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Nashwa H. Abdullah

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Evaluation of the biocontrol potential of a new native *Bacillus velezensis* isolate "NH1 OQ711964" against *Alternaria solani*

Nashwa H. Abdullah

Botany and Microbiology Department, Faculty of Science, Helwan University, Egypt

One of the current challenges is meeting the increasing food demand and providing sustainable food resources. Hence, controlling plant diseases, especially those caused by phytopathogens, has gained significant interest in reducing the annual loss of plant crops. Although the control programs have traditionally relied on the use of agrochemicals, there is an urgent need to shift to biological control techniques because of the developed environmental and health concerns as well as the rise of microbial resistance. *Bacillus velezensis* is an emerging biocontrol agent that has shown promising biocontrol potential. In the current study, a new native *B. velezensis* isolate has been isolated and tested for its antagonistic potential against *Alternaria solani*, the causal agent of a destructive disease affecting the solanaceous crops. The investigated isolate, *Bacillus velezensis* NH1 OQ711964, exhibited good antagonistic behavior against *Alternaria solani* in all tested methods. Its extracellular metabolites exerted a significant antifungal activity. Six phenolic, fatty acid, alkyne and pentadiene compounds have been estimated as the major metabolites in its extracellular ethyl acetate extract employing Gas Chromatography–Mass Spectrometry. These compounds in addition to Hydrocinnamic acid, a detected minor product, are well characterized by their antifungal activities which can explain the significant antifungal behavior of this isolate. Indeed, *A. solani* culture exposed to these metabolites has shown obvious mycelial growth inhibition, thinner hyphae with multiple fractures, coiling and distortions, in addition to cytoplasmic leakage combined with sparse cytoplasmic filling as illustrated by its scanning electron microscope imaging.

Keywords: *Bacillus velezensis; Alternaria solani;* biocontrol potential; antifungal; secondary metabolites; bioactivity

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Nashwa H. Abdullah
Botany and Microbiology Department,
Faculty of Science, Helwan University, Egypt
Email: nashwahamed_2010@yahoo.com
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INTRODUCTION

Annually, a significant crop loss is recorded as a result of plant diseases caused by plant pathogens. This issue limits the available food sources and seriously threatens global food security. Consequently, there is a critical need to provide more potent and efficient control systems to manage such plant diseases and secure sustainable production of economically important crops (Thambugala *et al.*, 2020; Ayaz *et al.*, 2023). Although most of the commonly used plant disease control systems rely intensely on agrochemicals, the wide use of such chemicals has led to serious environmental and health problems in addition to the development of an increased antimicrobial resistance (Kim, *et al.*, 2017; Gomes *et al.*, 2021). Thus, the concern is shifting again towards the use of alternative natural approaches to manage and control plant disease (Singh *et al.*, 2024). Among phytopathogens, fungi represent the most varied, common, and significant pathogens (Purcell, 2009). They are responsible for the most destructive plant diseases (El-Wakeil and Saleh, 2020). *Alternaria solani* is one of such significant pathogens. It is the causal agent of early blight disease affecting potatoes, tomatoes and other solanaceous crops (Cox and Simpson, 2010; Munyaneza and Bizimungu, 2022). Regarding *solanum* species, the diseased plant shows severe defoliation and produces sun-scalded and poor-colored fruits (Muimba-Kankolongo, 2018). Concerning the potato plant, it primarily affects the leaves and stems and can result in considerable defoliation leading to a significant reduction in crop yield, especially in the case of premature defoliation. The disease can also affect potato tubers (Munyaneza and Bizimungu, 2022). *Alternaria solani* is traditionally controlled using different fungicides (Deshmukh *et al.*, 2020). However, as its hosts represent widely cultivated commercial crops, that has resulted in an extensive and repeated use of such chemicals, leading to many environmental, water and soil pollution issues in addition to the development of significant resistance in the targeted pathogen, causing a reduction in control efficacy. On the other hand, the wide use of such chemical fungicides can result in health risks due to the presence of their residues on consumed fruits (Deshmukh *et al.*, 2020; Li *et al.*, 2024). Thus, there is an urgent and special need to provide alternative biological and ecofriendly methods for controlling *Alternaria solani* associated diseases.

One of the efficient and ecofriendly protocols used in the management of plant diseases is the use of biological control agents. The principle of such technique depends on the utilization of direct or indirect antagonistic behaviors of some microorganisms against other microbes that attack

the host plant in their natural environment (Wockenfuss *et al.*, 2024). Indeed, many different bacterial and fungal genera have been detected to exhibit strong biocontrol potential against various plant pathogens (Abdel Rahman et al., 2022; Korany et al., 2023; Kasem et al., 2024). One of the antagonistic activities of these organisms is maintained by their release of different natural compounds including secondary metabolites that can suppress the growth of plant pathogens (Ayaz *et al.*, 2023). These compounds are low molecular weight compounds with a diversified chemical structure. They are not produced for essential growth purposes but are excreted when the producing organism is exposed to unfavorable conditions as a survival tool (Ayaz *et al.*, 2023; Fouillaud and Dufossé, 2022).

Among the efficient biocontrol agents, *Bacillus* species are widely used for controlling of a wide range of plant pathogens. They exhibit strong antagonistic potential by competing with these pathogens for space or nutrients, producing inhibitory substances, or inducing plant defense mechanisms (Zhang *et al.*, 2021). Additionally, *Bacillus* species are endospore-forming bacteria which enhances their applicability as biocontrol agent by enabling them to exhibit good resistance to the abiotic stress including elevated temperatures (Baptista *et al.*, 2022). Additionally, *Bacillus* species have been well documented as potential producers of a wide range of bioactive secondary metabolites, which serves as an efficient targeting antagonistic mechanism against many plant pathogens (Zhang *et al.*, 2021; Pournejati, *et al.*, 2021). Indeed, a recent study has estimated that nearly 8% of *Bacillus* genomes encode the production of secondary metabolites (Pournejati, *et al.*, 2021). As a *Bacillus* species, *B. velezensis* is an emerging and promising biocontrol agent (Wockenfuss *et al.*, 2024). It is widely distributed in nature and characterized by its adaptation to different environmental conditions, significant resistance to variable abiotic and biotic stresses, genetic stability, and ease of cultivation (Li *et al.*, 2024). Recently, these strains have been reported to suppress many plant-pathogenic fungi due to their potential to secrete a wide variety of secondary metabolites with broad-spectrum antifungal activities. Hence, they are strongly recommended as a unique, efficient and eco-friendly biocontrol agent alternative to the chemical fungicides traditionally used for controlling many fungal crop diseases (Kim, *et al.*, 2017).

Lately, new *B. velezensis* strains have been isolated and their antagonistic behavior against many fungal plant pathogens has been evaluated. Indeed, *B. velezensis* has shown promising control potential against many phytopathogens, including *Verticillium dahlia* and *Colletotrichum gloeosporioides* (Yao *et al.*, 2025). In agreement, two rhizospheric *Bacillus velezensis* isolates have been reported to exert a strong antagonistic activity against two important fungal plant pathogens; *Ralstonia solanacearum* and *Fusarium oxysporum* (Cao *et al.*, 2018). Also, *Bacillus velezensis* strain BS1, isolated from a pepper rhizospheric soil, was found to inhibit the mycelial growth and the appressorium formation of *Colletotrichum scovillei* and reduce the disease developed by it in chili pepper (Shin *et al.*, 2021). Additionally, *B. velezensis* isolate TSA32-1 exhibited a significant antifungal activity against *Fusarium graminearum*, *F. fujikuroi*, *Alternatia alternate*, and *Diaporthe actinidiae* (Kim *et al.*, 2022).

However, very few studies have evaluated the antagonistic and biocontrol behavior of *B. velezensis* strains against *Alternaria solani*, the causal agent of early blight disease. This study aims to explore the biocontrol potential of a new native *B. velezensis* isolate against *Alternaria solani* and deduce its antagonistic behavior in relation to its secondary metabolites.

MATERIALS AND METHODS

Microorganisms

A native *Bacillus* isolate was isolated from soil samples collected from the rhizosphere of *Alternaria solani* resistant *Solanum lycopersicum* L. seedlings growing at Helwan University- Cairo- Egypt (29°51'52"N 31°19'00"E). Soil particles from the collected sample were spread directly under sterile conditions over the surface of Malt extract-Glucose-Yeast Extract-Peptone (MGYP) agar medium plates (Malt extract 3.00, Yeast extract 3.00, Peptone 5.00, Glucose 10.00 g/l, pH=6.2; Wickerham, 1951). The inoculated plates were incubated at 25 ˚C for 48 h. The developed colonies were purified and maintained on tryptone soy agar "TSA" (Merck, Germany) slants. *Alternaria solani* was kindly provided by Agricultural Research Center, Giza, Egypt.

Molecular identification of the isolated bacterium

Genomic DNA of the isolated bacterium was extracted using the Patho-gene-spin DNA/RNA extraction kit (Intron Biotechnology, Korea). The universal primers, 27F and 1492R, were utilized for the amplification of 16s rRNA sequences. PCR reaction, purification and sequencing of the PCR product were conducted at

SolGent Company, Daejeon-South Korea. The obtained sequences were analyzed utilizing Basic Local Alignment Search Tool (BLAST) provided by the National Center for Biotechnology Information (NCBI) database. Phylogenetic analysis was performed using (NCBI) and MegAlign (DNA Star) software version 5.05.

Antagonistic activity of the investigated bacterium against *Alternaria solani*

Agar double layer method

The antagonistic behavior of the isolated bacterium against *A. solani* was estimated by agar double layer method as described by Macedo-Raygoza *et al*, 2019 with slight modifications. Warm liquified tryptone soy agar medium was poured in sterilized petri dishes and left to be solidified. Single colony of the sub-cultured bacterium was inoculated on the center of the solidified tryptone soy agar plates, then soft Malt extract-Glucose-Yeast extract-Peptone agar medium seeded with *A. solnani* spore suspension was poured as an over lay layer. Plates were left to be solidified and incubated at 25 ◦C for 72 h. Soft Malt extract-Glucose-Yeast extract-Peptone agar plates inoculated with *A. solani* spore suspension were incubated at the same conditions and considered as control plates.

Agar block method

The antagonistic behavior was also evaluated by agar block method described by Ayuningrum *et al.*, 2017 and Calcagnile *et al.*, 2022 with slight modifications. Warm liquified soft Malt extract-Glucose-Yeast extract-Peptone agar medium inoculated with *A. solani* spore suspension was poured in sterilized petri dishes and left to be solidified. After solidification, the plates were inoculated with agar blocks (8 mm) obtained from the sub-cultured bacterium culture that was placed on the center of the plate. Soft Malt extract-Glucose-Yeast extract-Peptone agar plates inoculated with *A. solani* spore suspension were also used as control plates in this test. The plates were incubated at 25 ◦C for 72 h.

Dual culture method

Method 1: The tested bacterium was streaked on one side of a tryptone soy agar plate 2 cm away from the plate margin. A fungal disc (8 mm diameter) obtained from *A. solani* fresh culture was placed on the opposite side also 2 cm away from the plate margin. The plates were incubated at 25 ◦C for 11 days. Results were taken by measuring the radius of developed *A. solani* colony and calculating the growth inhibition percent as following:

GI%= [(R1-R2)/R1] × 100

Where, R2; is the radius of the developed colony in the direction of the antagonistic bacteria and R1 is the radius of the developed colony on the opposite side "control growth" (Haidar *et al.*, 2016).

Method 2: A mycelial disc is obtained from a fresh *Alternaria solani* culture and was inoculated on the center of Malt extract-Glucose-Yeast extract-Peptone plate. Three sterile filter paper discs (8 mm in diameter) were inoculated near the plate margins and loaded with 10 µl from fresh broth culture of the tested bacterium then the plates were incubated at 25 ◦C for 6 days. For control plates, the filter paper discs were loaded with 10 µl sterile saline instead of the bacteria broth culture. Results were taken by measuring the growth of the developed fungal colony and calculating the mycelial growth inhibition percent as following:

Mycelial growth inhibition (%) = (1 − TD/CD) × 100

Where, TD is the straight distance from the center of the fungal colony to fungal hyphae edges in the treatment plates while CD is this distance in the control plates (Shin *et al.*, 2021).

Extraction of bioactive metabolites

The tested bacterium was sub-cultured on Malt extract-Glucose-Yeast extract-Peptone broth media at 25 ◦C and 120 rpm for 24 h. One ml from the obtained bacterial culture was inoculated in 100 ml MYGP broth medium contained on 250 ml conical flask(s) and incubated at 25 ◦C and 120 rpm for 72 h. The obtained bacterial cells were collected from the fermentation broth by centrifugation at 4000 rpm for 10 min. Intracellular metabolites were extracted by resuspension of collected cells on ethyl acetate and its sonication for 3 min followed by the removal of cell debris by filtration process using 0.22 µm membrane filter. On the other hand, an equal volume of ethyl acetate was used to extract the extracellular metabolites from the obtained supernatant. The extracted metabolites were vacuum dried, and the dry residue was collected and weighted then dissolved in methanol for further tests.

Assessment of the antifungal activity of the extracted metabolites

Well diffusion method

The antifungal activity of the extracted metabolites was evaluated using well diffusion method (Valgas *et al.*, 2007). Warm liquified Malt extract-Glucose-Yeast extract-Peptone agar medium was seeded with *A. solani* spore suspension, poured in sterilized petri-

dishes and left to be solidified. After solidification, 8 mm diameter wells were made on the solidified plates using a sterile cork borer. 100 µl from the dissolved extract, in a concentration of 30 mg/ml, was inoculated in these wells. Wells inoculated with 100 µl methanol were used as control. Plates were incubated at 25 °C for 74 h and results were taken by measuring the inhibition zone diameter.

Dual culture technique

Dual culture technique was also utilized to estimate the *A. solani's* radial growth inhibition percentage that can be maintained by the metabolites of the tested bacterium. This test was carried out for the extracellular extract that showed the best result in the previous test according to the method described by Zhang *et al*, 2021. Warm liquified Malt extract-Glucose-Yeast extract-Peptone agar medium was poured in sterilized petri dishes and after their solidification, 8 mm diameter wells "3 cm away from each other's" were made using a sterile cork borer. 100 µl from the dissolved extract, in a concentration of 30 mg/ml, was inoculated in the wells. A fungal disc (8 mm diameter) obtained from a fresh *A. solani* culture was inoculated on the opposite site 3 cm away from these wells. Plates were incubated at 25 °C for 74 h and the results were taken by measuring the radius of developed colony on the direction that faces the extract's well (R2) and measuring that radius on the opposite direction (R1: control) and calculating the growth inhibition percent (%GI) as mentioned above.

Estimation of extracellular metabolites produced by the investigated bacterium using GC-MS

The nature of the extracellular metabolites produced by the investigated *Bacillus* isolate that may be correlated to its antifungal activities against *A. solani* was estimated by Gas Chromatography–Mass Spectrometry (GC-MS). The extracellular extract of the investigated bacterium has been analyzed by GC-MS using GC-TSQ Mass Spectrophotometer (Thermo Scientific, Austin,TX, USA) supplied with direct capillary column TG-5MS (30.0 m × 0.25 mm × 0.25 µm film thickness). Sample analysis was carried out according to the following heating program; the initial hold temperature was 60 °C then it increased by 5 °C /min to 250 °C with a hold of 2 min, then temperature was increased to 300 °C with 30 °C/min. Helium was used as the carrier gas. Samples were injected in split mode with 1 ml/min flow rate. The electron ionization mass spectra were obtained at 70 eV over 50-650 m/z scan range in full scan mode. The ionization source and transfer lines were set at 200 °C, and 280 °C respectively. Data of mass spectra were compared with data available at WILEY 09 and the National Institute of Standards and Technology (NIST14) databases for identification of separated compounds.

Estimation of the morphological changes stimulated by investigated bacterium's metabolites on *A. solani* using Scanning electron microscopy

Morphological changes that have been stimulated by the extracellular metabolites of the investigated bacterium on *A. solani* culture have been monitored using scanning electron microscopy (SEM). *A. solani* was co-cultured with the extracellular extract of the investigated bacterium as described in the dual culture technique. After incubation for 4 days, a part of the fungal mycelium that faces the well of the tested extract was taken. The mycelium was fixed with 2% glutaraldehyde then subjected to dehydration process using gradient ethanol concentrations (30, 50, 80, 90, and 100%). Finally, it was treated with 100% tertiary butylethanol and freeze-dried. After gold coating, it was imaged using Evo 15 - Zeiss UK field emission scanning electron microscope (Zeiss-United Kingdom).

Statistical analysis

Results are presented as a mean value of replicates with standard error (±Std. Error). Statistical analysis was performed by Minitab statistical package version 17.1.0.

RESULTS

Isolation and molecular identification of bacterial isolate

The investigated bacterium was isolated from the rhizospheric soil of *Solanum lycopersicum* L. seedlings that exhibited a significant resistance to *Alternaria solani* infection. Amplification and sequencing of 16S rRNA genes were utilized for its molecular identification. Analysis of the obtained sequences revealed that this isolate showed 99.89% similarity with 100% coverage to several *Bacillus velezensis* strains. Upon the phylogenetic tree analysis, this bacterial isolate was found in a common joining with *Bacillus velezensis* clusters as shown in Figure 1. Based on these results, this bacterial isolate was identified as a new native *Bacillus velezensis* isolate and the obtained sequence was submitted in GenBank as *Bacillus velezensis* isolate NH1 and released in GenBank under the accession number OQ711964.

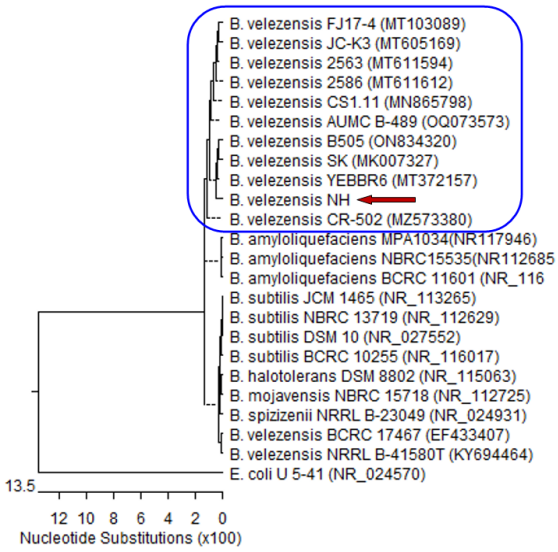

Figure 1. Phylogenetic tree analysis for the amplified 16S rRNA sequences of the investigated bacterium using MegAlign (DNA Star) software; the analyzed sequence is pointed by arrow.

Antagonistic activity of *Bacillus velezensis* NH1 against *A. solani*

The antagonistic behavior of the isolated *Bacillus velezensis* NH1 against *A. solani* was assessed by different plate assay methods namely agar double layer, agar block and dual culture techniques. Results of all tested methods have revealed the potent antagonistic activity of this new *Bacillus velezensis* isolate against *A. solani*. It resulted in a very sparse *A. solani* growth in the case of the agar double layer method and inhibits its growth almost completely in the case of the agar block method. On the other hand, it yields 45% mycelial growth inhibition for *A. solani* in the case of dual culture assay when performed by the first method and 63% mycelial growth inhibition in the case of the second method, Figure 2.

Assessment of antifungal activity of the extracted metabolites

Extracellular and intracellular metabolites of *Bacillus velezensis* NH1extracted by ethyl acetate were evaluated for their inhibitory effect against *A. solani* using well diffusion and dual culture method. Extracellular metabolites have shown significant antifungal activity, Table 1 & Figure 3.

Estimation of extracellular metabolites produced by *Bacillus velezensis*'s using GC-MS

GC-MS analysis has been utilized to estimate the types of secondary metabolites produced extracellularly by *Bacillus velezensis* NH1 to deduce

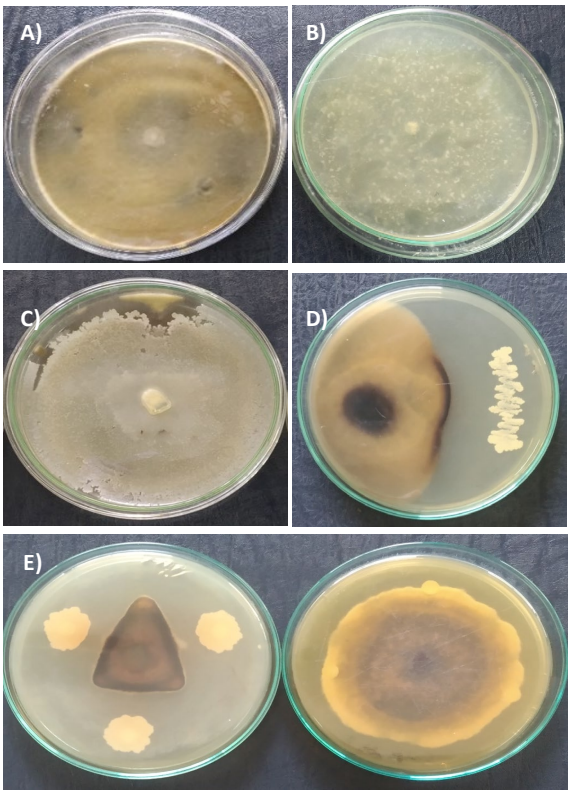

Figure 2. Assessment of *Bacillus velezensis* NH1's antagonistic effect against *A. solani*; (A) Control plate for Agar double layer and Agar block assays, (B) Result of Agar double layer assay, (C) Result of Agar block assay, (D) Result of Dual culture assay method 1, and (E) Result of Dual culture assay method 2 with a control plate on the right hand.

Table 1. Antifungal activities of *Bacillus velezensis* NH1 ethyl acetate extracts against *A. solani;* Results are presented as the main values of replicates followed by ± their standard error values

Tested Extract	Inhibition zone diameter (mm)	Mycelial growth inhibition (%)
Extracellular extract (30 mg/ml)	15.5 ± 0.5	50 ± 0.0 %
Intracellular extract (30 mg/ml)	11.8 ± 0.8	NI*
Control (Methanol)	0.0 ± 0.0	0 ± 0 %

***NI**; Not Investigated

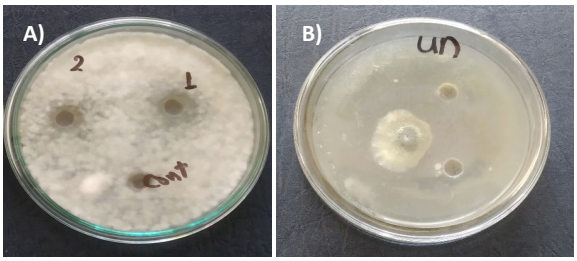

Figure 3. Assessment of the antifungal activity of *Bacillus velezensis* NH1's ethyl acetate extracts against *A. solani* using; A) well diffusion method [1; extracellular extract, 2; intracellular extract and cont.; methanol as control] and B) dual culture methods for extracellular extract.

their antifungal behavior. The test was carried out on the extracellular ethyl acetate extract and the obtained analysis results, Figure 4-b, were compared to the chromatogram of control sample "ethyl acetate extract of uninoculated medium; Figure 4-a," to exclude the common peaks. The GC-MS chromatogram of the extracellular ethyl acetate extract showed seven major peaks. Analysis of the obtained data utilizing WILEY 09 and NIST14 library databases' search has elucidated that six peaks correspond to a phenolic, fatty acids, alkyne and pentadiene compounds namely 2-Phenylphenol, 3,5-dimethoxy phenol, n-Hexadecanoic acid, Oleic Acid, 9-Octadecyne, 2,3-Dimethyl-1,4-pentadiene, Table 2. All these compounds are well characterized by their antifungal activities and that can explain the potent antifungal activities exerted by the *Bacillus velezensis* NH1's extracellular extract that has been tested. One major peak obtained in the retention time 32.18 min. was not detected by the utilized databases' search. Minor peaks also have been reported, Table 3. Among them a peak for an important antifungal compound, Hydrocinnamic acid, was detected.

Estimation of the morphological changes stimulated by *Bacillus velezensis* NH1's metabolites on *A. solani* using Scanning electron microscopy

Morphological changes that have been stimulated on *A. solani* culture by *Bacillus velezensis* NH1's extracellular metabolites, containing 2-Phenylphenol, 3,5-dimethoxy phenol, n-Hexadecanoic acid, Oleic Acid, 9-Octadecyne, 2,3-Dimethyl-1,4-pentadiene as major compounds, were investigated by scanning electron microscopy. In comparison to the control culture, *A. solani* culture exposed to such metabolites has shown obvious mycelial growth inhibition and higher sporulation rate. Additionally, the developed hyphae are thinner with multiple fractures, coiling and distortions and they show sparse cytoplasmic filling combined with cytoplasmic leakage, Figure 5.

Table 2. Compounds corresponding to the major peaks in the GC-MS analysis of the extracellular ethyl acetate extract of *Bacillus velezensis* NH1

The Identified compound	Molecular formula	Molecular weight	RT (min.)	Area* %	Chemical Nature	Biological activity
2-Phenylphenol	$C_{12}H_{10}O$	170	29.24	13.4	Phenolic	Antifungal
n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	26.5	12.6	Saturated fatty acids	Antifungal
Oleic Acid	$C_{18}H_{34}O_2$	282	29.6 /37.97	11.4	Monounsaturated fatty acids	Antifungal
9-Octadecyne	$C_{18}H_{34}$	250	29.5	9.3	long-chain alkyne	Antimicrobial- antioxidant
3,5-dimethoxy phenol	$C_8H_{10}O_3$	154	24.5	7.9	Phenolic	Antifungal
2,3-Dimethyl-1,4-pentadiene	C_7H_{12}	96	11.7	5.2	pentadiene	Antifungal

* Peak area was calculated after excluding the area of the control common beak "Bis(2-ethylhexyl) phthalate".

Table 3. Compounds corresponding to the minor peaks in the GC-MS analysis of the extracellular ethyl acetate extract of *Bacillus velezensis* NH1

The Identified compound	Molecular formula	Molecular weight	RT (min.)	Area* %
Hydrocinnamic acid	$C_9H_{10}O_2$	150	12.4	2.9
Octadecanoic acid	$C_{18}H_{36}O_2$	284	23.95	2.9
1-Nonadecene	$C_{19}H_{38}$	266	9.69	2.4
1-Decyne / 1-Dodecyne	$C_{10}H_{18}$	138	11.26	2.3
1-Octadecyne	$C_{18}H_{34}$	250	24.90	2.2
Cyclohexene, 4-methyl-1-(1-methylethyl)-	$C_{10}H_{18}$	138	5.23	2
Tributyl acetylcitrate	$C_{20}H_{34}O_8$	402	31.40	1.6
Cyclododecane	$C_{12}H_{24}$	168	20.76	1.4
Benzeneacetic acid	$C_8H_8O_2$	136	10.47	1.4
10-Undecyn-1-ol	$C_{11}H_{20}O$	168	37.85	1.4
1-Dodecanol, 3,7,11-trimethyl-	$C_{15}H_{32}O$	228	39.90	1.2
1-Tetradecanol	$C_{14}H_{30}O$	214	19.75	1
1-Hexadecanol	$C_{16}H_{34}O$	242	20.64	1
Isopulegol	$C_{10}H_{18}O$	154	28.67	0.9
1,2-Benzenedithiol, 4-methyl-	$C_7H_8S_2$	156	13.64	0.9
6-Hexyltetrahydro-2H-pyran-2-one	$C_{11}H_{20}O_2$	184	7.98	0.9
Benzoic acid	$C_7H_6O_2$	122	8.67	0.8
Pentadecanoic acid	$C_{15}H_{30}O_2$	242	22.47	0.6
10-Undecyn-1-ol	$C_{11}H_{20}O$	168	13.11	0.6
Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-, (1S)-	$C_{10}H_{16}O$	152	34	0.5
10-Undecenal	$C_{11}H_{20}O$	168	13.03	0.5
decyl-cyclopentane	$C_{15}H_{30}$	210	18.41	0.4

* Peak area was calculated after excluding the area of the control common beak "Bis(2-ethylhexyl) phthalate".

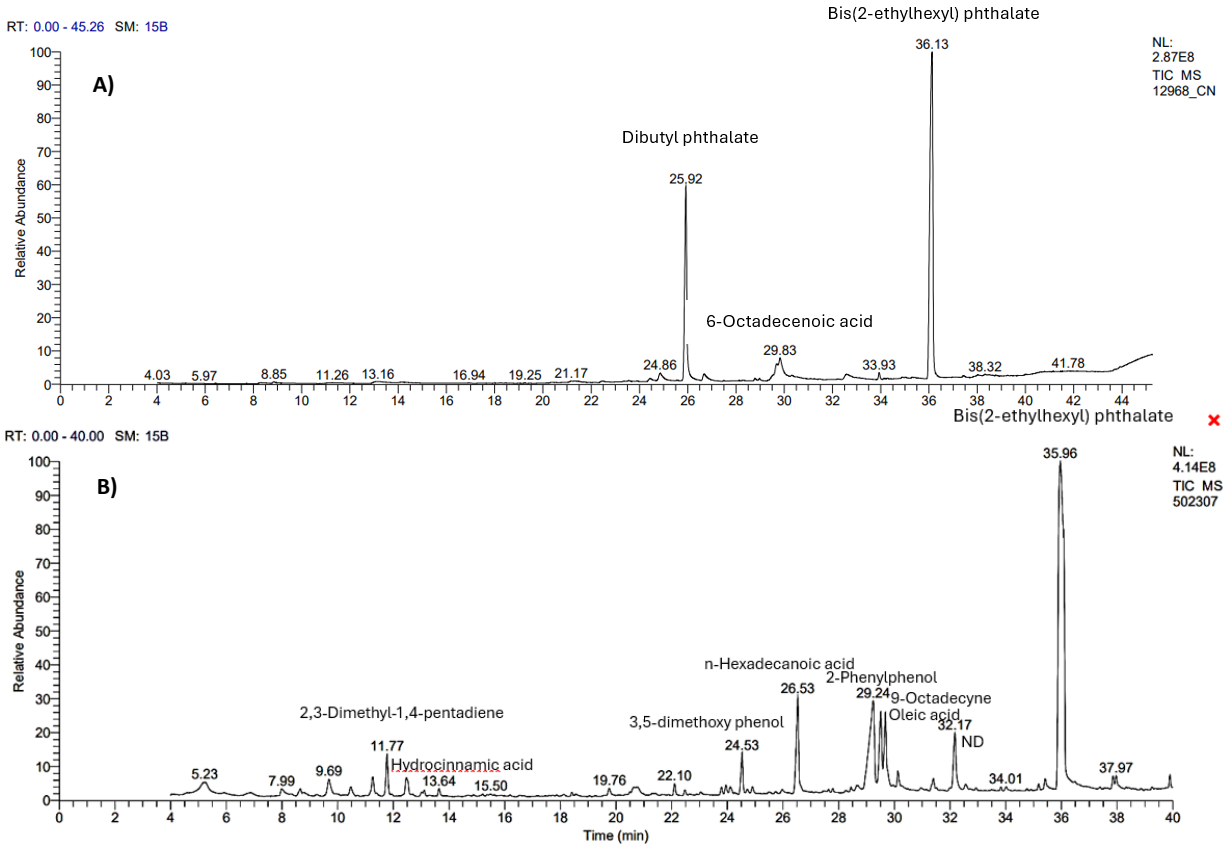

Figure 4. GC-MS chromatogram (A) Control sample "ethyl acetate extract of uninoculated culture medium, (B) ethyl acetate extracellular extract of *Bacillus velezensis* NH1.; ND=Not detected.

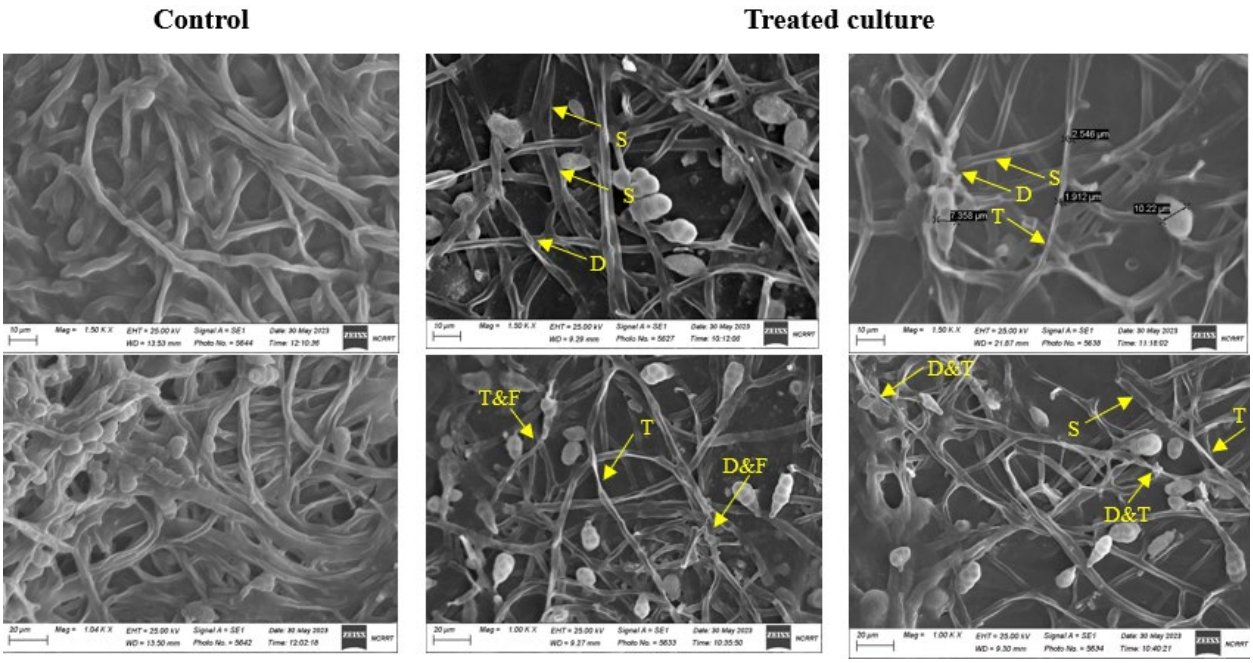

Figure 5. Scanning electron microscope imaging to monitor the damaging effect maintained by *Bacillus velezensis* NH1's extracellular metabolites, on *A. solani*; S= sparse cytoplasmic content; D= Distortion; T= Thinner hypha; F= Fractures.

DISCUSSION

Rhizosheric soil of *Solanum lycopersicum* L. seedlings showing good resistance to an *Alternaria solani* infection was used to isolate a *Bacillus* isolate. This bacterial isolate was identified molecularly by amplifying and sequencing its 16S rRNA genes. Analysis of the obtained sequences showed that, this isolate exhibited 99.89 similarity with 100% coverage to several *Bacillus velezensis* strains and was found with them in a common joining cluster as illustrated by its phylogenetic tree analysis. Hence, this isolation was identified as a new native *Bacillus velezensis* isolate with the identifier NH1 and its sequence was released in GenBank under the accession number OQ711964.

The antagonistic behavior of this *Bacillus velezensis* isolate against *Alternaria solani* was tested using different plate assay methods. Results of all tested methods confirmed the significant antagonistic potential of *Bacillus velezensis* NH1 against this fungal plant pathogen.

Recently, Chen *et al.*, 2025 have detected a significant biocontrol potential of a new *Bacillus velezensis* strain (strain SS-20) against *Rhizoctonia solani*, *Magnaporthe oryzae* and *Fusarium oxysporum* with inhibitory effects of 46.57 %, 82.07 % and 23.09 % respectively. The treated fungi showed abnormal fermentation products and swollen, deformed, and distorted hyphae.

Generally, biological control agents can antagonist the plant pathogens by different mechanisms. They may compete for space and nutritional resources, produce bioactive secondary metabolites that inhibit the growth of pathogens or induce the host defense mechanisms (Pandit *et al.*, 2022; Wockenfuss *et al.*, 2024; Ayaz *et al.*, 2023).

Bacillus velezensis, as one of *Bacillus* species, has been assumed as a strong producer of bioactive secondary metabolites. Indeed, many studies have detected its ability to produce a wide range of secondary metabolites with diverse bioactivities.

The current study aimed to deduce the role of secondary metabolites produced by the investigated *Bacillus velezensis* isolate in its antagonistic behavior against *Alternaria solani*, a plant pathogen that affects the solanaceous crops causing early blight disease. Hence, the extra and intracellular metabolites of this native isolate were extracted and tested for their inhibitory effect against *Alternaria solani*. The extracellular ethyl acetate extract exhibited a significant mycelial growth inhibition percentage for *Alternaria solani*. Type and nature of chemical compounds found in this extracellular extract was estimated employing Gas Chromatography–Mass Spectrometry (GC-MS). Results, after excluding the control peaks, showed the presence of 7 major peaks. The compounds corresponding to six peaks were identified using the applied library databases, while one peak (RT 32.18 min.) was not detected in the database' search. These compounds were found to be phenolic, fatty acids, alkyne and pentadiene in nature, all of which are well-documented bioactive compounds.

Two phenolic compounds have been detected among these secondary metabolites namely 2-Phenylphenol (RT; 29.24 min & peak area; 13.4%) and 3,5-dimethoxy phenol (RT; 24.5min & peak area; 5.2%). Generally, phenolic compounds are considered as the most widely distributed class of natural products in nature (Simonetti *et al.*, 2020). They are produced via the secondary metabolism of organisms (Kauffmann and Castro 2023), and exhibit diverse biological activities, including antifungal activity (Simonetti *et al.*, 2020). Geranylated phenol/methoxyphenol derivatives have been found to exhibit inhibitory effect against *Phytophthora cinnamomic*, and some studies have recorded a promising antifungal activity similar to that of Metalaxil®, a widely used commercial fungicide (Chavez *et al.*, 2018). Thus, they can be used as natural alternatives to synthetic fungicides.

Furthermore, two fatty acids have been detected among the major secondary metabolites produced by *Bacillus velezensis* NH1, n-Hexadecanoic acid (RT; 26.5 min & peak area; 12.6%) and Oleic Acid (RT; 29.6 min & peak area; 9.6%). Since 1945, studies have documented the successful use of fatty acids and their related compounds, particularly long-chain fatty acids, for inhibiting filamentous fungi and yeasts (Guimarães and Venânci, 2022). n-Hexadecanoic acid, Palmitic acid ($CH_3(CH_2)_{14}COOH$), is a saturated fatty acid. Like many other fatty acids, it has been characterized by its significant antibacterial and antifungal activities (Aparna *et al.*, 2012). Guimarães and Venânci (2022) have reported the antifungal activity of this fatty acid against *Alternaria solani* and other fungal plant pathogens including *Colletotrichum lagenarium*, *Fusarium oxysporum* f. sp. *Cucumerinum*, and *F. oxysporum* f. sp. *Lycopersici*. Concerning oleic acid, it is a monounsaturated fatty acid. Oleic acid has been reported to significantly reduce the mycelial growth of *Pythium ultimum* and *Crinipellis perniciosa*

at 100 µM and 1000 µM concentrations respectively (Walters *et al.*, 2004). Both n-Hexadecanoic acid and oleic acid have been detected among the antifungal metabolites released by *Bacillus velezensis* VB7 CP047587. The antifungal activity of this bacterium against *Fusarium oxysporum* f. sp. *cubense* was found to be linked to its production of a group of volatile and non-volatile organic compounds, including hexadecanoic acid, oleic acid, linoelaidic acid, octadecanoic acid, formic acid, succinamide, clindamycin, furanone, 4H-pyran and nonanol (Saravanan *et al.*, 2021).

In addition to the previously mentioned compounds, an alkyne, 9-Octadecyne (RT; 29.5 min & peak area; 9.3 %), was detected among the investigated *Bacillus velezensis* NH1's metabolites. 9-Octadecyne is a biologically active metabolite with antioxidant (Ahmed *et al.*, 2022; Vanitha *et al.*, 2019) and antimicrobial behavior (Vanitha *et al.*, 2019).

The final detected major compound is the 2,3-Dimethyl-1,4-pentadiene (RT; 11.7 min & peak area; 5.2 %). Pentadiene compounds are well reported as biologically active compounds. 1,3 pentadiene has been reported as the most abundant volatile organic compound produced by *Bacillus amyloliquefaciens* CPA-8s, and it was recorded to yield 50% mycelial growth inhibition for *Botrytis cinerea* (Gotor-Vila *et al.*, 2017). Also, 1,4-pentadiene-3-one, an important precursor in the biosynthesis of natural flavonoids, and its derivatives were found to exhibit antiviral, antibacterial and antifungal activities (Zhou *et al.*, 2023).

Hydrocinnamic acid (RT; 12.4 min & peak area; 2.9 %) was recorded among the obtained minor peaks for *Bacillus velezensis* NH1's metabolites. Cinnamic acid and its derivatives are well characterized by their strong antifungal potential (KoroŠec *et al.*, 2016). Cinnamic acid has been reported among the secondary antifungal metabolites produced by *Bacillus velezensis* HY19 which significantly inhibited the mycelial growth of *Penicillium digitatum* (Li *et al.*, 2024).

Zaid *et al.*, 2023 have detected 14 volatile organic compounds released by the rhizoshereric *Bacillus velezensis* HNA3 strain. Among these compounds, phenol,2,4-bis (1,1-dimethylethyl) and 1,2-benzenedicarboxylic acid are the major compounds known for their antifungal and plant growth-promoting properties. Additionally, 9-Octadecenoic acid (z)-, methyl ester, hexadecanoic acid, methyl ester and heptadecanoic acid, methyl ester have also been detected and they have shown antifungal effects on all tested phytopathogens.

Although the effectiveness of many *Bacillus velezensis* strains against many plant pathogenic fungi has been recently evaluated, limited data is available on their antifungal mechanisms against *A. solani* particularly in relation to their secondary metabolites. A study published in 2021 studied the antagonistic behavior of *Bacillus velezensis* C16 strain against *Alternaria solani* and explained the role of its non-volatile lipopeptides and volatile ketones in this antifungal effect (Zhang *et al.*, 2021). Similarly, Gao *et al.*, 2017 isolated an endophytic *Bacillus velezensis* isolate from Chinese catalpa which showed significant antifungal activity against *Alternaria solani* and other plant pathogens. This antifungal activity was found to be correlated with its productivity of a group of volatile organic compounds. Among these compounds, 2-tridecanone, pyrazine (2,5-dimethyl), benzothiazole, and phenol (4-chloro-3-methyl) showed the highest peak areas in the performed GC-MS analysis.

In an attempt to estimate the damaging effects stimulated by the investigated *Bacillus velezensis* NH1's metabolites on *Alternaria solani*, morphological changes that have been stimulated by its extracellular extract on *A. solani* culture have been monitored using Scanning electron microscopy (SEM).

In comparison to control culture, *A. solani* culture exposed to the extracted metabolites has shown obvious mycelial growth inhibition and an induced sporulation process as a resistance behavior. The developed hyphae appeared noticeably thinner than those in the control displaying multiple fractures, coiling and distortions. Additionally, sparse cytoplasmic filling was observed suggesting potential cytoplasmic leakage. All the previous observations confirm and validate the deduced antifungal potential of *Bacillus velezensis* NH1 in a relation to its secondary metabolites and recommend its applicability as a potential biocontrol agent against this potent fungal plant pathogen.

CONCLUSION

Bacillus velezensis NH1 OQ711964 was isolated as a new native isolate from the rhizosphere soil of *Solanum lycopersicum* L. seedlings that showed a good resistance to *Alternaria solani* infection. It exerted a considerable antagonistic potential against *Alternaria solani in vitro*. Its extracellular metabolites exhibited good mycelial growth inhibition for this plant pathogen. GC-MS analysis of its ethyl acetate

extracellular extract revealed the presence of 2-Phenylphenol, 3,5-dimethoxy phenol, n-Hexadecanoic acid, Oleic Acid, 9-Octadecyne and 2,3-Dimethyl-1,4-pentadiene as major compounds among its extracellular metabolites. Also, a cinnamic acid derivative, Hydrocinnamic acid, was detected as a minor component. Secondary metabolites that have been released by *Bacillus velezensis* NH1 OQ711964 greatly inhibited the vegetative growth of *Alternaria solani* and caused serious damaging effects on its hyphae as illustrated from Scanning electron microscopy imaging. In future prospective work, this *B. velezensis* isolate is intended to be evaluated under *in vivo* conditions to improve the agriculture of important crop plants.

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