

Isolation and Identification of *Pseudomonas viridiflava*, the causal agent of fruit rotting of *Cucumis sativus*

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ABSTRACT

Fruit rotting causes severe losses in cucumber crop grown in green houses in Jordan, rotting symptom is a common symptom for many bacterial infections. Therefore, the aim of this study was to precisely identify the bacterial causal agent which can be regarded as emergent causal agent in Jordan. Infected cucumber fruits with rotting symptom were sampled from different regions in Jordan, the samples were used in bacterial isolation and screening for putative plant pathogenic bacteria by LOPAT determinative tests. Nine representative Isolates were subjected to pathogenicity test, biochemical characterization and Identification by 16S-rDNA sequencing and *in silico* digestion by *SacI* and *HinfI* restriction enzymes. The isolates were characterized by production of yellowish mucoid materials on Mannitol-Glutamate (MG) medium, reproduce similar rotting symptoms on cucumber fruits as the field infection, typical LOPAT profile and similar biochemical profile to *P. viridiflava*, occurrence of seven sites for *HinfI* and absence of *SacI* site in the 16S-rDNA and -based on 16S-rDNA sequences- it had been clustered with *P. viridiflava* stains. Therefore, it can be concluded that the causal agent of cucumber fruit rotting in Jordan was *P. viridiflava*.

Keywords: *P. viridiflava*, fruit rotting, LOPAT, 16S-rDNA.

INTRODUCTION

Pseudomonas species are ubiquitous bacteria talented with special metabolism that enables them to live in a large variety of environmental niches (Sarris *et al.*, 2012). Different *Pseudomonas* species are important as pathogens of animals, insects and plants. There are about 21 *Pseudomonas* species reported to be phytopathogenic, *Pseudomonas viridiflava* has been grouped in oxidase negative Pseudomonads (Höfte and De Vos, 2007). Recently, *Pseudomonas viridiflava* has been reported to be phenotypically homologous to *P. syringae* group, and is considered to be a member of *P. syringae* species complex (Bartoli *et al.*, 2014).

P. viridiflava has been considered to be an epiphyte or

opportunistic pathogen. Since 1998, it has been frequently isolated from and associated with plant diseases. It has been reported to cause many plant diseases such as; fruit spot, stem soft rot, and pith necrosis of tomato (Goumas and Chatzaki, 1998; Goumas, *et al.*, 1999), leaf spot and necrosis of melon (Goumas and Chatzaki, 1998; Aysan, *et al.* 2003), leaf spot of blite, stem soft rot chrysanthemum, leaf spot of eggplant (Goumas and Chatzaki, 1998), leaf and stem necrosis of watermelon (Mirik *et al.*, 2004), leaf spot of the model plant species *Arabidopsis thaliana* (Goss *et al.*, 2005; Araki *et al.*, 2006), leaf blight of celery, leaf lesions and necrosis of artichoke (Sarris *et al.*, 2012), dark-reddish spot of soybean (González *et al.*, 2012), carrot rotting (Almeida *et al.*, 2013), and finally lettuce leaf rotting (Nuebling *et al.*, 2016).

In Greece, Gouman and Chatzaki (1998) reported that *P. viridiflava* caused severe tissue necrosis on melon and tomato crops, reducing foliage by more than 50%, while in Greek, it caused completely destruction of 10,000

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chrysanthemum plants and in Georgia-USA, it caused the total loss of sweet onion production (Gitaitis *et al.*, 1991).

Cucumber "*Cucumis sativus*" is widely grown in greenhouses in Jordan valley in winter and in highland regions in summer, the total cucumber cultivated area was around 5789 acre in 2014 with average production 48.29 ton/acre (DOS, 2014). General wilting and yellowing of the plants, black- brown spots with soft rot were observed on cucumber fruitlets in cucumber growing areas; the wilting and rotting symptoms prevent fruit maturation and cause huge economic loss in cucumber crop (Agrios, 2005). In spite of there are no accurate measurements of fleshy vegetables to bacterial soft rots, but they are estimated to vary between 15-30% (Bhat *et al.*, 2010). Hence, soft rotting is a common symptom for many bacterial infections; the aim of the present study was to precisely identify the bacterial causal agent which can be regarded as emergent in Jordan by molecular techniques such as 16S-rRNA amplification and sequencing.

Methodology

1. Plant materials and bacterial isolation

Samples of cucumber fruits with rotting symptoms were collected from different field locations in Jordan (Table 1). The samples were used for bacterial isolation on King's B medium (KB) (20 g peptone (BioBasic- Canada), 1.5 g K₂HPO₄ (Merck- Germany), 1.5 g MgSO₄ x 7H₂O (Merck- Germany), 10 ml glycerol (AppliChem- Germany), 15g agar (BioBasic- Canada) for 1 L, pH 7.2) as a complex medium, and Mannitol-Glutamate medium as minimal medium (10 g mannitol (BioBasic- Canada), 2 g L-Glutamic acid (Merck- Germany), 0.5 g KH₂PO₄ (Merck- Germany), 0.2 g NaCl (AppliChem- Germany), 0.2 g MgSO₄ x 7H₂O (Merck- Germany), 15 g agar (BioBasic- Canada) for 1 L, pH 7.0). The inoculated plates were incubated at 28 °C for 48 h. Single fluorescent colonies (isolates) were selected for re-cultivation and cryopreservation in 15-20% Glycerol for further uses (Swift, 1937).

Table 1. Origin and relevant features of typical LOPAT profile isolates analyzed in this study.

Isolate\ strains	Date of collection	Location	Colony morphology*	LOPAT profile					Pathogenicity on cucumber
				L	O	P	A	T	
NK1	Jun. 2014	Al-Balqa	not mucoid	+	-	+	-	+	+
NK2	Jun. 2014	Irbid	mucoid	-	-	+	-	+	+
NK3	Jul. 2014	Jerash	mucoid	-	-	+	-	+	+
NK4	Sep. 2014	Al-Mafraq	mucoid	-	-	+	-	+	+
NK5	Sep. 2014	Madaba	not mucoid	-	-	+	-	+	+
NK6	Oct. 2014	Amman	mucoid	-	-	+	-	+	+
NK7	Dec. 2014	Middle Jordan Valley		-	-	+	-	+	+
NK8	Dec. 2014	Northern Jordan Valley	mucoid	-	-	+	-	+	+
NK9	Jan. 2015	southern Jordan Valley	mucoid	-	-	+	-	+	+
Control strains									
<i>P. viridiflava</i> ATCC 13223				-	-	+	-	+	+
<i>P. syringae</i> pv. <i>lachrymans</i> GSPB 77				+	-	-	-	+	+

* Yellowish fluorescent isolates; +: indicates positive reaction; - : indicates negative reaction.

2. Screening for plant pathogenic bacteria

The LOPAT profile (Levan formation; Oxidase production; Pectinolytic activity; Arginine dihydrolase production; and Tobacco hypersensitivity) are the most widely used protocol for differentiation of plant pathogenic Pseudomonads (Hildebrand *et al.*, 1988). A 48 hr old fluorescent isolates were subjected to LOPAT determinative tests.

For levan formation, bacterial isolates were grown on MG for 48 h, and re-suspended in sterile 0.9% NaCl to an $OD_{600} \sim 1.0$ (corresponding to approximately 10^7 CFU/ml). Twenty microliters of the bacterial suspension were applied on MG supplemented with 5% sucrose as a hypersucrose medium. Formation of a dome shape colonies on agar plate after 48 h incubation at 28 °C was considered as a positive result for levan formation. In order to confirm the results, the isolates were grown in KB medium for 48 h at 28 °C; 250 rpm. The bacterial growth were adjusted to $OD_{600} \sim 1.0$, and centrifuged at 4000rpm for 30min. The supernatants were filtered with 0.22 μ m pore size filter. Cell free supernatants of 20 μ l volume were applied on MG medium supplemented with 5% sucrose (Srivastava *et al.*, 2012; Mehmood *et al.*, 2015).

Pectinolytic activity test was conducted on potato tubers. The tubers were surface sterilized with 1% NaOCl, rinsed three times with sterile water, dried, and sliced aseptically. Slices were inoculated by 20 μ l of $OD_{600} \sim 1.0$ bacterial suspension, and kept on a sterile wet filter paper in a petri-dish for 48 h at 28 °C. Tissue maceration of slice

was treated as positive, while the discoloration of slice at the point of inoculation was considered as negative control (Liao, 1994).

Tobacco hypersensitivity was conducted on tobacco plants (*Nicotiana tabacum* cv. Petit Havana SR1) grown at 20-25°C and 12h photoperiod. Tobacco plants were inoculated with bacterial suspensions of $OD_{600} \sim 1.0$ by syringe injection of leaf veins of the third and fourth leaf (Al-Karablieh *et al.*, 2009; Mehmood *et al.*, 2015). Infiltrated areas were monitored after 24 h for development of the hypersensitive reaction in form of necrosis.

In all tests, sterile 0.9% NaCl was used as negative control. *P. viridiflava* ATCC13223 and *P. syringae* pv. *lachrymans* GSPB 77 were used as reference strains. All experiments were repeated three times to confirm reproducibility of the results.

The oxidase and arginine dihydrolase tests of the LOPAT profile were conducted in parallel with other biochemical tests via analytical profile index (API) strips as described below.

3. Biochemical characterization

Bacterial isolates obtained from MG that showed the typical LOPAT profile for *P. viridiflava* were subjected to biochemical characterization, with *P. viridiflava* ATCC13223 and *P. syringae* pv. *lachrymans* GSPB 77. The biochemical tests were conducted by combination of API-E and API-NE (BioMérieux, Marcy-l'Étoile, France), according to the manufacturer's instructions.

Table 2. Some phenotypic and nutritional characteristics of typical LOPAT profile isolates analyzed in this study.

Characteristics	Reference strains		Isolates								
	<i>P. s. pv. lachryman</i>	<i>P. viridiflava</i>	NK1	NK2	NK3	NK4	NK5	NK6	NK7	NK8	NK9
Gram staining	-	-	-	-	-	-	-	-	-	-	-
Reduction of nitrates	+	+	+	+	+	+	+	+	+	+	+
Indole production	-	-	-	-	-	-	-	-	-	-	-
Tryptophane deaminase	+	+	+	+	+	+	+	+	+	+	+
Urease production	-	-	-	-	-	-	-	-	-	-	-
H ₂ S production	-	-	-	-	-	-	-	-	-	-	-
Esculin hydrolysis	+	+	+	+	+	+	+	+	+	+	+
Gelatin hydrolysis	+	+	+	+	+	+	+	+	+	+	+
Galactopyranoside	-	-	-	-	-	-	-	-	-	-	-
Acetoin production	-	-	-	-	-	-	-	-	-	-	-
Oxidative\ Fermentative	O	O	O	O	O	O	O	O	O	O	O
Lysine decarboxylase	-	-	-	-	-	-	-	-	-	-	-
Ornithine decarboxylase	-	-	-	-	-	-	-	-	-	-	-
Utilization of:											
D-Glucose	+	+	+	+	+	+	+	+	+	+	+
L-Arabinose	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+
Inositol	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	+	+	+	+	+	+	+	+	+	+	+
Rhamnose	-	-	-	-	-	-	-	-	-	-	-
Sucrose	+	+	+	+	+	+	+	+	+	+	+
Melibiose	+	+	+	+	+	+	+	+	+	+	+
N-acetyl-glucosamine	+	+	+	+	+	+	+	+	+	+	+
Potassium gluconate	+	+	+	+	+	+	+	+	+	+	+
Caprate	-	+	+	+	+	+	+	+	+	+	+
Adipate	-	-	-	-	-	-	-	-	-	-	-

Malate	+	+	+	+	+	+	+	+	+	+	+
Citrate	+	+	+	+	+	+	+	+	+	+	+
Phenyl-acetate	-	-	-	-	-	-	-	-	-	-	-
Amygdalin	-	-	-	-	-	-	-	-	-	-	-

+: indicates positive reaction; - : indicates negative reaction; O: indicates oxidative reaction.

4. Cucumber pathogenicity assay

Koch's postulates were used to test symptoms development caused by *Pseudomonas* isolates. Those isolates were collected from different locations, and showed the typical LOPAT profile. Cucumber plants (*Cucumis sativus* cultivar 189) were grown at 20 °C -25 °C, and 12 h photoperiod. Bacterial isolates were grown on MG agar plates for 48 h, re-suspended in sterile 0.9% NaCl, and adjusted to OD₆₀₀ ~1.0 for inoculation. Twenty microliters of the bacterial suspension were inoculated in the flower which attached to the fruit. The inoculated fruits were covered with a transparent plastic bag during the first 48 h period, and monitored for the development of rotting symptoms. When rotting symptoms appeared, part of the damaged tissues was processed, and used for bacterial isolation on MG medium. Each bacterial isolate was inoculated into six individual fruits, and compared with the reference strain of *P. viridiflava* ATCC13223. A 0.9% NaCl was used as a negative control.

5. 16S-rRNA sequencing and sequence analysis

Out of the bacterial isolates that show identical features, and collected from the same location at the same period of the year, only one representative was used (Table 1). 16S-rRNA amplification was conducted for nine bacterial isolates using the forward (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse (5'-TACGG(CT)TACCTTGTTACGCTT-3') primers (Heyer *et al.*, 2002). The total final volume of the reaction was 50 µl. The reaction composed of 1X KCl taq polymerase buffer, 1 mM d-NTP mix, 0.5 pmol of each primer, 6 mM MgCl₂, and 0.04 U/µl Taq DNA

Polymerase (Fermentas®, Thermo Scientific, USA). The volume was adjusted to 50 µl by sterile de-ionized water. One microliter of ~100ng/µl DNA was used as a DNA template. The reaction program steps were; initial denaturation at 94 °C for 4 min, 40 cycle of 94 °C for 1 min, 55 °C for 30 sec, 72 °C for 1 min, and final extension step for 7 min at 72 °C. Gel electrophoresis (Alpha innotech-Taiwan) was performed to visualize the pcr amplicons. Sterile de-ionized water was used as a negative control, and *P. viridiflava* ATCC13223 was used as a positive control. The amplicons were sequenced at Macrogen, South Korea.

To determine the phylogenetic affiliation, sequences were initially compared to the available databases by using nucleotide blast search. Phylogenetic tree was generated by MegAlign (DNASTAR lasergene 13 Madison, WI U.S.) by the ClustalW method as recommended by Burse *et al.*, (2004). The accession numbers of the 16S-rRNA sequences used for comparison were: *P. viridiflava* ATCC 13223, NR_114482; *P. viridiflava* CECT 458, NR_042764; *P. amygdali* LMG 2123T, Z76654; *P. cichorii* LMG 2162T, Z76658; *P. fluorescens* ATCC13525, Z76662; *P. marginalis* LMG 2210T, Z76663; *P. syringae* pv. *lachrymans* 361, KC860048; *P. syringae* pv. *actinidiae*, AB001439; *P. syringae* pv. *maculicola*, AB001444; *P. syringae* pv. *morsprunorum*, AB001445; *P. syringae* pv. *phaseolicola*, AB001448; *P. syringae* pv. *theae*, AB001450; *P. syringae* pv. *syringae* LMG 1247 t1T, Z76669; and *P. syringae* pv. *tomato* DC3000, NR_074597 (Moore *et al.*, 1996; Buell *et al.*, 2003).

6. In silico restriction enzyme digestion

Computer-simulated restriction digestion was conducted to detect occurrence of certain restriction enzymes site in the 16S rRNA genes (Wei *et al.*, 2007). The 16S rDNA sequences that were generated, and the relevant *Pseudomonas* sequences retrieved from databases, were examined by using the Restriction Site Analyses in Vector NTI advanced[®] 11.5 software (Thermo Fisher Scientific, USA). *Hinf*I and *Sac*I restriction enzymes were selected to distinguish between species following the suggestion of González *et al.*, (2003).

Results

Phenotypic characterization of *Pseudomonas* isolates.

Fluorescent bacterial isolates with typical LOPAT profile have been isolated from rotted cucumber fruits (Table 1). The samples were collected from cucumber plants that were grown in green houses from different geographical regions in Jordan. These regions were ; Al-Balqa, Irbid, Jerash, Al-Mafraq, Amman, northern-region of Jordan Valley, the middle-region of Jordan Valley, and the southern-region of Jordan Valley.

The isolates were differentiated by the levan formation test, and their pectinolytic activity. Colonies with mucoid

material of yellowish color (occasionally greenish) were produced from the tested isolates on medium supplemented with 5% sucrose, in contrast to the typical white mucoid dome shape colonies associated with levan producer strains, such as *P. syringae* pathovars (Fig. 1A). Similar mucoid materials were detected in sucrose-deficient medium (Fig. 1B). In order to avoid misleading of the results due to mucoid structure, cell free supernatants were loaded on MG medium supplemented with 5% sucrose. Transparent colony was formed by the supernatant of *P. syringae* pv. *lachrymans* GSPB77 only, whereas, neither *P. viridiflava* ATCC13223 nor the tested isolates have developed such transparent colony (Fig. 1C). The isolates showed rotting halos surrounding the inoculation site, when assayed on potato slices, which indicates variable pectinolytic activity (Fig. 1D), the rotting ability was similar or less than that of the control *P. viridiflava* strain. The fluorescent isolates were infiltrated into tobacco leaves for 24h to monitor the development of the hypersensitive response (HR) in form of necrosis, the fluorescent isolates were able to elicit typical HR during the incompatible reaction with tobacco plants (Fig. 1E).

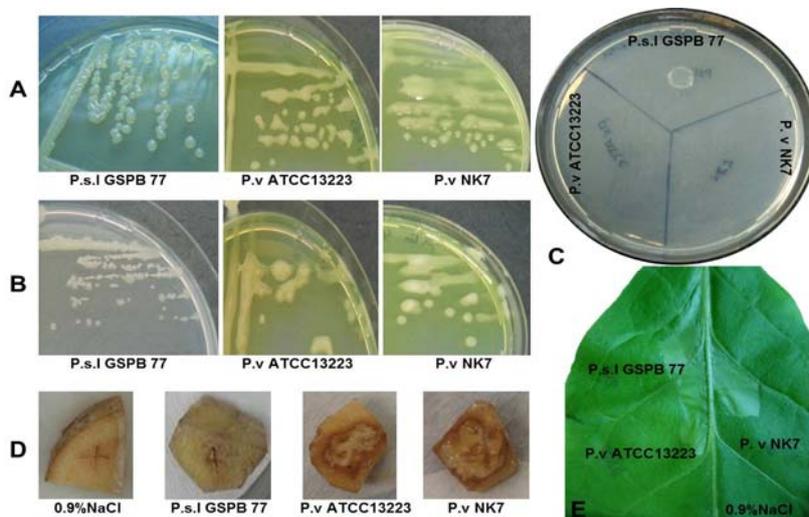


Fig. 1: Differential LOPAT tests of *Pseudomonas* isolates; A: Growth on MG medium supplemented with 5% sucrose; B: Growth on MG medium; C: Cell free supernatant on MG medium supplemented with 5% sucrose; D: Pectinolytic activity on potato slices and E: hypersensitive response on tobacco plant.

In regard of other LOPAT tests, the isolates were oxidase negative and arginine dihydrolase negative (Table 2). This LOPAT profile (group II) exactly fits that the characterized plant pathogenic bacterium *P. viridiflava* (Palleroni 1984; Hildebrand *et al.*, 1988).

The isolates had the same biochemical properties (Table. 2). For instance, all were gram negative, hydrolyzed esculin and gelatin, utilized the same set of carbon compounds. These biochemical properties were all shared by the reference strain *P. viridiflava* ATCC13223.

To prove that disease symptoms were due to *P. viridiflava*, the virulence of isolates, that showed the typical LOPAT profile as the reference strain *P. viridiflava*, was tested through inoculation on cucumber fruits as described in methodology. The isolates were able to reproduce the symptoms of the natural infection, darkening, soft rotting, and tissue maceration. In general, the damage caused by the typical isolates was similar to or less than that caused by the reference *P. viridiflava* strain (Fig. 2).



Fig. 2: Pathogenicity assay on cucumber fruits, darkening and rotting of the fruits with some ooze formation developed on the fruits after incubation at 28°C for 48h.

Genotypic identification of the isolates

Almost complete 16S rDNAs fragments from nine isolates with the typical LOPAT profile were sequenced after amplification (Table 1). Analysis of the 16S rDNA sequences revealed that those isolates with the typical LOPAT profile were related to each other (95.5% identity over 1,365 nucleotides), and they were closely related to

16S rDNA sequences from several strains of *P. viridiflava* (including *P. viridiflava* ATCC13223 and CECT 458). Sequences generated in this study have been deposited in GenBank under accession numbers KU686692-KU686700.

The sequence corresponding to hypervariable (hv) region 2 was found in the 16S rDNA genes of the new

isolates, this region was proposed by Moore *et al.*, (1996) as a signature of *P. viridiflava*. Moreover, the *P. viridiflava* sequences in hv regions 1 and 3, which distinguish this species from *P. syringae* or from *P. fluorescens*, respectively were found in the 16S rDNA genes of the new isolates (Fig. 3) (Moore *et al.*, 1996).

The results of *in silico* restriction digestion of the 16S rDNA genes of *P. viridiflava* ATCC 13223 and new isolates revealed that occurrence of seven *Hinf*I sites, which are absent from the 16S rDNA genes of *P. syringae* pathovars including; *lachrymans*, *actinidiae*, *maculicola*, *morsprunorum*, *theae*, *phaseolicola*, *tomato* and *syringae*. Whereas, the 16S rDNA genes from these pathovars contain a single *Sac*I site, which is absent from *P.*

viridiflava ATCC 13223 and new isolates. Examples of *in silico* *Sac*I and *Hinf*I digestions of 16S rDNAs from relevant strains are shown in Fig. 3. Such differential restriction sites were used to develop an *in vitro* RFLP ribotyping method that allows a rapid identification of the typical isolates when combined with phenotypical tests.

Based on sequence relatedness, we suggest that the 16S rDNA of the new isolates can be classified in the first group, which include *P. viridiflava* ATCC13223 and *P. viridiflava* CECT458, clustering of the typical LOPAT profile isolates 16S rDNA sequences with two control *P. viridiflava* sequences is revealed by the phylogenetic tree depicted in Fig. 4.

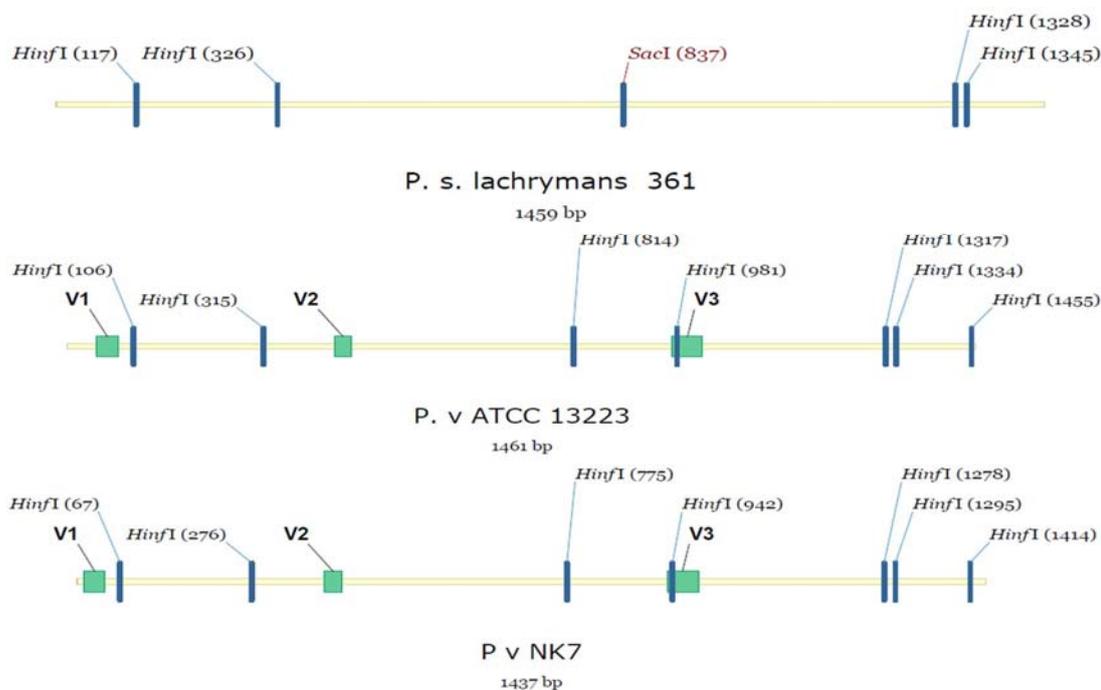


Fig. 3: In Silico *Sac*I and *Hinf*I restriction analyses conducted by restriction analyses by Vector NTI-Advance 11.5 (Thermo Fisher Scientific, USA), numbers in the brackets are the location of the restriction enzymes. V1, V2 and V3 indicate the sequences sites of variable region 1, 2 and 3 respectively in *P. viridiflava*.

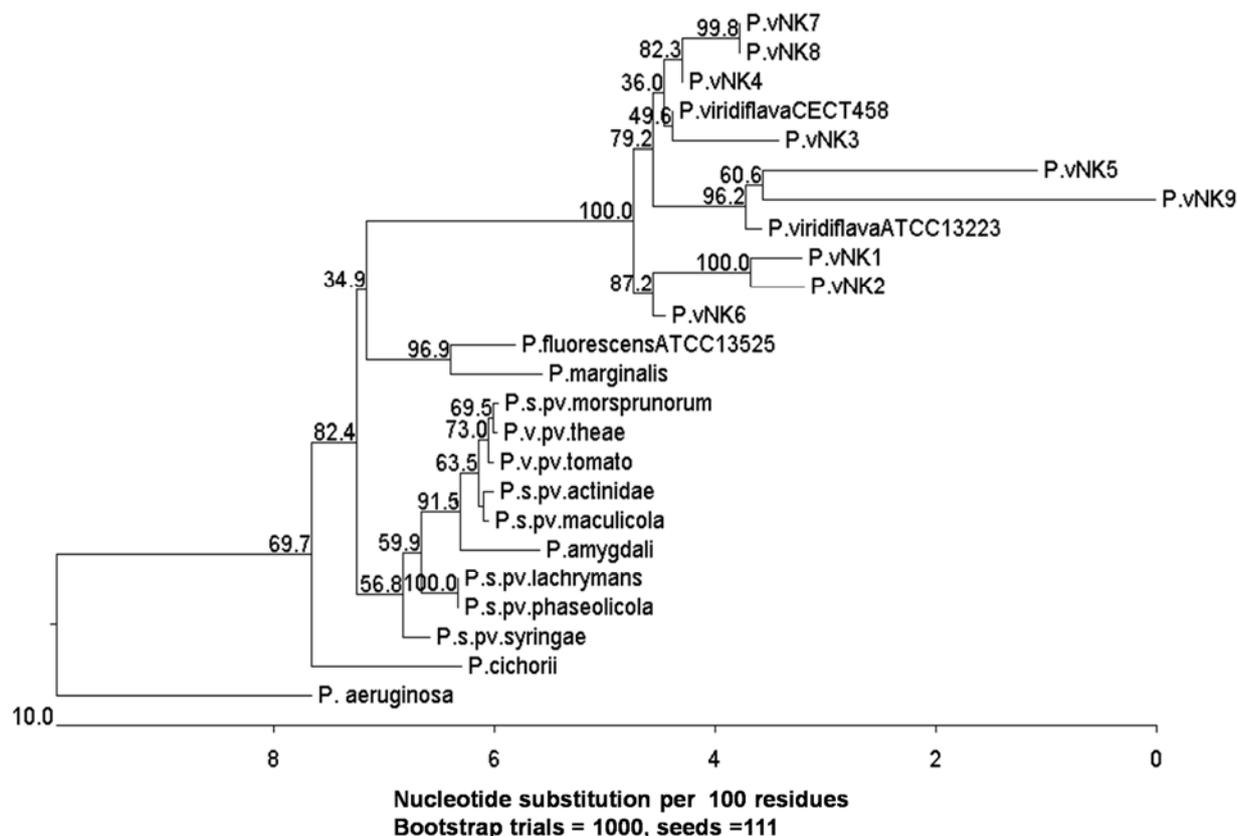


Fig. 4: Rooted Phylogenetic tree for 16S rDNA among the typical LOPAT profile isolates and members of the genus *Pseudomonas*. The dendrogram was generated in MegAlign version 13 (DNASTAR lasergene Inc.) by the ClustalW method. 16S rDNA of the new isolates belong to a cluster containing *P. viridiflava*.

Discussion

P. viridiflava is usually differentiated from *P. syringae* group by their pathogenicity, and by two biochemical tests represented in the LOPAT profile, namely; levan formation and pectinolytic activity which might be related to extracellular pectate lyase which is responsible for plant tissue maceration (Liao *et al.*, 1994). *P. viridiflava* is considered as an epiphytic or opportunistic pathogen. Whereas, *P. syringae*, apart from being epiphytic, includes true pathogenic pathovars that cause different types of damage, such as necrosis, blights, rots, and spots.

In regard of, the LOPAT tests, *P. viridiflava* strains

are negative in levan formation because they produce flat colonies on medium supplemented with 5% sucrose and positive in pectinolytic activity because they show strong pectinolytic activity on potato slices. In contrast, *P. syringae* pathovars are forming levan that has dome shape colonies on medium supplemented with 5% sucrose and negative in pectinolytic activity (Hildebrand *et al.*, 1988).

The isolated bacteria described here show a distinctive activity in both tests; levan formation and pectinolytic activity, with colonies characterized by a flat mucoid yellowish on MG medium supplemented with 5% sucrose, and a variable pectinolytic activity, ranging from negative or very weak to relatively strong activity similar

to the reference strain *P. viridiflava* ATCC13223.

It should be noted that in some tested isolates, some similar mucoid materials have been observed on KB and MG medium. This could be related to the nature of the exopolysaccharide released by some species of *Pseudomonas*, alginate (Schenk *et al.*, 2008). The production of mucoid materials on medium with and without sucrose, but not as a dome shape structure, strongly suggests that this mucoid materials could not be levan (Srivastava *et al.*, 2009); development of very clear transparent colony from the cell free supernatant of *P. syringae* pv. *lachrymans* on MG medium supplemented with 5% sucrose, while the cell free supernatants of *P. viridiflava* reference strain and the new isolates were unable to develop such transparent colony proves this suggestion.

All new isolates were able to elicit typical hypersensitive response during the incompatible reaction with tobacco plants. This demonstrates that these isolates possess the *hrp* type III secretion system encoding genes on their pathogenicity island and express the type III proteins secretion system, that involve in secretion of effector and avirulence factors into the host cell (Araki *et al.*, 2006; Araki *et al.*, 2007).

Based on the previous studies conducted on the bacterial diseases affecting cucumber in Jordan (Khlaif 1991a; Khlaif 1991b; Khlaif 1995), the damage and rotting caused by *P. viridiflava* to cucumber fruits reported in this study has not been previously reported in Jordan, in agreement with Young *et al.*, (1988), *P. viridiflava* causes remarkable damage on kiwi fruit during the flowering phase.

According to O'Brien and Lindow (1989), the population of the pathogen surviving epiphytically on the plant could represent an important source of inoculum; this population may cause damage under particular environmental conditions. Thus, mild temperatures and

high relative humidity values would facilitate their spread. These weather conditions are frequently found in green houses, where the *P. viridiflava* is causing severe damage, with huge economical loss.

The phenotypic profiling by pathogenicity and biochemical tests of the isolates were almost similar to the reference of *P. viridiflava*. However, this did not enable accurate identification of the isolates. Therefore, the accurate species identification of the plant pathogenic *Pseudomonas* isolates was conducted by analysis of the nucleotide sequences of 16S rDNA genes from representative isolates. Moore *et al.*, (1996) have previously demonstrated the potential of 16S-rDNA sequence analyses to distinguish *Pseudomonas* species via hypervariable regions, and to establish phylogenetic lineages within the genus. In addition, sequence comparisons led to the identification of hypervariable regions that can be regarded as signature of certain species. In this work, similar experiments revealed that the hypervariable regions which can be regarded as signatures of *P. viridiflava* were found in the new isolates.

In Silico restriction digestion developed in this study using 16S rDNA digestion by selected restriction enzymes (*SacI* and *HinfI*) could discriminate between *P. viridiflava* genome from closely related species genome, mainly the *P. syringae* pathovars and the *P. fluorescens*, the results of this experiments reveals that the new isolates were related to *P. viridiflava*. This technique could be adapted to constitute a rapid and accurate genetic procedure in *in vitro* identification of *P. viridiflava*.

Since, all signature of *P. viridiflava* that allow discrimination of *P. viridiflava* from other closely related *pseudomonas* species exist in the new isolates, it can be concluded that the causal agent of cucumber fruit rotting in Jordan was *P. viridiflava*.

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REFERENCES

- Agrios, G. N., 2005, *Plant Pathology*, 5th (ed), Elsevier Academic Press, Burlington, USA, pp. 656-662.
- Al-Karablieh, N.; H. Weingart; M. S. Ullrich, 2009, The outer membrane protein TolC is required for phytoalexin resistance and virulence of the fire blight pathogen *Erwinia amylovora*, *Microbial Biotechnology*, 4, pp. 465-475.
- Almeida, I. M. G.; K. W. Maciel.; R. J. Neto; L. O. S. Beriam, 2013, *Pseudomonas viridiflava* in imported carrot seeds. Australasian Plant Dis. Notes, 8, pp. 17-19.
- Araki, H.; H. Innan; M. Kreitman; J. Bergelson, 2007, Molecular evolution of pathogenicity-island genes in *Pseudomonas viridiflava*. *Genetics*, 177, pp. 1031-1041.
- Araki, H.; D. Tian; E. M. Goss; K. Jakob; S. S. Halldorsdottir; M. Kreitman; J. Bergelson 2006, Presence/absence polymorphism for alternative pathogenicity islands in *Pseudomonas viridiflava*, a pathogen of Arabidopsis. *Proc Natl Acad Sci* 15, pp. 5887-5892.
- Aysan, Y.; M. Mirik; A. Ala; F. Sahin; Cinar, 2003, First report of *Pseudomonas viridiflava* on melon in Turkey. *New Disease Reports* 7.
- Bartoli, C.; O. Berge; C. L. Monteil; C. Guilbaud; G. M. Balestra; L. Varvaro; C. Jones; J. Dangl; D. A. Baltrus; D. S. Sands; C. E. Morris, 2014, The *Pseudomonas viridiflava* phylogroups in the *P. syringae* species complex are characterized by genetic variability and phenotypic plasticity of pathogenicity-related traits. *Environmental Microbiology*, 16, pp. 2301-2315.
- Bhat, K. A.; S. D. Masood; N. A Bhat; M. A. Bhat; S. M. Razvi; M. R. Mir; S. Akhtar; M. Habib, 2010, Current status of post harvest soft rot in vegetables: *A review. Asian J. Plant Sci*, 9, pp. 200-208.
- Buell, C.R.; V. Joardar; M. Lindeberg; J. Selengut; I.T. Paulsen; M.L. Gwinn; et al., 2003, The complete genome sequence of the Arabidopsis and tomato pathogen *Pseudomonas syringae* pv. *tomato*. DC3000. *Proc. Natl. Acad. Sci*, 18, pp. 10181-10186.
- Burse, A.; H. Weingart; M. S. Ullrich, 2004, NorN, an *Erwinia amylovora* multidrug efflux pump involved in in vitro competition with other epiphytic bacteria. *Appl. Environ. Microbiol*, 70, pp. 693-703.
- Gitaitis R. D.; R. E Baird; R. W. Beaver; D. R. Sumner; D. A. Smittle, 1991, Bacterial blight of sweet onion caused by *Pseudomonas viridiflava* in Vidalia, Georgia. *Plant Dis*, 75, pp. 1180-1182.
- Department of Statistics. www.dos.gov.jo.
- González, A. J.; A. M. Fernández; M. S. José; G. González-Varela; M. R. Rodicio, 2012, A *Pseudomonas viridiflava*-related bacterium causes dark-reddish spot disease in *Glycine max*. *Appl Environ Microbiol*, 78, pp. 3756-3758.
- González, A.J.; M.R. Rodicio; M.C. Mendoza, 2003, Identification of an emergent and atypical *Pseudomonas viridiflava* lineage causing bacteriosis in plants of agronomic importance in a Spanish region. *Appl. Environ. Microbiol*, 69, pp. 2936-2941.
- Goss, E. M.; M. Kreitman; J. Bergelson, 2005, Genetic diversity, recombination and cryptic clades in *Pseudomonas viridiflava* infecting natural populations of *Arabidopsis thaliana*. *Genetics*, 169, pp. 21-35.
- Goumas, D. E.; A. K. Chatzaki, 1998, Characterization and host range evaluation of *Pseudomonas viridiflava* from melon, blite, tomato, chrysanthemum and eggplant. *Eur J Plant Pathol*, 104, pp. 181-188.
- Goumas, D. E.; N. E. Malathrakis; A. K. Chatzaki, 1999, Characterization of *Pseudomonas viridiflava* associated with a new symptom on tomato fruit. *Eur J Plant Pathol*, 105, pp. 927-932.

- Heyer, J.; V. F. Galchenko; P. F. Dunfield, 2002, Molecular phylogeny of type II methane-oxidizing bacteria isolated from various environments. *Microbiology*, 148, pp. 2831-2846.
- Hildebrand, D. C., H. N. Schroth; D. C. Sands, 1988, *Pseudomonas*, in N. W. Schaad (ed.), Laboratory guide for identification of plant pathogenic bacteria, 2nd ed., American Phytopathological Society, St. Paul, Minn, pp. 60-80.
- Höfte, M.; De Vos, P, 2007, Plant pathogenic *Pseudomonas* species, 2007, in S. S. Gnanamanickam (ed), Plant-associated bacteria, Springer, Dordrecht, The Netherlands, pp. 507-533.
- Khlaif, H., 1991a, Angular leaf spot of cucumber (*Cucumis sativus* L.) in Jordan: Disease development and effect on yield, *Dirasat*: 18, pp. 110-116.
- Khlaif, H., 1991b, Properties of *Pseudomonas syringae* pv. *lachrymans* (Smith and Bray) Carsner, the causal agent of angular leaf spot of cucumber (*Cucumis sativus* L.) in Jordan, *Dirasat*: 18, pp. 68-78.
- Khlaif, H., 1995, Varietal reaction and control of angular leaf spot disease on cucumber, *Dirasat*: 22, pp. 1201-1208.
- Liao, C. H.; D. E. McCallus; W. F. Fett, 1994, Molecular characterization of two gene loci required for production of the key pathogenicity factor pectate lyase in *Pseudomonas viridiflava*. *Molecular Plant-Microbe Interactions*, 7, pp. 391-400.
- Mehmood, A.; K. Abdallah; S. Khandekar; D. Zhurina; A. Srivastava; A. Al-Karablieh; G. Alfaro-Espinoza; D. Pletzer; M.S. Ullrich, 2015, Expression of extra-cellular levansucrase in *Pseudomonas syringae* is controlled by the in planta fitness-promoting metabolic repressor HexR. *BMC Microbiology*, 15, pp. 48.
- Mirik, M.; A. Aysan ; R. Cetinkaya-Yildiz ; F. Sahin ; H. Saygili, 2004, Watermelon as a new host of *Pseudomonas viridiflava*, Causal agent of leaf and stem necrosis, Discovered in Turkey. *Disease Notes*, 88, pp. 907.
- Moore, E. R. B.; M. Mau; A. Arnscheidt; E. C. Boettger; R. A. Hutson; M. D. Collins; Y. Van de Peer; R. de Wachter; K. N. Timmis, 1996, The determination and comparison of the 16s rRNA gene sequences of species of the genus *Pseudomonas* (sensu stricto) and estimation of the natural intrageneric relationships. *Syst. Appl. Microbiol*, 19, pp. 478-492.
- Nuebling, S.; H. Schmidt ; A. Weiss, 2016, Variation of the *Pseudomonas* community structure on oak leaf lettuce during storage detected by culture-dependent and independent methods. *Int J Food Microbiol*, 4, pp. 95-103.
- O'Brien, R. D.; S. E. Lindow, 1989, Effect of plant species and environmental conditions on epiphytic population sizes of *Pseudomonas syringae* and other bacteria. *Ecology and Epidemiology*, 79, pp. 619-627.
- Palleroni, N. J., 1984. Genus I. *Pseudomonas* Migula, 1894, in N. J. Krieg and J. G. Holt (ed.), *Bergey's manual of systematic bacteriology*, vol. 1. Williams & Wilkins, Baltimore, Md, pp. 141-199.
- Sarris, P. F.; E. A. Trantas; E. Mpalantinaki; F. Ververidis; D. E. Goumas, 2012, *Pseudomonas viridiflava*, a multi host plant pathogen with significant genetic variation at the molecular level. *Plos One* 7: e36090. doi:10.1371/journal.pone.0036090.
- Schenk, A.; H. Weingart; M.S. Ullrich, 2008, The alternative sigma factor AlgT, but not alginate synthesis, promotes in planta multiplication of *Pseudomonas syringae* pv. *glycinea*. *Microbiology*, 03, pp. 413-421.
- Srivastava, A.; A. Al-Karablieh; S. Khandekar; A. Sharmin; H. Weingart; M.S. Ullrich, 2012, Genomic Distribution and Divergence of Levansucrase-Coding Genes in *Pseudomonas syringae*. *Genes*, 3, pp. 115-137.
- Srivastava, A.; D. Zhurina; M. S. Ullrich, 2009, Levansucrase and levan formation in *Pseudomonas syringae* and related organisms. In *Microbial Polysaccharides: Current Innovations and Future Trends*. Ullrich, M. (ed). Great Britain: Caister Academic Press, pp. 213-222.
- Swift, H. F., 1937, A simple method for preserving bacterial cultures by freezing and drying. *J. Bacteriol*, 33, pp. 411-421.

Wei, W.; R. E. Davis.; I. Lee.; Y. Zhao, 2007. Computer-simulated RFLP analysis of 16S rRNA genes: identification of ten new phytoplasma groups. Int J Syst Evol Microbiol, 57, pp. 1855-1867.

Young, J. M.; G. J. Cheesmur; F. V. Welham; W. R. Henshall, 1988, Bacterial blight of kiwi fruit. Ann. Appl. Biol., 112, pp. 91-105.

عزل وتعريف *Pseudomonas viridiflava* المسبب المرضي لتعفن ثمار الخيار

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ملخص

تعفن الثمار يسبب خسائر فادحة لمحصول الخيار المزروع داخل البيوت المحمية في الأردن، حيث إن التعفن عرض شائع للعديد من الأمراض النباتية البكتيرية. لذلك، تهدف هذه الدراسة لتعريف البكتيريا المسببة للمرض والتي يمكن اعتبارها مسبباً مرضياً ناشئاً في الأردن. تم أخذ عينات من ثمار الخيار المصابة بأعراض التعفن من مناطق مختلفة في الأردن، استخدمت هذه العينات في عزل البكتيريا والكشف عن وجود بكتيريا ممرضة للنبات باستخدام فحوصات LOPAT. تعرضت تسع عزلات ممثلة لمناطق مختلفة لإختبار القدرة المرضية على ثمار الخيار، التعريف بواسطة الفحوصات البيوكيميائية، تحديد تسلسل النوكليوتيدات في جين 16S-rDNA والهضم بواسطة إنزيمي *SacI* و *HinfI* عن طريق المحاكاة بالكمبيوتر. تميزت العزلات الممثلة بإنتاج مواد مخاطية صفراء على البيئة الغذائية مانيتول-غلوتاميت، إحداث أعراض تعفن على ثمار الخيار مماثلة للإصابة في الحقل، أعطت نتائج نموذجية لـ *P. viridiflava* في فحوصات LOPAT وتشابه لنتائج *P. viridiflava* في الفحوصات البيوكيميائية، وجود سبعة مواقع لإنزيم الهضم *HinfI* وعدم وجود أي موقع لإنزيم الهضم *SacI* في تسلسل النوكليوتيدات في جين 16S-rDNA و - بناءً على تسلسل ترتيب النوكليوتيدات في جين 16S-rDNA- تجمعت العزلات الممثلة مع النوع البكتيري *P. viridiflava*. ولذلك، فإنه من الممكن الإستنتاج بأن المسبب المرضي لتعفن ثمار الخيار في الأردن كان النوع البكتيري *P. viridiflava*.

الكلمات الدالة: ثمرة الخيار، تعفن، النوع البكتيري.

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