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# Use of the RAPD-PCR Technique to Fingerprint of *Ralstonia solanacearum* and Its Phages

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BROWN ROT disease of potato is an important plant disease. It leads to significant decreases in potato yield in Egypt and other parts of the world. Therefore, the focus of this work aimed was an analysis of the causal agent of this disease *(Ralstonia solanacearum)* and its phages (from the rhizosphere of potato plants in Egypt) that infect an avirulent strain of this bacterium. The approach taken was to use random amplified polymorphic DNAs (RAPD) by the polymerase chain reaction (PCR) technology. Five decamer oligonucleotide primers OP-A13; OP-B02, OP-B03, OP-B08 and OP-B09 were used in this study to differentiate between the two bacterial isolates virulent (WRC1) and avirulent (DR1) isolates of *R. solanacearum* and three lytic phages growing on avirulent strains of this pathogen (RSP1, RSP2 and RSP3). Statistical analysis of RAPD-PCR polymorphisms results revealed a degree of similarity with ratio of 92.7 % between these bacterial isolates. In the case of phages, the statistical analysis of RAPD-PCR polymorphisms revealed a degree of similarities with ratio from 85.7 % to 94.7. Therefore, the study paid an attention for the use of RAPD-PCR technology as a new molecular tool for identification and classification of the bacteria as well as the phages.

**Keywords:** Brown rot disease, Potato, *Ralstonia solanacearum*, Phages, RAPD-PCR, Molecular markers and Identification.

Brown rot and wilt disease of potato is one of the most serious diseases that is widely distributed and causes crop damage in potato plantation in the tropical, subtropical, and warm temperature regions (Hayward, 1991; Smith *et al.*, 1995; Frey *et al.*, 1996; Poussier *et al*, 1999, Ramadan, 2000 and EL-Didamony *et al.*, 2002). Also, this disease found in cooler climatic regions of the world (Stead *et al.*, 1996). This disease causes economic loss of the potato production (Gunawan, 1987; Elphinstone, 1989 and Toth *et al.*, 1997) Moreover, this disease is a major reason why potato is withdrawn from the export market since the production affected by the disease is unuceptable; (Ramadan, 2000). This disease is caused by the pathogen *Ralstonia solanacearum* (synonym *Pseudomonas* or

*Burkholderia solanacearum*), which can infect plants other than potato. This pathogen can infect more than 200 plant species distributed in more than 50 botanical families (Hayward, 1994; Poussier *et al.*, 1999 and Salanoubat *et al.*, 2002). Some of these host plants have economic importance; such as tomato, tobacco, pepper, eggplant, groundnut and banana (Toth *et al.*, 1997).

Phages (bacteriophages or bacterial viruses) are obligate parasites to their bacterial hosts and potentially offer a way to control these diseases. There are a number of potential advantages of using bacteriophages to control infections; in particular, phages, which are highly specific for infection bacterium and thus are harmless to the crop plant that may be affected by these diseases. The initial dose of the phage can be low since the virus multiplies in the bacterial cells, releasing new phage particles on lysis. This process of enhancement of the number of phages should continue until all the bacteria have been destroyed. The problem of development of resistance can be reduced by concurrent administration of a number of different phages, each of which act on the same type of cells (Hanlon, 2001). The molecular study of *R. solanacearum* and their phages may help to understand these pathogens and how to control them. Moreover, molecular studies can be used to differentiate and classify the bacteria and phages. Therefore, this study was done to explore the use of the RAPD-PCR technique to fingerprint a virulent and an avirulent isolates of *R. solanacearum* and three phages to determine if it is possible to distinguish them using this technique.

## Material and Methods

*Bacterial strains*

A virulent and an avirulent culture (WRC1 and DR1) of *R. solanacearum* were isolated from infected tubers. These two isolates were identified as race 3 biovar 2 as reported by El-Didamony *et al.*, (2002).

*Phages*

Three lytic phages (RSP1, RSP2 and RSP3) growing on the avirulent strains (race 3 biovar 2) of *R. solanacearum* were used in this study. These three phages were isolated and identified by El-Didamony *et al.* (2003). They reported that all these three phages were monovalent; specific only to the avirulent isolates of *R. solanacearum* (race 3). Moreover, the two RSP1 and RSP3 belonged to group B and family Styloviridae while, RSP2 belonged to group B and family Podoviridae. The two phages (RSP1 and RSP3) gave clear plaques, while the phage RSP2 gave turbid plaques.

*R. solanacearum samples preparation*

The two isolates were grown in SPA broth medium (Jacobs and Gerstein, 1960). Fifty ml in 250-ml conical flask were inoculated separately with 5 ml of each of the two *R. solanacearum* strains and then were incubated at 28 ± 2°C for 24 hr on a rotary shaker (80 - 100 rpm), each culture was grown in duplicate. As control, a flask was left without inoculation.

*Extraction of nucleic acids from R. solanacearum strains*

Fifty ml of bacterial culture were suspended at 400rpm for 3min.The supernatants were decanted and the bacterial pellets were then resuspended in 20 ml of TE buffer containing SDS to a final concentration of 1%(Sambrook *et al.*, 1989).

*Extraction of phages DNA*

Phages DNA was extracted from three purified phage preparations based on the method of Sambrook *et al.*, (1989).

*Purification and determination of DNA concentration*

The purity and concentration of DNA extracts were determined as recommended by Brown (1990) using the ultraviolet absorbance spectrophotometer (Model Du-40 spectrophotometer Beckman). With a pure sample of DNA the $A_{260/280}$ is 1.8 – 2. The DNA concentration was then adjusted to 100 ng/µl for the molecular studies.

*III.A.4. Primer used for RAPD-PCR*

Five decamer oligonucleotide primers OP-A13; OP-B02, OP-B03, OP-B08 and OP-B09 from OPERON Technologies, Alameda, CA., kits A and B, were used for RAPD-PCR of *R. solanacearum* as well as phage isolates. The nucleotide sequences of the applied primers are presented in Table 1.

**TABLE 1. The nucleotide sequences of the primers used for RAPD-PCR analysis of *R. solanacearum* as well as its phages.**

| No. | Primer names | Sequences (5'----3') |
|---|---|---|
| 1 | OP-A13 | CAG CAC CCA C |
| 2 | OP-B02 | TGA TCC CTG G |
| 3 | OP-B03 | CAT CCC CCT G |
| 4 | OP-B08 | GTC CAC ACG G |
| 5 | OP-B09 | TGG GGG ACT C |

*RAPD-PCR*

RAPD-PCR was carried out according to the procedure given by Sambrook *et al.* (1989) using 100 ng genomic DNA template. The PCR amplification was performed in a Perkin-Elmer/DNA Thermal Cycler 480 (Norwalk, CT) for 35 cycles after initial denaturation for 4 min at 94°C. Each cycle consisted of denaturation at 94°C for 1 min, annealing temperature at 37°C for 2 min and extension at 72°C for 2 min. The final primer extension cycle was 5 min.

*PCR electrophoresis*

The amplified products were resolved by electrophoresis in 1.2 % agarose gel at 60 volts for 2.5 hr with TAE buffer (Sambrook *et al.* 1989). PCR products were visualized under UV light by staining gels with ethidium bromide (0.5 µg/ml) and photographed using a Polaroid camera.

*RAPD analysis*

Amplified products were visually examined and scored as presence (1) or absence (0) of bands of specific mobilities. The similarity coefficient (F) between isolates was defined by the formula of Nei and Li (1979). A dendrogram was derived from the distance by unweighted paired-group method, arithmetic mean (UPGMA) using the computer program package NTSYS 1.5 (Rohlf, 1990).

## Results and Discusion

*DNA fingerprinting analysis of R. solanacearum isolates*

Recently, new approaches to evaluate the diversity of bacteria using the polymerase chain reaction (PCR) have been developed (De Bruijn, 1993). Numerous PCR based procedures are routinely used to produce genomic fingerprints of prokaryotes. Using short primers of arbitrary nucleotide sequences, random amplified polymorphic of DNA (RAPD) has been used since it is a simple technique to detect polymorphisms that allow rapid identification and isolation of chromosome-specific DNA fragments (Judd *et al.*, 1993 and Nick & Lindstrom, 1994). Williams *et al.* (1990) were the first authors who described a new DNA polymorphism assay based on the amplification of random DNA segments with single primers of arbitrary nucleotide sequence called RAPD. Perret and Broughton (1998) used the targeted PCR fingerprinting (TPF) technique for rapid identification of *Rhizobium* strains as well as discriminating between *Rhizobium* species NGR234 and *R. fredii* USDA257 using PCR primers specific for the *nifH* and *recA* genes. Mohamed *et al.* (2001) used RAPD-PCR to differentiate between five *Streptomyces* species.

Fingerprinting studies of the two bacterial isolates based on RAPD-PCR analysis using five random oligonucleotides belonging to Operon kits A and B revealed that they were different (Table 2 and Fig. 1). No PCR products were observed in any of the negative controls, which indicated that the reaction mixtures were free from any strange DNA contamination. For each isolate, the number of amplified fragments differed with the different primers, which is expected, *i.e.* 20 and 21 out of 22 fragments amplified using 5 primers for the bacterial isolates WRC1 and DR1, respectively (Table 3).

The number and size of the amplified fragments differed from one isolate to the other when the same primers were used for PCR (Tables3 and 4). In addition, unique fragments 3963, 1245 and 3164 bp using the primers OP-A13, OP-B03 and OP-B08, respectively, distinguished the two *R. solanacearum* isolates. The use of OP-B02 and OP-B09 primers resulted in monomorphic fingerprints that were common for both isolates.

Statistical analysis of RAPD-PCR polymorphisms revealed the degree of similarity of the chromosomal DNA of the two bacterial isolates of *R. solanacearum* (virulent "WRC1" and avirulent "DR1" strains) with ratio of 92.7 % and a variation of 7.3 % between (Table 5). The variation of the chromosomal

DNA of the two *R. solanacearum* strains (virulent and avirulent strains) may be due to the virulence genes which described by Huang *et al.* (1993), Shell *et al.* (1994) and Chapman, & Kao (1998) and the complex network controlling the virulence genes which describing by Huang *et al.* (1995), Shell (1994) and Clough *et al.* (1997).

**TABLE 2. Scoring the a mplified fragments produced using OP-A13, OP-B02, OP-B03, OP-B08 and OP-B09 primers based on RAPD-PCR of DNA extracted from two *R. solanacearum* isolates (WRC1 and DR1).**

| Primer | *R. solanacearum* isolates | | | |
|---|---|---|---|---|
| | Fragment | $M_r$ (bp) | WRC1 | DR1 |
| OP-A13 | **F1** | 3936 | 1 | 0 |
| | F2 | 3116 | 1 | 1 |
| | F3 | 1965 | 1 | 1 |
| | F4 | 1652 | 1 | 1 |
| | F5 | 1150 | 1 | 1 |
| | F6 | 0874 | 1 | 1 |
| | F7 | 0569 | 1 | 1 |
| OP-B02 | **F8** | 2678 | 1 | 1 |
| | F9 | 1553 | 1 | 1 |
| OP-B03 | **F10** | 5566 | 1 | 1 |
| | F11 | 3237 | 1 | 1 |
| | F12 | 1756 | 1 | 1 |
| | F13 | 1245 | 0 | 1 |
| OP-B08 | **F14** | 3164 | 0 | 1 |
| | F15 | 2220 | 1 | 1 |
| | F16 | 1603 | 1 | 1 |
| | F17 | 1178 | 1 | 1 |
| | F18 | 0930 | 1 | 1 |
| | F19 | 0744 | 1 | 1 |
| OP-B09 | **F20** | 3746 | 1 | 1 |
| | F21 | 2660 | 1 | 1 |
| | F22 | 2031 | 1 | 1 |

**TABLE 3. Number of amplified fragments of two *R. solanacearum* isolates (WRC1 and DR1) based on RAPD-PCR analysis using five random primers.**

| Primers Used | Total amplified fragments | No. of amplified fragments | |
|---|---|---|---|
| | | *R. solanacearum* Strains | |
| | | WRC1 | DR1 |
| **OP-A13** | 7 | 7 | 6 |
| **OP-B02** | 2 | 2 | 2 |
| **OP-B03** | 4 | 3 | 4 |
| **OP-B08** | 6 | 5 | 6 |
| **OP-B09** | 3 | 3 | 3 |
| Total | 22 | 20 | 21 |

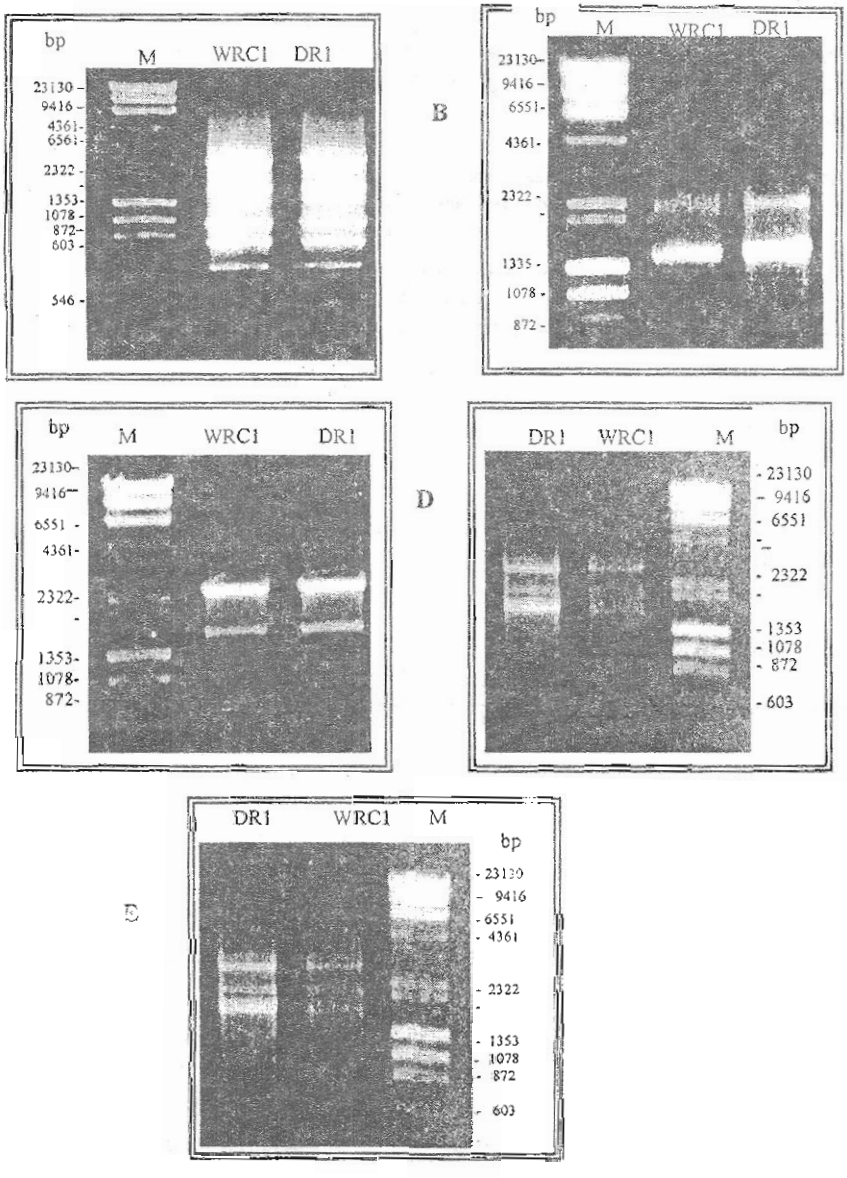


Fig. 1. 1%Agarose gel in TAE buffer stained with ethidium bromide showing RAPD-PCR polymorphism of DNA of *R. solanacearum* isolates (WRC1 and DR1) (Lanes 1 and 2, respectively) using A: OP-A13, B: OP-B02, C: OP-B03, D: OP-B08 and E: OP-B09 primers. M: DNA marker (Lambda *Hind* III/PhiX174 DNA marker, 23130, 6551, 2322, 1353, 872, 603 and 546 bp).

**TABLE 4. Number of polymorphic and unique fragments of two R. *solanacearum* isolates (WRC1 and DR1) based on RAPD-PCR analysis using five random primers.**

| Primers used | Total amplified fragments | $M_r$ (bp) of Unique band | Polymorphic and unique bands | |
|---|---|---|---|---|
| | | | *R. solanacearum* strains | |
| | | | WRC1 | DR1 |
| OP-A13 | 7 | 3936 | + | - |
| OP-B02 | 2 | * | * | * |
| OP-B03 | 4 | 1245 | - | + |
| OP-B08 | 6 | 3154 | - | + |
| OP-B09 | 3 | * | * | * |
| Total | 22 | 3 | 3 | 3 |

+ :Present - : Absent *: No unique bands were amplified.

**TABLE 5. Similarity between two *R. solanacearum* isolates (WRC 1and DR1) based on RAPD-PCR analysis using five random primers.**

| *R. solanacearum* Strains | Similarity between *R. solanacearum* (%) | |
|---|---|---|
| | WRC1 | DR1 |
| WRC1 | 100 | 92.7 |
| DR1 | 92.7 | 100 |

*DNA fingerprinting analysis of phages*

The RAPD-PCR analysis was successfully used to differentiate between the three phages (RSP1, RSP2 and RSP3) specific to avirulent strains of *R. solanacearum* based on the data represented in Table 6 and Fig. 2. The reaction mixture was free contamination, as no amplified bands were observed in any of the negative controls. Results in Table 6 showed that number of amplified fragments differed with different primers for each phage. On the other hand, the number and size of amplified fragments differed from one phage to the other for the same primer. Moreover, the phages were characterized by polymorphic and unique band(s) with the same primer used (Table 7). Whereas, five polymorphic and unique fragments 4097, 434 pb with OP-13 primer and 3263, 1481, 462 bp with OPB-08 primer were obtained. These fragments could be OP-A13, OP-B02, OP-B03, OP-B08 and OP-B09 successfully used as molecular markers for identification of these phages. However, some bands were common for all strains that considered as monomorphic bands. Statistical analysis of RAPD-PCR polymorphisms revealed a degree of similarities with ratio from 85.7 % to 94.7 % between the three phages under investigation (Table 8). This relationship was also apparent through the data of dendrogram shown in Fig. 3 as two phages RSP1 and RSP3 were to close to each other (94.7%, similarity). The two phages RSP1 and RSP2 had the lowest percent of similarity (85.7%).

The similarity ratios of the phages of this study are similar to the results of El-Didamony *et al.*, (2003). The highest similarity ratio was between the two phages RSP1 and RSP3 and was probably because both phages had similar clear plaque morphologies and were classified into the same family. The more dissimilar phage RSP2 belonged to another family and gave turbid plaques. The phages in this study were more similar than values reported by El-Helali (2001). The similarity in this study ranged from 85.7 to 94.7 %, while the phage similarity in El-Helali (2001) ranged from 40 to 70 %.

The RAPD-PCR technique provided a novel and effective method for distinguishing both *R. solanacearum* genomes as well as their phages according to the banding patterns. This is similar to Mohamed *et al.*, (2001) who applied the RAPD-PCR technique for the differentiation between five *Streptomyces* strains using ten different oligonucleotides as primers. El-Helali (2001) used the RAPD-PCR technique to fingerprint three rhizobiophages isolated from different soils. In addition, Barrangou *et al.*, (2002) used RAPD-PCR technique in order to differentiate between six bacteriophages active against *Leuconostoc fallax* that were isolated from industrial sauekraut fermentation.

**TABLE 6. Scoring the amplified fragments produced using OP-A13, OP-B02, OP-B02, OP-B03, OP-B08 and OP-B09 primers OP-B02 based on RAPD-PCR of DNA extracted from three phages (RSP1, RSP2 and RSP3) specific to avirulent strains of *R. solanacearum*.**

| primers | Fragment | $M_r$ (bp) | RSP1 | RSP2 | RSP3 |
|---|---|---|---|---|---|
| OP-A13 | **F1** | 4097 | 0 | 1 | 1 |
| | F2 | 3182 | 1 | 1 | 1 |
| | F3 | 1913 | 1 | 1 | 1 |
| | F4 | 1548 | 1 | 1 | 1 |
| | F5 | 1040 | 1 | 1 | 1 |
| | F6 | 0718 | 1 | 1 | 1 |
| | F7 | 0434 | 0 | 1 | 1 |
| OP-B02 | **F8** | 2971 | 1 | 1 | 1 |
| | F9 | 1715 | 1 | 1 | 1 |
| OP-B03 | F10 | 3076 | 1 | 1 | 1 |
| OP-B08 | **F11** | 4036 | 1 | 1 | 1 |
| | F12 | 3263 | 1 | 0 | 1 |
| | F13 | 1854 | 1 | 1 | 1 |
| | F14 | 1481 | 1 | 0 | 1 |
| | F15 | 1187 | 1 | 1 | 1 |
| | F16 | 0860 | 1 | 1 | 1 |
| | F17 | 0482 | 1 | 0 | 1 |
| OP-B09 | F18 | 3517 | 1 | 1 | 1 |
| | F19 | 2453 | 1 | 1 | 1 |
| | F20 | 2051 | 1 | 1 | 1 |

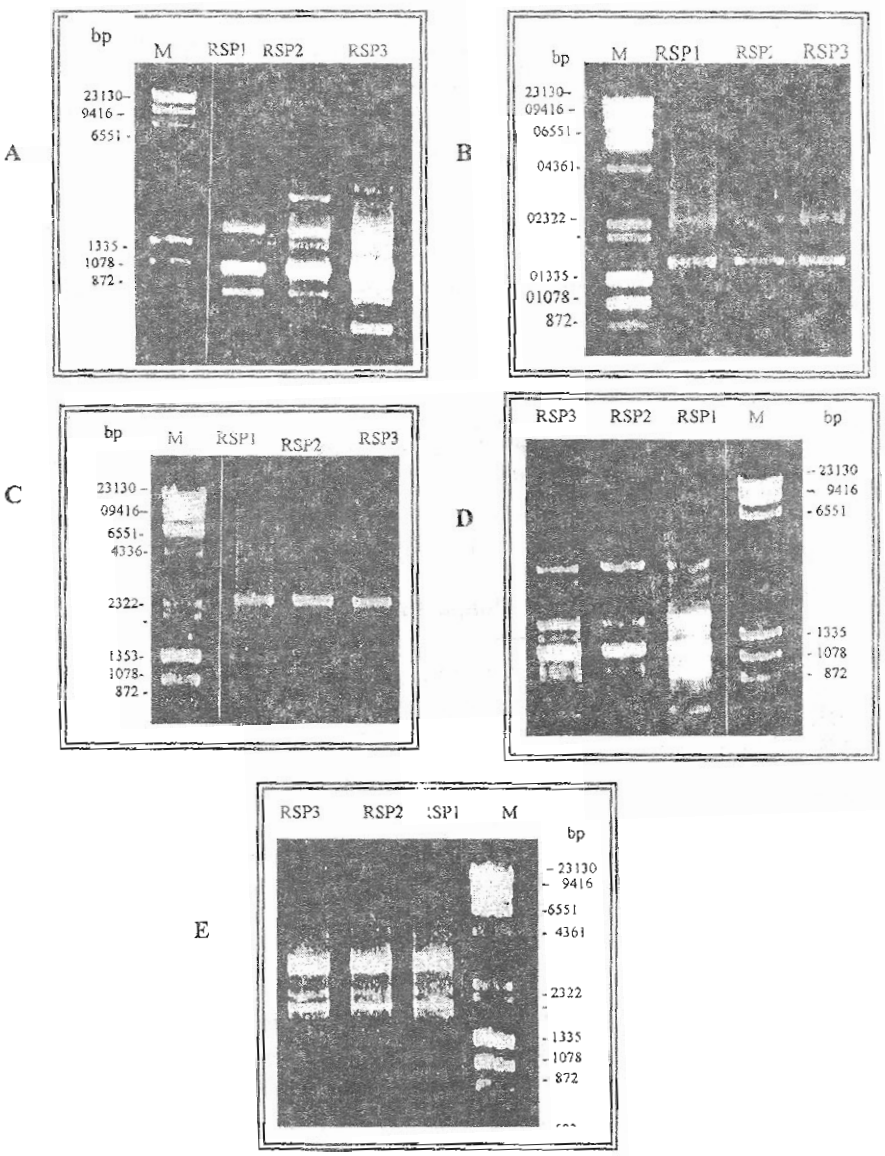


Fig. 2. 1%Agarose gel in TAE buffer stained with ethidium bromide showing RAPD-PCR polymorphism of DNA of three phages (RSP1, RSP2 and RSP3) specific to *R. solanacearum* (Lanes 1, 2 and 3, respectively) using A: OP-A13, B: OP-B02, C: OP-B03, D: OP-B08 and E: OP-B09 primers. M: DNA marker (Refer to Fig. 1).

**TABLE 7. Number of amplified fragments of three phages specific to *R. solanacearum* (RSP1, RSP2 and RSP3) based on RAPD-PCR analysis using five random primers.**

| Primers Used | Total amplified fragments | No. of amplified fragments | | |
|---|---|---|---|---|
| | | Phage isolates | | |
| | | RSP1 | RSP2 | RSP3 |
| OP-A13 | 7 | 5 | 7 | 7 |
| OP-B02 | 2 | 2 | 2 | 2 |
| OP-B03 | 1 | 1 | 1 | 1 |
| OP-B08 | 7 | 7 | 4 | 7 |
| OP-B09 | 3 | 3 | 3 | 3 |
| Total | 20 | 18 | 17 | 20 |

**TABLE 8. Number of polymorphic and unique fragments of three phages specific to *R. solanacearum* (RSP1, RSP2 and RSP3) based on RAPD-PCR analysis using five random primers.**

| Primer used | Total amplified fragments | $M_r$ (bp) of Unique band | Polymorphic and unique bands | | |
|---|---|---|---|---|---|
| | | | Phage isolates | | |
| | | | RSP 1 | RSP 2 | RSP 3 |
| OP-A13 | 7 | 4097 | - | + | + |
| | | 434 | - | + | + |
| OP-B02 | 2 | * | * | * | * |
| OP-B03 | 1 | * | * | * | * |
| OP-B08 | 7 | 3263 | + | - | + |
| | | 1481 | + | - | + |
| | | 462 | + | - | + |
| OP-B09 | 3 | * | * | * | * |
| Total | 20 | 5 | 5 | 5 | 5 |

+ :Present. -: Absent. * : No unique bands were amplified .

**TABLE 9. Similarity between three phage) RSP1, RSP2 and RSP3) specific to avirulent strains of *R. solanacearum* based on RAPD-PCR analysis using five random primers.**

| Phage isolates | Similarity between phages (%) | | |
|---|---|---|---|
| | RSP1 | RSP 2 | RSP 3 |
| RSP1 | 100 | 85.7 | 94.7 |
| RSP2 | 85.7 | 100 | 91.9 |
| RSP3 | 94.7 | 91.9 | 100 |

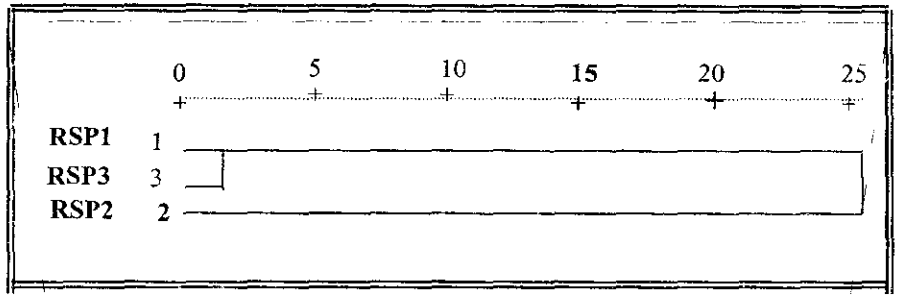


**Fig. 3. Dendrogram showing molecular relationship of the three phages (RSP1, RSP2 and RSP3) specific to avirulent strains of *R. solanacearum* based on RAPD-PCR analysis.**

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# استخدام تقنية الـ RAPD-PCR لعمل بصمة وراثية لبكتيريا *رالستونيا سولاناسيرام* واللاقمات الخاصة بها

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يعتبر مرض العفن البني في البطاطس من الأمراض النباتية المهمة التي تسبب نقصاً حاداً في محصول البطاطس في مصر ومناطق مختلفة من العالم، و قد استهدف هذا العمل إجراء دراسة على مستوى البيولوجيا الجزيئية للمسبب المرضى للعفن البني (*رالستونيا سولاناسيرام*) ، و للاقمات البكتيرية المتطفلة على السلالة الغير مميتة لهذه البكتيريا (المعزولة من التربة المحيطة بالنباتات المصابة) باستخدام تقنية الـ RAPD-PCR ، و قد تم استخدام خمس بادئات محدودة النيوكليتدات (OP-A13;OP-B02, OP-B03, OP-B08 & OP-B09) في هذه الدراسة للتمييز بين عزلة مميتة (WRC1) و أخرى غير مميتة (DR1) لهذا المسبب المرضي ، وبين ثلاث لاقمات (RSP1, RSP2 & RSP3) محللة للسلالات الغير مميتة لهذه البكتيريا ، ولقد أوضحت نتائج التحليل الإحصائي لنتائج الـ-RAPD PCRدرجة تشابه بين العزلتين البكتيريتين بنسبة ٩٢,٧ % ، بينما التشابه بين اللاقمات الثلاث يتراوح بين ٨٥,٧ % و ٩٤,٧ % .