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Bacillus velezensis VM54: A UV-tolerant Strain with Antifungal Activity against Diverse Phytopathogens

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Abstract. Fungal diseases significantly affect crop yield and product quality. The use of chemical fungicides to control fungal infestations can disturb the plant-associated microbiome and raise concerns about food security. Therefore, the use of biological antifungals, such as *Bacillus* species, is encouraged as they offer a sustainable alternative to chemical fungicides. However, the success of such biocontrol agents in the field depends on their ability to withstand environmental stressors like solar UV light and dryness. Here, we present a targeted strategy for selecting resilient biocontrol agents by first isolating endophytic microorganisms that are already adapted to extreme conditions, followed by screening them for antifungal activity. Using this approach, we investigated the chromium-tolerant, root nodule-associated strain *Bacillus velezensis* VM54 for its ability to antagonize fungal pathogens. *B. velezensis* VM54 exhibited strong *in vitro* and *in vivo* antagonism against *Fusarium fujikuroi*, *F. oxysporum*, and *Alternaria alternata*. Notably, the VM54 strain tolerated UV-C radiation up to 1824 J/m², a level comparable to that of the extremophile *Deinococcus radiodurans*, highlighting its robustness for potential field

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application. PCR-based genome analysis revealed the presence of biosynthetic gene clusters for several antimicrobial compounds, including surfactin, bacillaene, diffidin, and macrolactins. Mass spectrometry of cell-free culture extracts confirmed the production of putative fengycin A C16 and C17 lipopeptides.

These bioactive metabolites, along with volatile compounds produced by VM54, are likely responsible for its antifungal activity. This study supports the use of environmentally adapted endophytes as a promising source of resilient biocontrol agents for sustainable agriculture.

Resumen. Las enfermedades causadas por hongos afectan significativamente el rendimiento de los cultivos y la calidad del producto. El uso de fungicidas químicos para controlar las infestaciones fúngicas puede alterar el microbioma asociado a las plantas y generar riesgos para la seguridad alimentaria. Por lo tanto, el uso de antifúngicos biológicos es altamente recomendable, ya que ofrece una alternativa sostenible a los fungicidas químicos. Sin embargo, el éxito de estos agentes de biocontrol en el campo depende de su capacidad para resistir estresores ambientales como la luz solar UV y la sequía. En este trabajo, presentamos una estrategia para seleccionar agentes de biocontrol resilientes mediante el aislamiento de microorganismos endófitos ya adaptados a condiciones extremas, seguida de un escrutinio de la actividad antifúngica. Con este enfoque, investigamos la cepa *Bacillus velezensis* VM54, tolerante al cromo y asociada a nódulos radiculares, debido a su capacidad de antagonizar patógenos fúngicos. *B. velezensis* VM54 exhibió un fuerte antagonismo *in vitro* e *in vivo* frente a *Fusarium fujikuroi*, *F. oxysporum* y *Alternaria alternata*. Cabe destacar que la cepa VM54 toleró la radiación UV-C hasta 1824 J/m², un nivel comparable al de extremófilo *Deinococcus radiodurans*, lo que evidencia su robustez para su posible aplicación en el campo. El análisis genómico basado en ensayos de PCR reveló la presencia de grupos de genes biosintéticos para varios compuestos antimicrobianos, incluidos surfactina, bacillaeno, diffidina y macrolactinas. La espectrometría de masas de los extractos de cultivos confirmó la producción de los supuestos lipopéptidos C16 y C17 de fengicina A. Estos metabolitos bioactivos, junto con los compuestos volátiles producidos por VM54, probablemente son los responsables de la actividad antifúngica. Este estudio respalda el uso de endófitos adaptados al medio ambiente como una fuente prometedora de agentes de biocontrol resilientes para la agricultura sostenible.

Introduction

Fungal diseases threaten most crops by reducing yields and causing significant economic losses [1-3]. Fungal infections affecting tomatoes are damping off, septoria leaf spot, early blight, late blight, anthracnose, powdery mildew, southern leaf blight, *Fusarium* wilt, and *Verticillium* wilt [4]. *Fusarium fujikuroi* is known for causing bakanae disease in rice, stalk rot in maize, and grain mold in sorghum [2,3]. The genus *Fusarium* can colonize the vascular system, impairing the transport of water and nutrients from the roots to the rest of the plant [5]. Leaf spots and early blights of tomato and potato are diseases caused by the fungi *Alternaria alternata* and *A. solani*. This infection is characterized by dark spots on the lowest, oldest leaves of the plants, which may be brown or black [6].

Biocontrol prevents the growth of phytopathogens and compared with chemical control, can reduce environmental pollution and food toxicity [7]. Plant-beneficial microorganisms from the genera *Bacillus*, *Streptomyces*, *Pseudomonas*, and *Serratia* are commonly used as biological alternatives to chemical fungicides. *Bacillus velezensis*, a Gram-positive, aerobic, and endospore-forming bacterium, is capable of inhabiting soil, water, air, the plant rhizosphere, humans, animals, and fermented foods [8]. *B. velezensis* produces diverse secondary metabolites with proven antifungal activity, like cyclic lipopeptides (surfactin, fengycin, iturin, and bacilysin families), bacteriocins (amylocyclicin and amylolysin), and polyketides (diffidin, bacillaene, and macrolactin) [9]. Genes encoding surfactin (*srfA*), bacillaene (*baeR*), diffidin (*dfnD*), macrolactins (*mlnA*), and fengycin (*fenA*) have also been reported for this species [10].

However, the effectiveness of *Bacillus* spp. as biocontrol agents can be significantly limited by their sensitivity to ultraviolet (UV) radiation and other abiotic stresses commonly encountered in agricultural environments [11]. To address this limitation, we investigated a collection of chromium-tolerant *Bacillus* strains isolated from the root nodules of *Prosopis laevigata* shrubs growing in a heavy metal-contaminated region [12].

After surface-sterilization of nodules, 88 isolates were recovered from yeast mannitol agar plates. Bacterial isolates were tested with increasing concentrations of iron, chromium, and zinc. Only six strains were able to tolerate up to 10,000 mg/L of Cr^{6+} , including VM54, suggesting a high level of stress adaptation [12].

The *in vitro* screening of the *Bacillus* collection against *Fusarium oxysporum* identified *Bacillus velezensis* VM54 as the most effective strain. This isolate was further evaluated both *in vitro* and *in vivo* against *F. oxysporum*, *F. fujikuroi*, and *Alternaria alternata*. Mass spectrometry analysis of culture extracts revealed the presence of putative fengycin A C16 and C17, among the most abundant cyclic lipopeptides produced by VM54. Given its chromium tolerance and associated oxidative stress resistance [12], the strain was also tested for tolerance to UV-C radiation. Our findings showed that *B. velezensis* VM54 can survive under multiple abiotic stress conditions, supporting its potential as a robust biocontrol agent for sustainable agricultural applications.

Experimental

Preparation of bacterial and fungal cultures

Bacillus sp. strains, isolated from *P. laevigata* nodules [12], were streaked onto modified yeast extract-mannitol agar (YMA) plates and incubated for 24 h at 30 °C before use. *Bacillus* sp. VM54 grown in modified YM liquid medium was used to prepare bacterial suspensions in sterile distilled water for the plant or seed protection assay. The composition of modified YMA is as follows (g L⁻¹): yeast extract 1.0, mannitol 10, glutamic acid 0.05, and agar 14. The fungal pathogens *Fusarium oxysporum*, *Fusarium fujikuroi*, and *Alternaria alternata* were provided by the Center for Agroecology at the Universidad Autónoma de Puebla. Fungi were grown on potato dextrose agar (PDA) at 28 °C for one week before use in subsequent assays. PDA medium was prepared as follows: 200 g peeled potato, 20 g agar, and 20 g dextrose, adjusted with distilled water to a final volume of 1 liter, pH 6.

Sequence alignment of the *rpoB* gene and phylogenetic analyses

Bacillus sp. VM54 culture was grown overnight to extract total DNA using the Wizard Genomic DNA Purification Kit (Promega Corporation, USA), following the manufacturer's instructions. Amplification of the *rpoB* gene was performed as described by Ramírez et al. [13] using the primers reported by Martens et al. [14].

The *rpoB* gene sequence for *Bacillus* sp. strain VM54 was recorded in GenBank under the accession number OR999323.1. The known *rpoB* sequences of the genus *Bacillus* were retrieved from the Genbank/EMBL/DBJ databases. Multiple sequence alignment was performed using the CLUSTAL_X program [15], and the alignments were reviewed and edited with GeneDoc [16]. The phylogenetic relationships were inferred using the Minimum Evolution method [17] and executed with MEGA version 11 [18].

PCR amplification of genes encoding for secondary metabolite synthesis

Genes encoding secondary metabolite synthesis were detected by PCR using the primers described by Hazarika et al. [8]. *baeR*-F 5'-AGACTCCACCAAGGCAAATC-3'; *baeR*-R 5'-CAGCGGCTTCATGTCATACT-3'; *dfnD*-F 5'-CAGGCGGAATAGGAGAAGTATG-3'; *dfnD*-R 5'-CGGCAGCCGATTGAAATAAC-3'; *srfA*-F 5'-GCTGATGATGAGGAGAGCTATG; *srfA*-R 5'-GATGGTTCGATACGTCCGATAAA-3'; *mlnA*-F 5'-GGCAGGGTCATACCTCATAATC; *mlnA*-R 5'-AGCAGACTTTCGGTCTCATTC-3'. Internal fragments of the *baeR* (involved in bacillaene biosynthesis), *dfnD* (difficidin biosynthesis), *srfA* (encoding surfactin non-ribosomal peptide synthetase), and *mlnA* (biosynthesis of macrolactins) genes [19] were PCR amplified using the following cycling conditions: initial denaturation for 3 min at 95 °C followed by 35 cycles of denaturation (30 s at 95 °C), annealing (30 s at 59 °C), polymerization (90 s at 72 °C), and a final extension for 7 min at 72 °C. The amplified PCR products were visualized by electrophoresis on a 1% agarose gel. The expected PCR product sizes were 890 bp, 920 bp, 990 bp, and 900 bp for *srfA*, *mlnA*, *baeR*, and *dfnD*, respectively.

In vitro B. velezensis VM54-fungus antagonism assays

Dual culture method for chromium-tolerant *Bacillus* sp. LEM1004, LEM1054, MH778712, and MH778713.

The chromium-tolerant *Bacillus* sp. LEM1004, LEM1054, MH778712, and MH778713 were tested against *F. oxysporum* using the dual culture method. PDA plates (pH 6.0) were inoculated at two equidistant points with 10 μ L of a fungal suspension (1×10^6 conidia mL^{-1}). Then, a perpendicular streak of *Bacillus* sp. strains (LEM1004, LEM1054, MH778712, and MH778713) was inoculated along the diameter of the Petri dish. The plates were then incubated at 28 °C for 7 days. The experiment was conducted in triplicate, with each treatment having three replicates.

Dual culture method for *Bacillus velezensis* VM54

The antifungal activity of *B. velezensis* VM54 was screened against *F. oxysporum*, *F. fujikuroi*, and *A. alternata* using the dual culture assay. PDA plates (pH 6.0) were inoculated at the center with 20 microliters (μ L) of a fungal suspension (1×10^6 conidia mL^{-1}). Then, four equidistant streaks of *B. velezensis* VM54 were streaked along the perimeter of the Petri dish. All plates were incubated for 21 days at 28 °C to evaluate the bacterium's ability to inhibit the growth of fungal strains. *Escherichia coli* BL21 (DE3) streaks around the fungus were used as a negative control. Plates containing only the fungal suspension at the center served as controls to compare mycelial growth in the presence and absence of *B. velezensis* VM54. The experiment was conducted in triplicate, with each treatment having three replicates.

Antifungal activity of volatile compounds produced by *B. velezensis* VM54

The effect of volatiles produced by *B. velezensis* VM54 on the growth of fungal strains was evaluated in Bi-Plate partitioned Petri dishes. Ten ml of PDA and YMA were poured into each half of the partitioned Petri dish for the growth of fungi and bacteria, respectively. Twenty μ L of conidial suspension (1×10^6 conidia mL^{-1}) was inoculated on the PDA side of the Bi-plate, and 10 μ L of *B. velezensis* VM54 (1×10^8 CFU mL^{-1}) was inoculated on the YMA side. Bi-Plates were sealed with parafilm and incubated at 28 °C for 14 days. To compare the fungal growth in the presence and absence of *B. velezensis* VM54 volatiles, Bi-Plates seeded with the fungal strain alone were used as controls. Volatiles released by *E. coli* BL21 (DE3) were used as another negative control. Each experiment was performed in triplicate, three times.

Biocontrol assay on tomato seeds

Tomato seeds (*Solanum lycopersicon* var. *esculentum*) were used for the germination assay. To eliminate surface microbiota, tomato seeds were soaked in 0.6 % sodium hypochlorite for 15 min, followed by six washes with sterile distilled water. Ten surface-sterilized seeds were immersed in a 10 mL bacterial and/or fungal suspension for 24 h at 28 °C in a Falcon conical tube. The bacterial suspension was prepared by suspending 0.1 g of washed cells in 10 mL of 0.85 % NaCl buffer, while the fungal suspension was prepared with 1×10^6 conidia/mL in sterile 0.85 % NaCl buffer. Ten seeds immersed in 0.85% saline solution were used as controls. Subsequently, all seeds were incubated on agar plates for 24 h for germination. Each experiment was performed in triplicate.

Biocontrol assay on tomato plants

Optimax Roma tomato plants were used for the crop protection experiment. The plant variety used in the experiment had high resistance to *Alternaria* spp. stem canker; therefore, only *F. oxysporum* and *F. fujikuroi* were assayed for the plant protection experiment. When the tomato plants reached a height of 10 cm, they were transplanted into 8-cm-diameter pots containing 390 g of sterile substrate (vermiculite-peat moss, 1:2 v/v). One plant per pot was grown under greenhouse conditions for two weeks, with ambient temperatures ranging from 16 to 24 °C. At this point, five plants were inoculated, each with 3 mL of *B. velezensis* VM54 suspension containing 1×10^8 CFU mL^{-1} in sterile distilled water, while another five plants were inoculated with 3 mL of *F. fujikuroi* suspension containing 1×10^6 conidia mL^{-1} in sterile distilled water. Five plants remained untreated and served as controls. Additionally, five more plants were first inoculated with *B. velezensis* VM54 as described, and 24 h later, these plants were challenged with 3 mL of *F. fujikuroi* suspension and kept for an additional two weeks. A second infection experiment was performed with the same treatments and conditions as described before, but this time the pathogen was *Fusarium oxysporum* and the number of tomato plants was fifteen per treatment. Dry weight was determined by placing the plants in an oven at 68 °C for 3 days. During the experiments, plants were watered with sterile deionized water and not fertilized.

Biosurfactant production assay

B. velezensis VM54 grew for 24 h at 30 °C in modified YM liquid medium. The entire broth and the centrifuge-filtered extract were assayed for biosurfactant production using a modified drop-collapse technique [20]. Glycerol drops of 15, 30, 50 and 85 µL were placed on Parafilm® film, and the same volume of whole broth, centrifuge-filtered extract, deionized water or sterile YM medium was added to each drop. After 2 min, spread or drop-collapse was observed and recorded.

Protease activity on skim milk agar plate

To determine proteolytic activity, skim milk agar plates were prepared as follows: 100 g of skim milk and 14 g of agar were dissolved in 1 L of deionized water and then autoclaved at 121 °C for 15 min. *B. velezensis* VM54 and *E. coli* BL21 (DE3) were streaked in the middle of an agar plate and incubated for 24 h at 30 °C. Formation of a clear zone indicates proteolytic activity. The experiment was conducted in triplicate.

MALDI-TOF mass spectrometry analysis

B. velezensis VM54 was cultured in modified YM liquid medium for 24 h at 30 °C with agitation at 120 rpm. The cells were removed by centrifugation, and the cell-free supernatant was filtered through a 0.22 µm filter. Two samples were then submitted to the GC and LC Core Laboratory at the Institute of Chemistry of the Universidad Nacional Autónoma de México (UNAM) for MALDI-TOF mass spectrometric analysis. One mL of each sample was mixed with 5 mL of a saturated solution of 2,5-dihydroxybenzoic acid/acetonitrile/trifluoroacetic acid in a 60/40/0.1 composition (matrix). One µL of this mixture was placed onto the sample target and air-dried. Mass spectra in the range of m/z 1000 to 3000 were acquired using a Bruker Microflex MALDI-TOF instrument equipped with a 337-nm nitrogen laser for desorption and ionization. The instrument was operated in reflector-positive mode at an acceleration voltage of 20 kV. Putative lipopeptide assignments were made by comparing the observed masses to those reported in the literature for *Bacillus* cyclic lipopeptides.

UV-C radiation tolerance of *B. velezensis* VM54

The ability of *B. velezensis* VM54 to tolerate UV-C radiation (254 nm) was evaluated on modified YMA plates. Serial dilutions of an overnight culture of *B. velezensis* VM54 were stamped onto modified YMA plates using a replicator [21]. Subsequently, the plates were exposed to UV-C radiation for 2 minutes (1824 J/m²) or 1 minute (912 J/m²), left untreated, and then incubated at 30 °C for 24 h to assess growth. Serial dilutions of an overnight culture of *Bacillus subtilis* were treated as described before and used as controls. All experiments were conducted in triplicate.

Statistical analysis

The data obtained from seed germination and plant dry weight in the crop-protection experiment were subjected to a one-way analysis of variance (ANOVA). The effects considered significant were determined using the Tukey-Kramer post hoc tests in Microsoft 365 Excel. A p -value < 0.05 was used to indicate significant differences in the data.

Results

A collection of *Bacillus* strains isolated from the root nodules of *Prosopis laevigata* shrubs growing in arid, heavy-metal-polluted soil was described by Ramirez et al. [12]. *Bacillus* sp. LEM1004, LEM1054, MH778712, and MH778713 exhibited tolerance to stress caused by 10 000 mg Cr⁶⁺/L [12]. Since one of our research group's interests is the discovery of robust biocontrol agents capable of tolerating hostile field-crop conditions, further characterization of these Cr-tolerant *Bacillus* strains was desirable. Thus, in this work, the antagonistic activity of these *Bacillus* strains against *F. oxysporum* was performed in dual cultures (Fig. 1). After 7 days of incubation, *F. oxysporum* grown alone (Fig. 1(a)) or in interaction with *E. coli* BL21 (Fig. 1(b)) reached the center of the Petri plate with no appreciable growth inhibition. However, when *F. oxysporum* interacted with LEM1004 (Fig. 1(c)), MH778712 (Fig. 1(d)), or MH778713 (Fig. 1(f)), mycelial growth was

inhibited, though not as much as with the LEM1054 strain (Fig. 1(e)). Thus, the *Bacillus* sp. LEM1054 was selected for further characterization and renamed as *Bacillus* sp. VM54.

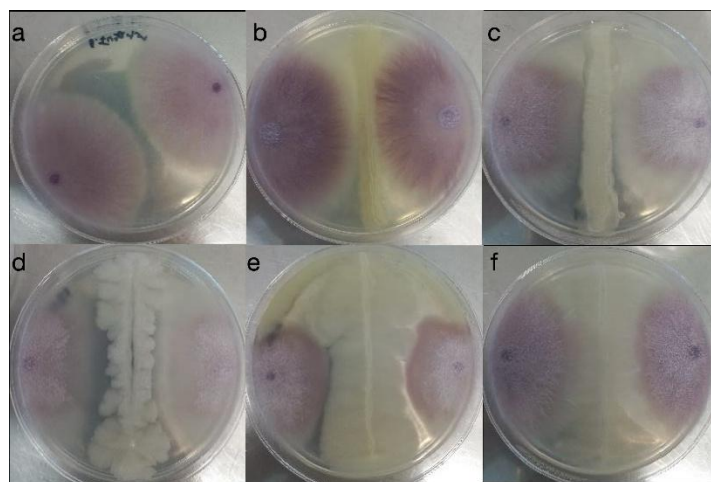


Fig. 1. Antagonistic activity of chromium-tolerant *Bacillus* sp. strains (LEM1004, LEM1054, MH778712, and MH778713) against *Fusarium oxysporum* inoculated at two equidistant points of PDA plates (pH 6.0). Plates were incubated for 7 days at 28 °C. (a) *F. oxysporum* alone. (b) *Escherichia coli* BL21 and *F. oxysporum*. (c) *Bacillus* sp. LEM1004 and *F. oxysporum*. (d) *Bacillus* sp. MH778712 and *F. oxysporum*. (e) *Bacillus* sp. LEM1054 and *F. oxysporum*. (f) *Bacillus* sp. MH778713 and *F. oxysporum*.

Molecular identification of the *Bacillus* sp. VM54

To identify the relationship between the VM54 strain and other *Bacillus* species, a phylogenetic analysis was conducted using the *rpoB* sequence. The minimum evolutionary tree showed that *Bacillus* sp. VM54 belongs to the *B. velezensis* species (Fig. 2). The *rpoB* gene sequence was submitted to NCBI under accession number OR999323.1.

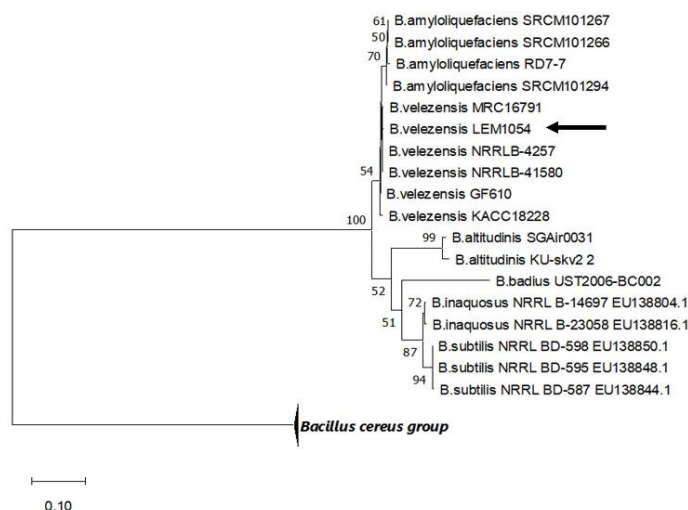


Fig. 2. Phylogenetic tree shows the relationships between *Bacillus velezensis* VM54 and other *Bacillus* species, based on the *rpoB* gene sequence. The evolutionary analyses were performed in MEGA 5. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are shown next to the branches.

Antifungal activity of *B. velezensis* VM54 against *Fusarium fujikuroi* and *Alternaria alternata*

Bacillus velezensis species have been used to control several phytopathogenic fungi; therefore, the ability of *B. velezensis* VM54 to antagonize *F. fujikuroi* and *A. alternata* were investigated in dual cultures for 7, 14 and 21 days (Fig. 3). *B. velezensis* VM54 had a marked inhibitory effect on the growth of *F. fujikuroi* at 7, 14, and 21 days (Fig. 3(G), (H), and (I) top panel) compared to its controls (Fig. 3(A), (B), and (C) top panel). *E. coli* BL21 was used as negative control (Fig. 3(D), (E), and (F) top panel) displaying a minor antagonist effect. A similar antagonism pattern of *B. velezensis* VM54 against *A. alternata* (Fig. 3(G), (H), and (I) bottom panel) and *F. oxysporum* (Fig. S1 (G), (H), and (I) top panel) was observed. These results indicate that *B. velezensis* VM54 displays antagonistic activity against those fungal strains.

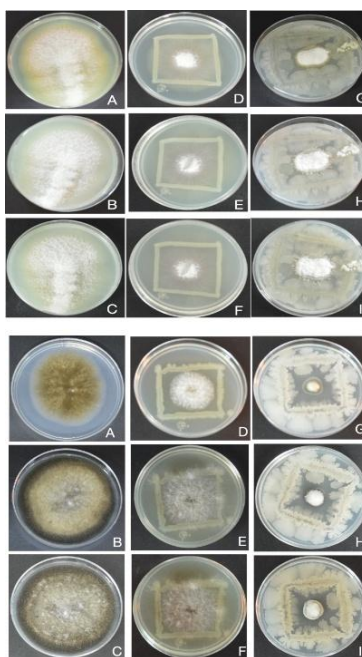


Fig. 3. Antagonistic activity of *B. velezensis* VM54 against *F. fujikuroi* (top panel) and *A. alternata* (bottom panel) in dual cultures at 7, 14 and 21 days. Inhibitory effect of *B. velezensis* VM54 on the growth of *F. fujikuroi* at 7, 14 and 21 days (G, H, I, top panel) compared to its controls at 7, 14, and 21 days (A, B, C, top panel). *Escherichia coli* BL21 was used as negative control (D, E, F top panel). Antagonistic activity of *B. velezensis* VM54 against *A. alternata* at 7, 14, and 21 days (G, H, I bottom panel) compared to its controls at 7, 14, and 21 days (A, B, C bottom panel) with *Escherichia coli* BL21 as negative control (D, E, F bottom panel) at the same points.

To investigate the inhibitory effect of volatiles released by *B. velezensis* VM54 on fungal strains, co-cultivations of *B. velezensis* VM54 with fungi were carried out in partitioned Petri dishes for seven and fourteen days (Fig. 4). The use of partitioned plates prevented bacteria and fungi from contacting, excluding the effects of non-volatile metabolites. Radial growth of *F. fujikuroi* controls without the bacteria (Fig. 4(A), (B), top panel) reached the barrier of the partitioned plate and passed it on days 7 and 14. However, when *B. velezensis* VM54 was co-cultivated on the other side of the partitioned plate, *F. fujikuroi* was unable to reach the plate division, suggesting that bacterial volatile compounds inhibited the mycelial growth of *F. fujikuroi* (Fig. 4(E), (F) top panel). On the contrary, the volatiles released by *E. coli* BL21 did not prevent the growth of *F. fujikuroi*, and after 14 days, the fungus reached the other side of the partitioned plate (Fig. 4(D) top panel). A similar inhibitory pattern was observed for *A. alternata* (Fig. 4(E), (F) bottom panel) and *F. oxysporum* (Fig. S1 (E), (F) bottom panel), while in negative controls, the radial growth of the fungus reached the other side of the partitioned plate (Fig. 4(B), (D) bottom panel and Fig. S1 (B), (D) bottom panel) after 14 days.

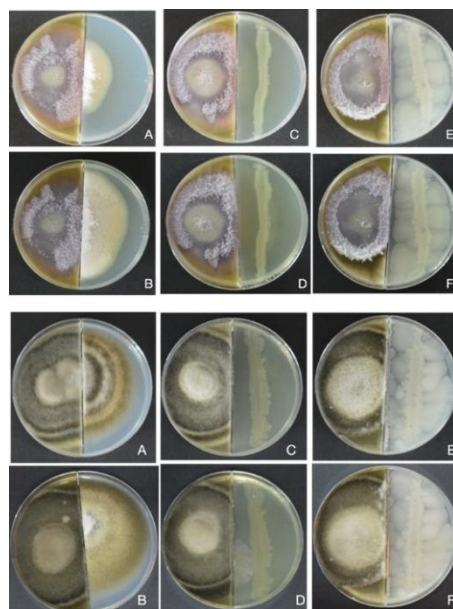


Fig. 4. Impact of volatile organic compounds (VOCs) released by *B. velezensis* VM54 on pathogen growth in partitioned Petri dishes for 7 and 14 days. **A, B top panel:** Radial growth of *F. fujikuroi* without *Bacillus* (control) at 7 and 14 days. **E, F top panel:** co-cultivation of *F. fujikuroi* with *B. velezensis* VM54 at 7 and 14 days. **C, D top panel:** co-cultivation of *F. fujikuroi* with *E. coli* BL21 at same point. **E, F bottom panel:** Inhibitory effects of VOCs produced by *B. velezensis* VM54 on *A. alternata* after 7 and 14 days. **C, D bottom panel:** co-cultivation of *A. alternata* with *E. coli* BL2 as control. **A, B bottom panel:** *A. alternata* grown alone after 7 and 14 days.

Protease activity, surfactant production, and putative fengycin A C16 detection

Bacillus species can inhibit plant pathogens using lytic enzymes such as chitinases, glucanases, and proteases. To test the protease activity of *B. velezensis* VM54, a skimmed milk agar assay was performed. *B. velezensis* VM54 displayed substantial protease activity (Fig. 5(B)) while *E. coli* BL21 (DE3) (negative control) showed no activity (Fig. 5(A)). To further elucidate the potential mechanisms used by *B. velezensis* VM54 against fungal strains, the presence of cyclic lipopeptide and polyketide genes in its genome was examined (Fig. 5(C) and Fig. S2, **original gel**). The gene *surfA* (encoding surfactin non-ribosomal peptide synthetase), *baeR* (involved in bacillaene biosynthesis), *dfnD* (difficidin biosynthesis), and *mlnA* (biosynthesis of macrolactins) were all detected by PCR (Fig. 5(C)). To validate primer specificity and PCR product sizes reported by Hazarika et al. [8], an alignment of the primer sequences against the *Bacillus velezensis* FZB42 genome was performed. The results confirmed primer specificity and PCR amplicon sizes. After obtaining the locus tags from NCBI, we searched the KEGG database to identify the corresponding genes, which confirmed the expected gene functions. Surfactin, a cyclic lipopeptide biosurfactant, is known to reduce the surface tension of water [22]. In Fig. 5(D), the drops containing broth culture (row labeled as S+B) or cell-free extract (row labeled as S-B) exhibited some degree of area expansion in comparison to control drops containing just water (row labeled as H₂O) or sterile medium (row labeled as YM), suggesting the production of biosurfactants. To confirm lipopeptide production, a culture extract of *B. velezensis* VM54 grown for 24 h was analyzed by MALDI-TOF MS (Fig. 6). The mass spectra ($[M+H]^+$) showed distinct groups of peaks within the following mass ranges: m/z 1463-1544 (putative fengycins), m/z 1022-1075 (putative surfactins), and m/z 1043-1096 (putative iturins), all of which are typical for *Bacillus* spp. (Fig. 6(A)). The most abundant peaks in the spectrum corresponded to the putative fengycin A C17 with $[M+H]^+$ m/z of 1478.017 and its potassium adduct (fengycin A C17 with $[M+K]^+$ m/z of 1516.031), followed by the putative fengycin A C16 with $[M+H]^+$ m/z of 1463.963 and its potassium adduct (fengycin A C16 with $[M+K]^+$ m/z of 1501.970) (Fig. 6(B)). Three families of lipopeptides produced by *Bacillus subtilis* or *B. velezensis* are iturins, fengycins, and surfactins. All of them were detected by the MALDI-

TOF analysis (Fig. 6(A)). Only surfactin non-ribosomal peptide synthase (*srfA*) was included in the PCR-based genome analysis, finding a match between the metabolite and the target gene. Although *baeR* (involved in bacillaene biosynthesis), *dfnD* (difficidin biosynthesis), and *mlnA* (biosynthesis of macrolactins) genes were detected by PCR-based analysis, they were not the principal compounds detected by MALDI-TOF analysis.

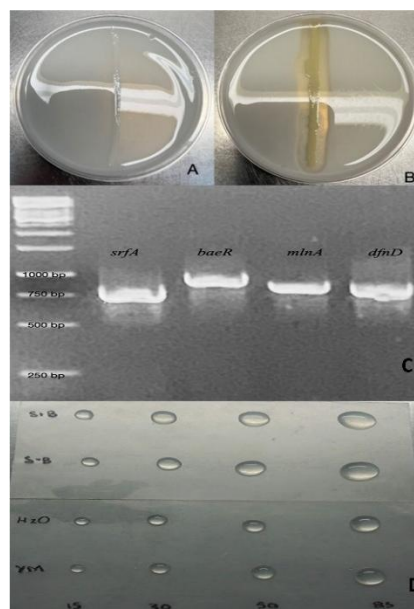


Fig. 5. Protease activity, surfactant production, and detection of genes encoding for secondary metabolite synthesis in *B. velezensis* VM54. **(A)** Protease activity of *E. coli* BL21 (DE3) as negative control. **(B)** Protease activity of *B. velezensis* VM54. **(C)** Detection of genes encoding cyclic lipopeptides and polyketides in *B. velezensis* VM54: *srfA* (surfactin synthetase), *baeR* (bacillaene biosynthesis), *mlnA* (macrolactin biosynthesis) and *dfnD* (difficidin biosynthesis). **(D)** Reduction of surface tension assay. Drops containing broth culture (S+B) or cell-free extract (S-B), compared with drops of water (H₂O) or sterile medium (YM), as controls.

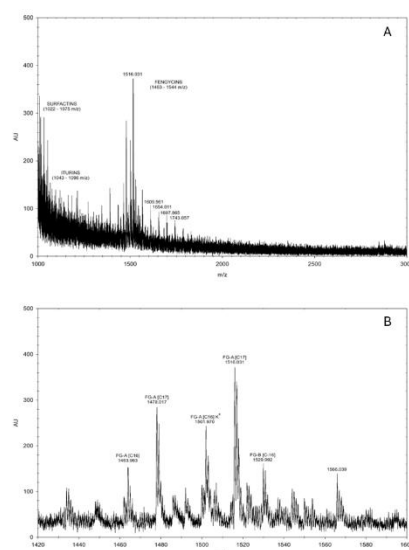


Fig. 6. MALDI-TOF mass spectra of cell-free extracts from *Bacillus velezensis* VM54 cultures **(A)**. Magnified view of the spectra in the 1420 – 1600 m/z range showing the presumed lipopeptide compounds.

Biocontrol potential of *B. velezensis* VM54 in seed germination and plants

Tomato seeds (*Solanum lycopersicon*) were used for the germination assay (Fig. 7(A)). The seeds were surface-sterilized and soaked in a bacterial and/or fungal suspension. Seeds immersed in 0.85 % saline solution were used as controls. The percentage of germination of tomato seeds inoculated with *B. velezensis* VM54 was higher than that of control seeds soaked with 0.85 % NaCl (Fig. 7(A)). Fungal inoculation (*F. oxysporum*, *F. fujikuroi*, *A. alternata*) inhibited germination, with a percentage of germination lower than 20 %, but co-inoculation of *B. velezensis* VM54 with *F. oxysporum* or *F. fujikuroi* on seeds increased seed germination by 3.3-fold or 2.9-fold compared to the seed germination of the pathogen alone (Fig. 7(A)). This result suggests that *B. velezensis* VM54 can protect seeds from these plant pathogens. *A. alternata* was the strongest pathogen inhibiting seed germination (Fig. 7(A)).

The protective effect of *B. velezensis* VM54 against *F. fujikuroi* was evaluated in plants for two weeks under greenhouse conditions (Fig. 7(B)). There was significant growth promotion (higher dry weight) in the bioagent control inoculated with *B. velezensis* VM54 compared to the control supplied with water (Fig. 7(B)), indicating that the VM54 strain was beneficial to tomato plants. Compared to the disease control, which was inoculated with *F. fujikuroi* alone, the coinoculation of *B. velezensis* VM54 and *F. fujikuroi* increased the dry weight (Fig. 7(B)), suggesting a better development of plants in the presence of *B. velezensis* VM54. Although the dry weight of the control supplied with water and the disease control were the same after two weeks of incubation (Fig. 7(B)), plants challenged with *F. fujikuroi* displayed (Fig. 7(F)) higher disease symptoms (wilting and chlorosis of leaves) than the water control (Fig. 7(C)). Plants co-inoculated with the pathogen and *B. velezensis* VM54 (Fig. 7(E)) did not show wilting and chlorosis of leaves, suggesting the potential protective effect of the VM-54 strain. In another experiment, tomato plants were challenged with *Fusarium oxysporum* for two weeks. Plants inoculated with *F. oxysporum* alone (Fig. 8(B)) displayed more symptoms of chlorosis compared to plants that were previously inoculated with *B. velezensis* VM54 and then challenged with *F. oxysporum* (Fig. 8(D)). The dry weight of infected but unprotected plants (0.338 ± 0.129 g) was statistically lower than that of challenged plants that were previously protected with *B. velezensis* VM54 (0.461 ± 0.141 g). The dry weight of control plants supplied with water (0.337 ± 0.118 g) and the disease control (0.338 ± 0.129 g) were not statistically different after two weeks of infection, but symptoms differed (Fig. 8(A), (B)). Again, plants inoculated with *B. velezensis* VM54 alone exhibited the highest dry weight (0.592 ± 0.220 g), suggesting the VM54 strain's potential growth-promoting trait.

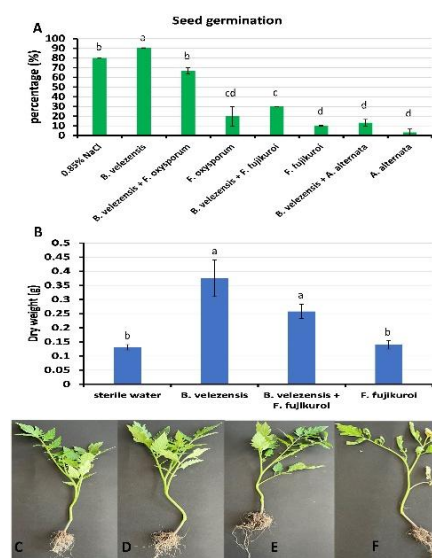


Fig. 7. Biocontrol potential of *B. velezensis* VM54 in seed germination and plant development. (A) Effect of co-inoculation of *B. velezensis* VM54 and fungal pathogens on seed germination, $n = 10$. Different letters indicate statistical differences ($p < 0.05$). (B) Protective effect of *B. velezensis* VM54 against *F. fujikuroi*, $n = 5$. The standard deviation is represented by the bars. (C) Control plants. (D) Plants inoculated with *B. velezensis* VM54. (E) Plants co-inoculated with *F. fujikuroi* and *B. velezensis* VM54. (F) plants inoculated with *F. fujikuroi* only.



Fig. 8. Protective effect of *B. velezensis* VM54 against *F. oxysporum*. (A) Control plants (non-treated), (B) Plants infected with *F. oxysporum*, (C) Plants inoculated with *B. velezensis* VM54, (D) Plants inoculated with *B. velezensis* VM54 and challenged with *F. oxysporum*. For each treatment 15 plants were used.

Tolerance of *Bacillus velezensis* VM54 to UV-C radiation

Robust biocontrol agents are essential for field applications, where UV radiation can compromise their survival and, consequently, their effectiveness. The ability of *B. velezensis* VM54 to tolerate UV-C radiation (254 nm) was investigated (Fig. 9(D), (E), (F)). After 2 min of UV-C radiation (1824 J/m^2), the initial bacterial count ($9 \times 10^8 \text{ CFU/ml}$) of *B. velezensis* VM54 dropped to $3 \times 10^7 \text{ CFU/ml}$, while *Bacillus subtilis* did not tolerate even 1 min of UV-C radiation (912 J/m^2) (Fig. 9(B)). This result highlights the UV-C tolerance of *B. velezensis* VM54 and its potential as a biocontrol agent for field applications.

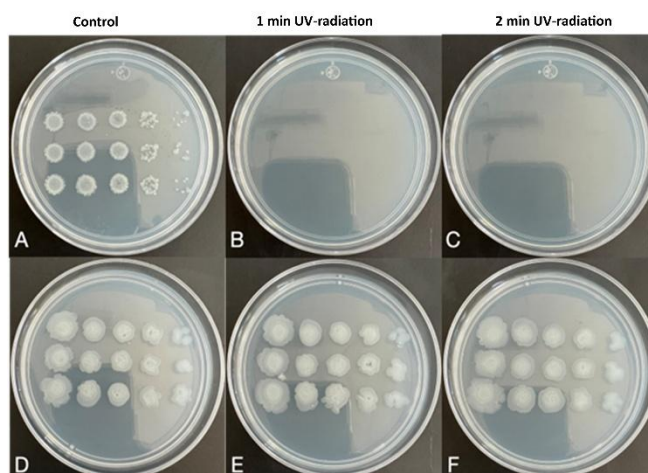


Fig. 9. UV-C radiation tolerance of *B. velezensis* VM54 and *B. subtilis*. YMA plates were inoculated with serial dilutions of *B. subtilis* and either left untreated (A), exposed to 1 minute (B) or 2 minutes (C) of UVC radiation. Panels (D), (E), and (F) show *B. velezensis* plates that were untreated, or exposed to 1 or 2 minutes of UV-C radiation, respectively.

Discussion

UV radiation tolerance is a critical trait for biocontrol agents, especially those intended for foliar applications where they are directly exposed to sunlight [11]. In this study, we identified a *Bacillus velezensis* strain, VM54, that combines strong antifungal activity with notable UV-C resistance, highlighting its potential for use under field conditions. VM54 exhibited antagonistic effects against *Fusarium oxysporum*, *F. fujikuroi*, and *Alternaria alternata*, while maintaining viability after exposure to 1824 J/m² of monochromatic UV-C (254 nm) radiation—suffering less than a 2-log reduction in CFU/mL (Fig. 9(F)).

This level of UV-C tolerance is particularly significant when compared to other *Bacillus* strains. For example, an endophytic *B. subtilis* isolated from papaya did not survive exposure to 912 J/m² (Fig. 9(B)), while commercial strains such as *B. thuringiensis* ATCC 10792 and *B. cereus* WIV 5958 tolerated only 396 J/m² and 144 J/m², respectively [23]. Remarkably, VM54's resistance is comparable to that of *Deinococcus radiodurans*, one of the most UV-resistant organisms known, which shows a 3–4 log reduction after 1500 J/m² [24]. While most solar UV exposure involves UV-B (280–315 nm), the ability to tolerate germicidal UV-C radiation suggests that VM54 possesses robust stress-response mechanisms that likely confer cross-protection under natural sunlight, where UV-B is less damaging than UV-C [24].

Importantly, both UV radiation and heavy metal exposure generate reactive oxygen species (ROS), leading to oxidative stress. The exceptional UV-C tolerance of *B. velezensis* VM54 may be linked to its prior adaptation to oxidative environments, as it was originally isolated from the root nodules of *Prosopis laevigata* in a chromium-contaminated site [12]. While most Cr-tolerant *Bacillus* strains reported in the literature tolerate between 600 and 1100 mg/L of Cr⁶⁺ [25–28], VM54 strain was able to grow at concentrations as high as 10,500 mg/L [12].

This study highlights a promising strategy for identifying resilient biocontrol agents: by first isolating endophytic microorganisms from extreme environments, such as heavy metal-polluted soils, researchers can pre-select strains with inherent stress tolerance. These naturally adapted organisms, such as *B. velezensis* VM54, are better equipped to withstand the challenging conditions of agricultural fields, thereby increasing their viability and effectiveness as sustainable biocontrol solutions.

Bacillus velezensis is well recognized for its dual role in suppressing plant pathogens and promoting plant growth. In this study, *B. velezensis* VM54 displayed some degree of protection to tomato seeds and plants from fungal infection (Figures 7 and 8), likely through the production of bioactive lipopeptides, particularly fengycins (Fig. 6). This aligns with findings by Lin et al [29], who demonstrated that among the lipopeptides produced by *B. amyloliquefaciens* JFL21 (iturins, fengycins, and surfactins), fengycins exhibited the strongest antimicrobial activity against *Listeria monocytogenes*, *Aeromonas hydrophila*, and *Colletotrichum gloeosporioides*.

To further confirm the role of fengycins in the antagonistic activity of VM54, future work should include targeted disruption of the *fenA* gene, a key biosynthetic gene in the *fenABCDE* cluster. Assessing the antifungal activity of a *fenA*-deficient mutant would help clarify whether fengycin production is indeed the primary mechanism underlying pathogen suppression. Furthermore, longer plant-infection experiments will be needed to validate the biocontrol potential of *B. velezensis* VM54.

Other *B. velezensis* strains, such as HN-Q-8 and WZ-37, have also demonstrated protective effects against fungal diseases, albeit through different metabolic strategies, including the activation of ethylene and jasmonic acid signaling pathways [1, 30]. These differences underscore the metabolic versatility of *B. velezensis* and highlight the importance of strain-specific characterization for developing targeted and effective biocontrol agents.

Conclusions

In summary, the findings of this study offer valuable insights for the development of biocontrol-based crop protection strategies, particularly under environmental stress conditions, such as soils contaminated with chromates. *Bacillus velezensis* VM54 demonstrated strong antagonistic activity against multiple fungal

phytopathogens, including *Fusarium oxysporum*, *F. fujikuroi*, and *Alternaria alternata*, both through the production of bioactive volatiles and lipopeptides. Importantly, its ability to tolerate high levels of UV-C radiation further supports its potential as a resilient biocontrol agent suitable for field applications in challenging environments.

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