In vitro Biological Control of *Pseudomonas viridiflava* by *Pseudomonas fluorescens* via Siderophore Competition

***Nehaya Al-Karablieh*[1], *Amani Al-Dokh*[1], *Ihsan Mutlak*[1], *Zainab Abdulhadi*[2]**

ABSTRACT

Pathogenic *Pseudomonas viridiflava* has been recently isolated from diseased cucumber plants with fruit rotting symptoms. The pathogen is an opportunistic pathogen with wide host range, producing pyoverdins siderophores type. The aim of this study was to isolate antagonistic bacteria for *P. viridiflava* from phyllosphere of healthy cucumber plants. Screening for antagonistic bacteria was conducted by bacterial isolation and agar plate antagonistic assay. The potential antagonistic isolates were subjected to pathogenicity test, biochemical characterization and identification by 16S-rDNA sequencing. Among the yellowish fluorescent bacteria isolated, four isolates showed potential antagonistic activity against *P. viridiflava*. One selected isolate NK2 was subjected to siderophore production analysis. Biochemical characterization and identification by 16S-rDNA sequencing revealed that the isolates belonged to non- pathogenic species *P. fluorescens* biovar 1 group. Qualitative and quantitative analysis of siderophore production demonstrated that *P. fluorescens* NK2 was powerful in siderophore production which inhibited *in vitro* growth of *P. viridiflava*.

Keywords: Pseudomonas fluorescens, Siderophore, LOPAT, 16S-rDNA.

INTRODUCTION

Pathogenic *Pseudomonas viridiflava* has been recently isolated from diseased cucumber plants with fruit rotting symptoms (Al-Karablieh *et al*., 2016). The pathogen is an opportunistic pathogen with wide host range, like most of fluorescent *Pseudomonas* species, it is producing pyoverdins siderophores type (Bultreys and Gheysen, 2000; González *et al*., 2012; Nuebling *et al*., 2016). However, soil and seed treatments and foliar spray with chemicals are commonly adopted to combat bacterial plant diseases (Agrios, 2005). Continuous use of pesticides to inhibit the outbreak of pathogens develops pathogens with resistant traits which can be transmitted to humans (Baker *et al*., 1997; McManus *et al*., 2002). Many agrochemicals cause environmental damage, thus have been prohibited. An alternative approach to control plant diseases is application of antagonistic organisms that are able to suppress pathogen development by the so called biological control (Wilson 1997).

Antagonistic bacteria belonging to the genus *Bacillus* and *Pseudomonas* have been widely used in controlling bacterial diseases (Ganeshan and Kumar, 2005; Chen *et al*., 2013). *Pseudomonas* has some good characteristics as antagonistic agent due to the production of secondary metabolites like siderophores, antibiotics, volatile compounds, hydrolytic enzymes and growth promoting hormones (Keel *et al*., 1992; Duffy and Défago, 1999; Gupta *et al*., 2001; Fogliano *et al*., 2002; Compant *et al*.,

[1] The University of Jordan, Hamdi Mango Center for Scientific Research. ✉n.alkarablieh@ju.edu.jo
[2]Karolinska Institutet, Biomedical Laboratory Science
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2005; Sayyed *et al*., 2005). A good number of fluorescent *Pseudomonas* species have been reported as effective biocontrol agents against wide range of phytopathogens *in vivo* (Wensing *et al*., 2010; Maleki *et al*., 2010; Haggag and El Soud, 2012; Akter *et al*., 2014).

As a successful colonizer, *Pseudomonas* can inhibit both rhizosphere and phyllosphere (Akter *et al*., 2014). *Pseudomonas* inhibiting rhizosphere are exposed to root exudates which are produced by the plant, create nutrient supply and increase microbial population (Thomashow, 1996); whereas *Pseudomonas* inhibiting the phyllosphere are exposed to hostile environmental conditions with low nutrient content, sudden changes in temperature and humidity, and exposure to UV-radiation (Ji and Wilson, 2003; Stromberg *et al*., 2004). These hard conditions resulted in predictions of diverse bacterial isolates having antagonistic potential. So far, the majority of isolated and identified antagonistic Pseudomonads have been isolated from the rhizosphere and few of them were reported from the phyllosphere (Behrendt *et al*., 2007; Maleki *at al*., 2010). Moreover, very little information on antagonistic phyllosphere bacteria for cucumber fruit rotting management is available (Barth *et al*., 2009). Therefore, the aim of the present study was to isolate, identify and characterize antagonistic bacteria from phyllosphere of healthy cucumber plants that can be used as potential biocontrol agents for management of *P. viridiflava*, the causal agent of cucumber fruit rotting.

Materials and Methods

Bacterial strains and cultivation conditions

Bacterial strains used in this study are listed in Table 1. *Pseudomonas* strains and isolates were routinely grown on King's B medium (KB) (20 g peptone (BioBasic- Canada), 1.5 g K_2HPO_4 (Merck- Germany), 1.5 g $MgSO_4.7H_2O$ (Merck- Germany), 10 ml glycerol (AppliChem- Germany), 15 g agar (BioBasic- Canada) for 1 L, pH 7.2) as a complex medium, and Mannitol-Glutamate medium (MG) as minimal medium (10 g mannitol (BioBasic- Canada), 2 g L-Glutamic acid (Merck- Germany), 0.5 g KH_2PO_4 (Merck- Germany), 0.2 g NaCl (AppliChem- Germany), 0.2 g $MgSO_4.7H_2O$ (Merck- Germany), 15 g agar (BioBasic- Canada) for 1 L, pH 7.0).

Cultivation of bacterial strains under iron limiting conditions was conducted in different minimal media including; Chrome azurol S (CAS), 5b and pipes media. The compositions of CAS medium were as follows per liter of demineralized water: 60.5 mg of Chrome azurol S (Sigma-Aldrich), 72.8 mg of Hexadecyltrime thylammonium bromide (Sigma-Aldrich) and 2.7 mg of $FeCl_3.6H_2O$ (Merck- Germany) dissolved in 100 ml for CAS indicator solution, 0.3 g of KH_2PO_4, 0.5 g of NaCl, 1 g of NH_4Cl (Merck- Germany), 30.24 g of pipes and 15 g of agar dissolved in 800 ml pH adjusted with 50%KOH to 6.8 for pipes solution, 2 g of glucose (AppliChem-Germany), 2 g of Mannitol, 493 mg of $MgSO_4.7H_2O$, 11.3 mg of $CaCl_2$ (AppliChem- Germany), 1.17 mg of $MnSO_4.7H_2O$ (DB_ Chemical-England), 1.4 mg of H_3BO_3 (Merck- Germany), 0.04 mg of $CuSO_4.5H_2O$ (Merck-Germany), 1.2 mg of $ZuSO_4.7H_2O$ (Sigma-Aldrich) and 1 mg of $Na_2MoO_4.2H_2O$ (Sigma-Aldrich) dissolved in 70 ml for carbon source, and 30 ml of 10% of filter sterilized Casamino acids, the solutions were autoclaved separately and mixed 1: 8: 0.7: 0.3 prior to use (Alexander and Zuberer 1991).

The compositions of 5b medium were as follows per liter of demineralized water: 2.6 g of KH_2PO_4, 5.5 g of Na_2HPO_4 (Merck- Germany), 2.5 g of NH_4Cl and 1 g of Na_2SO_4 (Merck- Germany) for solution A, as well as 10 g of glucose, 0.1 g of $MgCl_2.6H_2O$ (Merck- Germany) and 0.01 g of $MnSO_4.4H_2O$ (Merck- Germany) for solution B. Solutions A and B were autoclaved separately and mixed 1:1 prior to use. The compositions of the pipes medium were as follows per liter of demineralized water: 30.24 g

of piperazine-*N*-*N*'-bis-2-ethanesulfonic acid (Duchefa Biochemie- Netherlands), 0.3 g of KH_2PO_4, 1 g of Na_2HPO_4 and 1 g of NH_4Cl for solution A (pH 7.0), as well as solution B of 5b medium. Solutions A and B were autoclaved separately and mixed 1:1 prior to use (Schwyn and Neilands 1987).

Plant materials and bacterial isolation

Healthy cucumber plants were collected from different field locations in the middle-region of Jordan valley; the plants were grown in green house; the samples included leaves, flowers and fruits. Around 0.5 g of plant materials were macerated in mortar and pestle by one ml of sterile 0.9% NaCl, left at room temperature for 10 min, inoculated (loop-full of macerated tissues) was on KB and MG plates, and incubated at 28 °C for 48 h (Hildebrand *et al*., 1988). Single fluorescent colonies (isolates) were selected for re-cultivation and cryopreservation in 15-20% Glycerol for further use (Swift, 1937).

Screening for antagonistic bacteria

Screening for antagonistic bacteria was conducted by agar plate assay based on the modified protocol of Burse *et al*., (2004). *P. viridiflava* 13223 and *P. viridiflava* NK7 were used as plant pathogenic strains, while fluorescent bacterial isolates, *P. fluorescens* 13525 and *P. syringae* pv. *syringae* 22d/93 were used as antagonistic strains. The bacterial strains and the isolates were grown on KB media at 28 ºC for 48 h, re-suspended in sterile 0.9% NaCl to an OD_{600} ~1.0 (corresponding to approximately 10^7 CFU/ml). A 100 µl of OD_{600}~1 from the pathogenic strains; *P. viridiflava* 13223 and *P. viridiflava* NK7 were spread separately on KB plate. A10 µl of OD_{600}~1.0 from *P. fluorescens* 13525, *P. s* pv. *syringae* 22d/93 and the fluorescent bacterial isolates were applied separately on the inoculated KB plates with pathogenic strains. The plates were incubated 48 h at 28 °C. A 10 µl of 25 mg/ml of Chloramphenicol (Cm) was used as a positive control on five mm in diameter filter disc. Zones of growth inhibition caused by the isolates were examined by visual inspection (Burse *et al*., 2004).

In order to avoid selection of plant pathogenic bacteria as antagonistic agents, 48 h-old of the fluorescent bacterial isolates were subjected to LOPAT determinative tests as described in Al-Karablieh *et al* (2016). In all tests, sterile 0.9% NaCl was used as negative control. *P. fluorescens* 13525 and *P. viridiflava* 13223 were used as reference strains. All experiments were repeated three times to confirm reproducibility of the results.

Biochemical characterization

Bacterial isolates obtained from bacterial isolation form healthy cucumber plants and that showed typical LOPAT profile for *P. fluorescens* were subjected to biochemical characterization in comparison to *P. fluorescens* 13525. The biochemical tests were conducted by API-NE and API-E (BioMérieux, Marcy-l'Étoile, France), according to the manufacturer's instructions.

16S-rRNA amplification and sequencing

Out of the bacterial isolates that showed 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 four bacterial isolates using the forward primer 16S-rRNA Fwd: 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 16S-rRNA Rev: 5'-TACGG(CT) TACCTTGTT ACGCTT-3' (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 $MgCl_2$, 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. fluorescens* 13525 was used as a positive control. The amplicons were sequenced at Macrogen, South Korea.

Table 1: Bacterial strains used in this study.

Strain	Source	Reference
P. viridiflava 13223	ATCC* Type strain	
P. viridiflava NK7	Wild type isolated from rotted cucumber fruit	Al-Karablieh *et al*., 2016
P. fluorescens 13525	ATCC Type strain	
P. syringae pv. syringae 22d/93	Wild type isolated from soybean	Völksch *et al*., 1996

*ATCC: American Type Culture Collection.

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 as follows: *P. fluorescens* 13525, Z76662; P. *fluorescens* Pf0-1, NR_102835; *P. viridiflava* 13223, NR_114482; *P. aeruginosa* LMG 1242T, Z76651; P. protegens pf-5, NR_ 074599; *P. amygdali* LMG 2123T, Z76654; *P. cichorii* LMG 2162T, Z76658; *P. marginalis* LMG 2210T, Z76663; *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. *lachrymans* 361, KC860048; *P. syringae* pv. *syringae* LMG 1247 t1T, Z76669; and *P. syringae* pv. *tomato* DC3000, NR_074597 (Moore *et al*., 1996; Buell *et al*., 2003).

Growth under iron-limiting condition and detection of siderophore

Siderophores production was visualized on CAS agar plate (Alexander and Zuberer 1991). *P. viridiflava* 13223, *P. viridiflava* NK7, *P. s* pv. *syringae* 22d/93 and the newly identified *P. fluorescens* NK2 (the most close isolate to *P. fluorescens* Pf0-1) were grown in KB broth at 28 ºC for 48 h, bacterial growth was adjusted to an OD_{600} ~1.0, and 5 µl of each suspension was spotted on CAS agar plate, and incubated for 48 h at 28°C. Appearance of a reddish-brown zone surrounding the colony on CAS agar plate suggested siderophore production (Schwyn and Neilands 1987). The experiment was repeated three times to confirm reproducibility of the results.

Different media were used to investigate siderophore production by *P. fluorescens* NK2 under iron limiting conditions in comparison to *P. viridiflava* 13223, *P. viridiflava* NK7 and *P. s* pv. *syringae* 22d/93. The selected media were KB broth as complex medium, 5b and pipes as minimal media as suggested by (Wensing *et al*., 2010).

Siderophores content was quantified using the CAS assay as described by Schwyn and Neilands (1987). Briefly, 500 µl of CAS indicator solution containing 4 mM sulfosalicylic acid (Sigma-Aldrich) were mixed with the same volume of cell free supernatants. The reaction mixtures were incubated for 60 min at room temperature to allow complex formation, and the siderophore-dependent color change was determined at a wavelength of 630 nm. For quantification, deferoxamin mesylate (DFOM) (Sigma-Aldrich) was used as the standard, and there was a linear relationship between de-colorization and the DFOM concentration in the range from 0 to 100 µM.

Antagonistic assay by purified siderophore

Purification of siderophore was conducted by Amerlite XAD resin as described by Sayyed and Chincholkar (2006). Briefly, *P. fluorescens* NK2 was grown in pipes medium at 28ºC for 48 h, bacterial growth were adjusted to an OD_{600} ~1.0, cell free supernatant was desalted by XAD-4 resin (Sigma-Aldrich) and concentrated 10X by vacuum concentrator (Eppendorf-Germany) (Wensing *et al*., (2010).

Antagonistic assay by purified siderophore was conducted by agar plate diffusion assay as described by *Burse et al*., (2004), 100 µl of OD_{600}~1 from *P. viridiflava* 13223 and *P. viridiflava* NK7 were inoculated separately on KB plates. Twenty microliter of the 10X concentrated cell free supernatant was loaded in 5 mm in diameter well. Chloramphenicol was used as a positive control (10 µl of 12.5 mg/ml) and 10X concentrated desalted pipes medium was used as a negative control. The inoculated plates were incubated at 28 °C for 48 h, growth inhibition zones caused by the purified siderophore were monitored by visual inspection after 48 h incubation at 28°C (Burse *et al*., 2004). The experiment was repeated three times to confirm reproducibility of the results.

Statistical analysis

Analysis of ANOVA and Fisher's least significant differences at P = 0.05 have been conducted by IBM SPSS Statistics 24.

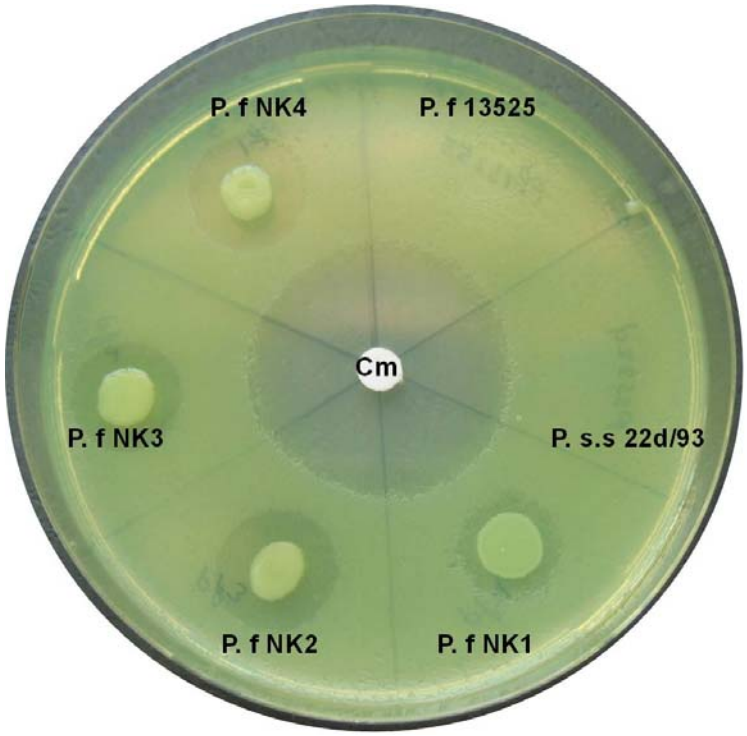

Fig. 1: Antagonistic assay by fluorescent bacterial isolates. Pathogenic *P. viridiflava* suspensions (100 µl100µl of OD_{600} ~1.0) were spread on KB medium, after drying bacterial suspension (10µl of OD_{600} ~1.0) of the potential antagonistic isolates/ strains were applied, Chloramphenicol (Cm) 10 µl10µl of 25mg/ ml was applied on 5 mm in diameter filter disc.

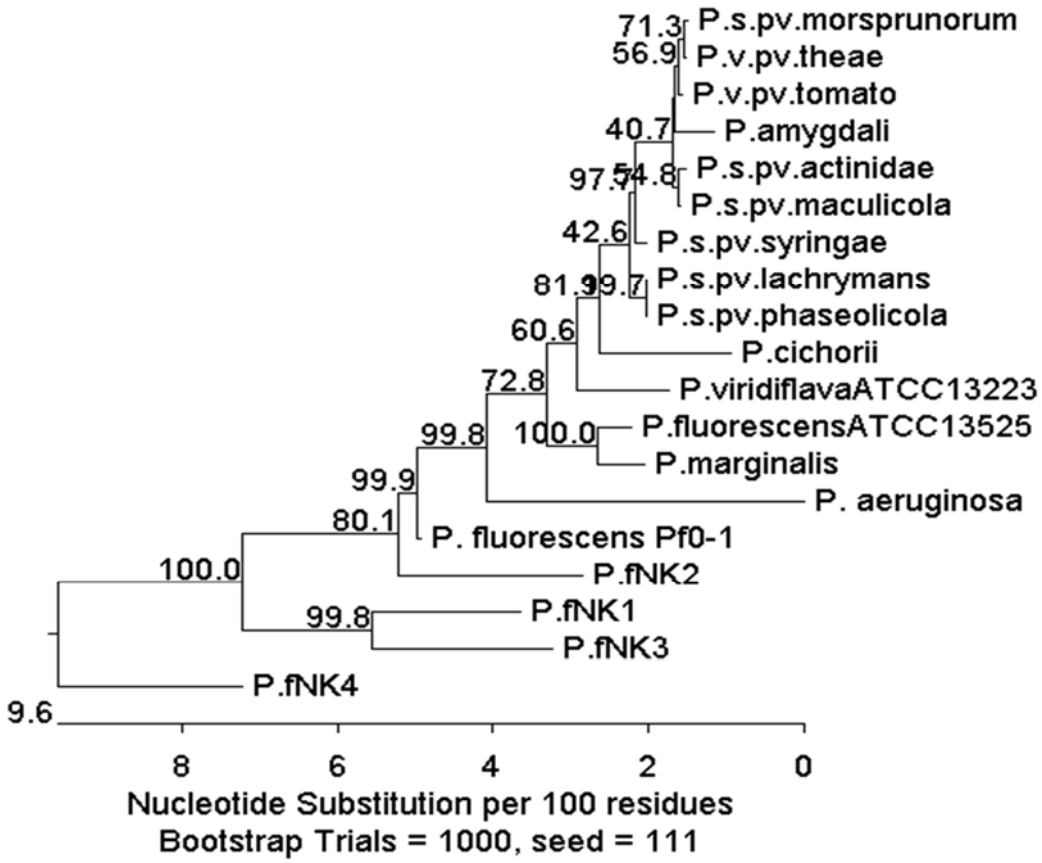

Fig. 2: Rooted Phylogenetic tree for 16S rDNA among the *P. fluorescens* isolates and members of the genus *Pseudomonas*. The dendrogram was generated in MegAlign version 13 (DNASTAR lasergene Inc.) by the ClustalW method. 16S rDNA of the new isolates belong to a cluster containing *P. fluorescens*.

Results

Bacterial isolation and screening of antagonistic agents

A total of 35 bacterial isolates have been isolated from the phyllosphere of healthy cucumber plants grown in greenhouses in the middle-region of Jordan valley, where cucumber is considered to be the most important crop. The bacterial isolates characterized by producing yellowish fluorescent pigments and varied between mucoid and non-mucoid colonies on KB and MG media.

Among them, only 4 isolates showed antagonistic activity against used pathogenic *P. viridiflava* strains by formation of clear inhibition zones on the plates inoculated by *P. viridiflava* 13223 and *P. viridiflava* NK7; whereas *P. fluorescens* 13525 and *P. s* pv. *syringae* 22d/93 were unable to form the inhibition zones (Fig. 1). The formed inhibition zones varied in mm in diameters in rage 13- 19 for NK4 and NK2 isolates respectively, there was no significant differences between NK1, NK2 NK3 isolates, but there was significant difference between them and NK4 isolate. It was notable that *P. viridiflava* NK7 was more sensitive to chloramphenicol than *P. viridiflava* 13223 (Table 2).

Table 2: Susceptibility of *P. viridiflava* strains to antagonistic[a] isolates on KB medium.

Inhibition zones (mm in diameter)[b]							
Pathogenic strains	**Antagonistic strain and isolates**						
	Cm	***P. s. s* 22d\93**	***P. f* 13525**	***P. f* NK1**	***P. f* NK2**	***P. f* NK3**	***P. f* NK4**
P. viridiflava 13223	30±0.6[a*]	0[d]	0[d]	16±0.5[b]	19±0.3[b]	16±0.4[b]	13±0.0[c]
P. viridiflava NK7	13±0.7[c]	0[d]	0[d]	17±0.6[b]	19±0.5[b]	16±0.6[b]	15±0.8[bc]

[a] Antagonistic assay by fluorescent bacterial isolates, bacterial suspension (100 µl of OD_{600} ~1.0) were spread on KB medium, after drying bacterial suspension (10 µl of OD_{600} ~1.0) of the isolates/ strains were applied. *P. viridiflava* 13223 was used as pathogenic isolates. Cm: Chloramphenicol (10 µl of 25 mg/ml) was applied on 5-mm in diameter filter disc. [b] Assay was repeated three times and the average of three replicates was recorded ± standard deviation. Data were evaluated with analysis of ANOVA at Fisher's least significant differences at $P = 0.05$.

In order to avoid selection of plant pathogenic bacteria as antagonistic agents, the four isolates that showed positive results as potential antagonistic isolates were subjected for LOPAT determinative tests in comparison to the *P. fluorescens* 13525 and plant pathogenic strain *P. viridiflava* 13223. The potential antagonistic isolates were similar to *P. fluorescens* 13525 in term of positive reaction in oxidase, arginine dihydrolase and levan formation tests, but negative in pectinolytic activity tests except NK1, which was able to cause rotting halos surrounding the inoculation site when assayed on potato slices. Finally neither *P. fluorescens* 13525 nor the putative antagonistic isolates were able to elicit the hypersensitive response on tobacco plant. In contrast to plant pathogenic strain *P. viridiflava* 13223 was negative in oxidase, arginine dihyrolase and levan formation, and positive in pectinolytic activity and able to elicit hypersensitive response on tobacco plant (Table 3).

Table 3: Isolates relevant features LOPAT profile analyzed in this study, isolated from healthy cucumber plants

Isolate/ strains	LOPAT profile				
	L	**O**	**P**	**A**	**T**
P. f NK1	+	+	+	+	-
P. f NK2	+	+	-	+	-
P. f NK3	+	+	-	+	-
P. f NK4	+	+	-	+	-
Control strains					
P. fluorescence 13525	+	+	-	+	-
P. viridiflava 13223	-	-	+	-	+

+: indicated positive reaction; - : indicated negative reaction.

Identification of the potential antagonistic isolates.

The phenotypic profiling by biochemical tests for the isolates that showed potential antagonistic activity revealed that those isolates had the similar biochemical properties (Table 4), for instance, all were gram negative, hydrolyzed gelatin, produced tryptophane deaminase, and the same set of carbon compounds. These biochemical properties were all shared by *P. fluorescens* 13525.

Table 4: Some phenotypic characteristics of *P. fluorescens* isolates, isolated from healthy cucumber plants.

Test	Tested antagonistic isolates				
	***P. fluorescence* 13525**	***P. f* NK1**	***P. f* NK2**	***P. f* NK3**	***P. f* NK4**
Gram staining	-	-	-	-	-
Reduction of nitrates	-	-	-	-	-
Indole production	-	-	-	-	-
Tryptophan deaminase	+	+	+	+	+
Urease production	-	-	-	-	-
H_2S production	-	-	-	-	-
Esculin hydrolysis	-	-	-	-	-
Gelatin hydrolysis	+	+	+	+	+
Galactopyranoside	-	-	-	-	-
Acetoin production	-	-	-	-	-
Oxidative\ Fermentative	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	+	+	+	+	+

Test	Tested antagonistic isolates				
	***P. fluorescence* 13525**	***P. f* NK1**	***P. f* NK2**	***P. f* NK3**	***P. f* NK4**
Citrate	-	-	-	-	-
Phenyl-acetate	-	-	-	-	-
Amygdalin	-	-	-	-	-

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

For accurate identification of the isolates, the complete 16S rDNA fragments from the four isolates with potential antagonistic activity were sequenced after amplification (Table 1). Analysis of the 16S rDNA sequences revealed that those isolates were related to each other (91.2% identity over 1385 nucleotides), and they were closely related to 16S rDNA sequences of *P. fluorescens* Pf0-1. These results clearly indicated an affiliation of the four analyzed isolates to the bacterial species *P. fluorescens.* The potential antagonistic isolates were designated as per described in Table 1; P. *fluorescens* NK1, *P. fluorescens* NK2, P. *fluorescens* NK3 and P. *fluorescens* NK4 respectively for the isolates; NK1, NK2, NK3 and NK4.

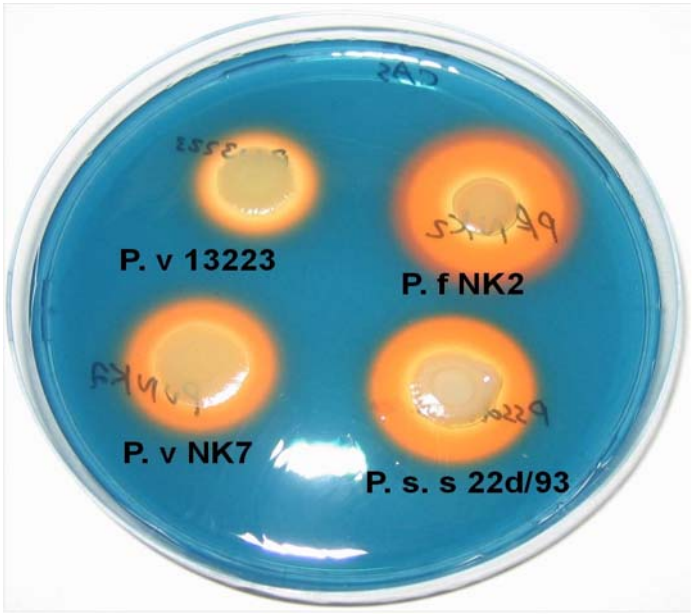

Fig.3: Detection of the siderophore by CAS agar plate assay. Bacterial suspensions (5 µl of OD_{600} ~1.0) from each bacterial strain were applied on CAS agar plate, formation of a reddish-brown zone surrounding the colony on CAS agar plate after 48 h at 28°C suggests siderophore production (Schwyn and Neilands 1987).

Nucleotide sequence accession numbers.

Sequences generated in this work have been deposited in the GenBank database under accession numbers KU686701- KU686704.

Siderophore production by *P. fluorescens* NK2 in different media.

Detection of siderophore production on CAS agar plates showed that there was a remarkable difference

between the antagonistic strains (*P. s* pv. *syringae* 22d/93 and *P. fluorescens* NK2) and the pathogenic strains (*P. viridiflava* 13223 and *P. viridiflava* NK7); a significantly larger siderophore halo was produced by antagonistic strains than by pathogenic strains (Fig. 3). The same tendency was observed in various low-iron liquid media when the siderophore activities of supernatants were determined by the CAS assay and normalized using cell density. In all media tested the siderophore production by *P. fluorescens* NK2 was greater than that by *P. viridiflava* strains (Fig. 4).

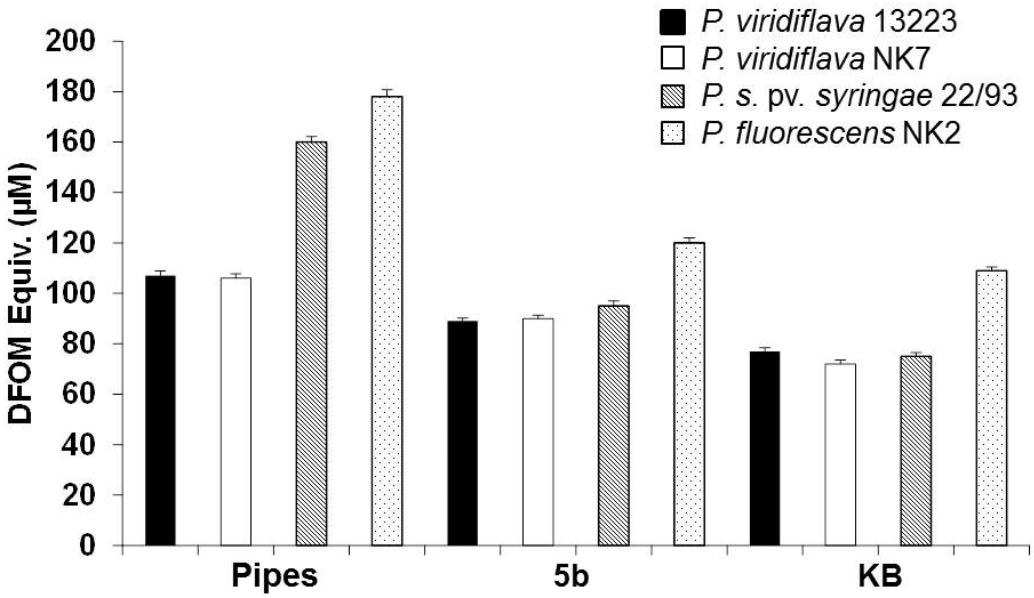

Fig. 4: Influence of culture media on siderophore production. *Bacterial strains* were cultivated in various low-iron media at 28°C for 48 h. The siderophore activity of supernatant was determined by the means and standard deviations of three independent experiments.

Antagonistic assay by purified siderophore

Antagonistic agar plate diffusion was conducted by purified siderophore produced by *P. fluorescens* NK2 in pipes medium against plant pathogenic strains of *P. viridiflava*. The results showed that the purified siderophore produced by *P. fluorescens* NK2 inhibited the *in vitro* growth of plant pathogenic strains of *P. viridiflava*, where clear growth inhibition zone was observed around the well filled with purified siderophore produced by *P. fluorescens* NK2 (Fig. 5).

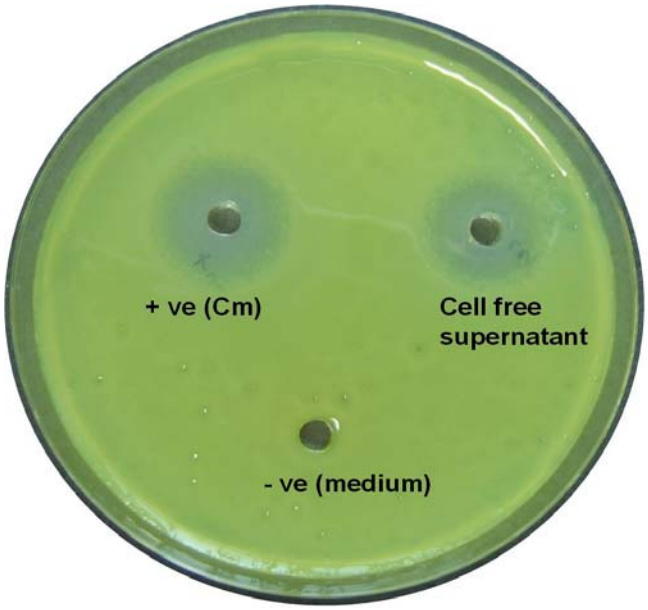

Fig. 5: Antagonistic assay by siderophore produced in pipes medium. KB plate was inoculated with 100 µl of OD_{600} ~1.0 of *P. viridiflava* 13223, after drying the plate; 10 µl of the concentrated XAD-4 treated cell free supernatant of *P. fluorescens* NK2 grown in pipes medium were loaded in 5mm in diameters well, 10 µl of 12.5 mg/ml of chloramphenicol and 10 µl of 10X concentrated desalted pipes medium.

Discussion

Over the last decades, many studies have reported on natural activity of some bacteria against plant pathogens, and this is considered as a very appealing alternative to the use of chemical fungicides (Gerhardson 2002; Welbaum *et al*., 2004). The rhizosphere microorganisms, especially fluorescent pseudomonads, have exceptional ability to promote the growth of host plant by different mechanisms (Joseph *et al*., 2007), and have different mechanisms to suppress plant diseases including production of antibiotics, efficient root colonization and production of powerful siderophores (Wensing *et al*., 2010; Maleki *et al*., 2010).

In the current study, 35 bacterial isolates were isolated from phyllosphere of healthy cucumber samples collected from greenhouses in the middle-region of Jordan valley. All the bacterial isolates produced yellowish fluorescent pigments on KB and MG media; thus it can be predicted as fluorescent *Pseudomonas*, this results isresult is in agreement with the results of Behrendt *et al*., (2003), where it was reported that fluorescent *Pseudomonas* was the typical inhabitants of the grasses phyllosphere.

All isolates were subjected to several screening procedures for selecting the best isolates for effective antagonistic agent against plant pathogenic *P. viridiflava* strains. Among tested isolates, four isolates namely; NK1, NK2, NK3 and NK4 were effective isolates against *P. viridiflava* strain and selected for further investigations. It is worth to mention that *P. viridiflava* 13223 was more susceptible to chloramphenicol than the Jordanian isolate *P. viridiflava* NK7, which might be related to heavy use of chemical pesticides in Jordan.

The potential antagonistic isolates were subjected to LOPAT determinative tests, the results of LOPAT tests demonstrated that NK2, NK3 and NK4 were missing important virulence factors, the *hrp* type III protein

secretion system that is involved in secretion of effector and avirulence factors into the host cell (Araki *et al*., 2006; Araki *et al*., 2007), and extracellular pectate lyase that is responsible for plant tissue maceration (Liao *et al*., 1994). Furthermore, isolates NK2, NK3 and NK4 showed typical LOPAT profile for *P. fluorescens* (Hildebrand *et al*., 1988).

The phenotypic profiling revealed that nutritional and physiological requirements of antagonistic isolates were almost similar to *P. fluorescens* 13525 which belonged to *P. fluorescens* biovar 1 (Palleroni 1984). The accurate species identification of the antagonistic isolates was conducted by analysis of the nucleotide sequences of 16S rDNA genes (Moore *et al*., 1996). The results revealed that the isolates were highly related to *P. fluorescens* Pf0-1, which has been described by Silby *et al*., (2009) as soil bacteria that interact with plant's nutrient cycling, pathogen antagonism and induction of plant defenses. The most highly isolate related to *P. fluorescens* Pf0-1 was NK2 (*P. fluorescens* NK2), thus, it was selected for further investigation.

However, the main problem of biological control is its low consistency and reliability under field conditions due to a high variability in efficacy (Ojiambo, 2006). The complex *in situ* condition in the natural phyllosphere influences survival, growth and production of secondary metabolites by the antagonistic strains (Lindow and Brandl, 2003). One of the important measures in developing reliable biocontrol systems is to identify the antagonistic mechanisms (Dong *et al*., 2007). In the present study, the ability of selected isolates for production of siderophores was investigated *in vitro*.

Siderophores chelates iron and other metals contribute to disease suppression by conferring a competitive advantage to antagonistic agents for the limited supply of essential trace minerals in natural habitats (Ahmed and Holmström, 2014). Siderophores may indirectly stimulate the biosynthesis of other antimicrobial compounds by increasing the availability of these minerals to the bacteria (Duffy and Défago, 1999). Results from the qualitative and quantitative estimations of siderophore produced by *P. fluorescens* NK2 showed that it is a powerful producer of siderophores, and the produced siderophores were able to *in-vitro* suppress growth of both tested *P. viridiflava* strains. Suppression of phytopathogens by *P. fluorescens* were reported; *P. fluorescens* ATCC17400 inhibited growth of *Pythium debaryanum*, the causal agent of damping off disease by pyoverdine siderophore (Cornelis *et al*., 1992), *P. fluorescens* fp-5 inhibited growth of *Botrytis cinerea* by its secondary metabolites (Haggag and Abo El Soud, 2012).

We report here that an antagonistic bacterial agent has been successfully isolated from healthy cucumber phyllosphere, identified as *P. fluorescens. P. fluorescens* NK2 has *in vitro* antagonistic activity against *P. viridiflava* and might resist the destructive effects of *P. viridiflava* in cucumber plants.

It was reported that *P. fluorescens* strains can produce different types of siderophores (pyoverdine and quinolobactin) under different environmental conditions which modulate biosynthesis of siderophores (Duffy and Defago, 1999; Mossialos *et al*., 2000). Therefore, the future aspect is to further investigate the antagonistic activity *in planta* and in field conditions, and to analyze the type of siderophores produced by *P. fluorescens* NK2 via mutational analysis of siderophore genes.

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المكافحة البيولوجية لبكتيريا Pseudomonas viridiflava بواسطة بكتيريا Pseudomonas fluorescens عن طريق التنافس على حاملات الحديد في ظروف المختبر

نهاية كرابلية[1]، اماني الدوخ[1]، احسان مطلق[1]، زينب عبد الهادي[2]

ملخص

تم عزل النوع البكتيري *Pseudomonas viridiflava* من نباتات خيار مصابة بأعراض تعفن الثمار. تهدف هذه الدراسة لعزل بكتيريا عدائية للنوع البكتيري *P. viridiflava*. واستخدمت عينات نباتية جمعت من المجموع الخضري لنباتات الخيار السليم في العزل البكتيري والبحث عن بكتيريا عدائية، بعد ذلك تم تعريض العزلات العدائية المتوقعة لفحص القدرة المرضية، والوصف البيوكيميائي والتعريف بواسطة تحديد تسلسل النوكليوتيدات في الحمض النووي لجين 16S-rDNA. أختيرت عزلة واحدة، تحديداً، NK2 لتحليل إنتاج حاملة الحديد. تميزت العزلات التي تم الحصول عليها بأنها ذات وميض أصفر، من ضمن هذه العزلات أظهرت أربعة فقط قدرتها العدائية ضد النوع البكتيري *P. viridiflava*. وأظهرت نتائج الفحوصات البيوكيمائية والحمض النووي أن العزلات تنتمي للنوع البكتيري *P. fluorescens* غير الممرض، (مجموعة 1). وأظهر التحليل الكمي والنوعي لحاملات الحديد المنتج من قبل العزلة المختارة أنها ذات قدرة لإنتاج حاملات حديد قادرة على تثبيط نموالنوع البكتيري *P. viridiflava* في المختبر. يمكن أن نخلص بأنه تم بنجاح عزل عامل عدائي وتم تعريفه على أنه *P. fluorescens* وأن العزلة المختارة NK2 *P. fluorescens* لها قدرة عدائية لتثبيط نموالنوع البكتيري *P. viridiflava* في المختبر، ومن المحتمل أن تقاوم التأثير المدمر لـ *P. viridiflava* في النبات.

الكلمات الدالة: النوع البكتيري *Pseudomonas viridiflava*، التنافس على الحاملات.

[1] مركز حمدي منكو للبحوث العلمية، الجامعة الأردنية.

[2] معهد كارولينسكا، العلوم الطبية المخبرية.

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