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Research Papers

## *Pseudomonas syringae* pv. *syringae* causes bacterial canker on Japanese quince (*Chaenomeles japonica*)

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**Summary.** Japanese quince trees are grown as ornamental plants in Iran, in parks and in orchards close to stone fruit and pome fruit trees. Shoots of Japanese quince (*Chaenomeles japonica*) showing sunken brown canker symptoms were observed and collected near Sari, the center of Mazandaran province in the North of Iran, during the 2016 growing season. Gram negative bacteria isolated from symptomatic tissues were similar to *Pseudomonas syringae* pv. *syringae* (*Pss*) were pathogenic on Japanese quince and on quince (*Cydonia oblonga*) seedlings after artificial inoculation, and were re-isolated from diseased hosts. Phylogenetic tree construction using partial sequences of ITS and *rpoD* genes showed that the Japanese quince isolates were in the same clade as *Pss* strains. The isolates had ice nucleation activity, and the *InaK* gene was amplified successfully. According to the results of phenotypic and genotypic characteristics, genomic DNA fingerprinting using REP-PCR, BOX-PCR and IS50-PCR and isolation of total cell proteins, we conclude that *Pss* is the causal agent of canker of the Japanese quince trees. Therefore, Japanese quince is a new host for *Pss* causing bacterial canker on many different host plants.

**Keywords.** Maule's quince, *rpoD*, BOX-PCR, REP-PCR, IS50-PCR.

### INTRODUCTION

Japanese quince (*Chaenomeles japonica* (Thumb.) Lindley ex Spach) is one of the four species of *Chaenomeles* Lindl. (*Maloidae*, *Rosaceae*). This small tree produces white to pink flowers in late winter or early spring. The flowers have five petals and can grow up to 4.5 cm in diameter. The tree is spiny and bears simple alternately arranged leaves with serrated margins. Being native to Eastern Asia and naturally grown in central and Southern Japan, it is also

cultivated in many countries including Iran (mostly together with forsythia), primarily as an ornamental plant for its showy flowers. Moreover, Japanese quince is also cultivated in Latvia, Lithuania, Russia, Poland, Belarus, Sweden, and Finland for its astringent apple-like pome fruits used in preserves and liqueurs, with potential as an alternate fruit crop (Jakobija and Bankina, 2018). Although Japanese quince is usually described as a healthy ornamental plant (Norin and Rumpunen, 2003), it can be attacked by a few pests and diseases, including aphids and scales, fungi and fire blight, reported as the main bacterial disease (Jewell, 1998).

*Pseudomonas syringae* is a heterogeneous group including more than 60 pathovars, belonging to the RNA homology group I of *Pseudomonas*, subclass  $\gamma$  from the Proteobacteria (Young, 2010). Its pathovars are host-specific on a range of plants, including beans, grasses and *Prunus* species (Little *et al.*, 1998). *Pseudomonas syringae* pv. *syringae* (*Pss*) (van Hall, 1902) causes bacterial canker and blast of stone fruit trees. It is one of the most important plant pathogens, causing foliar necrosis in host plants and hypersensitive responses (HR) in non-hosts (Bender *et al.*, 1999; Collmer *et al.*, 2000), leading to economic losses on more than 180 plant species (Bultreys and Kaluzna, 2010; Young, 2010). *Pss* is also an ice nucleation-active (INA<sup>+</sup>) pathogen causing frost injury to plants (Hirano and Upper, 2000).

In Iran, *Pss* strains were isolated and characterized as causal agents of bacterial canker of stone fruit hosts (cherry, peach, apricot), almond, olive, rose, mallow, pelargonium, pear, and quince, red streak of sugarcane, blast of mandarin, and leaf blight of wheat, rice, oat, barley, and cabbage, from different areas (Bahar *et al.*, 1982; Banapour *et al.*, 1990; Rahimian, 1995; Najafi Pour and Taghavi, 2011; Aeni and Khodakaramian, 2018; Khezri and Mohammadi, 2018; Vasebi *et al.*, 2019; Basavand *et al.*, 2021). Japanese quince trees showing sunken brown cankers were observed in the Sari county during the 2016 growing season. As these trees are grown in parks, often close to stone fruit and pome fruit orchards, the aim of the present study was to determine the causal agent of canker symptoms on Japanese quince using pathogenicity, phenotypic, biochemical, and molecular analyses.

## MATERIALS AND METHODS

### *Sampling and isolation of bacteria*

During the 2016 growing season, twigs of Japanese quince plants with canker symptoms and asymptomatic samples were collected from Sari in the Mazandaran

province in the North of Iran. To isolate bacterial strains, plant tissues were surface sterilized, ground in 10 mL sterilized distilled water for 30 min, and a loop-full of the resulting suspension was streaked on nutrient agar with 0.5% sucrose (NAS). Resulting colonies were purified on nutrient agar, and selected according to morphology, color, and shape (Rademarker *et al.*, 2000) for further biochemical, pathogenicity, and molecular tests.

### *Hypersensitive response and pathogenicity tests*

Hypersensitivity response tests were performed by infiltrating bacterial suspensions ( $1 \times 10^8$  cfu·mL<sup>-1</sup>) into tobacco and geranium leaves. Inoculated leaves were examined after 24 h (Klement *et al.*, 1964).

Bacteria grown on NAS for 24 h at 28°C were inoculated into liquid culture, reaching  $10^7$  cfu·mL<sup>-1</sup>. Ten  $\mu$ L of each suspension were inoculated into one-year-old Japanese quince and quince seedlings by subepidermal injections, infiltrating approximately 1 cm<sup>2</sup> of the bark tissue of each plant (Rahimian, 1995). For each bacterial isolate, ten plants were inoculated. Seedlings were incubated at 28°C in sterilized plastic boxes ( $\approx$ 80% moisture) for 1 month, and then observed for canker symptoms. One-year-old seedlings inoculated with sterile water were used as negative controls (Jones, 1971). To fulfill Koch's postulates, after development of typical bacterial canker symptoms on inoculated seedlings, bacteria from each of the inoculated plants were re-isolated as above described.

### *Biochemical and physiological tests*

All bacterial isolates were assessed for biochemical, physiological, and nutritional characteristics daily, for one month, as shown in Table 1 (Schaad *et al.*, 2001). Carbohydrate utilization from glucose, fructose, sorbitol, melibiose, galactose, cellobiose, lactose, maltose or mannose (Fahy and Pearsly, 1983) was determined using the basal medium (Ayers *et al.*, 1919). Nineteen isolates were selected for further analysis. The reference strain *Pss* ICMP 4916 was used in all tests as a positive control.

### *Isolation of total cell proteins and SDS-PAGE*

Bacterial isolates were cultured on nutrient agar at 25–28°C for 24 h and single colonies were re-suspended in distilled water. Optical density of the suspensions was adjusted to 2.0 at 600 nm using a spectrophotometer. Samples were centrifuged at 2,576 g for 5 min, the pellet was re-suspended in 1 mL SDW and then dissolved in

**Table 1.** Phenotypic and biochemical characteristics of *Pseudomonas* strains isolated from Japanese quince in Sari.

Characteristic	Identified strain			Reference isolate	
	JQ1	JQ2	JQ3	<i>Pss</i>	<i>P. viridiflava</i>
Gram reaction	-	-	-	-	-
Potato soft rot	-	-	-	-	+
Fluorescent induction on KB	+	+	+	+	+
Oxidase production	-	-	-	-	-
Catalase production	+	+	+	+	+
Tobacco hypersensitivity	+	+	+	+	+
Geranium hypersensitivity	+	+	+	+	+
Methyl red (MR)	-	-	-	-	-
Lecithinase	-	-	-	-	-
Arginine dihydrolase	-	-	-	-	-
Utilization of citrate	+	+	+	+	+
H <sub>2</sub> S production from cystein	-	-	-	-	-
Casein hydrolysis	+	+	+	+	+
Tween 80 hydrolysis	+	+	+	+	+
Reducing substrate from sucrose	+	+	+	+	-
Arbutin production	+	+	+	+	+
Indole production	-	-	-	-	-
Austin hydrolysis	-	-	-	-	-
Nitrate reduction	-	-	-	-	-
Fermentative metabolism	-	-	-	-	-
Oxidative metabolism	+	+	+	+	+
Ice nucleation	+	+	+	+	-
Esculin hydrolysis	+	+	+	+	+
Gelatin liquefaction	+	+	+	+	+
Syringomycine production	+	+	+	+	-
Urease production	-	-	-	-	-
$\alpha$ -methyl diglucoside	-	-	-	-	-
Trehalose	-	-	-	-	-
Ramnose	-	-	-	-	-
D-Fructose	+	+	+	+	+
Xylitole	-	-	-	-	-
Glucose	+	+	+	+	+
Sucrose	+	+	+	+	-
D-Manitol	+	+	+	+	+
Cellobise	-	-	-	-	-
Galactose	+	+	+	+	+
D-Mannose	+	+	+	+	+
D-Raffinose	-	-	-	-	-
D-Xylose	+	+	+	+	+
D-Ribose	+	+	+	+	+
Inositol	+	+	+	+	+
L-Arabinose	+	+	+	+	+
D-Arabitole	+	+	+	+	+
Adonitole	-	-	-	-	-
L-Serine	+	+	+	+	+
L-Asparagine	-	-	-	-	-
Melibiose	+	+	+	+	+

(Continued)

Table 1. (Continued).

Characteristic	Identified strain			Reference isolate	
	JQ1	JQ2	JQ3	<i>Pss</i>	<i>P. viridiflava</i>
Meso-Erithritol	+	+	+	+	+
Starch	-	-	-	-	-
Glycerol	+	+	+	+	+
Malonate	+	+	+	+	+
Citrate	+	+	+	+	+
D-Tartrat	+	+	+	+	+
L-Tartrat	-	-	-	-	-
Lactate	+	+	+	+	+
Succinate	+	+	+	+	+
Malic acid	+	+	+	+	+
Starch hydrolysis	-	-	-	-	-
Growth on 4% NaCl	+	+	+	+	+
Growth on 6% NaCl	-	-	-	-	-

+: positive, -: negative.

0.1 volume of 10% SDS (Sodium Dodecyl Sulphate (SDS) and denatured by boiling for 5 min. Cells were then fractured by sonication (UP200, Hielscher) for 1 min at low amplification in an ice-cooling bath. The resulting cell suspensions were incubated for 5 min on ice and centrifuged at 10,000 rpm for 10 min. The supernatants representing total cell protein extracts were analyzed by SDS-PAGE, following addition of an equal volume of glycerol. SDS-PAGE analysis was carried out in 10% polyacrylamide gels using the Hoefer mini-electrophoresis system (Amersham Biosciences), according to Ahmadvand and Rahimian (2005). Electrophoresis was carried out at a constant voltage of 18 mA for approx. 2 h. Gels were then stained overnight in 50% (v/v) methanol, 10% (v/v) acetic acid, 0.1% Coomassie brilliant blue and then de-stained with in 50% (v/v) methanol and 10% (v/v) acetic acid under constant shaking. Protein profiles of *Pss* ICMP 4916 and *P. viridiflava* isolated from citrus in Mazandaran were used for comparison.

#### Genomic DNA fingerprinting using REP-PCR, BOX-PCR and IS50-PCR

Bacterial genomic DNA was extracted according to Bertheau *et al.* (1995) and Mahmoudi *et al.* (2007). Following quality checks on 1% agarose gel, DNA samples were kept at -20°C until use. Repetitive PCR (REP-PCR) using REP1R/REP2 primers (Versalovic *et al.* 1991) and BOXAIR primers (Louws *et al.*, 1999), and Insertion sequence PCR (IS50-PCR) using IS50 primers (Weingart and Völksch, 1997) were carried out for genomic

DNA fingerprinting. Amplifications were performed in an Applied Biosystems 2720 thermal cycler (ThermoFisher) in 25 µL volumes, containing 200 mM of each dNTPs, 2 mM MgCl<sub>2</sub>, 1.5 pM primers, 1 U *Taq* polymerase, and 4 µL of DNA template. Thermal cycling was carried out with an initial denaturation cycle at 95°C for 5 min, followed by 35 cycles each consisting of denaturation at 94°C for 1 min, annealing at either 40°C (for REP primers), 52°C (for BOXAIR primers), or 44°C (for IS50 primers) for 1 min, extension cycle at 72°C for 3 min, with a final extension at 72°C for 7 min. Amplified products were separated by electrophoresis at 90 V in 2% agarose gels in Tris-Borate-EDTA (TBE) electrophoresis buffer (pH 8), and bands were visualized following ethidium bromide staining.

#### Data analysis

Data were statistically analyzed using a numerical taxonomy [amplified fragment of each strain scored as 1 (present) or 0 (absent)] and multivariate analysis system (NTSYS, version 2.1). Forty phenotypic characters were included in the analyses. Genetic relationships within and between bacterial strains were determined by cluster analysis, using the UPGMA method on distance calculated with the Jaccard coefficient matrices (Rohlf, 1990).

#### Partial ITS and *rpoD* gene amplification

The partial internal transcribed spacer (ITS) region and the RNA polymerase sigma factor D (*rpoD*)

gene were amplified using the primer pairs ITS-F (5' GGCTGGATCACCTCCTT 3') and ITS-R (5' TGC-CAAGGCATCCACC 3') and *rpoD*-F (5' ACCGCGCTG-GACACCGAAGGT 3') and *rpoD*-R (5' ACCACTTCT-GCTTGCTTGCGCTTGAGTG 3'). PCR amplification was carried out as described above. Thermal cycling was carried out with an initial denaturation cycle at 94°C for 5 min, 35 cycles each of denaturation at 94°C for 1 min, annealing at 58°C (ITS) and 54°C (*rpoD*) for 45 sec., extension at 72°C for 1 min, and a final extension at 72°C for 7 min (Versalovic *et al.*, 1991). PCR products were purified and directly sequenced on both strands by Macrogen (South Korea). The sequences were edited using BioEdit v.7.0.5.2 and compared to the *Pss* sequences deposited in the GenBank database using BLASTn (<https://www.ncbi.nlm.nih.gov>). Nucleotide sequence similarity and multiple alignment and phylogenetic tree construction of partial ITS and *rpoD* gene sequences were carried out using the neighbor-joining method and bootstrap analysis replicated 1000 times, employing MEGA6 software (Tamura *et al.*, 2013).

#### Ice nucleation activity

The ice nucleation activity of bacterial strains was conducted using the droplet-freezing method. Fifty  $\mu\text{L}$  droplets of bacterial suspension (each at  $1 \times 10^7$  cfu·mL<sup>-1</sup>) were placed on paraffin soluble in xylene-coated aluminum foil and kept at a range of temperature between -5 and -10°C. *Xanthomonas campestris* and *Pectobacterium carotovora* were used as negative controls. Ice nucleation activity was observed visually after 30 sec (Schaad *et al.*, 2001). The presence of the ice nucleation *InaK* gene was tested by PCR using *InaK* primers (forward 5' GGGCCACAAAAGTATCCTGA 3' and reverse 5' CTGTGACAAGGTCGCTGTGT 3') specific for *Pss*. Thermal cycling was carried out with an initial denaturation cycle at 94°C for 4 min, 30 cycles of denaturation each at 94°C for 1 min, annealing at 55°C for 1 min, extension cycle at 72°C for 45 sec., followed by a final extension cycle at 72°C for 6 min (Rashidaei *et al.*, 2012).

## RESULTS

#### Isolation and biochemical tests

The main symptoms of bacterial canker of Japanese quince in the surveyed area were blossom blight, twig wilting and dieback, blights around thorns, and sunken dark brown cankers, with no symptoms detected on leaves. Typical symptoms are shown in Figure 1.



**Figure 1.** Canker symptoms on Japanese quince plants collected from the Sari county, Mazandaran province, Iran.

#### Isolation and biochemical tests

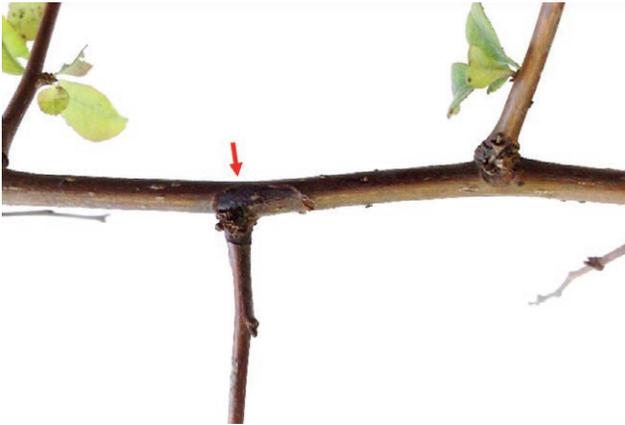
In total, 33 bacterial colonies were obtained, and 19 of them (designated JQ1 to JQ19) were chosen as representative isolates, based on the colour and morphology of the colonies and on biochemical assays. All 19 isolates were Gram negative, produced fluorescent pigment on King's Medium B, were levan-positive, oxidase-negative, potato soft rot-negative, arginine dehydrolase-negative, induced hypersensitivity-reactions on tobacco and pelargonium, with characteristics of *Pseudomonas* LOPAT group Ia. These 19 isolates were selected for further biochemical and pathogenicity tests and for ice nucleation tests; their genomic DNA was tested for REP-, BOX-, and IS50-PCR, ITS and *rpoD* gene analysis. The results of biochemical and nutritional tests are reported in Table 1. According to the morphological and biochemical assays, and to the LOPAT and GATTa identification tests, all 19 representative isolates were identified as *Pss*.

#### Pathogenicity tests

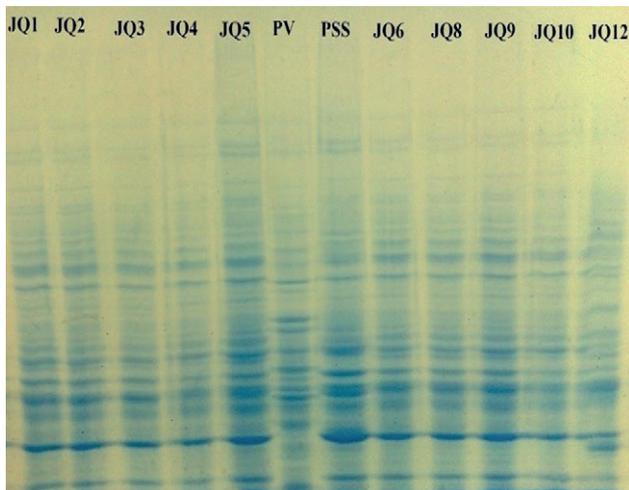
All 19 strains inoculated were pathogenic to Japanese quince and quince seedlings. All isolates caused water-soaked lesions on twigs within 6 d after inoculation, and developed necrotic sunken cankers after 9 d (Figure 2). No symptoms occurred on twigs inoculated with sterile water as control. The symptoms observed on the inoculated seedlings were identical to those collected from the surveyed area. Koch's postulates were confirmed following re-isolation from healthy and young Japanese quince and quince seedlings; indeed, bacterial strains with the same characteristics as those inoculated were successfully re-isolated from artificially inoculated and diseased host plants.

#### Analysis of total bacterial cell proteins electrophoresis

Protein profiles of ten randomly selected isolates were compared visually to the protein profiles of the *Pss*



**Figure 2.** Canker symptoms on Japanese quince one month after artificial inoculation



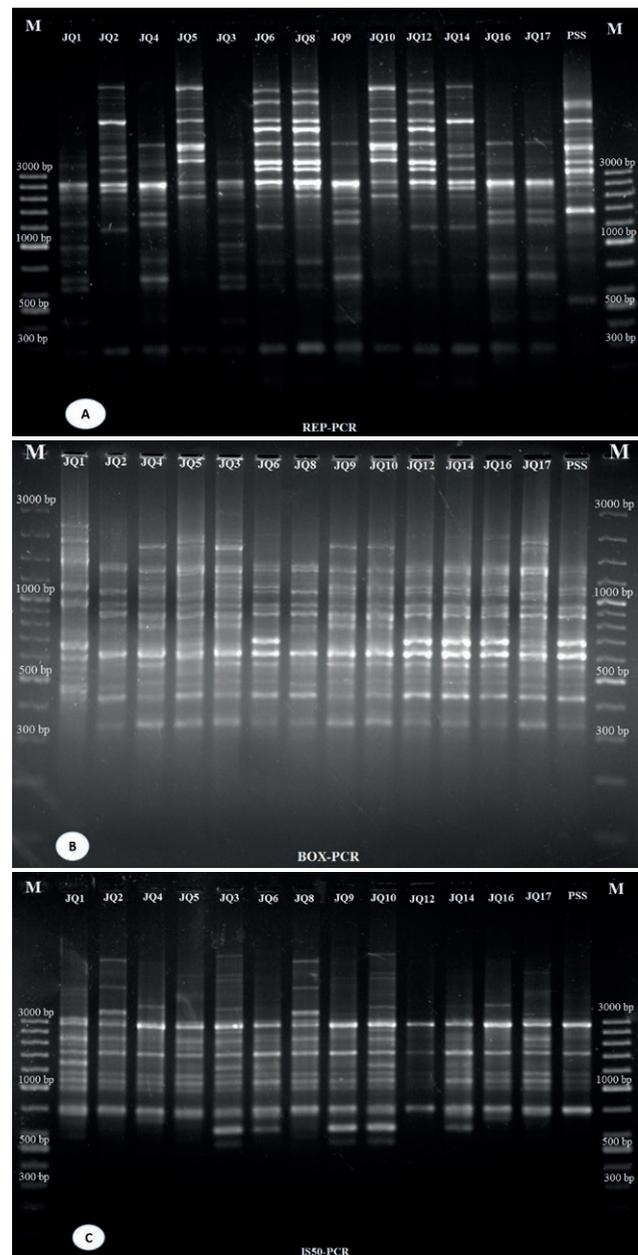
**Figure 3.** Electrophoretic pattern of total cell proteins of isolates JQ1, JQ2, JQ3, JQ4, JQ5, JQ6, JQ8, JQ9, JQ10, and JQ12 obtained from Japanese quince. PV, *Pseudomonas viridiflava* isolated from citrus; PSS, *Pseudomonas syringae* pv. *syringae* (reference strain ICMP 4916) isolated from citrus.

reference strain ICMP 4916 and to *P. viridiflava* isolated from citrus in the Mazandaran province. The protein profiles of the Japanese quince isolates were similar to *Pss* ICMP 4916 reference strain and differed from *P. viridiflava* (Figure 3).

#### Genomic DNA fingerprinting using REP-PCR, BOX-PCR and IS50-PCR

The genomic DNA fingerprints of 13 randomly selected *Pss* strains isolated from Japanese quince and of the reference isolate of *Pss* were determined using

BOX-, REP- and IS50-PCR products. Overall, the results showed approximately 721 fragments, with sizes ranging between 100 and 5000 bp (Figure 4). Five main clusters were obtained, with 67% similarity considering all the three PCR methods. In REP-PCR, JQ1 and JQ3 strains clustered together in cluster 1, JQ4 and JQ9



**Figure 4.** Fingerprints of genomic DNA from the bacterial isolates (JQ1, JQ2, JQ4, JQ5, JQ3, JQ6, JQ8, JQ9, JQ10, JQ12, JQ14, JQ16 and JQ17), obtained from Japanese quince and from *Pseudomonas syringae* pv. *syringae* (*Pss*) with REP-PCR (A), BOX-PCR (B), and IS50-PCR (C); M, 1kb DNA ladder (SMOBIOS).

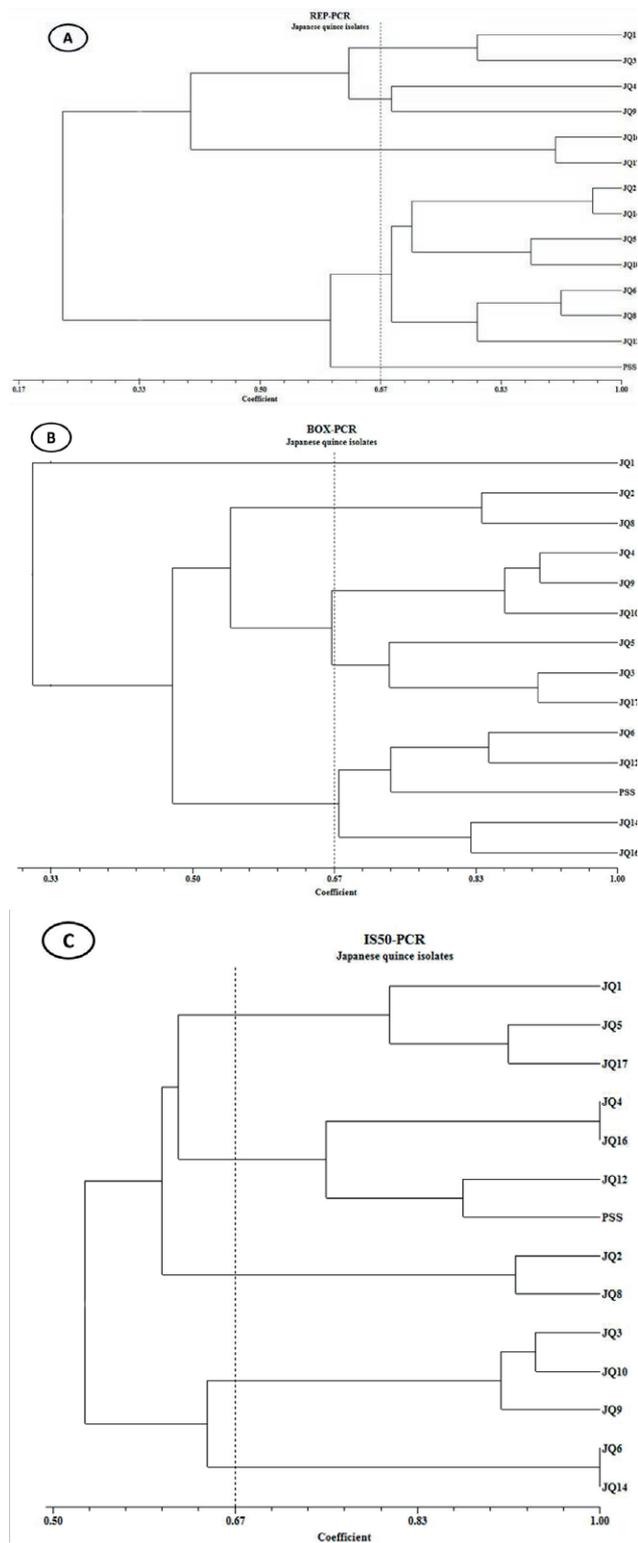
strains in cluster 2, JQ6 and JQ7 strains in cluster 3, and JQ2, JQ5, JQ6, JQ8, JQ10, JQ12 and JQ14 strains in cluster 4; the reference strain *Pss* made a separate cluster (Figure 5A). According to BOX-PCR, the JQ1 strain grouped into cluster 1, JQ2 and JQ8 were grouped in cluster 2, JQ9, JQ4 and JQ10 grouped in cluster 4, and JQ6, JQ12, JQ14, JQ16 and *Pss* strains were grouped in cluster 5 (Figure 5B). The strains were differentiated into five main clusters according to IS50-PCR. JQ1, JQ5 and JQ17 strains were grouped in cluster 1, JQ4, JQ12 and JQ16 in cluster 2, *Pss* strain in cluster 3, JQ3, JQ9 and JQ10 strains in cluster 4, and JQ6 and JQ14 strains in cluster 5 (Figure 5C). The combined dendrogram from REP, BOX and IS50-PCR was also obtained for 13 strains, and obtaining seven main clusters with 67% similarity and four main groups with 56% similarity, including JQ1 and JQ16 as two separate clusters, JQ3, JQ4, JQ9 and JQ17 in cluster 3, and eight other strains in cluster 4 (Figure 6).

#### ITS and *rpoD* gene amplification

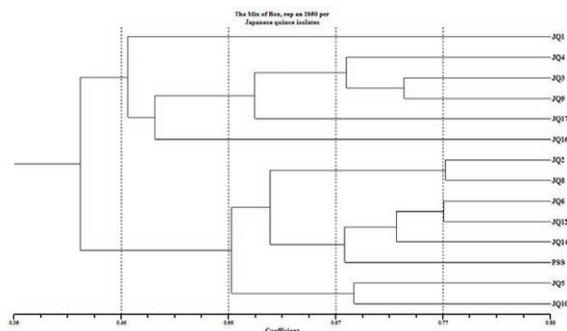
PCR amplification with specific primers for ITS and the *ropD* gene correctly amplified specific bands of 600 bp and 850 bp in all the assessed strains (Figure 7), while no amplification was obtained with negative control bacteria. The ITS sequences of two representative strains from Japanese quince, JQ4 and JQ9, had high similarity to each other (99%). The sequences of isolates JQ4 and JQ9 were deposited in the NCBI GenBank database under the respective accession numbers KJ754379 and KJ754378 (for the ITS gene) and KJ769138 and KJ769139 (for the *rpoD* gene). The results confirmed that these isolates belong to *Pss*. A Neighbor-joining phylogenetic tree with 1000 bootstrap replicates showed that isolates JQ4 and JQ9 clustered together with the *Pss* AY342179, based on the ITS gene sequence (Figure 8), and grouped together with the *Pss* isolates JX867789 and KC852112 based on the *rpoD* gene sequence (Figure 9). The isolates and strains used for phylogenetic tree construction and their GenBank accession numbers are listed in Supplementary Table 1.

#### Ice Nucleation activity

Ice nucleation activity was positive for all the tested strains obtained from Japanese quince, showing freezing of water droplets in less than 30 sec. In contrast, isolates of *X. campestris* and *P. carotovora* did not induce freezing of water droplets in the same timeframe (Figure 10A). Moreover, all PCR amplifications using Japanese



**Figure 5.** Dendrogram obtained by comparing bacteria isolated from Japanese quince JQ1, JQ2, JQ4, JQ5, JQ3, JQ6, JQ8, JQ9, JQ10, JQ12, JQ14, JQ16, and JQ17 and *Pseudomonas syringae* pv. *syringae* (*Pss*), with REP-PCR (A), BOX-PCR (B), and IS50-PCR (C). Five major clusters were categorized at the 67% similarity level.



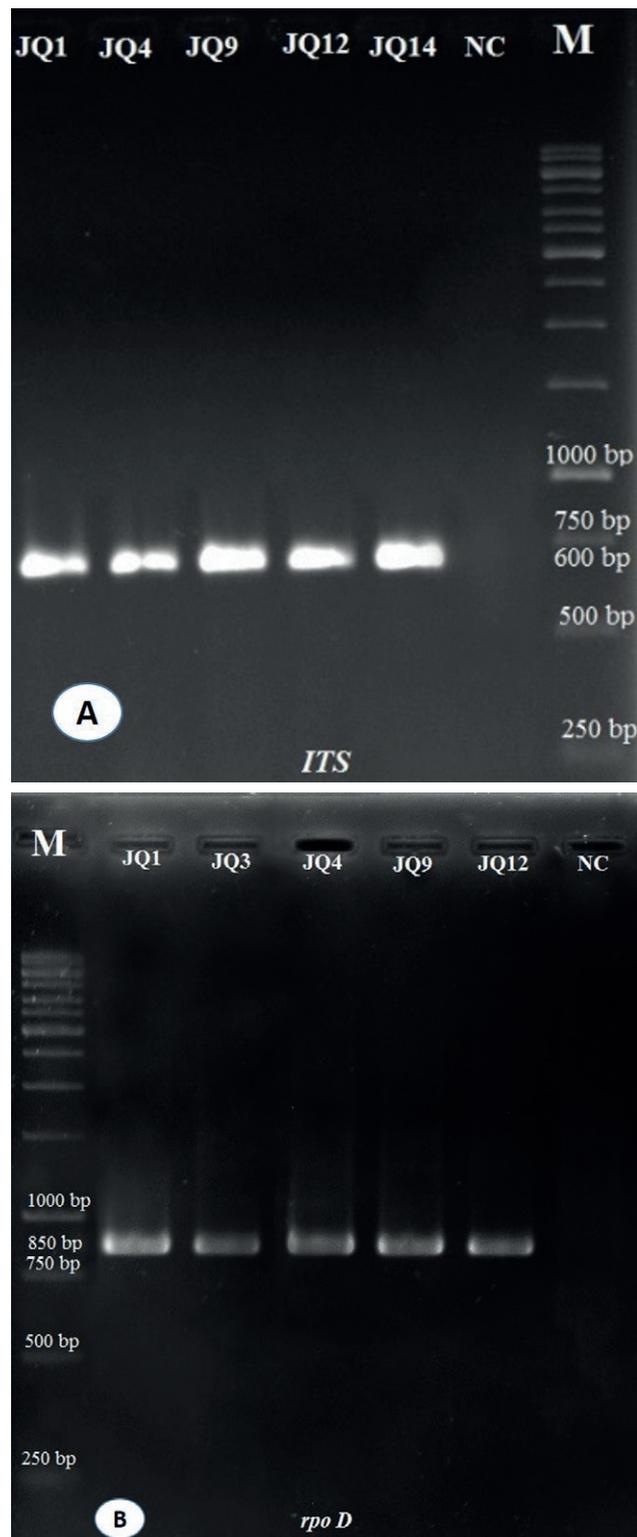
**Figure 6.** Dendrogram obtained by comparing the bacterial isolates derived from Japanese quince (JQ1, JQ2, JQ4, JQ5, JQ3, JQ6, JQ8, JQ9, JQ10, JQ12, JQ14, JQ16 and JQ17), and *Pseudomonas syringae* pv. *syringae* (*Pss*), by REP-PCR, BOX-PCR, and IS50-PCR assays.

quince strains resulted in products of the expected sizes (200 bp) of the *InaK* gene (Figure 10B).

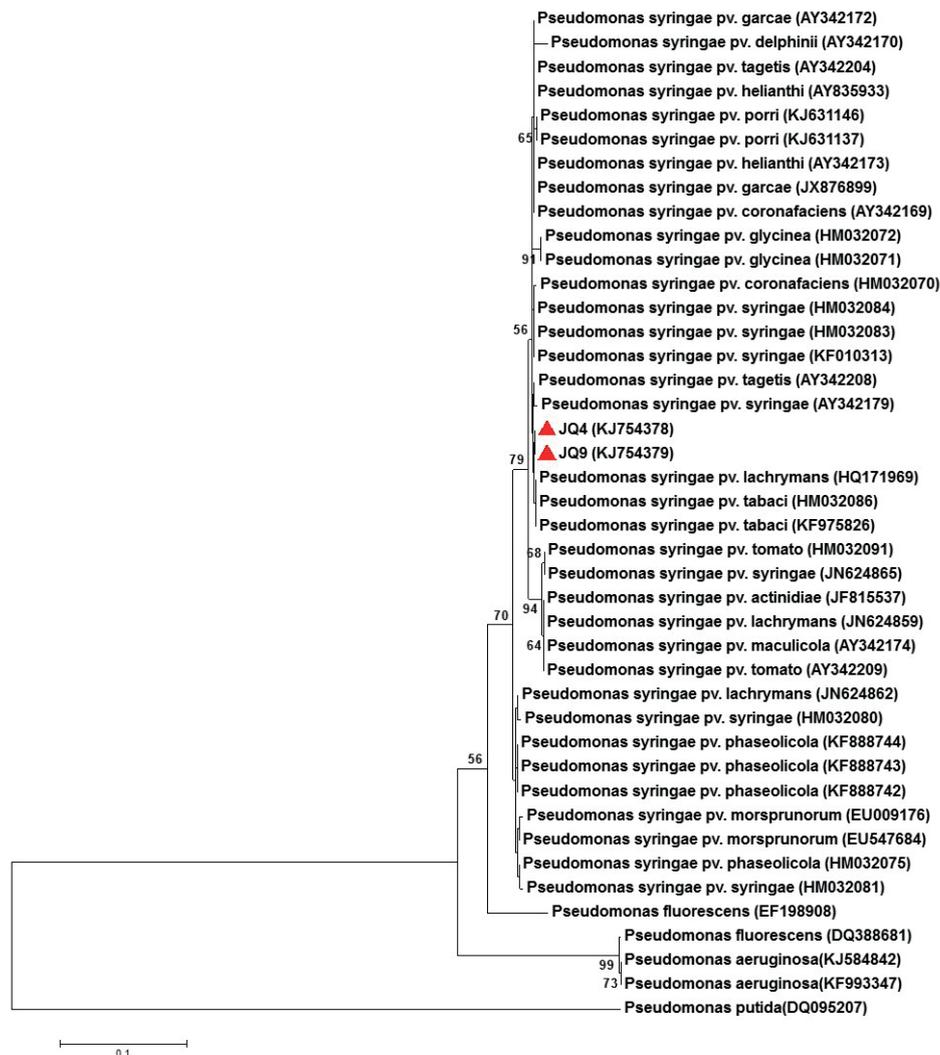
#### DISCUSSION

This study was carried out to identify the causal agent of canker of Japanese quince trees in the Sari County in the Mazandaran province located in the North of Iran. In total, 33 bacterial strains were isolated from canker lesions on plants located in different areas of the county. Characterization of the bacterial strains was achieved by biochemical, physiological and pathogenicity tests, indicating that the strains had phenotypic characteristics of *Pss*. Inoculations into quince and Japanese quince seedlings produced canker symptoms, consistent with previously described symptoms (Bahar *et al.*, 1982; Banapour *et al.*, 1990). Bacterial strains with the same features were also recovered from inoculated quince and Japanese quince seedlings, fulfilling Koch's postulates. However, as these tests do not precisely identify *Pseudomonas* strains (Peix *et al.*, 2018), in order to perform a phylogenetic analysis, molecular tests based on DNA fingerprinting and partial gene sequence analysis using ITS and the house-keeping *rpoD* gene were also conducted. Thus, following BLAST analysis of the sequences of two representative bacterial strains JQ4 and JQ9 we could conclude that *Pss* was the causal agent of Japanese quince canker in the North of Iran.

All bacterial isolates belonged to *Pss*, previously reported as the causal agent of bacterial canker of many plants, including stone fruits, pear, quince, almond, olive, rose, malva and pelargonium, or causing red streak of sugarcane, blast of mandarin and leaf blight of wheat, rice, oat, barley and cabbage from different



**Figure 7.** Agarose gel electrophoresis of the PCR products obtained with the *ITS* (A) and *rpoD* gene (B) specific primers (fragments of 600 bp and 850 bp, respectively). Japanese quince isolates (JQ1, JQ3, JQ4, JQ9, JQ12 and JQ14); NC, negative control; M, 1 kb DNA ladder.

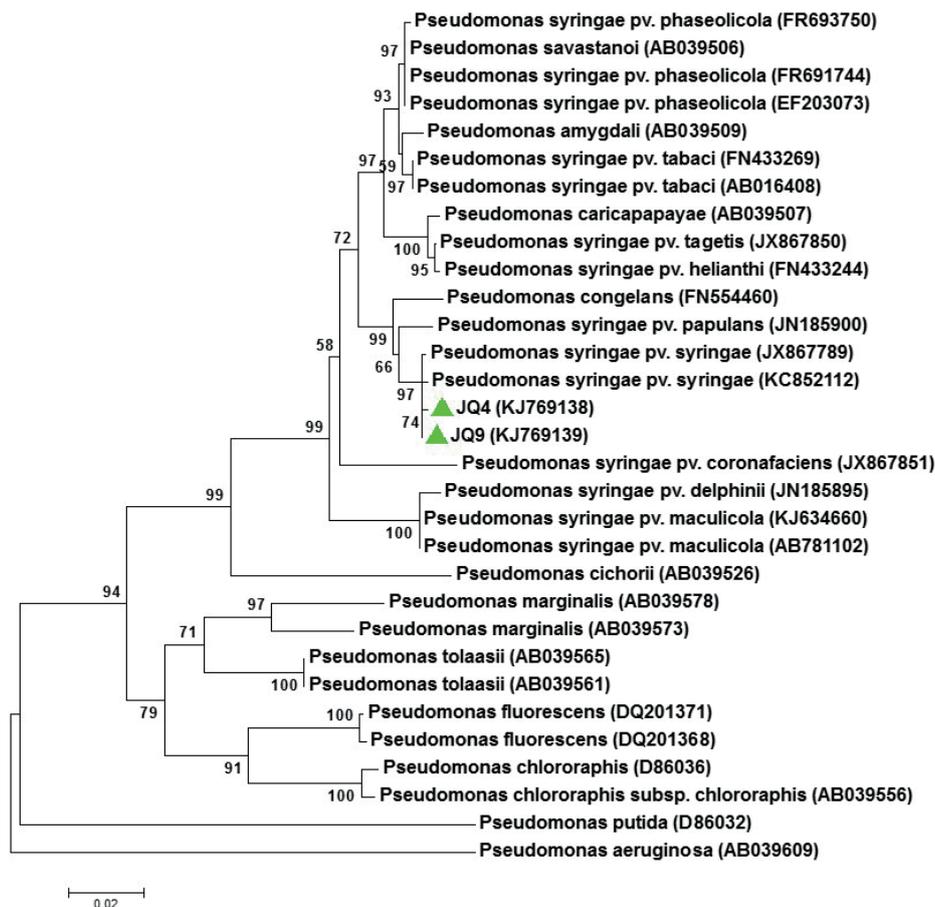


**Figure 8.** Phylogenetic tree obtained using the Neighbor Joining method of the ITS nucleotide sequence of Japanese quince isolates JQ4 and JQ9 (highlighted in red triangles), and of *Pseudomonas* strains available in GenBank. The GenBank accession numbers used in the analysis are shown in parentheses. Bootstrap values (expressed as percentage of 1000 replicons) are shown at the nodes.

areas of Iran (Bahar *et al.*, 1982; Banapour *et al.*, 1990; Rahimian, 1995; Najafi Pour and Taghavi, 2011; Aeni and Khodakaramian, 2018; Khezri and Mohammadi, 2018; Vasebi *et al.*, 2019; Basavand *et al.*, 2021). Genomic fingerprinting analyses, including REP-, BOX- and IS50-PCR showed some levels of variability among the *Pseudomonas* strains. The banding profiles for *Pseudomonas* isolates were also different and *Pseudomonas* isolates from the same region grouped into different clusters; therefore, genetic diversity among isolates was not related to their geographic origin. The methods here adopted are easy and rapid for distinguishing *P. syringae* at the pathovar level, with highly reproducible results. The present results agree with previous studies on genetic vari-

ation of *Pseudomonas* strains (Weingart and Völksch, 1997; Little *et al.*, 1998; Louws *et al.*, 1999; Najafi Pour and Taghavi, 2011; Cetinkaya Yildiz *et al.*, 2016; Abdelatif *et al.*, 2020).

Noteworthy, this is the first study comparing these three genomic fingerprint analyses for the genetic characterization of *Pss* strains isolated from Japanese quince. Sequence analysis of the ITS and of the housekeeping *rpoD* gene, and the use of repetitive elements including REP-, BOX- and IS50-PCR have been described as useful methods for identification, classification and characterization of diversity of *P. syringae* isolates (Little *et al.*, 1998; Najafipour and Taghavi, 2011). *P. syringae* can infect more than 200 plant species, due to a high degree



**Figure 9.** Phylogenetic tree obtained using the Neighbor Joining method comparing the *rpoD* nucleotide sequence data of Japanese quince bacterial strains JQ4, JQ9 (highlighted in green triangles) and *Pseudomonas* strains available in GenBank. The GenBank accession numbers used in the analysis is shown in parentheses. Bootstrap values (expressed as percentage of 1000 replicons) are shown at the nodes.

of genetic variability (Ruinelli *et al.*, 2019). The results of the present study show that a combination of different methods including biochemical, phenotypic, pathogenicity and molecular tests can be used for the identification of the causal agent of Japanese quince canker. Moreover, this is the first report of *P. syringae* as the causal agent of Japanese quince bacterial canker.

Bacterial canker of stone fruits, almond, olive, pear, and quince caused by *Pss* is a serious problem in all the areas where these crops are cultivated in Iran. Japanese quince, here reported as a new host for *Pss* in this country, can be grown within or near orchards and fields and can likely provide overwintering sites for *Pss*, serving as inoculum sources for disease development and outbreaks. The results of this study can facilitate further research on the ecology, epidemiology, diversity, and management of bacterial canker of Japanese quince and other host plants of *Pss*, not only in Iran but also in other countries. Further studies will allow to identify other

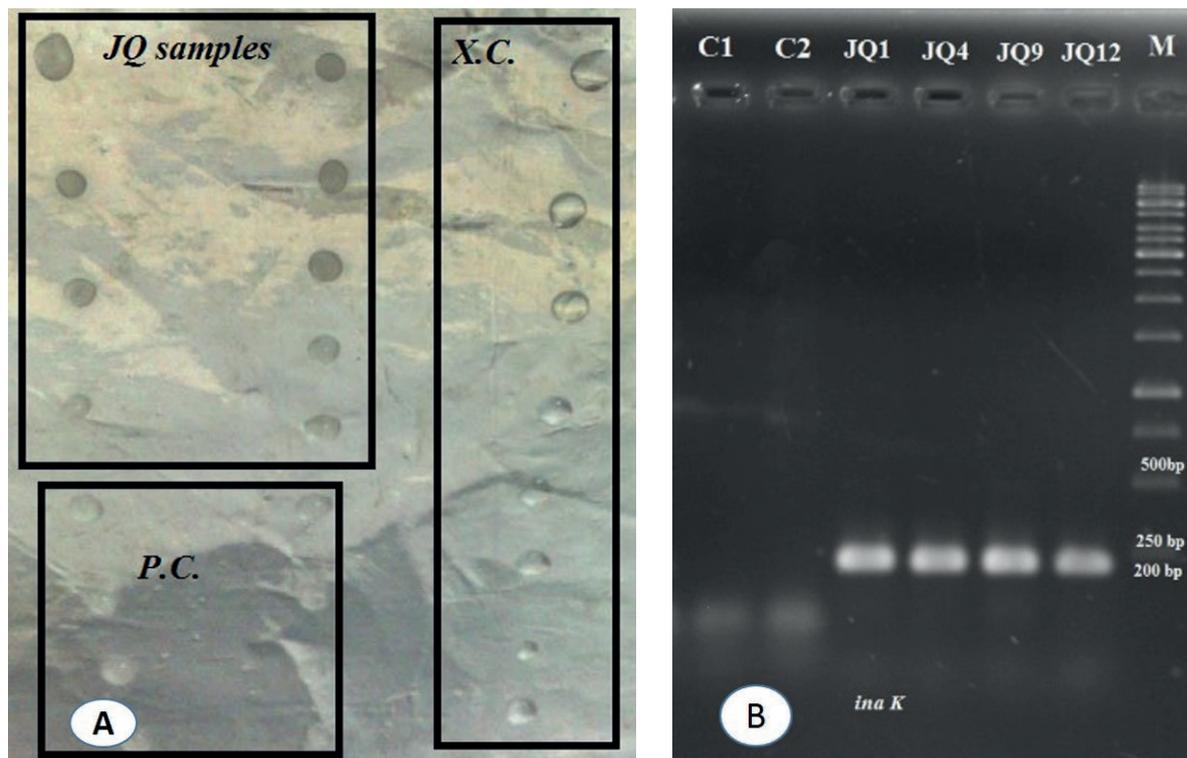
infested areas and to identify the etiology of bacterial canker of Japanese quince in Iran.

#### AUTHOR CONTRIBUTIONS

TA designed and conducted experiments as a Masters student and analyzed the data. MR advised on experimental design, analyzed the data and wrote the manuscript. HR advised on experimental design, assisted with sample collection, and provided technical advice. EN and SM reviewed, critically revised and edited the manuscript

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**Figure 10.** Ice nucleation assays of bacterial strains isolated from Japanese quince A. Ice nucleation test. Japanese quince isolates (JQ samples), *Xanthomonas campestris* (X.C.) and *Pectobacterium carotovora* (P.C.); B. Gel electrophoresis of *InaK* gene amplicon on 1.2% agarose gel. JQ1, JQ4, JQ9 and JQ12: isolates from Japanese quince, C1: *Xanthomonas campestris*, C2: *Pectobacterium carotovora*, M: 1 kb DNA Ladder.

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