using a sterile pestle and mortar. The resulting homogenate was subjected to a ten-fold serial dilution and 100 µL aliquots from appropriate dilutions (10^{-2} to 10^{-4}) were spread-plated onto NA plates. After incubation at 28 ± 2 °C for 48–72 hr, morphologically distinct colonies were purified by the multiple streak method. The colonies with morphology characteristic of the *Bacillus* genus (irregular, creamy-white, spreading) were selected for further characterisation.

Identification and molecular characterisation of endophytic bacterium

The genomic DNA of the endophytic bacterial isolates was extracted using a modified cetyltrimethylammonium bromide (CTAB) method. Bacterial pellets from overnight cultures were treated with TE buffer and 1-butanol to remove extracellular materials. The cells were then lysed with SDS, lysozyme and proteinase K at 65 °C. Subsequent purification involved chloroform-isoamyl alcohol extraction, followed by DNA precipitation with sodium acetate and cold isopropanol. The final DNA pellet was washed with ethanol, air-dried and resuspended in TE buffer. The purity and integrity of the genomic DNA were confirmed via agarose gel electrophoresis (19).

Polymerase chain reaction (PCR) was performed for the amplification of genomic DNA of five endophytic isolates using universal primers 27F (5'-AGAGTTTGATCTGGCTCAG-3) 1492R (5'-TACGGYTACCTGTTACGACT-3). The PCR reaction volume, 20 µL, contained 2.0 U of Taq polymerase (Sigma-Aldrich), 2µL of 10X buffer, 1.5 µL of 2.5 mM MgCl₂, 1 µL of 2.5 mM dNTP, 2 µL of 10 µM primer, 4 µL of genomic DNA and sterile distilled water. The thermocycling conditions consisted of an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 50 °C for 30 sec and final extension at 72 °C for 10 min. The successful amplification of the PCR product was verified on a 1 % agarose gel and the PCR products were sent for commercial sequencing (20).

The resulting 16S rDNA sequences were analysed using the BLAST tool in the GenBank database. Phylogenetic analysis, was accomplished by aligning the sequences with closely related

reference sequences using CLUSTAL-W. A phylogenetic tree was then constructed using the MEGA 11.0 software and the robustness of the tree topology was assessed through bootstrap analysis with 1000 replications (21).

In vitro antagonistic activity against *Xanthomonas citri* pv. *malvacearum*

Cross streak method

A modified dual-culture technique was employed to assess the antagonistic potential of all 5 *Bacillus* endophytes against *Xcm* under *in vitro* conditions (22). A single streak of a 24-hr-old culture of each *Bacillus* endophyte was made along the centre of a fresh NA plate. The plates were incubated at 30 °C for 24 hr to allow the diffusion of bacterial metabolites into the agar. Subsequently, a fresh 48-hr-old culture of *Xcm* was streaked perpendicularly to the *Bacillus* streak, maintaining a minimal gap of approximately 2–3 mm at the intersection to prevent direct contact. Plate streaked with pathogen alone served as control. Four replicates were maintained for each endophyte. All plates were incubated at 28 ± 2 °C for 48 hr. Antagonism was quantified by measuring the zone of inhibition (ZOI), defined as the clear area where *Xcm* growth was suppressed, from the edge of the *Bacillus* colony to the edge of the pathogen growth.

Agar well diffusion assay

The crude antibiotics were extracted from *B. velezensis* ER4 which showed the highest inhibition against *Xcm* and tested for its antibiotic potential using an agar well diffusion assay (23). A lawn of the pathogen was prepared by spreading 100 µL of a standardised *Xcm* suspension (10^8 CFU/mL, $OD_{600} = 0.1$) uniformly onto NA plates. Using a sterile cork borer, wells of 6 mm diameter were punched into the agar at four corners of each plate. Then, 50 µL of a cell-free culture filtrate of *B. velezensis* ER4 (prepared by growing the bacterium in LB broth for 72 h at 28 °C, followed by centrifugation at $10000 \times g$ for 15 min and filter sterilisation through a 0.22 µm membrane) was loaded into the wells. Methanol (50 µL) and streptomycin sulfate (10 µg/mL in sterile water) were used as negative and positive controls, respectively. The plates were sealed with parafilm to prevent the escape of volatile metabolites and incubated at 28 ± 2 °C for 48 hr. Antibacterial activity was evaluated by measuring the diameter (in mm) of the clear inhibition zone around each well.

Metabolite extraction and derivatisation

The extraction of extracellular metabolites was performed for three separate culture conditions *viz.*, *Xcm* alone, *B. velezensis* ER4 alone and their co-inoculated interaction. For each condition, a 1 L culture in Luria-Bertani (LB) broth was incubated in a shaking incubator at 150 rpm and 28 °C for 72 hr to reach the stationary phase and maximise secondary metabolite production. Following incubation, the cells were removed from the culture broth via centrifugation at $10000 \times g$ for 15 min at 4 °C and the supernatant was subsequently filtered through a 0.22 µm PES membrane. Extracellular metabolites were then extracted from the cell-free supernatant using liquid-liquid partitioning with an equal volume of ethyl acetate and this process repeated three times to ensure comprehensive recovery. The combined ethyl acetate fractions were dehydrated over anhydrous sodium sulphate and concentrated to complete dryness using a rotary evaporator at 40 °C. The resulting crude metabolite extracts were stored at -20 °C until analysis. For GC-MS analysis, a portion of

each dried extract was reconstituted in pyridine and derivatised with 50 µL of MSTFA at 70 °C for 30 min to enhance volatility (24).

Metabolomic profiling using GC-MS and LC-MS

Gas chromatography-mass spectrometry analysis

The derivatised samples were analysed using a Shimadzu QP2020 GC-MS system. Separation was achieved on an Rxi-5Sil MS capillary column (30 m $\times$ 0.25 mm i.e., 0.25 µm) with high-purity helium as the carrier gas at a constant flow rate of 1.0 mL/min. A 1 µL sample was injected in a 10 : 1 split mode with the injector temperature set at 250 °C. The oven temperature program initiated at 70 °C (held for 2 min), ramped to 300 °C at 10 °C/min and held for a final 10 min. The mass spectrometer operated in electron impact (EI) mode at 70 eV, with ion source and transfer line temperatures of 200 °C and 250 °C, respectively. Mass spectra were acquired across a 50–600 m/z range.

Raw data files were processed using Shimadzu GC-MS Post run Analysis software for peak picking, deconvolution and baseline correction. Metabolite annotation was performed by comparing mass fragmentation patterns and retention indices against the NIST v20 mass spectral library. A similarity index (SI) of ≥ 80 % was set as the threshold for confident identification. The annotated metabolites were subsequently used for statistical and pathway analysis.

Liquid chromatography-mass spectrometry analysis

The LC-MS analysis was carried out using a Thermo Scientific Q Exactive Plus Orbitrap mass spectrometer coupled to a Vanquish UHPLC system. Separation was achieved on a Hypersil GOLD C18 column (100 $\times$ 2.1 mm, 1.9 µm) with a mobile phase of (A) water with 0.1 % formic acid and (B) acetonitrile with 0.1 % formic acid. The gradient elution ran from 5 % B to 95 % B over 25 min, held for 5 min and then re-equilibrated. The flow rate was 0.3 mL/min, the column temperature was 40 °C and the injection volume was 5 µL. The HESI-II source parameters were: spray voltage, ± 3.5 kV; capillary temperature, 320 °C. Full MS scans (resolution 70000) from m/z 100–1000 were followed by data-dependent MS/MS scans (resolution 17500) of the top 5 ions.

The LC-MS raw data were processed using Thermo Xcalibur and Compound Discoverer 3.3 software for peak alignment, retention time correction and compound identification. Metabolite annotation was based on accurate mass (mass error < 5 ppm), MS/MS fragmentation patterns and comparison with databases such as HMDB and KEGG. Metabolite identification confidence was assigned according to the Metabolomics Standards Initiative (MSI) guidelines as follows: Level 1 (confirmed identification) based on comparison with authentic standards including retention time and MS/MS spectra; Level 2 (putatively annotated compounds) based on spectral similarity with public databases; Level 3 (putatively characterised compound classes) based on diagnostic fragmentation patterns; and Level 4 (unknown compounds) where insufficient structural information was available. Key bioactive metabolites identified in this study were primarily categorised under MSI Level 2, unless otherwise specified.

Statistical analysis

R software version 2.14.1 was used to statistically analyse the data. ANOVA followed by Duncan's multiple range test was used for mean comparison.

Results and Discussion

Isolation of endophytic bacterium

A total of five distinct bacterial endophytes were isolated from the internal root tissues of cotton. All the isolated colonies were purified by the multiple streak method and pure cultures were maintained on NA medium. The five potent isolates, designated as ER4, EI3, ER5, EP5 and EI4, were selected for further molecular identification and phylogenetic analysis (Fig. 1). Endophytic bacterial isolation from cotton was earlier performed by previous researchers (25, 26). Similarly, previous researchers isolated endophytic bacteria from the roots of cotton by serial dilution ranging from 10^{-2} to 10^{-5} (26).

Molecular identification and phylogenetic analysis of selected isolates

Molecular characterisation of five endophytic bacterial isolates was done by amplifying the 16S rRNA gene using universal primers 27F and 1492R, which yielded an amplicon approximately 1400 bp. Sequencing of PCR products of the five isolates followed by BLAST analysis revealed 97–99 % sequence identity with the *Bacillus* spp. The isolates ER4 and EI4 showed 99 % similarity to *B. velezensis* and *B. tequilensis*. The isolates EI3 and EP5 exhibited 97 % to *B. subtilis* and *Priestia endophyticus*, respectively. The isolate ER5 displayed 100 % similarity to *P. megaterium*. The partial sequences of the endophytic isolates were deposited in GenBank and accession numbers were obtained (Table 1). In addition, the *gyrB* gene sequence of *Bacillus velezensis* ER4 was deposited in GenBank under accession number PZ111316, providing additional resolution for species-level identification. The molecular characterisation and identification of the endophytic isolates correlate strongly with previous studies (11).

To validate species-level identification, we constructed phylogenetic trees using the neighbor-joining method in MEGA 11 software. Each isolate formed a well-supported cluster with its respective reference strain, confirming their evolutionary relatedness and distinct grouping within the *Bacillus* lineage (Fig. 2). Furthermore, phylogenetic analysis based on the *gyrB* gene for isolate ER4 showed consistent clustering with reference *B. velezensis* strains, further strengthening its taxonomic assignment. Our findings are consistent with established phylogenetic frameworks: the

Table 1. Accession numbers of endophytic bacterial strains

Sl. No.	Isolates	Bacterial endophytes	Accession number
1	ER4	<i>B. velezensis</i>	PQ423059
2	EI3	<i>B. subtilis</i>	PQ423061
3	ER5	<i>P. megaterium</i>	PQ423054
4	EP5	<i>P. endophyticus</i>	PQ423060
5	EI4	<i>B. tequilensis</i>	PQ436563



Fig. 1. Isolation of endophytic bacteria.

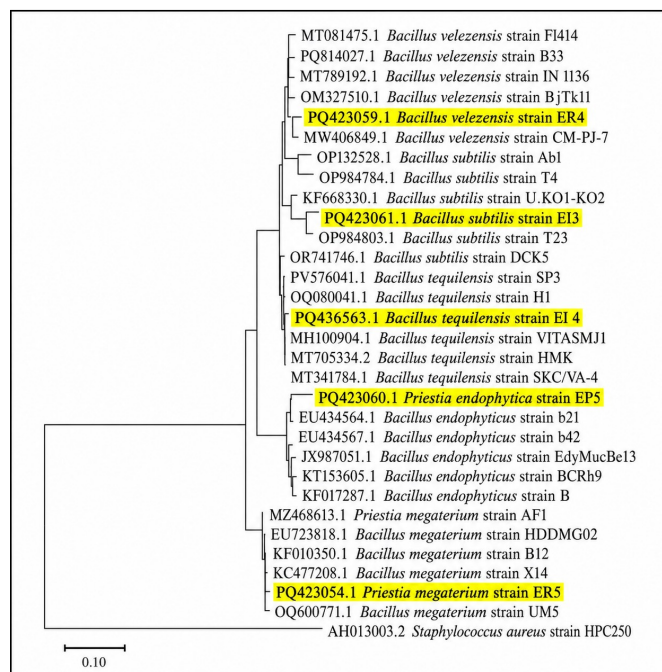


Fig. 2. Phylogenetic tree construction of all five endophytic strains.

clustering of *B. velezensis* ER4 aligns with (11), the robust position of *B. subtilis* EI3 mirrors (27) and the distinct monophyly of *Priestia megaterium* ER5 corresponds with (28). Furthermore, the identification of *P. endophyticus* EP5 and *B. tequilensis* EI4 is reinforced by previous researchers (29, 30). However, it is important to note that species-level identification based solely on 16S rRNA gene sequencing has limited discriminatory power within the *B. subtilis* species complex due to high sequence conservation among closely related taxa. In this study, the inclusion of the *gyrB* gene sequence for ER4 improves taxonomic resolution; however, other isolates lacking multilocus data should still be considered as putatively identified at the species level. Future studies incorporating multilocus sequence analysis (MLSA) using additional housekeeping genes (e.g., *rpoB*, *recA*) or whole-genome sequencing would provide higher taxonomic resolution and more robust confirmation of species identity.

Despite this limitation, the phylogenetic clustering and high sequence similarity observed in this study provide reasonable confidence in the taxonomic placement of the isolates and suggest that these endophytic *Bacillus* species maintain conserved genetic traits associated with a plant-associated lifestyle.

Cross streak method

The antagonistic potential of the isolated endophytic bacteria against *Xcm* was initially evaluated using the cross-streak method. All five endophytic bacterial isolates demonstrated noticeable inhibitory activity against the pathogen, evident from distinct

inhibition zones at the intersection of the two streaks. The degree of antagonism varied among isolates, indicating differential production of diffusible antimicrobial metabolites. Among them, isolate *B. velezensis* ER4 exhibited the strongest inhibitory effect, characterised by a broad and clear zone of growth suppression of 17 mm against *Xcm*. Isolates EI4 and ER5 also showed moderate to strong inhibition of 15 and 14 mm respectively, while EP5 and EI3 displayed comparatively lower, yet distinct, inhibitory zones of 13 mm (Table 2, Fig. 3).

The superior antagonistic activity of *B. velezensis* ER4 is consistent with the findings of earlier researchers who found that *B. velezensis* possess a remarkable capacity to synthesise a wide spectrum of lipopeptide antibiotics, such as surfactins and fengycins, which are highly effective against gram-negative bacteria like *Xanthomonas* (31). Another study demonstrated that *B. subtilis* strains can effectively inhibit the growth of *Xanthomonas axonopodis* through the production of the lipopeptide iturin A (32). A strain of *P. megaterium* from rice rhizosphere exhibited potent antibacterial activity against *Xanthomonas oryzae* (33). The antimicrobial potential of *B. tequilensis* was demonstrated by (34). The concordance between our experimental results and these previous studies confirms the significant biocontrol potential of these endophytic isolates, particularly *B. velezensis* ER4, for managing bacterial disease in cotton.

Agar well diffusion method

The antagonistic activity of the most efficient endophytic bacterial isolate, *B. velezensis* ER4, against *Xcm* was assessed using the agar well diffusion method. The cell-free culture filtrate of ER4 produced a clear inhibition zone surrounding the well inoculated with the pathogen, confirming the secretion of diffusible antimicrobial metabolites. The mean inhibition zone of 22.4 ± 0.58 mm was recorded, indicating strong antagonistic potential. In contrast, the control well containing only sterile nutrient broth exhibited no inhibition zone, confirming that pathogen suppression was exclusively due to metabolites produced by the isolate. The chemical control, namely streptomycin sulphate ($100 \mu\text{g mL}^{-1}$),

produced an inhibition zone of 25.1 ± 0.42 mm, serving as a positive reference standard. Although the inhibition zone formed by *B. velezensis* ER4 was slightly lower than that of streptomycin sulphate, the difference was statistically non-significant ($p > 0.05$), demonstrating the comparable efficacy of the bioactive compounds secreted by the isolate (Fig. 4).

The significant inhibition zone produced by the cell-free filtrate of *B. velezensis* ER4 confirms the secretion of potent diffusible antimicrobial compounds effective against *Xcm*. This finding is consistent with the report of earlier studies that documented model strain *B. velezensis* FZB42 synthesise a diverse arsenal of bioactive lipopeptides (31). The multi-metabolite nature of this bacterial antagonism, potentially involving a synergistic combination of compounds, presents a sustainable alternative to single-site synthetic antibiotics and may mitigate resistance development (11). These results strongly validate *B. velezensis* ER4 as a prime candidate for further development as a biocontrol agent for managing bacterial blight in cotton.

Metabolomic profiling- heat map

The hierarchical clustering heatmap generated from MetaboAnalyst revealed clear metabolomic distinctions among *B. velezensis* ER 4, *Xcm* and their interaction. The samples were grouped into three distinct clusters corresponding to the antagonist (*B. velezensis* ER 4) pathogen (*Xcm*) and interactions, indicating significant metabolic differentiation. Metabolites such as phytane, myristic acid, isopropyl

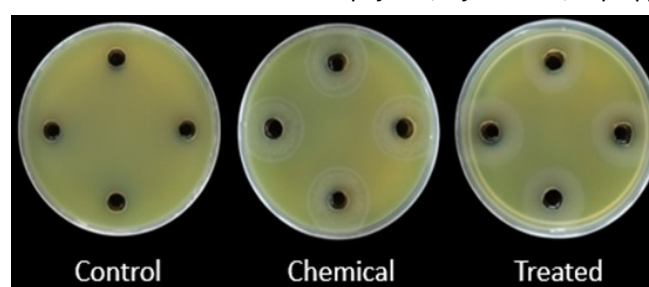


Fig. 4. Efficacy of crude metabolite of *Bacillus velezensis* ER4 against *Xanthomonas campestris* pv. *malvacearum* (Agar well diffusion assay).



Fig. 3. *In vitro* efficacy of endophytic bacteria against *Xanthomonas citri* pv. *malvacearum* by cross streak method.

Table 2. *In vitro* efficacy of bacterial blight against endophytic bacteria by cross streak method

Sl. No	Endophytic bacterial isolates	Inhibition zone length (mm)*
1	<i>Bacillus velezensis</i> ER4	17 ^a
2	<i>Bacillus subtilis</i> EI3	13 ^c
3	<i>Priestia endophytica</i> EP5	13 ^c
4	<i>Priestia megaterium</i> ER5	14 ^b
5	<i>Bacillus tequilensis</i> EI4	14 ^b
	SE(d)	0.23
	CV	2.03
	CD	0.52

*Mean of four replications.

In a column, mean followed by same superscript is not significantly different at $p = 0.05$.

myristate, stearic acid and palmitic acid showed high abundance in the *B. velezensis* ER 4 samples, suggesting an active production of hydrocarbons and fatty acids associated with bacterial metabolism and defence (35). These free fatty acids exhibit potent, broad-spectrum antibacterial activity, with their effectiveness linked to the disruption of microbial cell membranes (36). Similarly, the unique accumulation of lactones in *B. velezensis* ER 4 is a key finding and the antimicrobial role of lactones is well-established (37). In contrast, N-(2-acetylphenyl), valeraldehyde and phenylmalonic acid were more prominent in the pathogen samples, implying their potential role in pathogenicity or stress responses of *Xcm* (Fig. 5).

The interaction between *B. velezensis* ER4 and *Xcm* triggered a substantial metabolic shift, generating a unique signature characterised by elevated levels of delta-tetradecalactone, 2-hydroxy- γ -butyrolactone, diethyl phthalate and N-methyl-N-nitroso compounds. This profile indicates a profound biochemical reprogramming, with these molecules potentially functioning in signalling, quorum sensing interference, or direct antimicrobial activity. Gamma-butyrolactones have been identified as important signalling and antibiotic molecules in *Streptomyces*, suggesting that the 2-hydroxy- γ -butyrolactone observed in the present study may function in a similar manner (38). Furthermore, the presence of diethyl phthalate as a marker of the interaction is highly indicative of an antagonistic response. This is strongly supported by the work of previous researchers, who isolated diethyl phthalate from a *Streptomyces* species and confirmed its significant antibacterial activity against clinical pathogens, providing a clear precedent for its role as an antimicrobial agent in bacterial interactions (39). Broad-spectrum antibacterial activity of phthalate and di-n-butyl phthalate extracted from *Brevibacterium mcbrellneri* has been demonstrated (40). Antibacterial and antifungal activities of N-methyl-N-nitroso compounds, namely N-nitroso-2,6-diphenylpiperidin-4-ones, have also been reported (41). Additionally, the detection of pyrrolo[1,2-a]

pyrazine-1,4-dione derivatives further supports the antimicrobial potential of *B. velezensis* ER4. These diketopiperazine-type compounds are well-known for their antimicrobial, cytotoxic and quorum-sensing inhibitory properties, as demonstrated in *Bacillus* and *Streptomyces* species (42). Conversely, the suppression of specific pathogenic aldehydes and aromatic compounds during the interaction of *B. velezensis* ER4 and *Xcm* indicates a successful antagonistic influence, disrupting the pathogen's metabolic processes. The accumulation of fatty acids, lactones and phthalate derivatives suggests an induction of stress-related and defence-associated pathways, which are critical for membrane stabilisation, oxidative stress management and antimicrobial activity.

Metabolic differentiation and key metabolites

Metabolomic profiling conducted through MetaboAnalyst revealed significant metabolic variations among *B. velezensis* ER4, *Xcm* and their interactive system. Partial least squares discriminant analysis (PLS-DA) effectively differentiated the three groups and the variable importance in projection (VIP) score plot identified metabolites most responsible for this separation. Key metabolites with high VIP scores (> 1.0) including delta-tetradecalactone, N-(2-acetylphenyl), phytane, 2-hydroxy- γ -butyrolactone, phenylmalonic acid and diethyl phthalate were substantial contributors to the observed metabolic divergence. The high VIP score for diethyl phthalate is particularly revealing. Its role as an antimicrobial agent is well-documented; for instance, another researchers confirmed that phthalate esters exhibit measurable inhibitory effects against phytopathogens (43). Its upregulation here indicates a direct chemical countermeasure deployed by *B. velezensis*. Similarly, the significance of 2-hydroxy- γ -butyrolactone aligns with research on bacterial signalling interference (44). Furthermore, the presence of fatty acid-derived compounds like phytane and myristic acid among the top VIP metabolites points to alterations in lipid metabolism, a common stress response and source of

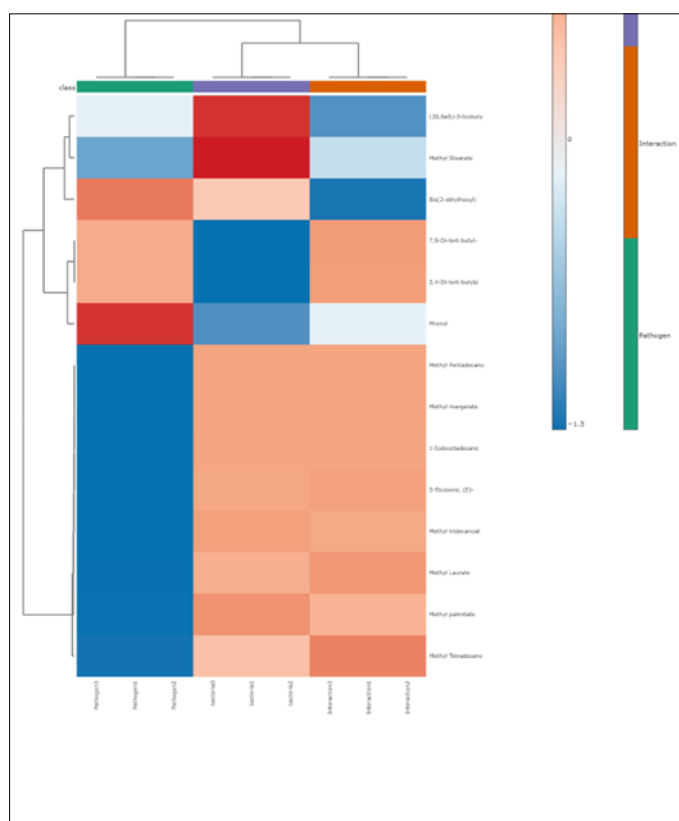


Fig. 5. Heat map of metabolomic profiling by GC-MS volatiles.

antimicrobials. While phytane itself is a derivative of bioactive isoprenoid pathways, its precursor, myristic acid, has a direct antibacterial mechanism (45). Fatty acids derived compounds have been reported to disrupt bacterial membranes and inhibit fatty acid synthesis in pathogens (36). In contrast, metabolites like 3-chloropropionic acid and E-2-tetradecen showed minimal influence based on their lower VIP scores. This variation in metabolite intensity underscores the biochemical diversity governing microbial interactions (Fig. 6).

Metabolic reprogramming and functional implications

The interaction between *B. velezensis* ER4 and *Xcm* induced a pronounced metabolic reprogramming in the antagonist, characterized by the differential accumulation of key metabolites. The elevated levels of phytane, phenylmalonic acid and delta-tetradecinal suggest an enhanced synthesis of antimicrobial hydrocarbons, phenolic derivatives and bioactive volatiles. Concurrently, the suppression of specific aldehyde and nitroso compounds indicates a successful disruption of the pathogen's metabolic state and stress responses. This metabolic shift represents a defined biocontrol strategy. The accumulation of phenolic acids, such as phenylmalonic acid, aligns with earlier findings, that *Bacillus* species upregulate shikimate and phenylpropanoid pathways to produce membrane-disrupting antimicrobials when challenged by pathogens (46). This suggests *B. velezensis* ER4 is deploying a conserved chemical defence playbook. Furthermore, the reduction in pathogen-associated metabolites is consistent with the mode of action of bacterial antagonists. The species' genome is enriched with biosynthetic gene clusters, and the metabolic changes detected represent the functional output of the activation of this genetic arsenal (31). These metabolites serve not only as biomarkers of this microbial confrontation but also underscore the dynamic biochemical interplay that underlies successful pathogen suppression.

Principal component analysis

The PCA score plot (Fig. 1) revealed a clear separation among the three groups, *B. velezensis*, *Xcm* and their interaction. The first two principal components, PC1 (93.1 %) and PC2 (6.9 %), together explained 100 % of the total variance, indicating strong variance capture by the model. However, such complete variance explanation with only two components is uncommon in biological metabolomics datasets and may indicate potential overfitting or a highly structured dataset. Therefore, caution must be exercised in interpreting this result. *B. velezensis* ER4 samples were positioned distinctly along the positive axis of PC1, while *Xcm* samples clustered on the negative side. The interaction samples formed a separate cluster away from both, highlighting a metabolomic shift specific to microbial confrontation. This pattern signifies that each condition possesses a unique metabolite composition with minimal overlap. The distinct clustering observed in the PCA plot demonstrates significant metabolic reprogramming during the interaction between *B. velezensis* and *Xcm*. The separation of groups suggests that *B. velezensis* ER4 alters its metabolic profile in response to the pathogen, potentially synthesising new bioactive compounds or modifying existing pathways. This metabolic divergence reflects a biochemical dialogue characteristic of antagonistic interactions, supporting the role of *B. velezensis* ER4 as a biocontrol agent capable of inducing strong metabolic differentiation (Fig. 7).

The distinct cluster of the interaction group suggests a targeted response, likely involving the upregulation of antimicrobial secondary metabolites. The genome of *B. velezensis* ER4 is equipped for such dynamic responses, containing numerous gene clusters responsible for the synthesis of lipopeptides, polyketides and other bioactive compounds that are often induced in the presence of a competitor or pathogen (31). The separate clustering of the pathogen along the negative PC1 axis further suggests that *Xcm* metabolism is also significantly altered, potentially as a result of stress or suppression induced by *B. velezensis* ER 4. Further validation using supervised models (e.g., PLS-DA) and permutation testing is recommended to confirm model robustness.

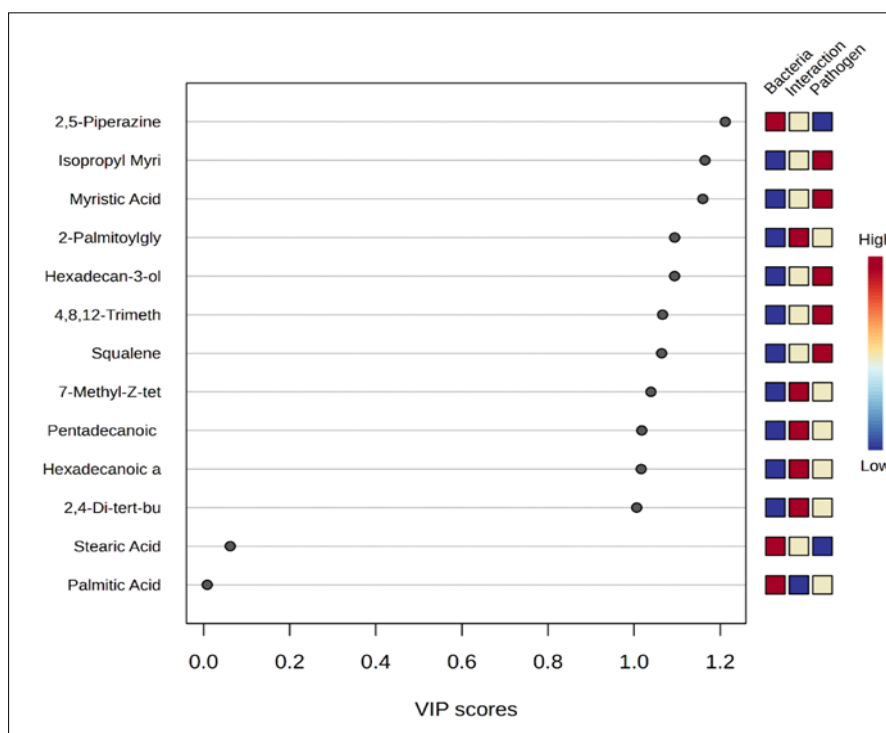


Fig. 6. Variable importance in projection (VIP) score plot analysis.

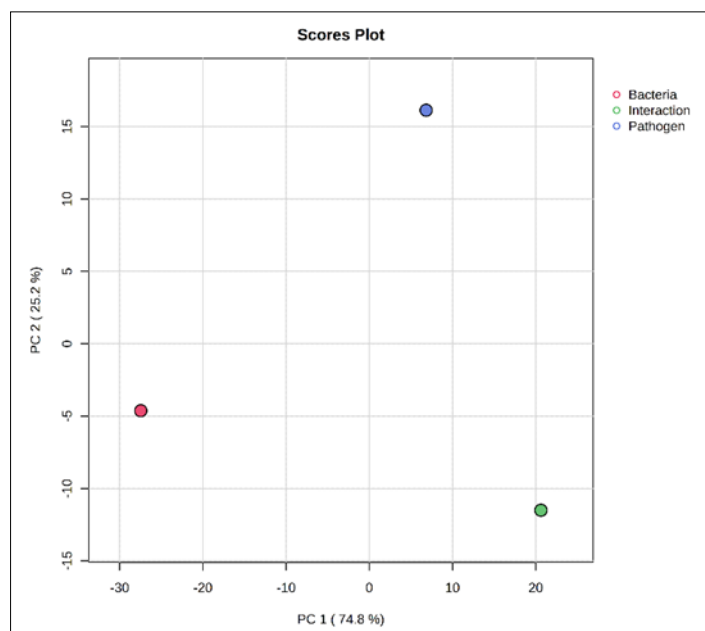


Fig. 7. Principal component analysis (PCA) plot of pathogen, antagonist and their interaction.

Pathway enrichment analysis

Pathway enrichment analysis of the differential metabolites revealed significant modulation in fatty acid biosynthesis and degradation, flavonoids, phenylalanine and aromatic compound metabolism and carbohydrate metabolism during the *B. velezensis*–*Xcm* interaction. Elevated levels of fatty acids and hydrocarbons such as phytane and delta-tetradecalactone indicate enhanced lipid remodelling and volatile production linked to antimicrobial activity. Concurrently, enrichment of phenolic and aromatic pathways suggests activation of defence-related secondary metabolism, while reduced nitroso and aldehyde derivatives imply suppression of pathogenic stress compounds. These metabolic shifts collectively point toward a biocontrol-driven reprogramming of *B. velezensis* under pathogen interaction (Table 3). Enhanced fatty acid biosynthesis and degradation, along with increased hydrocarbon and volatile compounds such as phytane and alkanes, have also been reported in well-characterised *B. velezensis* strains including FZB42 and SQR9, where lipid remodelling and volatile organic

compound production contribute directly to antimicrobial activity and pathogen suppression (14, 53). Similar enrichment of secondary metabolic pathways, particularly those involving phenolic, aromatic and flavonoid-related compounds, has been documented in multiple studies, underscoring their role in defence-related secondary metabolism and induced resistance against phytopathogens (14, 54). Alterations in phenylalanine and aromatic amino acid metabolism observed in the present study parallel findings from transcriptomic and metabolomic analyses of *B. velezensis*–plant–pathogen systems, where these pathways serve as key precursors for antimicrobial lipopeptides and phenolic compounds (55). Furthermore, modulation of carbohydrate metabolism has been widely reported as a critical adaptive response that supports energy demands and provides carbon skeletons for secondary metabolite biosynthesis during antagonistic interactions (35). The observed reduction in nitroso and aldehyde derivatives aligned with the findings of (56) suggesting that *B. velezensis* suppresses stress-related or pathogenic metabolites to stabilise its biocontrol niche.

Table 3. Summary of pathway enrichment analysis based on differential metabolites

Enriched pathway	Key metabolites involved	Biological role / interpretation	Previous reports
Fatty acid biosynthesis & degradation	Phytane, Palmitic acid, Myristic acid, Stearic acid	Indicates altered lipid metabolism during bacterial confrontation; linked to membrane remodeling and production of volatile fatty acids contributing to antimicrobial activity.	(47)
Phenylalanine & aromatic compound metabolism	Phenylmalonic acid, 2-Hydroxy-gamma compound	Associated with synthesis of phenolic derivatives and aromatic antimicrobials; suggests activation of defense-related secondary metabolism.	(48)
Carbohydrate metabolism (glycolysis/ gluconeogenesis, pentose phosphate pathway)	2,3-Dihydro-3-deoxyhexonic acid, 3-Deoxyhexonic acid	Reflects modulation of central carbon metabolism for energy redistribution during microbial interaction.	(49)
Nitroso & aldehyde compound pathways	N-methyl-N-nitroso compound, Valeraldehyde	Reduced abundance suggests suppression of stress or pathogenic metabolite synthesis during antagonistic interaction.	(50)
Glycerolipid metabolism	2-Palmitoylglycero, Isopropyl myristate	Implicates changes in lipid signalling and structural lipid production under interaction conditions.	(51)
Hydrocarbon biosynthesis	Delta-tetradec, Phytane	Enhanced synthesis of aliphatic hydrocarbons, possibly involved in volatile-mediated inhibition of pathogen growth.	(52)

Liquid chromatography–mass spectrometry profiling of pathogen

The LC-MS profiling of the *Xcm* metabolite extract revealed a complex array of compounds, with the total ion chromatogram showing numerous well-resolved peaks. The detected ions (m/z 148.08–1413.96) represented metabolites of varying polarity and complexity, corresponding to distinct metabolic pathways. Early-eluting, low-mass ions (e.g., m/z 148.08) were attributed to polar primary metabolites like amino acids, reflecting active cellular biosynthesis. Mid-retention compounds (e.g., m/z 276.14, 502.32) were identified as fatty acid and phospholipid derivatives, indicating active lipid metabolism for membrane integrity and synthesis of virulence-associated biosurfactants (Fig. 8). Lipids serve as precursors for biosurfactants that aid in host attachment and biofilm formation (33). Crucially, high-mass ions (m/z 1317.43, 1413.96) were detected, corresponding to cyclic lipopeptides or peptides synthesised by non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) pathways. These secondary metabolites are frequently implicated in bacterial virulence due to their membrane-permeabilising and antimicrobial activities. The NRPS-derived lipopeptides in *X. albilineans* are directly linked to pathogenicity (57).

Liquid chromatography–mass spectrometry profiling of *Bacillus velezensis* ER4

The LC-MS analysis of the endophytic *B. velezensis* ER4 revealed a rich metabolic profile, confirming its biosynthetic versatility. The total ion chromatogram displayed numerous well-resolved peaks, with major ions at m/z 146.04, 201.33, 960.27 and 1363.14 corresponding to a spectrum of bioactive compounds. The early-eluting ions (m/z 146.04, 201.33) are characteristic of polar primary metabolites like amino acids. Critically, the detection of medium- and high-molecular-weight ions confirmed the production of key secondary metabolites. The signal at m/z 960.27 is consistent with surfactin-type lipopeptides, while the high-retention ion at m/z 1363.14 strongly indicates fengycins or plipastatins (Fig. 8). This co-production of the three major lipopeptide families like surfactins, iturins (implied by the mass range) and fengycins is a hallmark of

B. velezensis and aligns with the genetic potential described for model strains like FZB42 (31). These lipopeptides act synergistically: surfactins permeabilise microbial membranes, facilitating the action of the more potent fungicidal iturins and antibacterial fengycins (58). The presence of this consortium provides a direct mechanistic explanation for the strong antagonism observed in our bioassays. Specifically, the high-molecular-weight fengycins are highly effective in suppressing Gram-negative pathogens like *Xcm* due to their potent antibacterial and anti-biofilm activities (59).

Liquid chromatography–mass spectrometry profiling of interaction

The LC-MS analysis of the *Xcm* and *B. velezensis* ER4 co-culture revealed a dynamic metabolic profile, indicating strong antagonism and biochemical cross-talk. The total ion chromatogram showed distinct peaks from 0.8 to 9.2 min, representing a wide array of metabolites. Key ions were detected at m/z 146, 193, 573, 734, 1160 and 1274. Early-eluting peaks (m/z 146, 193) corresponded to polar primary metabolites like amino acids. Critically, late-eluting ions in the m/z 1100–1400 range confirmed the presence of cyclic lipopeptides specifically surfactin, iturin and fengycin families characteristic of *B. velezensis* (Fig. 8). The synergistic action of these compounds, where surfactins permeabilise membranes to enhance the toxicity of iturins and fengycins (57), provides a direct mechanistic explanation for the observed suppression of *Xcm* growth.

The most significant finding was a distinct metabolic shift in the co-culture. Characteristic *Xcm* metabolites detected in monocultures were suppressed, while new, unique peaks emerged. This indicates that the interaction is not merely antibiotic but a complex biochemical dialogue. The suppression likely reflects a disruption of the pathogen's primary metabolism and virulence by *B. velezensis* ER4 which is consistent with transcriptomic studies of bacterial antagonism (60). The novel peaks may represent either induced antibacterial compounds or degradation products of *Xcm* cellular components.

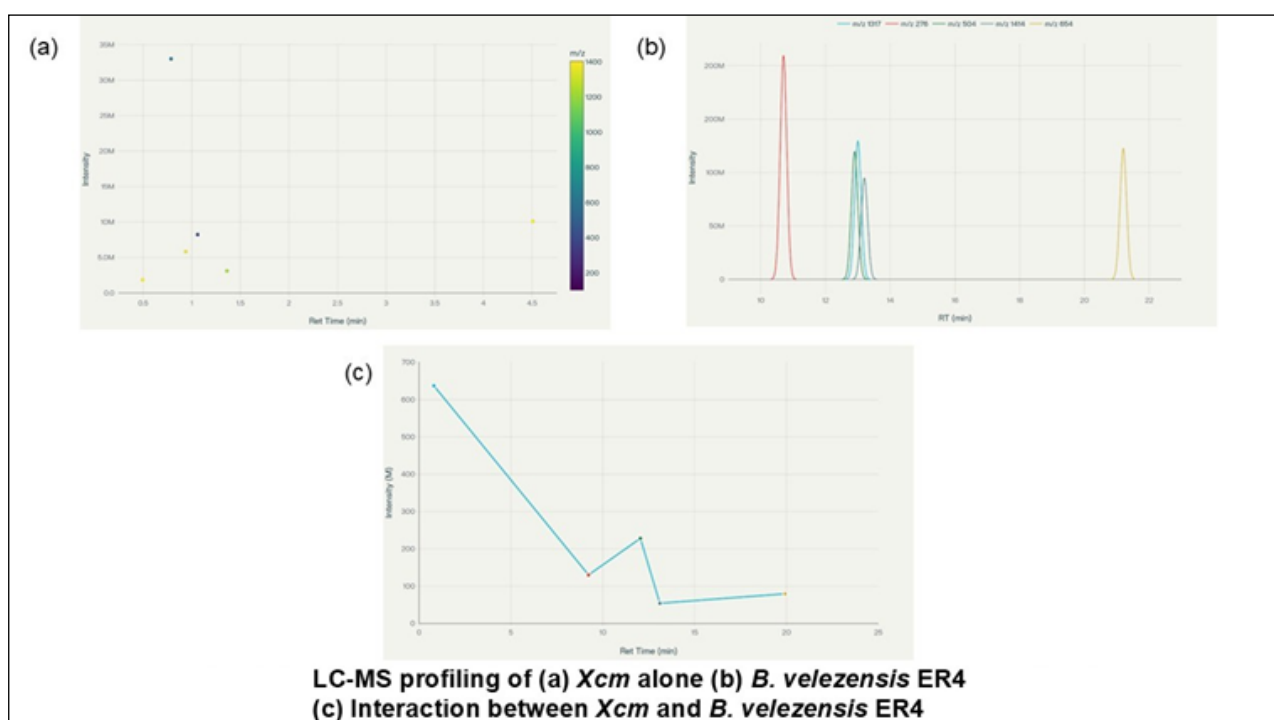


Fig. 8. Liquid chromatography-mass spectrometry (LC-MS) profiling of pathogen, *Bacillus velezensis* ER4 and interaction.

Conclusion

This investigation culminates in the identification and mechanistic validation of the endophytic bacterium *Bacillus velezensis* ER4 as a powerful biocontrol agent against cotton bacterial blight, caused by *Xanthomonas citri* pv. *malvacearum*. By employing a metabolome-driven workflow that integrated initial antagonistic assays with comprehensive GC-MS and LC-MS profiling, this study moved beyond simple phenotypic screening to achieve a deep functional characterisation of its mode of action. The research successfully decoded the biochemical basis for the observed potent antagonism, revealing a sophisticated arsenal of 147 metabolites, with key bioactive classes such as phenolic compounds, fatty acid derivatives and pyrrolo[1,2-a]pyrazine analogues identified as the major drivers of pathogen suppression through direct antimicrobial activity. This elucidated metabolome not only provides a chemical blueprint for its efficacy but also suggests a low risk of pathogen resistance due to the multi-faceted, synergistic action of these compounds. The findings thus provide a solid foundation for the next critical steps of translational application, where future work must focus on advanced formulation science to ensure the stability and delivery of these key metabolites, followed by large-scale field trials to validate the efficacy and any plant growth-promoting benefits in vivo. This research, therefore, marks a significant stride towards bridging the gap between a promising laboratory finding and the development of a practical, eco-friendly solution for cotton farmers, offering a sustainable alternative to chemical pesticides for integrated disease management.

Future prospects

The compelling findings of this study open several promising avenues for future research and development. Firstly, the logical next step involves advanced formulation and delivery systems. Research must focus on developing a stable, commercially viable product, such as a talc-based powder, liquid concentrate, or encapsulated formulation, that ensures the survival of *Bacillus velezensis* ER4 and the bioactivity of its key metabolites during storage, transport and field application. Secondly, extensive field validation and integrated pest management (IPM) integration are crucial. Multi-location, large-scale field trials over several growing seasons are needed to confirm the biocontrol efficacy under diverse agro-climatic conditions, assess its impact on cotton yield and determine its optimal integration with other IPM strategies, such as compatible chemical pesticides or organic amendments. Thirdly, the metabolomic blueprint provides a roadmap for mode-of-action and synergy studies. Future work should investigate the specific molecular targets of the key identified metabolites (e.g., pyrrolo[1,2-a]pyrazine analogues within the pathogen and explore the potential synergistic effects between different compounds in the metabolite suite. Finally, the plant-microbe interaction dimension warrants deeper exploration to validate the plant growth-promoting effects suggested by the metabolomic data and to understand how *B. velezensis* ER4, as an endophyte, colonises cotton plants and modulates the host's defence pathways. Collectively, these prospects will be instrumental in translating this promising laboratory discovery into a practical, sustainable and effective bio-solution for global cotton production.

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Authors' contributions

TMP conceptualised the study. TMP, along with RE, KR, SA, AR and JSSD, contributed to data collection, analysis and drafting of the manuscript. All authors read 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

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

During the preparation of this work, the authors used AI-assisted tools such as Grammarly for grammatical corrections and Quill Bot for paraphrasing to improve language clarity. The authors critically reviewed, edited and approved the final content and takes full responsibility for the accuracy, integrity and originality of the publication.

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