

Isolation and identification of bacterial wilt disease of alfalfa (*Clavibacter michiganensis* subsp. *insidiosus*) in Fars province

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ABSTRACT

In this study samples with stunting, chlorosis, leaf malformation and wilting symptoms from alfalfa fields in Fars province were collected and investigated in the lab. The bacterial agent was isolated, purified and identified using standard bacteriological tests. 32 strains were isolated from infected plants using NA and NBY media. The isolates were gram and catalase negative and oxidase negative and obligatory aerobic. All isolates induced hypersensitive reaction on tobacco. All strains produced acid from galactose and mannose did not produced from inulin, sorbitol, ribose, mannitol. None of the strains have been utilized sodium acetate and sodium succinate. The specific primers CMI-III/BB and CIRS1 CIRS2 were used in PCR test for final diagnosis. The isolates were able to amplify 218 bp and 132 bp DNA fragment with primer pairs CMI-III/BB1 and CMI-III/BB2 and primers pairs CIRS1 and CIRS2 in PCR test, respectively. Pathogenicity of the strains were confirmed on alfalfa plants under greenhouse condition. Based on symptoms, phenotypic characteristics and PCR test with specific primers, the bacterial isolates were identified as *Clavibacter michiganensis* subsp. *insidiosus*.

Keywords: Alfalfa bacterial wilt, Pathogenicity, Isolates, *Clavibacter michiganensis*

Introduction

Recently, Alfalfa (*Medicago sativa* L.) which is known as queen of forages is a primary forage crop and a major feed component used to feed sheep, dairy cattle, and other livestock production systems. [1,2] Because of its high feeding quality, edible value and straw yield, alfalfa is widely cultivated throughout the world including the United States of America, Argentina, Canada, Russia, Italy, China and Iran. [3]

Alfalfa, contains a broad range of components with probably health promoting effects, including antioxidant, anti-inflammatory, anticancer, antibacterial, immunomodulatory and memory-improving activities, etc. [4-6] On the other hand, A number of research projects on *M. sativa* have led to the extraction of some flavonoids, saponins, alkaloids, coumarins, fatty acids and essential oils. [6, 7] Due to high quality of the nutritional components and a variety of biological activities in

alfalfa, it has been approved by the European Food Safety Authority as a potential of dietary supplement. [8]

Disease can cause major yield reduction in alfalfa and reduce the feeding value of the forage. Leaves, stems, roots, and crown can be affected. Pathogens that cause alfalfa diseases include fungi, bacteria, viruses, and nematodes. Often the influence of diseases goes unrecognized, particularly root diseases, as symptoms can be subtle or mistaken for some-thing other than disease, and pathogens can be hard to detect. *Clavibacter michiganensis* spp. can significantly decrease fruit and vegetable yield and quality, thus causing considerable economic squandering both in greenhouses and in field. *Clavibacter michiganensis* ssp., are seed borne, persists in plant debris in soil and on contaminated greenhouse materials and infected seeds. [9] It infects host plants via roots or wound and invade the xylem vessels, followed by a systemic infection of the host which results in leaf lesions, wilting, fruit lesions and ultimately yield loss of fruits and vegetables. [10, 11] Bacterial wilt of alfalfa caused by *Clavibacter michiganensis* subsp. *insidiosus* (CMI), commonly occurs throughout the most of alfalfa growing areas of the world. This disease was reported for the first time by McColluch et al as a major limiting factor for alfalfa production. [12, 13] Alfalfa Bacterial Wilt is not identified by any spot or external wound. The most obvious features of completely diseased plants include having stunt and many abnormal stems. [14]

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Severely infection plants may only be a few centimeters in height while having abnormal and twisted leaves. ^[15, 16] As many soil bacteria, CMI produces extracellular polysaccharide that carry out important multi biological actions. ^[17] Considering pathogenic interactions with plant cells, extracellular polysaccharide possibly prevents the diagnosis of pathogen by the plants defense system. In doing so, it blocks the activity of lectins and agglutinin. ^[14, 16] Having high density of bacteria in xylem vessel, extracellular polysaccharide that is sticky and has high molecular weight causes water stress. ^[14, 16] Damage rarely occurs during the first two years after germination. The bacteria enter the plant through the root wounds caused by cold damage or through the cut-off ends caused while harvesting them and disseminate in the surrounding water and soil. ^[15, 16] Alfalfa Bacterial Wilt is reported from different areas of Hamadan in 2008. This was the first report from the existence of this disease in Iran. ^[12] The existence of this disease is also reported from alfalfa field of Bajgah area in Fars province. ^[18] The purpose of this research was the isolation and identification of the disease based on phenotypic, genotypic and pathogenicity tests and distribution of this disease in Fars province.

Materials and Methods

Sample collection and bacterial isolation:

The infected plants which had symptoms of dwarf and wilt were collected from the Shiraz suburbs including Sepidan (30.0451° N, 52.2762° E), Marvdasht (29.8787° N, 52.8206° E), Eghlid (30.9049° N, 52.6921° E), Nourabad Mamasani (30.1151° N, 51.5413° E), Rostam (30.1511° N, 51.2830° E), Shiraz (29.5926° N, 52.5836° E). Sampling was conducted from different parts of the collected shrubs including their roots, crowns, stems and soil surrounding the roots. Then collected samples putted in clean paper envelopes and were kept at 4 ° C in laboratory until further analysis. Isolation of presumptive pathogen bacteria:

In order to eliminate soils and contaminants, collected samples were sterilized by cotton imbrued with 70% ethanol. Then, the plant organs were placed in 5% sodium hypochlorite for five minutes and rinsed two times by sterilized water for five minutes and placed on a sterile filter paper. Then plant tissues were placed in a sterile mortar and pestle containing 50 milliliters of sterile water then they were thoroughly squished and shake for 20 minutes. After releasing of bacteria, 1 mL of suspension was cultivated in a petri dish containing Nutrient Agar (NA) supplemented with 5% Yeast extract which is crucial for promoting the growth of slow growing bacteria. Petri dishes incubated for 5-7 days at 25±1 ° C.

Phenotypic characteristics of isolates

Phenotypic characterization of isolated bacteria was performed as previously described by Tamang et al. (2005). ^[19] Presumptive

Clavibacter michiganensis isolates were stained for the gram reaction, tested for catalase production, oxidase carbohydrate fermentation.

Pathogenicity test

The pathogenicity of isolated bacteria was evaluated by the method presented by Osdaghi et al, (2018) with some modifications. ^[20] Briefly, Hamadan alfalfa seeds were purchased from local suppliers and sterilized by sodium hypochlorite 10 % for 15 minutes and rinsed for three times by sterile water. Three Seeds were planted in each pot containing 2 kilograms of loamy sandy soil and kept in the greenhouse. ^[15] The plants were grown in 20-cm diameter pots (10 plants/pot, and 3 pots/plant species) with one pot as control and they all were maintained in the greenhouse at ambient conditions (24-27°C, 14 h natural light). All plant species were directly sown in pots (not transplanted), watered when needed, and fertilized using nitrogen-phosphorus-potassium (NPK) fertilizer (2 g/l; Magical Silver®, Sabz Azar Caspian Co., Tehran, Iran) in ten-day intervals. All plants were inoculated after 45 days (or 6 to 7 weeks later) and when plants were at 3-4 leaf stage in Rostam region, emergence depending on the stage of growth of the species. In this step, 10 mL of bacterial suspension with density of 10⁸ CFU/ml was injected into crown and stem of plant using sterilized insulin syringe. Additionally, 200 mL of bacterial suspension with 10⁸ CFU/ml density was added to other pots (100 mL bacterial suspension per 1 kg of soil). Meanwhile, control sample was injected with 10mL still water via crown and stem, or 100mL bacterial suspension was added to the soil.

Bacterial suspensions of isolated bacteria were prepared according to 0.5 McFarland standard method. Fresh culture of isolated bacteria cultivated on Nutrient Agar (NA), and after 4-5 days fresh colonies suspended in sterile saline solution to reach an optical density of 0.1 at 600 nm (approximately 10⁸ CFU/mL). Inoculation was made using a sterile syringe injected freshly prepared bacterial suspension (1×10⁸ CFU/ml) through the stem and crown internodes of each plant; otherwise, by adding bacterial suspension into soil.

Molecular characterization of the isolated strains

The isolated bacterial strains were tested using PCR primers for *Clavibacter michiganensis* subsp. *insidiosus* using CMI-III/BB1 / CMI-III/BB2, ^[21] and CIRS-1/ CIRS-2. ^[15] Total DNA was extracted from bacterial isolates according to manufacturer's recommendations. For each strain, a 25 µl PCR including 100 ng total DNA and 1 µl of each primer (10 pmolµl⁻¹) were used. The annealing temperatures and the respective primer sequences are described in Table 1.

Table 1. Specific primers used to detect *Clavibacter michiganensis* subsp. *insidiosus* is the causal agent of Alfalfa Bacterial Wilt

Oligonucleotide primer	'Sequence of primers from 5' to 3'
CMI-III/BB1	5'- GAGGGACCGGACCGCATCTTTCGGGG - 3'
CMI-III/BB2	5'- GAT TGA TTC GTT TCG CCT CCC CTA GA- 3'
CIRS-1	5'- TTCAACCGCACCTCGCGAC - 3'
CIRS-2	5'-CGTCAGCCCGTGGCTCGAGT - 3'

The PCR reaction contained the primer (10 μM) 1 μM from each, PCR buffer (with final concentration 10X) 2.5 μl, MgCl₂ (50 mM) 0.75 μl, dNTPs (10 μM) 0.5 μl, Tag DNA polymerase (Cinnagen Co Iran) (5 unit/μl) 0.25 μl, Target DNA of each strain 1 μl and deionized sterile distilled water 18 μl; in a final volume of 25 μl. 34 cycles of PCR for primers CMI-III/BB1 and CMI-III/BB2 was followed as: initial denaturation at 95°C for 5 min, denaturation at 95°C for 1 min, annealing at 65°C for 1 min, elongation at 72°C for 2 min and final extension at 72°C for 10 min. 30 cycles of PCR for primers CIRS-1 and CIRS-2 was followed as: initial denaturation at 95°C for 4 min, denaturation at 94°C for 1 min, annealing at 58°C for 1 min, elongation at 75°C for 1 min and final extension at 72°C for 4 min. The PCR products were run on 1.4% agarose gels and the bands were visualized by ethidium bromide staining.

Results

Phenotypic characteristics of the isolates

Isolation resulted in 32 bacterial strains with colonies colored from yellow to orange and negative catalase from NA.

Morphological analysis of isolated bacteria is presented in Table 2. All strains were able to grow in aerobic condition, catalase negative, able to hydrolyze aesculin and induce hypersensitive reactions on tobacco. While, all of them were negative for hydrolysis of starch, gelatin hydrolysis, casein hydrolysis, tween (60 and 80) hydrolysis, and levan production. Most of them showed a negative oxidase. Fermentation test showed that all isolates were able to produce acid from Mannose, galactose and xylose, but were unable to produce acid from sorbitol, ribose, rhamnose, adonitol, dulcitol, erythritol, ethanol, mannitol and inulin. In addition, the isolates were able to use acetate, glutamate, malonate and succinate but were unable to use tartrate.

Table 2. Phenotypic and nutritional characteristics of the strain *Clavibacter michiganensis* subsp. *insidiosus* is the cause of alfalfa wilt

Tests	Reaction	Tests	Reaction
Gram	+	Galactose	+
Aerobic growth	+	Adonitol	-
Hydrolysis of Aesculin (growth in 6% NaCl)	-	Dulcitol	-
Grow in a medium contain TTC	+	Erythritol	-
Catalase	-	Ethanol	-
Oxidative/Fermentative	+	mannitol	-
Levan	-	Mannose	+
Oxidase	-	Xylose	+
Urease	-	Inuline	-
Casein hydrolysis	-	Use from:	
Starch hydrolysis	-	Succinate	-
Acid production from:		Fumarate	-
Sorbitol	-	Acetate	-
Rhamnose	-		
Ribose	-		

+: more than 80% strains showed the reaction. -: more than 80% strains did not show the reaction

Polymerase Chain Reaction (PCR) Test

Polymerase Chain Reaction (PCR) was conducted based on methodology that was done by using CIRS, CMI-III/B primers.^[22] Then electrophoresis of PCR products was done. The electrophoresis pattern of PCR products were shown in Figure 1. As expected, bands of 218 bp and 132 bp were observed and the presence of *Clavibacter michiganensis* subsp. *insidiosus* was identified as a main cause of bacterial wilt alfalfa.

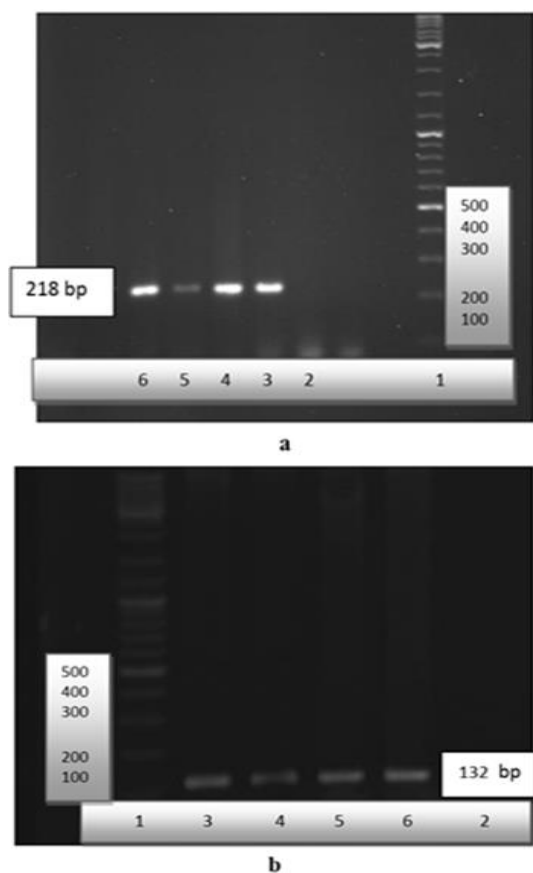


Fig.1. a: PCR amplified fragments of *Clavibacter michiganensis* subsp. *insidiosus* Isolated by CMI-III/BB1 and CMI-III/BB2, b: PCR amplified fragments of *Clavibacter michiganensis* subsp. *insidiosus* Isolated by CIRS-1 and CIRS-2

Discussion

As we visited the fields belonging to different areas of Fars province (Sepidan, Marvdasht, Eghlid, Nour Abad Mamasani, Rostam, and Shiraz), we identified that there were symptoms of *Clavibacter michiganensis* subsp. *insidiosus*, stunting, wilting, chlorosis, and browning of vessels (rarely). As Samac reported, *Clavibacter michiganensis* subsp. *insidiosus* was observed in fields aged more than three years^[15] which is in agreement with our observations. In most literatures, browning of vessels was also reported; however, this symptom was rarely observed during the sampling process of this study and it was mostly observed in plants with advanced symptoms.

From the infected plants, aerobic, gram positive, negative catalase, and negative oxidase bacteria were separated. Based on the results, This bacteria had negative reaction in the Production of levan, urease, starch hydrolysis, acid production from ribose, sorbitol, adonitol, dulcitol, erythritol, ethanol and inulin, using fumarate, Succinate and acetate, caseine hydrolysis, growth at 37 °C, and positive reaction in hydrolysis of aesculin, the use of acid production from galactose, negative inulin, mannitol, xylose and induction of hypersensitive reaction in tobacco.^[23]

The isolates did not show different phenotypic characteristics. Only in some characteristics such as acid production from adonitol, arabinose, ethanol, dulcitol, xylose, cellobiose, arabitol and starch hydrolysis were different. Since these differences were

not observed in the isolates of one particular area that was not the criteria of distinguish. Thirty isolates belonging to a certain area were put in one group based on the numerical analysis of phenotypic characteristics. The differences between these isolates were due to their nutritional differences.

In the pathogenicity test of alfalfa inoculated plants showed symptoms of *Clavibacter michiganensis* subsp. *insidiosus*, stunting, wilting, chlorosis after about 50-60 days, but there was no symptom of browning the ovens vessels. The observed symptoms were exactly the same as previous reports.^[14, 15, 20]

In the pathogenicity test of Hamadan isolates chlorosis were observed symptoms of chlorosis after 45 days.^[12] In the polymerase chain reaction, specific primers of CMI-III/BB1, CMI-III/BB2 and CIRS-1, CIRS-2 were used.^[14-16, 20] These primers were designed based on the intra-genome space between the 16S rRNA and 23rRNA genes and amplified 218 bp and 132 bp fragments, respectively. PCR test of the bacteria separated from alfalfa plants was identified *Clavibacter michiganensis* subsp. *insidiosus*.^[14, 16, 20]

Samac et al cultivated the seeds produced from the infected plants in the greenhouse condition. They placed the squashed seeds on the semi-selective Agar medium. Bacterial colonies appear on the medium if seeds are infected. After that, polymerase chain reaction (PCR) with specific initiator of CIRS was used for identifying these bacteria. As it was shown in this test, 6.3 to 7.7 percentages of the plants transferred their disease with their seeds.^[15] The separate examination of the seeds showed that 2.5 to 8.7 percentages of the seeds contained those bacteria.

In this study to determine seed born of the disease factor, 6 isolates were used whereas 4 pots were considered (one pot was considered as an evidence for each isolate). The seed born percentage of the desired bacteria has been stated 2.06 that was less than what Samac et al reported.

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