

Characterization of *Pseudomonas viridiflava* Causing Alfalfa Root Rot Disease in Hamedan Province of Iran

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Abstract

Alfalfa (*Medicago sativa*) is the most important forage crop in Hamedan province, located in west of Iran. Under field condition, plants showed growth stunting, chlorosis and wilting symptoms not previously reported. To survey the causal agent of the disease, symptomatic plants were collected from the main alfalfa growing area in this province. Plants samples were disinfected, grinded in phosphate buffer, and a loop full of the prepared suspension was stroked on nutrient agar medium containing 5% yeast extract and 1% glycerol. Isolation and characterization of the causal agent, especially from plants in primary growth stage of the disease, is difficult. Isolated bacteria caused wilting and chlorosis after 40 days on alfalfa plants, under green house condition. Phenotypic feature determination of the isolated bacteria, together with two defined strains, by standard bacteriological methods indicated that they belong to *P. viridiflava*. PCR assay confirmed results of identification of the causal bacterial agent, determined by classical methods. Tested representative bacterial isolates showed an 860 pb PCR band, using primers designed for 16S rDNA sequence of *P. viridiflava* LMG 2352T (type strain). This new reported bacterium is the second pathogenic bacterium agent, which causes alfalfa yield losses in Hamedan province of Iran.

Keywords: Stunting; Crown and root rot; Chlorosis; PCR

Introduction

Bacterial crown and root rot disease of alfalfa is caused by a Gram negative and fluorescent bacterium *Pseudomonas viridiflava* [1]. This is one of the important diseases of alfalfa, which causes crop quantity and quality losses. The bacterium was first recognized by Burkholder, then named as *Phytohomonas viridiflava* by Dowson, and finally changed to *P. viridiflava* [2]. *P. viridiflava* damages alfalfa plants at all growth stages. Infected seedling show symptoms which includes wilting, chlorosis, stunting, leaf yellowing and plant malformation. Plants in developed disease stage show dwarfing, and growth causal bacterium causes wound and rotting on plant roots and changes the root color. Wilting symptoms are very obvious, especially under warm condition. The bacterium is more active under humid and raining condition, and it can spread through the rain drops [3]. *P. viridiflava* has broad host range and causes leaf blight on melon, tomato, pumpkin, cauliflower, cabbage, grapevine, lettuce, carrot, peas, poppy, turnip and eggplant, wilt of sweet onion, and canker and nude blight of kiwi [4-6]. This bacterium was observed as second pathogen for pith necrosis of tomato and pepper, and has ability of transfer through the seeds in tomato and pepper. *P. viridiflava* is an agent of dwarfing in *Fabacea* plants and causes leaf necrosis, dwarfing and root rot of *Trifolium pretense* [1]. Also it assist *P. corrugate* to cause pith rot on several plants [2], and increase disease symptoms arising from soil borne nematodes such as *Meloidogyne hapla*, *pratylenchus penetrans*, *Ditylenchus dipsaci* and *Helicotylenchus dihystra* [7].

Using specific primers for detection and recognition of bacterial strains is very serviceable, and each organism could be recognized directly with PCR-based studies, in least time [8]. DNA-based methods, such as PCR have provided accurate description of genetic variation and phylogenetic relationships, among the pathogens. This technique is more sensitive than cultural or serological methods, and is rapid and specific for plant pathogens [9]. Alfalfa is one of the most important forage crops in Hamedan province of Iran. Climatic condition in this province is suitable for alfalfa growth but many diseases, including alfalfa bacterial crown and root rot disease, cause economic losses of this crop. As this disease is new, the main aim of

this study was characterization of the causal agent of the disease in Hamedan province, by classical and molecular methods.

Materials and Methods

Bacterial isolation

During 2009 spring, wilted and suspicious alfalfa plants were collected from the main growing areas in Hamedan province, located west of Iran. Plant roots samples were disinfected in 70% ethanol, following by 0.5% sodium hypochlorite for 5 minutes, washed twice in sterilized distilled water, grinded in phosphate buffer and shake (100 rpm) for 1 hr at room temperature [6]. A loop-full of bacterial suspension was stroked on the plates, containing nutrient agar medium enriched with yeast extract and glycerol, and they were kept at 25°C for 2-3 days. To purify the bacterial isolates, olive to golden brown color single colonies were selected and re-stroked on plates containing the above medium. Selected bacterial isolates were kept, either in sterilized distilled water [10] or on medium slants in test tubes, under sterilized liquid paraffin for further investigations.

Pathogenicity test

To prepare the plant seedlings for pathogenicity test, healthy alfalfa seeds were disinfected in 5% sodium hypochlorite for 10 minutes, and they were washed three times in sterilized distilled water. Fifteen seeds were planted in each pots containing two kilogram sterilized loam-sandy soil, under greenhouse condition in three replicates. Bacterial

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Received August 16, 2012; Accepted September 10, 2012; Published September 15, 2012

Citation: Heydari A, Khodakaramian G, Zafari D (2012) Characterization of *Pseudomonas Viridiflava* Causing Alfalfa Root Rot Disease in Hamedan Province of Iran. J Plant Pathol Microb 3:135. doi:10.4172/2157-7471.1000135

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strains were grown in nutrient yeast extract broth (NBY) and their pellet was obtained by centrifugation. Suspensions of bacterial strains with optical density 0.1 (OD=0.1) at 600 nm wavelength were prepared using spectrophotometer, and they were injected into the stem of alfalfa seedlings (3 to 4 leaves stage). At the same time, bacterial suspensions with the same concentration were added to the soil [1]. Sterilized distilled water was injected or added to the soil, as a control treatment. A total of 35 bacterial isolates which showed apparent characteristics of suspected causal agent of the alfalfa root rot disease, were used in pathogenicity test.

Phenotypic characterization of the bacterial strains

As bacterial isolates were uniform and showed similar pattern of pathogenicity, 15 representatives together with two defined *P. viridiflava* strains gifted by collection of laboratory of bacteriology, Iranian Plant Disease Research branch, were used for phenotypic feature characterization. Following physiological characteristics were determined, based on the standard bacteriological methods: Gram reaction using 3% KOH [11], Gram staining [10], hypersensitivity reaction on Tobacco, Geranium, Petunia and Tomato plants [12], oxidase [12] and oxidative/fermentative (O/F) test [13]. Also, gelatin liquefaction, levan formation, acid from trehalose, sucrose, D-arabinose, cellobiose, sorbitol, glucose, fructose and manitol, urease, growth at 37°C, fluorescent pigment on King's B medium, arginine dihydrolase, and pectinolytic activity on potato slice were performed [5,10,14].

PCR assay

DNA from bacterial isolates was extracted according to Ausubel et al. [15] with minor modifications. In brief, bacterial strains were grown in NBY medium for three days, and their cell pellet were precipitated by centrifugation. Bacterial pellets re-suspended in 100 μ TE buffer and boiled for 5 minutes, for DNA releasing. After cooling, bacterial debris was precipitated by centrifugation (10000 rpm), and 500 μ l from supernatant of each aliquot were kept at -20°C.

Amplification of target sequences of the bacterial DNA was performed using primers: Ten isolated bacteria together with two defined *P. viridiflava* strains gifted by collection of laboratory of bacteriology, Iranian Plant Disease Research branch, were used in primary PCR experiment. This experiment was conducted using a set of primers (PVF/PVR) designed for 16S rDNA sequence of *P. viridiflava* LMG 2352T (type strain), obtained from NCBI database with accession number Z76671. The primer sequence of PVF was 5'-TCAACCTGGAACTGCATCC-3' and PVR was 5'-ATCACACCGTGGTAACCGTC-3'. PCR experiment was conducted at 58°C annealing temperature, for 35 cycles. Then, two sets of primers were used for amplification of target sequences, only in 10 isolated bacteria from alfalfa plants in Hamedan province (Iran). Sequences of these primers sets were 7F (5'-GTTCCTTGAAGTGCCTGA-3') / 7R (5'-GTTTTTCGTAGCGGTTGCG-3') and 20F (5'-CGCCGTTTTTCGTTCTTGT-3') / 20R (5'-GCATGGAA-TACGCCGACA-3'). For these two sets of primers, PCR experiments were conducted at 50°C and 58°C annealing temperature, respectively, for 35 cycles. PCR reaction contained 1 μ l of primers 0.2 μ M, 2.5 μ l buffer 1X, 2 μ l MgCl₂ 2.5 mM, 0.5 μ l dNTPs 0.2 mM, 0.5 μ l Taq DNA polymerase, 1 μ l DMSO 2.5 mM, and 1 μ l of target DNA solution, in 25 μ l final reaction volume.

Results

Bacterial isolation

A total of 35 typical bacterial isolates were selected from bulk



Figure 1: Colonies of *Pseudomonas viridiflava* isolated from alfalfa plants in Hamedan province (Iran) on nutrient agar medium.

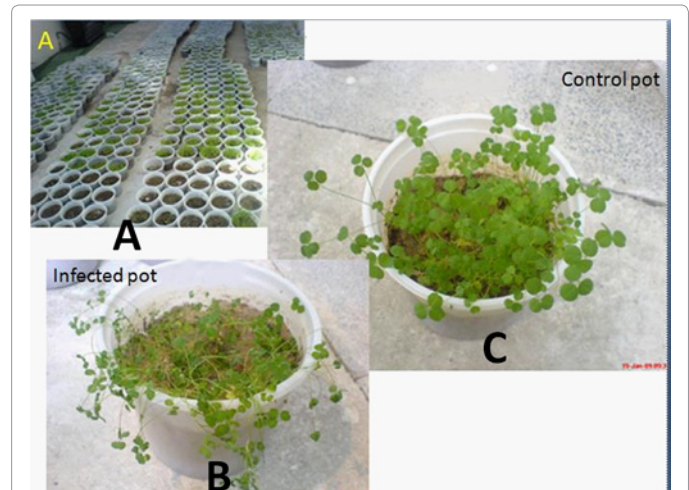


Figure 2: Pathogenicity of *Pseudomonas viridiflava* on alfalfa plants. A: green-house experiment; B: infected plants and C: healthy plants.

isolates, sampled from alfalfa growing area of Hamedan province. In 25°C, the olive to golden brown color colonies of isolated strains appeared after 3 days (Figure 1).

Pathogenicity test

After 40 days, infested alfalfa plants showed symptoms of the disease, including stunting and wilting. All of 35 bacterial isolates induced disease on alfalfa seedlings in green-house, and they showed similar pattern of pathogenicity, in both methods of inoculation, which includes plant stem injection and soil drenching. Under green house condition, disease symptoms were developed rapidly and severely, especially when bacterial suspension was injected into plant stem in comparison to soil drenching. In later method, disease symptoms appeared after 65 days (Figure 2).

Phenotypic characteristics of the bacterial strains

As 35 isolated strains showed identical features of colony color and shape, growth and pathogenicity pattern, 15 strains were randomly selected for phenotypic feature characterization. These strains together with two defined *P. viridiflava* strains gifted by collection

of laboratory of bacteriology, Iranian Plant Disease Research branch were characterized phenotypically. Gram reaction tests showed that all strains were Gram negative (Table 1).

All tested strains induced hypersensitive reaction (HR) on Tobacco, Geranium and Tomato leaves (Table 2). Based on the results of phenotypic feature determination, all tested bacterial strains were identified as *P. viridiflava* (Table 3). This new reported bacterium is less pathogenic than *Clavibacter michiganensis* subsp. *insidiosus* on alfalfa, in Iran. This disease and its causal agent previously has never been reported from other alfalfa growing area in Iran, even as an alfalfa plants associated bacteria.

PCR results

A set of primers PVF/PVR yielded a 860 pb PCR band in all tested bacterial isolates, including defined *P. viridiflava* strains as expected

Strain name	Place of isolation	Petunia	Tomato	Geranium	Tobacco	Time (hr)
AH4	Ghrovah	-	+	+	+	24
AH5	Ghrovah	-	+	+	+	24
AH8	Hamedan	-	+	+	+	36
AH11	Hamedan	-	+	+	+	24
AH12	Hamedan	-	+	+	+	24
AH16	Malayer	-	+	+	+	24
AH17	Malayer	-	+	+	+	24
AH18	Nahavand	-	+	+	+	24
AH19	Nahavand	-	+	+	+	36
AH20	Nahavand	-	+	+	+	24
AH21	Asad abad	-	+	+	+	24
AH22	Asad abad	-	+	+	+	24
AH23	Hamedan	-	+	+	+	24
AH24	Famenin	-	+	+	+	36
AH25	Famenin	-	+	+	+	24

+: Positive reaction and necrosis formation
 -: Negative reaction and chlorosis formation

Table 1: Hypersensitive reaction of *Pseudomonas viridiflava* strains isolated from alfalfa plants in Hamedan province (Iran) on some plants.

Test	Reaction
Gram reaction	-
Fluorescent pigment on King's B medium	+
Growth at 37° C	-
Pectolytic activity on potato and carrot	+
Oxidative/Fermentative	O
Levan formation	-
Oxidase	-
Arginine dihydrolyse	-
Urease	-
Gelatin hydrolysis	+
Acid from: Cellobiose	-
Trehalose	-
Sucrose	-
D-arabinose	-
Sorbitol	+
Glocose	+
Fructose	+
Manitol	+
Citrate test	+

+: Positive reaction and -: Negative reaction

Table 2: Some phenotypic characteristics of 15 representatives and defined *Pseudomonas viridiflava* strains isolated from alfalfa plants in Hamedan province (Iran).

Strain name	Place of isolation	Species
AH4	Ghrovah	<i>Pseudomonas viridiflava</i>
AH5	Ghrovah	<i>Pseudomonas viridiflava</i>
AH8	Hamedan	<i>Pseudomonas viridiflava</i>
AH11	Hamedan	<i>Pseudomonas viridiflava</i>
AH12	Hamedan	<i>Pseudomonas viridiflava</i>
AH16	Malayer	<i>Pseudomonas viridiflava</i>
AH17	Malayer	<i>Pseudomonas viridiflava</i>
AH18	Nahavand	<i>Pseudomonas viridiflava</i>
AH19	Nahavand	<i>Pseudomonas viridiflava</i>
AH20	Nahavand	<i>Pseudomonas viridiflava</i>
AH21	Asad abad	<i>Pseudomonas viridiflava</i>
AH22	Asad abad	<i>Pseudomonas viridiflava</i>
AH23	Hamedan	<i>Pseudomonas viridiflava</i>
AH24	Famenin	<i>Pseudomonas viridiflava</i>
AH25	Famenin	<i>Pseudomonas viridiflava</i>

Table 3: Identification of the 15 representative strains causing alfalfa root rot disease in Hamedan Province (Iran).

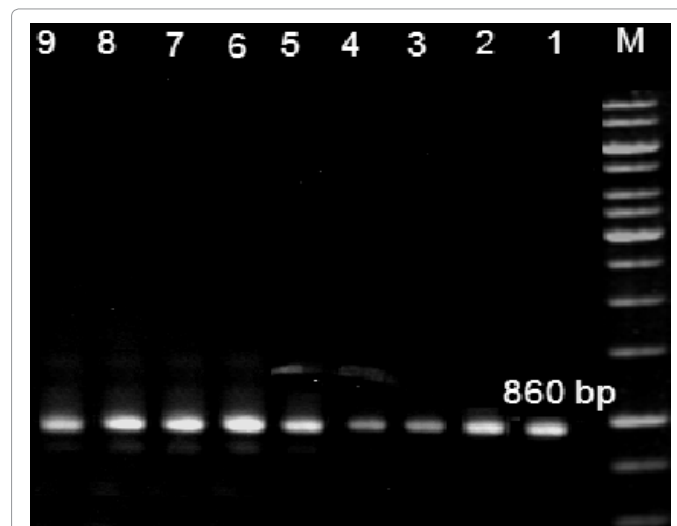


Figure 3: An 860 bp PCR amplified fragment of *Pseudomonas viridiflava* strains using PVF1/PVR1 primers designed for 16S rRNA sequence. From the right side: First lane DNA size marker; second and third lanes a defined *P. viridiflava* strains and other lanes AH4, AH8, AH11, AH16, AH18, AH23 & AH25 strains isolated from alfalfa plants in Hamedan province (Iran).

from 16S rDNA sequence of *P. viridiflava* LMG 2352T (Figure 3). This criterion confirms the causal agent of alfalfa root rot, to be *P. viridiflava*. The primers sets 7F/7R amplified nearly equal to 400 bp bands (Figure 4) and primers set 20F/ 20R amplified nearly equal to 440 bp band in all tested strains (Figure 5).

Discussion

P. viridiflava which causes alfalfa crown and root rot disease also affects melon, tomato, pumpkin, cauliflower, cabbage, grapevine, lettuce, carrot, peas, poppy, turnip, eggplant, sweet onion, kiwi, and clover [4,6,16]. The causal bacterium of the disease is found in the intercellular spaces of the seed parenchyma [17]. Bacterial crown and root rot disease is one of the important diseases of alfalfa, and causes economic losses in Hamedan province of Iran, where until now, no study was performed on the disease and its causal agent. All tested bacterial isolates in this study induced typical disease symptoms, caused by *P. viridiflava* on alfalfa seedling (Figure 2). The disease symptoms during sampling in Hamedan alfalfa growing area were

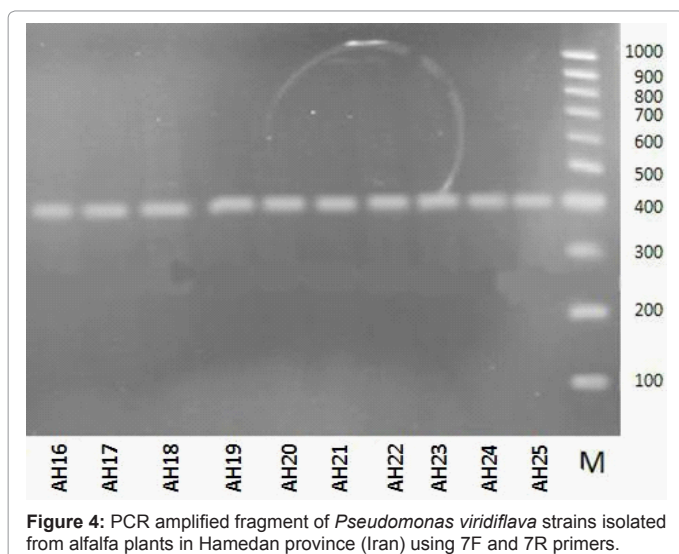


Figure 4: PCR amplified fragment of *Pseudomonas viridiflava* strains isolated from alfalfa plants in Hamedan province (Iran) using 7F and 7R primers.

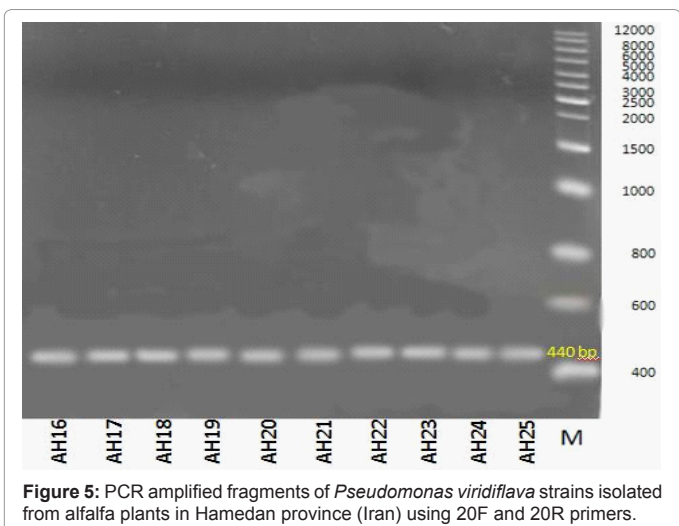


Figure 5: PCR amplified fragments of *Pseudomonas viridiflava* strains isolated from alfalfa plants in Hamedan province (Iran) using 20F and 20R primers.

same, as reported by Lukezic et al. [1] and Jones et al. [3], which include chlorosis, stunting and wilting. However severe stunted plants were observed rarely in diseased plants and this symptom was often observed in plants with high developed symptoms. This may indicate the importance of physical obstruction of vessels by bacterium, and inhibition of nutrient material transfer to upper parts of the plant. Some researchers showed observable symptoms developed by *P. viridiflava* on alfalfa, after 50 days after adding bacterial suspension into the soil, and after 30-40 days when they were injected into plants [1,3]. In our experiments, disease symptoms were observed after 40 days when bacterial suspension injected into the plants stem, and after 60 days when soil was infested by bacterial suspension. This result is in accordance with those reported by Lukezic et al. [1] and Jones et al. [3].

Hypersensitive reaction (HR) on tobacco leaves were observed as previously reported by other researchers [3,6]. However, this is the first report of HR on leaves of Geranium and Tomato by *P. viridiflava*. Results of phenotypic feature characterization, including LOPAT tests for bacteria isolated from diseased alfalfa plants, and two defined *P. viridiflava* strains were same, as described by others reports [10,14].

The PCR-based identification methods for *P. viridiflava* are very

rapid, sensitive and specific. In our study, the use of specific primers had lead to verification of the results of physiological tests, and the certainty of the presence of *P. viridiflava* in Hamedan province. An 860 pb expected PCR band in all tested bacterial isolates, including defined *P. viridiflava* strains using PVF/PVR primers set, confirms the causal agent of alfalfa root rot to be from *P. viridiflava*. This primer set was designed for amplification of 16S rDNA sequence, based on nucleotide database of *P. viridiflava* LMG 2352T derived from NCBI website. Also the 7F/7R and 20F/ 20R primer sets amplified nearly equal to 400 and 440bp, respectively. These observations in our PCR experiments were same to those reported by Goss et al. [18]. It is worthy to note that as this is the new reported bacterium causing alfalfa crown and root rot in Hamedan province, and there is no report on other alfalfa growing area, there is a possibility that the causal agent might be present in other area and caused crop losses. Therefore, survey for the presence of the bacterium causing alfalfa crown and root rot disease is very important and necessary, for disease control and management in future studies.

References

1. Lukezic FL, Leath KT, Levine RG (1983) *Pseudomonas viridiflava* associated with root and crown rot of alfalfa and wilt of birdsfoot trefoil. Plant Dis 67: 808-811.
2. Anzai Y, Kim H, Park JY, Wakabayashi H, Oyaizu H (2000) Phylogenetic affiliation of the *Pseudomonads* based on 16S rRNA Sequence. Int J Syst Evol Microbiol 50: 1563-1589.
3. Jones JB, Jones JP, McCarter SM, Stall RE (1984) *Pseudomonas viridiflava*: causal agent of bacterial leaf blight of Tomato. Plant Disease 68: 341-342.
4. Alippi AM, Dal Bo E, Ronco LB, Lopez MV, Lopez AC, et al. (2003) *Pseudomonas* populations causing pith necrosis of tomato and pepper in Argentina are highly diverse. Plant Pathol 52: 287-302.
5. Gitaitis R, Sumner D, Gay D, Smittle D, McDonald G, et al. (1997) Bacterial streak and bulb rot of onion: I. A diagnostic medium for the semiselective isolation and enumeration of *Pseudomonas viridiflava*. Plant disease 81: 897-900.
6. Goumans DE, Chatzaki AK (1998) Characterization and host range evaluation of *Pseudomonas viridiflava* from melon, blite, tomato, chrysanthemum and eggplant. European Journal of Plant Pathology 104: 181-188.
7. Brenner DJ, Krieg NR, Staley JT (2005) Bergey's Manual of Systematic Bacteriology, part B. (2ndedn), Springer Publication.
8. Babalola O (2003) Molecular techniques: An overview of methods for the detection of bacteria. African Journal of Biotechnology 2: 710-713.
9. Henson JM, French R (1993) The polymerase chain reaction and plant disease diagnosis. Annu Rev Phytopathol 31: 81-109.
10. Schaad NW, Jones JB, Chun W (2001) Laboratory Guide for Identification of Plant Pathogenic Bacteria. Minnesota, American Phytopathological Society Press.
11. Suslow TV, Schroth MN, Isaka M (1982) Application of a rapid method for Gram differentiation of plant pathogenic and saprophytic bacteria without staining. Phytopathology 72: 917-918.
12. Klement Z, Farkas GL, Lovrekovich L (1964) Hypersensitive reaction induced by phytopathogenic bacteria in the tobacco leaf. Phytopathology 54: 474-477.
13. Hugh R, Leifson E (1953) The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram negative bacteria. J Bacteriol 66: 24-26.
14. Burr TJ, Katz BH (1983) Isolation of *Agrobacterium tumefaciens* biovar 3 from grapevine galls and sap, and vineyard soil. Phytopathology 73: 163-165.
15. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, et al. (2002) A Compendium of Methods from Current Protocols in Molecular Biology. John Wiley & Sons Ltd.
16. Gitaitis R, MacDonald G, Torrance R, Hartley R, Sumner DR, et al. (1998) Bacterial Streak and Bulb Rot of Sweet Onion: II. Epiphytic Survival of *Pseudomonas viridiflava* in Association with Multiple Weed Hosts. Journal of Plant Disease 82: 935-938.

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17. Gaham JH, Frosheiser FI, Stuteville DL, Erwin DC (1980) Compendium of Alfalfa Diseases. American Phytopathological Society Press.
18. Goss EM, Kreitman M, Bergelson J (2005) Genetic diversity, recombination and cryptic clades in *Pseudomonas viridiflava* infecting natural populations of *Arabidopsis thaliana*. Genetics 169: 21-35.