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Occurrence of *Pseudomonas savastanoi* the Causal Agent of Winter Jasmine Gall in Iran

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Abstract-During the years 2007 and 2008, samples of winter jasmine (*Jasminum nudiflorum*) with symptoms of galls on shoots were collected from Shiraz, Fars province, Iran. Gram and oxidase negative, aerobic bacterium that produced fluorescent pigment on King's B medium was isolated from galls of infected trees. All isolates produced a hypersensitive reaction in tobacco. They were negative in arginine dihydrolase, gelatin hydrolysis, and potato rot slices. The strains were catalase positive, produced H_2S from cysteine and were able to grow at 37° C. Isolates were variable in levan production. All of the isolates were identified as *Pseudomonas savastanoi* based on morphological, physiological and biochemical characteristics, pathogenicity and with specific primer pairs iaaLf and iaaLr. In addition, the bacterium was detected directly by PCR in the gall from naturally infected winter jasmine plants.

Keywords: iaaLf and iaaLr primers, *Pseudomonas savastanoi*, winter jasmine

INTRODUCTION

Winter jasmine (*Jasminum nudiflorum*) is a member of the Oleaceae family and a shrub indigenous to China. The winter jasmine has yellow flowers which appear in winter and is an upward growing plant (24).

Pseudomonas savastanoi is the causal agent of gall disease of winter jasmine (33). This bacterium is commonly pathogenic on olive (*Olea europaea* L.), ash (*Fraxinus excelsior* L.), privet (*Ligustrum japonicum* Thunb), jasmine (*Jasminum* sp.), forsythia (*Forsythia* sp.), oleander (*Nerium oleander*), spanish broom (*Retama sphaerocarpa*), and *Phillyrea* sp. (1, 3, 4, 5, 9, 17 and 25). *Pseudomonas savastanoi* causes hyperplastic symptoms on olive and other minor host plants (9 and 33). Symptoms of the development of knots are dependent on the production of the phytohormone indol acetic acid (IAA) and cytokinins (6, 16, 29, 31 and 32). *P. savastanoi* pv.*savastanoi* isolates produce IAA from L-tryptophan via indoleacetamide as an intermediate. The two enzymes involved in IAA biosynthesis are tryptophan monooxygenase, catalyzing the conversion of L-tryptophan to indoleacetamide, and indoleacetamide to IAA and ammonium (19, 20 and 33). This

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bacterium can conjugate IAA with lysine to form 3-indoleacetyl-ε-L-lysine (IAA-lysine) (15). The enzyme involved in the conversion of IAA to IAA-lysine is (indol-3-acetyl)-L lysine synthetase (14). The genetic determinants for these enzymes have been identified as three genes termed *iaa*M, *iaa*H and *iaa*L (6, 14 and 27). Penyalver et al. (2000) described specific primers, *iaa*Lf/*iaa*Lr based on *iaa*L gene for the detection of *Pseudomonas savastanoi* in olive plants by PCR (26).

In Iran, olive knot disease was first reported from Mazandaran, in the north of Iran. The isolated bacteria were phenotypically similar to *Pseudomonas savastanoi* pv. *savastanoi* reported from Spain (11). Ghasemi and Taghavi (13) reported winter jasmine bacterial gall disease from Shiraz, Iran. Oleander knot disease has been reported from Tehran, Iran (12).

The aims of this study were to identify the causal agent of *P. savastanoi* of winter jasmine gall disease based on phenotypic characteristics and PCR test with specific primers for rapid, sensitive and direct diagnosis.

MATERIALS AND METHODS

Sampling and Isolation

Winter jasmine plants with gall symptoms on shoots and stems were collected from the city of Shiraz in Fars province, Iran. The galls were cut aseptically with a sterile scalpel, surface-sterilized with a 0.5% sodium hypochlorite solution, and macerated into 1ml of sterile distilled water. After 10 min, the obtained suspensions were streaked on King's medium B (18) and incubated at 25° C for 72h. A total of 23 strains were isolated from winter jasmine galls. Moreover, reference strains from Spain (IVIA-1657.8 and IVIA-1628.3) and strains isolated from olive and oleander galls (11 and 12) were used in this study.

Physiological and biochemical test

Physiological and biochemical characteristics of the isolates were determined by standard bacteriological methods including: gram stain, fluorescent pigment production on King's medium B (28), Colony morphology on nutrient agar (NA), LOPAT profile (21), indole production with Kovac's reagent, catalase reaction and nitrate reduction (10), starch hydrolysis (22), growth at 37° C in yeast salts broth growth in 3, 5 and 7% NaCl, susceptibility to antibiotics, gelatin liquefaction and H_2S production from L-cysteine (28). The isolates were compared to reference strains from Spain (IVIA-1657.8 and IVIA-1628.3) and strains previously isolated from olive and oleander galls (11 and 12).

Pathogenicity test

One and two-year-old winter jasmine (*Jasminum nudiflorum*), olive (*Olea europa* L.), oleander (*Nerium oleander*), ash (*Fraxinus excelsior* L.) and privet (*Ligustrum japonicum* Thunb) plants were used for inoculation. The bacterial suspensions were made in sterile distilled water (SDW), and concentration was adjusted to 10^8 cfu ml^{-1}. Five µl of the suspension was injected tangentially into the bark using a sterile needle and the hole was covered with parafilm for three days. The control plants were inoculated with SDW. The plants were kept in a greenhouse at 23-26° C and 75-80% RH. Symptom development was monitored up to 5 months after inoculation.

PCR amplification

Bacterial suspension was adjusted to 10^7-10^8 cfu/ml in SDW. The suspension was boiled for 10 min, and 2 µl of the boiled suspension was used as template for the PCR test. Oligonucleotide primers IAALf (5́-GGCACCAGCGGCAACATCAA-3́) and IAALr (5́-CGCCCTCGGAACTGCCATAC-3́) were used in a standard PCR assay (26). PCR reactions were performed in a 20 µl PCR cocktail containing 10×PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphates, 0.6 µM of each primer, 1.5U Taq DNA polymerase and 2 µl of template DNA. PCR amplification was carried out as follows: one cycle at 94˚C (5min), 35 cycles at 94˚C (30s), 62˚C (30s), and 72˚C (30s) and then one cycle at 72˚C for 5min in a 9600 Perkin Elmer thermocycler. Amplified products (6 µl) were separated by electrophoresis on a 1% agarose gel, stained with ethidium bromide and photographed by a gel documentation system.

Direct detection of *P. savastanoi* in winter jasmine gall

One gram of gall tissue was crushed in a sterile pounder and 1 µl of GES (guanidine thiocynate) and PVPP (Polyvinylpyrrolidone) buffers (0.1 M glycine-NaOH, pH 9.0, 50 mM NaCl, 1 mM EDTA) were added. The nylon membrane treated with these buffers was immersed in eppendeorf tubes filled with GES buffer, and then 2 µl of suspensions were boiled in a GES buffer at 95˚C for 10 min and used as template for PCR (8). Afterwards, PCR was performed with the specific primers iaaLf and iaaLr as mentioned above.

RESULTS AND DISCUSSION

Symptomatology

The symptoms of the disease in winter jasmine (*Jasminum nudiflorum*) plants are paranchymatic galls that appear on shoots. Galls usually appear in shoot branching or pruning sites (Fig. 1). These areas are suitable entrances for the bacteria. The galls were small, streaky and cream colored. However, upon disease progress, they enlarged, and became woody and dark.

Fig. 1. Symptoms of winter jasmine gall induced by *Pseudomonas savastanoi* on shoot

Isolation and identification of bacterial isolates

All of the 23 isolates recovered from the galls were gram-negative and colonies were grey to cream colored, circular or irregularly round, entire, flat or slightly raised on SNA medium at 25˚C after 5 days. All strains were negative for oxidase, potato rot, arginine dehydrolase, gelatin liquefaction, nitrate reduction, indole production, and hydrolysis of starch, tween80, lecithin, and aesculin. They showed positive reaction to tobacco hypersensitivity, catalase, utilization of citrate, sensitivity to tetracycline and amoxicillin, production of H_2S from L-cysteine, growth at 37˚C, and also ability to grow in 3, 5 and 7% (W/V) NaCl. The phenotypic, biochemical and nutritional characteristics of the isolates are listed in Table 1. The isolates showed homogeneity in their physiological, biochemical and nutritional characteristics but some of the isolates exhibited variation in the production of levan. Based on morphological, physiological and biochemical characteristics, the isolates were identified as *Pseudomonas savastanoi* (28). The strains were phenotypically similar to the reference strains of *Pseudomonas savastanoi* pv. *savastanoi* (IVIA-1657.8 and IVIA-1628.3) from olive, *P. s.* pv. *savastanoi*, and *P. s.* pv. *nerii* from olive and oleander respectively (11 and 12).

Pathogenic city test

Four months after inoculation, the symptoms began to appear as a small gall around the site of inoculation only on the stem of *winter jasmine* (Fig. 2). The bacterium was reisolated from the galls of artificially infected plants. None of the inoculated olive, oleander, ash and privet plants with the isolates produced gall on their shoots. None of the control plants inoculated with sterile distilled water developed gall symptoms. *P. savastanoi* pv. *nerii* from Tehran produced galls on the stem of oleander. The reference strains of *P. s.* pv. *savastanoi* caused similar symptoms on the stem of winter jasmine.

Fig. 2. Small gall on the stem of winter jasmine plant (*Jasminum nudiflorum*) inoculated with *Pseudomonas savastanoi* isolates

PCR assay

All of the 23 isolates from winter jasmine plants and reference strains produced the expected 454-bp product with the primer pairs *iaa*Lf/*iaa*Lr (26) in the PCR assay (Fig. 3). These results confirmed the identification of *P. savastanoi* isolates.

Table 1. Phenotypic, biochemical and nutritional characteristics of *Pseudomonas savastanoi* isolated from winter jasmine, oleander and olive

Characteristics	Bacterial strain[a]			
	IVIA-1628.3	Winter jasmine isolates	Olive	Oleander
Gram stain	-	-	-	-
Catalase	+	+	+	+
Potato soft rot	-	-	-	-
Oxidase	-	-	-	-
Gelatin liquefaction	-	-	-	-
Starch hydrolysis	-	-	-	-
Lecithinase	-	-	-	-
Flurecent pigment on KB	+	+	+	+
Aesculin	+	+	+	+
Levan	-	+/-	-	-
Hydrolysis of Tween80	-	-	-	-
Growth at 37°C	+	+	+	+
Growth in:				
3% (W:V) NaCl	-	-	-	-
5% (W:V) NaCl	-	-	-	-
7% (W:V) NaCl	-	-	-	-
Arginine dihydrolase	-	-	-	-
Tobacco hypersensitivity	+	+	+	+
H_2S production from cysteine	+	+	+	+
Nitrate reduction	-	-	-	-
Utilization of:				
Cellobiose	+	+	+	+
Sorbitol	-	-	-	-
Trehalose	+	+	+	+
Sucrose	-	-	-	-
Meso-tartrate	-	-	-	-
Sensitivity to:				
Amoxicilin	S	S	S	S
Erythromycin	R	R	R	R
Penicilin	R	R	R	R
Tetracycline	S	S	S	S

[a] +, positive; -, negative; KB, King's B medium; S, sensitive; R, resistance

Direct Detection of *P. Savastanoi* in Galls of Winter Jasmine by PCR

The PCR test with specific primers, *iaa*Lf/*iaa*Lr, enabled us to detect *P. savastanoi* in the gall of a naturally infected plant (Fig. 3). Detection of *P. savastanoi* is currently based on pathogenicity test and biochemical or serological techniques (5, 17, 30 and 35). The routine methods require a lot of time and are not quick and sensitive. There have been some reports on the specific detection of *P. savastanoi* in plants using preenrichment in PVF-1 and nested-PCR in a single closed tube (2 and 26).

P. savastanoi was detected in the inoculated and naturally infected samples by using a simple and rapid template preparation protocol reported by Dovas and Katis (8) (Fig. 3). This method is a rapid and sensitive detection method for *P. savastanoi* in naturally infected plants and reduces the effect of inhibitors present in plant materials.

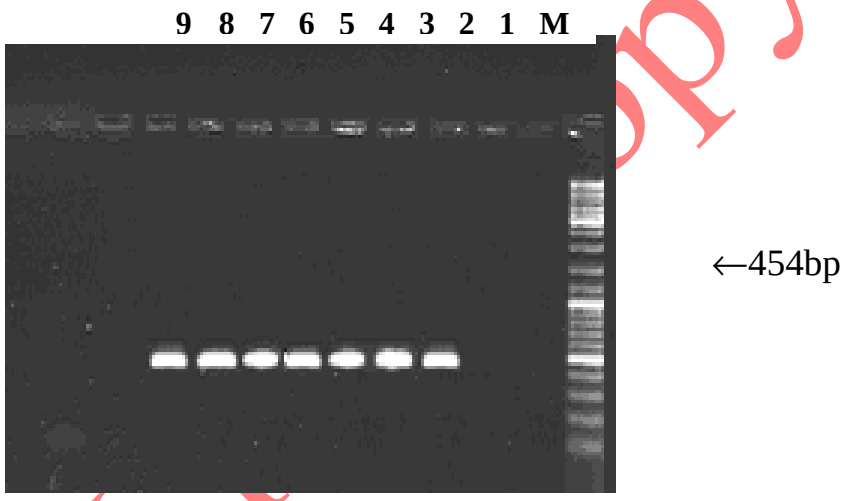

Fig. 3. PCR products of *Pseudomonas savastanoi* isolates amplified by specific primer iaaLF/iaaLR, M, 1Kb molecular marker; lanes 1, negative control; lanes 2, *Pseudomonas syrigae* pv. *syringae*; lanes 3,4; *Pseudomonas savastanoi* isolated from jasmine; lanes 5, *Pseudomonas savastanoi* isolated from olive; lanes 6, *Pseudomonas savastanoi* isolated from oleander; lanes 7,8,9; naturally infected winter jasmine gall extracts

In the present study, the isolated bacterium from winter jasmine galls, was identified as *P. savastanoi* and was similar to the reference strains from Spain and strains from olive and oleander in Iran on the basis of morphological, biochemical and pathogenicity tests (28), and specific PCR primers (26). This is the first report of specific, rapid and direct detection of *P. savastanoi*, the causal agent of winter jasmine gall by PCR with the specific primers in Iran.

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وقوع باکتری *Pseudomonas savastanoi* عامل بیماری یاسمن زمستانی در ایران

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طی سالهای 1386و 1387 از درختچه‌های یاسمن‌زمستانی دارای علائم گال روی سرشاخه‌ها و ساقه‌ها در شهرستان شیراز، استان فارس نمونه‌برداری گردید. یک باکتری هوازی، اکسیداز منفی که قادر به تولید رنگدانه فلورسنت روی محیط کشت KB بود، از گیاهان دارای علائم جدا گردید. کلیه‌ی جدایه‌ها قادر به تولید حساسیت فوق‌العاده روی توتون بودند ولی قادر به هیدرولیز آرژنین، ژلاتین و لهیدگی سیب‌زمینی نبودند. تمام جدایه‌ها کاتالاز مثبت، قادر به تولید H_2S از سیستئین و رشد در $37^{\circ}C$ بوده ولی در تولید لوان متغیر بودند. بر اساس خصوصیات مورفولوژیکی، بیوشیمیایی، فیزیولوژیکی و بیماری‌زایی و همچنین استفاده از آغازگرهای اختصاصی *iaa*Lf و *iaa*Lr در آزمون PCR، جدایه‌های مذکور به عنوان *Pseudomonas savastanoi* تشخیص داده شدند. همچنین باکتری عامل بیماری بوسیله PCR مستقیم از یاسمن‌زمستانی دارای علائم گال تشخیص داده شد.

واژه های کلیدی: **آغازگرهای اختصاصی، یاسمن زمستانی، iaaLr و iaaLf، *Pseudomonas savastanoi***

* به ترتیب استاد و دانشجوی پیشین کارشناسی ارشد

** مکاتبه کننده