

Full Length Research Paper

Identification and genetic diversity of Jordanian potato soft rot isolates, *Pectobacterium carotovorum* subspecies *carotovorum* (DYE 1969)

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Bacterial soft rot disease is one of the most important factors affecting potato production in Jordan. Based on biochemical and physiological analyses, Jordanian isolates of soft rot from potato were identified as *Pectobacterium carotovorum* subspecies *carotovorum* (DYE 1969). Phylogenetic analysis using *pmrA* gene sequence data showed that isolates from different regions were closely related to each other, whereas little genetic diversity was observed between these isolates. A DNA marker was developed from nucleotide sequences of the *pmrA* gene and a 318 bp fragment was polymerase chain reaction (PCR) amplified specifically from *P. carotovorum* subspecies *carotovorum* isolates, which could be used for detection of the disease in potato tubers.

Key words: Potato soft rot, *Solanum tuberosum*, genetic diversity, *pmrA* gene, *Pectobacterium carotovorum* subspecies *carotovorum*, Jordan.

INTRODUCTION

Potato (*Solanum tuberosum* L.) is one of the most common and important vegetable crops in Jordan, used for both consumption and export. During 2016, area planted with potato was 508600 hectares with production of 15858000 kg (Anonymous, 2016).

Bacterial soft rot disease is commonly found in potato growing regions worldwide (Perombelon, 2002; Czajkowski et al., 2009). Several studies have shown that the soft rot causal agents are too divergent to be included in one clade (Knwon et al., 1997; Hauben et al., 1998). The new classification grouped the genus *Erwinia* into *Pectobacterium* (formally *Erwinia carotovora*) and

Dickeya spp., according to the differences of pectolytic enzymes secreted through type II secretion system. Two species, *Dickeya chrysanthmi* and *Pectobacterium carotovorum* are particularly damaging to potato production (Toth et al., 2001; Czajkowski et al., 2009). *P. carotovorum* is a highly diverse species and is currently divided into five subspecies: *atrosepticum*, *betavascularum*, *carotovorum*, *odoriferum* and *wasabiae* (Hauben et al., 1998) with subspecies *carotovorum* and *atrosepticum* causing rotting of potato during vegetative growth and in stored tubers.

Control of bacterial diseases depends on the accurate

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detection and identification of the causal agent. Although, diagnosis of bacterial diseases based on symptoms is simple, symptoms are not always specific and can be confused with other biotic or abiotic factors. For preventive control measures, detection of the causal agent in symptomless plant material is necessary, but can be difficult, because the pathogen may be present in low population densities and in uneven distribution in the infected plants (Palacio-Bielsa et al., 2009). However, the diagnosis of the disease is not always related to field inspection, so, it is important to detect the pathogen in seed potato tubers before distribution to farmers in order to prevent spreading the disease. Efficient, low cost detection and identification methods are essential to investigate the ecology and pathogenesis of soft rot Enterobacteriaceae as well as in seed certification programmes (Czajkowski et al., 2014).

In a review by Czajkowski et al. (2014), more than 30 methods have been employed to detect, identify and differentiate soft rot causal agents to species and subspecies levels; including biochemical characters, serology and molecular techniques. Within the past 30 years, there has been a shift from microbiological and serological methods to molecular approaches (Palacio-Bielsa et al., 2009; Czajkowski et al., 2014). DNA markers have been used for the rapid detection of different strains of *P. carotovorum* subspecies *carotovorum* (*Pcc*) (Kang et al., 2003; Zhu et al., 2010; Rahmanifer et al., 2012; Azadmanesh et al., 2013). DNA sequence analysis of gene *pmrA*, which is linked to pathogenicity (response regulator), was used to evaluate the relationship among *Pcc* isolates collected from different regions of Morocco and results were equivalent to enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) and 16S rDNA sequencing (Kettani-Halabi et al., 2013).

In Jordan, *Erwinia carotovora* subsp. *carotovora*, which is recently known as *Pcc*, was identified as the causal agent of soft rot disease of vegetables, its detection and identification was carried out through traditional techniques. The pathogen infects and causes disease on a wide variety of hosts belonging to different families of vegetables either in field and storage in different areas including, Jordan Valley and Uplands. Soft rot of potatoes is a tuber borne disease where the contaminated mother tubers are reported to be the main source of inoculum. However, this bacterium was found to survive in the soil with population trends varying with the fluctuation in soil temperature (Rajeh and Khlaif, 2000).

Traditional techniques used for detection and identification of the causal agents are time consuming and relatively insensitive. Therefore, there is an urgent need for a sensitive and highly specific technique for rapid detection and identification of *Pcc*.

In this study, the detection, identification and genetic diversity of Jordanian isolates of *P. carotovorum* subspecies *carotovorum* were evaluated based on *pmrA*

gene sequencing analysis.

MATERIALS AND METHODS

Samples collections and bacterial isolation

Samples including stem and tubers suspected to be infected with soft rot were randomly collected throughout potato growing areas in Jordan during the growing seasons, fall and spring seasons and from storage facilities from different potato growing areas in Jordan.

Twenty potato growing regions and nine storage sites were surveyed and representative potato samples were collected from each site. Logan's and nutrient agar media were used for bacterial isolation (Fahy and Parsley, 1980; Schaad et al., 2001). The *P. carotovorum* subspecies *carotovorum* (*Pcc*) reference culture NCPPB312 was obtained from Food and Environment Research Agency, United Kingdom to identify *Pcc* isolates.

Biochemical and physiological tests

The 205 *Pcc* isolates were evaluated for oxidase and catalase reactions, potato soft rot, oxidative fermentative, ability to grow at 37°C, sodium chloride tolerance, reducing substances from sucrose, urease production and acid production from carbohydrates utilized as carbon source as described by Schaad et al. (2005) for characterization of the isolates.

Genomic DNA extraction from bacterial cultures

Pure bacterial cultures grown on NA media at 37°C for 24 h were used for DNA extraction. Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's instruction. DNA was quantified and stored at -20°C for further analysis.

Characterization of isolates with *pmrA* marker

The isolates were subjected to PCR amplification using *pmrA* primer set: F0145: TACCTGCAGATGAAATTATTGATTGTTGAAGA; E2477: TACCAAGCTTTGGTTGTTCCCTTTGGTCA (Kettani-Halabi et al., 2013). Primers were designed based on the sequence of *pmrA* gene in *Erwinia* species.

DNA amplification were performed in a BIORAD T100™ thermal cycler (BioRad, Hercules, CA) using the following protocols. PCR amplification was conducted in a 25 µl reaction containing 5.0 µl Crimson Taq buffer (5X), 1.1 µl MgCl₂ (25 mM), 0.5 µl of dNTPs (10 mM), 1.25 µl of each primer (10 µM), 0.13 µl of Taq polymerase (5U/µl) and 2 µl of the template DNA where its concentrations ranged between 450 and 600 ng/µl. The reaction involved initial denaturation (94°C for 3 min) followed by 32 cycles of denaturation (94°C for 1 min), annealing (47°C for 1 min), extension (72°C for 1 min) and final extension (72°C for 7 min). The amplified products were electrophoretically separated in 1.5% (w/v) agarose gel at 110 V for 35 min in TBE buffer and visualized with UV light after staining in a solution of ethidium bromide (0.5 µg/mL).

DNA sequencing of PCR amplification products

Samples were chosen after showing the specific bands. Taken into consideration resembling of different potato growing regions, bulk PCR amplification was conducted as described above for *Pcc*

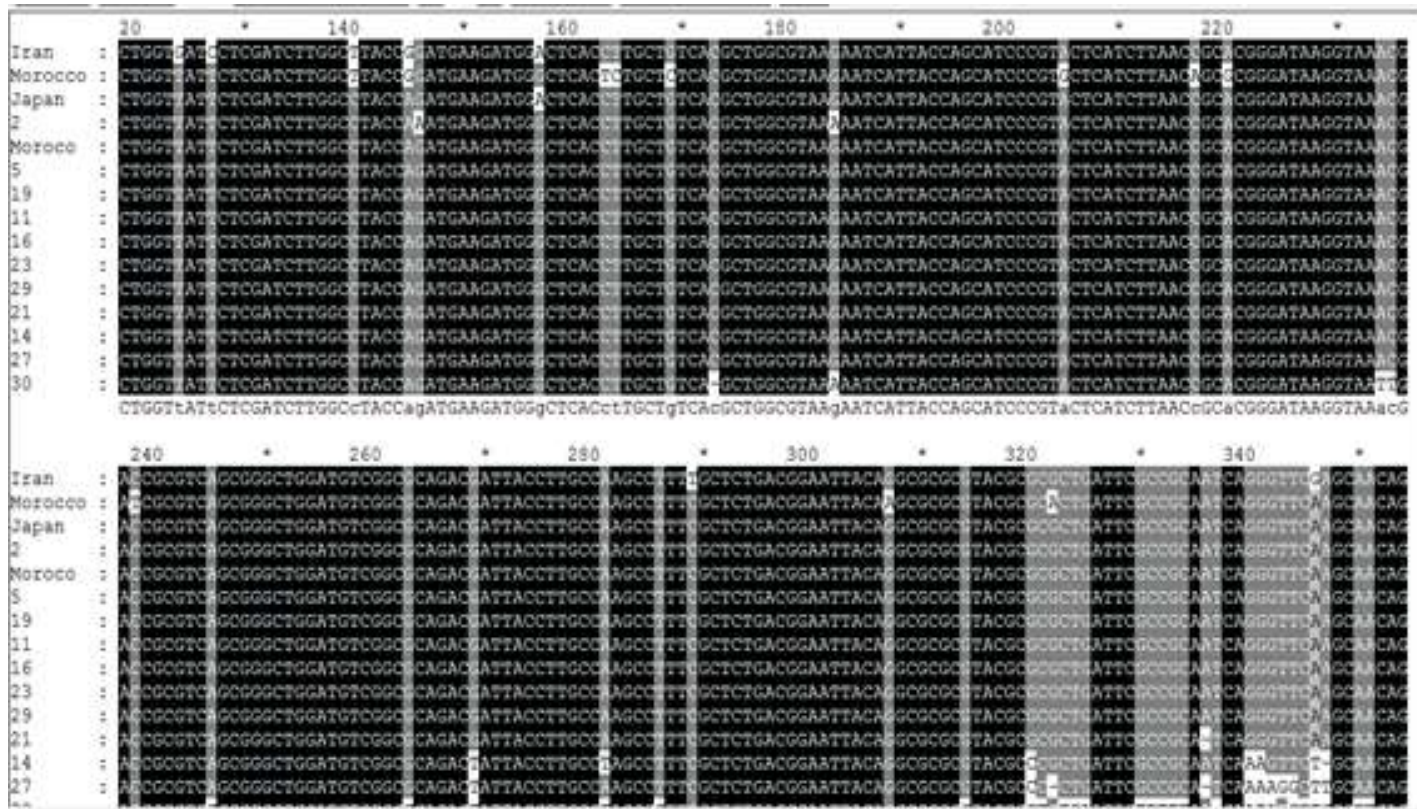


Figure 1. *pmrA* nucleotides sequences alignment used to design primers specific for PCR amplification of *Pectobacterium carotovorum* subsp. *carotovorum* Jo-isolates Q; 16, 27, 30, 19, 14, 21, 29, 23, A; 11, 2, 5 and Pcc reference strains; Pcc strain P603AH1 (Acc. no. JQ278721.1) and Pcc strain P116C2 (Acc. no. JQ278749.1) from Morocco; Pcc, Iran strain Irca3 (Acc. no. KJ634063.1) and Pcc Japan strain (Acc. no. AB447882.1).

isolates. DNA bands were excised from agarose gels and purified using Wizard SV Gel and PCR Clean Up System (Promega) according to the manufacturer’s instructions.

DNA fragments were ligated into the pGEM-T Easy Vector (Promega) according to the manufacturer’s instructions. Ligated products were transformed into JM109 high efficiency competent cells. Five recombinant colonies (white colonies), were selected for each reaction and grown in 4 mL LB broth containing ampicillin (100 µg/ml), at 37°C overnight with shaking at 200 rpm. Plasmid DNA was isolated from cultures using the Pure Yield Plasmid Miniprep Kit. Clones were tested for inserts and fragment size and two clones, from each *Pcc* isolate were sent for sequencing to Macrogen (Seoul, Rep. of Korea) and sequenced from both directions using SP6 and T7 primers.

Sequence analysis

The DNA sequence data were analyzed and homology search was performed using Basic Local Alignment Searching Tool (BLAST) at NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Multiple alignments were conducted using the ClustalX (Thompson et al., 1997). The sequences were then analyzed using GenDoc program from MEGA (Kumar et al., 2001).

Phylogenetic analysis

Evolutionary tree was reconstructed using the Neighbor-Joining

(NJ) program of MEGA (Kumar et al., 2001). The stability of relationships was assessed by performing bootstrap analyses of the Neighbor-Joining with a 1000 replicate bootstrap search.

Primers design

Comparisons of sequences of *pmrA* and other sequences of reference strains published in the GenBank were performed (Figure 1) and new specific primers were designed on the basis of similarities between *Pcc* Jo-isolates. Primer was designed using Primer3 software available at NCBI. Both specificity and sensitivity for set of primers were determined.

In order to assess the specificity of the designed primers, PCR was carried out for different isolates confirmed earlier as *Pcc* together with isolates belonging to other bacterial genera.

To assess the sensitivity of the designed primers, serial dilutions of different bacterial DNA extracts were prepared in NFW from 1x10⁻¹ to 1x10⁻⁷ diluted from 100 ng DNA as well as DNA without dilution, then 2 µl aliquots were directly used as templates for PCR reaction using set of primers.

RESULTS

Two hundred and five rotted potato samples suspected to be infected with soft rot disease were collected from fields and storage throughout potato growing areas in

Table 1. Locations, sampling date and number of samples collected.

Region	Location	Sampling date	Number of samples collected
Amman	Commercial Stores	26/07/2015	15
AR Ramtha	Torrah/Hallan Way	10/06/2014	15
	Torrah/Tanqeeh Station	16/04/2014	16
Jordan Valley	Ashshuna Al Janoubiyya /Wadi Al Abiad	20/01/2014	3
	Deir Alla	15/01/2014	5
	Deir Alla Station	08/04/2014	6
	Ghor Kabed	26/03/2014	25
	Karameh	29/01/2014	8
	North Ghor/Al Sleakhat	20/11/2013	5
	Sharhabeel	16/02/2014	25
Ma'an	Modawwarah	23/01/2014	5
	Modawwarah	29/05/2014	8
	Al Quwayra	08/05/2014	6
Madaba	Al Ariesh	03/07/2014	4
	Jrainah	13/11/2013	14
	Samek	23/04/2014	6
Mafraq	Mghayyer serhan	29/06/2014	2
	Sama serhan	15/01/2014	9
	Sama serhan	26/07/2015	20
	Thoghret El-Jobb	29/06/2014	8
Total number of samples			205

Jordan during summer, autumn and winter seasons, from November, 2013 to July, 2015 in 20 locations (Table 1).

Biochemical and physiological tests

Bacterial isolates were found to be oxidase negative, catalase positive, fermentation of glucose positive, rotting induced on inoculated potato slices, urease enzyme producing, develop growth on nutrient agar plates incubated at $37\pm 2^\circ\text{C}$ and on 5% NaCl. Also, all isolates were able to oxidize the alcoholic sugar and discharge it in the media to acidic reaction and were not able to reduce substances from sucrose. The reactions of the tested bacterial isolates to the different biochemical, physiological and nutritional tests were identical with the results of the same tests conducted for the reference bacterial culture of *Pcc* isolate NCPPB312.

Detection of *Pcc* using (PCR) with *pmrA* primer set

The DNA extracts of different isolates biochemically identified as *Pcc* were of good quality and quantity;

distinctive bands were detected when DNA extracts were ran in 1.0% agarose gel. Furthermore, the spectrophotometer readings indicated that the DNA concentrations ranged between 450 and 600 ng/ μl and the 260/280 ratio ranged between 1.8 and 2.0. Bacterial isolates showed band of about 666 bp (Figure 2) when tested using the specific set of primers *pmrA* (F0145/E2477).

Sequencing analysis

Maximum nucleotide similarity (BLASTn) with closely related species/subspecies ranged from 91 to 100% with *Pcc* strain P603AH1 (Acc. no. JQ278721.1) from Morocco. Similar results were obtained with maximum amino acid similarity (BLASTx).

Phylogenetic analysis

Most of the isolates from Mafraq (Jo-Q16, 19 and 23) and Amman (Jo-A5 and 11) formed a single cluster together with strains from Morocco, Iran and Japan with a

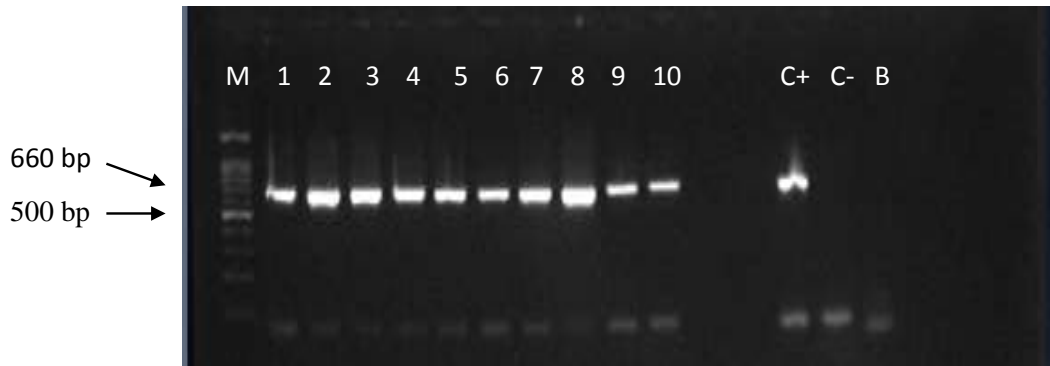


Figure 2. Agarose gel electrophoresis for PCR-amplified DNA of *Pectobacterium carotovorum* subsp. *carotovorum* isolates using *pmrA* primers set with the expected amplified product of 660 bp. Lane M represents Ladder 100 bp (GeneDirex). Lanes 1- 10, isolates Jo-Q16, Q19, A11, A2, Q14, Q21, Q29, A5, Q23 and Q27, respectively. Lanes C+, positive control (reference isolate NCPPB312), C-: Negative control isolate *Escherichia coli* and B: Buffer (Abu-Obeid et al., 2018).

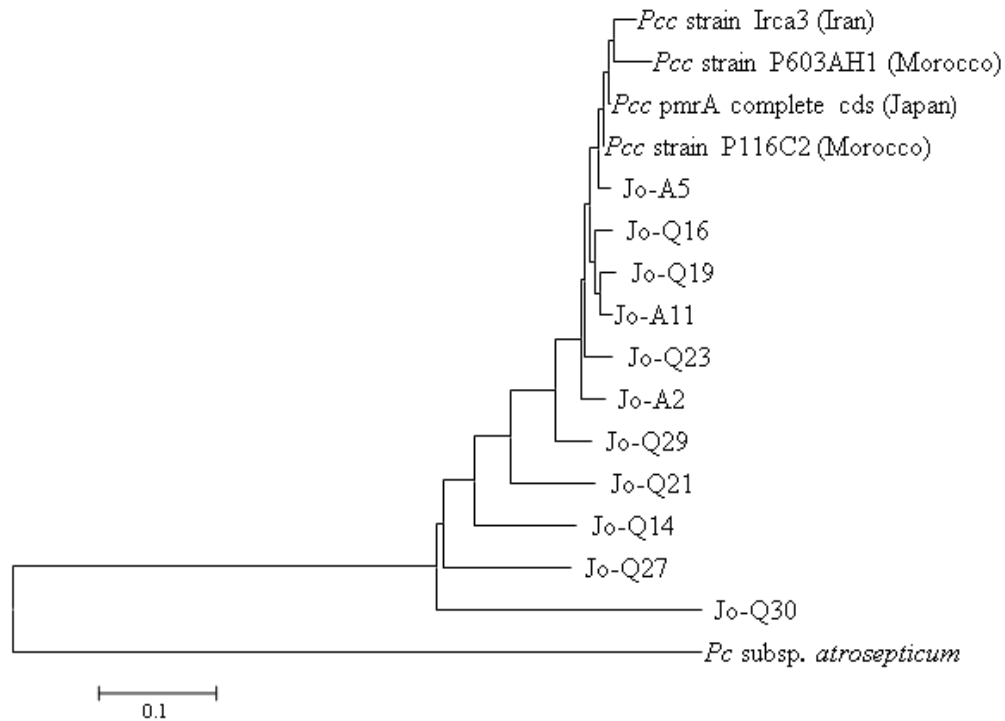


Figure 3. Phylogenetic analysis of nucleotide sequences of *Pectobacterium carotovorum* subsp. *carotovorum* Jo-isolates and reference strains based on *pmrA* partial gene sequences. The generation of the tree was conducted in MEGA program. The branching pattern was generated by the Neighbor-Joining method; stability of the tree was assessed by 1000 bootstrap replication. *Pectobacterium carotovorum* subsp. *atrosepticum* (Acc. no. AY209016.1) was used as out group.

bootstrap value of 92%, while the rest isolates clustered individually but were close to each other. Q30 isolate formed a single cluster, on the other hand, Q30 had lower similarity but higher E-value than the rest *Pcc* Jo-isolates which formed a single cluster clearly differentiating them from out group *P. carotovorum* subsp. *atrosepticum* (*Pca*) (Acc. no. AY209016.1); the results support the

classification of *Pcc* isolates as subspecies distinct from *Pca* (Figure 3).

PCR marker development

The new designed primer Jo-*pmrA*/For. GTCACGAAGG-



Figure 4. Agarose gel electrophoresis for PCR-amplified DNA for *Pectobacterium carotovorum* subsp. *carotovorum* isolates using designed *pmrA* primers set with the expected product size of 318 bp. Lane M represents Ladder 100 bp (GeneDirex). Lanes 1-10: isolates Jo-Q16, Q19, A11, A2, Q14, Q29, A5, Q30, Q23 and Q27; lanes 11 and 12: *Bacillus* spp. and *Listeria* spp., respectively. Lanes C+: Positive control (reference isolate NCPPB312) and B: Buffer.



Figure 5. Agarose gel electrophoresis for PCR-amplified DNA for *P. carotovorum* subsp. *carotovorum* Jo-isolates using designed *pmrA* primers set with the expected product size of 318 bp. Lane M represents Ladder 100 bp (GeneDirex). Lanes 1-8, isolates Jo-Q2 un-diluted, serial dilutions from 1×10^{-1} up to 10^{-7} ; lanes 9-15, isolates Jo-Q21 un-diluted, serial dilutions from 1×10^{-1} up to 10^{-6} , respectively. Lanes C+, positive control (reference isolate NCPP312) and B: Buffer.

TTATGCCTGC, Jo-*pmrA*/Rev. AGCACCACTGGCTTGT-CATC, was used to test PCR specificity and sensitivity to *Pcc* Jo-isolates; length of the primer was 20 bp, melting temperature (T_m) ranged from 56.3 to 57.9°C and GC ratio was 55% with an expected product size of 318 bp.

The marker was specific for detection of *Pcc* Jo-isolates: Jo-Q16, Q19, A11, A2, Q14, Q29, A5, Q30, Q23 and Q27, where it produced PCR product of 318 bp and no bands were observed when tested with other bacterial genera: *Bacillus* spp. and *Listeria* spp. (Figure 4). The marker was sensitive for detection of DNA concentration up to 1.0×10^{-4} ng/ μ l (Figure 5).

DISCUSSION

Seed potatoes planted in Jordan are imported each year from different countries, distributed worldwide in addition

to local production of many varieties. Potato tuber yield losses have increased due to soft rot which has spread in potato cultivation regions including Amman, AR Ramtha, Jordan Valley, Ma'an, Madaba and Mafraq.

Using specific primers for *pmrA* gene, isolates produced a 666 bp; results confirmed these isolates as *Pcc*. Furthermore, the phylogenetic analysis showed that all isolates clustered together with different *Pcc* reference strains available in the GenBank. The *Pcc* Jo-isolates were strongly differentiated from other *Pectobacterium* responsible for disease in potato including *Pca*.

Kettani-Halabi et al. (2013) indicated that *pmrA* sequence analysis was a reliable tool for detection and identification of *Pcc* and to determine genetic diversity. The *Pcc* Jo-isolates from different regions did not cluster according to locations and this could be attributed to the fact that potato seeds in Jordan are mainly from one source.

Terrta et al. (2011) did not observe any correlation between ERIC-PCR analysis, geographical areas and year when they studied the genetic distribution and epidemiological typing of *Pcc*.

Conclusion

Amplification of a specific region and using specific set of primer is a sensitive method for detecting *Pcc*. Little genetic diversity was found among isolates obtained from different regions and similarity was found between them.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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