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Implementation of 16S rRNA Gene and RAPD-PCR for Detection of *Ralstonia solanacearum* Isolates

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Six isolates of *Ralstonia solanacearum* were isolated from diseased potato tubers and three isolates of *Pseudomonas fluorescens* were recovered from the soil rhizosphere healthy potato plants. *R. solanacearum* isolates exhibited various degrees of wilting on aerial stems of three cultivars of potato. The antagonistic bacterium *P. fluorescens* had an inhibitory effect against *R. solanacearum* isolates. Identification of six isolates of *R. solanacearum* and two isolates of *P. fluorescens* were carried out using the total DNA isolated in polymerase chain reaction (PCR) analysis, where a region of the 16S rRNA gene had a molecular weight of 1550 bp was amplified by using universal primers. Eight primers (BAR, BAQ, 18, W, BIC 1, I, A9B4 and A9A10) were used in the differentiation between *R. solanacearum* as well as the *P. fluorescens* isolates. Primers produced considerable polymorphism among the studied isolates. The phylogenetic tree based on the similarity matrix was carried using the UPGMA method and revealed the existence of two main clusters, indicating the relationship among the eight examined isolates.

Keywords: Brown rot, *Ralstonia*, *Pseudomonas fluorescens*, RAPD-PCR and 16S rRNA gene.

Potato *(Solanum tuberosum* L.) is one of the major solanaceous crops. It is one of the most important vegetable crops in Egypt. Potato brown rot caused by *Ralstonia solanacearum* race 3, biovar 2 presents a major threat to the potato production worldwide, particularly in warm growing areas such as the Mediterranean region. Brown rot can be very destructive and cause considerable yield losses (Elphinstone, 2001; Kim *et al.*, 2008 and Denny, 2006). Characterization and differentiation of bacterial genome were studied by 16S rRNA technique and random amplified DNA technique by several primers (Araujo *et al.*, 2001). The results of biochemical identification were compared with 16S rRNA gene sequencing in 88 gram-negative bacteria by Wellinghausen *et al.* (2005). They found an agreement between the biochemical identification- results and 16S rRNA gene sequencing results so that identification of the bacterial cell by 16S rRNA considered as an excellent identification. *R. solanacearum* (race 3, biovar 2) could be reliably detected in 18 naturally infected potato tuber samples by 16S rRNA primers (Van der Wolf *et al.*, 2004). The polymerase chain reaction (PCR) allows biologists to sequence DNA from many species or individuals. DNA-based methods, particularly those exploiting the PCR, have enabled more precise understanding of

the genetic diversity of pathogens and of their phylogenetic relationships (Henson and French, 1993). Furthermore, PCR has provided a reliable method for the identification and detection of microorganisms. Biological control considered an alternative to the use of synthetic agrochemicals with the advantages of greater public acceptance, reduced environmental impact and involves the use of beneficial organisms, their genes, and or products, such as metabolites that reduce the negative effects of plant pathogens and promote positive responses by the plant (El-Mlegy, 2009). Some species of fluorescent Pseudomonas seem to be primary candidates for biological control because of their ability to synthesize a wide range of secondary metabolites, many of which possess antibacterial and antifungal activity (Mossialos *et al.*, 2002). The objectives of this study were: the isolation and identification of *R. solanacearum* affecting potato in the Behera governorate, Egypt, studying the susceptibility of different potato cultivars to *R. solanacearum* and the efficacy of certain isolates of *P. fluorescens* to control *R. solanacearum*.

Materials and Methods

1. Isolation of the associated bacteria:

Diseased potato tubers showing brown rot were collected from three different regions during the summer season, 2008. Tubers were firstly sterilized with 70% ethanol solution for 2 min, and then rinsed twice in sterile water. Sterilized tubers were cut across and loop-full of bacterial ooze exuding from the vascular bundle was suspended in 5 ml sterile water. A loop-full from each of the resulting suspension was streaked on glycerol nutrient agar medium (GNA) according to Abo El-Dahab and El-Goorani (1969). The triphenyl tetrazolium chloride (TTC) medium (GNA + 0.005% TTC) was used to differentiate the virulent from avirulent colonies according to Kelman (1954). Both virulent and avirulent isolates of *R. solanacearum* were purified through single colony isolation technique and routinely cultured and stored on GNA medium.

2. Identification of the causal bacterium:

2.1. Morphological and biochemical characterization of R. solanacearum isolates:

The morphological and biochemical characteristics of the isolated bacteria were identified using the standard recommended techniques (Cowan, 1974; Schaad, 1980; Fahy and Persley, 1983; Murray *et al.*, 1984 and Klement *et al.*, 1990).

2.2. Pathogenicity tests:

Virulence of the recovered isolates was tested on three potato cultivars, *i.e.* Ladybedfore, Diamont and Nicola. Surfaces of the aforementioned cultivars potato tubers were sterilized with 1% sodium hypochlorite for five minutes washed with sterile water and planted in plastic pots 15 cm diameter filled with sterile peat moss and clay. When plants reached 15-20 cm height, stems were inoculated by forcing a sterilized needle with a drop of bacterial suspension (10^9 cfu/ml A600, 0.5 into the stem 5 cm above the soil level (Prior and Steva, 1990). Plants were inoculated with three isolates and placed in a greenhouse at 25±2°C. Four replicates were used in the experiment and some plants were treated with sterile distilled water served as control. Severity of wilting was rated 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. Last reading was taken 3 weeks after inoculation.

2.3. Genotypic characterization:

2.3.1. Extraction of genomic DNA:

The bacterial genomic DNA was extracted using wizard Genomic DNA purification kit QIAGENE DNA purification kit (Germany), 1 ml of an overnight culture was added to 1.5 m1 micro-centrifuge tube, centrifugation at 16,000 x g for 2 minutes was carried out to pellet the cells and the supernatant was removed, 600 µl of Nuclei Lysis solution was added to the cell pellets and gently pipette until the cells are completely suspended, incubate at 80°C for *5* minutes was carried out to lyse the cells; then was cooled at room temperature, 3 µl of RNase solution was added to the cell lysate, then the tube was inverted 25 times to mix, sample was incubated at 37°C for 15-60 minutes, and then cooled to room temperature, 200 µl of Protein Precipitation Solution was added to the RNase-treated cell lysate, then it vortex vigorously at high speed for 20 seconds to mix the protein, precipitation solution with the cell lysate, the sample was incubated on ice for 5 minutes, sample was centrifuge at 13,000-16,000 x g for 3 minutes, transfer the supernatant containing the DNA to a clean 1.5 ml micro-centrifuge tube containing 600 µl of room temperature isopropanol, sample was mixed gently by inversion until the thread-like strands of DNA form a visible mass, sample was centrifuged at 13,000-16,000 x g for 2 minutes, carefully the supernatant was pour off and the tube was drain on clean absorbent paper. Also, 600 µl of 70% ethanol was added at room temperature and the tube was inverted gently several times to wash the DNA pellet, centrifuge at 13,000-16,000 x g for 2 minutes. Carefully ethanol was aspirate, drain the tube on clean absorbent paper was carried out to dry the pellet on air for 15 minutes, 100 µl of DNA rehydration solution was added to the tube and DNA was rehydrate by incubating at 65°C for 1 hour. The solution was periodically mixed by gently tapping the tube. Alternatively, DNA was rehydrated by incubating the solution overnight at room temperature or at 4°C. DNA was Stored at 28°C (Istock *et al.*, 2001).

2.3.2. Amplification of 16S rRNA gene:

According to (Hafez *et al.*, 2009) primers 1550 F and 1550 R, corresponding to the polymorphic region of *E. coil,* 16S rRNA conserved gene sequence, both of forward 5'AGAGTTTGATCMTGGCTCAG3' and reverse 5'TACGGYACCTTG-TTACGACTT3' were used to amplify approximately the 1550 bp of the 16S rRNA gene. The PCR reactions were performed in total volume 50µl containing approximately 2 µl 50 ng of bacterial genomic DNA, 5 µl buffer, 3 M dNT Ps ase, 4 µl 25 mM $MgCl_2$, 2 M primer F, 2 M primer R, 0.4 µl (5 unit µl) taq DNA polymerase (Promega Germany). The genomic DNA was subjected to PCR reaction with conditions, initial cycle with 95°C for 5 minutes and 34 cycles of 95°C for 1 minute, 58 for 1 minute and 72 for 10 min for final extension. Two µl of loading dye was added prior to loading of 10 µl per gel salt. Electrophoresis was performed at 100 volt with 0.5x TBE as running buffer in 1.5% agarose/0.5x TBE gels and then gel was stained in 0.5 µg/cm3 (w/v) ethidium bromide solution and distained in de-ionized water. Finally the gel was visualized and photographed using gel documentation system.

2.3.3. DNA sequencing for the amplified 16S rRNA gene:

2.3.3.1. Purification of PCR product:

QIA quick PCR purification kit (Qiagen. Germany) was used to purify the amplified products of 16S rRNA gene. Five volumes of the buffer PS were mixed with one volume of the PCR product and the mixture was loaded onto the PCR purification column placed into 2 cm^3 collection tube. The column was centrifuged at 9447 rpm for 1 min. After that, it was placed into a new collection tube in which 750 µl of PE buffer were pipetted. A centrifugation step at 9447 rpm for 10 min was conducted twice to ensure removal ethanol. The amplified fragment was eluted from the column using 50 µl sterile distilled water with a centrifugation step at 5590 rpm for 1 min.

2.3.3.2. Sequence alignment and phylogenetic analysis:

Pair wise and multiple DNA sequence alignment were carried out using CLUSTALW multiple sequence alignment programme version 1.82 (http://www.ebi.ac.uk/clustalw), (Thompson *et al.*, 1994). Bootstrap neighbour joining tree was generated using MEGA version 3.1 (Kumar *et al.*, 2004) from CLUSTALW alignment. Comparison with sequences in the Gene Bank database was achieved in BLASTN searches at the National Centre for Biotechnology Information site (http://ncbi.nlm.nih.gov). The Gene Bank accession numbers of I6S rRNA gene of bacterial isolates are listed in Table (1).

Table 1. The accession numbers of I6s rRNA gene of eight bacterial isolates in the Gene Bank

Isolation code*	Accession number
A	GU891463
B	GU891464
C	GU891465
D	GU891466
E	GU891467
F	GU891468
G	FJ9861004
H	FJ9861005

* A= *Rs* 1; B= *Rs* 2; C= *Rs* 3; D= *Rs* 4; E= *Rs* 5; F= *Rs* 6; G= *Pf* 1 and H = *Pf* 2.
Rs= *R. solanacearum* and *Pf*= *P. fluorescens.*

2.3.4. Random amplified polymorphic DNA (RAPD):

Eight random primers each consist of 20 bases were used to differentiate and fingerprint the isolates of *R. solanacearum* and *P. fluorescens* under study using genomic DNA sequences of all primers are illustrated in Table (2). For RAPD analysis, PCR amplification was carried out in total volume 25 µl containing 2.5 µl 10 x buffer, 2 µl 25 µl mM $MgCl_2$, 2 µl 2.5 µl mM dNTPs, 1 µl pmol primer,1 µl 50 ng of bacterial genomic DNA and 0.2 µl (5 units/ µl) Taq DNA polymerase (Promega Germany).

Table 2. List of primers name and their nucleotide sequences employed in the RAPD-PCR analysis

Number	Name	Sequence
1	Bic 1	CAG CCC CCT CCA GCA CCC AC
2	A9B4	GGT GAC GCA GGG GTA ACG CC
3	A9A10	GGA CTG GAG GTG GAT CGC AG
4	BAQ	GGT CTT GAA GTC GAG CGC AG
5	W	GAA ACG GGT GGT GAT CGC CC
6	18	CGC ATA GGA CCC GAT GCG AG
7	I	ATG TGA CCC GCG TAG GCC GC
8	BAR	CCA GGC AAT TTC ATC AAG CC

The following PCR programme was applied: initial denaturation at 95°C for 5 min, 40 cycles of 95°C for 1 min, annealing at 30°C for 1 min, finally, an extra final extension step at 72°C for 10 min (Istock *et al.*, 2001). Two µl of loading dye was added prior to loading of 10 µl per gel salt. 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 gel was stained in 0.5 µg/cm^3 (w/v) ethidium bromide solution and distained in deionised water. Finally the gel was visualized and photographed using gel documentation system. Presence and absence of RAPD bands produced from the use of eight primers were scored visually from the resulting photographs.

2.3.4.1. Dendogram construction based on the RAPD-PCR band patterns:

Data 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 (I) 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 and shared polymorphic products, was used to generate similarity coefficients according to Jaccard (1980). The similarity coefficients were used to construct a dendogram by UPGMA (Un-weighted pair-Group Method with Arithmetical Averages) using statistical program.

3. Pseudomonas fluorescens bacterium:

3.1. P. fluorescens isolates:

Fluorescent pseudomonads were isolated by serial dilution in King's B medium (KB) (King *et al.*, 1954) from healthy potato rhizosphere from El-Nubaria region, Behera governorate. Colonies were selected on King's B medium and incubated at 30°C for 24 hr. Then cultures were examined under ultra-violet (UV) lamp at 430 nm to detect the presence of fluorescent colonies.

3.2. Antagonistic effect:

Three isolates of *P. fluorescens* were tested *in vitro* for antibiosis against six isolates of *R. solanacearum*. Testing was done using King's B medium. The antagonistic and brown rot isolates were grown in GNA (glycerol nutrient agar) slants for 48 hr at 28°C, then suspended in 3-5 ml sterile distilled water, and

adjusted turbid metrically to approximately 10^9 cfu/ml (A_{540} 1.5) for *P. fluorescens* and 10^9 cfu/ml (A_{590} 0.7) for brown rot isolates. A loop-full of fresh culture of each antagonistic isolate was streaked as a single line at centre of the above mentioned media plates, and then incubated at 28°C for 24 hr. Inocula of the tested brown rot isolates, were streaked in lines perpendicular to those of the already grown antagonist. Three replicate plates were prepared for each combination of *R. solanacearum* isolates, then antagonistic-plates were incubated at 28°C for 48 hr. The resultant inhibition zones were measured, as the distances between the edge of antagonistic bacterial growth and the edge of tested bacteria. Data were recorded after 48 hr post streaking of antagonistic (Ciampi *et al.*, 1996).

3.3. Characteristics of P. fluorescens isolates:

The morphological, biochemical and physiological characteristics of *P. fluorescens* isolates were studied according to tests recommended by Murray *et al.* (1984) and Klement *et al.* (1990). Three isolates were chosen for the further studies.

3.4. Genotypic traits of P. fluorescens:

The genotypic traits, i.e. amplification of 16S rRNA and RAPD analysis were investigated as previously mentioned for *R. solanacearum*.

Results

1. Morphological, biochemical and physiological traits of the recovered R. solanacearum isolates:

Isolation trials during the 2008 season yielded six isolates of *R. solanacearum*. Data presented in Table (3) revealed that all the isolates were single rods, gram negative, non sporulating and motile. Meantime the isolates exhibited positive reaction for Kovac's oxidase, reducing substances from sucrose and acid production from maltose. However, the isolates showed negative reaction in case of gelatin liquefaction, starch hydrolysis, arginine hydrolysis and acid production from cellobiose, xylose and D (-) galactose. In this concern, most of isolates were positive for acid production from D (-) ribose. D (-) fructose, mannitol, lactose and sucrose. However, some of these isolates exhibited weak reaction.

2. Pathogenicity tests and varietal reaction:

Data presented in Table (4) showed that most of the *R. solanacearum* isolates recovered were either highly or moderately virulent. Two out of the six isolates tested were highly virulent as incited ≥ 70% plant leaflets wilt, while two isolates were moderately virulent as incited. ≥ 30 – < 70% leaflets wilt. Meanwhile, two isolates were weakly virulent as incited ≤ 30 leaflets wilt. On the other hand, the cv. Ladybedfore was the most resistant cultivar to the infection with the tested *R. solanacearum* isolates recovered from the surveyed area in Behera governorate, where exhibited 5 - 62.2% with mean of 26.11% wilt. However, cv. Nicola exhibited moderate resistance as incited 10-70.6% wilt with the different *R. solanacearum* isolates with the mean of 47.56% wilt. Meanwhile, cv. Diamont was the most susceptible cultivar as exhibited 30.32 - 98.7% leaflets wilt with the mean of 72.29% plant leaflets wilt.

Table 3. Morphological, physiological and biochemical traits of *Ralstonia solanacearum* isolates obtained from infected potato tubers in Behera governorate

Characteristics	Bacterial isolates					
	A	B	C	D	E	F
Cell shape (rods, single)	+	+	+	+	+	+
Gram staining	-	-	-	-	-	-
Sporulation	-	-	-	-	-	-
Anaerobic growth	-	-	-	-	-	-
Motility	+	+	+	+	+	+
Growth at 40°C	-	-	-		-	
Gelatin Liquefaction	-	-	-	-	-	-
Kovac's oxidase	+	+	+	+	+	+
Starch hydrolysis	-	..	-	-	-	..
Arginin hydrolysis	-	-	-	-		
Reducing substances from sucrose	+	+	+	+	+	+
Acid production from :						
Cellobiose	-	-	-	-	-	-
Xylose	-	-	-	-	-	-
D (-) ribose	+	+	+	+	W	W
D (-) fructose	+	W	W	+	+	+
D (-) galactose	-	-	-	-	-	-
Maltose	+	+	+	+	+	+
Mannitol	+	W	+	W	+	+
Lactose	+	+	W	+	+	+
Sucrose	+	+	+	W	+	+

+ = Positive reaction, - = Negative reaction and W= Weak reaction.

Table 4. Pathogenicity and cultivar reaction of potato against *Ralstonia solanacearum* isolates

Bacterial isolate	Cultivar			Mean effect	Virulence * of isolate
	Nicola	Ladybedfore	Diamont		
Rs1	49.24	62.2	98.07	70.05A	HV
Rs2	70.60	50.04	94.07	71.57A	HV
Rs3	65.25	23.02	90.04	59.44B	MV
Rs4	60.21	10.19	30.32	33.58C	MV
Rs5	30.05	6.18	50.21	28.82C	LV
Rs6	10.00	5.00	70.4	28.47C	LV
Cultivars mean effect	47.56B	26.11C	72.29A		

* HV= highly virulent; LV = low virulent and MV = moderately virulent.

- Each figure represents the mean of four replicates. Data are the average of percentage of infected leaflets.
- Values with the same letter on the same column or raw are not significantly different at p= 0.05 of probability.

3. Pseudomonas fluorescens bacterium:

3.1. Antagonistic effect of P. fluorescens against R. solanacearum:

Data presented in Table (5) showed that isolate of *P. fluorescens* (Pf1) exhibited strong inhibition (16.77 mm) on *R. solanacearum* isolates tested. However, the other two isolates, i.e. Pf2 and Pf3 showed weaker reaction (3.29-2.16 mm). This significant effect was observed 48 hours after inoculation. Besides, the tested *R. solanacearum* isolates were all sensitive to the tested *P. fluorescens* isolates with most tolerance was recorded for Rs6.

Table 5. The *in vitro* antagonistic effect of *P. fluorescens* isolates on *Ralstonia solanacearum* growth, 48 hr after inoculation.

R. solanacearum isolate	*P. fluorescens* isolate			*R. solanacearum* mean effect
	Pf1	Pf2	Pf3	
Rs1	22.00a	2.30b	1.00b	8.43A
Rs2	26.00a	2.31b	0.90b	9.74A
Rs3	23.00a	1.34b	0.83b	8.39A
Rs4	3.00b	10.01a	8.31a	7.10A
Rs5	21.60a	3.06b	1.40b	8.69A
Rs6	5.00b	0.70b	0.50b	2.06B
Mean effect	16.77A	3.29B	2.16B	

- Data were recorded 48 hr after inoculation as diameter (mm) of inhibition zones.
- Each figure represents the mean of three replicates.
- Values with the same letter on the same column or raw are not significantly different at p= 0.05 of probability.

3.2. Morphological and physiological traits of P. fluorescens:

Data presented in Table (6) revealed that all the isolates were gram negative, non-sporulating and motile. Meantime, the isolates exhibited positive reaction for fluorescence on KBA medium, growth at 4°C and pH 7, levan formation and gelatin liquefaction. However, the isolates showed negative reaction for growth at 41°C, in 7 % NaCl, and starch hydrolysis.

4. Genotypic characterization of R. solanacearum and P. fluorescens:

4.1. The 16S rRNA:

16S rRNA was used to study the genetic differentiation between the six isolates of *R. solanacearum* and two of *P. fluorescens*. Approximately 1550 bp was amplified using universal primers (16S F and 16S R). All the isolates tested exhibited the same banding pattern Fig. (1).

Table 6. Morphological and physiological characteristics of antagonistic fluorescent *pseudomonas*

Characteristic	G	H	I
Gram staining	-	-	-
Sporulation	-	-	-
Fluorescence on KBA	+	+	+
Motility	+	+	+
Growth at 41°C	-	-	-
Growth at 4°C	+	+	+
Growth at pH 7	+	+	+
Growth in 7% NaCl	-	-	-
Levan formation	+	+	+
Gelatin liquefaction	+	+	+
Starch hydrolysis	-	-	-

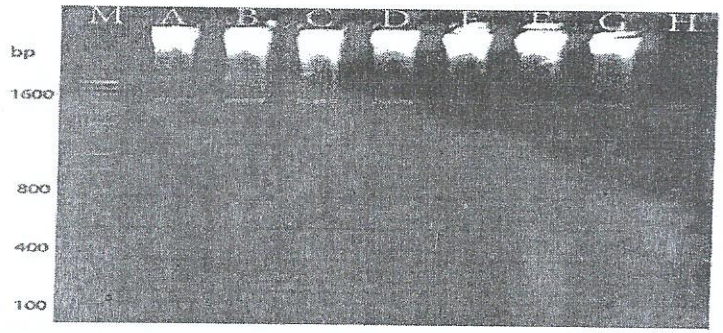

Fig. 1. Agarose gel electrophoresis pattern of 16S rRNA PCR product (1550bp), [M:DNA marker, Lanes A, B, C, D, E and F are *R. solanacearum* isolates and lanes G and H are *P. fluorescens* isolates].
- A= *Rs* 1, B= *Rs* 2, C= *Rs* 3, D= *Rs* 4, E= *Rs* 5, F= *Rs* 6, G= *Pf* 1, H= *Pf* 2.

4.2. Alignment and phylogenetic analysis:

Phylogenetic tree (Fig. 2) of the analyzed *R. solanacearum* and *P. fluorescens* isolates and bacterial isolates collected from Gene Bank revealed the existence of two clusters. Cluster 1 include two sub-clusters, sub-cluster 1 divided into two groups, group 1 divided into two sub-groups. Sub-group 1 include isolate Rs6, *R. solanacearum* strain GQ403791, *R. solanacearum* strain GQ505031, *R. solanacearum* strain EU4038965, Rs2, *R. solanacearum* strain FJ494776, *R. solanacearum* strain FJ210681 and *R. solanacearum* GU390462. Sub-group 2 include Rs5. Group 2 divided into two sub-groups, sub-group 1 include Pf1, Pf2, *P. fluorescens* strain FJ639838, sub-group 2 include *P. fluorescens* strain GU475123, *P. fluorescens* strain FJ950603, *P. fluorescens* strain GU198104, *P. fluorescens* strain FJ605510 and *P. fluorescens* strain FJ588704. Sub-cluster 2 consisted of one group include Rs3 and Rs4. Cluster 2 consisted of one group which included Rs1.

Alignment of 16S rRNA sequences of bacterial isolates investigated here with the 16S rRNA sequences of the *R. solanacearum* and *P. fluorescens* isolates from the Gene Bank was carried out. The phylogenetic tree of the analyzed six *R. solanacearum* revealed that these six isolates constituted two clusters. The highly virulent isolate Rs1 constituted a single cluster. The moderate and low virulent isolates, i.e., Rs3, Rs4, Rs5 and Rs6 constituted another cluster where Rs3 and Rs4 isolates constituted one group and Rs5 and Rs6 another group. (Fig.2). Data also, exhibited close relationships between *R. solanacearum* and *P. fluorescens* isolates. Meantime, the obtained banding pattern did not show differences between the most active Pf1 and the weak Pf2. Meantime, phylogentic tree showed that Pf1 and Pf2 were located in the same group.

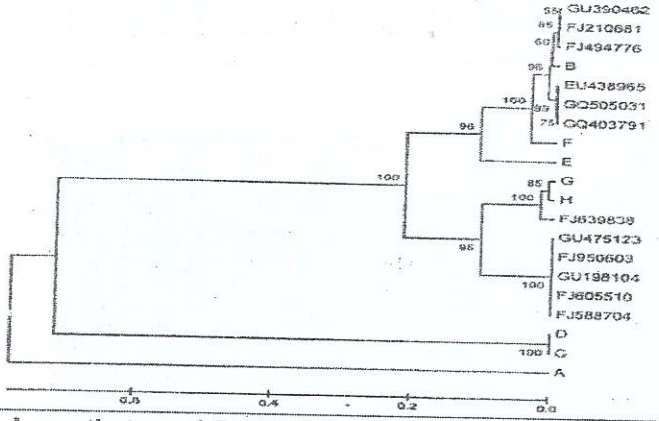

Fig. 2. Phylogenetic tree of *R. solanacearum*, and *P. fluorescens* isolates and related organisms from the alignment of 16S rRNA sequences.
A, B, C, D, E and F are *R. solanacearum* isolates, meanwhile G and H are *P. fluorescens* isolates.
- A= *Rs*1, B= *Rs*2, C= *Rs*3, D= *Rs*4, E= *Rs*5, F= *Rs*6, G= *Pf* 1, H= *Pf* 2.

4.3. Differentiation of R. solanacearum and P. fluorescens using Random Amplified Polymorphic DNA (RAPD):

Using of eight primers in RAPD-PCR showed clear difference among the eight isolates on the basis of amplified product band pattern observed with each primer (Fig 3 a, b, c, d, e, f, g and h). High similarity (84%) was observed between Rs1 and Rs2 isolates. Results indicated that 288 amplicons were produced by all primers. The number of amplified products produced by each primer varied from 41 with primer BAR to 27 with primer A_9A_{10}. The amplification profiles with the primers BAR, BAQ and W revealed that all of these primers succeeded to give polymorphic banding patterns, however, primer W failed to show any amplified products with isolate Pf1, (Fig a, b and d). The RAPD band patterns resulting from the use of the eight primers were analyzed using UPGMA method to construct a similarity matrix and to generate a dendogram indicating the relationship between the eight examined isolates. The presence or absence of any particular DNA bands was the only factor

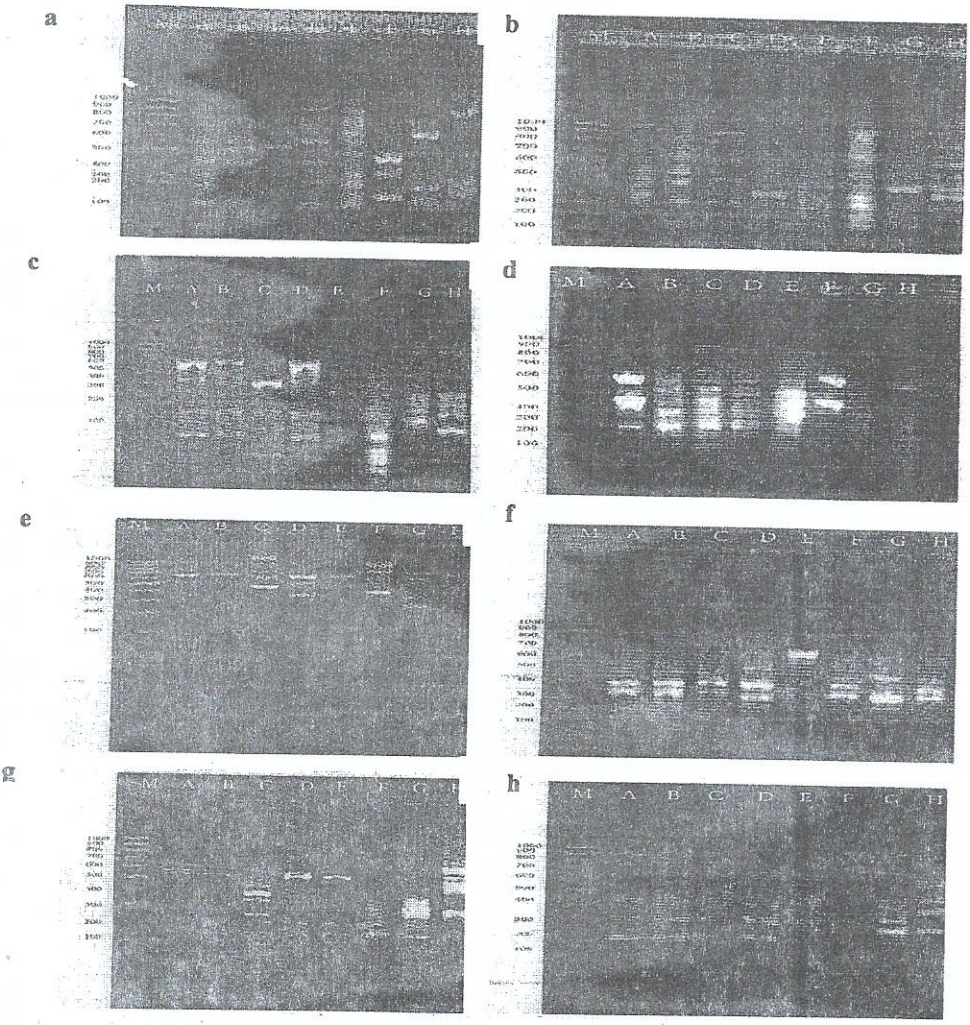

Fig. 3. Electrophoretic pattern of RAPD-PCR using primers (a: BAR, b: BAQ, c: 18, d: W, e: BIC 1, f: I, g: A_9B_4, and h, A_9A_{10}) [M: DNA marker. Lanes A, B, C, D, E and F are *R. solanacearum* isolates, lanes G and H are *P. fluorescens* isolates]. A = *Rs* 1. B = *Rs* 2. C= *Rs* 3. D= *Rs* 4. E= *Rs* 5. F= *Rs* 6. G= *Pf* 1. H= *Pf* 2.

considered in the computer analysis. The dendogram presented in Figure (4) indicated that the examined isolates were classified into two main clusters. Cluster A included two sub-clusters, sub-cluster A_1 included Pf1 isolate, sub-cluster A_2 include Pf2. However, cluster B divided into two sub-clusters, sub-cluster B_1 include one group (Rs5 and Rs6 isolates), sub-cluster B_2 divided into two groups, group 1 include Rs3 isolate, group 2 include two sub-groups, sub-group 1 include Rs4 isolate, sub-group 2 include two families Rs1 and Rs2 isolates.

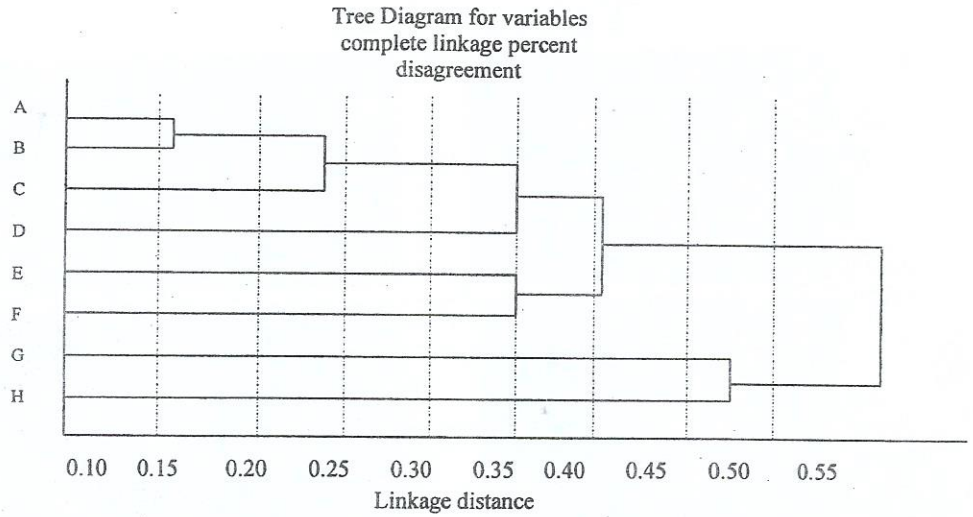

Fig. 4. Dendogram obtained by clustering (UPGMA method) based on the band pattern obtained by the RAPD-PCR analysis for eight isolates according to Jaccard index.
A= *Rs* 1, B= *Rs* 2, C= *Rs* 3, D= *Rs* 4, E= *Rs* 5, F= *Rs* 6, G= *Pf* 1, H= *Pf* 2.

The RAPD analysis conducted showed considerable variation for the banding patterns with the primers tested. This was most obvious with the primers BAR, BAQ, 18, 1, A_9B_4 and A_9A_{10}; however, BIC and W did not show obvious variation between the two tested *P. fluorescens* isolates.

Discussion

On the basis of the results obtained from inoculation tests performed on aerial stems of three cultivars of potatoes (Diamont, Nicola and Ladybedfore), Diamont was the most susceptible cultivar, however, Ladybedfore was the most resistant one. It was also noticed that, there were various degrees of wilting and *Ralstonia solanacearum* isolates differed in their virulence on different potato cultivars. Such results were in agreement with those reported by EL-Ariqi *et al.* (2008) as they showed that potato plants inoculated with *R. solanacearum* isolates exhibited partial wilting 19 days after inoculation. From data presented, it could be concluded that the isolates of *R. solanacearum* were morphologically monomorphic. Besides, the isolates partially differed in some physiological and biochemical traits. *In vitro* sensitivity of *R. solanacearum* to antagonistic bacterial isolates of *P. fluorescens* revealed that *P. fluorescens* isolate (Pf1) had more inhibitory effect toward *R. solanacearum* isolates than the two other isolates (Pf2 and Pf3). Such obtained data agreed with the findings of many investigators (Ciampi and Guaiquil, 1994) where they reported a considerable antagonistic action resulted from *P. fluorescens* against *R. solanacearum*. Additionally, such data were in accordance

with the findings of Ciampi *et al.* (1996) as they noticed that some isolates of *P. fluorescens* had the ability to synthesize a wide range of secondary metabolites. Among those are iron-chelating compounds called siderophores. The phenotypic cluster and genetic analysis revealed striking differences among strains from different areas. Identification of six isolates of *R. solanacearum* and two isolates of *P. fluorescens* were carried out using the total DNA isolated and used into polymerase chain reaction (PCR) analysis through amplifying 1550 bp region of the 16S r RNA gene using universal primers. Consequently, when the purified large region (1550 bp) of the 16S rRNA gene was subjected to sequencing, six isolates of bacteria were identified as *R. solanacearum* and two isolates as *P. fluorescens*. The sequences of 16S rRNA genes of *R. solanacearum* and *P. fluorescens* isolates were compared with other isolates from the Gene Bank database. The sequence of the 16S rRNA gene has been widely used as a molecular clock to estimate relationships among bacteria (phylogeny), but more recently it has also become important as a mean to identify an unknown bacterium to the genus or species level (Sacchi *et al.*, 2002). The phylogentic tree of *R. solanacearum, P. fluorescens* isolates and bacterial isolates collected from gene-bank revealed the existence of two clusters. Identity of the six pathogenic *R. solanacearum* isolates and *P. fluorescens* based on 16S rRNA gene sequences was supported by the above grouping characters based on phenotypic properties. Concerning the differentiation between *R. solanacearum* and *P. fluorescens* isolates, eight primes (BAR, BAQ, 18, W, BIC 1, I, A_9B_4, and A_9A_{10}) were used. All primers produced considerable polymorphism among the studied isolates. These results indicated that 288 amplicons were produced by all primers and the number of amplified products produced by each primer varied from 41 with primer BAR to 27 in case of primer A9A10. In addition, based on the dendogram generated to show linkage distance and the RAPD band patterns resulting from the use of eight primers, high similarity (84%) was observed between A and B isolates of *R. solanacearum*, consequently, indicated the relationship among the eight examined isolates. When 16S rRNA gene (1550 bp) was used in *R. solanacearum* and *P. fluorescens* detection, all the isolates gave one band at the right expected molecular weight. Such data indicated that the aforementioned results are in agreement with those obtained by Williams *et al.*, 1990; Kumar *et al.*, 2004; Van der Wolf *et al.*, 2004 and Gover *et al.*, 2006. These results confirmed the efficiency of RAPD procedure for correctly identifying the brown rot bacteria and *fluorescent pseudomonas*.

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استخدام الجين 16S rRNA والتضاعف العشوائي للـ DNA لاكتشاف عزلات البكتيريا رالستونيا سولانا سيرم

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تم عزل ست عزلات من البكتيريا رالستونيا سولانا سيرم من درنات بطاطس مصابة بالعفن البني وثلاث عزلات من بكتريا سيدوموناس فلورسينس من ريزوسفير نباتات بطاطس سليمة من منطقة النوبارية بمحافظة البحيرة. تم تعريف هذه العزلات باستخدام اختبارات فسيولوجية ومورفولوجية. تم عدوى السيقان الهوائية لثلاثة أصناف من البطاطس هي نيقولا ودايمونت وليدي بيدفور ببكتريا رالستونيا سولاناسيرم وكان أكثر الأصناف مقاومة لعدوى السيقان هو صنف ليدي بيدفور بينما كان صنف دايمونت هو الأكثر حساسية. تم إجراء عملية تعريف للبكتيريات المتحصل عليها باستخدام الـ DNA المعزول منها في تفاعل إنزيم البلمرة المتسلسل Polymerase Chain Reaction حيث تم تكبير منطقة من الـ DNA الكلي وزنها الجزيئي 1550 pb من جين 16S rRNA باستخدام بوادئ عالمية Universal primers . تم إجراء عملية Alignment sequencing لمعرفة تتابع النيوكليوتيدات في المنطقة المعزولة باستخدام جهاز DNA Sequencer . أمكن مقارنة تتابع منطقة الجين 16S rRNA للعزلات المتحصل عليها مع تتابعات لنفس الجين لعزلات أخرى متحصل عليها من الـ Gene Bank . تم التفريق بين عزلات رالستونيا سولانا سيرم وعزلات سيدوموناس فلورسينس عن طريق عمل مسح عشوائي للجينوم باستخدام ثماني بادئات وراثية. أظهرت النتائج وجود تباين بين العزلات وكذلك وجدت احزمة متشابهة وأخرى مميزة لكل عزلة .