PLANT SCIENCE TODAY
ISSN 2348-1900 (online)
Vol x(x): xx–xx
https://doi.org/10.14719/pst.7162

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RESEARCH ARTICLE

Biological control of tomato leaf curl New Delhi virus using cucurbitaceous endophytes in bitter gourd (*Momordica charantia*)

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OPEN ACCESS

ARTICLE HISTORY
Received: 11 January 2025
Accepted: 22 February 2025
Available online
Version 1.0 : 18 March 2025

Check for updates

Additional information
Peer review: Publisher thanks Sectional Editor and the other anonymous reviewers for their contribution to the peer review of this work.

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Indexing: Plant Science Today, published by Horizon e-Publishing Group, is covered by Scopus, Web of Science, BIOSIS Previews, Clarivate Analytics, NAAS, UGC Care, etc
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Sreegayathri E, Gopalakrishnan C, Karthikeyan G, Senthil A, Saraswathi T, Kokiladevi E. Biological control of tomato leaf curl New Delhi virus using cucurbitaceous endophytes in bitter gourd (*Momordica charantia*). Plant Science Today (Early Access).
https:/doi.org/10.14719/pst.7162

Abstract

Momordica charantia L., known as bitter gourd, is a vine species classified under the *Cucurbitaceae* family and is extensively cultivated across Southeast Asia. The *tomato leaf curl New Delhi virus* (ToLCNDV), a member of the *Begomovirus* genus and *Geminiviridae* family, significantly affects bitter gourd. In this study, endophytes were isolated from different cucurbitaceous crops. The germination potential of the bacterial and actinobacterial isolates was evaluated via the roll towel method. Notably, isolate B-BGR1 demonstrated a 100 percent germination rate with vigor index of 5636.00 compared with the sterile water control, which presented a vigor index of 1013.00. Subsequent pot culture experiments indicated that a 2 percent application of B-BGR1 resulted in the lowest disease incidence, with a 78.57 percent reduction over the control, followed by B-BGL1, which showed a 71.43 percent reduction over the control. The isolate B-BGR1 was molecularly confirmed as *Bacillus licheniformis* through sequencing. The presence of secondary metabolites in *B. licheniformis* was identified via gas chromatography-mass spectrometry (GC-MS). To further explore the mechanism of action, the ToLCNDV coat protein was designed via MODELLER software, yielding a model with the highest DOPE score of -22439.755859. Molecular docking experiments revealed strong binding affinities for compounds 7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-dien and Mandelic acid, with values of -5.0 and -4.9 kcal/mol, respectively. This study represents the first investigation to confirm the antiviral potential of *B. licheniformis* through molecular docking against the ToLCNDV coat protein. These results indicate that *B. licheniformis* is a potential biological control agent for managing ToLCNDV in bitter gourd.

Keywords

anti-viral activity; docking studies; endophytes; plant growth promotion; ToLCNDV

Introduction

The bitter gourd (*Momordica charantia* L.) is a significant vegetable crop worldwide and has considerable commercial importance. It is widely cultivated in India and across Southeast Asia. In India, cucurbitaceous vegetables constitute approximately 18% of the country's overall vegetable production (1). The crop is recognized for its antiviral, antimalarial and

immune-boosting properties. According to the second advance estimate for 2023-24, bitter gourd cultivation in India spans 13.48 million hectares. This cultivation yields a total production of 1.75 million metric tonnes, with an average output of 13 metric tonnes per hectare (2). The crop serves as a natural host for numerous DNA and RNA viruses, which significantly impacts its cultivation. *Begomoviruses,* which belong to the *Geminiviridae* family, are among the most important viruses in terms of causing more significant economic losses (3). The ToLCNDV is characterized by its single-stranded DNA genome and twinned icosahedral particles (4). The transmission of ToLCNDV occurs in a circulatory and persistent manner through the sweet potato whitefly *Bemisia tabaci* Gennadius, which belongs to the order *Hemiptera* and family *Aleyrodidae*. Globally, 45 morphologically identical cryptic species of *B. tabaci* have been identified, with nine species reported in India (5).

Viral disease management in crops presents significant challenges globally, with limited options currently available. Despite the necessity of using insecticides and other chemicals, their indiscriminate use has led to the development of resistance in insect populations and raised concerns regarding potential virus recombination. A number of limitations also affect genetic engineering, RNA silencing and viral resistance breeding approaches. There is a pressing need to identify effective bioagents that are both beneficial and environmentally safe. The combination of antagonistic bacterial strains that can combat viral infections is anticipated to stimulate host immune responses and promote growth in plants (6). Various studies have investigated the use of endophytes to combat viral infections in plants, including potato virus Y (PVY) and tomato spotted wilt virus (TSWV) in tomato plants (7), groundnut bud necrosis virus (GBNV) affecting tomato plants (8), cucumber mosaic virus (CMV) in tomato plants (9), tobacco streak virus (TSV) infecting cotton plants (10) and CMV infecting ridge gourd plants (11). With this information, the present study a) aimed to isolate effective endophytes from different cucurbitaceous crops, b) evaluated their efficacy in terms of plant growth promotion, c) explored the potential of ten effective *Bacillus* and *Streptomyces* strains for their efficacy in the management of ToLCNDV in bitter gourd under glasshouse conditions and d) investigated the antiviral potential of effective endophytes through molecular docking studies.

Materials and Methods

From 2023-2024, the study was conducted at the glass house, Department of Plant Pathology, Tamil Nadu Agricultural University (11°07'3.36" N 76°59'39.91" E), Coimbatore, Tamil Nadu, India.

Isolation of endophytes from cucurbitaceous crops

The endophytes were isolated from different cucurbitaceous crops, including bitter gourd, ivy gourd, bottle gourd, ash gourd, snake gourd, pumpkin, musk melon, cucumber and watermelon. Healthy plants were selected in the field and different plant parts, including roots, leaves, buds, flowers and young fruits, were collected. The samples were collected from different locations and three samples were collected for each plant part. For the isolation of endophytes, each sample was initially washed under running water. The samples were subsequently subjected to a surface disinfection procedure that included immersion in 70% alcohol for 1 minute, exposure to sodium hypochlorite (2.5% concentration) for 4 minutes, treatment with ethanol for 30 seconds and three rinses with sterilized distilled water. The disinfected plant material was then ground with a mortar and pestle with peptone salt buffer. Serial dilutions of the resulting tissue extracts were prepared and plated on nutrient agar media for *Bacillus* and starch casein agar for Actinobacteria. These isolation methods were adapted with slight modifications of a standardized protocol (12). The isolated plates were incubated at 28 ± 2°C for 24 hours and then purified by sorting and streaking morphologically distinct colonies onto Petri dishes. Afterwards, they were stored in glycerol stock media at -20°C for future use.

Evaluation of the plant growth-promoting activity of endophytes against bitter gourd plants

We investigated the growth-promoting effects of isolates obtained from various cucurbit crops on bitter gourd via the roll towel method in triplicate for each isolate. The optimized *Bacillus* isolate suspensions were adjusted to contain 1.33–2.4 × 10^{10} CFU/mL. The bitter gourd seeds were immersed in these suspensions for 30 minutes and then transferred to a rolled towel. The germination percentage of the emerged seedlings in each treatment, length of the roots and shoot, plant height and vigor index were assessed after 7 days. The following formula was used to calculate seedling vigor (13):

Vigor Index (VI) = (Mean shoot length + Mean root length) × Germination (%)…… (Eqn.1)

Maintenance of virus and vector sources

Samples of bitter gourd plants infected with ToLCNDV that exhibited mosaic symptoms were collected from cultivated fields in Thondamuthur village (11°00′35″N-76°49′41″E), which is located in the Coimbatore district, Tamil Nadu, India. These collected plants, which serve as a virus inoculum source, were subsequently maintained under separate polyhouse conditions. The *B. tabaci* vector was also collected from bitter gourd fields. Viruliferous and nonviruliferous whiteflies were kept separately on brinjal plants within insect cages. ToLCNDV infection-free whiteflies were maintained on healthy host plants within insect-proof enclosures. Nonviruliferous whiteflies that had been mass-generated were released into clip cages containing infected bitter gourd plants to obtain viruliferous whiteflies. The insects were allowed to feed on the host for a minimum of seven days. Whiteflies aged 20-25 days were used for the experiments.

Screening of effective endophytes against tomato leaf curl New Delhi virus

An evaluation of the effects of seven bacterial isolates and three actinobacterial isolates against ToLCNDV was conducted on the basis of their ability to promote plant growth. The assessment was conducted under greenhouse conditions via a preinoculation method, with three replicates maintained for each isolate. Inoculated and healthy plants

were maintained separately. At the two-leaf stage, bitter gourd seedlings were transferred to pots and a spore suspension was applied to them. The bacteria were cultured in Luria Bertanni broth for 24 hours, followed by incubation at room temperature (28 ± 2°C) for 48 hours with continuous shaking in an orbital shaker at 150 rpm. Furthermore, the culture broth was mixed with Tween 20 at 1% (10 mL), glycerol at 1% (10 mL), or polyvinylpyrrolidone at 1% (10 g) purchased from Sigma-Aldrich. After mixing, the mixture was shaken at 200 rpm for 5 minutes. The number of colony-forming units of the suspension was adjusted to a minimum of 2.5×10^{10} CFU mL^{-1} (14). After 24 hours, the treated plants were exposed to viruliferous whiteflies for infection. The percent disease incidence (PDI) was measured at regular intervals. To ensure reproducibility, the experiment was conducted twice.

Molecular confirmation of the bacterial isolate

The identification of effective bacterial strains was accomplished through 16S ribosomal RNA sequence analysis. Bacterial genomic DNA was extracted using a pre-published protocol (15). For amplification of the 16S rRNA gene, the universal primers 27F (5′ AGAGTTTGATCCTGGCTCAG 3′) and 1492 R (5′ GGTTACCTTGTTACGACTT 3′) were used. For PCR, an initial denaturation step was performed at 940°C for 4 minutes, followed by 35 cycles of denaturation (94^0C for 1 minute), annealing (55^0C for 1 minute) and extension (72^0C for 2 minutes). At 72^0C, the final extension was prolonged for 10 minutes, which produced a ~ 1.5 kb amplicon of the 16S ribosomal RNA region. Sequencing of the amplified PCR products in both the forward and reverse directions was performed in a single pass (Barcode Biosciences Pvt. Ltd.). The resulting sequences were analyzed and compared with similar bacterial DNA sequences using the BLAST search tool in the GenBank database. After confirming the genus and species with percent identity and query coverage with the previously submitted database, the obtained sequences were submitted to NCBI.

Metabolite profiling through gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry (Shimadzu Nexis 2030 linked to an nx8040 tandem mass spectrometer) was employed to examine the volatiles collected from various metabolic compounds. The device features a DB 5-MS capillary column as the stationary phase, measuring 30 mm × 0.25 mm ID × 0.25 µm and helium is used as the carrier gas. The injector was maintained at a steady temperature of 280° Celsius and 2 µL of the sample was introduced. In the oven, the temperature program was set to 70°C, held for 1 minute, then increased to 225°C at the rate of 5 °C per minute and maintained for 3 minutes. Eventually, the temperature reached 300°C and was maintained for five minutes. This mass spectrometer utilized an electron impact ionization detector with an electron impact energy of 70 eV with 230°C and 280°C temperatures for the ion source and interface, respectively. To identify compounds, scanning was conducted from 6.5-55 min, covering a range of 50-650 m/z.

Identification of bioactive compounds

To determine the relative percentage of each component present, the mean peak area of individual components was compared to the total area. This method allows the identification of specific metabolites by matching their characteristics with those found in the NIST mass spectra libraries and V3 library databases. The composition of the compound, its structure and its molecular weight were determined by using these libraries.

Molecular modeling of the ToLCNDV coat protein

The Coat protein of ToLCNDV has not been experimentally investigated. The computational structure of the ToLCNDV coat protein was developed via MODELLER 10.4 software. The ToLCNDV coat protein was recovered from the UniProt database and the amino acid sequences were analyzed via the BLASTp tool to search for template structures for homology modeling. The three-dimensional configuration of the protein was chosen according to the DOPE score among the five modeled structures of the ToLCNDV coat protein. We assessed the quality of our forecasted models via SAVES v5.0 tools (16) and ProSA (17). To ensure the precision and reliability of the predicted protein structures, multiple structural validation criteria were utilized. Furthermore, the Computed Atlas of Surface Topography (CASTp) was employed to examine the functional binding site of the coat protein (18).

Computational analysis of the effects of the ToLCNDV coat protein on bioactive molecules of B. licheniformis

To identify potential inhibitory compounds, the bioactive molecules extracted from *B. licheniformis* were subjected to molecular docking with the TOLCNDV coat protein. The PubChem database was used to obtain the 3D structures of all the molecules detected through GC-MS analysis (19). The Vina Wizard module in PyRx 0.8 was employed to perform molecular docking studies on these identified biomolecules. The "make macromolecule" feature in PyRx 0.8 was used to convert proteins into macromolecules. A United Force Field (UFF) was applied to minimize all ligands. The CastP server results were used to select binding sites and the Autogrid 4 module was utilized to configure the grid. In the subsequent step, molecular docking was performed and Biovia Discovery Studio software was used to visualize the interactions between ligands and proteins.

Statistical analysis

To compare the mean differences between treatments, one-way ANOVA with Duncan's multiple range test was conducted at a significance level of 5% (20). The results of the pot culture experiments were determined in a completely randomized design with three replications. The experimental data were analyzed via R Studio software.

Results and Discussion

Effect of cucurbitaceous endophytes on growth promotion

The growth-promoting effects of fifty bacterial isolates and twenty-two actinobacterial isolates were evaluated in bitter gourd seeds through the roll towel method. Among them,

seven bacterial isolates, B-BGR1, B-BGL1, B-RGR1, B-SGFL1, B-BOGB1, B-WMB1 and B-CB1, showed highest growth promotion activity. Specifically, B-BGR1 showed a 100 percent germination percentage with a vigor index of 5636.00, followed by B-BGL1, which possessed a vigor index of 5053.00 (Fig. 1). Among the actinobacterial isolates, isolates A-BGF1, A-PFR1 and A-SGF1 showed the highest vigor indices of 5401, 5333 and 5100, respectively, with 100 percent germination percentage (Fig. 2). The seeds treated with sterile distilled water served as a control showed lower vigor index of 1013. The isolates that exhibited better growth promotion ability were screened for their efficacy under glasshouse conditions. Multiple studies have shown the effectiveness of endophytes in enhancing plant growth through the roll towel technique. When this method was applied to eighteen *Bacillus* isolates from ridge gourd seeds, *Bacillus subtilis* (Bbv-57) demonstrated the highest vigor index at 2456.7, with isolates BST 8 and BSC 4 following closely behind. These isolates were subsequently tested in greenhouse settings to assess their antiviral capabilities against CMV in ridge gourd (11).

Screening the antiviral activity of selected endophytes under glasshouse conditions

In all the treatments, typical mosaic symptoms were expressed after 21 days of treatment, followed by viruliferous whitefly inoculation after 24 hours. A total of twelve treatments were conducted under glasshouse conditions. In the treatment, T_1 sprayed with 2 percent of isolate B-BGR1 had the lowest disease incidence, with a 78.57 percent reduction over the control. The treatment T_2 showed the next lowest disease incidence, with a 71.43 percent disease reduction over the control. On the other hand, the inoculated control group had the highest disease incidence, 93.33 percent. Overall, in comparison with the other treatments, treatment T_1 had the highest growth-promoting effect on bitter gourd (Table 1).

Additionally, disease progression in the treated plants was significantly different from that in the untreated control plants. A similar study carried out in tomatoes for the management of GBNV revealed that plants screened with various *Bacillus* isolates, *B. licheniformis* Soya 1 and *B. tequilensis* NBL6, had 16 percent disease incidence following *B. velezensis* VB7, which recorded 24 percent disease incidence (8). *Bacillus licheniformis* strain POT1 has demonstrated significant antiviral properties against *alfalfa mosaic virus* (AMV) in potato plants. Different treatment protocols were applied, including pre-inoculation, simultaneous inoculation and post-inoculation with bacteria and viruses. The pre-inoculation of *B. licheniformis* 24 hours before AMV inoculation resulted in an impressive 18.43-fold reduction in the viral titer compared with that of the inoculated control, which was associated with a 33.33-fold increase in the degree of virus accumulation. Additionally, treatment with *B. licheniformis* 24 hours before and 24 hours after inoculation also yielded positive results, resulting in a 4.40-fold reduction in viral levels (21). These findings indicate that *B. licheniformis* can be used for the management of viral diseases.

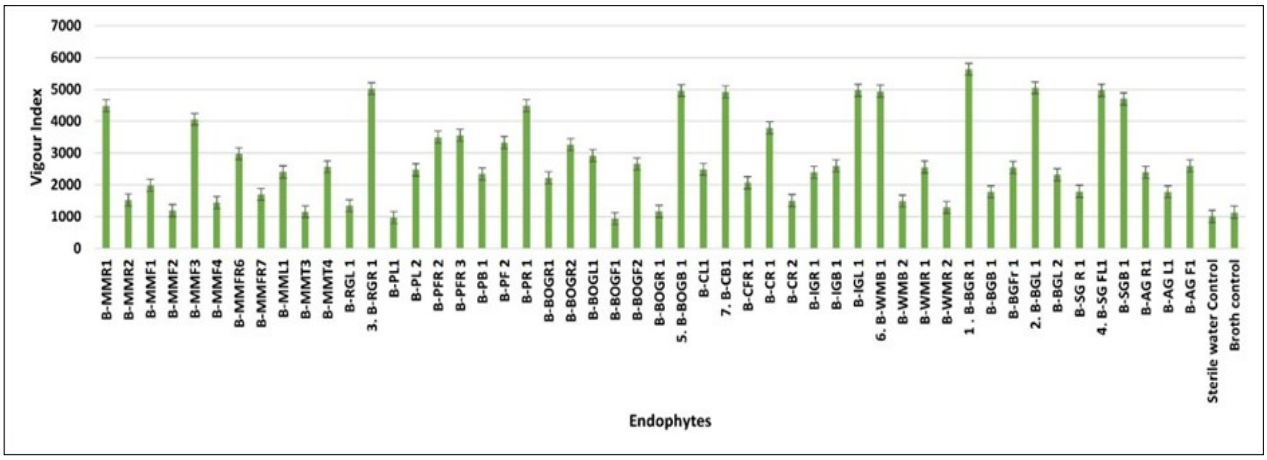

Fig. 1. Evaluation of the effects of bacterial isolates on the germination and growth promotion of bitter gourd via the roll towel method.

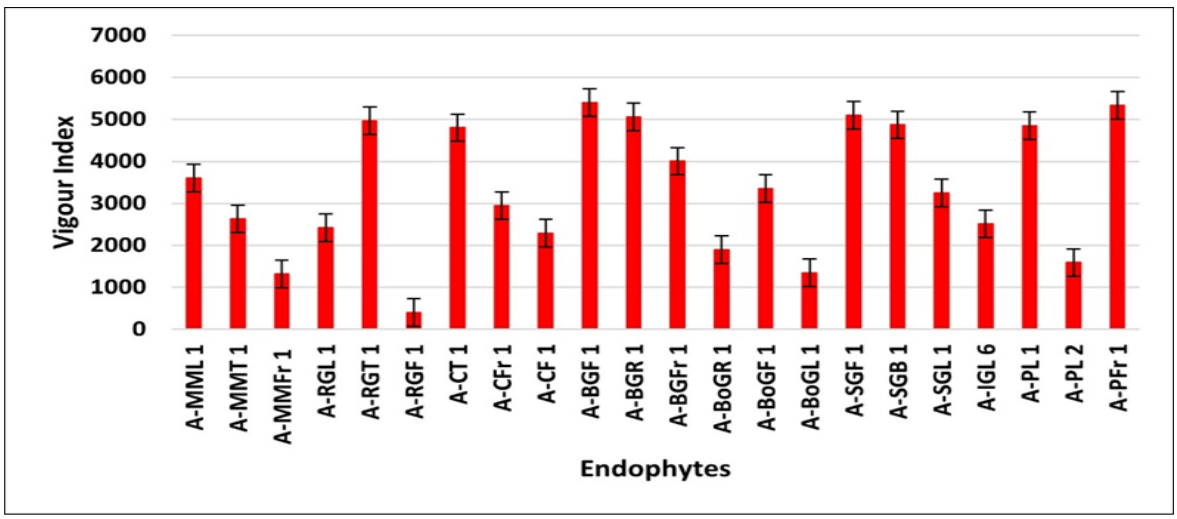

Fig. 2. Evaluation of the effects of actinobacterial isolates on germination and growth promotion of bitter gourd via the roll towel method.

Table 1. Assessment of the efficacy of selected bacterial isolates on the percent disease incidence of ToLCNDV in bitter gourd under glasshouse conditions

Treatments	Concentration	Mean Percent Disease Incidence	Percent reduction over control
T1 - B- BGR1	2%	20.00^{h} (18.665)	78.57066
T2 - B-BGL1	2%	26.66^{g} (29.534)	71.43469
T3- B- RGR1	2%	46.66^{d} (39.136)	50.00536
T4 - B- SG Fl 1	2%	40.00^{e} (40.814)	57.14133
T5 - B- BOGB1	2%	33.33^{f} (36.335)	64.28801
T6 - B-WMB1	2%	53.33^{c} (42.992)	42.85867
T7 - B - CB1	2%	53.33^{c} (47.047)	42.85867
T8 - A- BGF1	2%	40.00^{e} (41.806)	57.14133
T9 - A - SGF1	2%	53.33^{c} (43.896)	42.85867
T10 - A- PFr1	2%	60.00^{b} (50.017)	35.711
T 11 - Inoculated	-	93.33^{a} (66.739)	0
T12 - Uninoculated	-	-	-
CD (0.05)			1.9677
SEd			0.9534

*Values are the means of three replications.

Values in the parenthesis are arc sine transformed values. Means in a column followed by different superscript letters are significantly different according to DMRT at ρ ≤ 0.05.

Molecular characterization of the potential endophytes

The endophytic isolates B-BGR1, B-BGL1, B-RGR1, B-SGFL1, B-BOGB1, B-WMB1, B-CB1, A-BGF1, A-PFR1 and A-SGF1 selected via the roll towel method and glass house trial were further confirmed molecularly through the 16S rRNA gene, which was confirmed at an amplicon size of ~ 1.5 kb. The isolate B-BGR1, which has the highest antiviral potential among all the isolates, was further sequenced and a BLASTn search was carried out. The isolate showed 100.00% sequence similarity with *B. licheniformis* and the accession number was obtained (PV002722). The phenotypic and molecular characterization of effective bacterial strains of *Bacillus* was similar to recent studies on potential *Bacillus* species (10,21,22).

Metabolite profiling of B. licheniformis through gas chromatography-mass spectrometry

The GC–MS analysis revealed the presence of secondary metabolites such as pimelic acid, docosahexaenoic acid, stearic acid, methyl oleate, 2-propyl-glutaric acid, dihydrouracil, linoleic acid, methyl cis-10-pentadecanoate, lactic acid, palmitic acid, pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro, 7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-dien, 3-methyl-2,3,6,7,8,8a-hexahydropyrrolo[1,2-a], mandelic acid, anthranilic acid, 1-methoxy-3-phenylpropane, phthalic acid, benzyl 3-phenylpropyl ester, pentasiloxane, dodecamethyl, cyclotetrasiloxane, octamethyl, N6-acetyllysine, histidine, 4-aminobutyric acid, isovalerylglycine and tiglylglycine. Pyrrolo[1,2-a] pyrazine-1,4-dione and pyrazine-hexahydro presented an elevated peak area percentage of 1.49. In addition to these constituents, there were other compounds with varying retention times and peak areas. These results corroborate to a previously published report, where the GC-MS analysis of *B. licheniformis* produced 22 different bioactive compounds, of which pyrrolo[1,2-a] pyrazine-1,4-dione had the highest retention time. The efficiency of these compounds was further specifically analyzed through molecular docking (21).

ToLCNDV coat protein modeling and molecular docking studies

Using MODELLER software, five coat protein models were constructed based on the template. For subsequent docking analysis, the model that presented the lowest DOPE score of -22439.755859 was selected. (Fig. 3). The SAVE SERVER software was employed to verify this model, revealing that 95.6% of the residues were situated in the most favorable regions. Additionally, 3.9% were found in additional allowed regions, whereas 0.4% were located in marginally acceptable zones. Notably, no residues (0.0%) were detected in disallowed regions (Fig. 3). Further validation via PROSA yielded a Z score of -5.96, confirming the model's stability and reliability for further analysis. Protein structure prediction, molecular modeling of coat proteins and validation procedures were performed according to previous methods (23, 24). The commercial antiviral compound ningnanmycin served as a positive control (25).

The results of molecular docking carried out with PyRx software revealed that 12 metabolites from *B. licheniformis* that served as ligands against the ToLCNDV coat protein possessed higher binding affinities. The compounds 7,9-di-tert-butyl-1-oxaspiro (4,5)deca-6,9-dien, mandelic acid and pimelic acid had the highest binding affinities of -5.0, -4.9 and -4.3 kcal/mol against the virus coat protein, respectively (Fig. 4), which are slightly lower than that of ningnanmycin, which had a binding affinity of -5.8 kcal/mol. The number of hydrogen bonds and the number of residues involved in each interaction between proteins and ligands are displayed in Table 2. These results corroborate a previous study where the efficacy of 16 novel mandelic acid pyrazole amide derivatives was tested against tobacco mosaic virus (TMV) through the half-leaf method. They revealed that a 500 µg/L concentration provides better curative effects against the virus (26).

The antiviral activity of squalene and ganoderic acid against the coat protein and glycoprotein of GBNV has resulted in increased binding affinities with strong hydrophobic interactions, which block the binding of viral replication RNA with the coat protein (27). In a similar study, the secondary metabolites from *B. velezensis* and *Bacillus subtilis* were investigated against chitinase, transferase, endoglucanase I and proteinase K target proteins of *Macrophomina phaseolina*, resulting in the identification of the mechanism of action based on binding affinity (28).

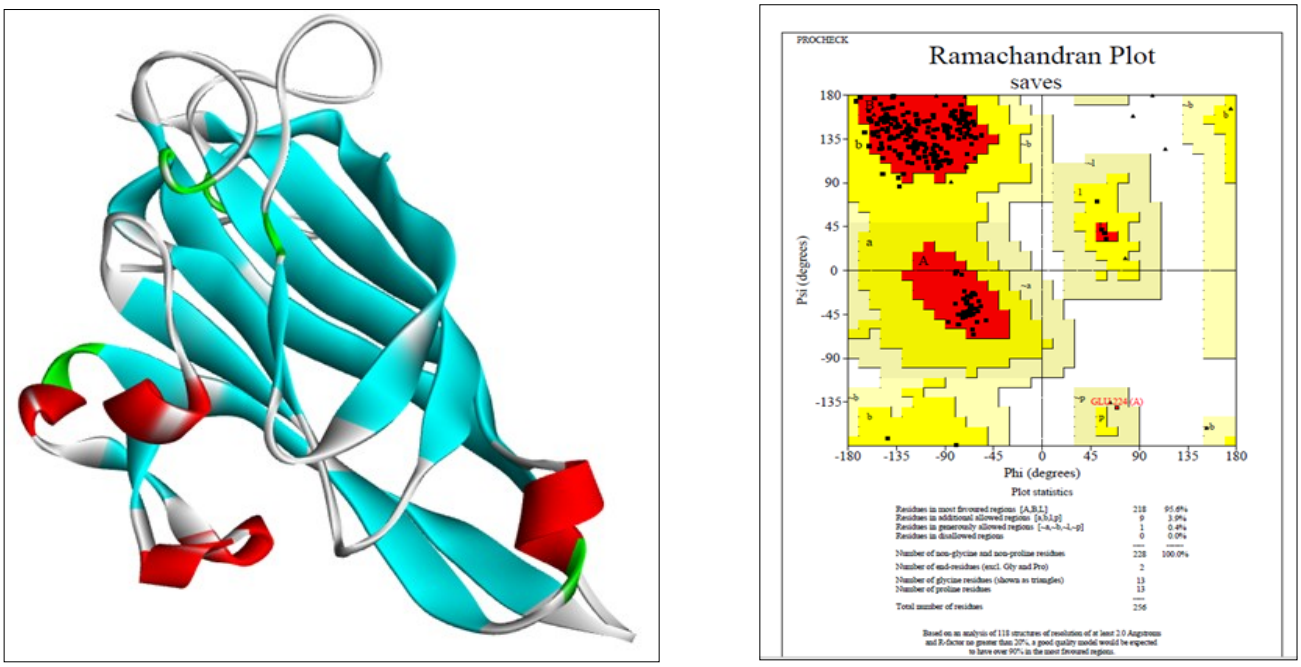

Fig. 3. A) 3D protein model of ToLCNDV coat protein B) Modeled coat protein validation through Ramachandran plot.

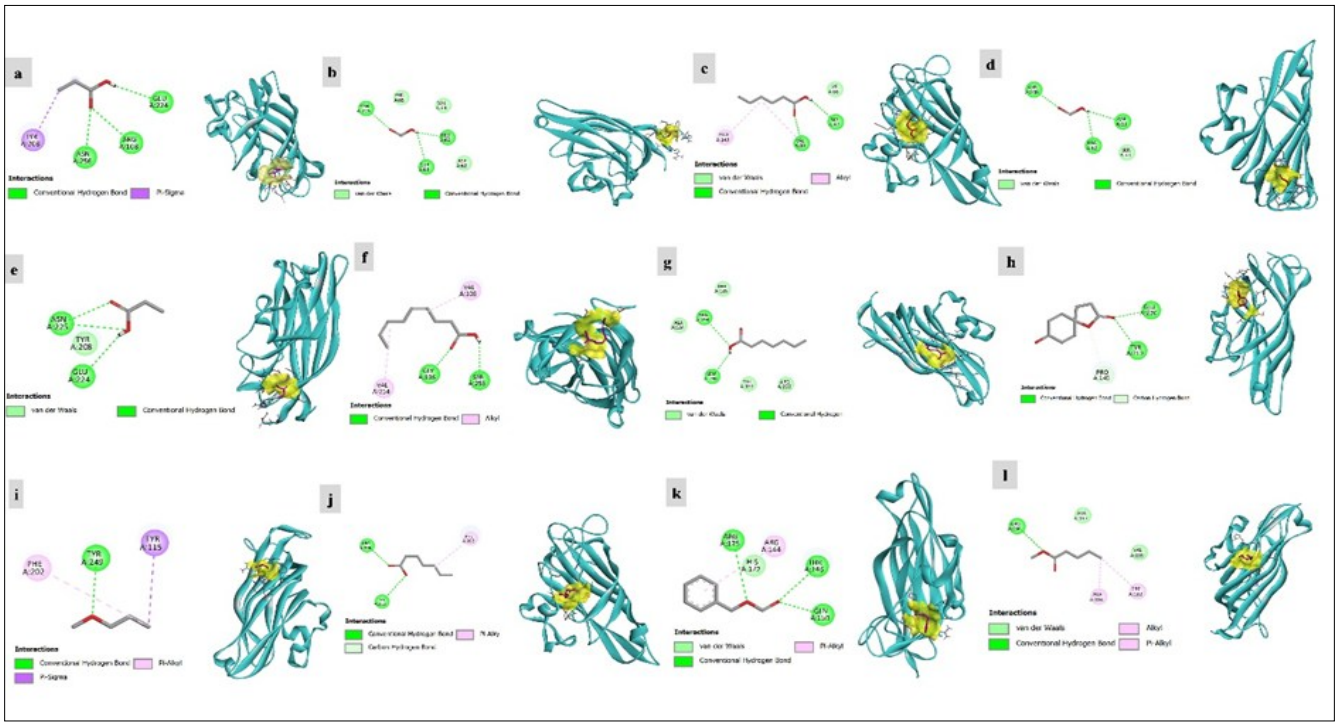

Fig. 4. Molecular docking interaction of ToLCNDV coat protein and secondary metabolites of *B. licheniformis.* a) Pimelic acid b) Lactic acid c) Palmitic Acid d) Mandelic acid e) 2-Propyl-glutaric acid f) Docosahexaenoic acid g) Oleic acid h) 7 ,9-Di-tert-butyl-1-oxaspiro (4,5) deca-6,9-dien i) 1-Methoxy-3-phenylpropane j) 1-Methoxy-3-phenylpropane k) Linoleic acid l) Phthalic acid, benzyl 3-phenylpropyl ester.

Table 2. Binding energy values and interacting residues of *Bacillus licheniformis* metabolites with the ToLCNDV target coat protein

Compound name	Binding affinity (Kcal/mol)	No. of H bonds	Interacting residues
Pimelic acid	-4.3	3	ASN 256, ARG 108, GLU 224, TYR 208
Lactic acid	-3.8	3	ASN 206, PRO 62, SER 61, PRO 65, VAL 64, ASP 63
Palmitic Acid	-3.9	2	GLY 87, VAL 89, LYS 88, PRO 149
Mandelic acid	-4.9	3	PRO 62, ASP 63, ASN 206, SER 61
2-Propyl-glutaric acid	-4.1	2	ASN 225, GLU 224, TYR 208
Docosahexaenoic acid	-3.9	2	GLY 106,SER 253, VAL 105, VAL 254
Oleic acid	-4.1	2	ARG 194, LEU 198, THR 185, ALA 184, VAL 199, ARG 200
7 ,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-dien	-5.0	2	TYR 219, GLU 220, PRO 149
1-Methoxy-3-phenylpropane	-4.2	1	TYR 249, PHE 202, TYR 115
Linoleic acid	-4.1	2	LYS 112, ARG 204, PHE 202
Phthalic acid, benzyl 3-phenylpropyl ester	-4.1	3	ARG 175, THR 146, GLN 150, ARG 144, HIS 172
Methyl cis-10-pentadecenoate	-3.5	1	ARG 194, ALA 197, VAL 199, ALA 184, TRP 182

Conclusion

The current investigation explored the antiviral efficacy of *Bacillus licheniformis* in managing ToLCNDV. These findings demonstrate that the use of *Bacillus licheniformis* not only promotes plant growth but also triggers the activation of defense-related genes, leading to the induction of systemic resistance in plants. Notably, the treatment significantly reduced the expression of symptoms associated with ToLCNDV. Molecular docking studies further corroborated the antiviral properties of *B. licheniformis*, suggesting its potential as a biocontrol agent. Further research is needed to optimize the application of *Bacillus licheniformis* under field conditions, which is essential for developing effective and sustainable strategies for virus management in crops. Optimization of the dosage for field application and consecutive sprays will prove the efficacy of *Bacillus licheniformis* under field conditions. Combining *B. licheniformis* with other proven antiviral plant extracts could provide a more comprehensive approach to the management of disease.

Acknowledgements

The authors are thankful to Tamil Nadu Agricultural University, Coimbatore for providing the necessary facilities during study.

Authors' contributions

SE carried out the experiment and recorded the data and wrote the original draft. GC formulated the research concepts, wrote the original draft, reviewed, edited and approved the final manuscript. KG formulated the research concepts, wrote the original draft and reviewed the articles. SA participated in laboratory techniques and wrote, reviewed and edited the manuscript. ST summarized and revised the manuscript. KE wrote, reviewed and edited the manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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