Molecular Identification of the *Erwinia carotovora* subsp. *carotovora*

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Abstract

Eight isolates of *Erwinia carotovora* (*Ec.c*) were isolated from infected Sponta potato tubers collected from different localities in Libya. All isolates were pathogenic, six isolates (*Ecc*1, *Ecc*3, *Ecc*4 and *Ecc*5), were highly pathogenic, while two isolates (*Ecc*2 and *Ecc*6) moderately pathogenic and three isolates (*Ecc*7 and *Ecc*8) were weakly pathogenic. Extraction of total DNA from the eight isolates of *E. carotovora* as well as identification of eight isolates used into Polymerase Chain Reaction (PCR) analysis and Random Amplified Polymorphic DNA (RAPD) technique were used. High similarity (45.8%, 45.1%, 41.4%)and (40.5%) were observed among the four *Erwinia carotovora* isolates (*Ecc*2, *Ecc*3, *Ecc*5, and *Ecc*7).Two specific primers (Y1, Y2) selected from the pectate lyase–encoding *pel* gene sequences of *E. carotovora* which is common to all strains of *E. carotovora* used to amplify or yielded a distinct band of 434 bp.

Key word: *Erwinia carotovora*, Molecular of *Erwinia carotovora*, soft rot

Introduction

Potato (*Solanum tuberosum* L.) is considered an economic field crop in many countries in the world including Libya. The soft-rot group *Erwinia* is the most important primary plant pathogen (7).

Erwinia spp. pathogen soft rot in potato have been studied extensively because of their economic importance. The different *Erwinia* spp. That are involved in potato diseases are *Erwiniacarotovora* subsp. *atroseptica*(*Eca*), *Erwinia carotovora* subsp. *carotovora* (*Ecc*) and *Erwinia chrysanthemi* (*Ech*) (13).They all can cause tuber soft rot *Erwinia carotovora*, an important gram-negative, rod-shaped bacteria (Family *Enterobacteriaceae*), causes soft rot diseases on wide variety of crop species, chiefly potato which is characterized by blackleg of potato plants and soft rot of its tubers during the storage condition, thus causing extreme yield losses worldwide. (14). Molecular techniques were also used to find out the possible presence of relationships and differences between *Erwinia carotovora* isolates such as; Random Amplified Polymorphic DNA (RAPD). RAPD technique is a useful technique for genetic studies based on the amplification of random DNA (19,21). This study, aimed to (i) Identification of *Erwinia carotovora* using Polymerase Chain Reaction (PCR), (ii) study the genotypic characterization of *Erwinia carotovora* isolates using Random Amplified Polymorphic DNA (RAPD).

Materials and methods

Isolation of soft rot, *E. carotovora* subsp *carotovora* pathogen

isolation of Sponta potato tubers contained soft rot symptoms, were collected from different locations (Alzahra and Alazizia) in Libya during

(2012-2013). Isolates of (*E.c.c*) were identified as (*Ecc*1,*Ecc*2, *Ecc*3, *Ecc*4, *Ecc*5, *Ecc*6, *Ecc*7 and *Ecc*8) by standard bacteriological techniques and pathogenicity test on tubers. Pure cultures of (*E.c.c*) isolated from diseased potato tubers were maintained by subculture on nutrient agar (NA).

Identification of *E. carotovora* by molecular methods

DNA extraction protocol from bacterial cells

Total DNA was extracted from *E. carotovora* isolates. Each isolate was grown overnight in LB medium (each 1 dm^3 contained 10 g tryptone, 5 g yeast extract, 10 g NaCl, and 15 g agar and had a pH of 7) at 30°C with constant shaking at 200 rpm. Cells harvested from 3 cm^3 culture were collected in a microfuge tube by centrifugation at 5590 g for 5 min (Biofuge primo, Heraeus, Germany), washed in Tris EDTA (10 mMTris pH 8.0, 1 mM EDTA), collected again as before and resuspended in a mixture of 567 µlTris EDTA + 30 µl of 10% sodium dodecyl sulphate (SDS) + 3 µl Proteinase K (20 mg / cm^3). After incubation at 37°C for 30 min, cells were collected and the supernatant was discarded, 600 µl of ten (10 mMTris pH 8.0, 0.4 M NaCl, 2 mM EDTA) was added, followed by 5 min incubation in a water bath at 75–80°C. An of 3 µl RNase A (10 mg / cm^3) was added, the mixture incubated for 15–60 min at 37°C, and then cooled to room temperature. A volume

of 0.2 cm^3 of saturated NaCl was added, followed by vortexing and centrifugation at 10956 g for 5 min. 0.6 cm^3 of absolute isopropanol was added to the supernatant, the mixture was vortexed and centrifuged again at 10956 g for 20 min. The isopropanol was poured off, and 0.6 cm^3 cold 70% ethanol was

added to wash the DNA. After gentle mixing and centrifugation, the ethanol was poured off and the tube was allowed to air dry. The DNA was resuspended in 100 µl of sterile distilled water (2,9).

Identification of *E. carotovora* by PCR:

Specific primers for DNA:-

Detection of *E. carotovora* was performed in this study using four primers were specific to *E. carotovora* (Y1,Y2) selected from the pectate lyase–encoding *pel* gene sequences of *E. carotovora* (5,11) described in (Table 1) were chosen amplification by PCR.

Table 1: Nucleotide sequences of primers used in PCR analysis.

PCR Code	Nucleotide sequence 5' to 3"
Y1	TTA CCG GAC GCC GAG CTG TGG CGT
Y2	CAG GAA GAT GTC GTT ATC GCG AGT

Amplification by PCR:

PCR amplification of DNA by using two specific primers as follows: primer Y1 together with primer Y2. A negative control was enclosed by running the PCR reaction with sterile water and the positive control was used as bacterial DNA template. Each PCR reaction was performed with a final volume of 25 µl reaction mixture containing 1 x PCR buffer with $MgCL_2$ (50 mM KCL, 10 mMTris - HCL, pH 9, 1.5 mM $MgCL_2$), 200 mM each of dATP, dCTP, dGTP, dTTP, 50 pmol primer, 2.0 µl template DNA and 2.5 units of Taq DNA Polymerase. Reaction mixtures were over laid with 15 µl sterile mineral oil subjected to PCR. Amplification was performed in a thermal cycler (Techne- Progene), and was achieved by three stages. The first stage was the initial cycle of denaturation at 95°C for 1 minute. The second

stage was performed by running forty cycles of a denaturating temperature at 94°C for 1 min, annealing temperature of 65°C for 1 min and an extension phase of 72°C for 45 seconds. The same temperature was used for both primer pairs. The third stage, an 8 minutes extension period, was added after the final cycle (1,2).

Analysis of the PCR product using agarose gel electrophoresis:

Gel electrophoretic analysis was performed by using 1.5% agarose NEEO Ultra-Quality gels in 1 x TBE separation buffer, pH 8.0, the gel electrophoresis apparatus used was a horizontal gel electrophoresis. Agarose was weighted, suspended in buffer and melted in a microwave until a transparent solution was achieved. After cooling until about 45-50°C, the agarose was poured into a plastic mold and allowed to solidify at room temperature. Air bubbles were removed with a pipette tip direct after pouring. After the gel was completely polymerized, it was transferred to a gel tank containing the electrophoresis running buffer (1 x TBE, pH 8.0) and the same buffer was added just enough to cover the

top of the gel to a depth of 1-2 mm (2,4).The samples were prepared as follows: Two µl of the loading buffer was pipetted onto a parafilm strip equal to the number of the performed PCR reactions and 8 µl from each of the PCR reaction were pipetted out by mixing with the loading buffer on the parafilm strip. The whole volume was transferred into the slots of the agarose gel. The lid of the electrophoresis tank was closed and the electrical leads connected to the power pack. A voltage of 100 V was set, and the sample migration from the anode to the cathode was

visualized by the migration of the 5 x loading dye solution along the gel, that was allowed to run for 60 minutes at room temperature. After DNA fragments were separated (in the electric field), the gel was removed carefully and put in 0.5 µg/ml ethidium bromide solution for 10 minutes, the gel was removed again and submersed in distilled water for 5 minutes, the gel was photographed with a Polaroid camera using a black and white film under UV apparatus. The gel was also visualized under UV transilluminator. The expected size of the PCR product was 434 bp for primers

Random Amplified Polymorphic DNA (RAPD)

RAPD assays

Four random primers each consists of 10 bases were used to differentiate the *E. carotovora* isolates. Sequences of random primers are illustrated in (Table 2). For RAPD analysis, PCR amplification was carried out in a total volume 25 µl containing 2.5 µl 10 x buffer, 2 µl of 25 mM $MgCl_2$, 2 µl of 2.5 mM

dNTPs, 1 µl of 10 pmol primer, 1 µl of 50 ng of bacterial genomic DNA and 0.2 µl (5 units/µl) Taq DNA polymerase. PCR amplification was performed in a thermal cycler programmed for one cycle at 95°C for 5 min followed by 30 cycles at 95°C for denaturation, 1 min at 36°C for annealing and 1 min at 72°C for elongation. Reaction mixture was then incubated at 72°C for 10 min for final extension (9).

Electrophoresis was performed at 100 volt with 0.5 x TBE as running buffer in 1.5% agarose/0.5 x TBE gels and then the gel was stained in 0.5 µg/cm^3 (w/v) ethidium bromide solutions and destained in deionized water (17). Finally the gel was visualized with a UV transilluminator. Presence and absence of

RAPD bands produced from the use of six primers were scored visually from the resulting photographs (2).

Table 2: Nucleotide sequences of primers used in (RAPD) analysis.

PCR Code	Nucleotide sequence 5' to 3"
Primer1	ACT TGG TAT T
Primer2	GGG CGC AAT A
Primr3	ATA GGC GGG T
Primer4	TTA CGC ATA C

Cluster analysis:

Data were scored for computer analysis on the basis of the presence or absence of the amplified products for each primer. A product present in a bacterial isolate, was designated (1) and when absent it was designated (0) after excluding common bands. Pair–wise comparison of bacterial isolates, based on the presence or absence of unique and shared polymorphic products, was used to generate similarity coefficients according to (15). The similarity coefficients were used to construct a dendrogram by UPGMA (6).

highly virulent isolates *E.c* 3,4were not present in all tested isolates of *E. carotovora* subsp. *carotovora* (Fig. 4).

Results showed that primer four produced two specific bands200,300bp for highly virulent isolates*E.c*4,5this band not found in all tested isolates of *E. c* subsp. *carotovora* and produced a specific bands150, 400bp for virulent isolate *E. carotovora*2 this band not found in all tested isolates of *E. carotovora* subsp. *carotovora* (Fig. 5).

Cluster analysis:

The RAPD band patterns resulting from the use of the four primers were analyzed using UPGMA method to construct a similarity matrix (Table3) and to generate a dendrogram indicating the relationship between the eight tested bacterial isolates (Fig.6).

The presence or absence of any particular DNA bands was the only parameter considered in the computer analysis.

The dendrogram generated in this study for the *E.carotovora* subsp. *carotovora* isolates (*Ecc*1,*Ecc*2,*Ecc*3,*Ecc*4,*Ec5*,*Ecc*6,*Ecc*7 and*Ecc*8) revealed that two main clusters do exist. Cluster 1contained only one isolate, namely *Ecc*8. Cluster 2 divided into two sub-cluster: Sub-cluster 1 divided into four Groups: Group1 included three isolates, *Ecc*3,*Ecc*1 and *Ecc*4and Group2 contained only one isolate *Ecc*5,Groups3 contained one isolate *Ecc*2,andGroup4 included one isolate *Ecc*6. Finaly, sub-cluster 2 indicated only one isolate *Ecc*7.

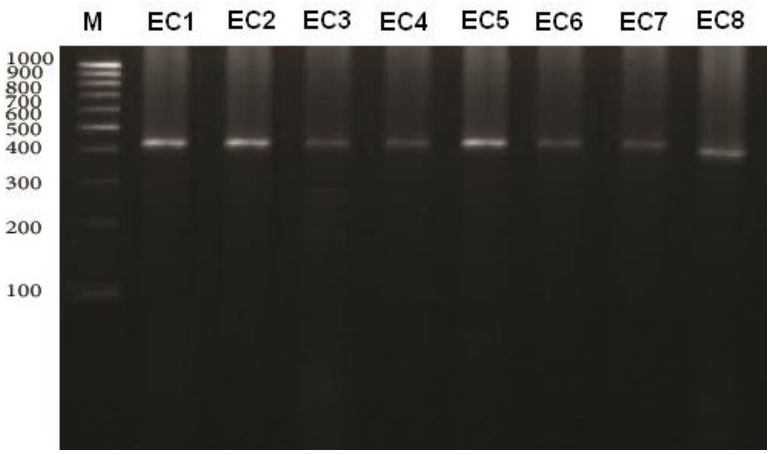

Fig.1. The PCR amplification products resulting from the use of two specific primers (Y1 and Y2).Analysis induction 8 isolates of *E. carotovora* subsp. *carotovora* obtained from potato tubers. Products were observed upon separation by agarose gel electrophoresis followed by staining with ethidium bromide and visualization under UV-light. Line 1, *Ecc*1;line 2, *Ecc*2; line 3, *Ecc*3; line 4, *Ecc*4; line 5, *Ecc*5;line 6, *Ecc*6; line7,*Ecc*7; and line8,*Ecc*8.M, a 100 bp DNA marker ladder

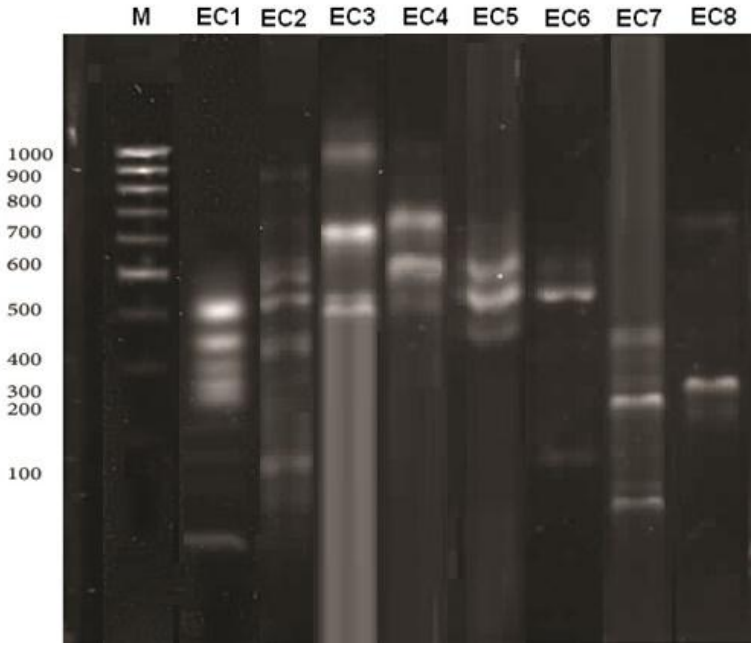

Fig.2. Separation of the *E. Carotovora* subsp. *carotovora* RAPD-PCR amplification product with primer (1) representing 8 isolates by agarose gel electrophoresis followed by staining with ethidium bromide and visualisation under UV-light Line 1, *Ecc*1;line 2, *Ecc*2; line 3, *Ecc*3; line 4, *Ecc*4; line 5, *Ecc*5; line 6, *Ecc*6*;* line7,*Ecc*7; and line8,*Ecc*8. M, a 100 bp DNA marker ladder

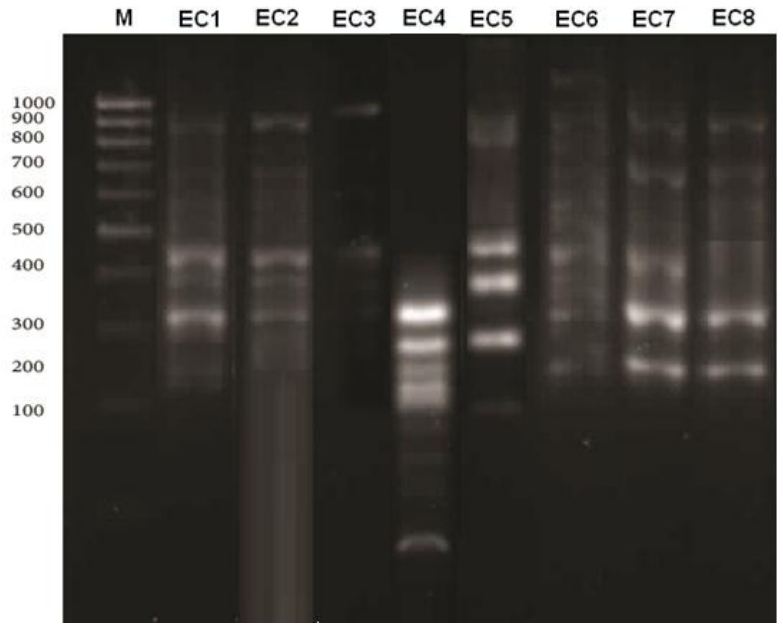

Fig.3. Separation of the *E. Carotovora* subsp. *carotovora* RAPD-PCR amplification product with primer (2) representing 8 isolates by agarose gel electrophoresis followed by staining with ethidium bromide and visualisation under UV-light Line 1, *Ecc*1;line 2, *Ecc*2; line 3, *Ecc*3; line 4, *Ecc*4; line 5, *Ecc*5; line 6, *Ecc*6*;* line7,*Ecc*7; and line8,*Ecc*8. M, a 100 bp DNA marker ladder

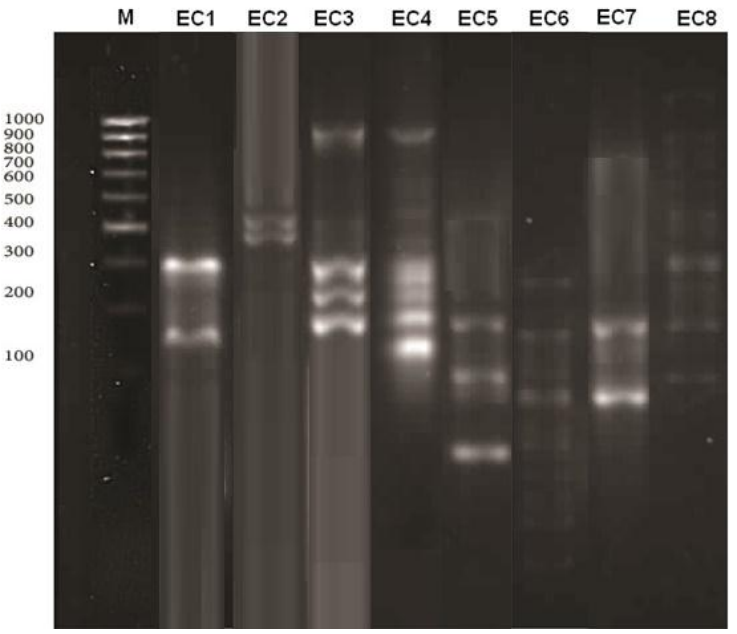

Fig.4. Separation of the *E. Carotovora* subsp. *carotovora* RAPD-PCR amplification product with primer (3) representing 8 isolates by agarose gel electrophoresis followed by staining with ethidium bromide and visualisation under UV-light Line 1, *Ecc*1;line 2, *Ecc*2; line 3, *Ecc*3; line 4, *Ecc*4; line 5, *Ecc*5; line 6, *Ecc*6*;* line7,*Ecc*7; and line8,*Ecc*8. M, a 100 bp DNA marker ladder

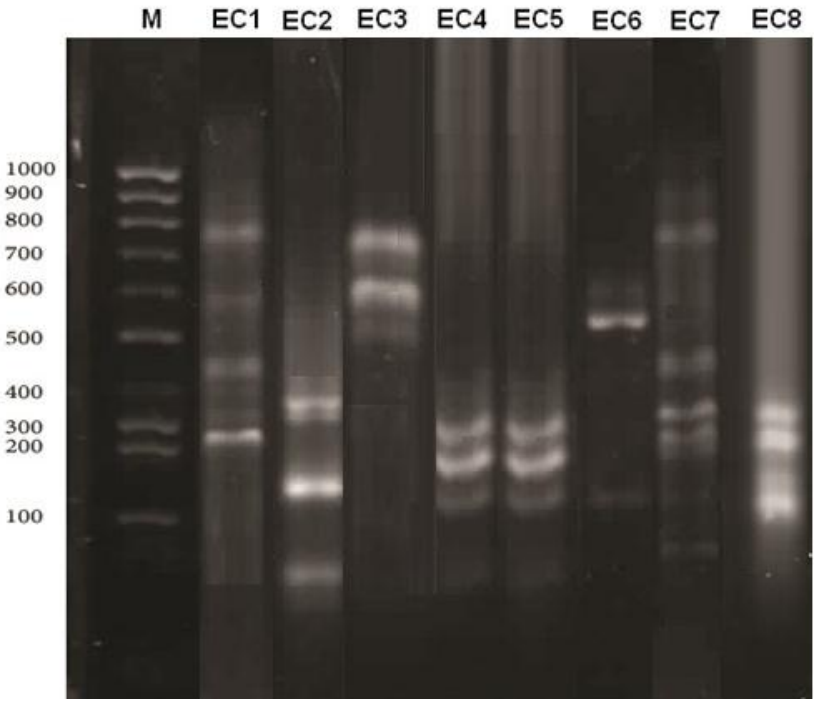

Fig.5. Separation of the *E. Carotovora* subsp. *carotovora* RAPD–PCR amplification product with primer (4) representing 8 isolates by agarose gel electrophoresis followed by staining with ethidium bromide and visualisation under UV–light Line 1, *Ecc*1;line 2, *Ecc*2; line 3, *Ecc*3; line 4, *Ecc*4; line 5, *Ecc*5; line 6, *Ecc*6*;* line7,*Ecc*7; and line8,*Ecc*8. M, a 100 bp DNA marker ladder

Table 4. Asmilarity matrix, in percentage, among *E. carotovora* subsp. *carotovora* isolates based on RAPD band pattern analysis and Jaccard index.

Bacterial isolates	*Ecc*1	*Ecc*2	*Ecc*3	*Ecc*4	*Ecc*5	*Ecc*6	*Ecc*7	*Ecc*8
*Ecc*1	100							
*Ecc*2	40.5	100						
*Ecc*3	30.2	45.1	100					
*Ecc*4	20.4	35	20.6	100				
*Ecc*5	45.8	17.2	23.4	25	100			
*Ecc*6	16.3	21.5	17.2	22.5	30.1	100		
*Ecc*7	41.4	23.3	26.2	17.5	20.1	22.3	100	
*Ecc*8	31.2	21.5	18.6	30.6	18.2	21.5	19.4	100

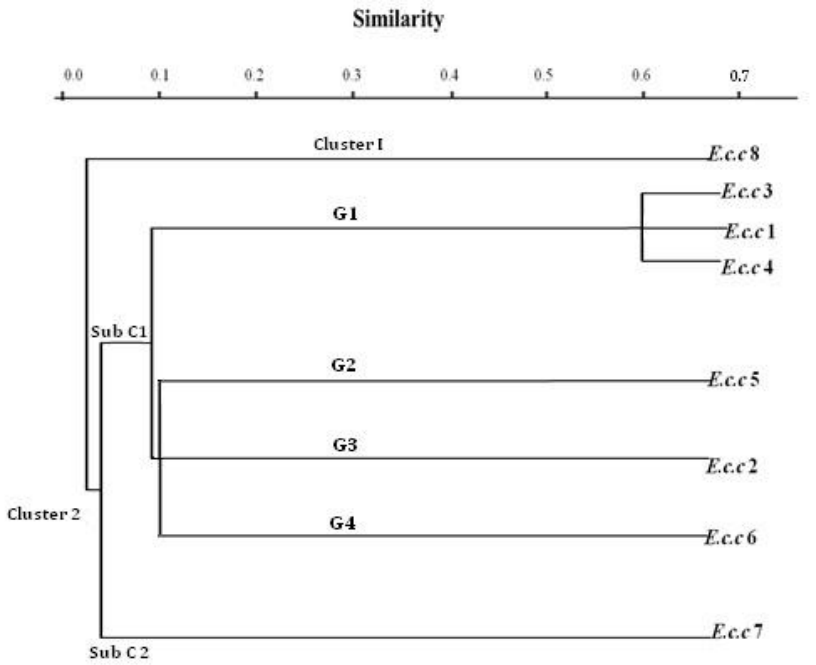

Figure 6:Dendrogram obtained by clustering (UPGMA method) based on the band pattern obtained by the RAPD-PCR analysis of eight *E. carotovora* subsp. *carotovora* isolates from different locations according to Jaccard similarity.

Discussion

Erwinia carotovora subsp. *carotovora* is the causal agent of the soft rot disease of potato tuber in stores and in the field (12,23).

In this study; pathogenicity tests of eight *E. carotovora* isolates *(Ecc*1, *Ecc*2, *Ecc*3, *Ecc*4, *Ecc*5, *Ecc*6, *Ecc*7 and *Ecc*8). All the potato tubers were infected by *Ecc,* isolates These results agreed with those reported by(16,18).

Molecular biology has provided a powerful tool to identify *Ecc*. The use of specific primers to amplify the 434 bp DNA fragment in the PCR (5,11). Identification of *E. carotovora*by DNA using the PCR technique. The differentiation between 8 isolates of *Ecc* from different areas was carried out using random amplified polymorphic DNA fragments $RAPD_s$ technique. Results showed that isolates of *Ecc* different banding patterns similar results were obtained by (10,22). Genetic in *Ecc* isolates is important in epidemiology. Data from genetic studies of pathogenic isolates, to identify possible sources of infection, to assist in gene mapping, to identification, and population genetics of species, and characters in molecular phylogenic studies (3,10).

A technique distinguish between *Ecc* isolates to provide information on relationships between them would be extremely valuable. Random Amplified Polymorphic DNA (RAPD) (20,21).

التعريف الجزيئي لعزلات البكتريا *Erwinia corotovora* subsp. *corotovora*

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الملخص

عزلات ثمانية عزلات من بكتيرياEcc من *Erwinia corotovora* subsp. *corotovora* من عينات درنات بطاطس صنف (سبوبتا) عليها أعراض التعفن الطري من مناطق مختلفة (الزهراء- العزيزية) جنوب غرب مدينة طرابلس (ليبيا)تتابين العزلات الثمان في امراضيتها أربعة منها (Ecc5,Ecc1,Ecc3,Ecc4) أظهرت أمراضية عالية، بينام العزلتين (Ecc2,Ecc6) كانت ذات أمراضية متوسطة، أما العزلتين (Ecc7,Ecc8) فكانتا ضعيفة الامراضية.

استخلص DNA الكلي من العزلات الثمانية وتم تعريفها باستخدام تقنية تفاعل سلسلة البولي ميراز (PCR) وكذلك تقنية متعدد التشكيل المتضخم العشوائي لـ DNA (RAPD) تم اختبار بادئين "Primers" نوعين (Y1,Y2) من ترميز ليبزا البكتات لتتاليات مورثة (Pel) لبكتريا Ecc والشائعة لجميع العزلات.

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