Detection and Identification of *Erwinia carotovora* subsp. *atroseptica* (Van Hall, 1902) the Causal Agent of Potato Blackleg by RFLP-PCR

Riyad Al-Zomor[1], Hamed Khlaif [1], and Muhanad Akash[2]

ABSTRACT

Potato blackleg disease is surveyed for spreading out in different potato growing areas in Jordan including; Jordan Valley (Al-Karama, Ashshuna Al-Janubiyya, Muthallath Al-Misri, Mu'addi, Deir Alla, Dirar, and Kurayyima), Amman (Al-Yadoda), Madaba (Om Al-Amad), Jerash (Tawahin Al-Udwan), Ma'an (Rum, and Al-Mudawwara). Forty six bacterial isolates of *Erwinia carotovora* subsp. *atroseptica* (*Eca*), the causal agent of the disease, were isolated and identified by different biochemical, physiological and nutritional tests from 145 diseased potato samples collected. Polymerase chain reaction (PCR)-based amplification of a 690-bp band was used to confirm the presence of *Eca* in the isolated bacteria estimated by the reference culture (ATCC 15713). PCR amplification was followed in isolation, identification and detection of *Eca* in infected and symptomless potato-stem and -tuber samples. The results showed that *Eca* was isolated from 30% of naturally infected potato samples with blackleg, while the 690-bp band was detected in 88% of DNA extracts from the naturally infected potato samples. In the other hand *Eca* was not isolated from the symptomless potato stem samples, while the 690-bp band was detected in 8% of the DNA extract from these stem samples. However; using symptomless-tuber samples have improved isolation efficiency. *Eca* was isolated directly from 4% of the samples when their suspension was streaked on Logan's medium plates however 690-bp band was detected from 32% of their DNA-extract amplified with PCR. Genetic variability among the *Eca* isolates has been able to detect 690-bp PCR products for 46 different isolates those were digested with the restriction enzymes *i.e. Alu*I, *Hae*II, *Hpa*II and *Sau*AI. Polymerase chain reaction-Restriction fragment length polymorphism (PCR-RFLP) analyses were able to cluster the *Eca* isolates into five PCR-RFLP groups with low genetic variability.

Keywords: *Erwinia carotovora* Subsp. *Atroseptica (Eca)*, Blackleg Of Potato, PCR-RFLP Analysis Survey, *Eca* Genetic Diversity, Detection.

INTRODUCTION

Potato (*Solanum tuberosum* L.) is considered an economic field crop in many countries in the world including Jordan. Potato ranks the second among the economic agricultural crops grown in Jordan, where the area planted with potato in 2008 was 56700 dunums with total production of 189100 tons. Of this area, 54.3 % was planted in the Jordan Valley (Annual Agricultural Statistics at http://www.moa.gov.jo).

Different bacterial diseases have been reported to attack potatoes: soft rot (*Erwinia carotovora* subsp. *carotovora* (Jones, 1901)), brown rot (*Ralstonia solanacearum* (Smith, 1896)), ring rot (*Clavibacter*

[1] The University of Jordan, Faculty of Agriculture, Department of Plant Protection, Amman 11942, Jordan
[2] The University of Jordan, Faculty of Agriculture, Department of Horticulture and Crop Science, Amman 11942, Jordan
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michiganensis subsp. *sepedonicus* (Spieckermann and Kotthoff, 1914)) and potato scab (*Streptomyces scabies* (Waksman and Henrici, 1948)). *Erwinia carotovora* subsp. *atroseptica* (*Eca*) the causal agent of blackleg is a seed born pathogen blackleg disease is one of the most important diseases attacking potato and drastically resulting in economic losses wherever it is grown (Elphistone, 1987; Cappaert *et al*., 1988, Bell *et al*., 2004). In USA, Kennedy and Alcorn (1980) estimated the annual losses in potatoes, due to blackleg disease, to be around 14 million dollars.

Blackleg has a restricted host range, where potato being the major host plant in cold to temperate regions (Perombelon and Kelman, 1987; George *et al*., 1991, Helias *et al*., 1998; Helias *et al*., 2000), sugar beet and sunflower were also reported as more hosts (Stanghellini *et al*., 1977; Mendonga and Stanghellini, 1979; Whiteney, 1982; Gudmestad *et al*., 1984; Jorge and Harrison, 1986; Schuerger and Bartz, 1993). Contaminated tubers, diseased or infected as propagative materials, are considered as primary inoculum sources for blackleg disease (Ward and De Boer, 1994; van der Wolf *et al*., 1996; Al-Masa'deh and Khlaif, 2003).

Blackleg, which is a seed-born pathogen, is one of potato diseases under quarantine system in Jordan. To restrict the spreading of the disease, different methods were followed to detect the pathogen from infected or contaminated potato tubers or latent infection, with varying degrees of specificity, sensitivity and suitability for large scale use (Perombelon and Hyman, 1995). As for these methods; direct isolation on common and selective media, such as crystal violet pectate (CVP), where the isolation of the causal agent could be seriously affected by the presence of large numbers of saprophytic bacteria (Jones *et al*., 1994). Immunological methods, such as enzyme-linked immunosorbent assay (ELISA), were found to be inefficient in detecting low population of the pathogen and can't detect all *Eca* different strains (De Boer and McNaughton, 1987; Gorris *et al*., 1994). Furthermore, these methods are time consuming, and relatively expensive. Therefore a rapid and efficient diagnostic technique for detection and identification of *Eca* is needed. Polymerase chain reaction (PCR) proved to be highly sensitive and specific method for the detection of blackleg pathogen in potatoes (De boer and Ward, 1995, Perombelon and Hyman, 1995).

Accordingly, this research was conducted in order to isolate and identify *Eca*, from different potato-growing areas surveyed in Jordan Valley and Uplands. Bacterial isolates, those are taken from diseased and symptomless tubers as well as plant-stem samples, were amplified using PCR. Genetic diversity, among different bacterial isolates, though PCR-RFLP was also aimed.

Materials and Methods

Samples collection: Potato samples, were randomly sampled including tubers and stems. Symptoms of blackleg-diseased samples were collected from different potato growing areas in Jordan (Table 1, Fig. 1). The collected samples were placed in an ice box and brought to the laboratory for further work.

Disease incidence: The disease incidence of the infected plants was estimated, using external symptoms, as percentage of infection. (Al-Masa'deh and Khlaif, 2004). Samples were collected in diagonal directions.

Pathogen isolation: The infected tubers or stem samples were placed in washing jars under running tap water to remove adhering soil particles. Surface was disinfected with sodium hypochlorite 0.5 % for 3 minutes, rinsed three times with sterile distilled water and then plotted to dry onto sterile filter paper. Sample of 10g of plant tissue was aseptically separated with sterile scalpel and then was cut into small pieces and was soaked and macerated in 90 ml of sterile distilled water in a sterile bottle and placed onto a shaker at room

temperature at 200 rpm till the suspension become turbid and homogenized. Ten fold serial dilutions were prepared from the suspension up to the 10^{-4}. A 0.1 ml volume from 10^{-3} and10^{-4} were separately spread by sterile glass rod onto the surface of dried Logan's medium plates (Nutrient agar 24g, yeast extract 5g, glucose 5 g and after autoclaving 10 ml of filter sterilized 0.5% of 2, 3, 5-triphenyl tetrazolium chloride were added). The inoculated plates were incubated at 25 ±2 ºC and checked periodically for the growth of colorless colonies that suspected to be for *Eca* within the first 24 hrs of inoculation. Single colonies were re-streaked onto new NA's plates with fresh nutrient agar. The obtained bacterial isolates were then grown on NA's slants and placed in a refrigerator for further identification (Schaad, *et al*., 2001).

Pathogen identification: Twenty four hours-old cultures, of the obtained bacterial isolates, were biochemically and physiologically characterized, identified and subjected to the following tests: oxidase test, catalase test, potato soft rot, oxidative fermentative test, growth at 37°C, sodium chloride tolerance test, starch hydrolysis test, nitrate reduction test, H_2S production test, indole production test, urease production test, reducing substances from sucrose, acid production from α-methyl-d-glucoside, and hypersensitive reaction to tobacco (Schaad *et al.*, 2001, Perombelon and van der Wolf, 2002). The same tests were run against a reference culture of *Eca* isolate (ATCC 33260).

DNA extraction from bacterial cultures: *Erwinia carotovora* subsp. *atroseptica* isolates were grown on NA plates at 25°C for 24 hrs, then from each isolate few bacterial colonies were picked up with a sterile loop and mixed thoroughly in 500 µl sterile ultra pure water and then heated at 50 °C for 3 hrs. DNA was precipitated from the supernatant fractions by adding 1 volume of isopropanol and then pelleted by centrifugation at 15,000 rpm for 4 min's. Pellets were washed with 70 % ethanol and dried at 58 °C for 10 min's, then re-suspended in 100 µl of nuclease-free water (Promega, USA), and stored at -20 °C for further PCR work (De Boer and Ward, 1995, Helias *et al*., 1998).

Bacterial DNA extraction from plant samples (infected and symptomless plant material): Plant tissues were macerated with sterile distilled water by using sterile mortar and pestle, and then stored on ice for 10 min's to allow debris and starch to settle. Then the supernatant was taken to new centrifuge tube and centrifuged at 5000 rpm to remove excess plant debris. After that the supernatant was transferred into new centrifuge tube and spun at 14000 rpm for five minutes to pellet the bacterial cells. Then the supernatant was discarded without disturbing the pellet and washed with 70 % ethanol, dried at 58 °C for 10 min, then re-suspended in 100 µl of nuclease-free water (Promega, USA), and stored at -20 °C (De Boer and Ward, 1995, Helias *et al*., 1998, Smid *et al.*, 1995).

PCR amplification: In order to detect the presence of the 690-bp DNA fragment of *Eca*, ECA1f forward primer (5'-CGGCATCATAAAAACACG-3') and ECA2r reverse primer (5'-GCACACTTCATCCAGCGA-3') were used for amplification (De Boer and Ward, 1995). Polymerase chain reaction mix contained 5 µl (10x) Taq DNA polymerase buffer, 4 µl (25 mM) $MgCl_2$, 0.5 µl of (10mM) dNTPs mixture, 0.3 µl of (50 µM) of each primers, 0.5 µl (1/10) of Tween 20, 0.2 µl of Taq DNA polymerase (Promega, USA), 2 µl of template DNA and ultra-pure water to a total volume of 50 µl. Amplification reaction was performed in a thermal cycler (9700 Perkin-Elmer) using the following protocol: initial denaturation at 95 ºC for 5 min, followed by 36 cycles of (30 s at 94 °C) for denaturation, (45 s at 62 °C) for annealing, and (45 s at 72 °C) for extension and an additional extension step at 72 °C for 8 min. After

amplification reaction 10 µl of the PCR product mixed with 2 µl of bromophenol blue as loading dye (6x) were separated by 1.2 % (w/v) agarose (Promega) electrophoresis in 1x TBE (Tris Borate EDTA) buffer. Ethidium bromide of 1 µl per 10 ml of agarose gel was used as detection agent and the 50 and 100bp DNA Step Ladder (Promega, USA) were used as a molecular marker. Electrophoresis reactions were performed at 100 V for 1 hrs. Then gels were photographed under ultra-violet (UV) light at 302 nm wavelength.

PCR-RFLP analysis: The PCR product DNA for each *Eca* isolate (690-bp DNA fragment) was taken, separately in a small Epndorf tubes, and cleaned and then digested separately with 4 different restriction endonuclease enzymes *Alu*I, *Hae*III, *Hpa*II and *Sau*3AI (BioLabs, USA) at 37 °C for 2 hrs as incubation period. The total volume of the digestion reaction for each enzyme was 20 µl, containing 8 µl from PCR product, 10 µl ultra-pure water and 1 U of the enzyme diluted in 2 µl of the appropriate buffer. The digested products were separated by electrophoresis (100 V, 1 hrs) on a 2.4 % (w/v) agarose gel and then photographed under UV light at 302 nm as described by Yahiaoui-Zaidi *et al*. (2003).

Data analysis: The digested DNA bands for the 46 bacterial isolates were scored as 1 for presence, 0 for absence and 9 for missing bands. Band size was identified in base pair. Similarities between isolates were estimated according to Dice (1945). Cluster analysis was conducted using the unweighted pair-group method, arithmetic average (UPGMA). All genetic analyses were conducted using a program in NTSYSpc 2.0 software (Rohlf, 1998).

Results

Samples collection and disease incidence:

One hundred forty five diseased potato samples shown the symptoms of blackleg disease were collected from different locations in Jordan including: Jordan Valley (Al-Karama, Ashshuna Al-Janubiyya, Muthallath Al-Misri, Mu'addi, Deir Alla, Dirar, and Kurayyima), Amman (Al-Yadoda), Madaba, Jerash (Tawahin Al-Udwan), Rum, and Al-Mudawwara (Table 1).

Table (1): Location, sampling date, disease incidence, number of collected samples and the obtained isolates of *Erwinia carotovora* subsp. *atroseptica*

Location	Sampling date	Incidence of disease at collection time	No. of collected samples	No. of Obtained *Eca* isolates	Isolates percentage from total isolates
Jordan Valley					
Al-Karama & Ashshuna Al-Janubiyya	4.3.2010	16%	8	2	4.3%
Muthallath Al-Misri	22.2.2010	26%	23	9	19.6%
Mu'addi	20.2.2010	14%	10	_	-
Deir Alla	7.3.2010	22%	20	4	8.7%
Dirar	23.2.2010	16%	8	2	4.3%
Kurayyima	8.3.2010	10%	5	3	6.5%
Amman					
Al-Yadoda	10.4.2010	20%	8	_	-

Location	Sampling date	Incidence of disease at collection time	No. of collected samples	No. of Obtained *Eca* isolates	Isolates percentage from total isolates
Madaba					
Om Al-Amad	10.4.2010	5%	5	_	-
Jarash					
Tawahin Al-Udwan	6.4.2010	24%	18	8	17.4%
Ma'an					
Rum	30.3.2010	-	15	9	19.6%
Al-Mudawwara	1.4.2010	20%	10	4	19.6%
	14.4.2010	28%	15	5	
Total			145	46	100%

The recorded disease incidence at the time of samples collection varied between the different locations and ranged from 5% to 28% for Madaba and Al-Mudawwara location respectively (Table 1).

Isolation, Identification and characterization of the causal agent:

When the suspensions of the natural diseased potato samples were streaked onto the surface of dried Logan's plates small, circular, and colorless bacterial colonies were developed after 24 hours. The results of the reaction of the obtained bacterial isolates (isolated from plants suspected to be infected with blackleg) against the different biochemical, physiological and nutritional tests are presented in the table (2). All the tested isolates were found to be catalase positive, oxidase negative, induce rotting on the inoculated potato slices, oxidative-fermentative positive, starch hydrolysis and growth at 37 ºC were negative, positive growth at 5% NaCl, nitrite produced from nitrate, Urease wasn't produced, H_2S was produced in the test tubes containing cystine broth. Indole was not produced from tryptophane. All isolates produced acid from α-methyl-d-glucoside, reduced substances from sucrose and have induced hypersensitive reaction on tobacco-seedling leaves (cultivar White Burley) injected with the suspension of the tested bacterial isolates.

The obtained results were similar to those of the reference culture (ATCC 15713).

Table (2). Laboratory tests for 46 different *Erwinia carotovora* subsp. *atroseptica* isolates collected from Jordan compared to (ATCC 15713) reference culture.

Isolate	Acid production from α-methyl-d-glucoside	Catalase	Growth at 37°C	H_2S production	Hypersensitive reaction to tobacco	Indole production	Nitrate reduction	Oxidase	Oxidative fermentative	Potato soft rot	Reducing substances from sucrose	Sodium chloride tolerance 5%	Starch hydrolysis	Urease production
Al-Karama & Ashshuna Al-Janubiyya (Ka29 and Ka30).	+	+	-	+	+	-	+	-	+	+	+	+	-	-
Muthallath Al-Misri (J1, J3, J4, J5, J6, J7, J8, J9 and J10).	+	+	-	+	+	-	+	-	+	+	+	+	-	-
Rum (Rm 71, Rm 72, Rm 74, Rm 75, Rm76, Rm 77, Rm 78, Rm 79 and Rm 80).	+	+	-	+	+	-	+	-	+	+	+	+	-	-
Kurayyima (Kr26, Kr27 and Kr28).	+	+	-	+	+	-	+	-	+	+	+	+	-	-
Tawahin Al-Udwan (Ta59, Ta60, Ta61, Ta62, Ta63, Ta64, Ta65 and Ta66).	+	+	-	+	+	-	+	-	+	+	+	+	-	-
Deir Alla (D15, D16, D20 and D23).	+	+	-	+	+	-	+	-	+	+	+	+	-	-
Dirar (Rb24 and Rb25)	+	+	-	+	+	-	+	-	+	+	+	+	-	-
Al-Mudawwara (April.14) (MM33, MM34, MM35, MM37 and MM41).	+	+	-	+	+	-	+	-	+	+	+	+	-	-
Al-Mudawwara (April.1) (MA85, MA86, MA88 and MA89).	+	+	-	+	+	-	+	-	+	+	+	+	-	-
Reference culture *Eca* (ATCC 15713).	+	+	-	+	+	-	+	-	+	+	+	+	-	-

Ref*: Reference culture *Eca* (ATCC 15713)

Detection of the 690-bp DNA band from bacterial cultures and PCR-RFLP analysis:

The DNA extract of the 46 *Eca* isolates was amplifie using PCR that produced the expected 690-bp band size when ECA1f and ECA2r were used (Fig. 2). Digestion of the PCR amplification products with restriction enzymes *Sau*3AI, *Hpa*II, *Hae*III, and *Alu*I for the tested bacterial isolates formed different fragment patterns (Fig. 3). Digestion with *Sau*3AI gave fragment sizes of 447, 126, 71 and 50-bp, *Hpa*II gave fragment sizes of 251, 282, 316, 355, 374 and 398-bp, *Hae*III gave fragment sizes of 126, 158, 199, 224 and 478-bp, while there was no digestion using *Alu*I enzyme.

Based on the genetic distances, isolates were clustered in five different groups as shown in figure 4. The first group included only one isolate (Rm80) where the digestion of the amplified PCR products of the 690-bp band resulted in bands formation of (447, 126, 71 and 50-bp); (251 and 374-bp); and (199 and 478-bp) using *Sau*3AI, *Hpa*II and *Hae*III enzymes respectively. In the other hand, the second group included the isolates (Ta59, Ta60, Ta61, Ta62, Ta63, Ta64, Ta65 and Ta66), these isolates produced similar bands (447, 126, 71 and 50-bp), (282 and 398-bp), and (126, 158 and 224-bp) using *Sau*3AI, *Hpa*II and *Hae*III restriction enzymes, respectively.

Isolates J1, J3, J4, J5, J6, J7, J8, J9, and J10 were clustered in group number 3. These isolates produced similar bands (447, 126, 71 and 50-bp); (282 and 316-bp); and (126,158 and 224-bp) using *Sau*3AI, *Hpa*II and *Hae*III restriction enzymes, respectively. Group number 4 included the isolates Rm71, Rm72, Rm74, Rm75, Rm76, Rm77, Rm78, Rm79, Rb24, Rb25, Kr26, Kr27, kr28, MA85, MA86, MA88, MA89, MM33, MM34, MM35, MM37, and MM41. All these 22 isolates produced the digested bands (447, 126, 71 and 50-bp); (251 and 374-bp); and (126, 158 and 224-bp), resulted from using *Sau*3AI, *Hpa*II and *Hae*III restriction enzymes, respectively (Fig. 4).

Finally, the isolates (Ka29, Ka30, D15, D16, D20 and D23) were fallen in the fifth group. The PCR amplified product of the 690-bp of these isolates resulted in bands formation of (447, 126, 71 and 50-bp) (251 and 355-bp); and (126,158 and 224-bp); when this region was digested with *Sau*3AI, *Hpa*II and *Hae*III restriction enzymes, respectively. The genetic similarities between the 46 *Erwinia carotovora* subsp. *atroseptica* isolates ranged from 1.00 between isolates within each group to 0.471 between group 1 and groups (2 & 3). Group 1 showed a genetic similarity of 0.60 with all other groups. It is showed a genetic similarity of 0.706 and 0.589 with group 4 and 5, respectively. Equal genetic similarity of 0.889 was observed between group 2 and 3, and between group 4 and 5. The genetic distance between groups (2 & 3) and groups (4 & 5) was 0.778 (Fig. 4).

Detection of *Eca* from naturally infected- and symptomless-potato plant tissues: Results of direct isolation on Logan's medium from symptomless potato tubers and stem samples are presented in Table (3). The results showed that *Eca* was not recovered from the symptomless potato stem samples, when their suspension was streaked on Logan’s medium plates, while the 690-bp typical for Eca was detected in 8% of the DNA extracted from the same symptomless potato stem samples amplified with PCR and horizontally separated by gel electrophoreses (Fig. 2). The results show that *Eca* was recovered only in 4% of the symptomless potato tuber samples when their suspension was streaked on Logan’s medium plates, while the 690-bp was detected in 32% of the DNA extracted from the same symptomless potato tuber samples amplified with PCR and horizontally separated by gel electrophoreses (Fig. 2).

Fig (1): Diseased potato samples infected with potato blackleg.

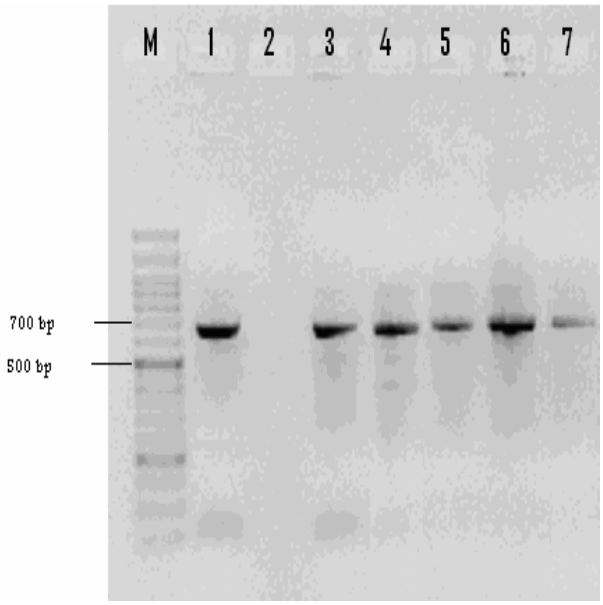

Fig (2): PCR-amplified *Eca* DNA (690-bp band) detection by gel electrophoresis: Lane (M), 100-bp molecular size marker, lane 1, Positive control using DNA extracted from bacterial culture of reference *Erwinia carotovora* subsp. *atroseptica,* Lane 2: water control, Lanes 3 and 4 for Rb24 and Rb25 isolates and Lanes 5-7 for Kr26, Kr27 and Kr28 isolates.

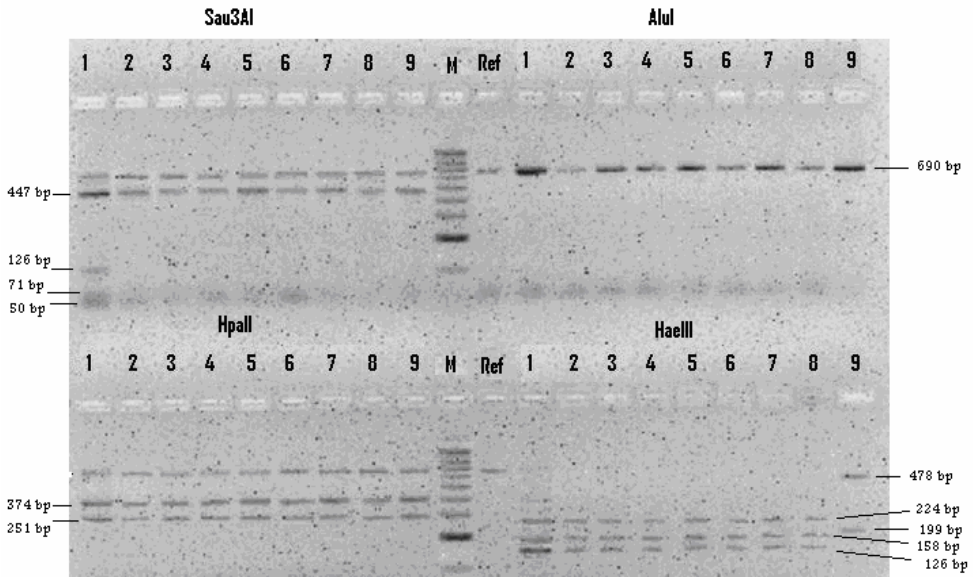

Fig (3): Restriction pattern of Rum *Eca* isolates for 690-bp band after digestion with (*Sau*3AI, *Alu*I, *Hpa*II *and Hae*III) enzymes. Lane M, 100-bp molecular size marker, Lane Ref, uncut PCR products (690-bp), Lane 1- 9 for (Rm 71, Rm 72, Rm 74, Rm 75, Rm76, Rm 77, Rm 78, Rm 79 and Rm 80) digested by *Sau*3AI, *Alu*I, *Hpa*II *and Hae*III enzymes.

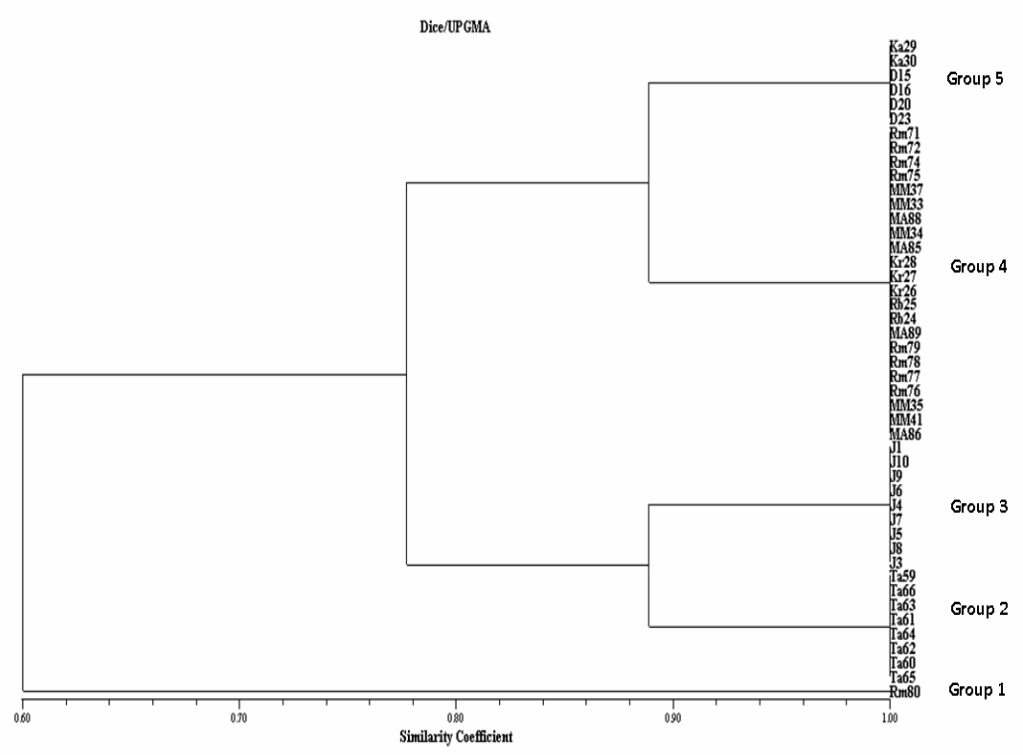

Fig. (4): Dendogram showing genetic relationships between the 46 isolates of *Erwinia carotovora* subsp. *atroseptica* based on PCR-RFLP analysis of the 690-bp band. The tree was constructed by using the UPGMA method.

Table (3): Sensitivity between different tests *i.e.* direct isolation on Logan's media and versus detection of the 690-bp DNA band with PCR.

a. From blackleg naturally infected potato tissue samples			
Method	**Number of tested samples**	**Number of positive result**	**Percentage**
PCR	50	44	88%
Logan's media	50	15	30%
b. From symptomless potato stem samples			
PCR	25	2	8%
Logan's media	25	0	0%
c. From symptomless potato tuber samples			
PCR	25	8	32%
Logan's media	25	1	4%

Discussion

Field trips to potato-growing areas revealed that blackleg disease is spreading in all surveyed areas including Jordan Valley (Al-Karama, Ashshuna Al-

Janubiyya, Muthallath Al-Misri, Deir Alla, Dirar, and Kurayyima), Jerash (Tawahin Al-Udwan), Rum, and Al-Mudawwara. Disease incidence, during sample collection, ranged from 5% up to 28% for Madaba and Al-Mudawwara locations respectively. Different sub species of *Erwinia* were reported to cause blackleg like symptoms *i.e. Erwinia carotovora* subsp. *carotovora* (*Ecc*) and *Erwinia chrysanthemi* (*Ech*) and *E. carotovora* subsp. *atroseptica* (Degefu *et al*., 2006).

The reaction, of the 46 obtained bacterial isolates those isolated from diseased potato samples and collected from different locations was recorded for biochemical, physiological and nutritional tests. Reaction results were found to be similar and identical to the reaction of the reference culture of *Erwinia carotovora* subsp. *atroseptica* (ATCC 15713). Schaad *et al*., (2001), Yahiaoui-Zaidi *et al*., (2003). This is confirming that *Erwinia carotovora* subsp. *atroseptica* is the causal agent of potato black leg in Jordan (Al-Masa'deh and Khlaif, 2003).

In this study, all the 46 bacterial isolates (*Eca*) produced the expected DNA band (690-bp) in PCR amplification. This result was in agreement with De Boer and Ward (1995) who used ECA1f (5'CGGCATCATAAAAACACG-3') and ECA2r (5'GCACACTTCATCCAGCGA-3') that specifically amplified a 690-bp DNA fragment of *E. carotovora* subsp. *atroseptica*, the causal agent of potato blackleg, but not other *E. carotovora* subspecies.

Our results showed that *Eca* was isolated on Logan's medium from 30% of naturally infected potato plant tissue, at the same time 88% of the DNA-extracted samples produced the 690-bp amplification by PCR. While in the case of symptomless potato tubers and stem samples, *Eca* was not recovered from the suspension of stem samples on Logan's media plates, and the 690-bp band was detected in 8% of their DNA extraction using PCR, and *Eca* was recovered in 4% of the symptomless potato-tuber samples on Logan's media plates, while the 690-bp was detected in 32% in the DNA-extracted samples using PCR. These results are in agreement with Frechon et al., (1998) who found that PCR was the most sensitive method for *Eca* detection (88%), followed by enrichment ELISA (72%) and normal ELISA, which was the least sensitive method (30%).

Using PCR was found to be more efficient in detecting *Eca* (Table 3) in comparison to isolation on Logan's medium and identification by biochemical, physiological and nutritional tests. The efficiency of PCR in detection 690-bp band could be related to that; 690-bp region could be easily detected from plant material without isolation from the expected healthy and infected plant parts within a short time, and the bacterial DNA sequence revealing for *Eca* can be amplified from viable, non-culturable, transformed plant cell. However, because *Eca* has a latent infection PCR could be a beneficial tool for detecting latent infection of *Eca* especially in imported potato-tuber seeds before distributing to the farmers.

Direct isolation results are in agreement with those of Toth *et al*., (2001) who reported that visual disease symptoms are not always sufficient to make an unequivocal identification of the pathogen latent infection. Thus, to ensure that a correct diagnosis is made and that steps are taken correctly toward reducing disease spread, identification systems are required. This can be achieved by using detection systems that identify the pathogen directly from plant samples like PCR technique. The results of PCR-RFLP of the 690-bp digestion band with (*Sau*3AI, *Alu*I, *Hpa*II *and Hae*III) enzymes, which obtained with ECA1f and ECA2r primers, indicated the occurrence of genetic relationship among the tested *Eca* isolates. The tested isolates from the same collected region were found to be identical,

since the genetic distance between them was found to be (0.0) as the same banding patterns were formed when their 690-bp bands were digested with the four different restriction enzymes (Fig. 4). These results are in agreement with Yahiaoui-Zaidi *et al*., (2003), where molecular fingerprints showed low genetic variability within *Eca* strains using PCR-RFLP after analysis of the restriction patterns of the PCR products obtained with the Y1 (5'-TTA CCG GACGCC GAG CTG TGG CGT-3') and Y2 (5'-CAG GAA GAT GTC GTT ATC GCG AGT-3') primers. This result could reflect the adaptation of *Eca* to local environmental conditions (Yahiaoui-Zaidi *et al*., 2003).

Our RFLP results agreed with the results of De Boer and McNaughton, (1987) that showed that 13 strains from *Eca* belong to only two specific PCR-RFLP groups. And the low variability in this subspecies is in agreement with serological studies that indicated the presence of only a few serogroups (De Boer *et al.*, 1979). Moreover, Costa *et al*., (2006) found low molecular variability between 10 tested isolates of *Eca* collected from Portuguese fields.

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الكشف عن تعريف مرض الساق السوداء على البطاطا (*Erwinia carotovora subsp. atroseptica* (Van Hall, 1902)) باستخدام الـ RFLP-PCR

رياض الزمر[1]، حامد خليف[1]، ومهند العكش[2]

ملخـص

في هذه الدراسة، تم تسجيل انتشار مرض الساق السوداء في البطاطا ودراسته في مناطق زراعة البطاطا المختلفة في الأردن شملت: وادي الأردن (الكرامة، الشونة الجنوبية، مثلث المصري، المعدي، دير علا، ضرار، كريمة)، عمان (اليادودة)، مأدبا (أم العمد)، جرش (طواحين العدوان)، معان (رم، المدورة)، حيث تم عزل وتعريف 46 عزلة من البكتيريا *Eca*، وهي المسبب المرضي لمرض الساق السوداء في البطاطا من المناطق المختلفة .

استخدم تفاعل البلمرة المتسلسل (PCR) في تضخيم حزمة الـ DNA ذات الحجم (690-bp) لتأكيد وجود بكتيريا *Eca* بعد عزلها وتمت مقارنة هذه الطريقة مع طريقة الكشف عن البكتيريا بالعزل المباشر على بيئة لوغان الغذائية (Logan's medium) من سيقان ودرنات البطاطا التي تظهر عليها الأعراض، وأخرى لا تظهر عليها الأعراض، حيث أشارت النتائج إلى أن عزل المسبب المرضي تم في 30% من عينات البطاطا المصابة، بينما تم الكشف عن 690-bp band في 88% من مستخلصات الـ DNA للعينات نفسها باستخدام الـ PCR. كما تم إجراء الكشف عن وجود المسبب المرضي في عينات نباتات البطاطا لا تظهر عليها أعراض المرض ولم يعزل المسبب المرضي مباشرة على البيئة، بينما تم كشف الـ 690-bp band في 8% من العينات. وعند إجراء الكشف عن وجود المسبب المرضي في درنات بدلاً من سوق لا تظهر عليها أعراض الإصابة تم عزل *Eca* من 4% من هذه العينات على بيئة لوغان، في المقابل تم الحصول على 690-bp band في 32% من مستخلص DNA للعينات نفسها. وعليه؛ فإن استخدام تفاعل البلمرة المتسلسل هو الاكثر حساسية للكشف عن هذا المرض من أجل الكشف عن التباين الوراثي بين الـ 46 عزلة من الـ *Eca* استخدمت طريقة RFLP-PCR، حيث تم هضم الحزمة الـ DNA ذات الحجم (690-bp) بوساطة استخدام الأنزيمات *Alu*I, *Hae*III, *Hpa*II and *Sau*AI وقسمت العزلات إلى خمس مجموعات، وقد أظهرت النتائج أن هذه المجموعات ذات تباين وراثي قليل.

الكلمات الدالة: مرض، الساق السوداء، البطاطا.

[1] قسم وقاية النبات، كلية الزراعة، الجامعة الأردنية

[2] قسم البستنة والمحاصيل، كلية الزراعة، الجامعة الأردنية

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