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Phenotypic and molecular characterization of the bacterial causal agents of sugar beet soft rot and vascular necrosis disease in Southeastern Iran

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Abstract The bacterial soft rot and vascular necrosis disease in sugar beet (*Beta vulgaris* var. *saccharifera*) can be caused by different bacteria such as *Pectobacterium betavasculorum* and *P. carotovorum* subsp. *carotovorum*. During the 2018-2019 growing season, tissue samples from symptomatic sugar beet plants were collected from sugar beet growing areas in Kerman province, southeastern Iran. Totally, thirty-one bacterial isolates were isolated from the symptomatic tissues, of which nineteen isolates were identified as *Pectobacterium* spp., nine isolates as *Bacillus* spp. and three isolates as *Flavobacterium* spp. based on the phenotypic and biochemical tests. *Pectobacterium* isolates were further characterized by PCR amplification (using the EXPCCF/EXPCCR primer set) and genomic fingerprinting by rep-PCR. Only 27% of the *Pectobacterium* isolates were identified as *P. carotovorum*, while 73% of the isolates were identified as *P. betavasculorum*. Artificial inoculation of isolates into the wounded sugar beet petioles led to black streaking, root rot and vascular necrosis from which the same bacterial isolates were re-isolated and identified. PCR assays using BOX and ERIC primers, and combined assays clearly differentiated the bacterial isolates, indicating high variability among the isolates. The results shed light on the bacterial causal agents of the sugar beet soft rot and vascular necrosis disease in southeastren Iran, identifying Pectobacterium isolates as the main causal agents (61% of all isolates), and uncovered the phenotypic/genetic variability among the bacterial isolates.

Keywords: Sugar beet, Bacterial soft rot, *Pectobacterium*, *Flavobacterium*, *Bacillus*, rep-PCR

Introduction

Sugar beet (*Beta vulgaris* var. *saccharifera*) is an important economic crop in diverse industries dealing with production of foods, animals, ethanol etc. (Sheikholeslami, 2006). It is a major crop in southeastern Iran, with approximately 309 hectares planted and 6,030 tons harvested in 2016 (Anonymous, 2016). Soft rot and vascular necrosis is one of the most important

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diseases that limits sugar beet production in different areas worldwide, which can be caused by bacterial pathogens such as *Pectobacterium betavasculorum* and *P. carotovorum* subsp. *carotovorum* (*Pcc*) (Syn. *Erwinia carotovora* subsp. *carotovora*) (Thomson *et al.,* 1981), plant-pathogenic oomycetes such as *Pythium aphanidermatum* and *Phytophthora drechesleri*, and fungal pathogens such as *Rhizoctonia solani* (Jacobsen, 2006). The bacterial genus *Pectobacterium*, with broad geographic distribution and host range is an important Gram-negative plant pathogen (Enterobacteriaceae), which induces major losses to different commercial crops in the field as well as during the storage phase (Pérombelon, 2002; Toth *et al.,* 2003; Charkowski, 2006). *Pectobacterium* can produce several types of plant cell-wall degrading enzymes (PCWDEs) such as pectinase, polygalacturonase, and cellulose (Lee *et al.,* 2014). The maceration of cell walls by PCWDEs results in soft rot symptoms in host plants. *Pectobacterium carotovorum* was classified into five subspecies including *atrosepticum, betavasculorum, carotovorum, odoriferum* and *wasabiae* (Hauben *et al.,* 1998). Recently, some of these subspecies were moved to the species level, including *atrosepticum, betavasculorum* and *wasabiae* (Gardan *et al.,* 2003). It was shown that *P. carotovorum* subsp. *carotovorum* (*Pcc*) has a wide host range compared to *P. atrosepticum, P. betavasculorum, P. wasabiae* and *P. carotovorum* subsp. *odoriferum* that have a relatively narrow host range including potato, sugar beet, horseradish, and chicory, respectively (Seo *et al.,* 2003; Charkowski, 2006; Lee *et al.,* 2014). However, there are also some other bacteria that cause soft rot in different plants such as *Pseudomonas* (Cuppels and Kelman, 1974; Liao and Wells, 1987b), *Xanthomonas* (Liao and Wells, 1987a), *Cytophaga* (Liao and Wells, 1986), *Clostridium* (Campos *et al.,* 1982), *Flavobacterium* (Lund, 1969) and *Bacillus* (Dowson, 1943). The sugar beet bacterial soft rot disease was shown to be caused by *P. betavasculorum* in sugar beet fields of Isfahan province, Iran (Samavatian, 2006), however, the status of the disease in southeastern parts of Iran (especially in Kerman province, as one of the main sugar beet producing regions) is unknown. This study was carried out to characterize the bacterial causal agents of the sugar beet soft rot and vascular necrosis disease in in Kerman province (southeastern Iran) using phenotypic and molecular techniques.

Materials and methods

Plant sampling and bacterial isolation

During the 2018-2019 growing season, leaf, petiole and root samples of sugar beet plants showing soft rot and vascular necrosis symptoms as well as

those without symptoms were collected from seven different regions (Bardsir, Dashtkar, Negar, Narp, Bahram Jerd, Ganatsir and Rain) in Kerman province located in southeastern Iran (Table 1). Bcagterial isolates were isolated from margin of the necrotic vascular bundles of leaves, petioles and roots. For this purpose, plant tissues were surface sterilized first with ethanol, ground in 10 mL sterilized distilled water (SDW) and a loop-full of the suspension was streaked on plates containing eosin methylene blue (EMB) agar or nutrient agar (NA). Then, the isolated colonies were purified on NA plates according to their morphology, color, and shape. Hypersensitivity test was performed on tobacco plants for all the bacterial isolates (Kelement *et al.*, 1964). Reference strains of Pcc (EccSCRI 193) and Pb (SCRI 479) were provided by the Scottish Crop Research Institute (SCRI, Dundee, UK) and used as controls in this study. Totally, 31 bacterial isolates (Table 1) were isolated, and subsequently grown on Luria-Bertani (LB) medium containing 20% (v/v) glycerol (ratio of 1:1) for long-term storage at -80 ℃ (Janse, 2006).

Table 1. List of the *Pectobacterium* isolates, the sampling areas, and the plant tissue types used to isolate each given isolate

Plant tissue	City	Sampling area	Bacterial isolate
Root	Bardsir	Dashtkar	A_1
Root	Bardsir	Dashtkar	A_2
Root	Bardsir	Ganat sir	B_1
Root	Bardsir	Ganat sir	C_1
Root	Bardsir	Ganat sir	C_2
Leaf	Bardsir	Ganat sir	C_3
Root	Bardsir	Negar	D_1
Root	Bardsir	Negar	D_2
Root	Bardsir	Negar	D_3
Leaf	Bardsir	Negar	D_4
Leaf	Bardsir	Negar	D_5
Root	Bardsir	Narp	E_1
Root	Bardsir	Narp	E_2
Root	Kerman	Rain	F_1
Root	Bardsir	Bardsir	G_1
Root	Bardsir	Bardsir	G_2
Leaf	Bardsir	Bardsir	G_3
Root	Bardsir	Bahram Jerd	H_1
Root	Bardsir	Bahram Jerd	H_2

Pathogenicity test

All bacterial isolates were grown on NA plates for 48 h at 28 C° followed by preparation of the bacterial suspensions (108 cfu mL-^{1}SDW). Then, 10 µL of each suspension was inoculated onto the crown of the two-month-old sugar beet seedlings (cv. P22). Ten individual plants were inoculated with each bacterial isolate. The inoculated seedlings were incubated at 28 ℃ in sterilized plastic boxes (~80% moisture) for one month and monitored for soft rot symptoms. The seedlings inoculated with SDW were used as negative control. In another experiment, six-month-old sugar beet plants were inoculated by the same method and grown in greenhouse conditions at 28 ℃. Plants were harvested three to five weeks after inoculation, sliced and examined for vascular necrosis and root rot symptoms.

The pathogenicity test was also performed on sterile sugar beet disks in Petri dishes using bacterial suspension (108 cfu mL-^{1}) at 28 C° for 48 h (Pérombelon and Van Der Wolf, 2002).

Biochemical and phenotypic tests

All 31 bacterial isolates were compared based on their biochemical, physiological and nutritional characteristics daily over a one-month period as shown in Table 2 (De Boer and Kelman, 2001; Schaad *et al.,* 2001). Carbohydrate utilization capacity of the isolates was determined using the basal medium (Ayers *et al.,* 1919) supplemented with different carbohydrates including glucose, fructose, sorbitol, melibiose, galactose, cellobiose, lactose, maltose and mannose (Fahy and Persley, 1983).

DNA Extraction

Bacterial DNA was extracted using the SDS-boiling method as described by Mahmoudi *et al.* (2007). The DNA quality was assessed by electrophoresis on 1% agarose gel and stored at -20 ℃ until analysis.

Species-specific PCR for identification of Pcc

To identify the bacterial species Pcc, we used the primers EXPCCR (5'-CC-GTAATTGCCTACCTGCTTAAG-3') and EXPCCF (5'-AACTTCGCACCGCCGACCTTCTA-3') in our PCR assay (Kang *et al.,* 2003). The PCR reaction was performed in a Bio-Rad I-cycler (Hercules, CA, USA) in PCR reaction mixture (25 µL) containing 10 mM Tris-HCl, 1.5 mM

MgCl2, 200 mM of each dNTP mix, 100 ng of primers, 1 unit of Taq polymerase (Cinnagen, Germany) and 1 µL of the bacterial DNA template using the following program: 94 ℃ for 4 min, for initial denaturation; 30 cycles of 94 ℃ for 1 min; 60 ℃ for 1 min; and 72 ℃ for 2 min, followed by a final elongation step of 72 ℃ for 7 min. The PCR products were visually inspected on 1% agarose gel.

REP-PCR

Repetitive PCR (REP-PCR) was used with ERIC (ERIC 1R: 5'-ATGTAAGCTCCTGGGGATTCAC-3'/ ERIC2:5'-AAGTAAGTGACTGGGGTGAGCG-3') and BOX (BOXA1R:5'-CTACGGCAAGGCGACGCTGACG-3') primers (Versalovic *et al.*, 1994). Amplification was performed in a Bio-Rad I-cycler (Hercules, CA, USA) in 25 µL PCR reaction mixtures containing 200 mM of each dNTP mix, 2 mM MgCl2, 1.5 pM primers, 1 U of Taq polymerase and 4 µL of DNA template. Thermal cycling was carried out as an initial denaturation cycle at 95 ℃ for 7 min, 30 cycles of denaturation at 94 ℃ for 1 min, annealing at 52 ℃ (ERIC), 53 ℃ (BOX) and 44 ℃ (REP) for 1 min, followed by the extension cycle at 65 ℃ for 8 min, and a final extension cycle at 65 ℃ for 15 min (Versalovic *et al.*, 1994). The PCR products were separated on 1.5% agarose gel and viewed under UV illumination.

Data analysis

Data were statistically analyzed using the numerical taxonomy and multivariate analysis system (NTSYS, version 2.1). Forty phenotypic characteristics were included in the analysis. Genetic relationships within and between bacterial isolates were determined by cluster analysis using the UPGMA method with a squared Euclidean distance metric (Rohlf, 2000).

Results

Phenotypic characterization of bacterial isolates

In total, 31 bacterial isolates were isolated from sugar beet symptomatic tissues, and identified based on their phenotypic and biochemical properties; nineteen isolates as *Pectobacterium*, three isolates as *Flavobacterium* and nine isolates as *Bacillus* (Table 2).

Table 2. Phenotypic and biochemical characteristics of *Pectobacterium*, *Flavobacterium* and *Bacillus* isolates from Kerman province

Test No.	Characteristics	*Pectobacterium*						*Flavobacterium*					*Bacillus*											
		Identified Strains			Reference strain			Identified Strains			Reference strain		Identified Strains								Reference strain			
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	*O*	*P*	*Q*	*R*	*S*	T	U	V	W
1	Fermentative metabolism	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	Gram reaction	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
3	Potato soft rot	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	nr	nr	nr	
4	Oxidase production	-	-	-	-	-	-	-	+	-	nr	nr	+	+	+	-	+	+	-	+	+	+	+	-
5	Growth at 37 ℃	-	+	+	-	+	+	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
6	Growth at 25 ℃	*	*	*	*	*	*	+	+	+	+	+	*	*	*	*	*	*	*	*	*	*	*	*
7	Growth at 45 ℃	*	*	*	*	*	*	*	*	*	*	*	+	+	+	+	+	+	+	+	nr	nr	nr	
8	Growth at 50 ℃	*	*	*	*	*	*	*	*	*	*	*	+	+	+	+	+	+	+	+	+	+	+	-
9	Growth at 65 ℃	*	*	*	*	*	*	*	*	*	*	*	-	-	-	-	-	-	-	-	-	-	-	-
10	Nitrate reduction	+	+	+	+	+	+	-	+	-	+	+	+	-	+	-	+	-	+	-	-	+	-	-
11	Flourescent production on KB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	Catalase production	+	+	+	+	+	-	+	+	+	nr	nr	+	+	+	+	+	+	+	+	+	+	+	+
13	Lecithinase	-	-	-	-	-	+	-	+	+	nr	nr	+	+	+	+	+	+	+	+	-	-	-	
14	Aestuin hydrolysis	-	-	-	-	-	-	-	-	-	nr	nr	+	+	+	-	+	+	+	+	+	+	-	
15	3-ketolactose production	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
16	H_2S production from cysteine	+	+	+	+	+	+	-	-	+	+	nr	+	+	+	+	+	+	+	-	nr	nr	nr	
17	Arginine dihydrolae	+	+	+	-	-	-	+	-	-	nr	nr	-	-	-	-	-	-	-	-	-	-	-	
18	Tween 80 hydrolysis	-	-	-	+	+	+	+	+	+	nr	nr	-	+	-	-	+	-	-	+	-	nr	nr	nr
19	Litmus milk																							
	Acidification	-	-	-	-	-	-	-	+	+	nr	nr	-	-	+	-	-	-	+	-	+	nr	nr	nr
	Coagulation	+	+	-	+	+	+	-	-	+	nr	nr	+	+	-	+	+	+	-	+	-	nr	nr	nr
	Peptonisation	60	-	-	-	-	-	-	-	-	nr	nr	-	-	-	-	-	-	-	-	-	nr	nr	nr
20	Gelatin liquefaction	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
21	Methyl red(MR)	+	-	-	+	+	+	+	+	+	nr	nr	-	-	-	+	-	-	-	-	-	-	-	+
22	Gas from glucose	80	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
23	Indole production	-	-	-	-	-	-	-	-	-	nr	nr	*	*	*	*	*	*	*	*	*	*	*	*
24	Erythromycine sensitivity	+	-	+	-	-	+	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
25	Growth at pH 5.7	*	*	*	*	*	*	*	*	*	*	*	+	+	+	+	+	+	+	+	nr	nr	nr	
26	Urease production	-	-	-	-	-	-	-	+	-	nr	nr	-	-	-	-	-	+	-	-	-	-	-	+
27	Acid production from																							
	α-methyl diglucoside	-	+	-	+	+	+	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	Sorbitol	-	+	+	+	+	+	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	Lactose	+	+	+	+	+	-	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	Fructose	+	+	34	+	+	+	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	Maltose	+	+	+	+	+	+	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	Glucose	+	+	+	+	+	+	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	Sucrose	+	50	-	+	+	+	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	Manitol	+	+	+	+	+	+	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	Cellobise	+	+	+	+	-	+	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	Galactose	+	+	+	+	+	+	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	Mannose	-	+	+	+	-	-	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	Starch	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	Glycerol	-	-	6	-	-	+	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

28	Ammonium production	+	+	7+	+	-	-	-	-	-	nr	nr	+	+	-	+	+	+	-	-	+	nr	nr	nr
29	casein hydrolysis	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	+
30	Reducing substrate from sucrose	+	+	+	+	+	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
31	Growth on 3% NaCl	*	*	*	*	*	*	*	*	*	*	*	+	+	+	+	+	+	+	+	+	+	+	+
32	Growth on 5% NaCl	+	+	+	+	+	+	*	*	*	*	*	+	+	+	+	+	+	+	+	+	+	+	+
33	Growth on 7% NaCl	*	*	*	*	*	*	*	*	*	*	*	+	+	+	+	+	+	+	+	+	+	+	+
34	Starch hydrolysis	-	-	-	-	-	+	-	+	+	+	+	+	-	+	+	+	+	-	-	-	+	-	+
35	Utilization of citrate	80	+	84	+	-	+-	-	-	nr	nr	-	-	-	-	-	-	-	-	+	-	+	+	+
36	Mobility	*	*	*	*	*	*	*	*	*	*	*	+	+	+	+	+	+	+	+	+	+	+	+

A: D_2 (n=5) **B:** H_2B (n=2)**, C:** C_3 (n=12)**, D:** Pa, **E:** Pb, **F:** Dda, **G:** G_2**, H:** H_2A **, I:** E_2 **, J:** *F. s***, K:** *F. p***, L:** A_3 **, M:** G_4A **, N:** M_2**, O:** C_6 **, P:** G_6 **, Q:** D_8**, R:** C_7 **, S:** G_4C**, T:** G_1 **, U:** *B. s* **, V:** *B. p***, W:** *B. m*

The nineteen Iranian *Pectobacterium* isolates isolated from sugar beet tissues were grouped in three clusters based on 90% similarity in their phenotypic characteristics (Figure 1A). *P. betavascolurum, P. atrosepticum* and *Dickeya didantii* were used as strain references.

Cluster 1 corresponded to 12 isolates (C3 as the representative strain) and had 52%, 55%, and 43% similarity with *P. betavascolurum, P. atrosepticum* and *Dickeya didantii*, respectively. Cluster 1 also had 55% and 53% similarity with other *Pectobacterium* isolates in clusters 2 and 3, respectively.

The isolates in cluster 1 were different from *P. atrosepticum* based on methyl red, arginine dehydrolase, growth at 37 ℃ and 5% NaCl, reaction to litmus milk and acid production from sucrose and α-methyl diglucoside tests. Also, they were different from *P. betavascolurum* based on methyl red, arginine dehydrolase, reaction to litmus milk, ammonium production, utilization of citrate and acid production from sucrose, cellobiose and α-methyl diglucoside tests. Furthermore, the isolates in cluster 1 were different from *Dickeya didantii* based on methyl red, arginine dehydrolase, reaction to litmus milk, ammonium production, lecithinase and acid production from sucrose, lactose and α-methyl diglucoside tests (Table 2).

Cluster 2 inlcuded two isolates (H2B as the representative strain) and had 52%, 58% and 43% similarity with *P. betavascolurum, P. atrosepticum* and *Dickeya didantii*, respectively. It also had 55% and 53% similarity with other *Pectobacterium* isolates in clusters 1 and 3, respectively. The isolates in this cluster were different from *P. betavasculorum* based on methyl red, arginine dehydrolase, ammonium production, utilization of citrate and acid production from mannose and cellobiose tests. Also, they were different from *P. atrosepticum* based on methyl red, arginine dehydrolase, growth at 37 ℃ and 5% NaCl and acid production from mannose tests, and they were different from *Dickeya didantii* based on methyl red, arginine dehydrolase, growth at 5%

NaCl, ammonium production, lecithinase, erythromycine sensitivity, catalase, starch hydrolysis, gelatin liquefaction and acid production from lactose and glycerol tests (Table 2).

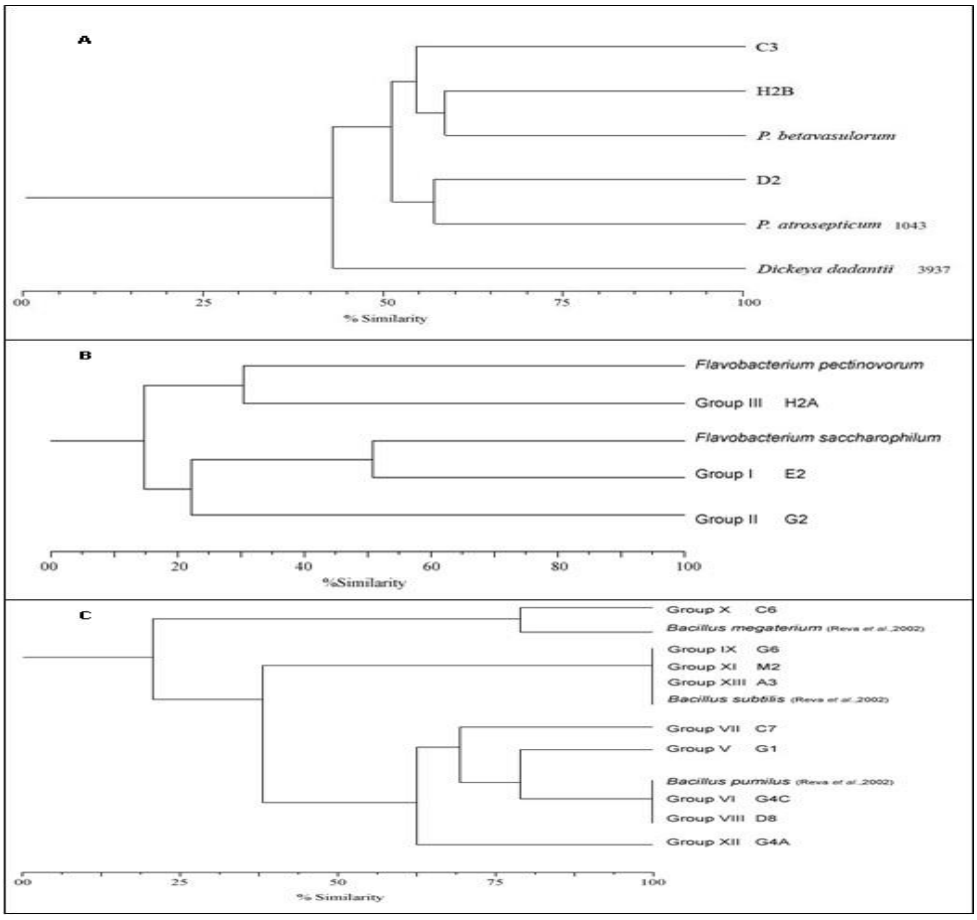

Figure 1. Dendrogram constructed based on the phenotypic characteristics of 19 Iranian *Pectobacterium* isolates (A), 3 *Flavobacterium* isolates (B) and 9 *Bacillus* isolates associated with sugar beet soft rot and vascular necrosis disease, based on 90% similarity. The isolates with codes are the representative isolates for each group

Cluster 3 corresponded to five isolates (D2 as the representative isolate) and had 57%, 53% and 43% similarity with *P. betavascolurum, P. atrosepticum* and *Dickeya didantii*, respectively. Cluster 3 had 53% similarity with other *Pectobacterium* isolates in clusters 1 and 2. The isolates in this cluster were different from *P. betavasculorum* based on arginine dehydrolase, growth at

37 ℃ and 5% NaCl, ammonium production, erythromycine sensitivity, utilization of citrate, gelatin liquefaction and acid production from sorbitol, mannose, and cellobiose and α-methyl diglucoside tests. Also, they were different from *P. atrosepticum* based on arginine dehydrolase, erythromycine sensitivity, gelatin liquefaction and acid production from sorbitol and α-methyl diglucoside tests and they were different from *Dickeya didantii* based on arginine dehydrolase, growth at 37 ℃, ammonium production, catalase, lecithinase, starch hydrolysis and acid production from α-methyl diglucoside, mannose, lactose, and glycerol tests. The results of the phenotypic characteristics (Table 2) and cluster analysis (Figure 1A) showed high variation among the Iranian *Pectobacterium* isolates associated with sugar beet.

The three Iranian *Flavobacterium* isolates were separated into three clusters based on 90% similarity (Figure 1B). Cluster 1 corresponded to one strain (H2A) and had 30% and 14% similarity with *Flavobacterium pectinovorum* and *F. saccarophilum*, respectively. Also, it had 14% similarity with the other two Iranian *Flavobacterium* isolates (E2 and G2) isolated from sugar beet. Cluster 2 corresponded to one strain (E2) and had 14% and 51% similarity with *Flavobacterium pectinovorum* and *F. saccarophilum*, respectively. It also had 14% and 22% similarity with the other *Flavobacterium* isolates (H2A and G2). Cluster 3 corresponded to one strain (G2) and had 14% and 22% similarity with *Flavobacterium pectinovorum* and *F. saccarophilum*, respectively, and it had 14% and 22% similarity with the other *Flavobacterium* isolates (H2A and E2).

The cluster analysis of nine Iranian *Bacillus* isolates (isolated from sugar beet tissues) resulted in six clusters based on 90% similarity (Figure 1C). Cluster 1 corresponded to one strain (C6) and had 78%, 20% and 20% similarity with the refernec strains *B. megaterium, B. subtilis* and *B. pumilus*, respectively. Also, it had 20% similarity with the other *Bacillus* isolates in the other clusters. Cluster 2 corresponded to isolates G6, M2 and A3 that had 20%, 100% and 38% similarity with *B. megaterium, B. subtilis* and *B. pumilus*, respectively. Also, this cluster had 20%, 38%, 38%, 38% and 38% similarity with clusters 1, 3, 4, 5 and 6, respectively. Cluster 3 corresponded to one strain (C7) and had 20%, 63% and 70% similarity with *B. megaterium, B. subtilis* and *B. pumilus*, respectively. Also, this cluster had 20%, 38%, 70%, 70% and 63% similarity with clusters 1, 2, 4, 5 and 6, respectively. Cluster 4 corresponded to one strain (G1) and had 20%, 38% and 78% similarity with *B. megaterium, B. subtilis* and *B. pumilus*, respectively. Also, this cluster had 20%, 38%, 70%, 78% and 63% similarity with clusters 1, 2, 3, 5 and 6, respectively. Cluster 5 corresponded to two isolates (G4C and D8) and had 20%, 38% and 100% similarity with *B. megaterium, B. subtilis* and *B. pumilus*, respectively. Also,

this cluster had 20%, 38%, 70%, 78% and 63% similarity with clusters 1, 2, 3, 4 and 6, respectively. Cluster 6 corresponded to one isolate (G4A) and had 20%, 38% and 63% similarity with *B. megaterium*, *B. subtilis* and *B. pumilus*, respectively, and it also had 38%, 20%, 38%, 63% and 63% similarity with clusters 1, 2, 3, 4 and 5, respectively.

Pathogenicity tests

Sugar beet bacterial vascular necrosis is accompanied by both wilt and root rot symptoms. Disease symptoms were observed in all inoculated two-month-old sugar beet plants. Root soft rot was developed 3–5 weeks after inoculation (Figure 2A). Black streaking along the petioles and frothing around the crown area were observed 3–5 days after inoculation under the same conditions (Figure 2B). To confirm the presence of the bacterial isolates, Koch's rules were followed and bacterial isolates with same characteristics as those inoculated were successfully re-isolated from the symptomatic inoculated plants.

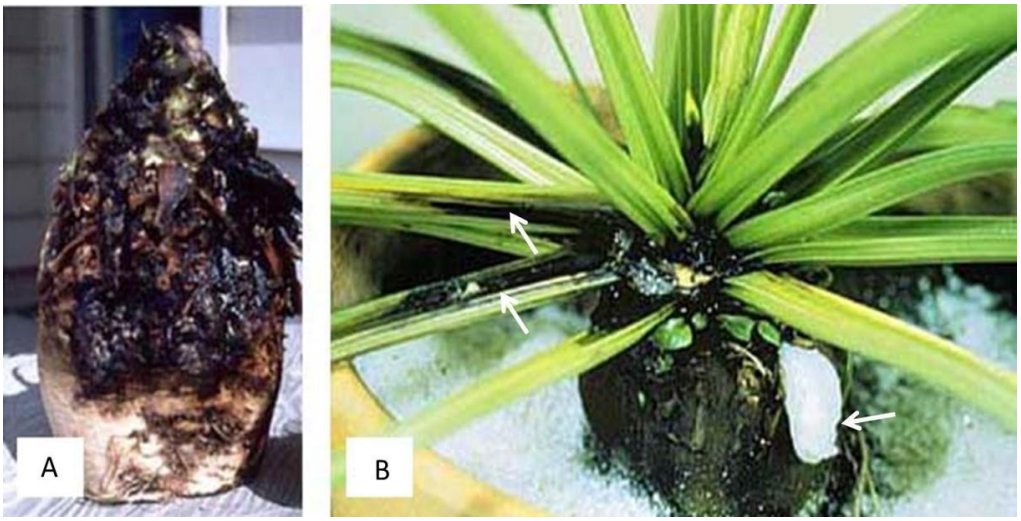

Figure 2. Symptoms of sugar beet bacterial vascular necrosis and root rot caused by *Pectobacterium carotovorum* in artificially inoculated plants: (A) infected root/hypocotyl showing soft rot (B): infected plant with black streaking along the petioles and frothing in the crown area

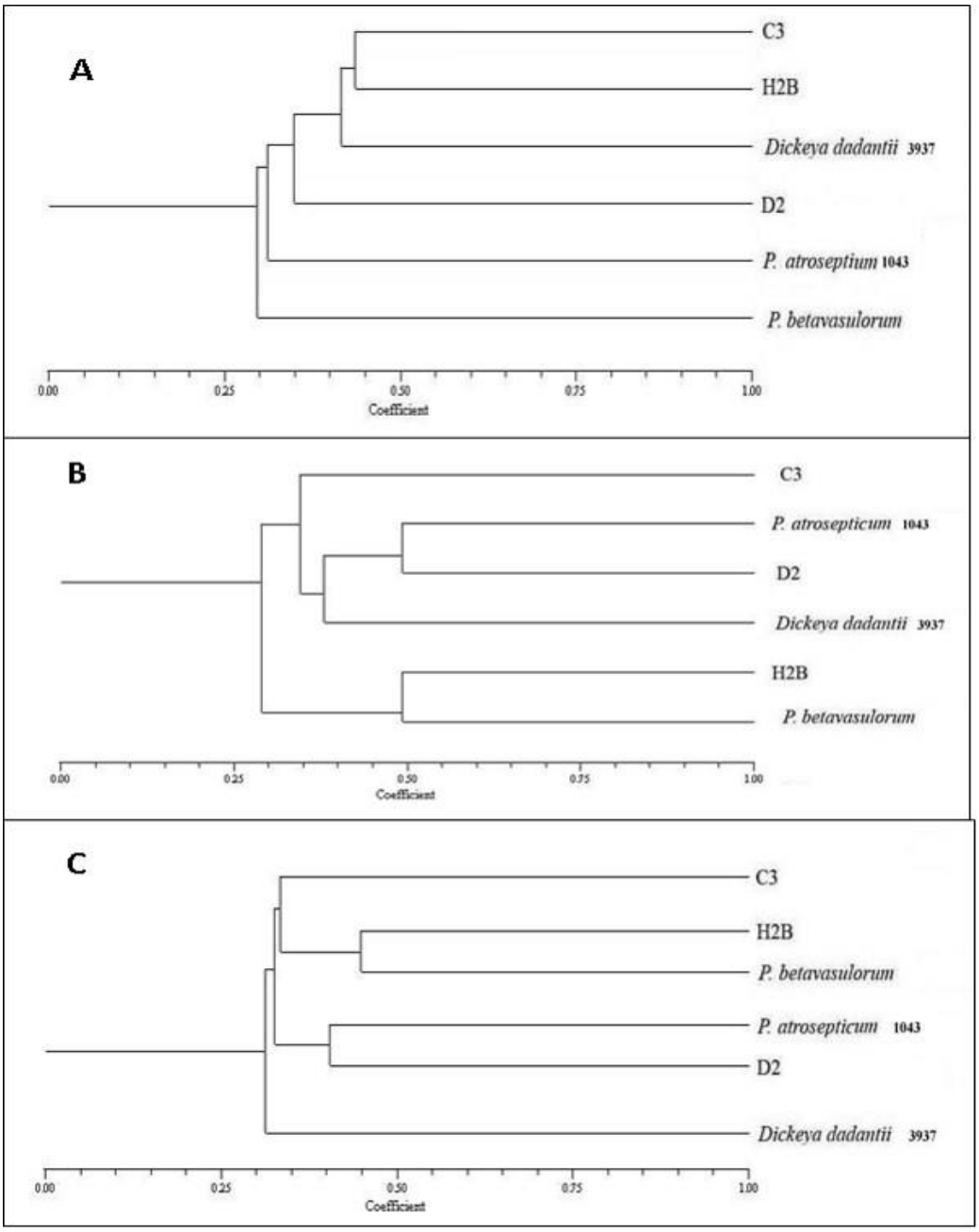

Figure 3. Dendrogram of bacterial isolates causing soft rot and vascular necrosis in sugar beet based on BOX primers (A), ERIC primers (B) results, or combined BOX and ERIC primers (C). The isolates with codes are the representative isolates for each group

Molecular characterization of Pcc

The PCR test was conducted on all isolates, leading to amplification of a 550bp fragment in *Pcc* isolates, using EXPCCF and EXPCCR primers, confirming the suitability of these primers for identification of the Iranian *Pcc* isolates. The primers designed for conserved sequences of the ERIC and BOX elements annealed to genomic DNA and generated unique genomic fingerprints for the *Pcc*, *Pb*, *Pa* and *Dda* isolates. BOX and ERIC-PCR clearly differentiated the Iranian soft rot bacterial isolates (Figure 3). The PCR bands were compared based on the presence or absence of fragments at a specific position, and the similarity coefficients of isolate pairs were calculated to determine the genetic relationship among the bacterial isolates. There were significant differences in the intensity of some amplified fragments as well as in the presence/absence of some polymorphic bands. Results of BOX-PCR analysis showed five distinctive groups (Figure 3A). Group I comprised C3 and H2B isolates with 44% similarity. C3 was the dominant isolate in Bardsir region and had 29%, 32% and 42% similarity with *P. betavasculorum, P. atrosepticum,* and *D. dadantii*, respectively. Group II comprised the isolate D2, which was isolated from Negar region and had 29%, 32% and 35% similarity with *P. betavasculorum*, *P. atrosepticum* and *D. dadantii,* respectively. Group III comprised the standard reference isolate *D. dadantii* with 29% and 32% similarity with the reference strains *P. betavasculorum* and *P. atrosepticum*, respectively. Group IV comprised the reference strain *P. atrosepticum* with 29% and 32% similarity with the reference strains *P. betavasculorum* and *D. dadantii*. Group V comprised the reference strain *P. betavasculorum* with 29% similarity with the reference strains *P. atrosepticum* and *D. dadantii*. The ERIC-PCR analysis separated isolates into four groups (Figure 3B). Group I consisted the isolate H2B and the reference strain *P. betavasculorum* with 49% and 29% similarity with *P. atrosepticum* and *D. dadantii*, respectively. Group II comprised isolate D2 and the reference strain *P. atrosepticum* with 49% similarity. This group had 29% and 38% similarity with *P. betavasculorum* and *D. dadantii*, respectively. Group III consisted isolate C3 with 34%, 29% and 34% similarity with the reference strains *P. atrosepticum, P. betavasculorum* and *D. dadantii*, respectively. Group IV comprised the reference strain *D. dadantii* with 29% and 38% similarity with the the reference strains *P. betavasculorum* and *P. atrosepticum*. Data analysis on the combined results of BOX-PCR and ERIC-PCR assays separated the bacterial isolates into six distinctive groups based on 90% similarity and four groups based on 49% similarity (Figure 3C). Isolate H2B and the reference strain *P. betavasculorum* were placed in Group I with 49% similarity. Isolate D2 and the reference strain

P. atrosepticum were placed in Group II with 42% similarity. Group III comprised isolate C3 with 34%, 33% and 32% similarity with the reference strains *P. betavasculorum, P. atrosepticum* and *D. dadantii*, respectively. Group IV comprised the reference strain *D. dadantii* with 33% similarity with the reference strains *P. betavasculorum* and *P. atrosepticum*. Reproducible genomic PCR profiles consisted of approximately 15 bands ranged 250–4000 bp for BOX primers and 11 bands ranged 250–4000 bp for ERIC-PCR primers. Overall, the molecular analysis separated the bacterial isolates into two distinctive clusters. Isolates in cluster 1 were more closely related to *P. betavasculorum*. This cluster was subdivided into two subclusters; the isolate C3 belonged to subcluster I1 and the isolate H2B was placed in subcluster I2. Cluster II contained isolate D2, which was more closely related to *P. carotovorum* subsp. *Carotovorum*. The molecular analysis also showed a high variability among the Iranian *Pectobacterium* isolates of different sampling regions, as also revealed by biochemical tests.

Discussion

This present study led to identification of the bacterial isolates associated with sugar beet soft rot and vascular necrosis in the Kerman province (southeastern Iran). Totally, 31 pectolytic bacterial isolates were isolated from roots and petioles of sugar beet plants showing disease symptoms. Bacterial inoculation of host plants through wounded crowns produced soft rot and vascular necrosis symptoms, which were consistent with those described elsewhere (Thomson *et al.,* 1977). Also, the same bacterial isolates were recovered (Koch's rules) from the inoculated plants. Biochemical and physiological characteristics as well as the pathogenicity test showed that the bacterial isolates belonged to *Pectobacterium carotovorum* subsp. *carotovorum*, *Pectobacterium betavasculorum, Flavobacterium* spp. and *Bacillus* spp. In addition, physiological, biochemical and molecular analyses showed that 61% of the isolates were *Pectobacterium*. Physiological, biochemical and molecular analyses of 19 sugar beet *Pectobacterium* isolates showed that the population was very heterogeneous in southeastern parts of Iran and only 27% of the isolates could be identified as *P. carotovorum*. *Pectobacterium carotovorum* subsp *carotovorum* was previously reported as the major soft rot causal agent on vegetable crops and ornamental plants in Iran (Soltani-Nejad *et al.,* 2005; Firoz *et al.,* 2007; Baghaee *et al.,* 2011).

In the present study, most *Pectobacterium* isolates were identified as *P. betavasculorum* (73%), which has previously been reported as the main cause of sugar beet soft rot in Iran (Fassihiani and Nedaienia, 2008; Samavatian 2006; Rezaei and Taghavi, 2010). Likewise, Saleh *et al.* (1996) identified *P. betavasculorum* as the main bacterial causal agent of beet soft rot in Egypt. Our results showed a high variability among *Pectobacterium* isolates among the sampling regions. In addition, the banding profiles of PCR products for *Pectobacterium* isolates were different in ERIC-PCR and BOX–PCR techniques, i.e. and the *Pectobacterium* isolates from the same region grouped into different clusters using BOX-PCR and ERIC-PCR analysis, indicating that the genetic diversity among the bacterial isolates was not merely related to their geographic location (Dana *et al.,* 2015). Here, we could also isolate *Bacillus* spp. and *Flavobacterium* spp. isolates from sugar beet as the causal agents of the sugar beet soft rot. Isolates of *Bacillus* spp. and *Flavobacterium* spp. were shown to cause soft rot on several plants, such as potato (Pérombelon, 2002; Elbanna *et al.,* 2014), dieffenbachia (Mikiciński *et al.,* 2010) and some fruits like peach and apple (Saleh *et al.,* 1997). However, there are no reports showing that these bacteria can cause sugar beet soft rot in Iran and, to the best of our knowledge, the current study reports *Flavobacterium* and *Bacillus* as the causal agents of sugar beet soft rot in Iran for the first time. The occurrence of these bacteria in other sugar beet growing regions of Iran as well as other crop fields needs to be investigated.

In the present study, *Pectobacterium, Flavobacterium* and *Bacillus* isolates were identified as the bacterial causal agents of the sugar beet soft rot and vascular necrosis in southeastern parts of Iran (Kerman province) using a combination of biochemical and molecular analyses and pathogencity test, and *Pectobacterium betavasculorum* was identified as the main causal agent. The results of this study can facilitate future studies on ecology, diversity, epidemiology, and disease management of sugar beet vascular necrosis and soft rot in Iran.

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