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RAPD ANALYSIS OF SOME LOCAL *R. SOLANACEARUM* ISOLATES AND STUDY THE RELATIONSHIP BETWEEN POLYGALACTURONASE ACTIVITY AND INFECTION OF DIFFERENT POTATO CULTIVARS

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ABSTRACT: ***Seven bacterial isolates were isolated from infected potato tubers collected from different governorates of Egypt. Pathogenicity test of isolates on Spunta potato cultivar which was known to be highly susceptible to the infection by R. solanacearum showed that the isolates (Rs2, Rs5 and Rs3) differed in their virulence (high, moderate and weak), respectively. R. solanacearum produces several extracellular plant cell wall-degrading enzymes that are suspected to be virulence factors. The Seven isolates were used to determine the activity of Polygalacturonase (PG) enzyme. Rs2, Rs5 and Rs3 isolates showed high, moderate and low enzyme activity, respectively. These isolates were tested on potato cultivars Spunta, Kara and Nicola. Nicola cultivar was the most resistant, while Spunta was the most susceptible. Kara proved to be moderately susceptible. Using of six primers in RAPD-PCR showed clear differences among the seven isolates on the basis of amplified product band patterns. All primers showed polymorphic bands among all the isolates. The dendrogram generated showed that the examined isolates were classified into two main clusters, the isolates Rs1, Rs5, Rs6 and Rs7 in cluster A, while, the isolates Rs2, Rs3 and Rs4 in cluster B.***

Key words: ***Pathogenicity, RAPD, R. solanacearum, potato cultivars.***

INTRODUCTION

Ralstonia **(Pseudomonas)** ***solanacearum*** **is soil borne, gram-negative bacteria that can wilt more than 200 plant species in 50 families. Important hosts of** ***R. solanacearum*** **include tomato, potato, tobacco, geranium, and banana, and it routinely causes significant yield losses in commercial and subsistence agriculture world-wide.** ***R. solanacearum*** **normally invades plant roots via wounds or where secondary roots emerge, penetrates the xylem, and then, systemically colonizes the vascular system. Extensive colonization**

disrupts vascular function and the plants rapidly wilt and die. It is only during the later stages of the disease that nonwoody stem tissue is obviously macerated (Hayward 2000 and Schell 2000). Numerous studies have assessed the genetic diversity of *R. solanacearum* strains collected from diverse geographic areas and host species (Cook *et al.,* 1989). There are also studies on the phenotypic and genotypic variation of *R. solanacearum* populations within localized geographic regions (Dookun *et al.,* 2001; Horita and Tsuchiya 2001; Smith *et al.,* 1995).

***R. solanacearum* virulence factors are quantitative characters, each one adding a degree of pathogen aggressiveness (Roberts *et al.,* 1988). Many of the known virulence factors are secreted enzyme including an extracellular polysaccharide (EPS I), an endoglucanase, and three PGs (Denny and Baek 1991; Huang and Allen 1997 and Schell *et al.,* 1988). A consortium of three plant cell wall-degrading PGs apparently facilitates plant invasion by *R. solanacearum*. Although all three PGs hydrolytically cleave the pectic polymer, they have distinct reaction products. *PehA* (also known as *PglA*), an endo-PG (E.C. 3.2.1.82), cleaves the pectic polymer internally at random, releasing large oligomers, mainly trimers of galacturonic acid (galUA) (Allen *et al.,* 1991 and Roberts *et al.,* 1988). *PehB*, an exo-poly-α̃-D-galacturonosidase (E.C. 3.2.1. .15), and *PehC*, an exo-PG (E.C. 3.2.1.67), cleave the pectic polymer at the nonreducing end of pectin, releasing dimers and monomers of galUA, respectively (Huang and Allen 1997).The optimum polygalacturonase activity was at pH 5.5 (Ofuya 1984).**

Random amplification of polymorphisms (RAPD) is a DNA polymerase assay based on the amplification of random DNA segments with single primers of arbitrary nucleotide sequence (Dobrowolski and O'Brien, 1993). RAPD-PCR produces DNA profiles of varying complexity depending on the primer and template used. Each amplification products is expected to result from the existence of two annealing sites in inverted orientations, 3' ends facing each other within the amplified distance. Polymorphisms could be caused by differences in nucleotide sequences at the priming sites (e.g. point mutation), or by structural rearrangements within the amplified sequence (insertion, deletions and inversions). Richard *et al*., (2009) developed a sensitive, selective and rapid protocol for detecting *R. solanacearum* from soil and plant tissues based on the integration of the rapid self-replicating ability of bacteriophages with quantitative PCR (q-PCR). Clain *et al.,* (2004) confirmed that the homogeneity between accessions regarding high bacterial wilt tolerance by the genetic homogeneity (0% polymorphism) was revealed using 168 RAPD-marker. Gover *et al.,* (2006) studied a total of 44 field isolates *in vitro* generated clones of *R. solanacearum* for genotypic diversity by RAPD technique. It was done using 30 primers and the isolates were identified as distinct genotypes at 70% similarity level.

The aim of this work is to the isolate, purify and identify some isolates of bacterial potato brown rot disease. Isolates were collected from diverse geographic regions (governorate) in Egypt, to determinate Polygalacturonase enzyme activity in *R. solanacearium* isolates. Furthermore study the susceptibility of the three potato cultivars (Spunta, Nicola and Kara) to the isolated *R. solanacearum* by detecting the relationship between polygalacturonse activity and infectivity

MATERIALS AND METHODS

1.Sample collection

Spunta, Nicola and Kara potato cultivars were obtained from the International Potato Center (IPC) Kafr El-Zayat, Egypt.

Five bacterial isolates were obtained from four infected potato cultivars (Kara, Diamont, Nicola and Spunta) Table (1). These potato tubers were collected from five governorates in Egypt; El-Monofiya (Rs1), El-Menia (Rs2), Kafr El-Sheik (Rs3), Alexandria (Rs4), El-Behiera (Abou-Homus (Rs5), Abou El-Matamir (Rs6) and kafr El-Dawar (Rs7).

Table (1): The infected potato tubers collected from the different governorates in Egypt

Governorates	Potato cultivars	Isolates
El-Monofiya	Nicola	Rs1
El-Menia	Spunta	Rs2
Kafr El-Sheik	Kara	Rs3
Alexandria	Diamont	Rs4
El-Behiera		
1.Abou-Homus	Kara	Rs5
2.Abou El-Matamir	Kara	Rs6
3.kafr El-Dawar	Spunta	Rs7

2. Isolation and purification of the causal organmism:

Oozes and discoloured vascular tissues of infected potato tubers were placed in sterile water, a loopful of bacterial suspension was streaked on tetrazolium agar medium (TZC) (Abo-El-Dahab and El Goorani, 1969). Media were composed of Peptone (5 g), beef extract (3 g), glycerol 20 ml, agar (15 g) distilled water 1 litre and 0.05%TZC (pH 7.0). Plates were incubated for 48 hr at 28 ^{o}C. The TZC medium was used in differentiating fluidal colonies of virulent isolates (Kelman, 1954). Both virulent and avirulent strains of *Ralstonia solanacearum* were routinely cultured on glycerol nutrient agar (GNA) medium at room temperature

3.Pathogenicity test:-

R. solanacearum **isolates were tested for pathogenicity on Spunta potato cultivar which was known to be highly susceptible. Severity of wilting was rated daily on the scale of He *et al*., (1983) as follow: 1 = no symptoms, 2 = one leaf wilted, 3 = two or three leaves wilted, 4 = four or more leaves wilted, and 5 = plant dead, the last reading was taken 5 weeks after inoculation.**

4. Determination of Polygalacturonase (PG) activity produced by *R. solanacearium* isolates.

4.1.Polygalacturonase (PG) enzyme preparation.

The isolates were grown in Nutrient broth (NB) medium supplemented with 0.2% polygalacturonic acid (PGA) as the sole carbon source for 48 h at 30^{o}C ±2. Bacteria were removed from cultures by centrifugation at 12000 rpm for 20 min at 4 ^{o}C (Universal 32 R centrifuge, Hettich-Zentrifugen, Germany). Ammonium sulfate crystals were added to supernatant to about 60 – 80% saturation and the mixture was stirred overnight at 4^{o}C. Precipitates were collected by centrifugation redissolved in minimal volumes of 0.05 M citrate-phosphate buffer pH 5.5 and dialyzed against the same buffer.

4.2.Polygalacturonase (PG) assays:

Polygalacturonase (PG) activitiy was determined using the thiobarbituric acid (TBA) method (Lei *et al.,* 1985a and Lei *et al*., 1985b) with some modifications. PG was assayed by adding the crude enzyme to tubes containing 2.5 ml 0.5% polygalacturonic acid in 0.05 M sodium acetate buffer, pH 5.5 and 0.1 M NaCl (Nasuno and Starr, 1966). The reaction mixture was incubated for 2 hrs at 30 ^{o}C and the reaction was stopped by heating the tubes at 100 ^{o}C for 10 min. After cooling at room temperature the reaction mixture was centrifuged at 12000 rpm for 10 min, 2 ml of the supernatant were mixed with an equal volume of TBA reagent (Ayers *et al.,* 1966) and heated at 100 ^{o}C for 30 min. The activity was determined by the increase in the absorbance (optical density) at 515 nm (OD_{515}). Zero time reaction mixture containing active enzyme was used as control.

5. Susceptibility and resistance of different potato cultivars to *R. solanacearum* isolates under greenhouse condition.

The pathogenicity test of three isolates of *R. solanacearum* (*Rs2*, *Rs3* and *Rs5*) showed variation in pathogenicity ability, Rs2 (strong isolate), Rs5 (intermediate isolate) and Rs3 (weak isolate). Isolates were tested for pathogenicity on potato cultivars i.e. Spunta , kara and Nicola. Potato tubers of the three cultivars were surface sterilized with (1% sodium hypochlorite for five minutes, then washed with sterile water), and planted in plastic pots 15 cm diameter filled with sterile peat moss(one tuber per pot). When plant

reached 15-20 cm tall, stems were inoculated by forcing a sterilized needle 0.25 ml of bacterial suspension (10^9 cfu/ml (A600, 0.5) injected into the stems at height of 5 cm above the soil level (Prior and Steva, 1990). Inoculated plants were placed in a green house at 25 + 2ºC. Four replicates were used in the experiment and plants injected with sterile distilled water served as control. Severity of wilting was rated daily on the scale of (He *et al.,* 1983)

6.Random Amplified Polymorphic DNA (RAPD):

Six random primers each consist of 20 bases were used to differentiate and finger print tested the *R. solanacearum* isolates. The DNA primers sequences are illustrated in Table (2).

Table (2): List of primers (numbers and their nucleotide sequences) used in the RAPD-PCR analysis.

Primer number	Nucleotide sequence(5′ to 3′)
A9B10	GGA CTG GAG GTG GAT CGC AG
BAQ	GGT CTT GAA GTC GAG CGC AG
BAR	CCA GGC AAT TTC ATC AAG CC
BIC1	CAG CCC CCT CCA GCA CCC AC
I	ATG TGA CCC GCG TAG GCC GC
A9B4	GGT GAC GCA GGG GTA ACG CC

For RAPD analysis, PCR amplification was carried out in total volume of 25µl containing 2.5µl 10 x buffer, 2.5 µl 50 mM $MgCl_2$, 2.5 µl 4 mM dNTPs,7 µl 50 pmol primer,1 µl 10 ng of bacterial genomic DNA and 0.2 µl (5 units/ µl) Taq DNA polymerase (Promega Germany).

The following PCR programme was applied; initial denaturation at 95ºC for 5 min, 40 cycles of 95ºC for 1 min, annealing at 30ºC for 1 min and extension at 72ºC for 1 min finally, an extra final extension step at 72ºC for 10 min (Istock *et al*., 2001) Two µl of loading dye were added prior to loading of 10 µl per gel slot. Electrophoresis was performed at 100 volt with 0.5x TBE as running buffer in 1.5% agarose and then gel was stained in 0.5 µg/cm3 (w/v)

ethidium bromide solution and destained in deionized water. Finally the gel was visualized and photographed using gel documentation system. Presence and absence of RAPD bands produced from the use of six primers were scored visually from the resulting photographs.

6.1.Dendogram construction based on the RAPD-PCR band patterns

Data obtained by RAPD-PCR band patterns were scored for computer analysis on the basis of the presence and absence of the amplified products for each primer. A product present in a bacterial isolate was designated (1) and when absence it was designated (0) after excluding common bands. Pair-wise comparison of bacterial isolates, based on the presence or absence of unique bands was used to generate similarity coefficients according to Jaccard (1980). The similarity coefficients were used to construct a dendrogram by UPGMA (Un-weighted pair-Group Method with Arithmetical Averages) program.

RESULTS

1. Pathogenecity test:

Pathogenicity test of the seven isolates of *R. solanacearum* was performed on Spunta potato cultivar which was known to be highly susceptible. Data presented in Fig (1) show that all isolates were virulent, however the isolates No. *Rs2* and *Rs6* were highly virulent, while the isolates No. *Rs7, Rs1* and *Rs5* were moderately virulent and the isolates No. *R3* and *Rs4* were weakly virulent according to He *et al*., (1983).

Fig. (1): Response of Spunta potato cultivar to infection by *R. solanacearum* isolates

2. Determination of polygalacturonase (PG) enzyme activity in *Ralstonia solanacearum* isolates.

The data illustrated in Fig (2) showed that the all isolates of *R. solanacearium* produce (PG) enzyme, the maximum enzyme activity was observed with El-Menia isolate *Rs2* followed by Abou El Matameer isolate *Rs6* (virulent isolates), while the minimum enzyme activity was observed in case of Kafr El-Sheik isolate *Rs3* followed by Alexandria isolate *Rs4* weakly virulent isolates). But, Abou-Homus isolate *Rs5* showed moderate activity followed by El Monifyia isolate *Rs1* (moderately virulent isolates). Results of the production and activity of polygalacturonase enzyme showed a strong relation to pathogenicity.

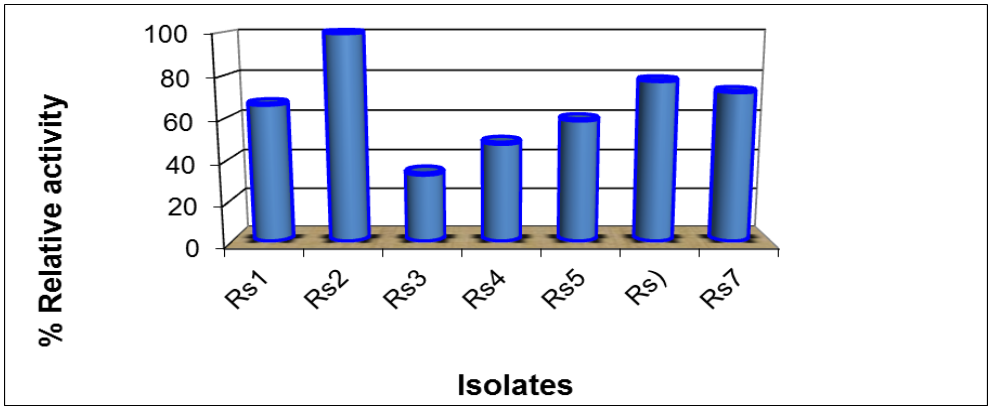

Fig. (2): Activity of Polygalcturonase (PG) enzyme produced by the seven isolates of *R. solanacearum.*

3. Susceptibility and resistance of different potato cultivars to *R. solanacearum* isolates under greenhouse condition

The isolates of *R. solanacearum* (*Rs2*, *Rs 5* and *Rs3*) which differed in their virulence were chosen depending on data from Fig (2). These isolates were tested on potato cultivars Spunta, Kara and Nicola. Data shown in Fig (3) revealed different susceptibility of potato cultivars. Nicola cultivar was the most resistant, while Spunta cultivar was the most susceptible to the infection. On the other hand, Kara cultivar proved to be moderately susceptible to the infection with three tested bacterial isolates according to He *et al*., (1983).

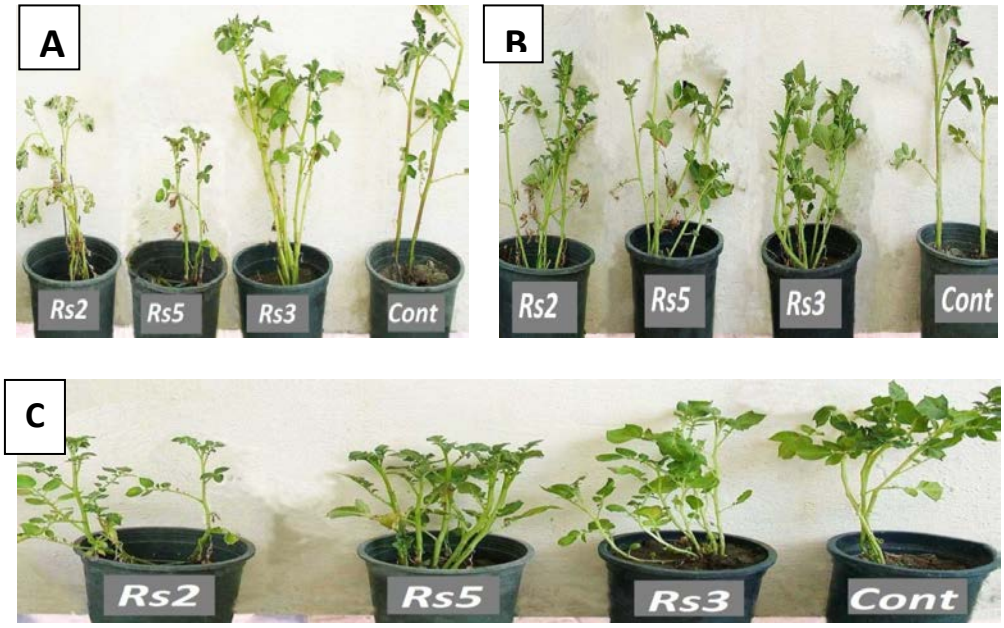

Fig. (3): Artificially inoculated aerial stems of(A) Spunta (B)Kara (C) Nicola with *R. solanacearum* 19 days after inoculation. Cont=control, *Rs2, Rs3, Rs5* three isolates of *R. solanacearum*

4. Fingerprinting of isolates of *R. solanacearum* using Random Amplified Polymorphic DNA (RAPD)

Using of the RAPD-PCR analysis with six random primers the result showed clear difference among the seven isolates on the basis of amplified produced band patterns observed with each primer (Fig.4). Results indicated that 256 amplicons were produced by all primers. The number of amplified products produced by each primer varied from 36 with primer BAQ to 52 with primer A9B4. The amplification profiles with the primers BAR, A9B10, BICI and I are shown in Fig (4) revealing that all of these primers succeeded to give polymorphic patterns among all the isolates. Also, high similarity was observed between *Rs3* and *Rs4* isolates.

4. 1.Cluster analysis of RAPD results

The RAPD band patterns were analyzed using UPGMA method to construct a similarity matrix (Table 3 and Fig 5) and to generate a dendrogram indicating the relationship between the seven examined isolates. We could classify the tested isolates on analysis of RAPD to two main clusters. Cluster A included two sub- clusters, sub-cluster A1 was

divided into two groups, group1 which include *Rs1* and *Rs5* isolate, group2 which include *Rs6* isolate. Sub-cluster A2 include *Rs7* isolate. Moreover cluster B was divided into two sub-clusters, sub-cluster B1 include *Rs2* and sub- cluster B2 include *Rs3* and *Rs4* isolates.

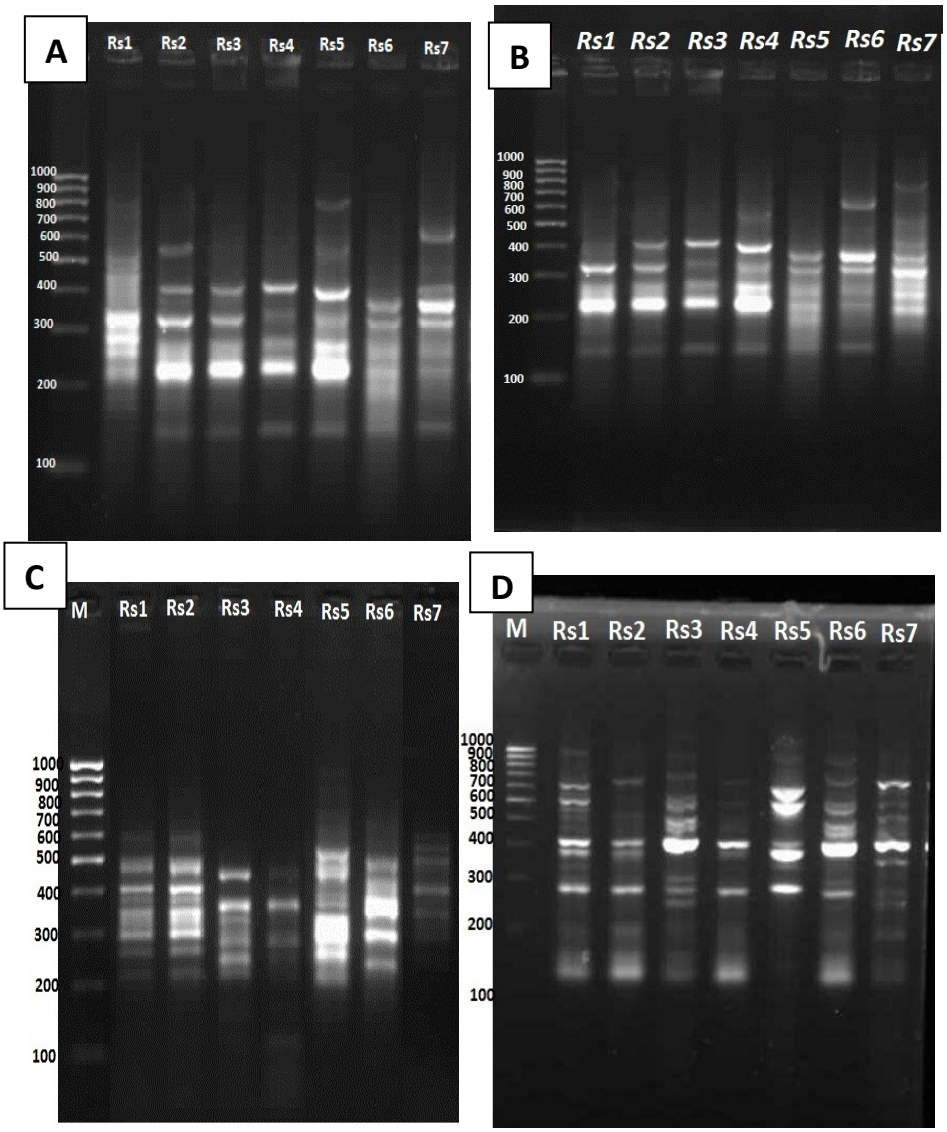

Fig.(4): RAPD-PCR using primer A (A9B10), B (BICI) , C (BAR) and D (I), M: DNA marker. Lanes *Rs1, Rs2, Rs3, Rs4, Rs5, Rs6* and *Rs7* are *R. solanacearum* isolates

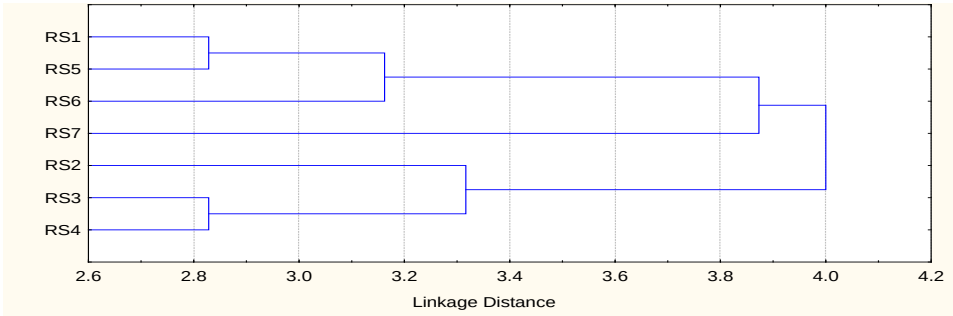

Fig.(5): Dendrogram obtained by clustering (UPGMA method) based on the band pattern obtained by the RAPD-PCR analysis for seven isolates according to Jaccard index.

Table (3): A similarity matrix, in percentage, among the examined bacterial isolates based on RAPD band pattern analysis and Jaccard index.

	Rs1	Rs2	Rs3	Rs4	Rs5	Rs6	Rs7
Rs1	**100**	**60**	**58**	**60**	**72**	**68**	**61**
Rs2		**100**	**67**	**67**	**59**	**60**	**60**
Rs3			**100**	**72**	**60**	**60**	**58**
Rs4				**100**	**60**	**60**	**60**
Rs5					**100**	**68**	**61**
Rs6						**100**	**61**
Rs7							**100**

DISCUSSION

To lessen the huge losses *R. solanacearum* causes in subtropical and tropical areas, the rapid identification of the pathogen is the most important for disease management. To our knowledge, there was no specific and sensitive real-time PCR assay detecting directly the pathogen *R. solanacearum* from samples of soils and plants without culture, although many related methods and marker genes were reported Kang *et al.,* 2007; Kutin *et al.,* 2009; Poussier *et al.*,2000 and Thammakijjawat *et al.*, 2006. Isolates of *R. solanacearum* were isolated from infected different potato cultivars tuber from different locations in Egypt.

The results obtained from pathogencity test on aerial stems of Spunta cultivar indicated that all isolates of *R. solanacearum* were virulent, however

the isolates No. *Rs2, Rs5 and Rs3* were highly, moderately and weakly virulent, respectively.

The isolates are able to produce polygalacturonase (PG) enzyme. These findings are in agreement with several studies (González & Allen, 2003, Schell, 2000). The Optimum PG activity was observed at pH 5.5. These findings were in agreement with several studies where a pH optimum of 5.5 for PG from *E. carotovora subsp. carotovora* (Saarilahti *et al.,* 1990) and *P. solanacearum* (Ofuya,1984). The results obtained from inoculation tested performed on aerial stems of three cultivars of potatos (Nicola, Spunta and Kara) indicated that the infection by *R. solanacearum* caused various degrees of wilting and that isolates differed in their virulence. Such results were in conformity with those reported by EL-Ariqi (2008) and EL-Gayar (2003) who showed that potato plants inoculated with *R. solanacearum* isolates exhibited partial wilting 19 days after inoculation. It was also noticed that Spunta was more susceptible, while Nicola was more resistant to the infection than other cultivars. Kara cultivar proved to be moderately susceptible to the infection with the tested isolates. In current study RAPD – PCR using six primers was used to study the differentiation between the seven isolates. Such data, agreed with those obtained by Gover *et al.,* (2006). All primers produced high polymorphism among the studied isolates. The dendrogram generated indicated that the examined isolates were classified into two main clusters, (Cluster A and Cluster B). In General the results showed that the isolates differed in their virulence on potato cultivars, also differed in the PG enzymatic activity and the isolates different in their genotype.

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استخدام تكنيك ال RAPD لتحليل عزلات رالستونيا سولانا سيرم المحلية و دراسة العلاقه بين نشاط انزيم البولى جالاكتيورنيز والقدرة على العدوى لاصناف بطاطس مختلفة

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الملخص العربى

تم عزل سبع عزلات من بكتريا العفن البنى فى البطاطس (رالستونيا سولانا سيرم)) من درنات بطاطس مصابه من محافظات مختلفة فى مصر.

تم إختبارالسبع عزلات لمعرفة قدرتها المرضيه على صنف حساس للمرض وهو سبونتا اظهرت كل العزلات قدرة مرضيه حيث اوضحت النتائج ان العزلات Rs2 و Rs5 و Rs3 اختلفت فى قدرتها المرضيه حيث تتدرج من شديده إلي متوسطه إلي ضعيفة القدرة المرضية على التوالى.

تفرز هذه البكتيريا انزيم البولىجالاكتيورنيز الذى تقوم بتكسير الجدر الخلوية والتى تعتبر من العوامل المساهدة على احداث المرض حيث اختبرت قدرة السبع عزلات على انتاج هذا الانزيم المحلل للبكتين فى الجدر الخلوية للنبات وتم تقدير نشاطة وتبين ان كل العزلات تنتج الانزيم و اظهرت العزلة Rs2 اعلى نشاط للانزيم بينما العزلة Rs3 اقل نشاط للانزيم بينما العزله Rs5 اظهرت نشاط متوسطا.

هذه العزلات الثلاثة اختبرت على ثلاث أصناف من البطاطس هى سبونتا وكارا ونيكولا ووجد ان أكثر الأصناف مقاومة هو صنف نيكولا والصنف سبونتا هو أكثر الأصناف حساسية بينما اظهر الصنف كارا مقاومة متوسطة .

بأستخدام تكنيك التضاعف العشوائي للمشابهات الوراثيه (RAPDS) تم التفرقه بين العزلات البكتيريه باستخدم ستة بوادئ عشوائيه حيث أوضحت النتائج ان هناك اختلاف بين العزلات على اساس عدد الحزم الناتجة وان كل البوادى اعطت اختلافات بين العزلات.

تم دراسه شجره النسب التى عملت وضعت للعزلات المختبره وانقسمت العزلات الى مجموعتين أساسيين. حيث العزلات Rs1 و Rs5 و Rs6 و Rs7 تقع فى مجموعه ا بينما العزلات Rs2 و Rs3 و Rs4 تقع فى مجموعة ب.