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DIFFERENTIATION AMONG *RALSTONIA SOLANACEARUM* ISOLATES CAUSING POTATO BACTERIAL WILT BY RANDOM AMPLIFIED POLYMORPHIC DNA

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Abstract

Ten *Ralstonia solanacearum* isolates were obtained from Spunta and Nicola potato cultivars . PCR-based RAPD analysis was carried out, using four primers, on agarose and polyacrylamide gels as support media. Only, the primer 6-d (AACGCGCAAC)-3 differentiated the isolates recovered from the two cultivars, others did not. In general, separation on poly-acrylamide and staining with silver nitrate gave better resolution than those on agarose and stained with ethidium bromide. Further investigations are needed using greater number of isolates, from different potato cultivars and /or different sources, to confirm the result as well as the possible effect of the host cultivars on the properties of bacterial DNA.

Key words (*Ralstonia solanacearum,* potato cultivars, polyacrylamide, silver nitrate and RAPD)

INTRODUCTION

Ralstonia solanacerarum (Yabuuchi *et al.*, 1995), the cause of bacterial wilt on solanaceous crops, has been reported in Egypt many years ago (Briton-Jones,1925). The disease has created a lot of economic problems in potatoes exported to Europe in winter, and banning of Egyptian potatoes has been proposed by certain Europe community member states (N. S. Farag, personal communication). Although the disease is favored by the warm climates, serious outbreaks have been reported in Europe (Grousset *et al.*, 1998). Surveys and race determination(s) made in Egypt revealed that race 3 (biovar 2), or the so-called potato race, is the strain recorded in Egypt . On the other hand, race 1 has not been recorded in Egypt either on solanaceous crops or other hosts (Hayward, A.C., 1964; Holt *et al.*, 1994 and Schaad, 1988).

In the last ten years, the interest in nucleic acid-based techniques for the detection of microorganisms has dramatically increased. Identification of specific micro-

organisms is important for risk analysis of the disease. Molecular biological techniques provide highly specific methods for the detection and identification of such bacteria. The application of Random Amplified Polymorphic DNA (RAPD) (Welsh and McClelland, 1990, Williams *et al.*, 1990) to produce isolate-specific DNA fingerprints is especially promising (Hadrys *et al.*, 1992). This technique has the advantages that no DNA sequence information of the organism is needed, and the bacteria do not have to be genetically marked. The objective(s) of the present work is to define the most reliable primer for detection and differentiation of *Ralstonia solanacearum* isolates as well as the influence of host plants on DNA pattern.

MATERIAL AND METHODS

Isolation of bacteria:

The bacterium was isolated from Spunta and Nicola potato tubers collected from fields in Qalyubia governorate, near Cairo. Isolation was made on modified selective media of South Africa (SMSA medium). Single colonies were selected 4 days after plating and incubation at 28-30 °C. Five isolates from each cultivar were tested for pathogenicity on 4- leaves potted tomato plants in the greenhouse. Re-isolation was made from the wilted plants on SMSA, 6 - 8 days after inoculation . Identification of the original isolates was made according to Bergey's Manual of Determinative Bacteriology (Holt *et. al.*, 1994) and Hayward (1964).

DNA isolation and RAPD technique:

DNA was isolated from 50 mg of the organism using Qiagen Kit for DNA extraction(cat. no. 69104). The extracted DNA was dissolved in 100 ul of the elution buffer. The concentration and purity of the obtained DNA was determined using "Gene quant" system-pharmacia Bio-tech. The purity of the DNA for all samples in this study ranged between 90 - 97% and with ratio between 1.7 and 1.8. Concentration was adjusted to 6 ng/ ul for all samples using TE tris-buffer, pH. 8.0.

Random amplified polymorphism DNA technique(RAPD):

Thirty ng from the extracted DNA were used for amplification reaction. The polymerase chain reaction (PCR) mixture contained PCR beads tablet (Amessham Pharmacia

Biotech), containing all the necessary reagents except the primers and the DNA.

The Amessham Pharmacia Biotch kits contain six primers with the following sequences:

RAPD Analysis Primer 1:6-d (GGTGCGGGAA)-3
RAPD Analysis Primer 2:6-d (GTTTCGCTCC)-3
RAPD Analysis Primer 3:6-d (GTAGACCCGT)-3
RAPD Analysis Primer 4:6-d (AAGAGCCCGT)-3
RAPD Analysis Primer 5:6-d (AACGCGCAAC)-3
RAPD Analysis Primer 6:6-d (CCCGTCAGCA)-3

Primers 3, 4, 5 and 6 were used in this study. Five microliters of the primer (10 mer) were used and the total volume was completed to 25 ul ,using sterile distilled water. The amplification protocol was carried out as follows using PCR unit II Biometra.

a. Denaturation at 95 °C for 5 min.
b. 45 cycles each consists of the following steps:
    1. Denaturation at 95 °C for 1 min.
    2. Annealing at 36 °C for 1 min.
    3. Extension at 72 °C for 2 min.
c. Final extension at 72 °C for 5 min.
d. Hold at 4 °C

Seven ul of 6 X tracking buffer (manufactured by Qiagen Kit) were added to 25 ul of the amplification product.

Amplification product analysis:

Two methods were used for elctropheresis:

1. The amplified DNA for all samples (15ul) were electrophoresed using electrophoresis unit (WIDE mini-sub-cell GT Bio-RAD) on 1% agarose containing ethidium bromide as a stain (0.5 µg/ml). Constant power of 75 volt was applied and the bands were determined with an UV transilluminator .

2. The PCR products was separated and determined using polyacrylamide gel electrophorsis slabs according to Pieter *et. at.*, (1995). The gel matrix consisted of the following components:

TBE 10 x pH 8.3

Dissolve in water	200 ml
Boric Acid	11.0 g
Tris Base	21.6 g
EDTA anhydrous	1.168 g
Tris base, Boric acid, EDTA(TBE) 10 x pH 8.3	4.0 ml
Acrylamide 40%	4.7 ml
Bisacrylamide 2%	3.0 ml
10% (W/V) Ammonium persulphate (APS)	300 ul
Water	28 ml
Temed	20 ul

Eight ul of each sample were added to the well of the electrophoresis unit (Hoefer SE 600 series, Pharmacia). Running was made at 2 mA per sample for about 2 hours. The amplified pattern was developed as described by Cairns and Murray (1994) using silver stain. The protocol consisted of the following steps.

- Incubation in fixing solution for 3 min. (10% ethanol, 0.5% acetic acid).
- Incubation in fixing solution containing 0.2% silver nitrate for 5 min.
- Washing once for 20 sec and again for 2 min in sterile distilled water.
- Incubation in the developing solution (3.0% NaoH, 0.5% formaldehyde) until band development.

Gel analysis:

Gel documentation system (AAB Advanced American Biotechnology) was used. The similarity levels were determined by unweighed pair-group method based on arithmetic mean (UPGMA) clustering using Pearson product .

RESULTS AND DISCUSSION

Isolation and Identification

The bacteria isolated from Spunta and Nicola potato tubers were all pathogenic on tomato seedlings (artificial inoculation) under greenhouse conditions. The developed pathogen showed weak reaction in stained preparations . No spores could be detected. Two bipolar staining bodies can be easily seen. No fluorescence in Kings medium B could be detected, (Table 1) All cultures produced brown diffusible pigments with different intensity. All cultures produced acids from maltose, and cellobiose. No acids were produced from mannitol, sorbitol and dulcitol. Other tests are shown in table (1). The results revealed no variation among isolates, which showed the basic characteristics of Race 3, biovar 2 of *Ralstonia solanacearum*. All isolates were identical according to Bergey's manual of determinative bacteriology (Holt *et al.*, 1994)

PCR- studies :

Four primers were used in the RAPD analysis of ten isolates of *Ralstonia solanacearum* derived from the potato cultivars Spunta and Nicola. Agarose and polyacrylamide gels were employed as supporting media for electropheresis.

Different primers yielded variable results as evidenced by the dendograms of the PCR products. Also, the results were obviously affected by the type of medium used for electrophoresis. Accordingly, clustering based on similarity levels among isolates varied markedly.

Primer No. 3 (G-d (GTAGACCCGT)-3) yielded banding patterns for the ten isolates on agarose (Fig. 1, A & 1, B) showing that spunta isolates separated into 4 subclusters with an overall similarity level of 87.12 %. However, the Nicola isolates separated into 3 subclusters including isolates 6 and 7 with similarity level of 78.22; isolate 8 showed a similarity level of 74.17 with the Spunta isolates and 53.09 % with the subcluster 6 and 7. All ten isolates showed an overall similarity level of 22.03 %. Using polyacrylamide gel for electrophoresis separation, a completely different picture was seen (Fig. 2, A & 2, B). Isolates 2 and 3 from Spunta formed a subcluster having 92.43 % similarity which clustered with isolate 1 at 30.92 % similarity level. Isolate 4 came in a separate subcluster which showed a similarity level of 52.67 % with isolates 6, 9 and

Table 1. physiological characteristics of the isolated bacteria.

source of isolates / Tests	Spunta					Nicola				
	1	2	3	4	5	6	7	8	9	10
Fluorescent, diffusible pigment	-	-	-	-	-	-	-	-	-	-
Diffusible brown pigment	+	-	+	+	+	+	+	-	+	-
Accumulation levan formation from sucrose	-	-	-	-	-	-	-	-	-	-
Arginine dihydrolase	-	-	-	-	-	-	-	-	-	-
catalase	+	+	+	+	+	+	+	+	+	+
O/F	0	0	0	0	0	0	0	0	0	0
Utilization of :	acid production									
Maltse	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	+	+	+
Mannitol	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-
Salicin	-	-	-	-	-	-	-	-	-	-
Glucose	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+
Raffinose	-	-	-	-	-	-	-	-	-	-
Arabinsoe	-	-	-	-	-	-	-	-	-	-
Xylose	-	-	-	-	-	-	-	-	-	-
Fructose	+	+	+	+	+	+	+	+	+	+
Glycerol	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+

(+) Positive reaction or acid production in Sugar utilization tests (-) negative reaction.

(+) Brown pigment produced (-) no pigmentation.

(0) oxidation in (o/f) test.

10 originating from Nicola variety. The latter three isolates clustered in two subcluster; 6 and 9 at 96.67 % and both clustered with isolate 10 with 91.87 % similarity. Isolates 7 and 8 from Nicola subclustered at 96.43 % similarity level and which were quite distant from the rest of Nicola isolates showing only a 21.73 % similarity level. Still isolate No. 5 from Spunta formed a very distant subcluster from their sister isolates as well as those isolates derived from Nicola and showed only a 13.48 % similarity. In view of the above, primer No. 3 seems inappropriate for either the detection or the differentiation among isolates from different host cultivars.

Primer No. 4 (G-d (AAGAGCCCGT)-3) revealed, when using polyacrylamide (Fig. 4-A and 4-B), that the five isolates originating from the variety "Spunta" had an overall similarity level of 96.62 % (with three subclusters.) The five isolates originating from the variety "Nicola" showed two subclusters; isolates 7 and 6 with 99.35 % similarity and isolates 9 and 10 with 98.98 % similarity, whereas isolates 8 was somewhat distinct from the other four isolates of Nicola and close to the isolates of Spunta showing a similarity level of 94.58 %. Similar trend was expected with agarose medium (Fig. 3, A & 3, B); however, values of similarity level were somewhat lower.

Using primer No. 5 (G-d (AACGCGCAAC)-3) with polyacrylamide being the separation medium (Fig. 6, A & 6, B), the five Spunta isolates separated as isolates 1 and 2 at 96.40 % similarity, 3 and 4 at 96.71 % similarity and isolates 5 linked with isolate 1 and 2 cluster at 93.34 % similarity. Isolates 3 and 4 were linked with the 1, 2 and 5 at a similarity level of 92.19 %, which represented the overall similarity level of the five (Spunta) isolates. This primer revealed 4 subclusters among the (Nicola) isolates showing an overall similarity of 89.70 %. Isolates 7 and 9 subclustered at 94.30 % similarity and linked with isolates 8 at 92.08 % similarity. These three isolates (7, 9 and 8) linked with isolate 10 at a similarity level of 90.96 %. Isolates 6 clustered with the other four isolates with a similarity level of 89.70 %. It is obvious, that this primer separated the isolates originating from (Spunta) cultivar from those isolates from (Nicola) without any overlapping. The two groups showed a similarity level of 86.15 % representing the overall similarity level among the ten isolates under investigation. Employing agarose as a support medium (Fig. 5, A & 5, B) allowed the separating of the five Spunta isolates into 3 subclusters with an overall similarity of 92.19 %; however, the Nicola isolates showed more variation and lower levels of similarity within, and with an overall similarity

among all ten isolates of only 3.09 %.

Employing primer No. 6 (G-d (CCCGTCAGCA)-3) and using polyacrylamide gel for separation of PCR-products, (Fig. 8, A & 8, B), revealed subclusters between isolates 2 and 3 at 98.97 %, which linked with isolates 5 at 98.48 % similarity. The three isolates 2, 3 and 5 linked with isolates 1 at 96.89 % similarity. Isolates 4 of Spunta subclustered with isolate 7 from Nicola at 97.45 % similarity level. The latter subcluster linked with isolates 1, 2, 3 and 5 at 96.37 % similarity and with which isolate 6 linked at 94.87 %, then isolate 8 clustered with all the above isolates at 94.26 % similarity. Isolates 9 and 10 of Nicola subclustered at 96.68 % similarity and this subcluster was linked with all other isolates at 92.62 % which represented the overall similarity level for all ten isolates for electrophoresis, (Fig. 7, A & 7, B). The five isolates from Spunta subcluster very closely at an overall similarity level of 89.54 %, whereas the five isolates originating from Nicola showed somewhat lower similarities with an overall similarity level of 32.78 %. The two sets of isolates from the varieties linked together at a 15.31 % level of similarity.

These results are in agreement with those of Hansen and Winding (1997) who demonstrated that a fragment obtained by RAPD can be used as an isolate-specific marker for the detection of *Pseudomonas putida* B in the rhizosphere. The same conclusion was observed when Dristig and Dianese (1990) who characterized *P. solanacearum* biovars based on membrane protein patterns, where strains of the same biovar from the same host showed high similarity levels among themselves. However, when differentiating biovars from the same host were compared with each other, similarity was much lower.

When the primer 6 was used with the ten isolates in concern, different subcluster with different similarity levels were demonstrated either with agarose or polyacrylamide. The results of primer 6 are in agreement with those of 3 and 4 without differentiating isolates of the two potato cultivars.

It could be concluded that Primer 5:6-d (AACGCGCAAC)-3 was the most reliable for detection and differentiation of *R. solanacearum* isolates at the cultivar level. Primers (4, 5 and 6 on polyacrylamide) showing high levels of similarity among isolates (on polyacrylamide), regardless of their origin could possibly be used for detection of *R. sol-*

anacearum. However, to confirm such a conclusion, more isolates representing different *Pseudomonas* spp. have to be tested using such primers. More investigations are needed on greater number of isolates from different cultivars and / or different sources, to confirm the feasibility of employing such a technique in detection and differentiation of bacteria.

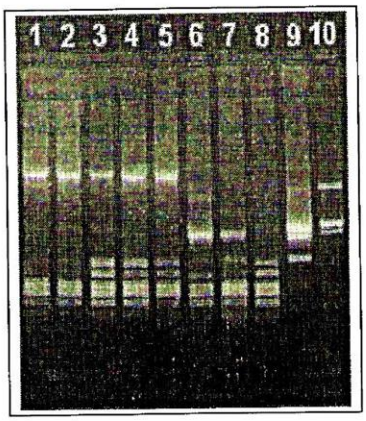

Fig.1-A: Polymorphism of DNA of isolates obtained by PCR-based RAPD (Primer 3) on a agarose as a support medium. Isolates 1, 2, 3, 4 and 5 from Spunta cultivar, Isolates 6, 7, 8, 9 and 10 from Nicola cultivar.

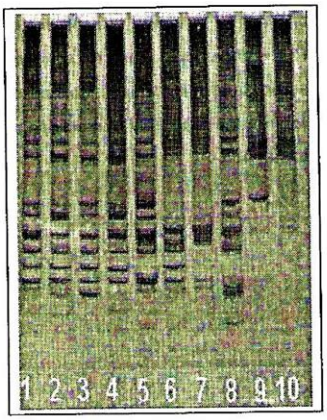

Fig.2-A: Polymorphism of DNA of isolates obtained by PCR-based RAPD (Primer 3) on a polyacrylamide as a support medium. Isolates 1, 2, 3, 4 and 5 from Spunta cultivar, Isolates 6, 7, 8, 9, and 10 from Nicola cultivar.

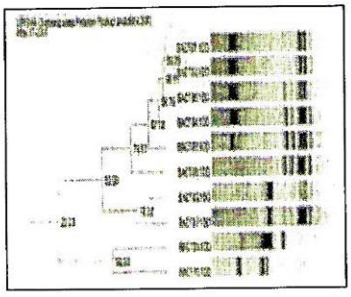

Fig.1-B: Dendrograms of DNA of isolates obtained by PCR-based RAPD (Primer 3) on a agarose as a support medium.

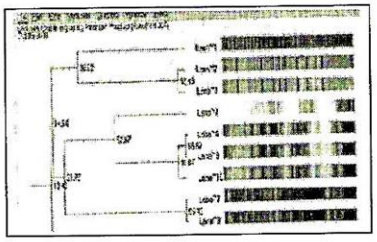

Fig.2-B: Dendrograms of DNA of isolates obtained by PCR-based RAPD (Primer 3) on a polyacrylamide as a support medium.

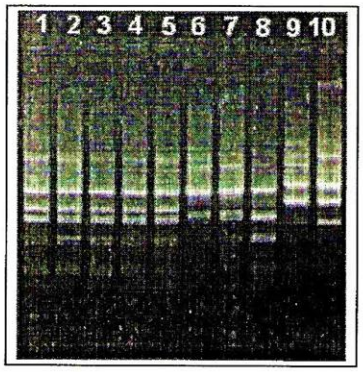

Fig.3-A: Polymorphism of DNA of isolates obtained by PCR-based RAPD (Primer 4) on a agarose as a support medium. Isolates 1,2,3,4 and 5 from Spunta cultivar, Isolates 6,7,8,9, and 10 from Nicola cultivar.

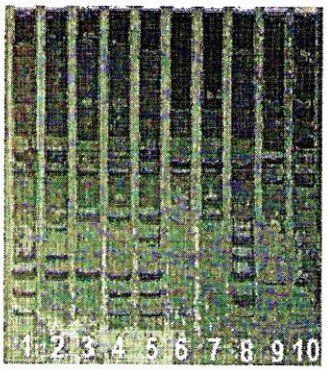

Fig.4-A: Polymorphism of DNA of isolates obtained by PCR-based RAPD (Primer4) on a polyacrylamide as a support medium. Isolates 1,2,3,4 and 5 from Spunta cultivar, Isolates 6,7,8,9, and 10 from Nicola cultivar.

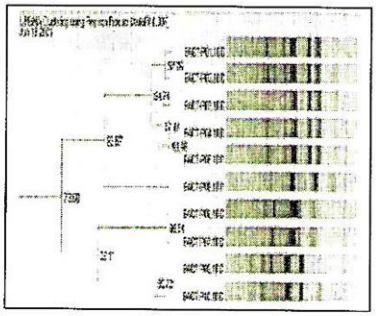

Fig.3-B: Dendrograms of DNA of isolates obtained by PCR-based RAPD (Primer 4) on a agarose as a support medium.

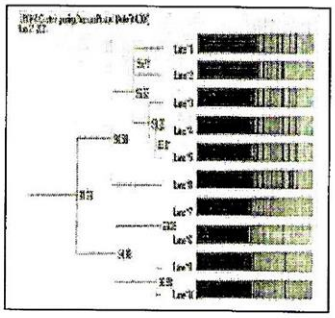

Fig.4-B: Dendrograms of DNA of isolates obtained by PCR-based RAPD (Primer 4) on a polyacrylamide as a support medium.

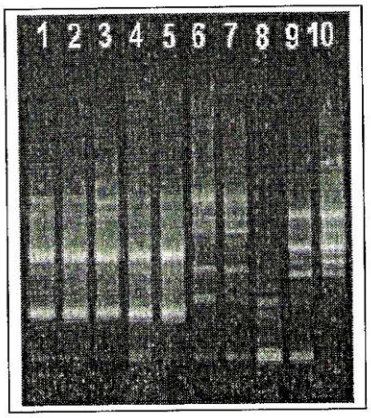

Fig.5-A: Polymorphism of DNA of isolates obtained by PCR-based RAPD (Primer 5) on a agarose as a support medium. Isolates 1,2,3,4 and 5 from Spunta cultivar, Isolates 6,7,8,9, and 10 from Nicola cultivar.

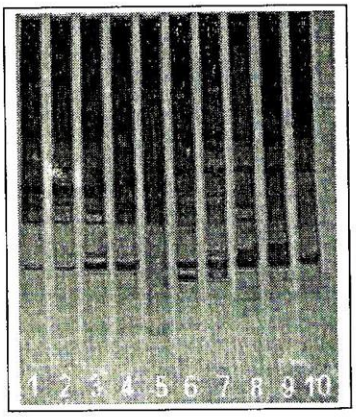

Fig.6-A: Polymorphism of DNA of isolates obtained by PCR-based RAPD (Primer5) on a olyacrylamide as a support medium. Isolates 1,2,3,4 and 5 from Spunta cultivar, Isolates 6,7,8,9, and 10 from Nicola cultivar.

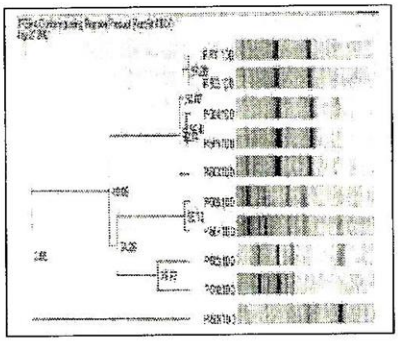

Fig.5-B: Dendrograms of DNA of isolates obtained by PCR-based RAPD (Primer 5) on a agose as a support medium.

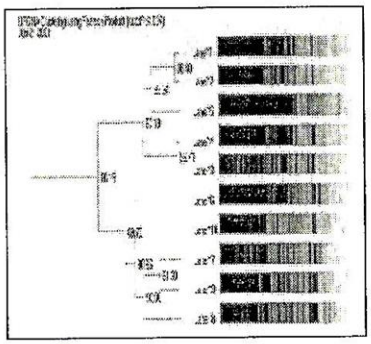

Fig.6-B: Dendrograms of DNA of isolates obtained by PCR-based RAPD (Primer 5) on a olyacrylamide as a support medium.

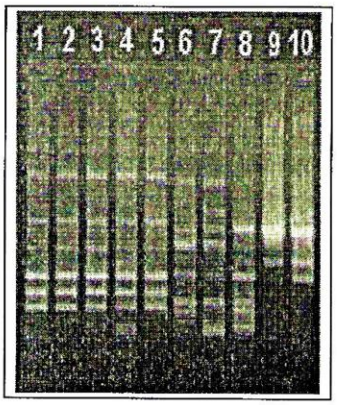

Fig.7-A:Polymorphism of DNA of isolates obtained by PCR-based RAPD (Primer 6) on a agarose as a support medium. Isolates 1,2,3,4 and 5 from Spunta cultivar, Isolates 6,7,8,9, and 10 from Nicola cultivar.

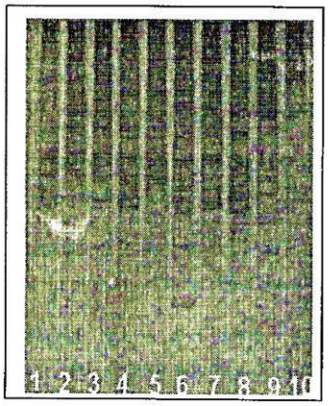

Fig.8-A: Polymorphism of DNA of isolates obtained by PCR-based RAPD (Primer 6) on a polyacrylamide as a support medium. Isolates 1,2,3,4 and 5 from Spunta cultivar, Isolates 6,7,8,9, and 10 from Nicola cultivar.

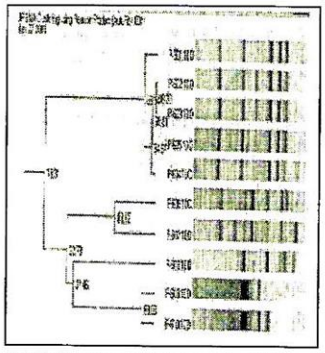

Fig.7-B: Dendrograms of DNA of isolates obtained by PCR-based RAPD (Primer 6) on a agarose as a support medium.

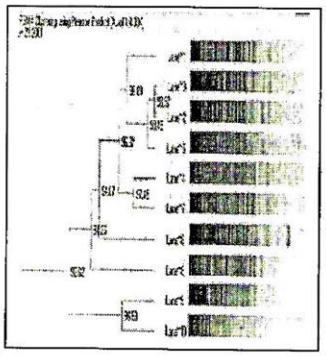

Fig.8-B: Dendrograms of DNA of isolates obtained by PCR-based RAPD (Primer 6) on a polyacrylamide as a support medium.

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التفرقة بين عزلات رلستونيا سولانسيرم المعزولة من البطاطس والمسببة للذبول البكتيرى بواسطة التضاعف العشوائي للحامض النووى

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استعملت عشر عزلات من بكتريا ريلستونيا سولانسيرم وتم عزلها من أصناف البطاطس نيقولا وسبونتا . وباستخدام تكنيك التضاعف العشوائي لمناطق متباينة من الدنا وباستعمال البادئات رقم 3-(GTAGACCCGT)، 4 (AAGAGCCCGT)، 5 (AACGCGCAAC)، 6 (CCCGTCAGCA) والفصل على كل من الاجاروز و البولياكرلاميد كوسط للفصل . و الدراسة توضح وجود علاقة بين تكنيك التضاعف العشوائي لمناطق متباينة من الدنا بأستحدام البادئ رقم ٥ و التفرقة بين عزلات البكتريا المعزولة من أصناف البطاطس النيقولا و السبونتا اتضح أن البادئ رقم ٥ هو الوحيد من هده المجموعة القادر على تميز مجموعة العزلات على أساس صنف البطاطس حيث أن جميع البادئات الأخرى المستخدمة لم تستطع الفصل بين العزلات على أساس الصنف . كما أن، البادئات التي أظهرت درجة عالية من التجانس بين العزلات (٤، ٥، ٦ على البولياكرلاميد) بغض النظر عن مصدرها يمكن استعمالها في الكشف عن بكتريا ريلستونيا سولانسيرم و لأثبات هذا التصور على مختلف العزلات التي تتضمن جنس البسيدومونس يجب اختبارها باستعمال نفس البادئات. و قد اتضح أن الفصل على البولياكرلاميد و الصبغ بنترات الفضة أعطى نتائج افضل من الفصل على الاجاروز و الصبغة بالاثيديم بروميد. و قد أوضحت الدراسة ضرورة أجراء مزيد من البحوث على عدد اكبر من العزلات و الأصناف و المصادر.