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Research Article

Survey of fluorescent pseudomonads from rhizosphere and rhizoplane of tomato for biocontrol of *Clavibacter michiganensis* subsp. *michiganensis*

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Abstract: Tomato bacterial wilt and canker caused by *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) is an economically important seed-borne disease in the major tomato growing regions all over the world. Also, this disease is present in northern, northwestern, and central provinces of Iran. This study aimed to isolate fluorescent pseudomonads with efficient antagonistic activity against Cmm from tomato rhizosphere and rhizoplane. In the present study, ninety six fluorescent pseudomonads were isolated from rhizosphere and rhizoplane of tomato plants using King's medium B agar and tested for antagonistic activity against Cmm by co-inoculation culture *in vitro*. Seed and roots of tomato transplants were inoculated with representative antagonistic strains and planted in the soil infected by Cmm in greenhouse. The incidence and severity of the disease were assessed on tomato as well as growth parameters. Nineteen strains exhibited inhibitory activity against Cmm *in vitro* and two selected strains (M1R1 and H1R1) were identified as *Pseudomonas putida* based on their phenotypic characteristics and partial 16S rRNA gene sequences. These two strains produced siderophore and hydrogen cyanide. In greenhouse, strain M1R1 reduced the disease incidence by 30% and 40% in Superluna and Falat cultivars, respectively. Strain H1R1 reduced the disease incidence by 20% only in Falat cultivar. Strains M1R1 and H1R1 decreased the disease severity by 52.93 % and 10.60 % in Superluna and 47.90 % and 42.88 % in Falat cultivars, respectively. Strain M1R1 increased the growth parameters, including fresh and dry weight, and height of the inoculated plants significantly in comparison with control. These results indicated that rhizospheric fluorescent pseudomonads could be effective agents in the biocontrol of tomato bacterial wilt and canker disease.

Keywords: Antagonistic activity, Biological control, *Pseudomonas putida*, Siderophore, Tomato bacterial canker

Introduction

Bacterial wilt and canker of tomato caused by *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) is an important disease due to its considerable economic losses (nearly 100% crop loss) (Gitaitis, 1990; Chang *et al*., 1992c; Boudyach *et al*., 2001). The disease was initially reported from Michigan in 1909 (Smith, 1910). But to date, the disease has been reported from major tomato growing areas all over the world (Tsiantos, 1987; Gitaitis, 1990; Fatmi *et al*., 1991; Chang *et al*., 1992a, 1992b; Boudyach *et*

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al., 2001; Myung *et al*., 2008; Ftayeh *et al*., 2010). Moreover, Cmm can affect other economically important crops, such as pepper and tobacco (Gleason *et al*., 1993). In Iran, bacterial wilt and canker of tomato was first observed in Urmia, West Azerbaijan province (Mazarei and Orumchi, 1993), and then the disease has been reported from Golestan, West Azerbaijan East Azerbaijan, Zanjan, Qazvin, and Kohgiluyeh-Boyer-Ahmad provinces (Nazari *et al*., 2008; Osdaghi *et al*., 2018). But no severe outbreaks of the disease were detected in these areas (Osdaghi *et al*., 2018).

Symptoms of bacterial wilt and canker disease consist of stunting, wilting, curling of leaflets, browning of leaves, vascular discoloration, fruit lesions, and development of stem cankers (EPPO, 2016). Spots on fruits have the dark brown centers with a white halo called "bird's-eye" spots. The causal pathogen can occur on the tomato seed coat (externally) through fruit lesions or within the seeds (internally) via the vascular system of fruit tissues (Ribeiro *et al*., 2016). Infected seeds are a source of the bacterium for long periods. Also, the pathogen can survive for months to years in soil, greenhouse structures, and pieces of equipment (Chang *et al*., 1991). Seed treatment with biocides reduces the percentage of infected seeds, but the internally-infected seeds will not be completely free of bacteria (de León *et al*., 2011). Wilting and death in tomato plants, reduction of photosynthetic capacity, reduction in the quality and quantity of fruit are yield losses in the field, and greenhouses (Xu *et al*., 2012). Successful control of tomato bacterial wilt and canker is a serious problem for tomato cultivation worldwide. The possible methods to reduce the considerable damage caused by Cmm are removing and destroying infected plants and providing seeds and seedlings free of the pathogen (Chang *et al*., 1991; Mansfeld-Giese, 1997).

Few commercially grown cultivars of tomato have significant tolerance or resistance to Cmm (Gleason *et al*., 1993). Biological control using plant growth-promoting rhizobacteria (PGPR) is a promising alternative to protect tomato plants against Cmm (Boudyach *et al*., 2004). PGPRs have been strongly considered as potential biocontrol agents that can control many bacterial and fungal diseases and improve plant growth indirectly (Kloepper, 1983; Liu *et al*., 1995; Walsh *et al*., 2001; Amkraz *et al*., 2010). Also, their direct effects include either producing or changing the concentration of plant growth regulators or solubilization of mineral phosphates and other nutrients. Fluorescent pseudomonads (FLPs) belonging to PGPRs exhibit an enormous metabolic versatility and produce a vast array of extracellular secondary metabolites including phenazines, 2, 4-diacetylphloroglucinol, rhizoxins, HCN, siderophores, and others (Haas and Défago, 2005; Oni *et al*., 2015) which reduce or prevent the deleterious effects of phytopathogenic microorganisms (Ahmad *et al*., 2008). So far, fluorescent pseudomonads were used as seed and root treatment before transplanting for biological control of Cmm (Boudyach *et al*., 2004). Also, the reduction of the incidence of tomato bacterial canker disease has been evaluated by seed treatment with *Pseudomonas fluorescens* in the field (Umesha, 2006) and seedling treatment with *Bacillus subtilis* and *Trichoderma harzianum* (Root Shield) under greenhouse conditions (Utkhede and Koch, 2004). Given the important role of antibiotics in the biocontrol activity of fluorescent pseudomonads, some genes such as *pltC* (pyoluteorin), *prnD* (pyrrolnitrin) (de Souza and Raaijmakers, 2003), *phzCD* (phenazine-1-carboxylic acid), and *phlD* (2, 4-diacetylphloroglucinol) have been studied in different species (Raaijmakers *et al*., 1997). Therefore, it is valuable to isolate beneficial and eco-friendly strains of these bacteria, which have antagonistic activity against phytopathogens to use as a safe alternative for the harmful pesticides. Thus, the present study focused on the isolation of fluorescent pseudomonads from the rhizosphere and rhizoplane of tomato plants as well as *in vitro* and *in vivo* evaluation of their antagonistic potential towards Cmm.

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Materials and Methods

Sampling and isolation of fluorescent pseudomonads

To isolate fluorescent pseudomonads, a total of 60 soil and root samples of healthy tomatoes were collected from several fields in East and West Azerbaijan provinces, north-west Iran. One gram of rhizosphere soil and root segments was added to 100 ml of sterile distilled water and shacked at 120 rpm for 30 min. The resulted suspensions were serially diluted (10^{-1}- 10^{-3}), spread 20 µl of each dilution on King's medium B (KB) agar and incubated at 27 ºC for 48 h. The bacterial colonies with fluorescent pigments were subcultured on nutrient agar (NA) medium. The isolated strains were stored in the nutrient broth medium containing 25% (v/v) glycerol at -70 ºC for further studies.

Phenotypic characterization of fluorescent pseudomonad strains

Phenotypic characteristics of the bacterial strains were determined based on various tests, including gram reaction, oxidase and catalase activity, soft rot on potato slices, hypersensitive reaction on tobacco plant (HR), gelatin and starch hydrolysis, arginine dihydrolase, levan formation, fluorescent pigment on KB agar, growth at 4 and 41 °C (Fahy and Persley, 1983; Schaad *et al*., 1988). These tests were conducted with three replications.

Pathogenicity tests

Seven Cmm strains (namely 4, 21, 45, 41, 60, 64, and 83) were donated by the Iranian Research Institute of Plant Protection, Tehran, Iran. Pathogenicity of the Cmm strains was evaluated on tomato seedlings (cv. Superluna) growing in pots containing a mixture of sand and peat (1:2 v/v) at the second true leaf stage. The suspensions of the Cmm strains (OD_{600nm} = 0.4, approx. 10^7-10^8 CFU/ml) were prepared from overnight cultures grown on Nutrient-Broth Yeast Extract Agar (NBYA) medium at 28 ºC. The seedlings were inoculated by injection of 50 µl of each bacterial suspension into their stem. Plants inoculated with sterile distilled water served as negative control. Each treatment was carried out with three replications. Plants were kept in a greenhouse at 26 ºC and 80% relative humidity with a 16-h light/8-h dark and closely supervised for occurrences of wilting and stem canker for seven weeks. To fulfill Koch's postulates, the bacteria were isolated from 2 cm above the inoculation points of the inoculated plants (Kado, 2010).

In vitro antagonistic activity of the fluorescent pseudomonads

The strains were screened against the Cmm strain 64 which was selected in the pathogenicity tests, using a co-inoculation technique as described by Ryan *et al*., (2004). Suspensions of fluorescent pseudomonads (10^8 CFU/ml) were spotted onto KB agar and incubated at 26 ºC for 24 h. The bacterial colonies on the agar plates were cleaned by sterile cotton and killed with chloroform vapors for 30 min. Plates were aerated for 30 min and a suspension of Cmm (10^8 CFU/ml) was spread on the medium. The plates were incubated at 26 ºC for 24 h, and the diameter of the inhibition zones was measured. Antagonistic activity was tested in a completely randomized design with three replications.

Antibiotic production test

The fluorescent pseudomonads were spotted onto 1% Nutrient Agar Glucose (NAG) medium saturated with $FeCl_3$ (1000 µmol/ml) and incubated at 26 °C for 24 h. Then colonies on agar plates were cleaned by sterile cotton and sterilized with chloroform vapors for 30 min. Petri plates were aerated in sterile conditions for 30 min. A suspension of the Cmm strain 64 (10^8 CFU/ml) selected in the pathogenicity test was spread on the medium. The presence of the inhibition zone representing antibiotic production was monitored after 24 h of incubation (Weller and Cook, 1983).

[DOR: 20.1001.1.22519041.2020.9.3.5.6]

Siderophore production test

The selected antagonist strains were spotted onto 1% Nutrient Agar Sucrose (NAS) and NAS containing $FeCl_3$ (1000 µmol/ml) media and incubated at 26 ºC for 24 h. Then the bacterial colonies were cleaned by sterile cotton and killed with chloroform vapors for 30 min. Petri plates were aerated in sterile conditions for 30 min and incubated at 60 ºC for four h to inactivate any likely produced antibiotics. A suspension of the Cmm strain 64 (10^8 CFU/ml) was spread on the media, and the plates were incubated for 24 h. The absence of the inhibition zone in the NAS medium containing $FeCl_3$ and the presence of the inhibition zone in the NAS medium was regarded as representing siderophore production.

Hydrogen cyanide (HCN) production

The selected antagonistic strains were examined for the production of hydrogen cyanide by adapting the method of Lorck, (1948). Briefly, the nutrient broth medium was amended with 4.4 g glycine/l, and the strains were streaked on the modified agar plate. A Whatman filter paper no. 1 was saturated with 0.5% picric acid solution and socked with 2% sodium carbonate solution. Then, the paper was placed at the top of the plate. Plates were sealed with parafilm and incubated at 28 ºC for four days. One plate without inoculation of bacteria was considered as control. HCN production was determined by the change in color of the filter paper from orange to red. Color changes, according to intensity, were considered as low, medium, high, and very high ability in producing HCN.

Cotyledon test for screening of antagonistic fluorescent pseudomonads

The cotyledons of four-day-old tomato seedlings were dusted with carborundum powder, lightly abraded between the fingers and simultaneously inoculated with a suspension containing approx. 10^7-10^8 CFU/ml of the Cmm strain 64 and the antagonist strains H1R1, M1R1, G2R1, G2R4, and H3R1. Also, infection of the cotyledons with the Cmm strain 64 was performed two hours after inoculation of the antagonistic strains (discontinuous inoculation). The incidence of the spots was recorded by counting the total number of plants and the number of plants showing the characteristic symptoms (white blisters) two days post-inoculation (Chaldecott and Preece, 1983). The positive and negative controls were separately inoculated with the Cmm strain 64 and sterile distilled water respectively. The experiments were conducted in a completely randomized design with three replications.

Antagonistic activity of the representative strains under greenhouse conditions

Suspension of the Cmm strain 64 (final concentration of ~ 109 CFU/ml in sterile distilled water) was prepared from overnight cultures grown on NBYA medium at 26 ºC for 48 h. Tomato seeds of Superluna and Falat cultivars were surface-sterilized in 2.5% (v/v) sodium hypochlorite solution for 3 min (Mew and Rosales, 1986), washed three times in sterile distilled water and air-dried for 12 h. The seeds were separately treated with 100 ml of antagonistic strains suspension (H1R1, M1R1 and G2R4) (final concentration of ~ 10^8 CFU/ml) containing 1% methylcellulose as an adhesive as described by Boudyach *et al*., (2001) and planted in sterile cell trays containing a mixture of sand and peat (1:2 v/v). The seedlings were pulled out from soil in the third leaf stage and their roots were soaked for 3 min in a suspension (10^8 CFU/ml) of the representative strains. The seedlings were transplanted into plastic pots (one seedling/pot) containing a sterile mixture of sand/peat inoculated with 20 ml of suspension of the Cmm strain 64 (10^8 CFU/ml). The plants were placed in an experimental greenhouse at 15 °C night and 26 °C day with relative humidity (RH) 80% under a 12 h photoperiod. The experiments were conducted in a factorial arrangement and a completely randomized design with 10 replications. The pots were watered daily, and plants were monitored for 60 days, and the disease symptoms were recorded. The incidence and severity of the disease as well as growth promotion efficacy were evaluated eight weeks

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after inoculation. The disease incidence (%) was calculated by the following formula: $[(N_I / N_T) \times 100]$ where N_I is the number of infected plants, and N_T is the total number of plants (Guo *et al*., 2004). The disease severity of the treated plants was evaluated on a scale of 0-4 shown in Table 1 (Kabas *et al*., 2016). Biological control efficacy (BCE) of representative strains was calculated according to the following formula: $BCE = [(D_C - D_T) / D_C] \times 100$, which D_C is disease severity of the control, and D_T is disease severity of the treatment (Guo *et al*., 2004). The growth promotion efficacy by the antagonistic strains was calculated by $GPE = [(G_T - G_C) / G_C)] \times 100$, where G_T is growth promotion by the treatment group, and G_C is growth promotion by the control (Aliye *et al*., 2008).

Table 1 Evaluation of disease severity in tomato plants treated with antagonistic strains and *Clavibacter michiganensis* subsp. *michiganensis* on a scale of 0-4 (Kabas *et al*., 2016).

Scale	Wilting area of the plant (%)
0	No disease symptoms
1	1-25% wilting of plant and stunting
2	26-50% wilting of plant and necrotic leaf lesions
3	51-75% wilting of plant and yellowish-brown discoloration of the vascular element
4	76-100% wilting of plant or dead plant

Molecular identification of the selected antagonistic strains

DNA extraction from the selected antagonistic strains was performed as described by Arabi *et al*., (2006). The partial sequence of 16S rRNA gene was amplified using the primers pA/pH* (Edwards *et al*., 1989) (Table 2) in 50 µl of a reaction mixture containing 25 µl PCR master mix (2x), 1 µl forward primer (1 µM), 1 µl reverse primer (1 µM), 2 µl template DNA (10pg -1µg) and 21 µl nuclease-free water with the PCR program consisting of an initial denaturation at 95 °C for 5 min, followed by 30 cycles at 94 °C for 60 s, annealing at 60 °C for 60 s, extension at 72 °C for 90 s, and a final extension at 72 °C for 10 min. PCR products were purified and sequenced directly for both strands by Bioneer Corporation (Daejeon, South Korea). The BLASTn program was used to compare the nucleotide sequences obtained (1480 bp) with other sequences available in GenBank database. Phylogenetic analysis was conducted using MEGA 6 software (Tamura *et al*., 2013). The phylogenetic trees were constructed using the maximum likelihood method with 1000 bootstrap replications.

Detection of antibiotic-coding genes in selected antagonists

DNA extraction from antagonistic fluorescent pseudomonads was performed as described by (Arabi *et al*., 2006). Table 1 shows the Phl2a/Phl2b and PCA2a/PCA3b primer sequences used in this study. Polymerase chain reaction (PCR) was performed in 12.5 µl of a reaction mixture containing 6 µl of master mix (2X), 1 µl of each primer (10 pmol) and 1 µl of the template DNA. PCR amplification was performed using a Thermo cycler (Bio-Rad, MJ Mini, USA) with the following program; initial denaturation for 5 min at 95 °C, 35 cycles of denaturing at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min and followed by a final extension at 72 °C for 10 min.

Statistical analyses

The analysis of variance (ANOVA) and mean comparison were carried out by SAS software (version 9.4) and Duncan□s multiple range test at 0.05 probability levels.

Results

Isolation and *in vitro* selection of antagonistic fluorescent pseudomonads

A total of 96 fluorescent pseudomonads strains were isolated from tomato rhizosphere and rhizoplane. Strains with negative reaction in the HR and soft rot tests were examined for the antagonistic activity towards Cmm. Nineteen strains were able to produce inhibition zones that had significant statistical differences at the 5% probability level ($P \leq 0.05$). Strain L4R2 with 11 mm and strains M1R1 and G2R4 with 48 mm inhibition zone had the lowest and highest inhibitory effect, respectively (Fig. 1; Table 3).

[DOR: 20.1001.1.22519041.2020.9.3.5.6]

Table 2 Target genes and primers used in this study.

Target gene	Target group	Primer	Sequence	Length	Reference
phlD(2,4-DAPG, diacetylphloroglucinol)	Fluorescent pseudomonads	Phl2a Phl2b	GAGGAGTCGAAGACCACCA ACCGCAGCATCGTGTATGAG	745bp	(Raaijmakers *et al*. 1997)
PhzCD(phenazine-1-carboxylic acid)	Fluorescent pseudomonads	PCA2a PCA3b	TTGCCAAGCCTCGCTCCAAC CCGCGTTGTTCCTCGTTCAT	1.15kb	(Raaijmakers *et al*. 1997)
16S rRNA		pA pH*	AGAGTTTGATCCTGGCTCAG AAGGAGGTGATCCAGCCGCA	1.5kb	(Edwards *et al*., 1989)

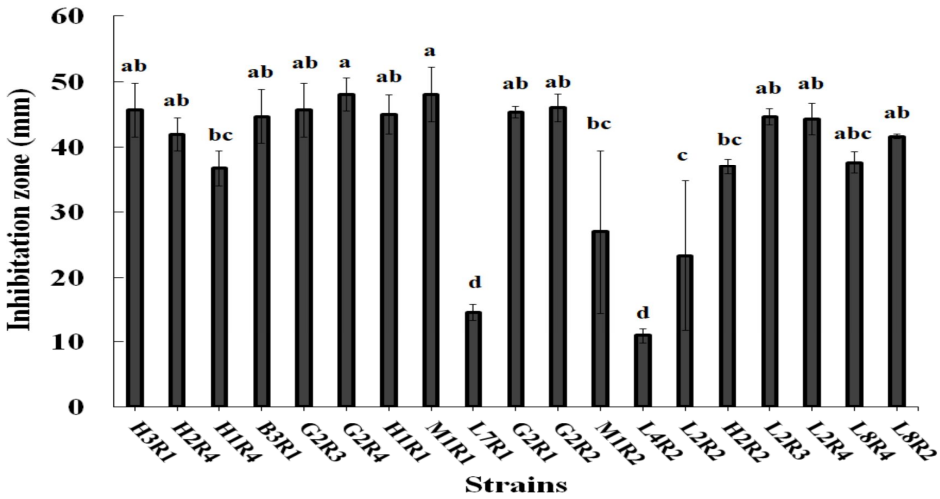

Figure 1 Mean comparison of inhibition zones exhibited by antagonistic fluorescent *Pseudomonas* strains against *Clavibacter michiganensis* subsp. *michiganensis in vitro*. Error bars represent the standard error of the means. Means in a column followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).

Table 3 Antagonistic fluorescent *Pseudomonas* strains isolated from tomato rhizosphere and rhizoplane in this study

Strain codes	Origin
G2R1, G2R2, G2R3, G2R4	Gharakhezer-Malekan-East Azerbaijan
L2R2, L2R3, L2R4, L4R2, L7R1, L8R2,L8R4	Laklar-Malekan-East Azerbaijan
H1R1, H1R4, H2R2, H2R4, H3R1	Hoseinabad-Malekan-East Azerbaijan
B3R1	Bayghout-Malekan-East Azerbaijan
M1R1, M1R2	Mamadel-Miandoab-West Azerbaijan

Identification of antagonistic strains and phylogenetic analysis

The antagonistic strains showed the highest similarity to *P. fluorescens, Pseudomonas* sp. and *P. putida* based on physiological and biochemical characteristics. Strains were gram-negative, able to produce fluorescent pigment on KB agar, aerobic, oxidase-positive, catalase-positive, and negative in the HR on tobacco and potato soft rot tests. Table 4 shows the results of physiological and biochemical tests for the obtained strains. According to the sequence data of 16S rRNA gene, the strains M1R1 and H1R1 showed 99.12% similarity with *P. putida* A1 (MK680517.1) and OBS-2 (KT253976.1) strains in the GenBank (Fig. 2). The nucleotide sequences of the strains M1R1 and H1R1 were deposited in the GenBank database under the accession numbers MK878727.1 and MK878726.1.

[DOR: 20.1001.1.22519041.2020.9.3.5.6]

Table 4 Physiological and biochemical characteristics of fluorescent *Pseudomonas* strains identified in this study.

Tests	*Pseudomonas fluorescens*											*Pseudomonas* sp.		*Pseudomonas putida*					
	G2R2	H1R1	L2R3	L8R2	L8R4	H2R2	H1R4	M1R2	L2R2	L7R1	L4R2	G2R4	G2R1	M1R1	H3R1	G2R3	B3R1	L2R4	H2R4
Gram reaction	G-	G-	G-	G-	G-	G-	G-	G-	G-	G-	G-	G-	G-	G-	G-	G-	G-	G-	G-
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fluorescent pigment on KB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Levan formation	-	-	+	-	-	+	+	-	-	-	+	-	-	-	-	-	-	-	+
Hypersensitive reaction on tobacco	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arginine dihydrolase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Potato soft rot	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hydrolysis of:																			
Gelatin	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
Starch	+	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-
Tween 80	+	-	-	-	-	-	+	+	-	+	+	-	+	-	+	+	+	-	+
Nitrate reduction	-	+	-	-	-	+	-	+	-	+	+	+	+	-	-	-	-	-	-
Growth at 4 ºC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 37 ºC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 41 ºC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OF	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
Sorbitol*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Adonitol*	+	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	ND	-	-
Meso-inositol*	+	+	+	-	-	-	-	-	-	+	+	+	+	-	-	-	ND	-	-
D-galactose*	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	ND	+	+
Lecithinase	-	+	-	+	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-

OF: Oxidation/ fermentation of glucose, Acid production from *, ND: Non-determined, +: Positive, -: Negative.

Pathogenicity tests

All tested strains induced symptoms of wilting, browning of the leaves, and vascular discoloration on tomato seedlings during 14-49 days post-inoculation (Fig. 3A). Cmm was re-isolated from tissues 2 cm above the inoculated section and the roots of wilted seedlings. The control plants inoculated with sterile distilled water showed no symptoms. Finally, the Cmm strain 64 which caused severe wilting and vascular discoloration was selected for *in vitro* and *in vivo* experiments.

Cotyledons tests

Strains H1R1, M1R1, G2R1, G2R4, and H3R1which had the antagonistic activity against Cmm strain 64 *in vitro*, were used in this experiment. All the strains decreased the white spots on cotyledons of tomato (cvs. Superluna and Falat) in comparison with control (treated with Cmm strain 64). The strains H1R1 and M1R1 indicated the best efficiency and the incidence of the white spots on cotyledons was decreased by 33% in simultaneous inoculation of the strain H1R1 or M1R1 and Cmm strain 64. Also, when the Cmm strain 64 was inoculated on cotyledons

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two hours after inoculation of the strain H1R1 or M1R1, the incidence of the spots was reduced by 66% (Fig. 3B).

Siderophore, antibiotic and HCN production

All 19 selected strains inhibited the growth of Cmm strain 64 and produced inhibition zone in the siderophore production test. However, none of the selected fluorescent pseudomonads exhibited an inhibition zone in the antibiotic production test. Also, in the semi-quantitative HCN production test, strains H1R1 and M1R1 changed the color of filter paper to light brown and showed a low ability to produce HCN.

Antagonistic activity of the representative strains under greenhouse conditions

The strains H1R1 and M1R1 (identified as *P. putida*) that were effective against Cmm strain 64 in laboratory and cotyledon tests, were selected for evaluating their effects on the incidence and severity of the disease in tomato plants (cvs. Superluna and Falat) under greenhouse conditions. In these experiments, the first symptoms of wilt were observed after 20 days of inoculation in the control plants. Wilting of leaves in plants treated with antagonistic strains appeared after 30 days.

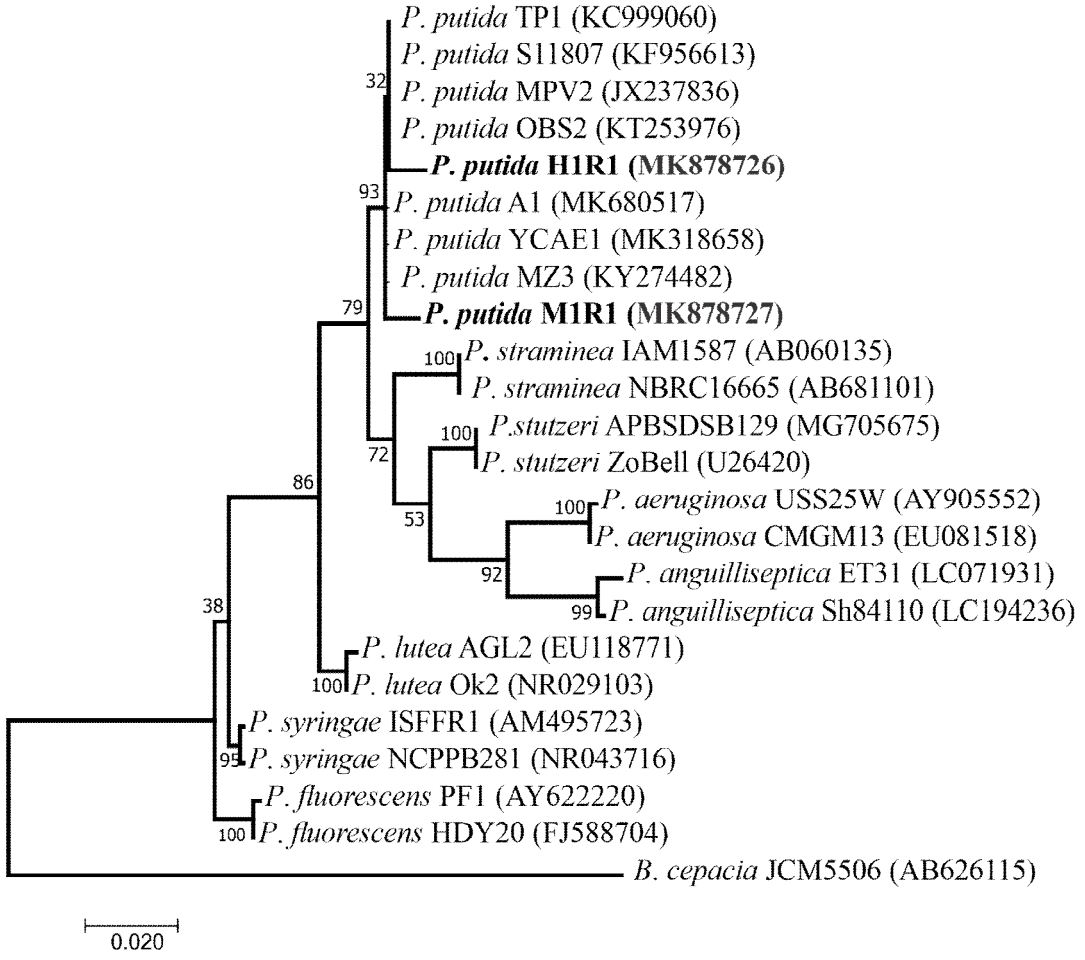

Figure 2 The phylogenetic tree constructed using the maximum likelihood method based on partial sequences of the 16S rDNA encoding gene of the two antagonists studied (in bold) and some strains of *Pseudomonas* spp. from the GenBank database. Bootstrap values (1,000 replicates) are shown on branches. *Burkholderia cepacia* JCM 5506 was used as the out-group.

[DOR: 20.1001.1.22519041.2020.9.3.5.6]

Figure 3 Pathogenicity and cotyledon tests. Injection of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) strain 64 on tomato seedling stem and discoloration of vascular tissues on wilting plant seven weeks after inoculation (A). The white spots reduced on cotyledons of four-day-old tomato seedlings inoculated with the strain H1R1and Cmm strain 64. The yellow arrow points to the white blister on the cotyledon surface (B, right). The control plant inoculated with sterile distilled water (B, left)

According to the analyses of variance of data, there was a significant influence of bacterial inoculation on the incidence and severity of the disease (P = 0.05). Results showed that *Pseudomonas putida* strain M1R1 reduced the disease incidence by 30% and 40% in Superluna and Falat cultivars, respectively. *Pseudomonas putida* strain H1R1 reduced the disease incidence by 20% only in Falat cultivar and had no effect on Superluna cultivar. Also, disease severity was significantly decreased by antagonistic strains in both Superluna and Falat cultivars ($F_{5, 54}$ = 3.5, P < 0.0082). The strains M1R1 and H1R1 reduced the disease severity by 52.93 % and 10.60 % in Superluna cultivar and 47.90 % and 42.88 % in Falat cultivar, respectively (Fig. 4). None of the two strains protected the tomato plants completely against Cmm when seeds and roots were treated.

Effects of representative antagonist strains on growth parameters

Two strains, *P. putida* H1R1 and *P. putida* M1R1, increased the growth of tomato plants. According to the analyses of variance in Superluna and Falat cultivars, there was significant difference between treatments in fresh weight (F_2 = 18.96, P < 0.0001) and dry weight (F_2 = 3.49, P < 0.0467), and plant height (F_2 = 2.88, P < 0.0755) was near to significant difference. *Pseudomonas putida* strain M1R1 increased the fresh weight, dry weight, and height of tomato plants 51.24%, 67.84%, and 20.60% in Superluna cultivar and 32.52%, 43.11% and, 23.52% in Falat cultivar respectively as compared with control. These growth parameters were increased by *P. putida* strain H1R1 about 49.46, 61.77, and 35.52% in Superluna cultivar and 17.24, 8.41, and 13.74% in Falat cultivar respectively (Fig. 5).

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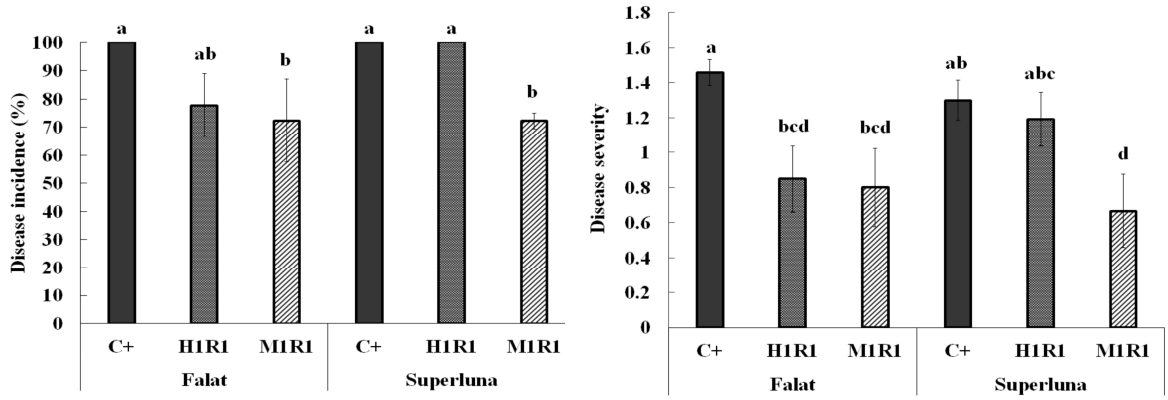

Figure 4 Effect of the antagonistic strains M1R1 and H1R1 on the incidence and severity of the disease in tomato plants eight weeks post-inoculation with *Clavibacter michiganensis* subsp. *michiganensis*. Error bars represent the standard error of the means. Means in a column followed by the same letter(s) are not significantly different according to Duncan's multiple range test ($P = 0.05$).

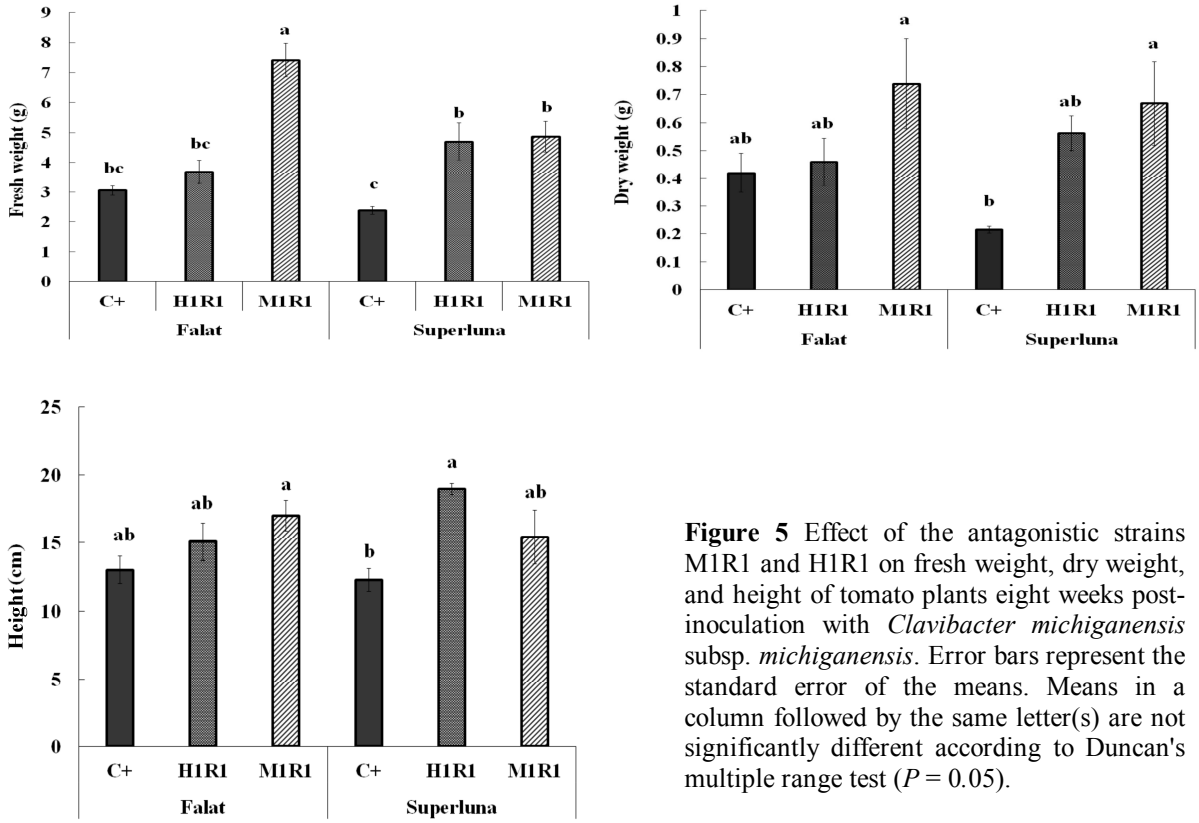

Figure 5 Effect of the antagonistic strains M1R1 and H1R1 on fresh weight, dry weight, and height of tomato plants eight weeks post-inoculation with *Clavibacter michiganensis* subsp. *michiganensis*. Error bars represent the standard error of the means. Means in a column followed by the same letter(s) are not significantly different according to Duncan's multiple range test ($P = 0.05$).

Detection of antibiotic-coding genes in selected strains

Antibiotic coding genes were not detected in any strains obtained in this experiment.

Discussion

Fluorescent pseudomonads are considered in the sustainable agricultural systems as effective

[DOR: 20.1001.1.22519041.2020.9.3.5.6]

biocontrol agents that can improve the growth and health of desired plants.

In this study, some isolated fluorescent pseudomonads showed antagonistic activity against Cmm *in vitro*. Strain H1R1 was identified as *P. fluorescens* based on its phenotypic features, whereas the partial sequencing of the 16S rRNA gene revealed it as *P. putida*. This result showed that phenotypic discrimination using the traditional methods in some cases is inconsistent with molecular methods. Therefore accurate identification has become more difficult just based on biochemical tests. Evaluation of siderophore and antibiotic production by tested strains *in vitro* showed that inhibition zone was only observed in siderophore production strains which indicates the important role of siderophore in increasing the antagonistic ability of these strains against Cmm.

Detection of antibiotic-encoding genes in representative antagonists was consistent with the antibiotic production test and the results of Kim *et al*., (2013) and Oni *et al*., (2015), which could not detect the *PhzCD* and *DAPG* genes in their studies on *Pseudomonas* spp. The role of antibiotics and siderophores in biocontrol of Cmm has been not reported, but for the other pathogens have been described (Leong, 1986; Loper and Ishimaru, 1991; Dowling and O'Gara, 1994; Raaijmakers and Weller, 1998; Wright *et al*., 2001). *Pseudomonas fluorescens* strains CHAO, Pf-5, Q2-87, F113 and other fluorescent pseudomonads have been reported to produce a wide variety of antibiotics such as phenazine-1-carboxylic acid (PCA) and other derivatives, 2,4-diacetylphloroglucinol (DAPG), pyrrolnitrin (Prn), and pyoluteorin (Plt) against soil-borne pathogens including *Thielaviopsis basicola*, *Rhizoctonia solani*, *Gaeumannomyces graminis* var. *tritici*, *Pythium ultimum*, *Globodera rostochiensis*, *Pectobacterium carotovorum* subsp. *atrosepticum*, etc. (Weller, 2007).

The biocontrol potential of fluorescent pseudomonads against Cmm was preliminarily determined by the modified cotyledon test, whereby antagonistic strains M1R1 and H1R1 reduced (significant at 0.05%) white spot on cotyledons of tomato plants (cvs. Superluna and Falat) compared with control. This result could be due to the rapid colonization of the cotyledon surface, competition for nutrients, and prevention of pathogen growth by producing secondary metabolites (Dowling and O'Gara, 1994; Rosales *et al*., 1995). *Pseudomonas putida* strains M1R1 and H1R1, which showed the best antagonistic ability against the pathogen in the preliminary tests, were selected for assessment in greenhouse conditions. The present study showed that two fluorescent *Pseudomonas* strains (M1R1 and H1R1) significantly reduced the incidence and severity of tomato bacterial canker disease and increased the growth parameters of tomato plants in comparison with controls. According to the results of this research and previous studies (Dowling and O'Gara, 1994; Rosales *et al*., 1995), antagonistic activity of fluorescent pseudomonads could be due to the colonization of roots, production of siderophores, antibiotics, hydrogen cyanide, and competition for nutrients and space. On the other hand, these bacteria can increase plant resistance to pathogens by activating resistance pathways such as ISR (Induced systemic resistance) (Bakker *et al*., 2007), which should be investigated in *P. putida* M1R1 and H1R1 strains. Previous researchers showed that seed treatment followed by root treatment, completely protected tomato against Cmm (Boudyach *et al*., 2001), but in the present study, none of these strains completely protected tomato plants against bacterial wilt and canker disease in seeds or roots inoculation in greenhouse condition. Combination of two strains may lead to better results which should be tested. Also, these strains promoted tomato growth parameters, which could be due to the improvement of nutrient uptake and phytohormone production (Chunhaleuchanon, 2008). In this study, strain G2R4 which showed effective antagonistic activity *in vitro* did not exhibit desired results under the greenhouse condition (data not shown), which could be due to its incompatibility with soil conditions and

[DOR: 20.1001.1.22519041.2020.9.3.5.6]

low root colonization. Isolation of effective antagonists from the rhizosphere and rhizoplane of a specific crop against pathogens could lead to the collection of antagonistic strains that could be adapted to the plant species as well as the particular environmental conditions (Cook, 1993). In general, results of this research were consistent with previous studies that indicate rhizospheric bacteria can be effective agents in the biocontrol of soil-borne pathogens such as Cmm, and they could be viable alternative agents for synthetic pesticides and organic fertilizers.

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Statement of conflicting interests
The authors declare that they have no conflict of interest.

Authors' Contributions
Hosein Hamidi contributed to the acquisition of data, performed of the data analyses and interpretation of data, Fatemeh Shahryari was responsible for supervising the work, conception, design, interpretation of data and drafting of the manuscript, Abolghasem Ghasemi contributed to the revision of the manuscript, donated the Cmm strains and material support.

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بررسی سودوموناده‌ای فلورسنت ریزوسفر و ریزوپلن گوجه‌فرنگی جهت کنترل زیستی باکتری *Clavibacter michiganensis* subsp. *michiganensis*

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چکیده: شانکر و پژمردگی باکتریایی گوجه‌فرنگی ناشی از *Clavibacter michiganensis* subsp. *michiganensis* (Cmm)، بیماری بذربرد مهم و اقتصادی در مناطق عمده کشت گوجه‌فرنگی در سراسر دنیا است. این بیماری در استان‌های شمال، شمال غرب و مرکزی ایران نیز وجود دارد. این پژوهش جهت جداسازی سودوموناده‌ای فلورسنت از ریزوسفر و ریزوپلن گوجه‌فرنگی و بررسی فعالیت آنتاگونیستی آن‌ها جهت کنترل زیستی بیماری شانکر باکتریایی گوجه‌فرنگی انجام شد. در این مطالعه، ۹۶ استرین سودوموناد فلورسنت روی محیط کشت King's B آگار جداسازی و فعالیت آنتاگونیستی آن‌ها علیه Cmm در شرایط آزمایشگاه بررسی شد. در آزمایشات گلخانه‌ای بذر و ریشه نشاءهای گوجه‌فرنگی با استرین‌های آنتاگونیست منتخب مایه‌زنی و در خاک آلوده با Cmm کاشته شدند. سپس وقوع و شدت بیماری روی گیاه گوجه‌فرنگی و هم‌چنین شاخص‌های رشدی ارزیابی شدند. در شرایط آزمایشگاه، ۱۹ استرین از رشد Cmm ممانعت کردند که دو استرین منتخب M1R1 و H1R1 براساس خصوصیات فنوتیپی و توالی‌یابی بخشی از ژن 16S rRNA، به‌عنوان گونه *Pseudomonas putida* شناسایی شدند. این دو استرین توانایی تولید سیدروفور و سیانید هیدروژن را داشتند. در آزمایش گلخانه‌ای استرین M1R1 وقوع بیماری را در رقم‌های سوپرلونا و فلات به‌ترتیب ۳۰ و ۴۰ درصد کاهش داد. با استفاده از استرین H1R1 وقوع بیماری فقط در رقم فلات ۲۰ درصد کاهش یافت. شدت بیماری توسط استرین‌های M1R1 و H1R1 به‌ترتیب ۵۲/۹۳ و ۱۰/۶۰ درصد در رقم سوپرلونا و ۴۷/۹۰ و ۴۲/۸۸ درصد در رقم فلات کاهش یافت. هم‌چنین استرین M1R1 شاخص‌های رشدی از قبیل وزن تر، وزن خشک و ارتفاع گیاهان مایه‌زنی شده با Cmm را در مقایسه با شاهد به‌طور معنی‌داری افزایش داد.

واژگان کلیدی: سیدروفور، *Pseudomonas putida*، شانکر باکتریایی گوجه‌فرنگی، فعالیت آنتاگونیستی، کنترل زیستی

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