Identification and Characterization of Bacterial Agents Causing Moderate Damage and Souring of the Fig Fruits

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ABSTRACT

In order to determine the factors of decay and sourness of Fig fruits, in the summer of 2016-17, 60 leaf, fruit and stem samples from different regions of Tehran, Varamin (Qal'e No), Mazandaran (Amol, Noor and Sari), Lorestan and a branch of Fig fruit sample from Italy were collected. The samples were surface disinfected with 70% alcohol, washed and macerated in DH_2O and one loopful of each suspension was cultured on NA and KB media. The dominant colonies from each culture were further selected and purified. The pathogenicity tests were fulfilled by Hypersensitivity reaction on geranium leaves and artificially by Fig fruit inoculation. Thirty isolates were considered as pathogenic bacteria and key phenotypic traits were performed. This was confirmed by *16S rRNA* gene PCR assay using a pair of universal P1/P6 primer. The amplified PCR products were sequenced by Macrogene Inc. and were Blast in NCBI database (www.ncbi.nlm.nih.gov). The bacteria, *Stenotrophomonas maltophilia, Pseudomonas aeruginosa, Pseudomonas fulva, Brevibacterium linens, Pseudomonas fragi, Bacillus licheniformis, Bacillus paralicheniformis* and *Bacillus cereus* were identified. None of the isolates caused fruit rot but typical disease symptoms were observed on Fig leaves and fruits. This is the first report of the presence of pathogenic bacteria on Fig trees in Iran.

Keywords: Fig trees; Bacteria; Iran; *16S rRNA* gene; Sequencing

INTRODUCTION

Among the agricultural products, fruits are the most important horticultural products that play an important role in supplying human nutrition and health. Among these, the Fig (*Ficus carica* L.) is one of the earliest cultivated fruit trees in the world [1]. The tree is cultivated in warmest and temperate Mediterranean climates with fifty percent of the world's fig production concentrated in the Mediterranean area. Fresh and dried figs are consumed and appreciated world-wide not only for their unique taste and distinct flavor, but also for their health benefits. Figs have been popular not only because of their pleasant taste but possibly also because of their medicinal properties. The short postharvest life of fresh fruits is a critical point for the marketability in respect to dried fruits. The fresh figs are highly perishable fruits being susceptible to postharvest deterioration, such as softening, bruising, splitting, and pathogens growth. In Iran, Fars province and Estahbanat city are considered as the poles of Fig production [2]. According to the Agricultural Jihad statistics, 86685.5 hectares of dry and rainfed agricultural lands of the country were allocated to the commercial Fig production [3]. FAO statistics also show that Iran's rank in terms of production and cultivation of Fig is fifth and third, respectively

[4]. Many biotic factors have been forced to reduce the production of Figs in gardens and in warehouses. These include the fungi, Cercospora leaf spot (*Cercospora fici*), Fig souring (*Hanseniospora osmophila*), Fig brown rot (*Fusarium moniliforme*) and Fig mosaic virus (unknown virus) [2,5] Some other Fig fungal diseases include Botrytis blight (*Botrytis cinerea*), Thread blight (*Pellicularia koleroga*) results in necrosis of stems and matted foliage, *Botryosphaeria dothidea* which cause necrosis of leaves and stems and *Rhizopus stolonifer* (smut) as the causal of fruit drop of cultivars with an open eye. *Fusarium* spp. and *Aspergillus niger* are also attack ripe fruits [6].

Few bacterial diseases were also reported on *Ficus* spp. particularly on edible Figs (*Ficus carica*). These include crown gall (*Agrobacterium tumefaciens*), Pseudomonas leaf spot (*Pseudomonas cichorii*) and Xanthomonas leaf spot caused by *Xanthomonas campestris* pv. *fici* [7,8]. *Rhizobium larrymoorei* (=A. *larrymoorei*) was isolated from gall of weeping Fig (*Ficus benjamina*) [9].

Apart these, various yeasts, fungi, and bacteria apparently responsible for the souring disease of the fruit of the Fig. Organisms are carried into the fruit by the dried fruit beetle, *Carpophilus hemipterus*. It is a preharvest problem resulting from yeasts and bacteria carried

into the Figs by insects, especially vinegar flies, resulting in odors of alcohol or acetic acid [10-12]. Many types of Fig fruit rot were observed, from the soft, watery, fermented type to the typical dry-rot type [13]. The aim of this study was to identify new bacterial pathogens and some bacteria affecting fruit souring of Fig trees.

MATERIALS AND METHODS

Isolation and purification of bacteria

In the summer and late autumn of 2016, 60 plant samples including branch, leaf and fruit of infected Fig trees with brown spot symptoms on leaves and rot and deformity of fruits and necrotic spots on fruit and stems were collected from different regions of Tehran, Mazandaran and Varamin. A peculiar Fig samples were collected from a tree grown in Modarres hospital yard in Tehran. All samples were washed with distilled water and then sterilized with 70% alcohol for 1 minute. Small pieces from border of infected and healthy tissues were prepared and each was transferred to tubes containing 5 ml sterilized distilled water. A loopful of sample suspension was streaked on Nutrient agar (NA) and King's (KB) media. Petriplates were incubated at 25°C for 48 hours. The typical single colonies were transferred to new culture media and purified. Pathogenicity tests were fulfilled and all suspected pathogens were preserved in nutrient broth containing 15% glycerol at -20°C and in NA slants under liquid paraffin.

Pathogenicity tests

Hypersensitivity reaction (HR): The hypersensitivity reaction test was performed on geranium leaves. At first, a suspension of bacteria with an approximate concentration of 10^6 cfu/ml was prepared and then injected with a sterile syringe to underside of the young leaves. The reaction was evaluated for 24 to 48 hours [14].

Potato soft rot test: Healthy and fresh potato tubers were washed thoroughly with tap water, surface sterilized with 70% alcohol. The tuber skin was peeled and internal tissue was cut into small slices. Each slice was placed into sterile petri dish containing about 1/8 inch of sterile water and the surface was of the tissues were inoculated with a single colony of each isolate. Distilled water was used as negative controls. Petri dishes were kept at 25-30°C for 24-24 hours. All rotten and decay slices were recorded [15].

Pathogenicity test on detached Fig fruits and leaves: The fruits and leaves of two Fig varieties (desert King Fig and Adriyatic Fig) were first disinfected with alcohol and then were put into sterilized petri dishes containing sterile distilled water. The leaves and Fig fruits were inoculated with individual bacterial suspension using straight wire [15]. Distilled water is used as a negative control. The negative treatments were checked daily for development of any disease symptoms.

Phenotypic characterization of the isolates

The isolates were characterized based on certain key tests. These include, gram test, aerobic/anaerobic growth, NaCl tolerance, spore formation, fluorescent pigmentation, levan production, oxidase, catalase, gelatinase, amylase, β-glucosidase, urease and nitrate reductase tests. Citrate utilization, casein and tween hydrolyses, MR-VP tests were also tested [15].

Identification based on PCR analysis

DNA extraction: Bacterial DNA was extracted by simple boiling method modified after Sambrook et al. [16]. Bacteria were grown in agar media at 28°C for 2 days. A loopful of cells was suspended in 25 µl of sterile distillated water and followed an addition of 25 µl of freshly prepared lysis buffer containing 0.1 N NaOH and 0.5% SDS. The mixture was boiled in a water bath for 15 min. 200 µl of TE (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) was added to the mixture and centrifuged at 13,000 rpm for 5 minutes. Each supernatant was transferred to a clean tube and stored at -20°C.

Preparation of mixture reaction for PCR test: The final volume of the reaction mixture was 25 µl for each tube, containing 23 µl of the reaction mixture and 1 µl of genomic DNA equivalent to 100 ng for each isolate. The primer pair P1 (5′-AGAGTTTGATCCTGGTCAGAACGCT-3′) and P6 (5′-TACGGCTACCTTGTTACGACTTCACCCC-3′) was used [17]. The reaction mix was placed on a thermocycler and subjected to PCR cycles: 94°C for 2 min, followed by 94°C for 45 seconds, annealing at 58°C for 45 seconds, primer extension at a temperature of 72°C for 1 minute and final extension by 35 cycles at a temperature of 72°C for 20 minutes. PCR amplified fragments were electrophoresed in an agarose gel (1%) and were visualized using loading buffer dye. PCR products of 10 strains were sent to Macrogen Inc. (Seoul, South Korea) for sequencing. The DNA sequences are compared with existing sequences in the NCBI GenBank. Phylogenetic tree from aligned sequences was constructed using Mega-6 software.

RESULTS

Isolation and characterization of the isolates

Sixty Fig plant samples with different diseased and abnormal symptoms from 7 cities and one from Italy were collected (Figure 1). Thirty bacterial isolates were selected in the first stage that were capable of causing HR on tobacco leaves and to some extent disease symptoms of Fig leaf and fruit. Of these, 10 isolates with sever disease symptoms were selected for PCR analysis.

Pathogenicity tests

Hypersensitivity reaction: Thirty isolates were able to cause hypersensitivity and necrosis on pelargonium leaves (Figure 2).

Potato soft test: None of the 30 isolates that showed necrosis on the pelargonium leaves were able to soft rot on the potato slices.

Testing for pathogenicity on Fig fruits and leaf: All 10 selected isolates caused various disease symptoms including necrosis and chlorosis in leaves; rot and deformity of fruits and brown spots on fruit skin and stem. The four bacteria namely, *Pseudomonas fragi*, *Brevibacterium linens*, Serratia marcescens, *Pseudomonas aeruginosa* could have severe symptoms of the disease on Adriatic fruits rather than desert king Fig fruits (Figure 3).

Identification of bacterial isolates

The ten selected bacteria were identified based on certain morphological, physiological and biochemical tests (Table 1).

Determination of the *16S rDNA* gene Sequences

All 10 selected bacteria produced a single sharp 1500bp fragment (Figure 4). The PCR products were sent to the Macrogene Inc for sequencing. Sequencing was done in both directions of Sense and

Figure 1: Fig fruits and leaves suspected of bacterial contamination collected from different regions. a) Fig fruit deformation, b) Wrinkling and sagging of fig fruit, c) Brown spots on figs fruits, d) Fig fruit soreness and wrinkles.
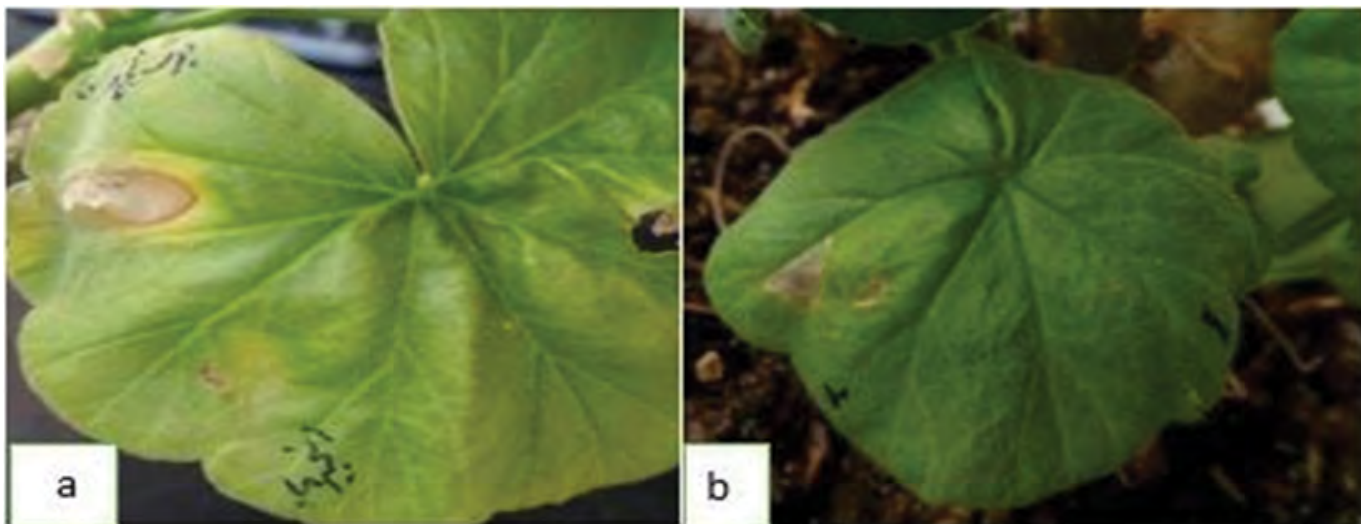

Figure 2: The two representative isolates that caused necrosis symptoms on pelargonium leaves. a) *Stenotrophomonas maltophilia* AM2, b) *Serratia marcescens* AM15.
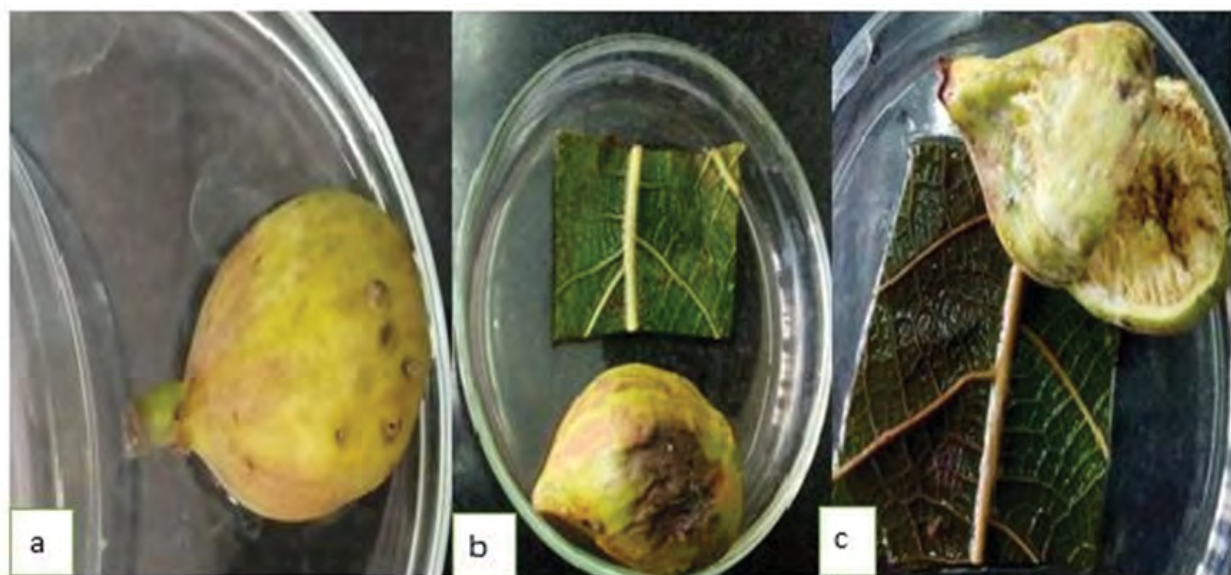

Figure 3: Incidence of necrosis and chlorosis symptoms on leaves and fruits by isolates a) *Stenotrophomons maltophilia* AM2, b) *Pseudomonas aeruginosa* AM22, c) *Brevibacterium linens* AM12.

Antisense using P1/P6 primers. The sequence alignments were done using BLAST software in NCBI database (www.ncbi.nlm.nih. gov). The results were indicated that the strains AM2, AM5 and AM11 by 100% of homology were belonged to *Stenotrophomonas maltophilia*, *Bacillus paralicheniformis* and *Pseudomonas fragi*, respectively. Likewise, the strains AM13, AM15 and AM26, AM22 and AM27 by 100% of homology were assigned as *Bacillus licheniformis*, *Serratia marcescens*, *Pseudomonas aeruginosa* and *Bacillus cereus*, respectively. Percentage of homology 96% and 84% ensured that The *16S rRNA* gene sequences of the strains AM12 and AM18 were also showing 96% and 84% identity to *Brevibacterium linens* and *Pseudomonas fulva*, respectively.

Their GenBank accession numbers of the strains are as follows:

MH001477, MH012180, MH012187, MH012195, MH012196, MH012198, MH013188, MH014944, MN461457 and MN461460.

Phylogenetic analysis

Phylogenetic tree based on the *16S rRNA* gene of 10 pathogenic bacteria of Fig plants (Figure 5). The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. The numbers at the nodes are bootstrap values based on 1000 replications. Evolutionary analyses were conducted in MEGA-6 [18].

DISCUSSION

Despite the importance of Fig for Iran, which is economically

Table 1: Physiological and biochemical tests for differentiating 10 selected fig fruits. 1) *Stenotrophomons maltophilia* AM2; 2) *Bacillus licheniformis* AM 5; 3) *Pseudomonas fragi* AM11; 4) *Brevibacterium linens* AM12; 5) *Bacillus paralicheniformis* AM13; 6) *Serratia marcescens* AM15; 7) *Pseudomonas fulva* AM18; 8) *Pseudomonas aeruginosa* AM22; 9) *Serratia marcescens* AM26; 10) *Bacillus cereus* AM27; M: Milky; Y: Yellow; C: Creamy; W: White; O/F; O: Aerobe; F: Facultative anaerobe.

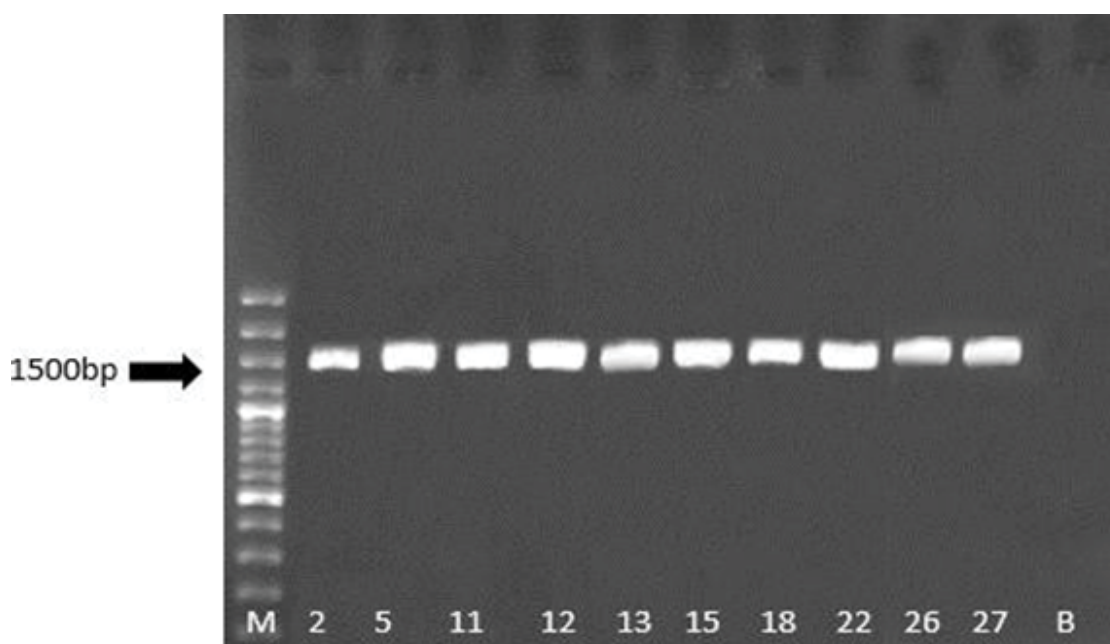| Isolates /Characteristic	1	2	3	4	5	6	7	8	9	10
Gram reaction	-	+	-	+	+	-	-	-	-	+
Colony color	Y	W	W	C	W	W	Y	M	W	W
Fluorescent pigment on KB	+	-	+	-	-	-	-	+	-	-
O/F test	O	F	O	O	F	F	O	O	F	F
Catalase	+	+	-	+	+	+	-	+	+	+
Nitrate reduction	+	+	-	-	+	+	-	+	+	+
Oxidase	-	-	+	+	-	-	+	+	-	-
Arginine dihydrolase	-	+	-	+	+	+	-	+	+	+
Hydrolysis:										
Starch	-	+	-	-	+	-	-	+	-	+
Gelatin	+	-	-	+	-	+	-	+	+	-
Tween 80	+	-	+	-	-	+	-	+	+	-
Levan production	-	-	-	-	-	-	-	-	-	-
Potato soft rot	-	-	-	-	-	-	-	-	-	-
HR on geranium	+	+	+	+	+	+	+	+	+	+

Figure 4: A PCR amplification products of 1500bp for all 10 selected strains. Lane designations: M, 100 bp ladder; 2) *Stenotrophomons maltophilia* AM2; 5) *Bacillus licheniformis* AM5; 11) *Pseudomonas fragi* AM11; 12) *Brevibacterium linens* AM12; 13) *Bacillus paralicheniformis* AM13; 15) Serratia marcescens AM15; 18) *Pseudomonas fulva* AM18; 22) *Pseudomonas aeruginosa* AM22; 26) *Serratia marcescens* AM26; 27) *Bacillus cereus* AM27.
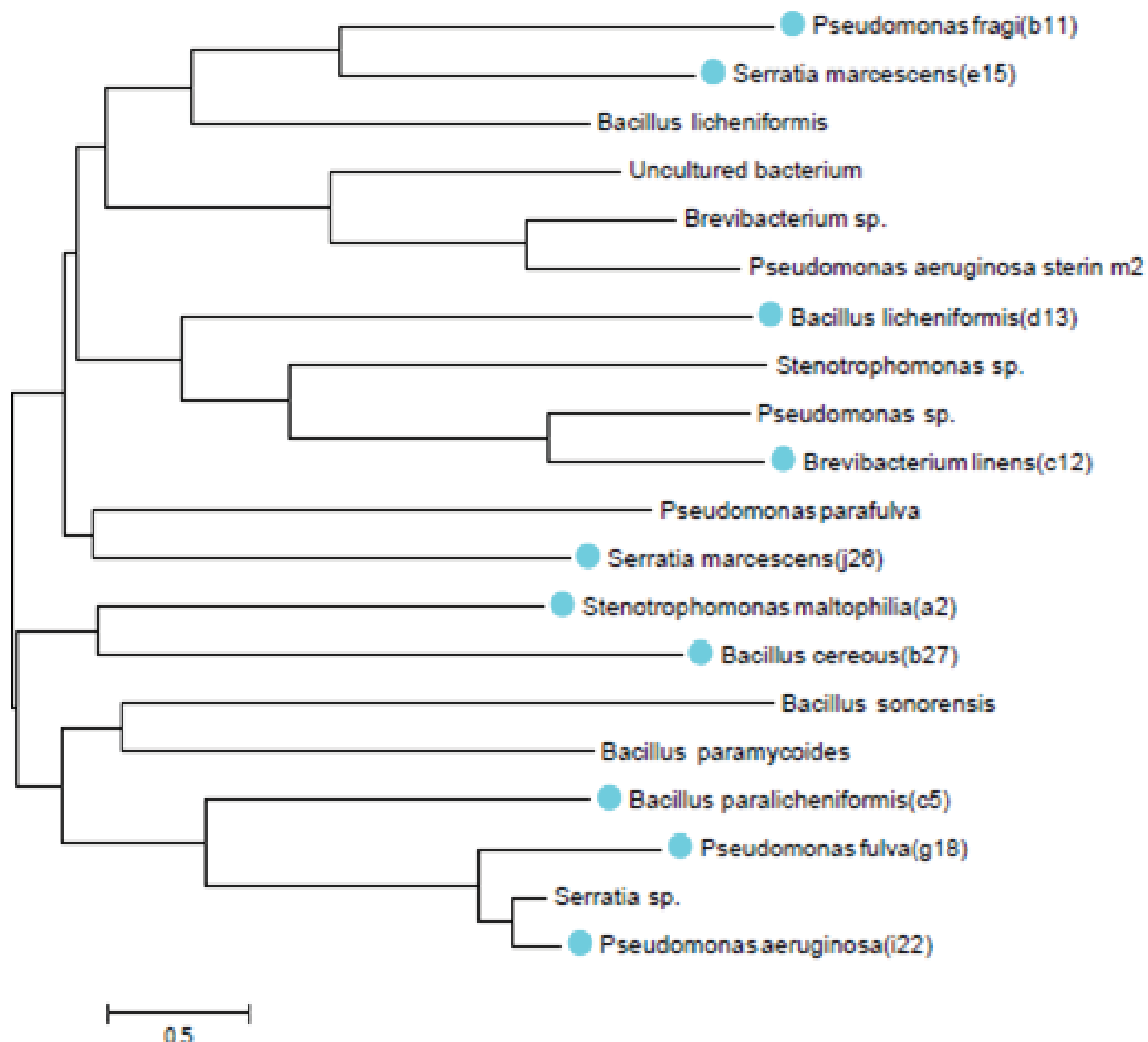
Figure 5: Dendrogram on the phylogenetic relationships of 10 selected isolates based on the sequences of the16S rRNA gene and a number of closest strains in the NCBI GenBank. The tree was constructed by Neighbor-Joining method with 1000 bootstrap in the Mega-6 software.

valuable, the bacterial diseases of the Fig trees, has remained silent until now. Perhaps one of the reasons is that there are a few reports of bacterial diseases on this host worldwide. Most diseases are caused by fungi and most prevalent fruit infection is souring which has been caused by various yeasts, fungi, and bacteria, carried into the fruit by the dried fruit beetle, *Carpophilus hemipterus* [8,13]. In this research, it was attempted to collect all contaminated samples from gardens of seven cities and one from the hospital yard and one from Italy in order to isolate some pathogenic bacteria. Overall of 60 samples were collected, of which 30 isolates were cultured and purified. 10 isolates with severe virulence were selected and further analyzed by PCR method. Pathogenicity tests were included HR on geranium leaves, potato soft rot and direct injection of bacterial suspensions into leaf midribs and immature Fig fruits. Fruit skins were pierced by needles infected with bacterial colonies. Necrosis and water-soaking were the two characteristic leaf symptoms were observed. Fruit symptoms varied from discoloration and decay in the internal tissue of Fig fruits to brown spots on the fruit skins.

Apart these, *Pseudomonas aeurginosa* AM22 caused the wrinkling

of the fruits, as it had been seen in natural conditions. On the other hand, *Serattia marcescens* AM15 and AM26, *Brevibacterium linens* AM12, *Stenotrophomonas maltophilia* AM2 and *Pseudomonas fragi* AM11, caused brown spots, water-soaking lesions and fruit spoilage. The three more isolates namely *Bacillus licheniformis* AM13, *Pseudomonas fulva* AM18 and *Bacillus paralicheniformis* AM5 could cause brown spots and water-soaking lesions without fruit spoilage.

All bacteria were negative to potato soft rot test. This indicates that their pathogenic potency was not dependent on the activity of the cellulolytic and pectiolytic enzymes. All isolates were identified on the basis of biochemical traits and then confirmed by PCR analysis using P1/P6 as a universal primer pair for amplification of *16S rRNA* gene.

The distribution of these bacteria and the areas where these isolates were isolated are as follows: *P. aeruginosa* and *S. marcescens* were isolated from leaf and fruits of the Fig trees located in Modarres hospital in Tehran. *P. fragi* and *S. maltophilia* were isolated from

leaves and ripe fruits of Fig trees in green space of Varamin Research Institute, respectively.

S. maltophilia and *B. paralicheniformis* were isolated from ripe fruits and leaves of Figs trees in Varamin city, respectively. *P. fulva* from leaf and *B. linens* from fruit samples of Amol city. *B. licheniformis* was the only isolate which was collected from Eram Park-Tehran.

Among these, *B. cereus* was the only isolate obtained from a single Fig sample of the Bari-Italy. An important finding in this study was the isolation of *Serattia marcescens, Stenotrophomonas maltophilia, Pseudomonas aeruginosa, Pseudomonas fulva, Bacillus licheniformis, Bacillus cereus* as common bacteria in humans and plants from Fig trees in different regions.

According to numerous reports, these bacteria cause an infection in humans, especially in patients with immune deficiency [19,20]. It was reported by Kamar, 1989 that Bacillus cereus is the cause of two types of poisoning (diarrhea and vomiting). In the case of *Brevibacterium linens* and *Pseudomonas fragi*, these two species have been isolated from plant and dairy products and have been caused the consumer illness [21] whereas; *Pseudomonas fragi* was isolated from lettuce [22]. *Pseudomonas aeruginosa*, a common opportunistic bacterium between humans and plants was isolated from diferent sources such as rhizosphere of corn [23] and tobacco leaf in China [24]. Another important species was *Pseudomonas fulva*, a clinical isolate which has been reported mostly from aquatic environments including rice seed and fennel plants [22]. *Serattia marcescens*, a ubiquitous bacterium has been isolated from plant materials such as watermelon and nutty trees [25]. The latter, was first isolated from Figs in New York, USA [22].

Bacillus licheniformis, has a wide range of hosts and is isolated from soil containing vegetable waste [26], wheat fields of central provinces, Golestan and Mazandaran [27], cucumber farrowing in Jiroft [28], soil and water around the hot springs in Semnan [29]. *Bacillus paralicheniformis* like most of the above bacteria, has been isolated on various plants rather than the Figs. For example, from soybean paste [30], soil containing citrus waste soil in several regions of Golestan province [31], wheat fields of central provinces of Golestan and Mazandaran [27], rhizosphere of orange trees in Kerman province [28] and also from asparagus plant in Iran [29] and in New York [22,31].

CONCLUSION

In conclusion, it should be noted that all bacteria except *Serratia marcescens* are reported for the first time on Fig plants in Iran. There is no doubt that water, food and vegetables play an important role in the spread of pathogens and secondary infections. Given that some of these causal agents are transmitted through consumption of fresh fruits and vegetable, planting fruit trees in open gardens of hospitals is a major mistake and potentially very dangerous for the incidence and spread of diseases with a common human-plant hosts. And finally, due to the abundance of microbial populations in soft rot and damaged fruits, their consumption should be avoided to maintain the health of the body.

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