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Characterization of *Pseudomonas mediterranea* and *Pseudomonas viridiflava* strains associated with tomato pith necrosis in the Al Taif region, Saudi Arabia

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Tomato pith necrosis is a damaging disease affecting greenhouse and field-grown tomato production globally, often associated with environmental stress and excessive nitrogen fertilization. This study investigates the bacterial etiology and the (inter)national context of disease outbreaks during the 2023–2024 growing seasons in Al Taif, Saudi Arabia (SA). Sixteen bacterial isolates were recovered from symptomatic plants, and phenotypic and molecular analyses identified *Pseudomonas mediterranea* (n = 12) and *Pseudomonas viridiflava* (n = 4) as the causal agents, although *Pseudomonas corrugata* and *Pseudomonas fluorescens* biotype I have also been reported from SA in the past. *P. mediterranea* was the most prevalent species, consistent with earlier findings in southern Europe. Genetic characterization using multilocus sequence typing and minimum spanning tree analysis revealed high genetic diversity in *P. viridiflava*, including a novel singleton lineage, indicating potential local adaptation, and all SA strains belonged to *Pseudomonas syringae* (sensu *latu*) phylogroup 7a. In contrast, *P. mediterranea* isolates exhibited lower genetic diversity, but six novel sequence types (ST8–ST13) were identified exclusively in SA strains, forming a distinct phylogenetic clade. Pathogenicity assays showed that *P. viridiflava* caused severe necrosis, while *P. mediterranea* caused internal browning without wilting, suggesting a difference in virulence. The co-occurrence of both species in the same greenhouses highlights the complexity of disease dynamics and potential interactions between these pathogens. Given the global relevance of *P. syringae* phylogroup 7a, mainly formed by *P. viridiflava* strains, and the risk of pathogen spread via plant trade, this study underscores the importance of stringent phytosanitary controls and accurate diagnostics tailored to regional pathogen variants.

Keywords Genetic variation, Greenhouse, Multi-locus sequence typing, Epidemiology

Tomato (*Solanum lycopersicum* L.) cultivation in Saudi Arabia is particularly significant, especially under controlled greenhouse conditions. In 2023, greenhouse vegetable production in Saudi Arabia reached 721,000 metric tons, with tomato accounting for 36.7% of the total greenhouse area and yielding approximately 300,000 tons[1].

Bacterial pith necrosis affects tomato across various regions worldwide (see Supplementary Table 1, S1) and occasionally other crops, such as pepper (*Capsicum annuum*), chrysanthemum (*Chrysanthemum morifolium*), geranium (*Geranium* spp.), and eggplant (*Solanum melongena*). This disease can occur at any developmental stage and in various cultivation systems, including greenhouses, open fields, and hydroponic farming[2,3], causing severe wilting and collapse of plants. Brown necrotic patches develop on the stem, accompanied by internal pith necrosis, soft rot, and browning of the vascular tissues, leading to plant collapse of the plant[4]. It typically develops in environments characterized by cool temperatures, high humidity, and limited sunlight, especially in plants subjected to excessive nitrogen fertilization[2].

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The disease was first observed in New Zealand, where it was associated with *Pseudomonas cichorii*[5], and shortly thereafter in the United Kingdom (UK), where it was linked to *Pseudomonas corrugata*[4], the pathogen most frequently implicated. Since then, outbreaks have been documented in several other countries (Table S1). Other pathogens involved either as a sole agent or in combination include *Pseudomonas marginalis, Pseudomonas mediterranea*, and *Pseudomonas viridiflava*, while *Pseudomonas fluorescens* biotype I and *Pseudomonas putida* have also been associated with pith necrosis, although they typically cause much milder symptoms. In Italy, *Xanthomonas perforans* has also been identified as a causal agent[6]. *P. corrugata*, *P. mediterranea,* and *P. viridiflava* can cause extensive pith necrosis, whereas the other pathogens mainly cause chlorosis and internal stem discoloration with little or no pith rot[7].

The main species worldwide associated with pith necrosis belong to two major groups: *P. corrugata* and *P. mediterranea* are members of the oxidase-positive *P. fluorescens* complex, mainly comprising saprophytic and/or weakly plant pathogenic species[8,9]. *P. viridiflava*, belongs to the oxidase-negative *Pseudomonas syringae* complex[10,11], which includes important and virulent plant pathogens that may also occur as harmless epi- or endophytes[12–14].

Tomato pith necrosis (TPN) is one of the most serious diseases affecting tomato in Saudi Arabia, and earlier studies identified *P. fluorescens* (biotype I) and *P. corrugata* as the primary pathogens[15,16]. Mixed infections involving multiple pathogens occur and can significantly exacerbate severity of TPN[3,17–19].

Traditionally, species of the genus *Pseudomonas* have been differentiated using standard biochemical schemes such as the LOPAT profile[20,21], which provides a preliminary framework for taxonomic identification before molecular confirmation.

Advancements in molecular tools such as (real-time) PCR, multilocus sequence typing (MLST) and whole-genome sequencing have become essential for taxonomic classification, phylogenetic analysis, and epidemiological tracking of plant-associated bacteria[22,23]. MLST, in particular, provides high-resolution bacterial typing by sequencing multiple housekeeping genes, generating a detailed genetic fingerprint for precise strain differentiation[24–26]. This technique has been successfully applied to various phytopathogens, including members of the *P. syringae* complex[10,27,28]. In the present study, we focused on the molecular characterization of *Pseudomonas* spp. associated with TPN symptoms in Saudi Arabia. Our objectives were to identify the *Pseudomonas* species responsible for the disease using phenotypic, biochemical, and pathogenicity tests, to assess the genetic diversity of *Pseudomonas* strains, and to analyse their phylogenetic relationships using MLST with five housekeeping genes. This report is the first to identify *P. mediterranea* and *P. viridiflava* as causal agents of TPN in the Al Taif region, Saudi Arabia, deepening our understanding of the etiology and epidemiology of this disease and its possible control.

Materials and methods

Sampling, isolation, and purification of bacteria

Surveys were conducted from March to June during the 2023–2024 growing seasons to determine the incidence of TPN disease and the identity of the bacterial pathogens causing pith necrosis in tomato grown in commercial greenhouses across various locations in the Al Taif region of Saudi Arabia. All diseased tomato plants that exhibited typical symptoms of stem and pith necrosis (Fig. 1) were collected during March (10)–June (12), and the average disease incidence for each location was calculated. A small piece of infected pith was soaked in 0.6% sodium hypochlorite for 1 min, placed in a sterile mortar, and macerated in sterile distilled water (SDW) with a pestle. Loopfuls of the suspension were streaked on plates of nutrient agar containing 5% D+ glucose (NGA) and King's medium B (KB) plates[29]. The plates were incubated at 28 °C and examined after 48 h. Pure cultures were obtained from these colonies[20] and stored at − 20 °C in 20% glycerol.

Fig. 1. Symptoms of pith necrosis observed in tomato plants (natural infection): (**A**) Early external symptoms, showing longitudinal brown streaks and stem cracking, (**B**) progression of symptoms with severe stem collapse and darkened necrotic tissues, and (**C**) internal stem sections displaying characteristic pith degradation, hollowing, browning, and complete necrosis of internal tissues.

Biochemical identification

All 16 isolates and reference strains of *P. mediterranea* (CFBP5447T) and *P. viridiflava* (PV273 and CFBP2107T) were characterized using a set of biochemical and physiological tests, following a previously established protocol[30]. These assays included Gram staining, fluorescence on King's B, levan production, oxidase activity, pectolytic activity, arginine dihydrolase activity, nitrate reduction, gelatine and lecithin hydrolysis, poly-β-hydroxybutyrate accumulation, and the ability of isolates to grow at 37 °C after 48 h in nutrient broth (NB; Difco). The pectolytic activity was tested on potato tuber slices. Each test was performed in duplicate.

Hypersensitivity and pathogenicity tests

Tobacco leaves (*Nicotiana tabacum*) were infiltrated with a 24-h-old bacterial suspension (10^8 CFU ml^{-1}) in SDW, using the procedure described by[20]. Necrosis that developed 24–48 h after infiltration was recorded as a positive hypersensitivity reaction. A pathogenicity test was carried out on 6-week-old tomato seedlings cv. Sun Gold grown in 20-cm pots containing sterile peat. The inoculation was performed on the axil of the first true leaf, using a hypodermic syringe with 50 µl inoculum/plant (two injections for each plant). Five tomato plants were used for each isolate. Briefly, the inoculum was grown for 24 h at 28 °C on King's B medium and suspended at a concentration of 10^8 cells/ml in SDW. SDW was used as a negative control[31]. After inoculation, tomato plants were covered with plastic bags for 48 h and then maintained at 70% relative humidity and 25–28 °C for 48 h under a 16 h photoperiod. After two days, the cover was removed, and the plants were grown under greenhouse conditions. Disease symptoms were evaluated, and plants were sectioned to determine the presence of pith necrosis and vascular discoloration 30 days post-inoculation. Bacteria were re-isolated and characterized morphologically, physiologically, and biochemically to fulfil Koch's postulates.

DNA extraction

Genomic DNA was extracted from bacterial cultures grown on NGA for 24 h at 28 °C. Cells were harvested with a sterile loop and suspended in 500 µl of extraction buffer (2% CTAB, 100 mM Tris–HCl pH 8.0, 25 mM EDTA, 1.4 M NaCl, 1% polyvinylpyrrolidone, 2% β-mercaptoethanol followed by an hour incubation at room temperature and five minutes centrifugation at 10,000 g. The clear supernatant was transferred to a new microcentrifuge tube and the aqueous phase was purified and extracted using an equal volume of chloroform-isoamyl alcohol (24:1)[32]. The DNA was precipitated with isopropyl alcohol, washed with 70% ethyl alcohol, resuspended in 75 µl of nuclease-free water and stored at – 80 °C until use.

PCR amplification and sequencing

Partial sequences of five housekeeping genes: *rpoB* (encoding RNA polymerase beta subunit), *rpoD* (encoding sigma factor 70), *cts* (encoding citrate synthase), *pfk* (encoding phosphofructokinase), and *gyrB* (encoding gyrase)[25] were amplified and sequenced. The PCR reactions were performed in a 25 µl volumes containing 2 µl of DNA template (50 ng); 0.2 µM of each primer (Table 1); 12.5 µl of 2 × ready mix PCR reaction mixture amaR OnePCR ™ (Simply Biologics, San Diego CA, USA). PCR reactions were run on a Nexus Gradient Master Cycler [Eppendorf, Hamburg, DE] using the following profile: initial denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 2 min, annealing temperature at primer-specific temperatures (Table 1) for 1 min, and extension at 72 °C for 1 min.

MLST was carried out by concatenating the sequences of the five housekeeping genes (*rpoB, rpoD, cts, pfk*, and *gyrB*) into a 3545 bp alignment for 16 local isolates plus 49 reference sequences retrieved from GenBank representing *P. mediterranea, P. viridiflava, P. syringae, P. corrugata*, and *P. cichorii*) (Table S2).

Sequence types (STs) were defined based on unique allelic profiles across the analyzed loci following established MLST methodology[24]. Each distinct allele at a locus was assigned a number, and isolates sharing identical allele combinations were grouped into the same ST. Novel allelic combinations not previously reported were designated as new STs. Allele designations and reference profiles were retrieved from the Plant-Associated and Environmental Microbes Database (PAEMD) (http://genome.oows.vt.edu) (Table S3).

Genes	Primer (5′–3′)		Annealing temperature (°C)	References
gyrB	Fwd	MGGCGGYAAGTTCGATGACAAYTC	63	25
	Rev	TRATVKCAGTCARACCTTCRCGSGC		
Pfk	Fwd	ACCMTGAACCCKGCGCTGGA	63	
	Rev	ATRCCGAAVCCGAHCTGGGT		
rpoD	Fwd	AAGGCGARATCGAAATCGCCAAGCG	63	
	Rev	GGAACWKGCGCAGGAAGTCGGCACG		
rpoB (*laps*)	Fwd	CGGCTTCGTCCAGCTTGTTCAG	56	35
	Rev	TGGCCGAGAACCAGTTCCGCGT		
Cts	Fwd	AGTTGATCATCGAGGGCGCWGCC	56	25
	Rev	TGATCGGTTTGATCTCGCACGG		

Table 1. List of primers used in this study.

Minimum spanning tree (MST) analysis

To assess genetic relations among identified STs, minimum spanning trees (MSTs) were generated using BIONUMERICS v7.6 (Applied Maths, bioMérieux, France). MSTs based on allelic profiles to infer evolutionary connectivity and visualize clustering patterns. Separate MSTs were constructed for *P. mediterranea* (five genes) and *P. viridiflava* (three genes), with Saudi isolates highlighted to illustrate regional clustering patterns.

DNA polymorphism and neutrality tests

Sequences were aligned using CLUSTAL W, Bioedit[33]. To assess within-species genetic diversity, DNA polymorphism parameters including the number of segregating sites (S), haplotype diversity (Hd), and nucleotide diversity (π) were estimated using DnaSP v6.12.03[34]. Neutrality was evaluated using Tajima's D, Fu and Li's D*, and Fu and Li's F* statistics to detect departures from neutral evolution. These analyses were performed separately for *P. mediterranea* and *P. viridiflava*.

Results

Incidence, symptomatology, and phenotypic characterization of Saudi isolates associated with TPN in Al Taif, Saudi Arabia

Based on observable external symptoms, the average disease incidence attributed to *Pseudomonas* species was estimated at approximately 20–25% across two hectares representing ten nurseries/production entities. Affected plants exhibited yellowing and wilting of the lower leaves, irregular dark brown to black lesions of varying sizes on the stem, and internal browning of the pith, consistent with the symptoms described in the Materials and Methods section (Fig. 1). These symptoms were most frequently observed in tomato plants grown under glasshouse conditions in the Al Taif region of Saudi Arabia. In total, sixteen isolates were recovered from symptomatic stems showing characteristic pith necrosis.

Based on colony morphology and fluorescence on King's B medium, all these 16 isolates formed smooth, circular, creamy-white to light-yellow colonies and were initially suspected and later confirmed to belong to the genus *Pseudomonas*. Subsequent physiological, biochemical, and molecular characterization confirmed this preliminary identification (see below and Table 2, Figs. 3 and 4). All 16 isolates were first physiologically and biochemically characterized and compared with reference strains *P. mediterranea* (CFBP5447T) and *P. viridiflava* (PV273 and CFBP2107T) (Table 2). All isolates were Gram-negative and catalase-positive. Twelve isolates (Ps_SA_1.1 to Ps_SA_1.4, and Ps_SA_8.1 to Ps_SA_8.8) showed consistent oxidase positivity and gelatine hydrolysis, but lacked fluorescence on King's B medium and did not produce levan. All 12 isolates exhibited arginine dihydrolase activity, grew at 37 °C, and were non-pectolytic, aligning with the profile of *P. mediterranea* CFBP5447T (Table 2). In contrast, four isolates (Ps_SA_4.1 to Ps_SA_4.4) displayed a distinctly different phenotype. They were oxidase-negative, hydrolysed gelatine, induced potato rot and lacked arginine dihydrolase activity. This combination of traits matched those of the reference strains of *P. viridiflava* PV273 and CFBP2107T. The observed phenotypic profiles support their identification as *P. viridiflava*-like strains.

Pathogenicity test

Tomato plants were inoculated with the 16 *Pseudomonas* spp. isolates from Saudi Arabia, representing both fluorescent and non-fluorescent groups. Within 15 days, plants inoculated with isolates identified as *P. viridiflava* developed characteristic symptoms of pith necrosis, including wilting and brown discoloration of the pith tissue, which progressed to plant death at advanced stages of disease. Necrotic lesions on stems extended approximately 1.5 cm above and below the inoculation site (Fig. 2A–C). In contrast, plants inoculated with isolates identified as *P. mediterranea* showed no wilting, even at 45 days post-inoculation. Longitudinal stem sections revealed only

	Isolated strains		*Pseudomonas* spp. reference strains	
Biochemical test	**Group I**	**Group II**	***P. mediterranea* (CFBP5447T)**	***P. viridiflava* (PV273 and CFBP2107^T)**
Number of isolates	12	4	1	1
Gram reaction	–	–	–	–
Fluorescence on King's B	–	+	–	+
Levan production	–	–	–	–
Oxidase reaction	+	–	+	–
Pectolytic activity (potato soft rot)	–	+	–	+
Arginine dihydrolase	+	–	+	–
Tobacco hypersensitivity	+	+	+	+
Growth at 37 °C	+	+	+	+
Nitrate reduction	+	+	+	+
Lecithinase	–	+	–	+
Gelatine hydrolysis	+	+	+	+
Poly-β-hydroxybutyrate accumulation	+	+	+	+

Table 2. Physiological and biochemical characteristics of *Pseudomonas* spp. isolates from Saudi Arabia compared with reference strains of *P. mediterranea* (CFBP 5447^T) and *P. viridiflava* (PV273 and CFBP 2107^T).

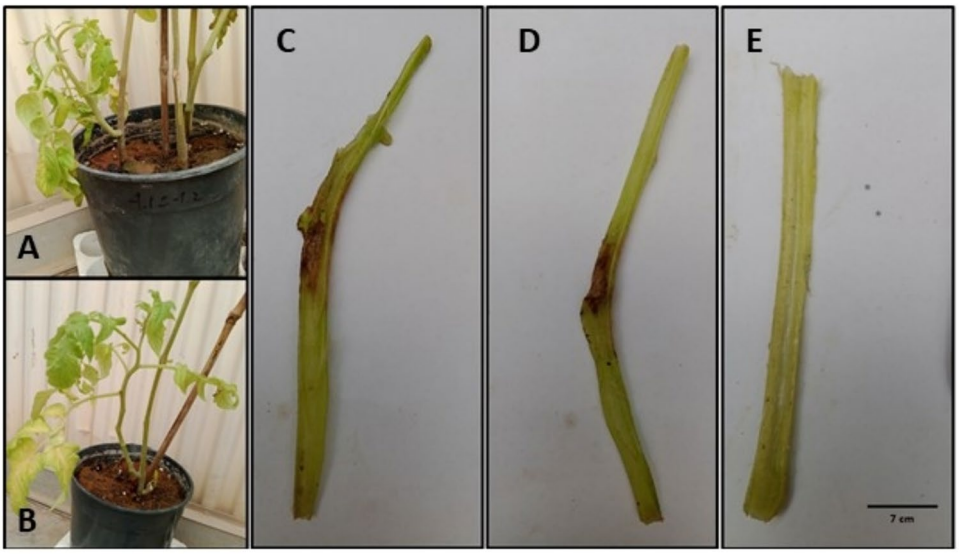

Fig. 2. Symptoms of pith necrosis on *Solanum lycopersicum* cv. Sun Gold following artificial inoculation with *Pseudomonas* isolates obtained from naturally infected plants. (**A**) Wilting of leaves and (**B**) chlorosis of basal foliage. (**C**) Longitudinal stem section showing internal pith necrosis caused by *P. viridiflava* isolate SA 4.1; (**D**) pith browning caused by *P. mediterranea* isolate SA 8.8; and (**E**) healthy stem tissue of a control plant, inoculated with sterile distilled water.

Species	Number of sequences	Length (bp)	M	P	S	P	h	Hd	Pi	T*	Fu and Li's	
											D test **	*F test* ***
P. viridiflava	88	3548	2876	666	88	578	58	0.98	0.023	−1.61	0.5	−0.5
P. mediterranea	23	3546	3517	28	6	22	13	0.94	0.002	−0.55	0.41	0.13

Table 3. Nucleotide polymorphism analysis of *Pseudomonas viridiflava* and *Pseudomonas mediterranea* isolates from Saudi Arabia, along with selected global strains retrieved from the NCBI GenBank complete genome database. The study was based on a concatenated sequence of five housekeeping genes: *rpoB, rpoD, cts, pfk*, and *gyrB*. M: monomorphic site; P: Polymorphic site; S: singleton site; P: Parsimony site; h: Haplotype; Hd: haplotype diversity; Pi: Nucleotide diversity; T: Tajima's D test; *P. viridiflava* (*): $0.10 > P > 0.05$; (**): $P > 0.10$; (***): $P > 0.10$; *P. mediterranea* (*): $P > 0.1$; (**): $P > 0.1$; (***): $P > 0.1$.

internal browning of the pith extending up to 0.9 cm at 21 days post-inoculation (Fig. 2D). However, healthy stem tissue was observed in tomato plants inoculated with SDW, serving as a negative control (Fig. 2E).

Nucleotide polymorphism analysis

Analysis of concatenated sequences of five housekeeping genes (*rpoB*, *rpoD*, *cts*, *pfk*, and *gyrB*) revealed significant differences in genetic variation between *P. viridiflava* and *P. mediterranea* (Table 3). For *P. viridiflava* (88 sequences, 3548 bp), 2876 monomorphic and 666 polymorphic sites were identified, including 88 singletons and 578 parsimony-informative sites. The population exhibited high haplotype diversity (Hd = 0.98) and moderate nucleotide diversity ($\pi = 0.022$), reflecting substantial sequence variation. Neutrality tests showed a slightly negative Tajima's D (−1.61; $0.10 > P > 0.05$) and non-significant Fu and Li's D and F statistics ($P > 0.10$), suggesting neutral evolution with a possible weak signal of population expansion or purifying selection. In contrast, *P. mediterranea* (23 sequences, 3546 bp) displayed markedly lower variability, with 3517 monomorphic and only 28 polymorphic sites. Despite high haplotype diversity (Hd = 0.94), nucleotide diversity was low ($\pi = 0.002$), indicating overall genomic conservation. The non-significant ($P > 0.10$) neutrality tests (Tajima's D = −0.55; Fu and Li's D = 0.41; Fu and Li's F = 0.13) for this species were consistent with a stable population structure and absence of recent selective pressures. These results demonstrated that *P. viridiflava* populations are genetically more diverse, while *P. mediterranea* represents a more conserved lineage.

Multilocus sequence typing (MLST) of *Pseudomonas mediterranea*

MLST analysis of *P. mediterranea* was conducted using sequences from 23 isolates, comprising 12 Saudi isolates and 11 reference strains obtained from the NCBI database (Table 4). A total of 22 alleles were identified across the five housekeeping genes analysed—*rpoB* (4 alleles), *rpoD* (1 allele), *cts* (3 alleles), *pfk* (7 alleles), and *gyrB* (7 alleles). Twelve (12) distinct sequence types (STs) were defined, represented in Table 4. Six sequence types were found exclusively among the Saudi isolates: ST8 (2 isolates), ST9 (3 isolates), ST10 (1 isolate), ST11 (1 isolate),

Sequence type	Isolate/strain	Country	Isolation source	Allele					Source
				rpoB	*rpoD*	*cts*	*pfk*	*gyrB*	
1	DSM 16733	Italy	Infected tomato	1	1	1	1	1	NCBI
1	CFPB 5447	Italy	Infected tomato	1	1	1	1	1	
2	EDOX	Netherlands	Tomato endophyte	4	1	1	1	2	
3	PVCT 3C	Italy	Tomato	2	1	1	1	3	
3	TEIC1105	Greece	Infected tomato	2	1	1	1	3	
3	CFBP5444	Italy	Infected tomato	2	1	1	1	3	
3	CFBP5404	Spain	Infected pepper	2	1	1	1	3	
4	B21-060	Canada	Soil	1	1	1	1	6	
5	TEIC1022	Greece	Infected tomato	4	1	1	1	5	
6	S 58	China	Tobacco rhizosphere	2	1	2	1	4	
7	Bi112	UK	Soil rhizosphere	3	1	1	2	3	
8	Ps_SA_8.5	Saudi Arabia	Infected tomato	4	1	3	1	7	
8	Ps_SA_8.6	Saudi Arabia	Infected tomato	4	1	3	1	7	
9	Ps_SA_8.1	Saudi Arabia	Infected tomato	4	1	3	7	7	
9	Ps_SA_8.3	Saudi Arabia	Infected tomato	4	1	3	7	7	
9	Ps_SA_8.4	Saudi Arabia	Infected tomato	4	1	3	7	7	
10	Ps_SA_8.2	Saudi Arabia	Infected tomato	4	1	3	3	7	
11	Ps_SA_1.4	Saudi Arabia	Infected tomato	4	1	3	6	7	
12	Ps_SA_1.1	Saudi Arabia	Infected tomato	4	1	3	5	7	
12	Ps_SA_1.2	Saudi Arabia	Infected tomato	4	1	3	5	7	
12	Ps_SA_1.3	Saudi Arabia	Infected tomato	4	1	3	5	7	
13	Ps_SA_8.7	Saudi Arabia	Infected tomato	4	1	3	4	7	
13	Ps_SA_8.8	Saudi Arabia	Infected tomato	4	1	3	4	7	
Number of alleles				4	1	3	7	7	

Table 4. Multilocus sequence typing (MLST) data for *Pseudomonas mediterranea* isolates from Saudi Arabia and reference strains from global sources. Sequence types were determined based on allelic profiles of five housekeeping genes: *rpoB, rpoD, cts, pfk*, and *gyrB* identified from 23 isolates/strains.

ST12 (3 isolates), and ST13 (2 isolates). Notably, ST9 was the most common among Saudi strains, with three isolates sharing an identical allelic profile.

Multilocus sequence typing (MLST) of *Pseudomonas viridiflava*

MLST analysis of 120 *P. viridiflava* isolates (4 Saudi and 116 global) revealed extensive allelic diversity, comprising 31 *cts*, 46 *gyrB*, and 54 *rpoD* alleles, defining 82 distinct STs (Supplementary Table S5). The Saudi isolates (Ps_SA_4.1–Ps_SA_4.4) all belonged to ST60, which displayed a novel allelic profile not reported in current MLST databases, indicating a potentially new lineage. ST60 was closely related to isolates from Switzerland and the USA by sharing *cts* and *gyrB* alleles while differing in *rpoD*, which suggests localized diversification. Globally, isolates from France, Italy, and New Zealand accounted for the majority of STs, consistent with the ecological versatility of *P. viridiflava*. Several STs (e.g., ST3, ST33, ST35, ST41, ST42, ST75) contained isolates from multiple countries and hosts, indicating widespread distribution and possible clonal expansion.

Phylogenetic analysis of *Pseudomonas viridiflava* and *Pseudomonas mediterranea*

MLSA-based phylogenetic analysis of concatenated gene sequences from 194 representative strains revealed distinct and well-supported clades (bootstrap = 100%) for both species (Fig. 3). The Saudi *P. viridiflava* isolates formed a monophyletic clade with strains from the USA, France, and New Zealand, closely related to isolates Pv98_04 (USA) and P12_DOA (New Zealand), but distinct from isolates from Chile, Turkey, and Switzerland. Similarly, Saudi *P. mediterranea* isolates clustered tightly with reference strains from Italy, Spain, Greece, and the UK, forming four subclusters that reflected intra-regional genetic diversity. The separation of the two species into strongly supported clade highlights their clear genetic distinction and underlying diversity.

Phylogenetic grouping of *Pseudomonas viridiflava*

A multilocus sequence analysis -based phylogenetic tree was constructed using concatenated gene sequences from 183 *P. viridiflava* and *P. syringae* complex globally distributed reference strains, and four newly sequenced isolates from Saudi Arabia. The Maximum Likelihood (ML) analysis revealed significant phylogenetic diversity and delineated 13 well-supported phylogroups (PGs), consistent with previously established clades (Fig. 4). All four Saudi isolates Ps_SA_4.1 to Ps_SA_4.4 clustered within Phylogroup 7a (PG 7a). These isolates formed a distinct monophyletic sub-cluster supported by a 100% bootstrap value, suggesting a shared evolutionary origin and potential local adaptation or recent diversification. PG 7a also included strains from diverse geographical regions such as France, Italy, Turkey, the USA, Switzerland, Chile, and China, reflecting its broad distribution

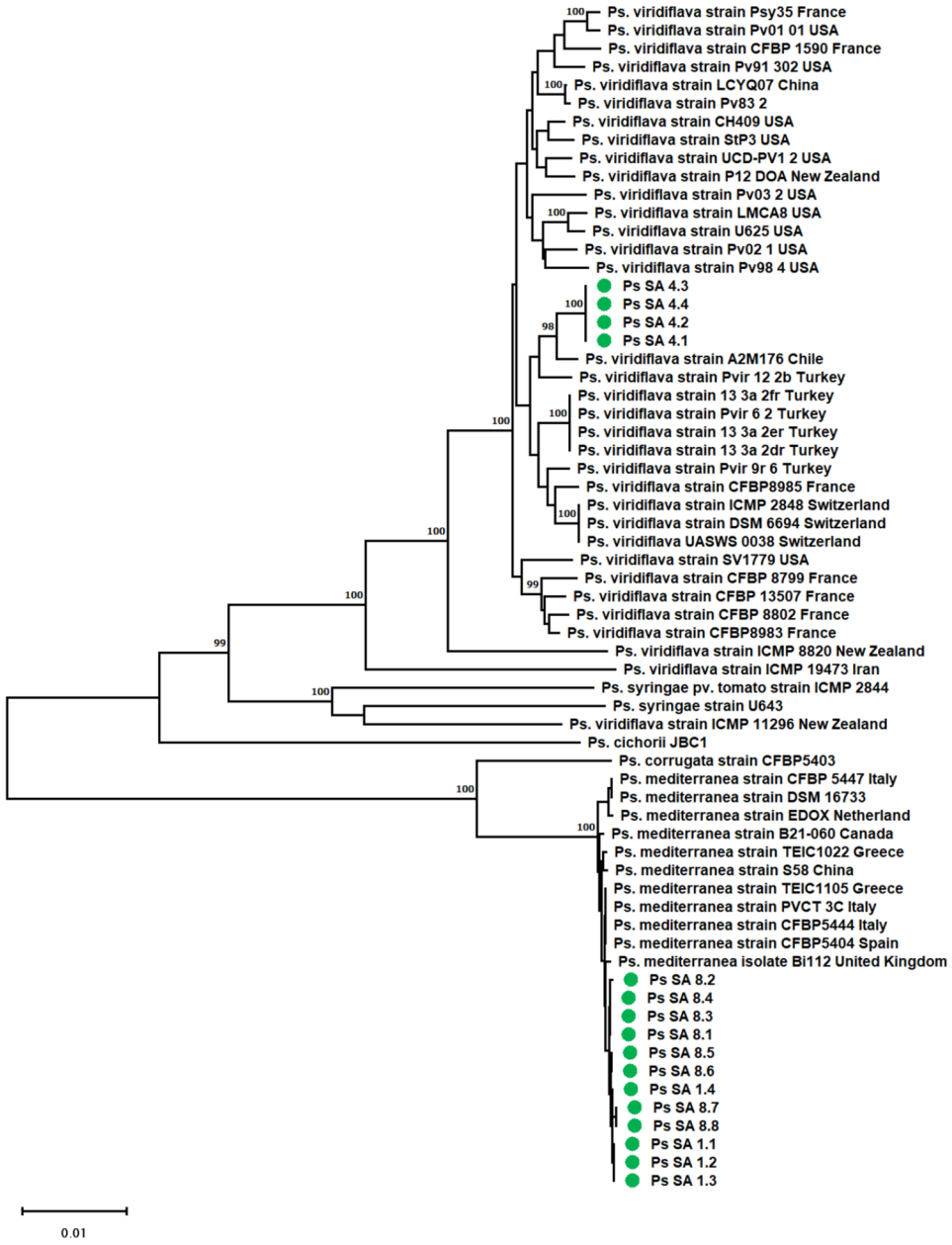

Fig. 3. Multilocus sequence typing (MLST) phylogenetic tree of *Pseudomonas viridiflava* and *P. mediterranea* isolates from Saudi Arabia and reference strains obtained from the NCBI database. The tree was constructed based on a 2472 bp concatenated alignment of five partial housekeeping genes (*rpoB, rpoD, cts, pfk*, and *gyrB*) using the Maximum Likelihood method in MEGA version 11.0.13. Saudi isolates are highlighted with green circles. Bootstrap values (≥70%) are shown at major nodes to indicate branch support. The tree reveals high sequence diversity among *P. viridiflava* isolates and the clustering of Saudi isolates as a separate phenon. *P. mediterranea* is more homogeneous, although the Saudi isolates clustered into multiple distinct sequence types, separate from the reference strains.

and ecological versatility. The Saudi PG 7a cluster was most closely related to the French strain CFBP9895 and the reference strain CSB0072, indicating possible genetic relatedness between populations from the Middle East and Europe populations. This relationship may reflect historical dispersal events or shared environmental niches. Among the global dataset, PGs 7, 8, and 10 were the most represented, indicating wide distribution and host adaptability. In contrast, PGs 1, 4, 6, 11, 12, and 13 were sparsely represented and separated by long branch lengths, suggesting greater evolutionary divergence. PGs 2, 3, 5, and 9 showed moderate representation, primarily from Europe and New Zealand. Our analysis also showed for the first time that *P. viridiflava* strains ICMP 3272 and ICMP 11296 both belong to PG 3.

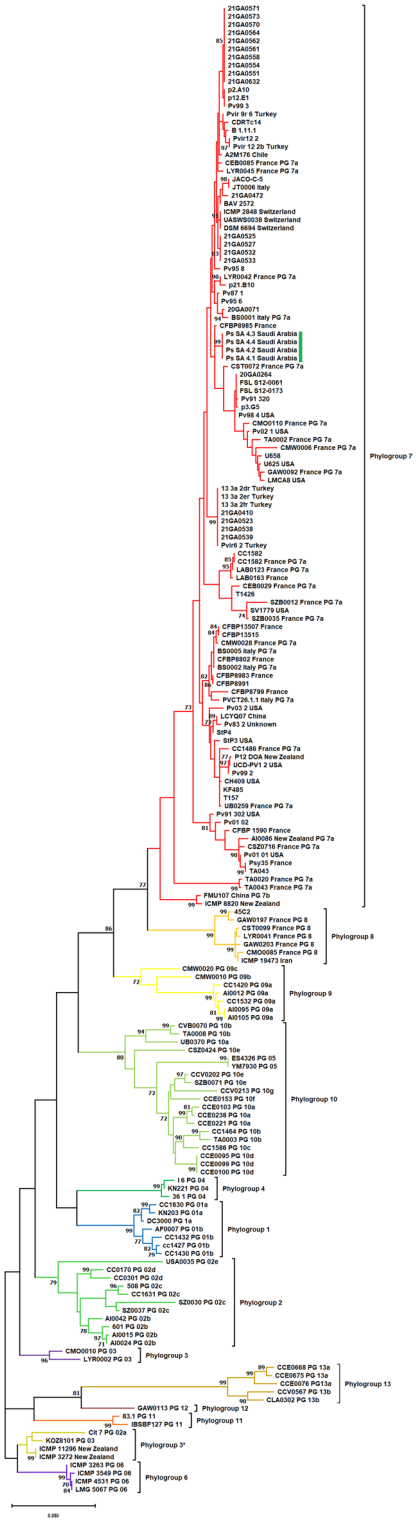

Fig. 4. Maximum likelihood phylogenetic tree based on concatenated multilocus sequence data from 183 *Pseudomonas viridiflava* and *P. syringae* complex globally distributed reference strains, including four isolates from Saudi Arabia (Ps SA 4.1, Ps SA 4.2, Ps SA 4.3, and Ps SA 4.4, shown in green). Bootstrap values ≥ 50% from 1000 replicates are shown at branch nodes. Thirteen well-supported phylogroups (PGs) are indicated on the right and color-coded for clarity. The Saudi isolates form a distinct, highly supported sub-clade within Phylogroup 7a. The tree is rooted and scaled; the scale bar represents 0.02 nucleotide substitutions per site.

Population structure of *Pseudomonas mediterranea* based on MLST

A minimum spanning tree (MST) was constructed from concatenated sequences (3545 bp) of the five housekeeping genes to evaluate the (within-species) genetic diversity among *P. mediterranea* isolates and reference strains (Fig. 5). The analysis included six Saudi isolates (sequence types ST_8 to ST_13) and seven representative global strains (ST_1 to ST_7), revealing a distinct separation between Saudi and global sequence types. The Saudi STs formed a unique cluster, with allelic distances ranging from 3 to more than 40 nucleotide differences, indicating the presence of both closely related and highly divergent relationships among STs. Notably, ST_11 and ST_13 shared the shortest allelic distance (3), suggesting a recent common ancestry, while ST_8 showed the greatest divergence from global types. The robustness of clustering was supported by bootstrap analysis (1000 replicates), with key branches showing support values exceeding 85%, affirming the distinctiveness of Saudi strains. Corresponding sequence type assignments and metadata are detailed in Table 4.

Population structure of *Pseudomonas viridiflava* based on MLST

Minimum spanning tree (MST) analysis of 120 *P. viridiflava* isolates, including four strains from Saudi Arabia and 116 global references, revealed a highly diverse (within-species) population structure comprising 82 distinct sequence types (STs) (Fig. 6). Most STs were represented by single isolates, highlighting extensive genetic heterogeneity across the species.

The Saudi isolates clustered within ST60, forming a unique lineage distinct from major clonal groups. ST60 was closely linked to ST52 (Switzerland) and ST80 (France), suggesting possible evolutionary relationships between Middle East and Europe populations. To our knowledge, ST60 has not been previously described in international MLST databases, supporting its designation as a novel lineage.

Globally, several STs demonstrated clonal expansion. ST75 (USA, France) was the largest clonal group, comprising multiple isolates across continents, while ST63 and ST48 also represented frequent types with international distribution. Other notable STs included ST35, ST66, and ST74, which were shared among isolates from Europe and North America, indicating both regional diversification and long-distance dispersal. Geographic clustering patterns were apparent in several cases: isolates from France and Italy were distributed across multiple STs, whereas isolates from Turkey, Germany, and China were narrowed to fewer lineages. Despite this geographical structuring, no consistent host specialization was observed, with isolates from tomato, kiwifruit, and environmental sources frequently overlapped within the same clonal groups.

MST analysis confirmed *P. viridiflava* populations are genetically diverse and widely dispersed, with frequent occurrence of unique STs and occasional clonal expansion. The identification of a new Saudi Arabian lineage (ST60) emphasizes the importance of regional sampling for capturing uncharacterized diversity and monitoring pathogen evolution.

Discussion

This study provides the first molecular characterization of *P. mediterranea* and *P. viridiflava* strains associated with TPN in the Al Taif region of Saudi Arabia. Sixteen isolates were collected, of which twelve were identified as *P. mediterranea* and four as *P. viridiflava*. The predominance of *P. mediterranea* aligns with earlier reports

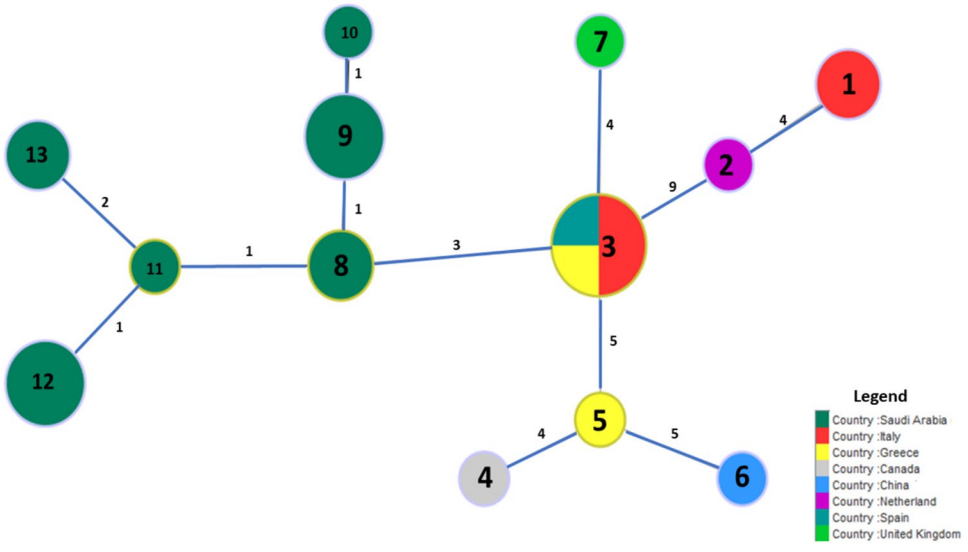

Fig. 5. Minimum spanning tree (MST) of *Pseudomonas mediterranea* sequence types (STs) based on concatenated sequences (3545 bp) of five housekeeping genes: *rpoB, rpoD, cts, pfk*, and *gyrB*. Saudi Arabian isolates (ST_8 to ST_13) are shown in distinct clusters relative to selected global isolates (ST_1 to ST_7). Bold numbers represent absolute allelic differences between sequence types. The size of each node corresponds to the number of isolates sharing the same ST. High-confidence branches (bootstrap ≥ 85%) are indicated by thick connecting lines.

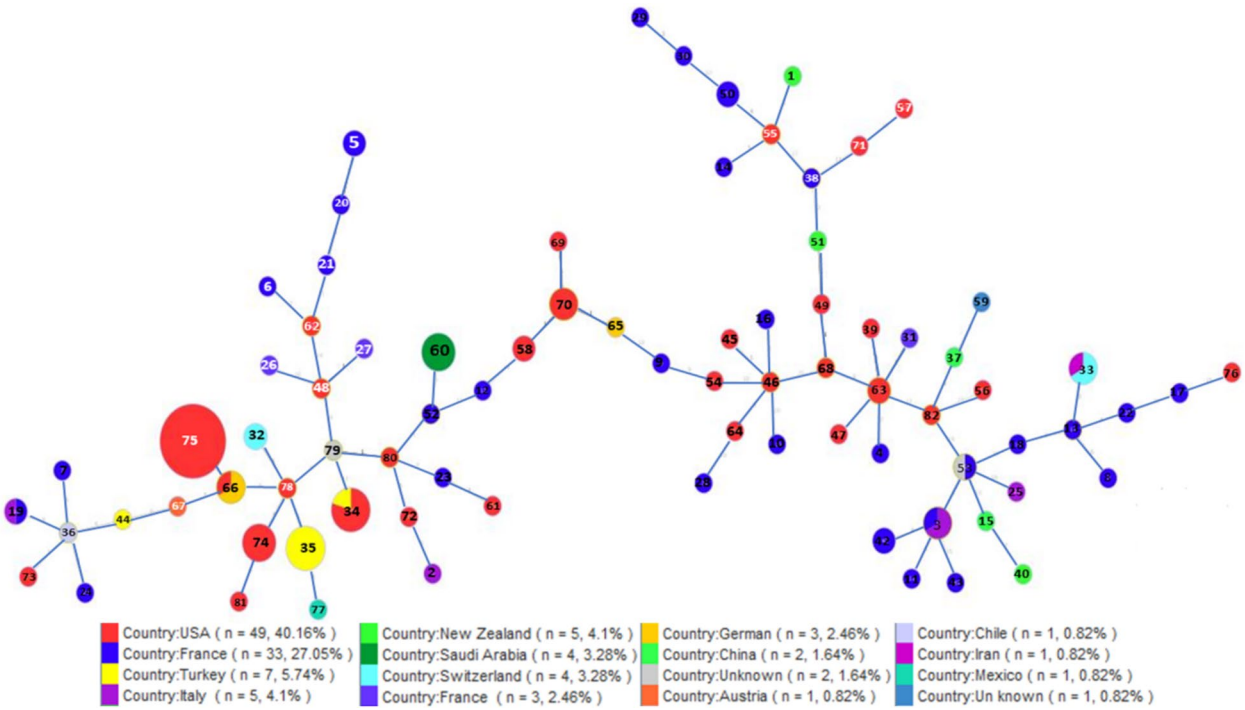

Fig. 6. Minimum spanning tree of 120 *Pseudomonas viridiflava* isolates based on concatenated sequences of three housekeeping genes (*cts*, *gyrB*, and *rpoD*). Each circle represents a unique sequence type (ST), with circle size proportional to the number of isolates. Colors indicate country of origin, and connecting lines represent single-locus or double-locus variants. The four Saudi Arabian isolates grouped within ST60, forming a distinct lineage related to European strains (ST52, ST80).

indicating that it is more frequently isolated than *P. corrugata* in central and south-western tomato-growing regions[36,37]. Together with previous records of *P. fluorescens* and *P. corrugata* from the region[15], these findings confirm the presence of multiple *Pseudomonas* species associated with TPN, consistent with global patterns (Supplementary Table S1)[38]. This highlights the importance of strict plant quarantine and biosecurity measures.

Species differentiation can be achieved using MLST of the housekeeping genes employed in this study (Table 2), along with species-specific primers PC5/1-PC5/2 for *P. mediterranea* and PC1/1-PC1/2 for *P. corrugata*[31]. *P. viridiflava* strains belong to subclades 7 (7a and b based on *cts* gene sequence analysis) and 8 of the *P. syringae* complex, and can be detected using primers developed by[39]. Additional tools include a real-time TaqMan PCR for phylogroup 7 *P. viridiflava* strains causing kiwifruit blight[40] and another PCR assay developed specifically for this species[41]. These molecular methods collectively demonstrate the genetic complexity pf *Pseudomonas* populations involved in TPN.

The pathogenicity assays showed clear differences between the two species. *P. viridiflava* isolates were highly virulent, inducing pith necrosis and wilting, consistent with previous reports describing this species as a necrogenic bacterium[39,42]. In contrast, *P. mediterranea* caused only internal vascular and pith browning without wilting. This pattern reconfirms that *P. mediterranea* may function as an opportunistic pathogen that elicits internal symptoms under certain environmental and/or physiological plant stress[43].

Nucleotide polymorphism analysis revealed pronounced differences in genetic diversity between the two species. *P. viridiflava* exhibited high haplotype and nucleotide diversity, along with a significant negative Tajima's D, suggesting purifying selection or recent population expansion. These findings agree with several earlier studies reporting *P. viridiflava's* evolutionary adaptability and its ability to persist across diverse ecological niches[39,44–47]. In contrast, *P. mediterranea*, showed low nucleotide diversity, consistent with a more conserved genetic structure, yet six new sequence types (ST8–ST13) were identified among Saudi isolates, indicating intra-regional diversification and potential clonal adaptation. Similar clustering patterns have been reported in southern Europe[31,43,48].

The identification of novel STs in both species strengthens the evidence for regional diversification in SA. A unique lineage, ST60, was identified as a new *P. viridiflava* sequence type whose allelic profile was absent from PAEMDB and NCBI, suggesting recent local differentiation comparable to region-specific signatures observed in P. *syringae* pv. *tomato* population[49]. Most European of *P. mediterranea* isolates, particularly those from France and Italy, clustered within *P. syringae* phylogroup 7a, consistent with their association with outbreaks in temperate areas[39].

Saudi *P. viridiflava* strains primarily belonged to subclades 7a, 7b, and 8 of the *P. syringae* complex, reflecting the phylogenetic diversity previously described for this species. In contrast, several South American isolates did not align with known phylogroups, indicating unresolved taxonomic boundaries within the *P. syringae* complex.

Recent genome-based studies emphasized the need for deeper genomic analysis to refine species delimitation and phylogenetic resolution[10,50,51].

MLST analysis confirmed that *P. viridiflava* populations are both highly diverse and widely dispersed, with clonal complexes such as ST75, ST63, and ST35 displaying broad geographic distribution, whereas others remained more localized. Phylogenetic analysis placed the Saudi *P. viridiflava* isolates within phylogroup 7a, which includes strains from North America, Europe, and New Zealand[88], suggesting long-distance dissemination via international seed or plant material movement. Meanwhile, Saudi *P. mediterranea* isolates grouped with strains from southern Europe, indicating shared evolutionary origins with Mediterranean populations.

The coexistence of *P. mediterranea* and *P. viridiflava* in the same production systems has important implications. Mixed infections of different *Pseudomonas* species can complicate symptom expression and disease diagnosis[19,52,53]. Some strains have been described as potential biocontrol agents[2,54,55], while others cause severe necrosis depending on host interactions and environmental conditions[39,42,56–59]. This wide functional variability highlights the ecological complexity of TPN-associated Pseudomonas communities.

The ecological flexibility of *P. viridiflava*, which can persist as an epiphyte on weeds[12,13,58,60] and as a post-harvest pathogen in cold-stored vegetables[12,58,60], suggests that non-crop reservoirs may facilitate pathogen persistence and reintroduction. Variability in hrp pathogenicity islands composition and type *III* secretion system genes, similar to those observed in natural *Arabidopsis thaliana* populations[10,39,61–63], may also contribute to differences in virulence and host adaptation. Comparative genomic studies in *Pseudomonas* species have revealed extensive diversity and modularity in type *III* effector repertoires, which play central roles in host specificity and pathogenicity[49,64–67]. Understanding effector variation in Saudi isolates may therefore provide insight into their pathogenic potential. Furthermore, the coexistence of virulent and weakly pathogenic or commensal *P. viridiflava* strains suggests potential host-priming or cross-protection effects that merit further study.

Overall, this study demonstrates that *P. viridiflava* is more virulent and genetically diverse than *P. mediterranea*, which appears to represent a more conserved yet regionally relevant lineage. The identification of novel sequence types in both species highlights the importance of local surveillance to uncover hidden diversity and understand pathogen evolution. Our findings highlight the roles of both geography and man-made factors, such as seed trade and nursery practices, excessive nitrogen fertilization, and high humidity conditions in greenhouses may shape disease outbreaks and pathogen population's structure[10,48–50].

Integrated disease management remains essential, focusing on balanced fertilization, careful substrate use, temperature and humidity control, and strict hygiene practices[68–71]. Additional practical recommendations are provided in the PlantWisePlus CABI datasheet for *P. corrugata*[72]. Considering the limited efficacy of chemical control options and copper resistance in *P. viridiflava*[56,73], preventive measures, improved diagnostics, and continued monitoring are critical for protecting tomato production in Saudi Arabia and beyond.

Future research should incorporate whole-genome sequencing and expanded pathogenicity tests to better interpret the implications of this diversity and to strengthen surveillance efforts, especially in underrepresented regions where advisory campaigns are underway.

Data availability

The datasets generated and/or analysed during the current study are available in the National Center for Biotechnology Information repository, [https://www.ncbi.nlm.nih.gov], accession number PQ045909-PQ045988.

Received: 23 July 2025; Accepted: 18 December 2025
Published online: 08 January 2026

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Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Yasser E. Ibrahim, Arya Widyawan, Ali A. Al Masrahi, Abdullah F. Al Hashel, and Mohammed A. Al Saleh. The first draft of the manuscript was written by Yasser E. Ibrahim and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding

This research was funded by the National Plan for Science, Technology, and Innovation (MAARIFAH), King Abdul-Aziz City for Science and Technology, Kingdom of Saudi Arabia, Grant No. 2-17-04-001-0029.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-33480-y.

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