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## Harnessing *Bacillus amyloliquefaciens* for sustainable control of pomegranate bacterial leaf blight: Insights from GC-MS to targeting protein XopN molecular docking

S. Naresh\*, L. Karthiba\*\*♦, S. K. Manoranjitham\*, D. Vidhya\*\*\* and M. Gnanachitra\*\*\*\*

\*Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore-641003, Tamil Nadu, India

\*\* Department of Pulses, Tamil Nadu Agricultural University, Coimbatore-641003, Tamil Nadu, India

\*\*\* Department of Fruit Science, Horticulture College and Research Institute, Tamil Nadu Agricultural University, Coimbatore-641003, Tamil Nadu, India

\*\*\*\* Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore-641003, Tamil Nadu, India

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## Abstract

Bacterial leaf blight (BLB), caused by *Xanthomonas axonopodis* pv. *punicae* (XAP), poses a significant threat to pomegranate cultivation in India. This study surveyed major pomegranate-growing districts of Tamil Nadu, with Theni and Tirupur districts recording the highest BLB incidence. Eight XAP isolates were identified and confirmed for pathogenicity. Twenty-five native endophytic and rhizosphere bacterial isolates were screened for their antagonistic activity, with *Bacillus amyloliquefaciens* (UBL-8), *Bacillus subtilis* (UBL-7), and *Bacillus velezensis* (TBL-21) showing strong inhibition against XAP. GC-MS analysis of UBL-8 revealed 40 bioactive compounds; pentenyl angelate and 3-methylbenzothiothiophene exhibited potent antimicrobial activity. Molecular docking showed these compounds bind effectively to XopN, a key virulence protein of XAP, with no interaction with host proteins, indicating targeted action. The results highlight *B. amyloliquefaciens* (UBL-8) as an eco-friendly biocontrol agent for managing BLB in pomegranate, reducing reliance on chemical pesticides and supporting sustainable agriculture.

## 1. Introduction

Pomegranate (*Punica granatum* L.), a favourite table fruit crop of the Lythraceae family is extensively cultivated in India across 227.1 ha, producing 2882.5 MT with a productivity of 12.7 MT/ha in 2023-24 (FAO, 2023-24). Key growing states include Maharashtra, Andhra Pradesh, Karnataka, Gujarat and Tamil Nadu. Bacterial leaf blight (BLB), caused by *Xanthomonas axonopodis* pv. *punicae* (XAP), severely impact pomegranate cultivation with yield losses of 60-70% under favourable conditions and 30-40% on average (Chand and Kishun, 1991). First reported in New Delhi in 1952 (Hingorani and Mehta, 1953). BLB was later documented in Tamil Nadu by Rangaswamy (1962). The pathogen affects leaves, fruits and stems causing significant economic, nutritional, and postharvest losses (Dhandar *et al.*, 2004; Thakur *et al.*, 2021). BLB pathogenicity validated through biochemical and molecular analyses underscores its virulence (Harshitha *et al.*, 2018). Warm and humid conditions exacerbate disease spread particularly in regions like Theni and Tirupur (Mondal and Singh, 2008). Secondary metabolites from UBL-8 were analysed via GC-MS, identifying bioactive compounds with antimicrobial properties including pentenyl angelate and 3-

methylbenzothiothiophene. Molecular docking studies confirmed their selective binding to the XopN protein of XAP with strong binding affinities (-7.5 and -7.1 kcal/mol) demonstrating their potential biocontrol agents. This integrated approach supports the development of eco-friendly strategies to manage BLB and reduce dependence on chemical pesticides. This study surveys BLB prevalence in Tamil Nadu pomegranate growing districts (Theni, Coimbatore, Erode, Tirupur and Nilgiris) characterizes XAP isolates and evaluates native bacterial antagonists *Bacillus* sp. as a biocontrol agent by identifying effective bioagents, this research aims to develop sustainable disease management strategies by reducing usage of chemical pesticide and enhancing pomegranate productivity.

## 2. Materials and Methods

## 2.1 Field survey and isolation of bacterial strain

Field survey conducted in major pomegranate growing districts of Tamil Nadu like Theni, Coimbatore, Erode and Tirupur along with Nilgiris, during the months of October to December 2024 and prevalence of pome BLB was recorded in all these regions. The samples were collected from the respective pomegranate fields. Totally 8 isolates of *X. axonopodis* pv. *punicae* were isolated from different parts of the plant, viz., leaves, fruits and stems. Samples being initially surface sterilized with 1.0 % sodium hypochlorite (NaOCl) and sterile water wash followed by 70% ethanol and subsequent washes with sterile distilled water, the surface sterilized tissue were subjected to tissue segmentation method for isolation of the pathogen in nutrient glucose agar (NGA) medium (beef extract: 3

## Corresponding author: Dr. L. Karthiba

Assistant Professor, Department of Pulses, Tamil Nadu Agricultural University, Coimbatore-641003, Tamil Nadu, India

E-mail: [karthiba.l@tnau.ac.in](mailto:karthiba.l@tnau.ac.in)

Tel.: +91-9443861248

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g, D-G-anhydrous: 2.5 g, peptone: 5 g, agar: 20 g, distilled water: 1000 ml), subsequently purified using the single colony method and maintained aseptically for 48 to 72 h at 28°C on NGA medium. The cultures were regularly sub-cultured and stored at 4°C.

## 2.2 Cultural and biochemical characterization

Cultural characters observed after 72 h of incubation, the morphological characteristics including colony color, elevation, cell shape, colony shape, texture, appearance and margin were investigated and recorded. In order to identify and characterize the BLB, the bacterial isolates were subjected to a number of biochemical assays including gram staining, (KOH) solubility test, starch hydrolysis, gelatin liquefaction (Hilder brand and Schroth, 1972), cellulase activity, growth on asparagine medium (Bradbury, 1984), Methyl red test, Vogesproskauer test and Catalase tests (Lelliott and Stead, 1987).

## 2.3 Proving Koch's postulate

The spray method was used to test pathogenicity of the *X. axonopodis* pv. *punicae* strains. This technique involved inoculating the pomegranate leaves with bacterial leaf blight pathogen. The leaves were sprayed with a known concentration of an aqueous solution of bacterial inoculum ( $10^{6-8}$  cfu). The plants sprayed with sterile distilled water act as control and maintained at 70-80% humidity. Two replications were maintained for each treatment. Before the inoculation, the plants prior 48 h of period were covered with polythene bags and after inoculation the plants were subjected to polythene bags to create a humid chamber and pathogen-inoculated plants were kept at  $28 \pm 2^\circ\text{C}$  and maintained 90% humidity. We recorded the emergence of bacterial leaf blight symptoms on the leaves in different interval of days. In order to validate the Koch postulate, the inoculated culture and the re-isolated pathogen from infected leaves were examined (Chand and Kishun, 1991).

## 2.4 Isolation of native bacterial antagonist

During survey, healthy pomegranate plant samples and rhizosphere soil were collected for the isolation of the native endophytic bacterial strains. The collected leaves and root samples were cleansed thrice using sterile distilled water and one gram of the sample weighed separately, cut into 2-3 cm pieces and surface-sterilized for 4 min using 2.0% sodium hypochlorite solution (4.0% w/v) (Maroof Ahmed *et al.*, 2012), followed by 70% ethanol for 2 min and three sterile water washes. 0.1 ml aliquot of the final wash was plated on nutrient agar (NA) medium as a sterilization check and no colonies were observed. Under aseptic condition with use of sterile pestle and mortar the surface sterilized samples were macerated with 9.0 ml of phosphate-buffered saline to homogenize (Aravind *et al.*, 2009). The mixture was serially diluted to  $10^5$  CFU, each dilution ( $10^1$  to  $10^5$ ) of the mixture was spread on tryptic soy agar (TSA) media and the plates were incubated at 28°C for 48-72 h. Well-isolated colonies from each dilution were chosen and sub-cultured on TSA media as a pure culture.

## 2.5 Molecular characterization and confirmation of *X. axonopodis* pv. *punicae* and bacterial endophytes using PCR-based methods

Genomic DNA from *X. axonopodis* pv. *punicae* isolates were extracted using a CTAB method as described by (Kumar *et al.*, 2004). For the amplification of 16S rDNA, the prokaryotic universal primers 27F and 1492R were employed. The reaction was carried out in 10  $\mu\text{l}$

reaction mixtures containing 30 ng of genomic DNA, 0.2 mM dNTPs, 1X PCR buffer, 1.5 mM  $\text{MgCl}_2$ , 1.25 U of Taq DNA polymerase, and 0.4  $\mu\text{M}$  primers. The PCR amplification was carried out in a master cycler gradient, Eppendorf PCR. The PCR protocol described by (Williams *et al.*, 1990) was used with some modifications by using 16S rRNA diagnostic primer for confirmation of 8 strains of *X. axonopodis* pv. *punicae*. Unpurified products were subjected to bidirectional sanger sequencing to achieve comprehensive gene sequence coverage. The sequences were processed, trimmed at the end and contigs were constructed using BioEdit. Then aligned with GenBank sequences through BLAST analysis, sequence similarities were evaluated using the NCBI database with bacterial identity determined by the closest match (Altschul *et al.*, 1997; Shukla *et al.*, 2012).

## 2.6 In vitro screening of antagonistic bacteria against *X. axonopodis* pv. *punicae*

Totally of 25 bacterial strains were isolated from the phyllosphere and rhizosphere regions of the pomegranate. Using the paper disc assay method, *in vitro* screening was carried out under aseptic condition. Initially, 25 bacterial isolates were inoculated in tryptic soy broth and kept in a shaker cum incubator at 28°C for 24-48 h. Then, pure culture of *X. axonopodis* pv. *punicae* streaked over nutrient glucose agar medium and sterile filter paper discs were immersed in 48 h old bacterial broth and placed over the streaked pathogen and plates were incubated at room temperature for 24-48 h. After the incubation period, the inhibition zones were observed and recorded.

## 2.7 GC-MS profiling of antagonist bacterial metabolites

The bacterial culture filtrate of the promising endophytic *B. amylo-liquefaciens* strain was analysed using gas chromatography-mass spectrometry (GC-MS) to identify the secondary metabolites for validating its antimicrobial property. For that 48 h old bacterial culture broth was first centrifuged to remove precipitates and the supernatant was collected. This supernatant was then mixed with ethyl acetate in a 1:1 volume ratio. The mixture was vigorously agitated on an orbital shaker at 200 rpm for overnight to ensure thorough extraction. Using a separating funnel, the organic (ethyl acetate) layer was carefully separated from the aqueous phase. The organic extract was then concentrated using a rotary evaporator at 80°C and dissolved with methanol. The concentrated sample was subjected to GC-MS analysis. Helium served as the carrier gas at a constant flow rate of 1/ ml/min. The sample was run for 53 min at an ionization energy of 70 electron volts (eV) and the resulting mass spectra were compared against entries in the GC-MS library for compound identification.

## 2.8 Retrieval of XopN sequence

The XopN protein sequence was retrieved from UniprotKB database. UniprotKB is the central hub for the collection of functional information of proteins.

### 2.8.1 Ligand preparation and analysis

The bioactive compounds of the best isolate (UBL 8) were examined using GC-MS, based on the confrontation assay results from the forty bioactive compounds, the six compounds with a significant peak area% compounds; namely, 1H-indole-3-acetic acid, 5-hydroxy; pentenyl angelate 2Z; 3-hydroxy-4-methoxybenzoic acid; 3methylbenzothiophene; 4-phosphonobutyric acid and N-acetyl-d-glucosamine were chosen for molecular docking. Using Open Babel software, six bioactive compounds were obtained in SDF format from the Pubchem, database and converted to PDB file format.

### 2.8.2 *In silico* screening and docking studies

The Auto-Dock vina module in PyRx 0.8 was used to carry out molecular docking. Protein preparation was carried out using the PyRx software to make macromolecule function. All ligand structures (UFF) were reduced by 200 steps of conjugate gradient optimization using a unified force field and commercial molecular mechanics parameters. Pockets at the target binding position were found using CASTp3.0. AutoDock4 and AutoGrid4 assisted in setting up the ligands for grid and docking.

During docking, ligands could take on several orientations and conformations when the exhaustiveness was set to 8. The target protein and ligand structures were transformed into PDBQT format. BIOVIA Discovery Studio Client 2021 was used to visualize the docked protein-ligand complex interactions.

## 3. Results

*X. axonopodis* pv. *punicae* is the most devastating pathogen of pomegranate causing both qualitative and quantitative losses through oily spot (bacterial leaf blight) disease. The economic losses were believed to be 40% on average (Chand and Kishun, 1993).

### 3.1 Survey, collection, and isolation of bacterial strain

Survey was conducted to study the incidence of BLP in pomegranate. The results of the survey given in Table 1 indicating that the disease incidence % of BLB was higher in Periyakulam (37%) and Palarpatti (36%) regions of Theni District, followed by Udumalaipet (34%)

regions of Tirupur and Thalavadi (29%) region of Erode. Pomegranate orchards in Periyanaickenpalayam, Seeranaickenpalayam and TNAU of Coimbatore observed BLB incidence of 15-22%, whereas the Nilgiris District of Coonoor (13%) recorded lower disease incidence. Infected leaves and fruits were collected from different pomegranate-growing districts of Tamil Nadu. The pathogen from the infected samples were isolated on NGA medium. These cultures were frequently sub-cultured and stored at 4°C for further studies.

### 3.2 Cultural and biochemical characterization of *X. axonopodis* pv. *punicae* isolates

In nutrient glucose agar medium, the isolates TNIXP, TVLXP, PKMXP and SNPXP produced dark yellow colonies whereas CBEXP, UDPXP, CNRXP and PNPXP produced pale yellow colonies. The colonies of the *X. axonopodis* pv. *punicae* isolates on NGA medium range from 1.0 to 2.0 mm in size and circular in shape. The elevation of all the colonies is convex. Cell shape was observed as a single rod with an entire margin. Isolates CNRXP, CBEXP, and SNPXP exhibited high mucoid texture, and the remaining isolates were slightly mucoid. Biochemical characteristics of XAP isolates shows positive result to the catalase test, (KOH) solubility, starch hydrolysis, cellulase activity and gelatin liquefaction, whereas negative result for gram staining, methyl red, voges proskauer and growth on asparagine medium. The data of biochemical tests of all *X. axonopodis* pv. *punicae* isolates were presented on Figure1 and Table 2.

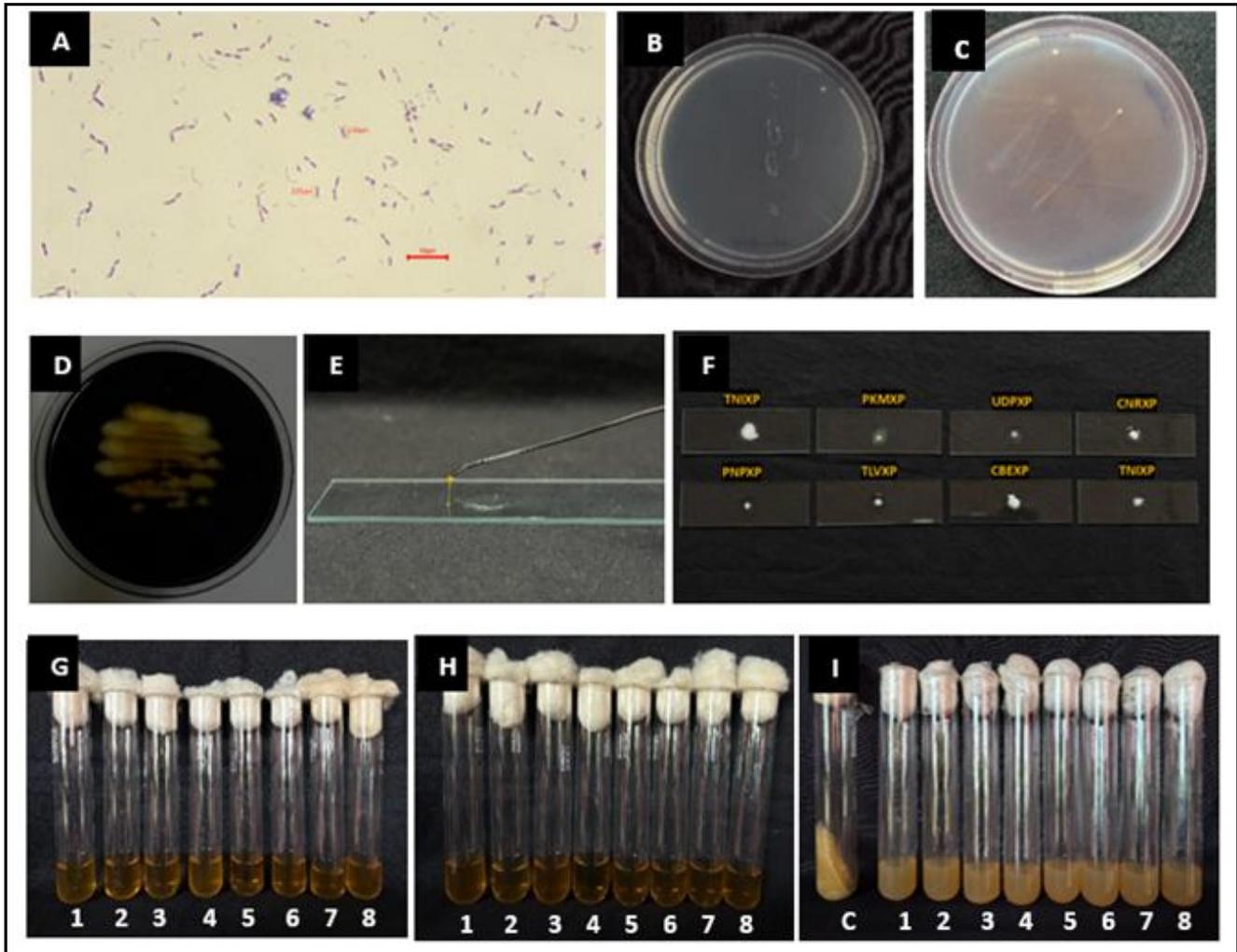
**Table 1: Survey conducted on different districts of Tamil Nadu with disease incidence**

S.No.	Location	District	Latitude and longitude	Disease incidence (%)
1.	TNAU orchard (CBEXP)	Coimbatore	11.0069° N, 76.9309° E	15
2.	Periyanaickenpalayam (PNPXP)	Coimbatore	11.1465° N, 76.9445° E	22
3.	Seeranaickenpalayam (SNPXP)	Coimbatore	11.0106° N, 76.9225° E	19
4.	Palarpatti (TNIXP)	Theni	9.9392° N, 77.4006° E	36
5.	Periyakulam (PKMXP)	Theni	10.1239° N, 77.5475° E	37
6.	Udumalaipet (UDPXP)	Tirupur	10.5855° N, 77.2513° E	34
7.	Thalavadi (TVLXP)	Erode	11.7782° N, 77.0052° E	29
8.	Coonoor (CNRXP)	Nilgiris	11.3439° N, 76.7945° E	13

**Table 2: Biochemical variations of *X. axonopodis* pv. *punicae* isolates**

S.No.	Biochemical tests	<i>X. axonopodis</i> pv. <i>punicae</i> isolates							
		CBEXP	PNPXP	SNPXP	TNIXP	PKMXP	UDPXP	CNRXP	TLVXP
1.	Gram staining	+	+	+	+	+	+	+	+
2.	Catalase test	+	+	+	+	+	+	+	+
3.	KOH test	+	+	+	+	+	+	+	+
4.	Asparagine test	-	-	-	-	-	-	-	-
5.	Starch hydrolysis	+	+	+	+	+	+	+	+
6.	Cellulase activity	+	+	+	+	+	+	+	+
7.	Methyl red test	-	-	-	-	-	-	-	-
8.	Voges proskauer test	-	-	-	-	-	-	-	-
9.	Gelatin liquefaction	+	+	+	+	+	+	+	+

Note: (+) positive; (-) negative

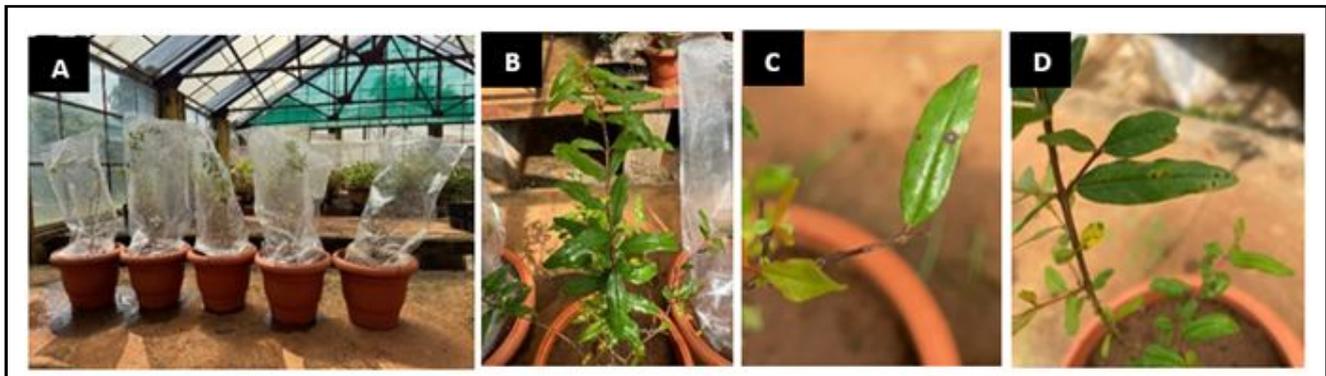


**Figure 1:** (A) Gram staining, (B) Asparagine test, (C) Cellulase activity, (D) Starch hydrolysis, (E) KOH test, (F) Catalase test, (G) Methyl red, (H) Voges-proskauer, (I) Gelatin liquefaction.

### 3.3 Pathogenicity test

Pomegranate plants inoculated with eight isolates exhibited the typical symptoms of bacterial leaf blight under glasshouse conditions. Observations were made from 6 to 19 days after inoculation. The

symptoms recorded as irregular, water soaked lesions on the leaves after 8 days of inoculation. On the 18<sup>th</sup> DAI, the spots are surrounded with yellow 'halos' on the abaxial side, followed by drooping of leaves (Figure 2). Using the single colony method, the pathogen was reisolated from infected leaves and compared to the original culture.



**Figure 2:** (A) Induction of humidity by covering polythene covers for 48 h, (B) Healthy plant, (C) Irregular water-soaked spots on 8<sup>th</sup> DAI, (D) Black spots with yellow 'halos' on 18<sup>th</sup> DAI, \*(DAI - days after inoculation).

### 3.4 Bacterial identification through 16S rRNA molecular confirmation

The PCR amplification reaction was optimized by varying the concentration of PCR components. Comparison of the 16S rRNA gene sequence (1500 bp) (Figure 4) with GenBank records confirmed the isolate's identity, showing over 99.0% similarity to *X. axonopodis* pv. *punicae* entries in the NCBI database. The 16S rRNA sequence of

the isolate was submitted to GenBank, assigned with accession number PV523272 and published. Promising bacterial antagonists; namely, UBL-7, UBL-8 and TBL-21 isolates also molecularly identified as *B. subtilis*, *B. amyloliquefaciens* and *B. velezensis* respectively and the isolate were submitted to GenBank, assigned with accession numbers PV616838, PV616839 and PV616840 (Figure 3).

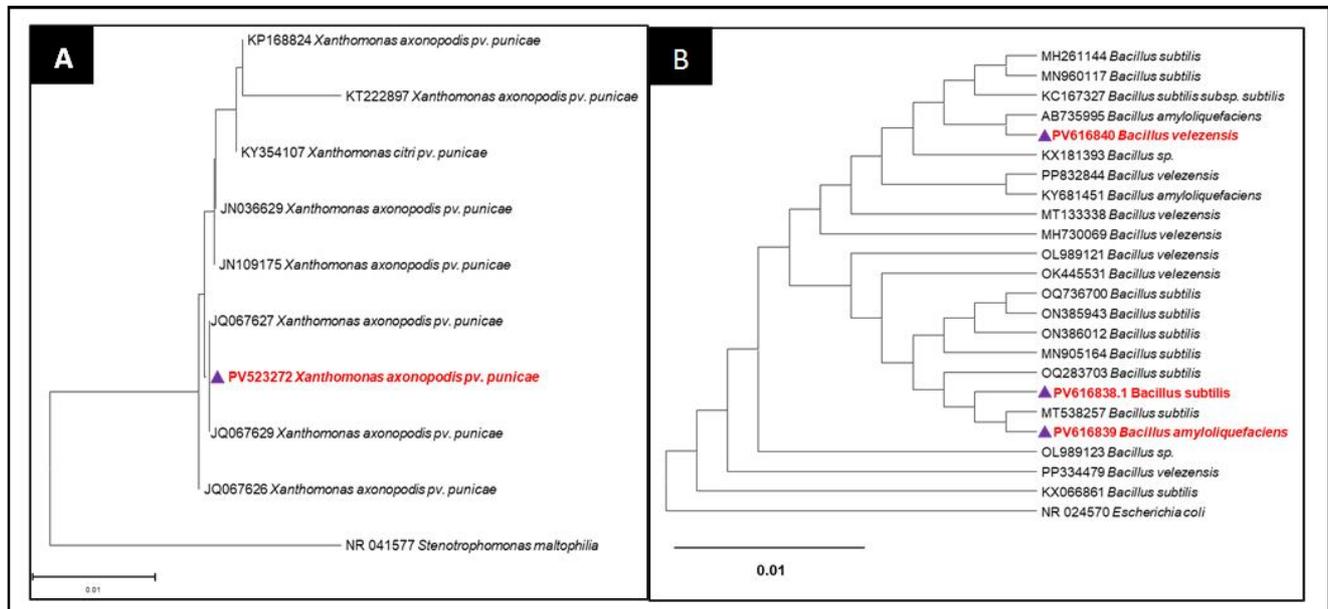


Figure 3: Phylogenetic analysis of (A) *X. axonopodis* pv. *punicae* and (B) *Bacillus* sp.

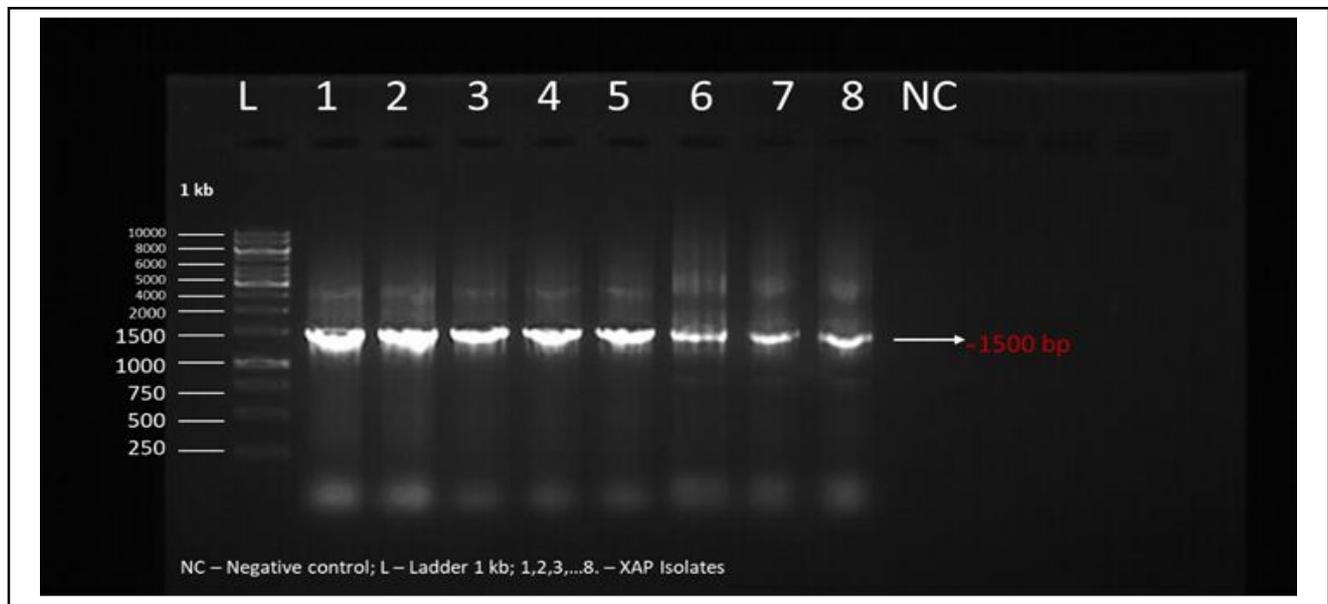


Figure 4: Molecular detection of *X. axonopodis* pv. *punicae* using 27F and 1492R primers.

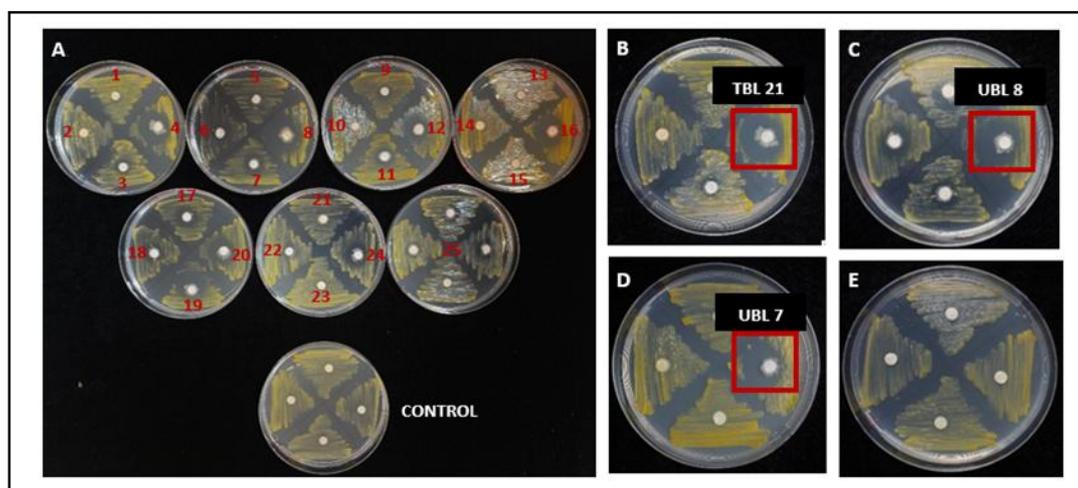
### 3.5 In vitro evaluation of bacterial antagonists against *X. axonopodis* pv. *punicae*

A total of 25 bacterial isolates were 16 isolates shown antagonistic property. Among them, three isolates; namely, UBL-8, TBL-21 and UBL-7 recorded maximum inhibition zones of 21.7 mm, 21.3 mm

and 18.7 mm against the pathogen *X. axonopodis* pv. *punicae* (Table 3 and Figure 5). These isolates were molecularly identified as *Bacillus* sp. and with the results obtained from the *in vitro* assay, it is evident that the bacterial endophytes isolated from the phyllosphere region (leaves) were more predominant in zones of inhibition when compared to the bacterial isolates obtained from the rhizosphere soil.

**Table 3:** *In vitro* screening of antagonistic bacteria against *X. axonopodis* pv. *punicae* pathogen

S.No	Isolate code	Source of isolation	Inhibition zone (mm)			Mean value	SD value
			R1	R2	R3		
1.	UBS1	Rhizosphere-Udumalaipettai	0	0	0	0.0	0.0
2.	UBS2		9	6	6	10.7	1.5
3.	UBS3		13	12	9	15.3	1.2
4.	UBS4		17	15	16	10.7	8.4
5.	UBS5		0	0	0	0.0	0.0
6.	UBL6	Leaf endophytes-Udumalaipettai	0	0	0	13.3	2.1
7.	UBL7		7	8	4	18.7	1.5
8.	UBL8		10	13	16	21.7	1.5
9.	UBL9		21	18	18	5.3	1.5
10.	UBL10		22	23	19	15.3	0.6
11.	CBL11	Leaf endophytes-Coimbatore	20	12	16	16.0	4.0
12.	CBL12		6	15	16	15.7	0.6
13.	CBL13		0	0	0	0.0	0.0
14.	CBL14		0	0	0	0.0	0.0
15.	CBR15	Root endophytes-Coimbatore	11	13	22	15.3	5.9
16.	CBR16		0	0	0	0.0	0.0
17.	PBL17	Root endophytes-Coimbatore	0	0	0	0.0	0.0
18.	PBL18		0	0	0	0.0	0.0
19.	PBR19	Root endophytes-Periyakulam	5	4	5	4.7	0.6
20.	PBR20		0	0	0	0.0	0.0
21.	TBL21	Leaf endophytes-Theni	21	23	20	21.3	1.5
22.	TBL22		15	11	13	13.0	2.0
23.	TBR23	Theni-leaf endophytes	15	14	15	14.7	0.6
24.	TBR24	Rhizosphere-Theni	0	0	0	0.0	0.0
25.	UBS25	Rhizosphere-Udumalaipettai	10	10	11	10.3	0.6

**Figure 5:** (A) *In vitro* screening of 25 bacterial antagonist against *X. axonopodis* pv. *punicae*, (B, C, D) maximum inhibition zone by isolate TBL 21, UBL 8, UBL 7(E) Control.

### 3.6 Identification of secondary metabolites produced by *B. amyloliquefaciens*

The GC-MS analysis is carried out for the promising endophytic bacterial isolate, which revealed that the ethyl acetate extract of the *B. amyloliquefaciens* contained a total of 40 compounds comprising various biologically active aliphatic (17) and aromatic compounds (23) (Figure 6). The three aromatic compounds; namely, N-acetyl-d-glucosamine, 1H-indole-3-acetic acid, 3-hydroxy-4-methoxybenzoic

acid showed the highest concentration (peak area %) of 6.103, 5.389 and 4.228, respectively, followed by other three aliphatic compounds; namely, pentenyl angelate, 2Z-, 3-methylbenzothiophene, 4-phosphonobutyric acid with peak area concentration of 18.29, 7.522 and 3.022, respectively (Figure 7). *B. amyloliquefaciens* (UBL 8) with peak bioactive compound pentenyl angelate, 2Z shows antibacterial property which aligns with the studies of Chen *et al.* (2010), demonstrating its role in disrupting bacterial cell walls and inhibiting pathogen growth.

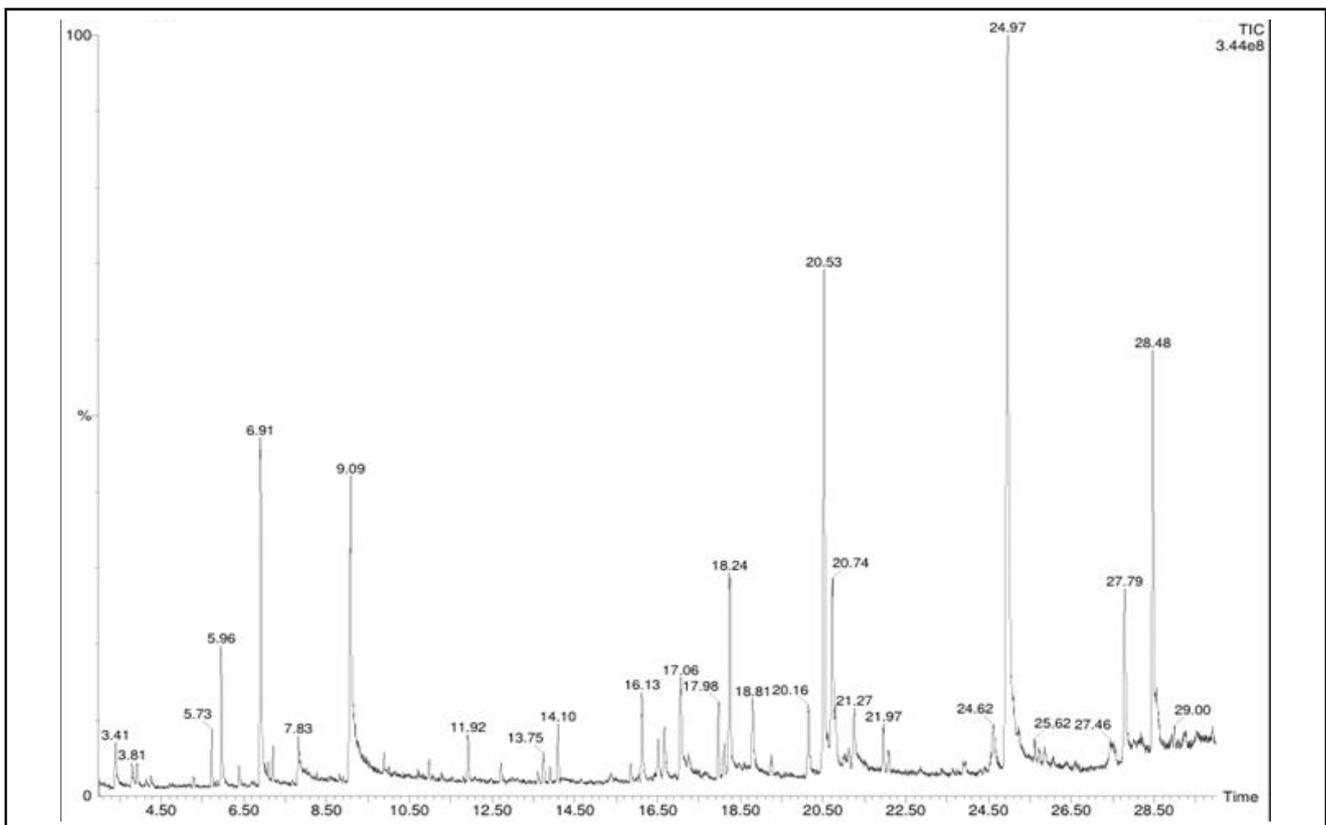


Figure 6: GC-MS fractionation of ethyl acetate extract of the culture filtrates of *B. amyloliquefaciens*.

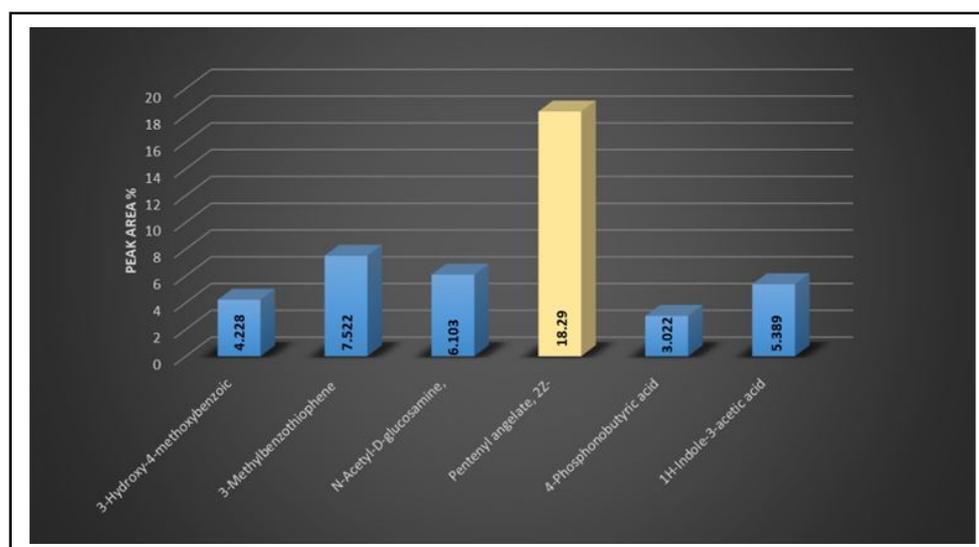


Figure 7: GC-MS bioactive compound of *B. amyloliquefaciens* with high peak area concentration.

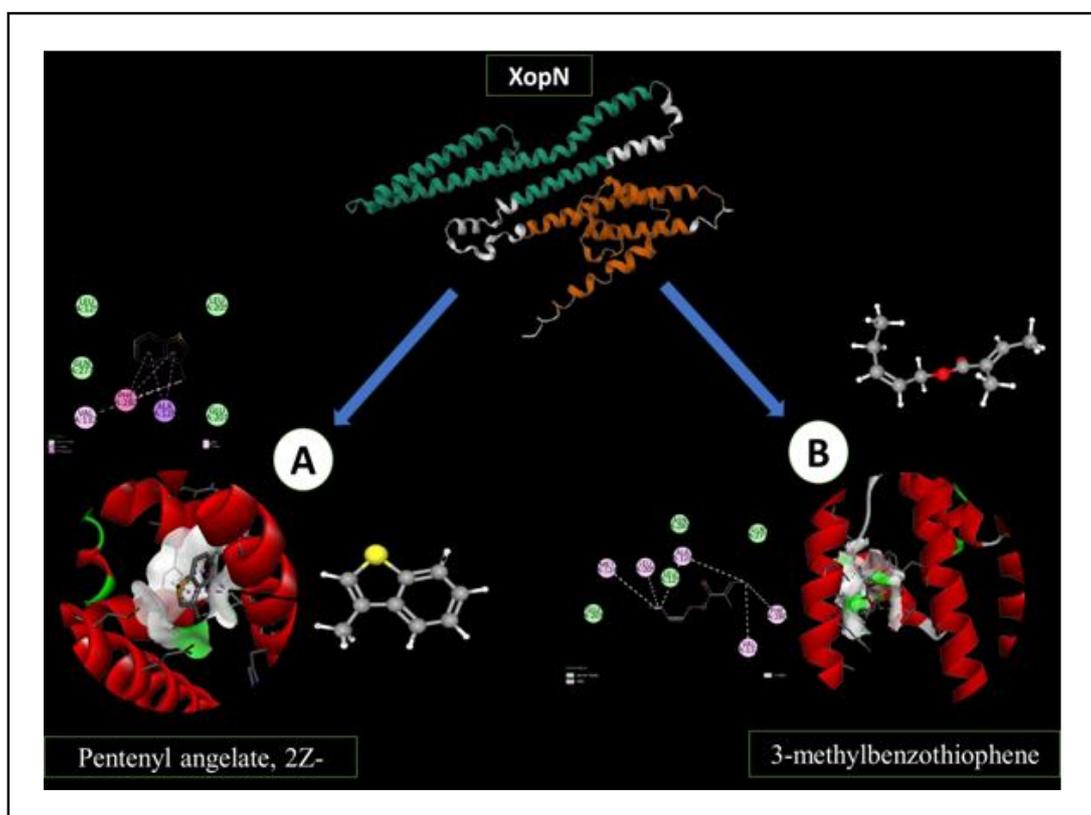
### 3.7 Molecular docking - sequence similarity analysis

Sequence similarity was performed using the BLASTP tool for the XopN outer protein as a query against the pomegranate genome proteins to see whether there are any comparable proteins in the pomegranate genome. No single hit or similar sequences were discovered during the similarity search. Thus, it was demonstrated that pentenyl angelate, 2Z, 3-methylbenzothiophene bound to the XopN protein targets selectively while avoiding the other pomegranate protein targets.

#### 3.7.1 Digital screening and molecular interaction analysis

XopN protein structure was docked with six bioactive compound to

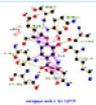
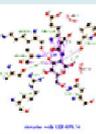
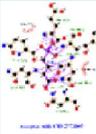
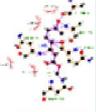
determine their binding mechanism. The binding affinity values of pentenyl angelate, 2Z, and 3-methylbenzothiophene with the XopN protein were -7.5 kcal/mol and -7.1 kcal/mol, which exhibited the highest binding affinity value measuring other biocompounds (Table 4 and Figure 8). The presence of hydrogen bonds in all the complexes demonstrated the strength and endurance of pentenyl angelate, 2Z, and 3-methylbenzothiophene's attraction for the XopN target. Hydrogen bonds are established between the side chain and backbone of binding site residues in the docked complexes. Other contact types such as, vander waals and hydrophobic interactions were also seen in the docked complex.



**Figure 8:** Molecular docking of XopN protein target with ligand molecules pentenyl angelate, 2Z and 3-methylbenzothiophen.

**Table 4:** PyRx energy values for bio-molecule compound with target protein and hydrogen bonds

S. No.	Compound	Ligand	Target	Binding energy	Interacting H- bonds	Binding site structure (2d)	Properties	References
1.	Pentenyl angelate, 2Z-	91692561	XopN	-7.5	Gln139, Ala140, Lys141, Gln142, Glu143, Ala144, Asn145, Asn146, Leu148.		Antibacterial, anti-inflammatory and antioxidant activities.	Guo <i>et al.</i> , 2020
2.	1H-indole-3-acetic acid, 5-hydroxy-	1826	XopN	-5.1	Arg80, Gln139, Ala140, Lys141, Gln142, Glu143, Asn145, Asn146, Lys147, Asn149		Antimicrobial activity	Jayaraman <i>et al.</i> , 2023

3.	3-hydroxy-4-methoxybenzoic acid	12575	XopN	-6.5	Ala140, Lys141, Gln142, Glu143, Ala144, Asn145, Asn146, Lys147, Asn149.		Antimicrobial activity	Farag <i>et al.</i> , 2015
4.	N-acetyl-D-glucosamine	439174	XopN	-6.2	Arg 80, Gln 139, Ala140, Lys141, Gln142, Glu143, Ala144, Asn149		Antibacterial property	Boulangier <i>et al.</i> , 2014
5.	4-phosphonobutyric acid	2773805	XopN	-5.6	Ala140, Gln142, Glu143, Ala144, Asn1454, Asn146, Lys147, Asn149		Plant growth regulation, Stress tolerance	Wilson <i>et al.</i> , 2023
6.	3-methylbenzothioephene	73817	XopN	-7.1	Gln139, Ala140, Lys141, Gln142, Ala144, Asn146, Lys147, Leu148		Antimicrobial property	Lai <i>et al.</i> , 2023

\*Gln-Glutamine; Ala-Alanine; Lys-Lysine; Asn- Asparagine; Leu-Leucine; Arg - Arginine; Glu-Glutamic acid,

#### 4. Discussion

In the present study, the pomegranate bacterial leaf blight pathogen is focused, which is a major threat over pomegranate growing farmers. The disease is prevalent throughout India in many states like Maharashtra, Gujarat, Karnataka, Rajasthan and Tamil Nadu. The survey conducted in pomegranate growing districts of Tamil Nadu, outcome with the results recorded higher disease incidence in Theni District. Observation correlates with the report of Mondal *et al.* (2013), indicating that warm and humid conditions favour the proliferation of *X. axonopodis* pv. *punicae*. In contrast, the lower disease incidence in Coonoor attributed to cooler temperature and lower humidity, which are less conducive to *X. axonopodis* pv. *punicae* infection (Hingorani and Mehta, 1953).

The cultural characterisation of XAP isolates varied from smooth to profuse texture, variation in size and shape, colour of the strain ranges from pale to typical yellow, these variations are similar with earlier studies on *Xanthomonas* sp., where colony morphology differences are linked to genetic diversity and environmental adaptations (Bradbury, 1984). The biochemical tests conducted in this study showed variation among the isolates in substrate utilization tests. Starch hydrolysis (+ve) stating the ability of producing higher quantity extracellular enzyme amylase by the XAP isolates responsible for hydrolyzing starch, as reported by Manjula (2002). The positive reaction of catalase, KOH solubility, and cellulase activity are consistent with its classification as a Gram-negative bacterium capable of producing extracellular enzymes. Catalase activity indicates the bacterial ability to decompose hydrogen peroxide, a common metabolic byproduct. The KOH test confirms its Gram-negative nature characterized by a thin peptidoglycan layer. Additionally, XAP produces cellulolytic enzymes such as endo- $\beta$ -1,4-glucanase and xylanase, which aid in the degradation of plant cell wall components, facilitating infection and colonization in pomegranate tissues as reported by Amat *et al.* (2014). These biochemical characteristics are supported by studies of Kale *et al.* (2012). The inability of XAP to grow on asparagine medium (-ve) is a key diagnostic feature distinguishing it from other yellow-pigmented bacteria like *Enterobacteriaceae* and *Pseudomonads* (Bradbury, 1984;

Vauterin *et al.*, 1995). The pathogenicity conducted in glasshouse conditions recorded symptoms emerging from 8 to 18 DAI, these symptoms support earlier description of XAP induced BLB in pomegranate (Gholami *et al.*, 2018; Hingorani and Mehta, 1953; Kishun, 1993).

*In vitro* study of the bacterial antagonist against *X. axonopodis* pv. *punicae*, outcome notably the phyllosphere isolates exhibited more pronounced antagonistic effects compared to those from the rhizosphere strains. Singh *et al.* (2022) isolated potential endophytic bacteria from pomegranate plants and identified them as *Bacillus*, *Burkholderia*, and *Lysinibacillus* species, which were significantly reduced the *in vitro* growth of XAP and decreased disease incidence and severity *in planta*. The report by Sharma *et al.* (2023). Singh *et al.* (2022) states *B. amyloliquefaciens* has been shown to induce systemic resistance in plants by activating defence-related enzymes such as peroxidases and phenylalanine ammonia-lyase, contributing to enhanced plant immunity and produces a range of antimicrobial compounds, including lipopeptides and bacteriocins, which inhibit the growth of bacterial blight and various phytopathogens (Yuvarani *et al.*, 2024). These findings underscore the potential of utilizing *B. amyloliquefaciens* as an effective biocontrol agent for managing bacterial leaf blight in pomegranate.

XopN is a type III effector protein of *Xanthomonas*, known for suppressing plant immunity, especially PAMP-triggered immunity (PTI), thereby facilitating bacterial colonization and disease progression reported by Mo *et al.* (2020). Studies by Mondal *et al.* (2020) have shown that deletion of XopN from XAP results in reduced bacterial growth and diminished virulence, as evidenced by smaller water-soaked lesions and lower bacterial populations in infected leaves. Targeting the protein with the peak concentration of GC-MS compound of *B. amyloliquefaciens* and the binding affinity revealed that the ability of *Bacillus* strain to suppress the XAP pathogen. These findings are consistent with studies on biocontrol agents, where phyllosphere bacteria like *Bacillus* and *Pseudomonas* species have shown promise in suppressing *Xanthomonas* infections (Muthukumar *et al.*, 2017). The identification of effective antagonists opens avenues for developing eco-friendly biocontrol strategies

reducing reliance on chemical pesticides, which can have adverse environmental impacts (Kumar *et al.*, 2021).

## 5. Conclusion

The study identified *B. amyloliquefaciens* as the most potent bacterial antagonist against *X. axonopodis* pv. *punicae*, the causative agent of bacterial leaf blight in pomegranate. *In vitro* assays revealed significant inhibition zones underscoring its strong antagonistic activity. GC-MS profiling of UBL-8's culture filtrate revealed 40 bioactive compounds including pentenyl angelate and 3-methylbenzothiophene with notable antimicrobial properties. Molecular docking of these compounds with the XopN virulence protein demonstrated high binding affinities, suggesting selective and stable interaction while avoiding host targets. These findings highlight the potential of UBL-8 and its metabolites as eco-friendly biocontrol agents offering a sustainable alternative to chemical pesticides for managing bacterial leaf blight in pomegranate. The integrated approach combining microbiological, chemical and *in silico* methods reinforces the feasibility of using native endophytes in developing effective, targeted disease management strategies.

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## Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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