Research Article

Antiviral Activity of Selected *Bacillus* Isolates against Tobacco Mosaic Virus and Their Molecular Identification Using 16S rRNA Sequencing and Start Codon Targeted Markers

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Abstract | Biological control using *Bacillus* species has gained increasing attention as an environmentally friendly strategy for managing plant viral diseases. Understanding the genetic diversity of the microbial isolates is essential for explaining variations in their biological activities and identifying effective biocontrol candidates. Therefore, this study aimed to assess the genetic diversity of five *Bacillus* isolates using Start Codon Targeted (SCoT) markers and evaluate their antiviral activity against Tobacco mosaic virus (TMV). Genomic DNA from the isolates was analyzed using nine SCoT primers to determine genetic polymorphism, similarity coefficients, and phylogenetic relationships. SCoT analysis revealed substantial genetic diversity, characterized by high levels of polymorphism, variable amplification profiles, and the presence of unique DNA fragments. The SCoT-based genetic similarity coefficients among the investigated isolates ranged from moderate to high, with the highest similarity observed between isolates B-02 and B-04 and the lowest between B-01 and B-02. Cluster analysis separated the isolates into two major groups: B-01, B-03, and B-05 formed one cluster, whereas B-02 and B-04 constituted a distinct cluster. The antiviral activity of the isolates against TMV was assessed by measuring reductions in necrotic local lesion numbers on inoculated plants. All isolates substantially inhibited TMV infection, although their effectiveness varied. *Bacillus subtilis* (B-02) and *Bacillus cereus* (B-04) exhibited the strongest antiviral activity, achieving inhibition percentages of 96.62% and 96.09%, respectively, after 48 h of incubation at 4°C. The maintenance of high antiviral efficacy under refrigerated conditions suggests that the bioactive metabolites produced by these isolates possessed considerable stability. Molecular identification based on *16S rRNA* gene sequencing identified isolates B-01, B-03, and B-05 as *Bacillus thuringiensis*, B-02 as *Bacillus subtilis*, and B-04 as *Bacillus cereus*. Phylogenetic analysis supported these identifications and showed clustering patterns consistent with SCoT analysis. Collectively, the integration of SCoT profiling, 16S rRNA sequencing, and antiviral bioassays demonstrated remarkable genetic variability among the studied isolates and highlighted *B. subtilis* (B-02) and *B. cereus* (B-04) as promising candidates for the bio-control of TMV. These findings provide a foundation for future studies aiming at identifying antiviral metabolites and elucidating their mechanisms of action.

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Introduction

Tobacco mosaic virus (TMV) is one of the most important plant viruses affecting crops in the *Solanaceae* family, including tomatoes, due to its impact on productivity (Hančinský *et al*., 2020; García-Estrada *et al*., 2022). Because TMV is highly stable and mechanically transmitted easily, its control requires specific strategies, the most important of which are the preventative measures. The lack of direct treatments against plant viruses has prompted scientists to search for biological agents (Anikina *et al*., 2023; Trojak-Goluch, 2024).

Several members of the genus *Bacillus* have gained increasing attention as potential biocontrol agents against plant viral infections (Villarreal-Delgado *et al*., 2018; Karačić *et al*., 2024; dos Santos Lobo *et al*., 2026). Although traditionally recognized for their antibacterial and insecticidal properties, certain *Bacillus* species have been reported to exert antiviral effects through indirect mechanisms, including the induction of systemic resistance, enhancement of antioxidant defense systems, and modulation of plant immune signaling pathways (Guo *et al*., 2019; Wang *et al*., 2023). Species such as *Bacillus subtilis* and *B. amyloliquefaciens* have been widely studied for their ability to reduce symptom severity and viral accumulation in infected plants. In particular, *Bacillus*-derived compounds have been shown to suppress the replication and systemic movement of several plant RNA viruses under experimental conditions (Lee and Ryu, 2016; Beris *et al*., 2018; Rajamanickam and Nakkeeran, 2020; Amin *et al*., 2023; Hemmati *et al*., 2025). The antiviral potential of *Bacillus* spp. remains less explored compared to their antibacterial and insecticidal applications, highlighting the need for further studies to elucidate their strain-specific activity and underlying molecular mechanisms (Vinodkumar *et al*., 2018; Hemmati *et al*., 2025). Recently, increasing attention has been given to microbial metabolites and bio-agents with potential antiviral properties. Among these, *B. thuringiensis* has been primarily recognized for its insecticidal activity; however, emerging evidence suggests that some bacterial metabolites may also exhibit indirect antiviral effects, either through induction of plant defense responses or interference with viral infection processes (Zhou *et al*., 2008; Andreeva *et al*., 2014, 2020).

The use of *16S rRNA* gene sequencing has become a fundamental approach for the accurate identification and taxonomic classification of members of the genus *Bacillus*. This molecular marker is widely employed due to the *16S rRNA* gene presence in all bacteria, along with the coexistence of conserved and hypervariable regions that allow both broad-range amplification and fine-scale phylogenetic discrimination (Mohkam *et al*., 2016; Xu and Kovács, 2024). In *Bacillus* taxonomy, 16S rRNA sequencing has been extensively applied to resolve species identity, clarify evolutionary relationships, and differentiate the closely related taxa within the complex groups such as the *B. cereus* group. It is particularly useful for confirming the identity of environmental and clinical isolates, where phenotypic characteristics alone may be insufficient for reliable classification (Goto *et al*., 2000; Bavykin *et al*., 2004). Additionally, comparison of 16S rRNA sequences with curated databases enables robust phylogenetic placement and facilitates the discovery of novel or divergent strains (Church *et al*., 2020; Nunes Ramos *et al*., 2025). However, due to the high genetic similarity among some *Bacillus* species, particularly at the strain level, 16S rRNA analysis is often complemented with additional molecular tools to achieve higher

resolution in the bacterial discrimination (Miranda *et al*., 2008; Ki *et al*., 2009).

Genetic fingerprinting techniques such as start codon targeted (SCoT) markers have emerged as powerful tools for assessing genetic diversity and differentiating closely related bacterial isolates. SCoT analysis is based on the amplification of genomic regions flanking the conserved ATG start codon, and generating reproducible and highly polymorphic DNA fragment's patterns that reflect the functional gene-associated variability (El-Sayed *et al*., 2022; Tekin *et al*., 2024). SCoT technique has been increasingly applied to discriminate among the bacterial strains that exhibit limited variability when assessed using classical molecular markers (Kumari *et al*., 2026). Within the genus *Bacillus*, SCoT markers have proven particularly useful for revealing intraspecific polymorphism among closely related isolates, including members of the *B. cereus* group (El-Shaer, 2022). Moreover, SCoT analysis provides a cost-effective and reliable alternative for exploring genetic relationships without requiring prior genomic information, making it suitable for diverse microorganism's populations (Jedrzejczyk, 2020; Rai, 2023). Consequently, SCoT markers represent a valuable complementary approach to sequence-based identification methods for comprehensive bacterial characterization (Tekin *et al*., 2024).

Despite advances in molecular characterization and plant virus control strategies, few studies have integrated antiviral bioassays with both 16S rRNA-based identification and SCoT fingerprinting of the bacterial isolates. This integrated approach is essential for understanding the relationship between genetic variability and antiviral potential, particularly under virus–plant–microbe interaction systems. Therefore, this study aimed to evaluate the antiviral activity of selected *Bacillus* isolates against TMV, confirm their molecular identity using *16S rRNA* gene sequencing, and assess their genetic diversity using SCoT markers, to identify promising candidates with potential antiviral applications.

Materials and Methods

Bacterial isolates, culture conditions, and preparation of culture filtrate

Five *Bacillus* isolates were isolated from soil samples and kindly provided by the Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University. The bacterial isolates were cultured in Luria–Bertani (LB) broth and incubated at 30°C for 48 h under shaking conditions to ensure optimal growth (Rashki *et al*., 2021). Following incubation, the bacterial cultures were centrifuged at 3000 g for 10 min. The supernatant (culture filtrate) was carefully collected and used as a source of bioactive metabolites in the subsequent antiviral assays (Zhou *et al*., 2008).

Morphological and Gram staining characterization of Bacillus isolates

All *Bacillus* isolates were characterized by Gram staining (Coico, 2006) and microscopic examination, which were performed independently to confirm cellular morphology and ensure consistency of identification across the different experimental applications.

Virus source and inoculum preparation

An identified TMV isolate was kindly provided by the Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University, and used as a model virus in this study. The viral inoculum was prepared from infected plant leaves by homogenization in phosphate buffer (pH 7.0) to obtain a crude viral extract (Rabie *et al*., 2024).

Evaluation of antiviral activity

The bacterial culture filtrate was mixed with the viral inoculum at a ratio of 1:1 (v/v) and the mixture was incubated for 2 h at 4°C (Abdelkhalek *et al*., 2022). Subsequently, the treated inoculum was mechanically inoculated onto the leaves of suitable host plants, *i.e*., *Datura metel*. Control treatments included plants inoculated with the virus alone and plants inoculated with the bacterial culture filtrate alone. After an appropriate incubation period (6–7 d), the number of necrotic local lesions (NLL) per leaf was recorded. The percentage of inhibition was calculated using the following equation:

$$\text{Inhibition (\%)} = \text{C-T/C} \times 100$$

Where; C= number of lesions in the control. T= number of lesions in the treatment.

Molecular characterization of the Bacillus isolates

Molecular identification and genetic fingerprinting of the bacterial isolates

Genomic DNA was extracted from pure bacterial

cultures of the five *Bacillus* isolates following the protocol described by El-Sayed *et al.* (2022). The quality and concentration of the extracted DNA were assessed using agarose gel electrophoresis and spectrophotometric analysis prior to downstream molecular applications.

Start codon targeted –polymerase chain reaction (SCoT-PCR) analysis and genetic diversity assessment

SCoT primers (Table 1) were used to evaluate the genetic diversity among the *Bacillus* isolates. These primers are designed based on conserved regions flanking the ATG translation start codon within functional genes, enabling the amplification of reproducible and polymorphic DNA amplified fragments (DAFs). The primers (18 nucleotides in length) were used under optimized annealing temperatures ranging from 52–60°C depending on the primer sequence, to ensure clear and consistent banding patterns.

Genetic polymorphism among the bacterial isolates was assessed using ten SCoT primers. PCR amplification was performed in a 25 µl reaction mixture containing 12.5 µl of 2× PCR Master Mix, 2.5 µl of template DNA (approximately 10 ng), 2.5 µl of primer (10 pmol), and 7.5 µl of double-distilled nuclease-free water. The amplification protocol was carried out following a modified version reported by Tekin *et al.* (2024), with minor adjustments in reaction conditions. PCR amplification reactions were carried out using a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems, Foster City, California, USA). The thermal cycling program started with an initial denaturation at 94°C for 5 min, followed by 40 amplification cycles. Each cycle included denaturation at 94°C for 45 s, primer annealing at 50°C for 55 s, and extension at 72°C for 1 min. A final extension step was then performed at 72°C for 7 min to ensure complete amplification of all PCR products.

Scoring, visualization, and statistical analysis of SCoT markers

PCR products were separated by electrophoresis on 1.5% agarose gels and stained with ethidium bromide (0.5 µg/ml) in 1× TBE buffer. Electrophoresis was performed at 95 V. The DAFs were visualized under ultraviolet (UV) light and documented using a gel documentation system (Bio-Rad 2000, Bio-Rad Laboratories, Hercules, CA, USA). Only clear and reproducible DAFs were manually scored across all *Bacillus* isolates as present (1) or absent (0) (El-Sayed *et al.*, 2022). Both polymorphic and monomorphic bands were included in the analysis. Based on the scoring results, a binary data matrix was constructed from the banding patterns. Genetic similarity among the isolates was estimated using Dice's similarity coefficient. Cluster analysis was performed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). A dendrogram illustrating the genetic relationships among the bacterial isolates was generated using PAST software (version 1.91), based on similarity indices (Qasim *et al.*, 2018).

Molecular identification of high antiviral Bacillus isolates based on 16S rRNA gene analysis

Molecular identification of the selected *Bacillus* isolates was performed using *16S rRNA* gene sequencing. The most potent isolates, which exhibited the highest antiviral activity were selected for species-level molecular confirmation.

Table 1: *SCoT primers with nucleotide composition and annealing conditions (Collard and Mackill, 2009).*

Primer	Sequence (5'→3')	Length (nt)	A+T	G+C	GC %	AT %	Annealing (°C)
SCoT-03	ACGACATGGCGACCCACA	18	07	11	61.1	38.9	58
SCoT-04	ACCATGGCTACCACCGCA	18	07	11	61.1	38.9	58
SCoT-05	CAATGGCTACCACTAGCG	18	08	10	55.6	44.4	56
SCoT-06	CAATGGCTACCACTACAG	18	10	08	44.4	55.6	52
SCoT-07	ACAATGGCTACCACTGAC	18	09	09	50.0	50.0	54
SCoT-09	ACAATGGCTACCACTGCC	18	08	10	55.6	44.4	56
SCoT-10	ACAATGGCTACCACCAGC	18	08	10	55.6	44.4	56
SCoT-12	CAACAATGGCTACCACCG	18	08	10	55.6	44.4	56
SCoT-13	ACCATGGCTACCACGGCA	18	07	11	61.1	38.9	58
SCoT-14	ACCATGGCTACCAGCGCG'	18	06	12	66.7	33.3	60

DNA extraction and PCR amplification of 16S rRNA gene

Genomic DNA was used as a template for PCR amplification of the *16S rRNA* gene using universal bacterial primers: 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) (Janda and Abbott, 2007). PCR amplification was carried out in a 25 µl reaction mixture containing 12.5 µl of 2× PCR Master Mix, 1 µl of each primer (10 pmol), 2 µl of genomic DNA (approximately 10–20 ng), and 8.5 µl of nuclease-free water. The amplification was performed under optimized cycling conditions consisting of an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 1.5 min, with a final extension step at 72°C for 7 min to ensure complete amplification. The PCR products were analyzed by agarose gel electrophoresis to confirm the expected amplicon size prior to sequencing (Sergeant *et al*., 2012).

Sanger sequencing and sequence analysis

The purified PCR products were subjected to Sanger sequencing to determine the exact nucleotide sequence of the amplified *16S rRNA* gene fragments (Sanger *et al*., 1977). The obtained sequences were analyzed using the BLAST (Basic Local Alignment Search Tool) (Altschul *et al*., 1990). The percentage identity and query coverage were used as key parameters for species-level confirmation.

Phylogenetic analysis

For phylogenetic analysis, the confirmed sequences were aligned with reference sequences retrieved from databases using multiple sequence alignment tools (Felsenstein, 1985). Evolutionary relationships among the selected bacterial isolates and closely related bacterial strains were inferred, and a phylogenetic tree was constructed to illustrate genetic relatedness and divergence (Weisburg *et al*., 1991). This analysis provided a clearer understanding of the evolutionary position of the bacterial isolates within the *Bacillus* group.

Statistical analysis

All experiments were conducted with three replicate, and the results are expressed as mean ± standard deviation (SD). Data were analyzed using one-way analysis of variance (ANOVA) in SAS software (Lohr, 2021). Mean comparisons were performed using the Least Significant Difference (LSD) test at $p < 0.05$.

Results

Microscopic and gram staining characteristics of the Bacillus isolates

Figure 1 illustrates the microscopic morphology of the Gram-stained bacterial isolates. Based on microscopical examination, all isolates were initially characterized as belonging to the genus *Bacillus*. All isolates displayed typical Gram-positive bacillary cells, with noticeable differences in cell organization and chain formation patterns. Photomicrographs were obtained using a light microscope at ×1000 magnification under oil immersion.

Antiviral activity of the Bacillus isolates against TMV under low-temperature incubation conditions

The data presented in Table 2 and Figures 2–7 demonstrated remarkable differences in the antiviral activity of the five *Bacillus* isolates against TMV under low-temperature incubation conditions (4°C), as evidenced by reductions in the number of necrotic local lesions (NLLs), compared to the untreated control. All tested isolates considerably inhibited TMV infection; however, their antiviral efficiencies varied markedly. Among the evaluated isolates, B-02 and B-04 expressed the strongest antiviral activity, recording the lowest numbers of necrotic local lesions (NLLs) and the highest inhibition percentages after both incubation periods. Following 2 h of incubation at 4°C, B-02 reduced the NLLs to 15 lesions, corresponding to 93.33% inhibition, whereas B-04 resulted in 21 lesions with 90.67% inhibition. After 48 h of incubation, the antiviral efficacy of both isolates increased, with B-02 reducing the NLLs to 8 and achieving 96.62% inhibition, while B-04 recorded 9 NLLs with 96.09% inhibition. Isolate B-01 also displayed a considerable antiviral activity, reducing the NLLs to 44 lesions after 2 h and 33 lesions after 48 h, corresponding to inhibition percentages of 80.44% and 85.65%, respectively. In contrast, B-03 exhibited the weakest antiviral activity, recording 98 lesions after 2 h and 66 lesions after 48 h, with inhibition percentages of 56.44% and 71.30%, respectively. Isolate B-05 showed intermediate inhibitory effects, recording 72 NLLs after 2 h and 68 NLLs after 48 h, corresponding to 68% and 70.43% inhibition, respectively. As expected, the untreated control recorded the highest NLLs (225 and 230 lesions after 2 and 48 h, respectively) and displayed no inhibitory effect.

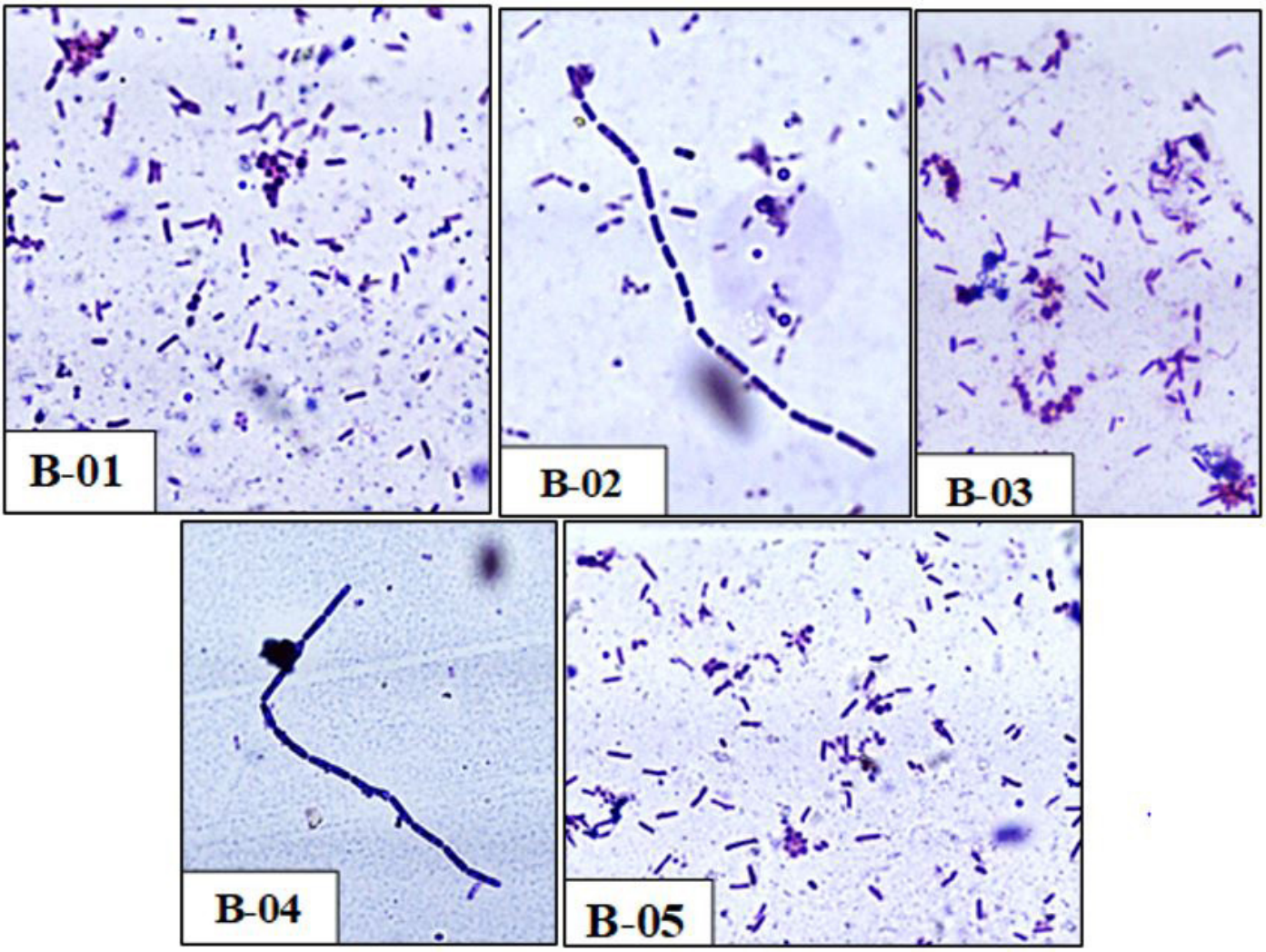

Figure 1: *Morphological characteristics of Gram-stained Bacillus isolates observed under a light microscope. Isolates B-01, B-03, and B-05 were identified as Bacillus thuringiensis, isolate B-02 as Bacillus subtilis, and isolate B-04 as Bacillus cereus. The isolates showed typical Gram-positive rod-shaped cells with variations in cellular arrangement and chain formation. Images were captured at ×1000 magnification under oil immersion.*

Table 2: *Evaluation of the antiviral activity of the Bacillus isolates against TMV under greenhouse conditions (30± 2°C).*

Bacillus **isolate**	**No. of NLL 2 h post incubation at 4°C**	**%inhibition 2 h post incubation at 4°C**	**No. of NLL 48 h post incubation at 4°C**	**%inhibition 48 h post incubation at 4°C**
B-01	044.00^{d}±2.73	80.44^{c}±0.55	033.00^{c}±3.03	85.65^{b}±0.51
B-02	015.00^{f}±6.67	93.33^{a}±0.48	07.00^{d}±14.29	96.62^{a}±1.01
B-03	098.00^{b}±1.02	56.44^{e}±0.79	066.00^{b}±1.52	71.30^{c}±0.61
B-04	021.00^{e}±4.76	90.67^{b}±0.49	09.00^{d}±11.11	96.09^{a}±0.45
B-05	072.00^{c}±1.39	68.00^{d}±0.65	068.00^{b}±1.70	70.43^{c}±0.62
Control	225.00^{a}±0.44	00.00^{f}±0.00	230.00^{a}±0.75	00.00^{d}±0.00

*Where; *Means ± standard deviation (±SD) within a column followed by the same letter are not significantly different by Duncan's multiple range test at p < 0.0001. NLL: Necrotic local lesions.*

Molecular identification and genetic fingerprinting of the Bacillus isolates

Using *16S rRNA* gene sequencing, the species-level identification of the five isolates was as follows: *Bacillus* thuringiensis B-01 (Acc. no. LC933331.1), B-03 (Acc. no. LC933332.1), B-05 (Acc. no. LC933334.1), *Bacillus* subtilis B-02 (Acc. no. LC933372.1), and *Bacillus* cereus B-04 (Acc. no. LC933333.1), respectively.

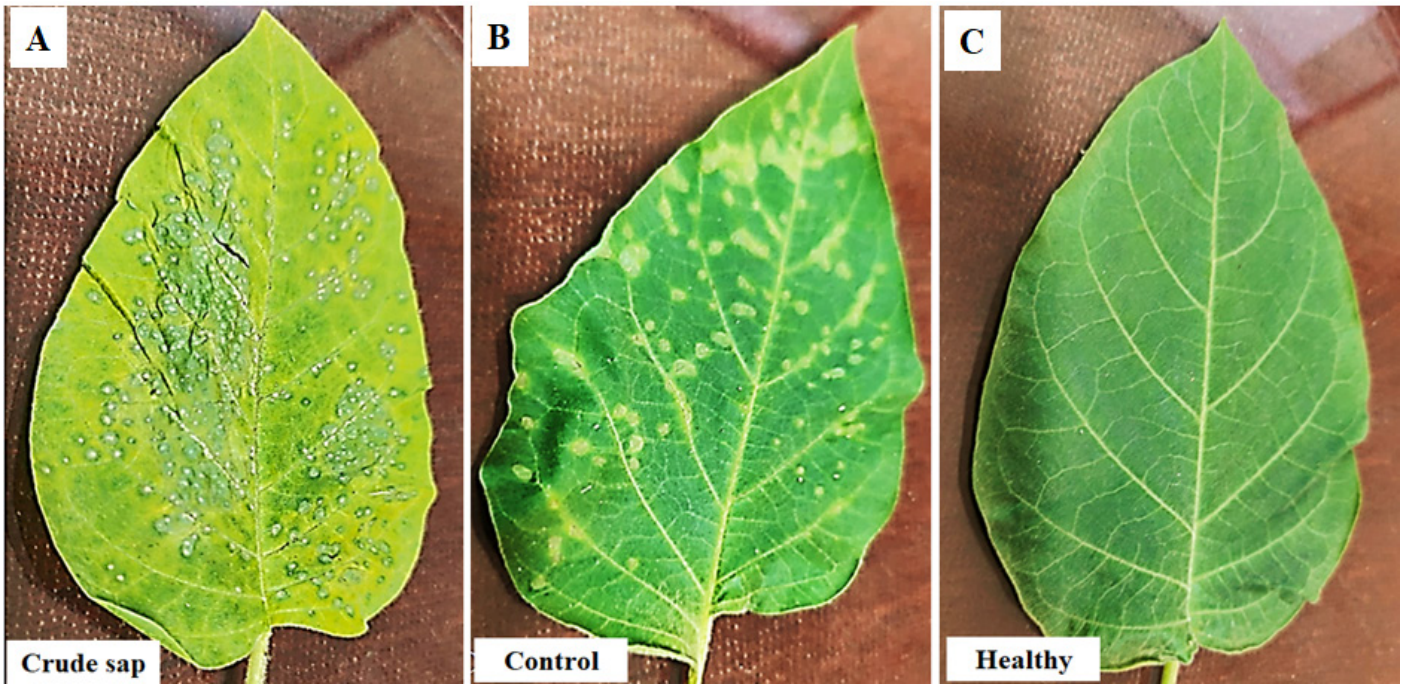

Figure 2: *Necrotic local lesions induced on Datura metel leaves following TMV inoculation. (A) TMV sap diluted 1:1 (v/v), (B) crude TMV sap, and (C) healthy uninoculated control plants.*

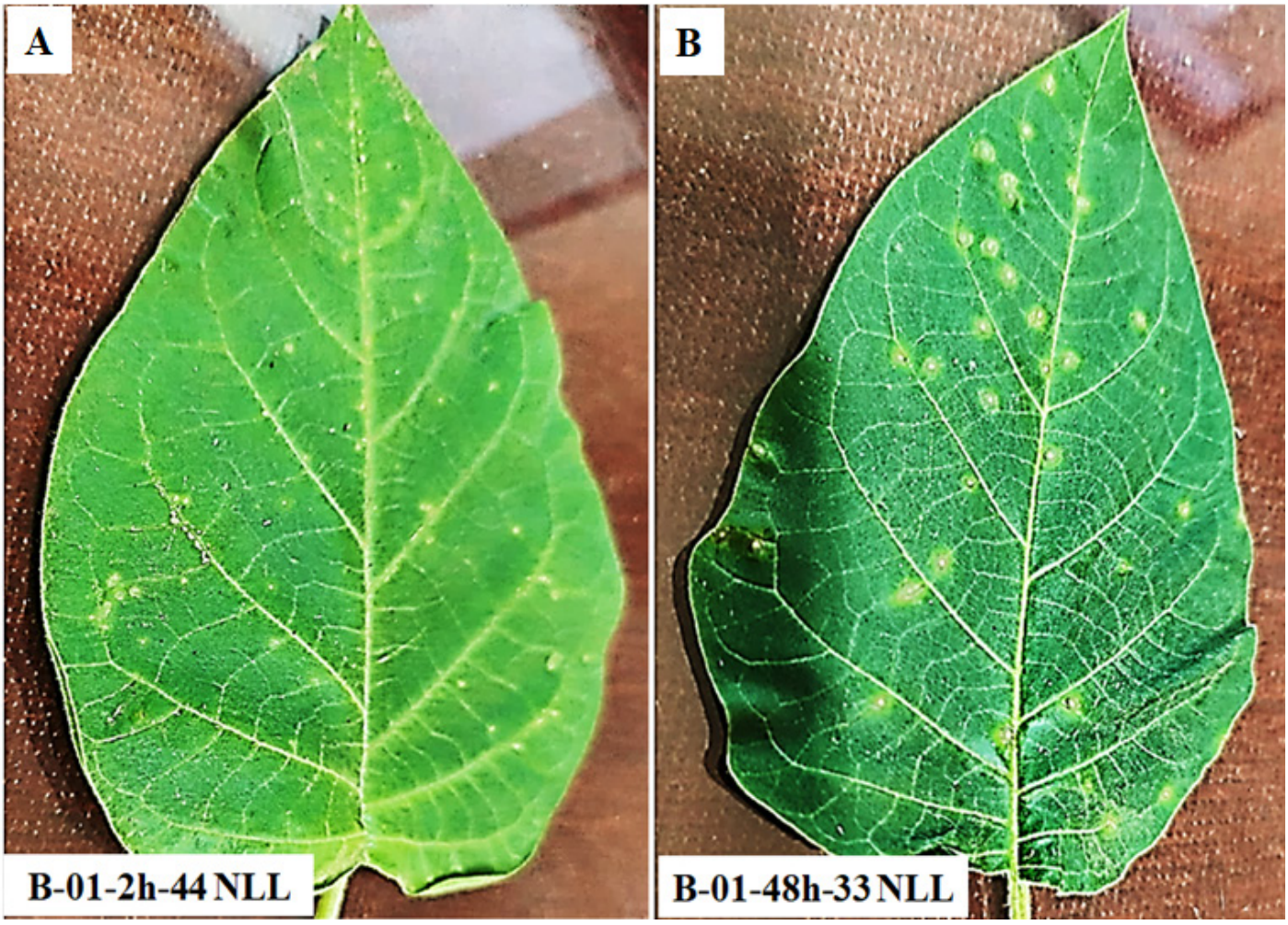

Figure 3: *Number of necrotic local lesions developed on Datura metel leaves after inoculation with TMV sap mixed with culture filtrate of isolate B-01 following incubation at 4°C for 2 h and 48 h. (A) After 2 h incubation, and (B) after 48 h incubation.*

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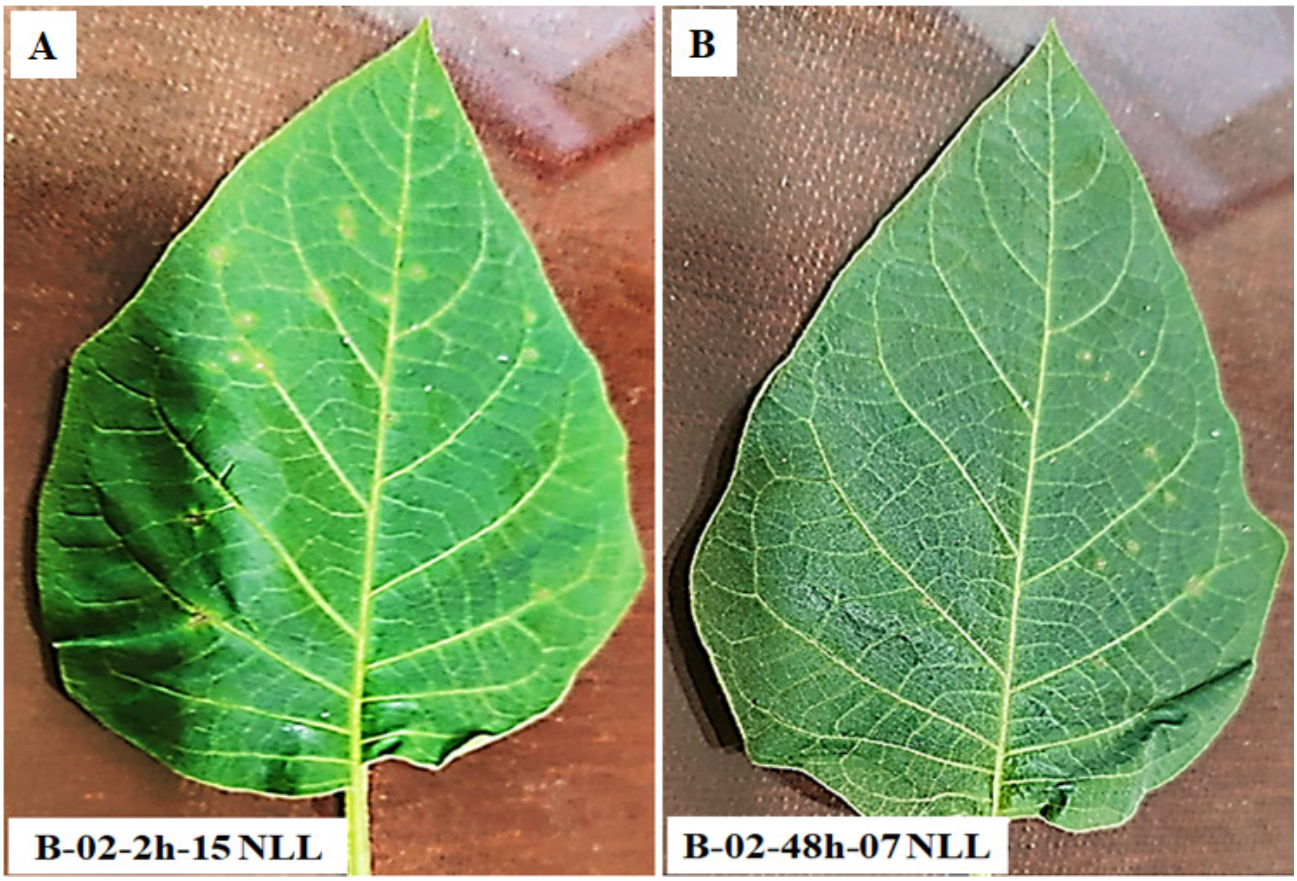

Figure 4: *Number of necrotic local lesions developed on Datura metel leaves after inoculation with TMV sap mixed with culture filtrate of isolate B-02 following incubation at 4°C for 2 h and 48 h. (A) After 2 h incubation, and (B) after 48 h incubation.*

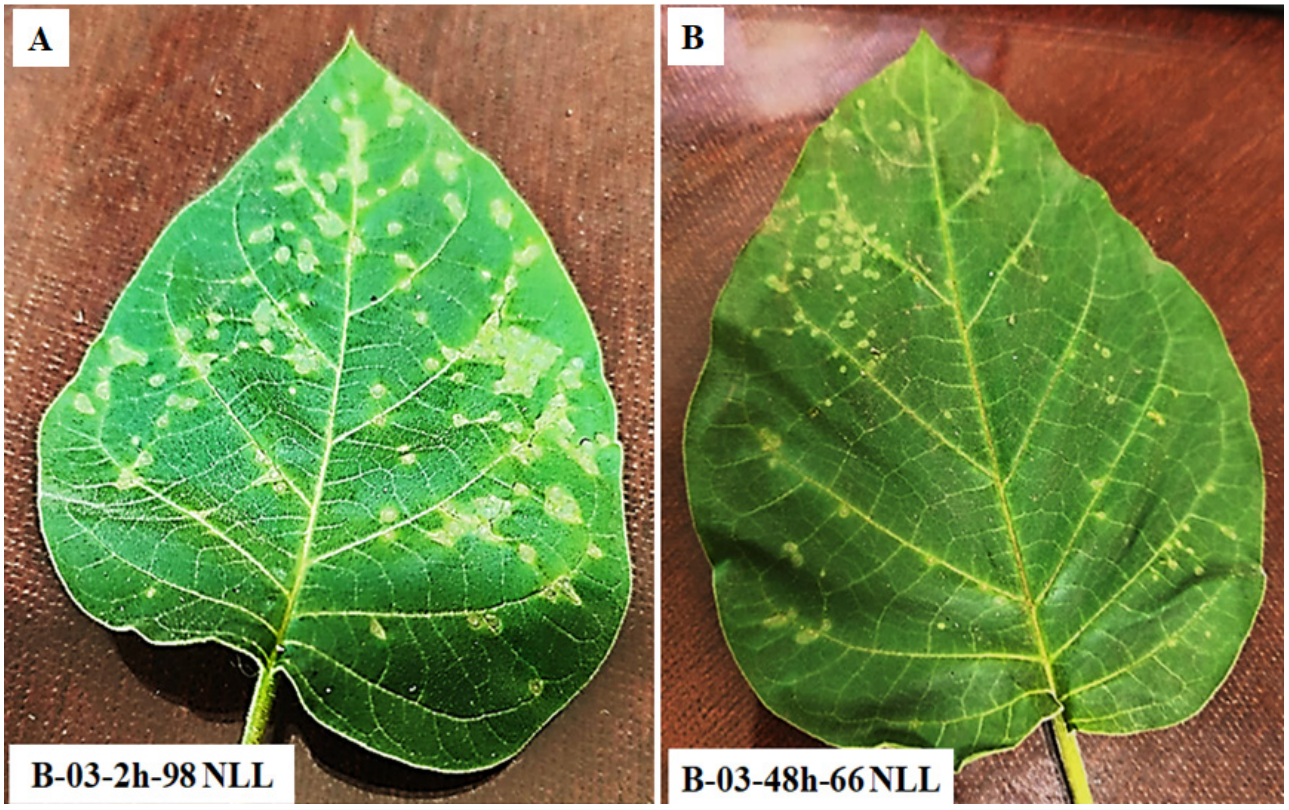

Figure 5: *Number of necrotic local lesions developed on Datura metel leaves after inoculation with TMV sap mixed with culture filtrate of isolate B-03 following incubation at 4°C for 2 h and 48 h. (A) After 2 h incubation, and (B) after 48 h incubation.*

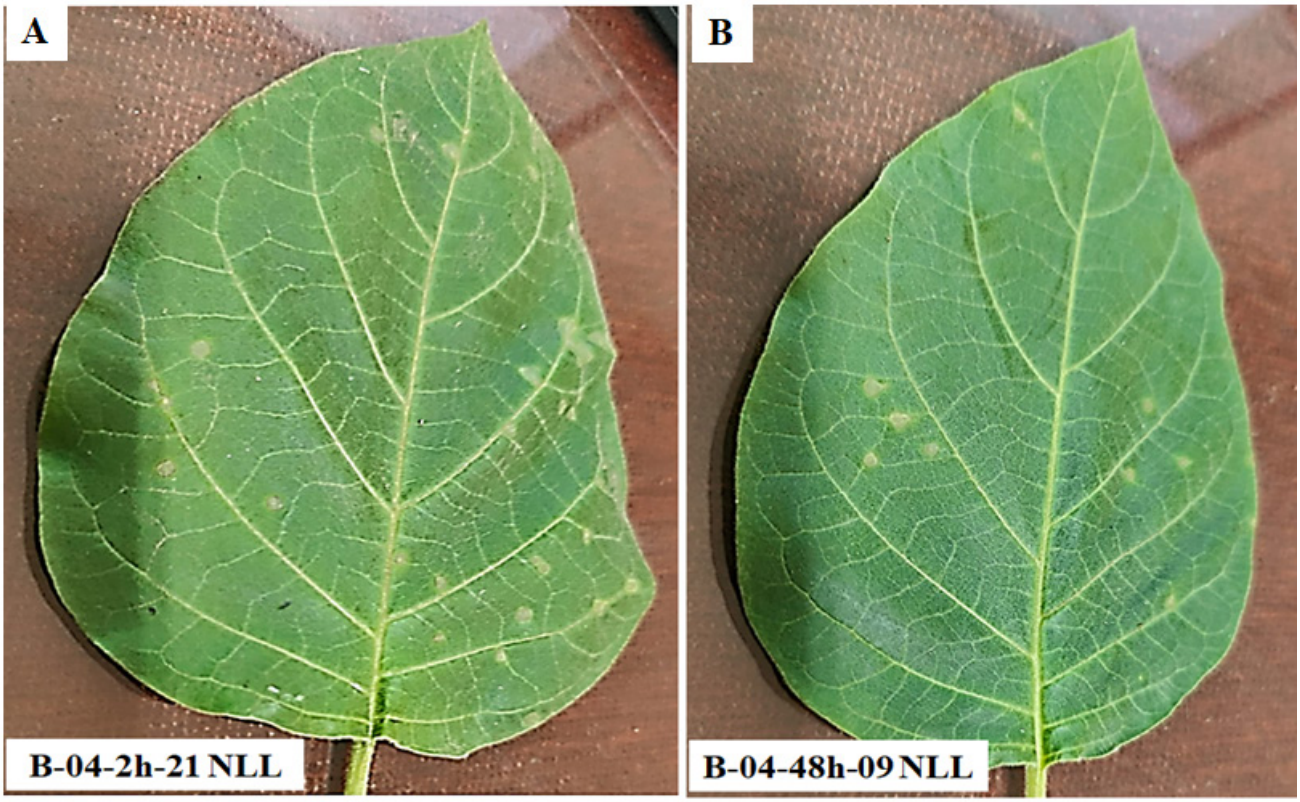

Figure 6: *Number of necrotic local lesions developed on Datura metel leaves after inoculation with TMV sap mixed with culture filtrate of isolate B-04 following incubation at 4°C for 2 h and 48 h. (A) After 2 h incubation, and (B) after 48 h incubation.*

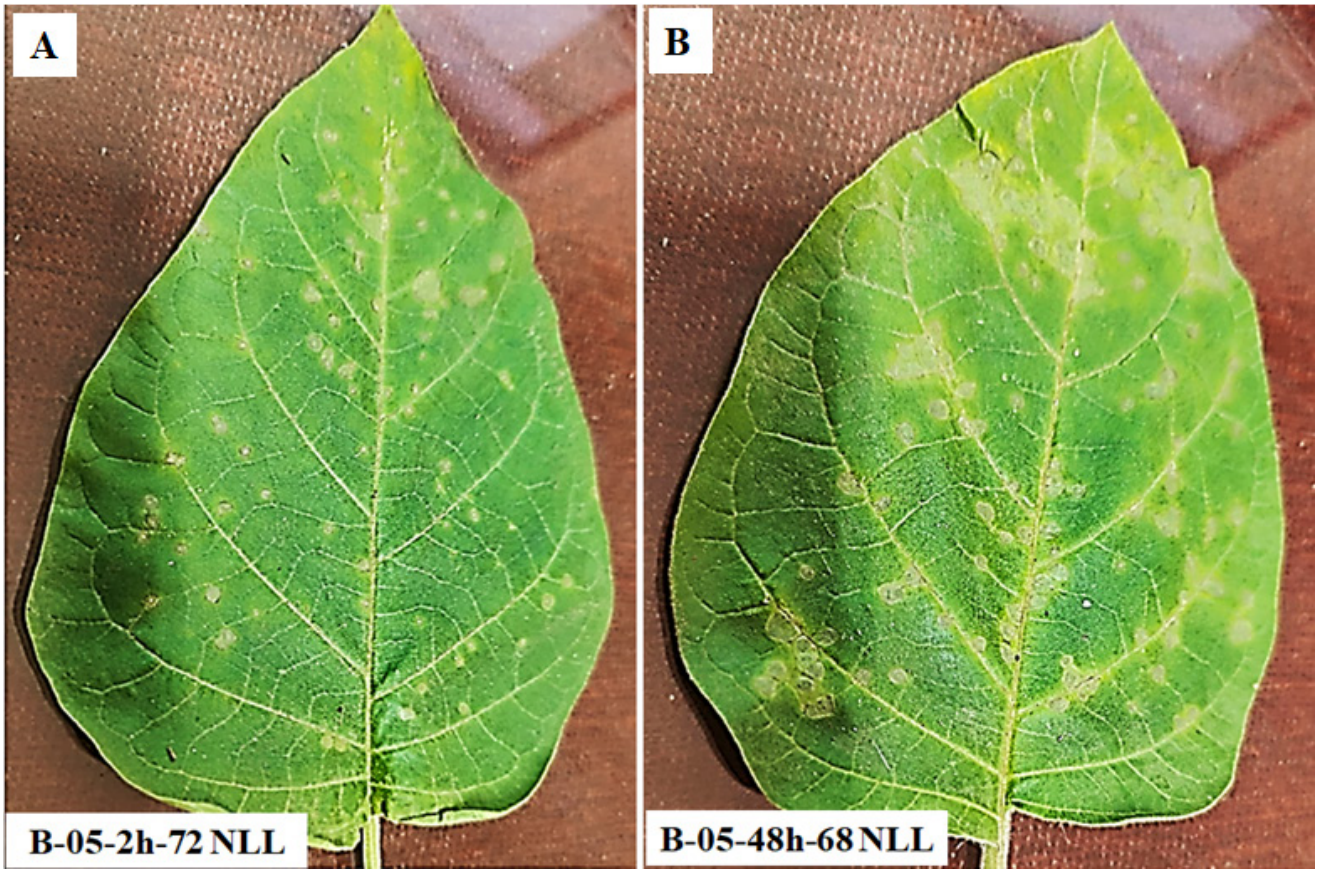

Figure 7: *Number of necrotic local lesions developed on Datura metel leaves after inoculation with TMV sap mixed with culture filtrate of isolate B-05 following incubation at 4°C for 2 h and 48 h. (A) After 2 h incubation, and (B) after 48 h incubation.*

Genetic diversity assessment of the Bacillus isolates using SCoT markers

SCoT-PCR amplification profiles and polymorphism analysis

Data presented in Supplementary Table S1 and illustrated in Figures 8-10 revealed that the SCoT analysis showed considerable genetic polymorphism among the investigated *Bacillus* isolates. A total of multiple amplified DNA fragments with different molecular weights were generated using the selected SCoT primers, indicating a high level of genetic diversity among the isolates. Several monomorphic DNA amplified fragments (DAFs) with a frequency of 1.0 were detected, reflecting conserved genomic regions shared among all isolates, whereas polymorphic DAFs with lower frequencies demonstrated a clear genetic variation. Primers SCoT-04, SCoT-12, and SCoT-13 produced a relatively high number of polymorphic and informative DAFs, suggesting their efficiency in discriminating among the studied isolates. The variation in banding patterns confirmed the usefulness of SCoT markers as a reliable molecular tool for assessing genetic diversity and phylogenetic relationships among the *Bacillus* isolates.

The data presented in Table 3 demonstrate a substantial genetic variability among the investigated *Bacillus* isolates as revealed by SCoT markers. A total of 127 amplified DNA fragments varied among the primers, ranging from 9 DAFs for SCoT-10 to 23 DAFs for SCoT-04, indicating differences in primer efficiency and genome coverage. The highest level of polymorphism was recorded with primer SCoT-09 (100%), followed by SCoT-03 (93%), and SCoT-05 (88%), suggesting their high discriminatory power among the isolates. In contrast, SCoT-14 exhibited the lowest polymorphism percentage (10%), reflecting a high degree of band conservation.

The number of unique DAFs also differed markedly among the primers, where SCoT-05 generated the highest number of unique fragments (9 DAFs), followed by SCoT-09 (8 DAFs), indicating the presence of isolate-specific genetic markers. Monomorphic DAFs were most abundant in SCoT-14, SCoT-12, and SCoT-13, suggesting conserved genomic regions among the tested isolates. Furthermore, the mean band frequency ranged from 0.33 to 0.94, confirming the variability in allele distribution among the bacterial isolates. Overall, the obtained results highlighted the effectiveness of SCoT markers in detecting genetic polymorphism and assessing genetic relationships among the *Bacillus* isolates.

Polymorphism, unique markers, and discriminatory power of SCoT primers

The obtained results of SCoT-PCR presented in Table 4 demonstrated a clear genetic variation among the investigated *Bacillus* isolates through differences in total amplified fragments, polymorphic markers, and unique DNA bands. The total amplified DNA fragments (TADFs) ranged from 9 fragments in SCoT-10 to 23 fragments in SCoT-04, indicating variable amplification efficiency among the primers. Primer SCoT-04 produced the highest number of polymorphic markers (19), followed by SCoT-05 (15), and SCoT-03/SCoT-09 (14 each), reflecting their strong capability for detecting the genetic diversity. Unique DNA amplified fragments (UDAFs) varied

Table 3: *Summary* of SCoT primer amplification characteristics across the five Bacillus isolates (B-01–B-05).*

Items	Type of DNA fragments amplified by SCoT PCR primers using data obtained from all the five *Bacillus* isolates								
	SCoT-03	**SCoT-04**	**SCoT-05**	**SCoT-06**	**SCoT-09**	**SCoT-10**	**SCoT-12**	**SCoT-13**	**SCoT-14**
Total DNA amplified fragments	15	23	17	14	14	9	14	11	10
Monomorphic DNA fragments	1	4	2	2	0	2	7	7	9
Polymorphic DNA fragments (with Unique)	14	19	15	12	14	7	7	4	1
Polymorphic DNA fragments (without Unique)	11	12	6	5	6	3	7	2	1
Unique DNA fragments	3	7	9	7	8	4	0	2	0
Polymorphism percent	93	83	88	86	100	78	50	36	10
Mean of band frequency	0.47	0.48	0.40	0.47	0.33	0.49	0.74	0.80	0.94

*Where; *The presented data summarize the amplification efficiency and polymorphism parameters of each SCoT primer calculated from the combined banding profiles of the five analyzed Bacillus isolates (B-01–B-05).*

Table 4: *Distribution of unique and polymorphic DNA amplified fragments generated by SCoT primers among the Bacillus isolates.*

SCoT PCR primers	TADFs	PM	UDAFs	*Bacillus* isolates				
				B-01	B-02	B-03	B-04	B-05
SCoT-03	15	14	3	(1) 680/+ve	0	(1) 1000/+ve	(1) 1150/+ve	0
SCoT-04	23	19	7	(3) 1400/+ve 370/+ve 180/+ve	(0)	(2) 1150/+ve 1050/+ve	(1) 1100/+ve	(1) 770/+ve
SCoT-05	17	15	9	(1) 410/+ve	(4) 1250/+ve 680/+ve 600/+ve 300/+ve	(1) 340/+ve	(2) 470/+ve 250/+ve	(0)
SCoT-06	14	12	7	(6) 710/+ve 650/-ve 570/+ve 520/+ve 490/+ve 250/+ve	(0)	(0)	(0)	(1) 820/+ve
SCoT-09	14	14	8	(3) 460/+ve 330/+ve 170/+ve	(2) 840/+ve 720/+ve	(1) 360/+ve	(1) 230/+ve	(1) 510/+ve
SCoT-10	9	7	4	(0)	(0)	(3) 750/+ve 650/+ve 330/+ve	(1) 250/+ve	(0)
SCoT-12	14	7	0	(0)	(0)	(0)	(0)	(0)
SCoT-13	11	4	2	(1) 460/+ve	(1) 420/-ve	(0)	(0)	(0)
SCoT-14	10	1	0	(0)	(0)	(0)	(0)	(0)

Where; TADFs: Total DNA amplified fragments, PM: Polymorphic, UDAFs: Unique DNA amplified fragments. +ve: Present. –ve: Absent.

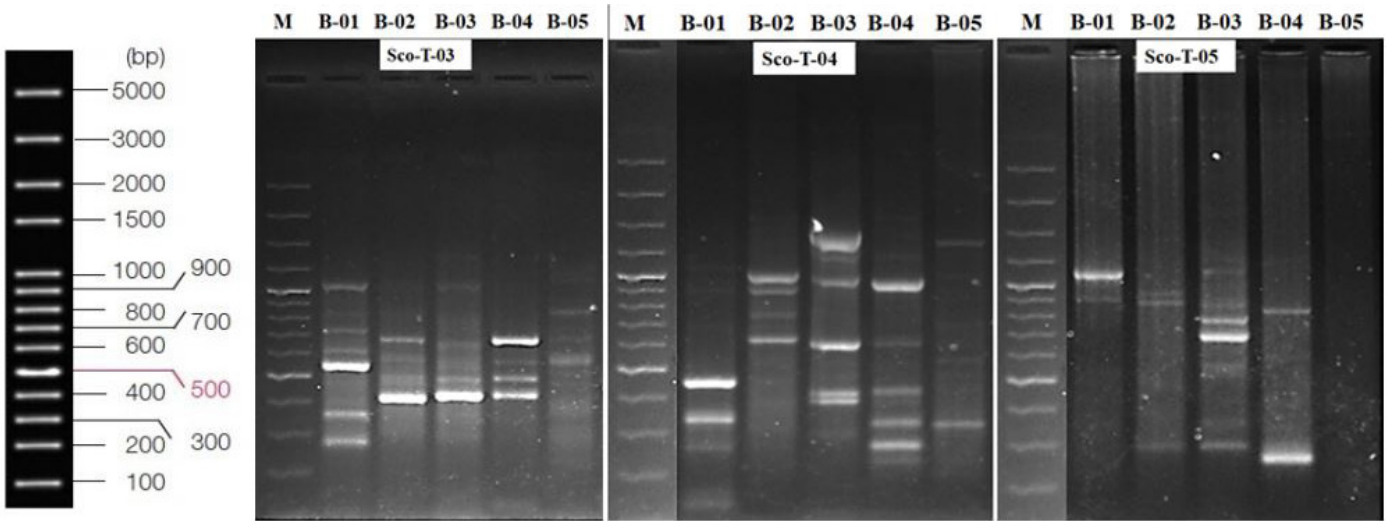

Figure 8: *Agarose gel electrophoresis (1.5%) showing SCoT-PCR amplification patterns of the five Bacillus isolates (B-01 to B-05) generated using the primers Sco-T-03, Sco-T-04, and Sco-T-05. Lane M represents the 0.1–5 kbp DNA marker, while lanes B-01 to B-05 correspond to the tested Bacillus isolates. The obtained banding patterns revealed both polymorphic and shared DNA fragments among the isolates, reflecting genetic diversity detected by the SCoT markers.*

Links Researchers

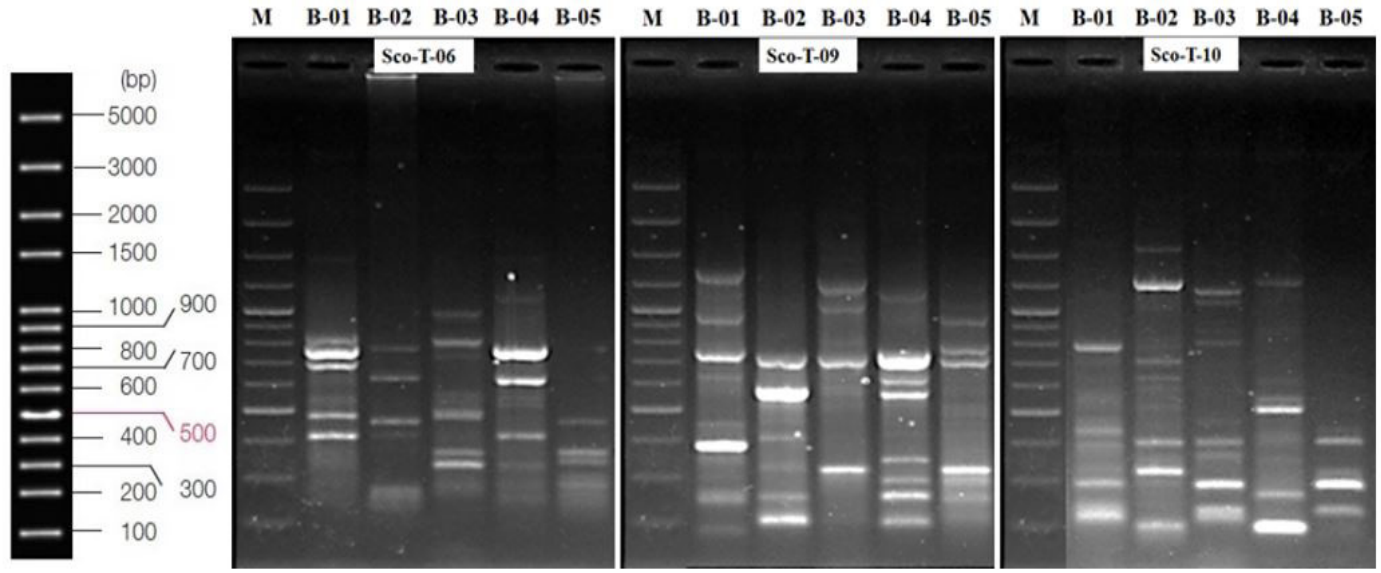

Figure 9: *Agarose gel electrophoresis (1.5%) showing SCoT-PCR amplification patterns of the five Bacillus isolates (B-01 to B-05) generated using the primers Sco-T-06, Sco-T-09, and Sco-T-10. Lane M represents the 0.1–5 kbp DNA molecular weight marker, while lanes B-01 to B-05 correspond to the tested Bacillus isolates. The obtained banding patterns revealed both polymorphic and shared DNA fragments among the isolates, reflecting genetic diversity detected by the SCoT markers.*

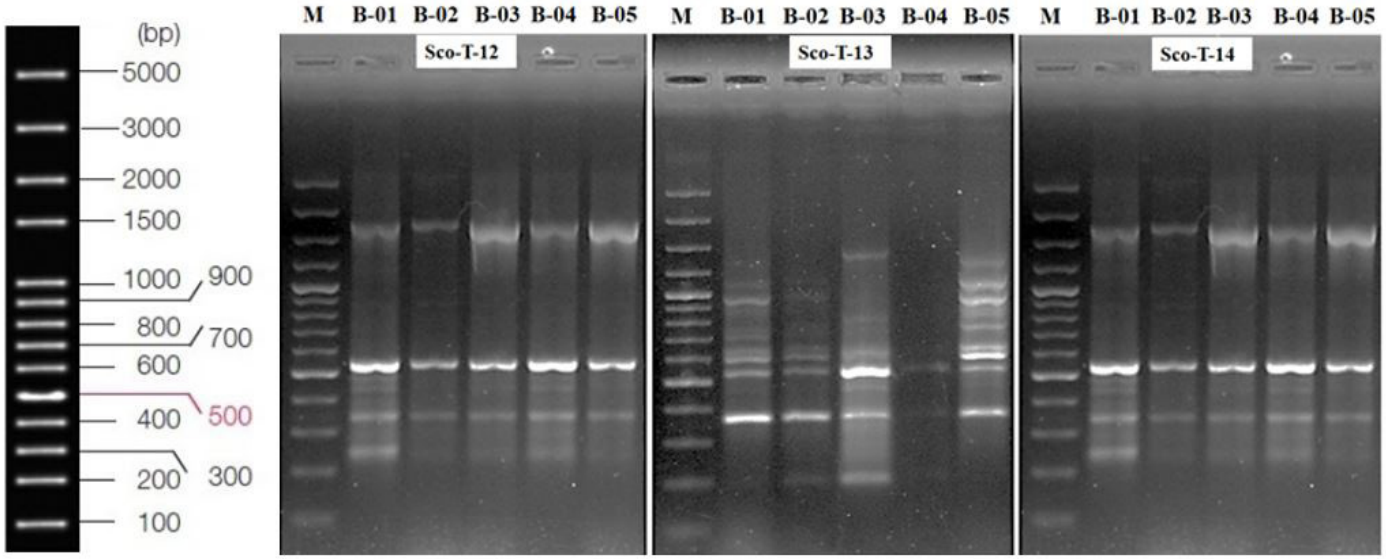

Figure 10: *Agarose gel electrophoresis (1.5%) showing SCoT-PCR amplification patterns of the five Bacillus isolates (B-01 to B-05) generated using the primers Sco-T-12, Sco-T-13, and Sco-T-14. Lane M represents the 0.1–5 kbp DNA molecular weight marker, while lanes B-01 to B-05 correspond to the tested Bacillus isolates. The obtained banding patterns revealed both polymorphic and shared DNA fragments among the isolates, reflecting genetic diversity detected by the SCoT markers.*

among the isolates and primers. SCoT-05 generated the highest number of unique fragments (9), followed by SCoT-09 (8), and SCoT-04/ SCoT-06 (7 each), suggesting the presence of isolate-specific molecular markers. In contrast, primers SCoT-12 and SCoT-14 did not produce any unique fragments, indicating a highly conserved amplification patterns among the tested isolates.

Distribution of unique SCoT markers among the Bacillus isolates

Among the isolates, B-01 showed the highest number of unique positive markers across the several used primers, particularly with SCoT-06 and SCoT-09, whereas some isolates showed either few or no unique markers. The presence of positive (+ve) and negative (–ve) unique bands emphasized the genetic divergence among the studied isolates. Overall, these

findings confirmed that SCoT markers are efficient and informative tools for molecular characterization and differentiation of the *Bacillus* isolates.

Genetic similarity analysis based on SCoT markers

The similarity matrix obtained from SCoT marker analysis revealed varying degrees of genetic relatedness among the investigated *Bacillus* isolates (Table 5). The similarity coefficients ranged from 0.56 to 0.78, indicating moderate to high genetic diversity among the tested isolates. The highest similarity value (0.78) was observed between isolates B-02 and B-04, suggesting a close genetic relationship between these two isolates. In contrast, the lowest similarity coefficient (0.56) was recorded between B-01 and B-02, indicating a substantial genetic divergence. Isolate B-05 showed a relatively high similarity with B-03 (0.71) and B-01 (0.70), reflecting a partial genetic relatedness, whereas the remaining comparisons displayed intermediate similarity values. Overall, the obtained results demonstrated that SCoT markers successfully differentiated among the *Bacillus* isolates and provided a reliable information about their genetic relationships and diversity patterns.

Table 5: *Genetic similarity coefficients among the Bacillus isolates based on SCoT analysis.*

***Bacillus* isolates**	**B-01**	**B-02**	**B-03**	**B-04**	**B-05**
B-01	1.00				
B-02	0.56	1.00			
B-03	0.63	0.65	1.00		
B-04	0.61	0.78	0.65	1.00	
B-05	0.70	0.64	0.71	0.64	1.00

Phylogenetic clustering of the Bacillus isolates based on SCoT data

The phylogenetic tree analysis based on SCoT marker data (Figure 11) revealed the presence of two major clusters among the investigated *Bacillus* isolates. The first cluster included isolates B-01, B-03, and B-05, indicating a relatively close genetic relationship among these isolates. In contrast, isolates B-02 and B-04 were grouped together in a separate cluster, reflecting a higher degree of genetic similarity between them, compared to the other isolates. These clustering patterns were consistent with the similarity coefficient analysis and confirmed the effectiveness of SCoT markers in distinguishing the genetic relationships among the studied *Bacillus* isolates.

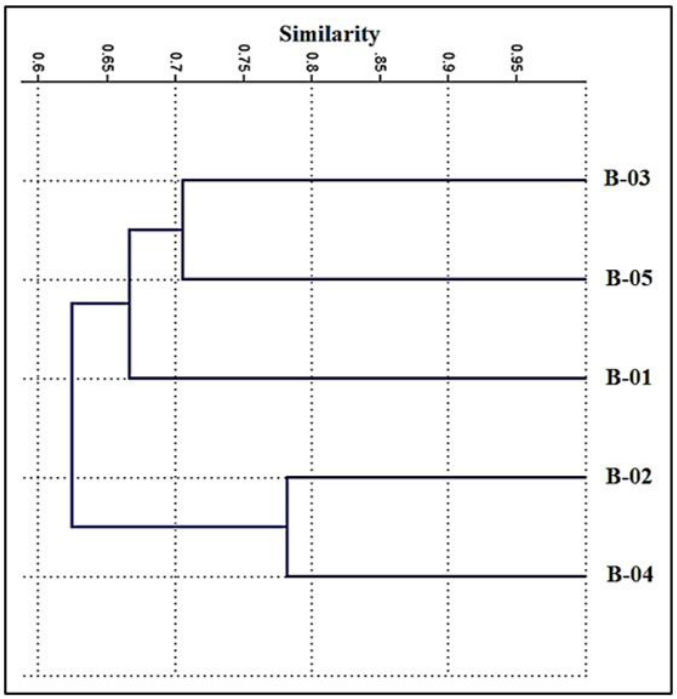

Figure 11: *UPGMA dendrogram illustrating the genetic relationships among the five Bacillus isolates based on SCoT marker analysis. The dendrogram divided the isolates into two major clusters. The first cluster included isolates B-01, B-03, and B-05, demonstrating a high level of genetic similarity among them. In contrast, the second cluster comprised isolates B-02 and B-04, indicating a distinct genetic relationship compared to the first cluster.*

Molecular identification and phylogenetic analysis of the Bacillus isolates based on 16S rRNA gene sequences

Molecular identification based on *16S rRNA* gene sequencing confirmed the taxonomic affiliation of the selected *Bacillus* isolates. Isolates B-01, B-03, and B-05 were identified as *B. thuringiensis*, whereas B-04 and B-02 were identified as *B. cereus* and *B. subtilis*, respectively. These results confirmed that the studied isolates belonged to well-established *Bacillus* species and provided a reliable taxonomic basis for subsequent genetic and biological investigations. Furthermore, pairwise comparison of the partial *16S rRNA* gene sequence of *B. thuringiensis* isolate B-01 (LC933331.1) with the other selected isolates revealed a high degree of sequence similarity among most isolates. The highest sequence identity was recorded between *B. thuringiensis* B-01 and *B. thuringiensis* B-05 (99.62%), followed by *B. thuringiensis* B-03 (99.44%) and *B. cereus* B-04 (99.34%), with query coverage values ranging from 89% to 99% and E-values of 0.0. In contrast, *B. subtilis* B-02 exhibited a substantially lower sequence identity (82.95%) and the lowest query coverage (62%), indicating a greater genetic

Table 6: *Pairwise sequence similarity between Bacillus thuringiensis isolate B-01 and the selected Bacillus isolates based on partial 16S rRNA gene sequences.*

Description	Query Cover (%)	E value	Identity (%)	Accession no.
Bacillus thuringiensis B-01 gene for 16S rRNA, partial sequence	100	0.0	100.0	LC933331.1
Bacillus cereus B-04 gene for 16S rRNA, partial sequence	99	0.0	99.34	LC933333.1
Bacillus thuringiensis B-03 gene for 16S rRNA, partial sequence	98	0.0	99.44	LC933332.1
Bacillus thuringiensis B-05 gene for 16S rRNA, partial sequence	89	0.0	99.62	LC933334.1
Bacillus subtilis B-02 gene for 16S rRNA, partial sequence	62	6e-88	82.95	LC933372.1

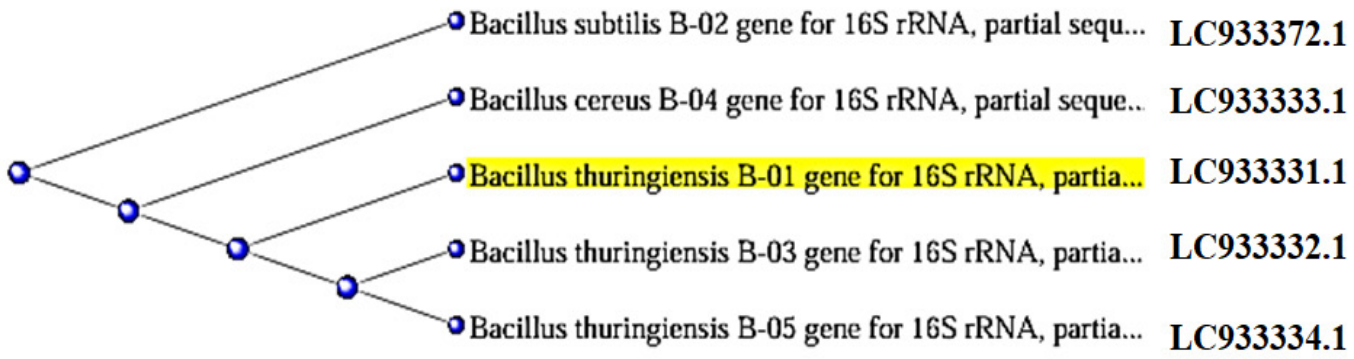

Figure 12: *A phylogenetic tree based on partial 16S rRNA gene sequences showing the evolutionary relationships among the selected Bacillus isolates. The tree illustrates the genetic relatedness of Bacillus thuringiensis isolates B-01, B-03, and B-05, together with Bacillus cereus B-04 and Bacillus subtilis B-02. Accession numbers assigned by DDBJ are indicated for each isolate.*

divergence from B-01 than that observed among the other isolates (Table 6). The phylogenetic analysis based on partial *16S rRNA* gene sequences revealed clear relationships among the selected *Bacillus* isolates (Figure 12). The tree clustered *B. thuringiensis* isolates B-01, B-03, and B-05 within the same lineage, indicating a close genetic relationship among them. Isolate B-05 appeared to be the closest relative to B-01, followed by B-03, which is consistent with the high sequence identity values obtained from the pairwise sequence comparisons. *B. cereus* B-04 was positioned near the *B. thuringiensis* cluster, reflecting the close taxonomic relationship between these species. In contrast, *B. subtilis* B-02 formed a separate branch, indicating a greater degree of genetic divergence from the other *Bacillus* isolates.

Discussion

The present study provides an integrated view of genetic diversity and biological activity among five *Bacillus* isolates using SCoT markers, *16S rRNA* gene sequencing, and antiviral assays against TMV. The combined molecular and biological approaches revealed a clear variability at both the genetic and functional levels, highlighting the importance of linking genotypic diversity with phenotypic performance (Vasemägi and Primmer, 2005; Govindaraj *et al.*, 2015). SCoT marker analysis revealed a high degree of polymorphism among the tested isolates, indicating a substantial genomic diversity. The variation in total, polymorphic, and unique DNA fragments suggested differences in the genomic regions potentially associated with the metabolic and functional genes. The presence of unique DNA amplified fragments in certain isolates further confirmed their genetic distinctiveness and supported the usefulness of gene-targeted markers for assessing intra-species variation. In this context, gene-targeted marker systems such as SCoT have been widely recognized as efficient tools for detecting the genetic variability and functional divergence among the microbial strains (Masny and Plucienniczak, 2001; El-Sayed *et al.*, 2022; Tekin *et al.*, 2024; Kumari *et al.*, 2026).

The similarity coefficient matrix showed a moderate to high genetic relatedness among the isolates, reflecting their shared taxonomic background at the genus level, while simultaneously revealing a strain-level divergence. The highest similarity observed between B-02 and B-04 indicated a closer genetic proximity, whereas the lowest similarity between B-01 and B-02 confirmed

a marked genomic differentiation. These findings were further supported by phylogenetic clustering, which separated the isolates into two main groups, confirming the robustness of the molecular dataset in resolving the fine-scale genetic relationships. Such clustering patterns are consistent with the reliability of DNA-based marker systems in distinguishing among the closely related bacterial strains (Yıldırım *et al*., 2011; Sharma *et al*., 2020). The phylogenetic analysis further emphasized the genetic structure of the bacterial isolates and suggested an evolutionary divergence that was likely driven by adaptation to different ecological niches or selective environmental stresses. The observed congruence between similarity coefficients and phylogenetic grouping strengthened the reliability of SCoT markers in discriminating among the closely related bacterial isolates and aligned with the previous reports on microbial genetic structuring using molecular markers (Wu *et al*., 2013).

The antiviral activity assays demonstrated that all *Bacillus* isolates exhibited inhibitory effects against TMV, although with varying efficiencies. The superior performance of B-02 (*B. subtilis*) and B-04 (*B. cereus*) suggested their enhanced capacity to produce potent antiviral metabolites, and other secondary metabolites capable of interfering with viral infection and replication processes (Chowdhury *et al*., 2015). These findings agree with several previous studies highlighting the pivotal role of *Bacillus*-derived lipopeptides in plant disease suppression and induction of host resistance responses (Ongena and Jacques, 2008; Saiyam *et al*., 2024).

The variation in inhibition percentages among the investigated isolates may be associated with differences in their genetic background, gene expression profiles, metabolic capabilities, and biosynthetic gene clusters involved in secondary metabolite production (Chen *et al*. 2009; Liu *et al*., 2017). Genetically, the diverse isolates often express distinct metabolic potentials and varying capacities to synthesize bioactive compounds, which may explain the observed differences in antiviral efficacy (Raaijmakers *et al*., 2010; Dinesh *et al*., 2017; Li *et al*., 2023). This observation is consistent with the substantial genetic polymorphism revealed by SCoT analysis, suggesting that genomic diversity among the *Bacillus* isolates may contribute to functional variations, including differences in the production of bioactive metabolites and antiviral performance (Stein, 2005; Fira *et al*., 2018; Caulier *et al*., 2019).

Interestingly, extending the incubation period at 4°C from 2 to 48 h further enhanced the antiviral activity, particularly in isolates B-02 and B-04, indicating that their bioactive metabolites remained stable and biologically active during the refrigerated incubation. Similar observations have been reported for *Bacillus* spp., whose cyclic lipopeptides, including surfactins, iturins, and fengycins, exhibited a remarkable physicochemical stability while retaining their biological activity under the diverse environmental conditions (Ongena and Jacques, 2008; Andreeva *et al*., 2014; Beris *et al*., 2018; Abdelkhalek *et al*., 2022; Amin *et al*., 2023; Karačić *et al*., 2024; Hemmati *et al*., 2025). These metabolites contribute to plant protection through direct antimicrobial effects and the activation of host defense pathways (Fira *et al*., 2018; Caulier *et al*., 2019).

Furthermore, Pan *et al*. (2025) demonstrated that the *Bacillus*-derived hydrolase P1 can directly suppress TMV infection while simultaneously inducing antiviral resistance in host plants. Therefore, the sustained inhibitory activity currently observed in B-02 and B-04 following prolonged cold incubation may reflect the presence of similarly stable antiviral metabolites. Such characteristics are particularly advantageous for the development of *Bacillus*-based biocontrol formulations, as they indicate that refrigerated storage and transportation may not compromise the antiviral efficacy.

Collectively, these findings suggested that the outstanding antiviral performance of B-02 and B-04 isolates was likely attributable to both their genetic attributes and ability to produce stable antiviral metabolites. Consequently, these isolates represent promising eco-friendly candidates for the biological management of TMV and potentially other plant viral diseases. Notably, *16S rRNA* gene sequencing confirmed the taxonomic identity of the most active isolates, identifying B-01 as *B. thuringiensis*, B-02 as *B. subtilis*, and B-04 as *B. cereus*. This molecular confirmation provided a solid taxonomic framework linking the species identity with the biological activity (Vynne *et al*., 2011). The strong antiviral performance associated particularly with *B. subtilis* and *B. thuringiensis* is consistent with previous studies reporting their roles in plant defense activation and viral suppression (Andreeva *et al*., 2014; Wang *et al*., 2023; Isaia *et al*., 2025).

The high sequence similarity (> 99%) observed among *B. thuringiensis* isolates B-01, B-03, and B-05, along with their close relationship to *B. cereus* B-04, reflected the conserved nature of the *16S rRNA* gene within the *B. cereus* species complex. In contrast, *B. subtilis* B-02 displayed a lower sequence identity and query coverage, indicating a greater genetic divergence. Phylogenetic analysis confirmed these relationships by clustering B-01, B-03, and B-05 together while placing B-02 and B-04 separately. Notably, this clustering pattern closely matched that obtained from SCoT marker analysis, highlighting the consistency between sequence-based and marker-based approaches and demonstrating the value of combining 16S rRNA sequencing with SCoT markers for reliable assessment of genetic diversity among the *Bacillus* isolates.

Overall, the integration of molecular characterization, phylogenetic analysis, and biological evaluation demonstrated that the genetic diversity within the *Bacillus* isolates was closely associated with the variability in antiviral activity. This supports the hypothesis that strain-level genomic variation contributes to functional differences in secondary metabolite production and antiviral efficacy (Stein 2005; Caulier *et al*., 2019). Similar conclusions have been reported in previous studies emphasizing the link between microbial diversity and functional biocontrol traits (Ongena and Jacques, 2008; Raaijmakers *et al*., 2010; Seipke, 2015).

These findings collectively highlight the potential application of selected *Bacillus* strains as eco-friendly biocontrol agents against plant viral diseases, particularly TMV, and provide a strong foundation for future studies aiming at characterizing the specific antiviral metabolites and their mechanisms of action.

Conclusions and Recommendations

The present study demonstrated the effectiveness of SCoT markers in assessing the genetic diversity of five *Bacillus* isolates, revealing 97.67% polymorphism and clear phylogenetic separation into two major clusters. Molecular identification based on *16S rRNA* gene sequencing confirmed the classification of the isolates as *Bacillus* thuringiensis, *B. subtilis*, and *B. cereus*, supporting the genetic relationships inferred from the SCoT analysis. All isolates exhibited antiviral activity against TMV; however, *B. subtilis* (B-02) and *B. cereus* (B-04) were the most effective, achieving inhibition percentages of 96.62% and 96.09%, respectively, after 48 h of incubation at 4°C. The integration of genetic diversity analysis, molecular identification, and antiviral bioassays identified *B. subtilis* (B-02) and *B. cereus* (B-04) as the most promising eco-friendly biocontrol agents against TMV and provided a solid basis for the future studies to identify the antiviral metabolites responsible for their activity and elucidate their mechanisms of action. Based on the promising antiviral activity observed in *B. subtilis* (B-02) and *B. cereus* (B-04), future studies should focus on the isolation and characterization of the bioactive metabolites responsible for TMV inhibition. Further investigations under greenhouse and field conditions are recommended to evaluate the efficacy, persistence, and practical applicability of these isolates as biological control agents. In addition, transcriptomic and metabolomic analyses could provide deeper insights into the mechanisms underlying their antiviral activity and interactions with the host plants.

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Novelty statement

This study presents an integrated approach combining antiviral bioassays with molecular genetic characterization (SCoT markers and *16S rRNA* gene sequencing) of the *Bacillus* isolates. The novelty of this study lies in integrating the genetic diversity with the antiviral efficiency against TMV, providing a dual perspective for selecting the highly effective biocontrol *Bacillus* strains.

Author's Contribution

FMB: Conceptualization, methodology, and supervision. SMO: Methodology and data analysis. AT: Methodology. AM: Methodology, data interpretation, reviewing and editing. ASS: Design, supervision, writing and revision. AHA: Methodology, data analysis, and investigation. All authors have read

and approved the final version of the manuscript.

Ethical approval
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Generative AI and AI assisted technology statement
The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Conflict of interests
The authors have declared no conflicts of interest.

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