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Full Length Research Paper

Using virulence genes *hrpB*, *egl* and *fliC* in differentiation between virulent and avirulent isolates of *Ralstonia solanacearum*, the causal agent of potato brown rot

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To characterize the genetic variation between virulent and avirulent isolates of *Ralstonia solanacearum* race 3 (biovar II), the causal agent of potato brown rot. Nine isolates of *R. solanacearum* recovered from the natural habitats (potato tubers, weeds, soil and water) were used. Six virulent and three avirulent isolates were characterized in terms of pathogenicity and molecular level using PCR technique with specific primers targeting three-virulence related genes located on the megaplasmid which was involved directly or indirectly in the disease process. Both virulent and avirulent forms of *R. solanacearum* were pathogenic to potato plants causing different symptoms. The two forms of the pathogen were containing the megaplasmid which carry three genes *hrpB*, *egl* and *fliC*. Detection and digestion of the amplification PCR of the three genes by two restriction endonuclease enzymes EcoRI and DraI showed differences between the two forms of *R. solanacearum*, whereas digested *egl* gene by the DraI enzyme gave two bands with all virulent isolates, while it gave only one band with the avirulent isolates. On the other hand, there were no differences in bands with the two genes *hrpB* and *fliC*.

Key words: *Ralstonia solanacearum*, virulent, avirulent, pathogenicity, potato, PCR, megaplasmid, restriction enzymes.

INTRODUCTION

Potato (*Solanum tuberosum*) is an important vegetable crop in Egypt; it is the fourth most important staple food after wheat, rice and maize. Egypt is the largest potato producer in Africa, the total cultivation area of potatoes reached 197,250 feddans (Abd-Elgawad and Youssef, 2008).

Potato brown rot caused by *Ralstonia (Pseudomonas) solanacearum* was reported for the first time in Egypt at El-Gemeiza farm, Gharbia governorate based on symptomatology only by Briton-Jones (1925). The disease is considered to be a severe obstacle to the production of solanaceous plants in tropical, subtropical and temperate regions. As a diverse species complex, *Ralstonia solanacearum* has developed an extremely broad host range throughout the world, including over 450 host species representing 54 plant families (Allen et al., 2005).

R. solanacearum is a Gram-negative, non-fluorescent, non spore forming, non-capsulating but slime forming rod shaped bacterium. *R. solanacearum* spontaneously undergoes a phenotypic conversion (PC) from a pathogenic wild-type to a non-pathogenic form shifting from a mucoid to non mucoid colony morphology (Denny and Baek, 1991).

From the phytopathological point of view, the bacterium is found in nature in two forms: virulent (vi) and avirulent (av). Both forms may be recovered from diseased tissues

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on isolation plates, though the interrelation between them is not yet well understood. In media containing 2, 3, 5-triphenyltetrazolium chloride Colonies of virulent wild types are irregularly rounded and are white with pink centers. Colonies of avirulent variants are uniformly round, butyrous, and deep red (Kelman, 1954; Krieg and Holt, 1984).

Using various nucleic acid based techniques that showing highly specific tools in the detection and identification of microorganisms has been dramatically increased recently. Restriction fragment length polymorphism (RFLPs) and specific DNA fingerprints are especially promising techniques (Welsh and McClelland, 1990). The present study was conducted to differentiate between the two forms of *R. solanacearum* in terms of pathogenicity and the genetic variation.

MATERIALS AND METHODS

Isolation and identification of the pathogenic bacteria

Infected potato tubers and weed plants, also infested soil and water samples were collected from Ismailia and El -Minufiya governorates. Isolation procedures were carried out using a semi selective medium of south Africa SMSA (Elphinstone et al., 1996) under incubation conditions according to OEPP/EPPO (1990). Pathogenicity test was determined on 3 leaves tomato plants. Inoculation was made by stem puncture technique according to Janse (1988). Morphological, cultural, physiological and biochemical characteristics were determined according to Hayward (1964) and Palleroni (1984).

The immunofluorescence antibody staining (IFAS) described by Janse (1988) as a serological method for rapid detection and presumptive identification of bacteria was also tested.

Plant material

Potato tubers used in this study were provided by El-Domiaty group and Maba for import and export companies. Three potato cultivars currently cultivated in Egypt namely Diamant, Spunta and Hermes were used for planting.

Bacterial strains and growth conditions

Nine isolates (six virulent and three avirulent) recovered from different habitats were tested periodically for their pathogenicity, these isolates used for further studies. All isolates were propagated separately in glucose nutrient agar medium and incubated for 3 days at 28°C, the growing bacterial cells were removed using swab and the collected cells were suspended in sterilized water. The inoculum density was adjusted colorimetric to give about 10^7 cells/ml.

Pathogenicity test and data scoring

Standardized eye-pieces of each cultivar were dipped into the bacterial suspension of each virulent or avirulent isolate of the pathogen separately, for 30 min. followed by 10 min. for partial drying before planting in sterile pots (20 cm in diameter) filled with autoclaved soil under greenhouse conditions.

Control treatments were prepared by applying sterile water instead of bacterial suspension; five replicates were made for each isolate. Irrigation of plants was kept regular throughout the growth period.

The disease index was determined periodically according to the key proposed by Winstead and Kelman (1952) describing the wilt symptoms in the plant as follow:

0 = no symptoms; 1 = one or 2 leaves wilted; 2 = three leaves wilted; 3 = four or more leaves wilted and 4 = plant died.
Disease index (DI) was calculated by the following formula:

$$\text{Disease index (DI)} = \frac{\sum R\,T \quad X\,100}{4N}$$

Whereas:
R = disease severity scale (0, 1, 2, 3 and 4); T = number of wilted plants in each category and N = total number of tested plants.

Difference between virulent and avirulent isolates of *R. solanacearum* using molecular characterizations

PCR technique using specific primer

Molecular techniques were also applied to identify and differentiation between virulent and avirulent isolates of *R .solanacearum*. Three virulence related – genes (*hrpB* gene – *egl* gene and *fliC* gene) located on the megaplasmid were PCR amplified from total DNA by using specific primers.

i. DNA isolation: Total DNA was extracted from pure broth culture of the bacteria by using the Qiagen DNA isolation kits.
ii. Primers: Primer sequences for PCR were generated as described by (Castillo and Greenberg, 2007).
iii. PCR amplification: The PCR reaction was carried out in a 25 µl volume containing 0.4 U of Taq DNA polymerase (Bioron), 2.5 µl of 10 × PCR buffer, 2.5 µl of 10 mM $MgCl_2$, 0.25 µM of each primer, 1 µl of 10 mM dNTPs and 25 ng of genomic DNA.

Thirty five cycles of amplification were performed with a denaturation temperature of 94°C for 1 min, the appropriate annealing temperature for 1 min was varied depend on the genes (Table 1) and an extension step at 72°C for 1 min. A preceding denaturation step and a final extension step were carried out at 94 and 72°C for 5 min each and hold on 4°C.
iv. Gel analysis: The PCR fragments were analyzed on 1.5% agarose at 75 V in TAE (40 mM Tris/acetate, 1 mM EDTA, pH 8.0) and stained with ethidium bromide (10 mg/ml). The gel was analyzed using gel documentation system (AAB Advanced American Biotechnology 1166 E. Valencia Dr. Unit 6 C, Fullerton CA 92631).

DNA fragments were scanned for band Rf according to 1Kb DNA ladder marker used with M.W. 250/253 – 500 – 750 – 1,000 – 1,500 – 2,000 – 2,500 – 3,000 – 4,000 – 5,000 – 6,000 –8,000 and 10,000 bp (promega catalog G 5711).

The molecular weight of DNA bands was determined against the DNA marker using the Un-weighted pair-Group Method based on Arithmetic mean (UPGMA).

Restriction enzyme digestion

Following amplifications, the products were cut with two restriction endonucleases enzymes EcoR I (promega cat. # R6271) and Dra I (promega cat. # R6011).

For EcoRI digests approximately 1 µg/µl of amplified DNA was added to a restriction digest buffer containing 16.3 µl of sterile

Table 1. Primer sequences used in PCR.

Gene	Primer designation[a]	Primer sequence (5 ' ⟶ 3')	Annealing temperature
hrpB	RShrpBF	TGCCATGCTGGGAAACATCT	64
	RShrpBR	GGGGGCTTCGTTGAACTGC	
egl	EglF	AAATCCAGATATCGAATTGCCAA	57
	EglR	GCGTGCCGTACCAGTTCTG	
fliC	Rsol_fliCF	GAACGCCAACGGTGCGAACT	63
	Rsol_fliCR	GGCGGCCTTCAGGGAGGTC	

[a]F, forward primer; R, reverse primer.

deionized water, 2 µl of 10 X buffer H (promega), 0.2 µl of acetylated Bovine Serum Albumin (BSA) 10 µg/µl and 0.5 µl of restriction enzyme 10 u/µl up to total volume 20 µl. All digests were placed in a water bath at 37°C for 1 h.

Total reaction volume for DraI digests were 20 µl, including 1 µg/µl of amplified DNA, 16.3 µl of sterile deionized water, 2 µl of 10 X buffer B (promega), 0.2 µl of acetylated BSA 10 µg/µl mixed by pipetting and then 0.5 µl of restriction enzyme 10 u/µl was added. All digests were placed in a water bath at 37°C for 1 h.

The cutting DNA fragments were then separated by gel electrophoresis on 1.5% agarose at 75 V in TAE (40 mM Tris/acetate, 1 mM EDTA, pH 8.0) and stained with ethidium bromide (10 mg/ml).

The gel was analyzed using gel documentation system (AAB Advanced American Biotechnology 1166 E. Valencia Dr. Unit 6 C, Fullerton CA 92631).

Statistical analysis

The greenhouse experiment was carried out in a completely randomized design. The obtained data were subjected to the analysis of variance (ANOVA). Data were compared at 5% level using least significant differences (L.S.D.) according to Gomez and Gomez (1984).

RESULTS

Isolation and identification of the pathogenic bacteria

Fluidal, irregular, white and/or white with pink centers colonies, typical for *R. solanacearum* virulent form, were picked. Two isolates from potato tubers (S1, S2), two isolates from soil (S3, S4), one isolate from weed (S5) and one isolate from water (S6) were selected and used as virulent isolates. Developed less fluidal, uniformly round and completely pink to deep red colonies typical for *R. solanacearum* avirulent form were also picked, three isolates from potato tubers (S7, S8 and S9) were used as avirulent isolates or phenotypic conversion type mutants (PC-type mutants) .Virulent and avirulent isolates recovered from different habitats showed an agreement with *R. solanacearum* race 3 biovar 2 characteristics according to Bergey's Manual of systematic Bacteriology (Palleroni, 1984). Serological testing by means of Immunofluorescence antibody staining (IFAS) did not show any variation between the isolates either in morphology or in the degree of fluorescence.

Pathogenicity of virulent and avirulent isolates on different potato cultivars

Pathogenicity of the nine isolates (six virulent and three avirulent isolates) was tested in the greenhouse; all isolates were found pathogenic to the three different cultivars of potato plants namely Spunta, Hermes and Diamant. It is worth to note that a variable degree in wilting was noticed between the six virulent isolates (S1 to S6) with different cultivars as shown by the disease onset that ranged between 39-43 days and the disease index as shown by a complete wilting of the inoculated plants .On the other hand, the three avirulent isolates (S7, S8 and S9) were still pathogenic to the three cultivars of potato plants causing stunting, chlorosis and adventitious root formation (Figure 1).

Disease index results in Table 2 show that the most two pathogenic isolates were found to be those of the tuber origin (S1 and S2). They produced the higher disease indexes with all cultivars along with an early disease onset (39 days).However, the water isolate (S6) produced the least disease indexes in the three cultivars and showed late disease onset (43 days).While, soil and weed isolates (S3, S4 and S5) were intermediate in this regard.

Characterization of *R. solanacearum* virulent and avirulent strains by using DNA-based techniques

PCR - technique using Specific primer

DNA amplification of all isolates (six virulent and three avirulent) was performed using specific primers targeting the three virulence related- genes (*hrpB* – *egl* and *fliC*) which located on the megaplasmid of the bacteria. Using PCR specificity test yielded DNA fragments with the expected size 810, 686 and 318 bp when the primer pairs

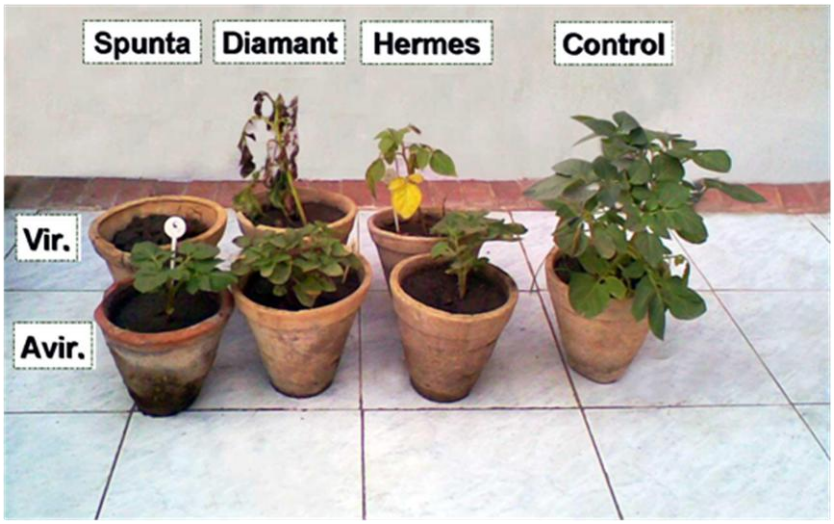

Figure 1. Effect of virulent and avirulent isolates on different potato cultivars.
Vir: Virulent isolates; Avir: Avirulent isolates.

Table 2. Disease index (D.I %) of bacterial wilt on different potato cultivars after inoculation with *R. solanacearum.*

Source of isolates	No. of isolate (virulent isolates)	Disease index (%) on different potato cultivars (after 63 days)			Mean
		Spunta	Diamant	Hermes	
Tuber	S1	100	86.4	43.2	76.53
Tuber	S2	93.2	73	39.8	68.66
Soil	S3	73	56.4	23	50.8
Soil	S4	86.4	69.6	33	63
*Weed	S5	69.8	52.8	23	48.53
Water	S6	59.6	56.4	19.8	45.27
	Control	0	0	0	0
	Mean	68.86	56.37	25.97	

*Weed: stems of (Amaranthus viridis)
L.S.D at 5% level for: Cultivars (A) 7.88; Isolates (B) 11.16 and Interaction (AB) N.S (non-signifigant).

(RShrpBF, RShrpBR); (EglF, EglR) and (Rsol_fliCF, Rsol_fliCR) were used, respectively in both virulent and avirulent isolates as shown in Figures 2, 3, 4 and Table 3.

These results show that the existence of megaplasmid in both virulent and avirulent isolates of *R. solanacearum* contains the three genes which involved in the disease process causing different symptoms.

DNA digestion with endonuclease restriction enzymes

For differentiation between virulent and avirulent isolates of *R. solanacearum* using restriction enzymes, the three amplified fragments of genes *fliC*, *hrpB* and *egI* were digested with two restriction enzymes EcoRI and Dra I. EcoRI digestion gave no differences between virulent and avirulent isolates. Digested *hrpB* gene by EcoRI enzyme gave two bands of 955bp and 593 bp in both virulent and avirulent isolates (Figure 5). Digested *egI* gene fragment and *fliC* gene fragment by EcoRI enzyme gave one band in both virulent and avirulent isolates at 1022 and 428 bp, respectively. As shown in Figures 6, 7 and Table 4.

Restriction digested of the three genes by DraI enzyme gave variation between virulent and avirulent isolates only with one gene, while it had no variation with the other two genes.

Only one band for each gene was obtained from digested *hrpB* and *fliC* genes by DraI enzyme at 1485 and 376 bp (Figures 8 and 10), respectively in both virulent and avirulent isolates. On the other hand, digested *egI* gene by DraI enzyme gave two bands with all virulent isolates (S1 to S6) the first band at 698 bp and the second one at 613 bp .While it gave only one band with avirulent isolates (S7 to S9) at 657 bp (Figure 9) , as shown in Table 4.

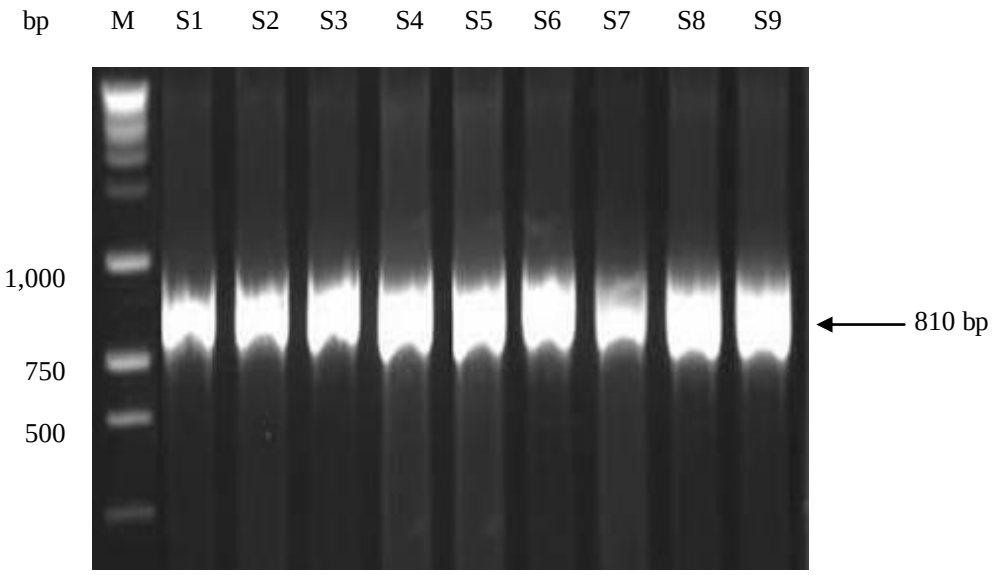

Figure 2. Genomic DNA amplification of *R. solanacearum* using primer pair (RShrpBF, RShrpBR), Lane (M): DNA marker, Lane (S1, S2, S3, S4, S5 and S6): amplified products from total DNA of the six virulent isolates, Lane (S7, S8 and S9): amplified products from total DNA of the three avirulent isolates.

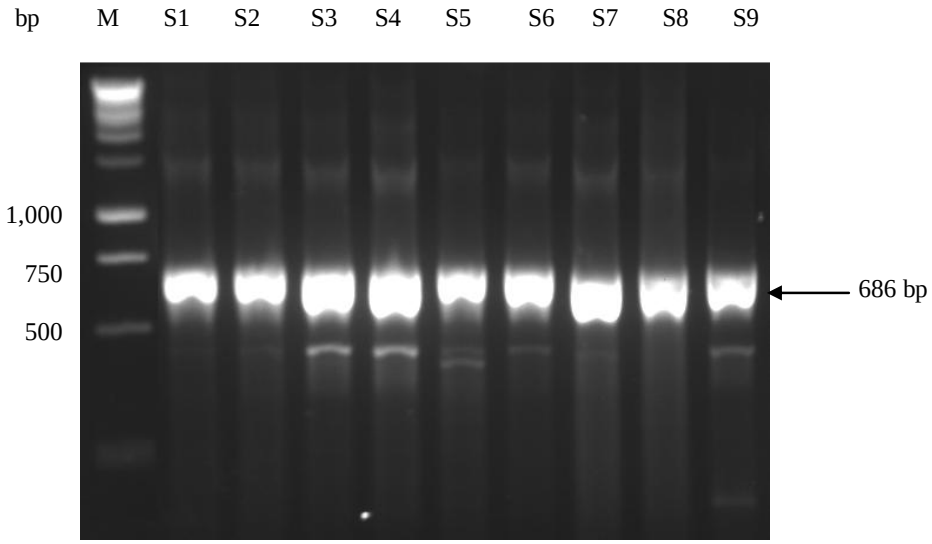

Figure 3. Genomic DNA amplification of *R. solanacearum* using primer pair (EglF, EglR), Lane (M): DNA marker, Lane (S1, S2, S3, S4, S5 and S6): amplified products from total DNA of the six virulent isolates, Lane (S7, S8 and S9): amplified products from total DNA of the three avirulent isolates.

DISCUSSION

Potato brown rot, caused by *R. solanacearum* (Yabuuchi et al., 1995) has created a lot of quarantine problems during the course of exportation of table potatoes to Europe (Farag, 2000). The shift from high typical virulent to avirulent form of *R. solanacearum* can be routinely detected on the basis of mucoid (highly virulent) and non mucoid (avirulent) colony morphologies on Kelman's agar medium (Kelman, 1954), this phenomenon is now known as phenotypic conversion (Kelman, 1954; Denny and Baek, 1991). The possible transformation of one form to the other is still controversy (Balabel, 2006).

In the present study, the obtained isolates were found

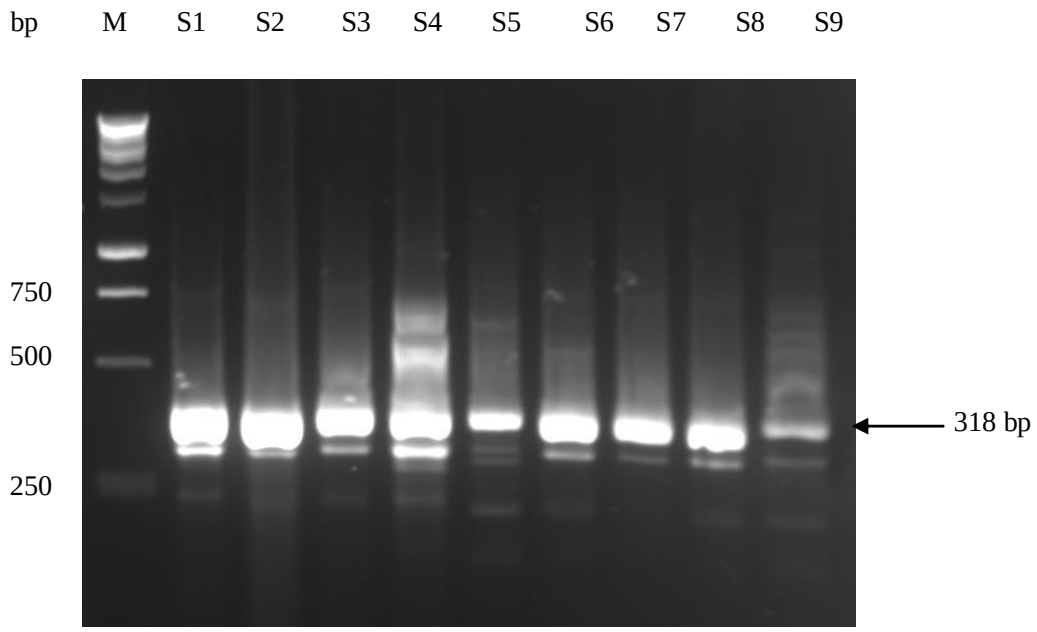

Figure 4. Genomic DNA amplification of *R. solanacearum* using primer pair (Rsol_fliCF, Rsol_fliCR), Lane (M): DNA marker, Lane (S1, S2, S3, S4, S5 and S6): amplified products from total DNA of the six virulent isolates, Lane (S7, S8 and S9): amplified products from total DNA of the three avirulent isolates.

Table 3. The three virulence- related genes located on the megaplasmid in virulent and avirulent isolates of *R. solanacearum.*

Gene	Fragment length (bp)	Virulent isolates					Avirulent isolates			
		S1	S2	S3	S4	S5	S6	S7	S8	S9
hrpB	810	+	+	+	+	+	+	+	+	+
egl	686	+	+	+	+	+	+	+	+	+
fliC	318	+	+	+	+	+	+	+	+	+

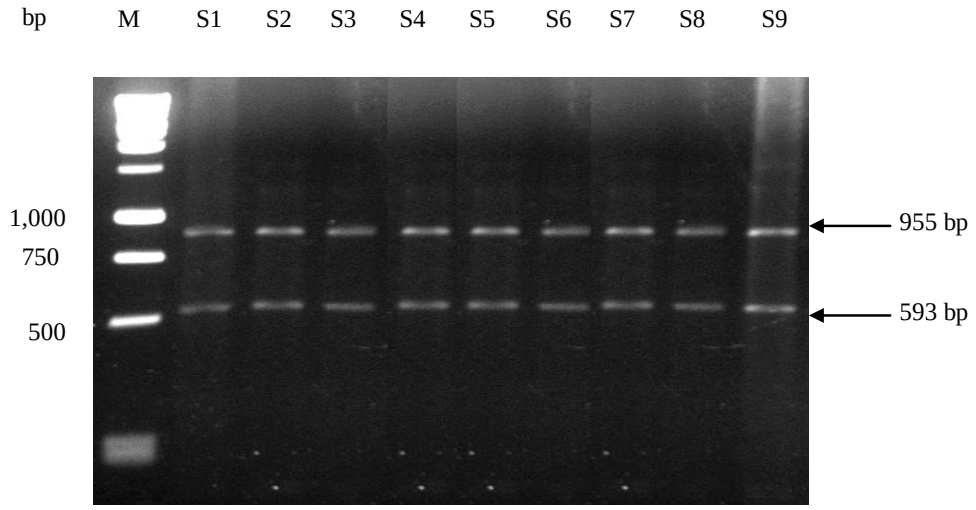

Figure 5. *hrpB* gene digested by EcoRI enzyme, Lane (M): DNA marker, Lane (S1, S2, S3, S4, S5 and S6): amplified products from the six virulent isolates, Lane (S7, S8 and S9): amplified products from the three avirulent isolates.

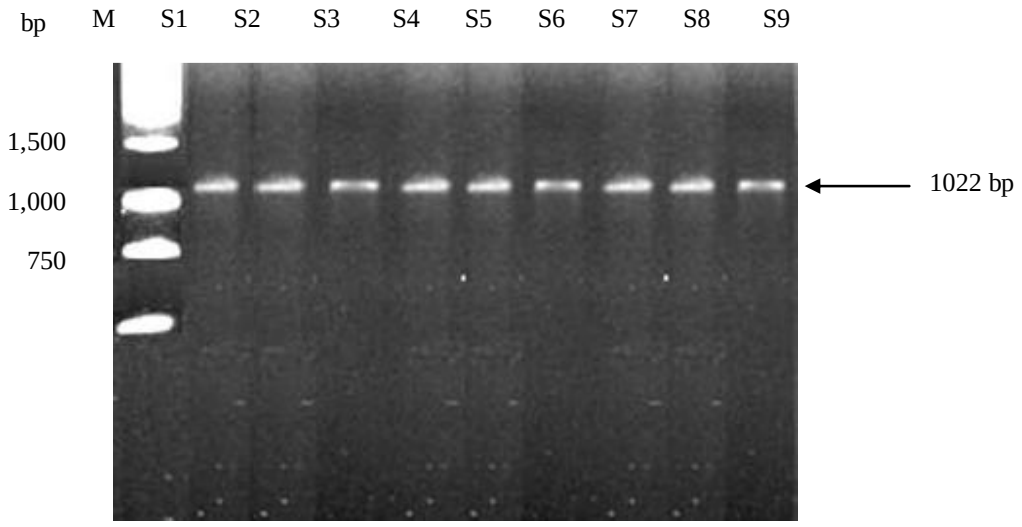

Figure 6. *egl* gene digested by EcoRI enzyme, Lane (M): DNA marker, Lane (S1, S2, S3, S4, S5 and S6): amplified products from the six virulent isolates, Lane (S7, S8 and S9): amplified products from the three avirulent isolates.

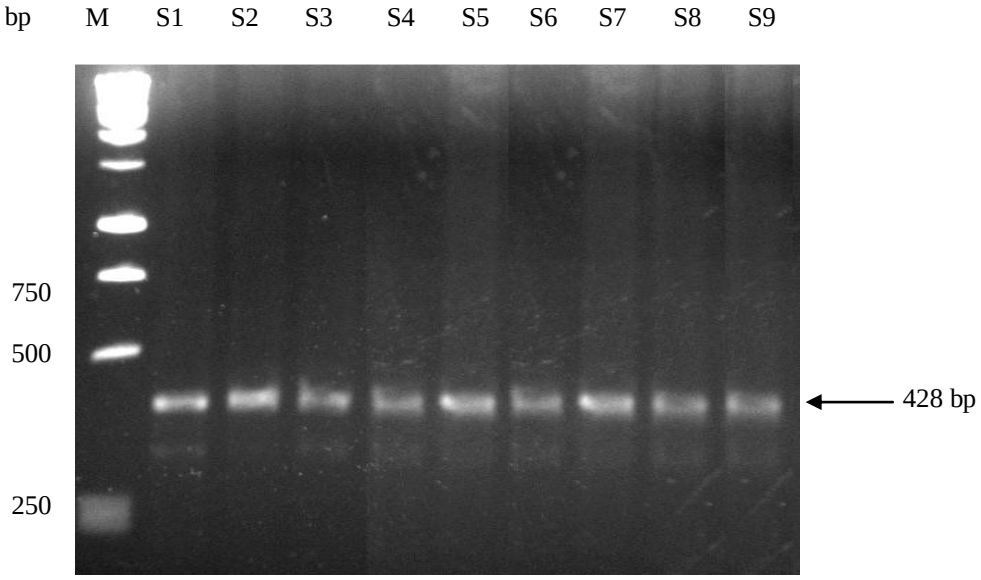

Figure 7. *fliC* gene digested with EcoRI enzyme, Lane (M): DNA marker, Lane (S1, S2, S3, S4, S5 and S6): amplified products from the six virulent isolates, Lane (S7, S8 and S9): amplified products from the three avirulent isolates.

similar to those described for Race 3, biovar II of *R. solanacearum*. No variation could be noticed between isolates from different sources. The results in general are similar to those reported by Palleroni (1984) .Serological testing for all isolates revealed no variation in either morphology or the degree of fluorescence on examination with the immunofluorescence antibody staining(IFAS) method (Janse, 1988).

From obtained isolates there were six virulent and three avirulent ones. The loss of virulence of *R. solanacearum* under laboratory growth conditions might be attributed to the development of avirulent or weakly virulent forms as a result of the repetitive culturing *in vitro* or certain growth conditions such as maintaining in broth culture (Kelman and Hruschka, 1973) or prolonged culture on agar plates (Buddenhagen and Kelman, 1964). The difference between the virulent wild types and avirulent form was demonstrated as difference in colony morphology when

Table 4. Digestion of the three virulence genes by EcoRI and DraI enzymes in the virulent and avirulent isolates of R. *solanacearum*.

Restriction enzyme	Genes	M.W (bp)	Virulent isolates						Avirulent isolates		
			S1	S2	S3	S4	S5	S6	S7	S8	S9
EcoRI	*hrpB*	955	+	+	+	+	+	+	+	+	+
		593	+	+	+	+	+	+	+	+	+
	egl	1022	+	+	+	+	+	+	+	+	+
	fliC	428	+	+	+	+	+	+	+	+	+
DraI	*hrpB*	1485	+	+	+	+	+	+	+	+	+
		613	+	+	+	+	+	+	--	--	--
	egl	657	--	--	--	--	--	--	+	+	+
		698	+	+	+	+	+	+	--	--	--
	fliC	376	+	+	+	+	+	+	+	+	+

M.W: molecular weight of bands.

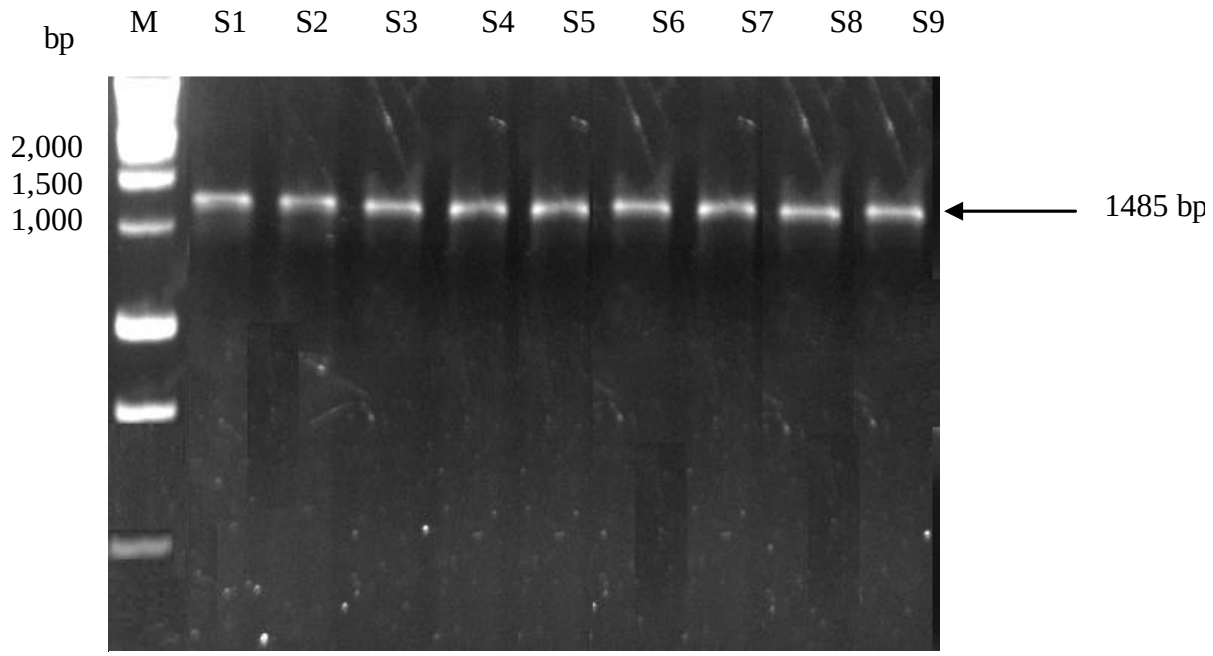

Figure 8. *hrpB* gene digested by DraI enzyme, Lane (M): DNA marker, Lane (S1, S2, S3, S4, S5 and S6): amplified products from the six virulent isolates, Lane (S7, S8 and S9): amplified products from the three avirulent isolates.

grown on peptone glucose (PG) media supplemented with 2, 3, 5 - Triphnyl tetrazolium chloride. Also, Shekhawat et al. (1992) found that the differences in colony morphology of *R. solanacearum* may be attributed to conversion of colonies from mucoid to non-mucoid this phenomenon is known as phenotypic conversion (PC). Consequently, the pathogen undergoes spontaneous phenotype shift from fluidal wild type to non fluidal avirulent or weakly virulent phenotype. The virulent colonies developed on semi selective medium of south Africa SMSA were milky, white, irregular and fluidal with blood red coloration in the center. The avirulent forms less fluidal or afluidal colonies were completely pink to red on that medium. The results are similar to those described by Elphinstone et al. (1996) and Anonymous (1997).

The virulent and avirulent isolates (PC- type mutants) were tested for their ability to cause wilting in three different potato cultivars. Pathogenicity test on the potato cultivars showed that all isolates (virulent and avirulent ones) were found pathogenic to potato plants causing different symptoms.

The six virulent isolates caused significantly wilting to the plants. Though the frequency of recovery from

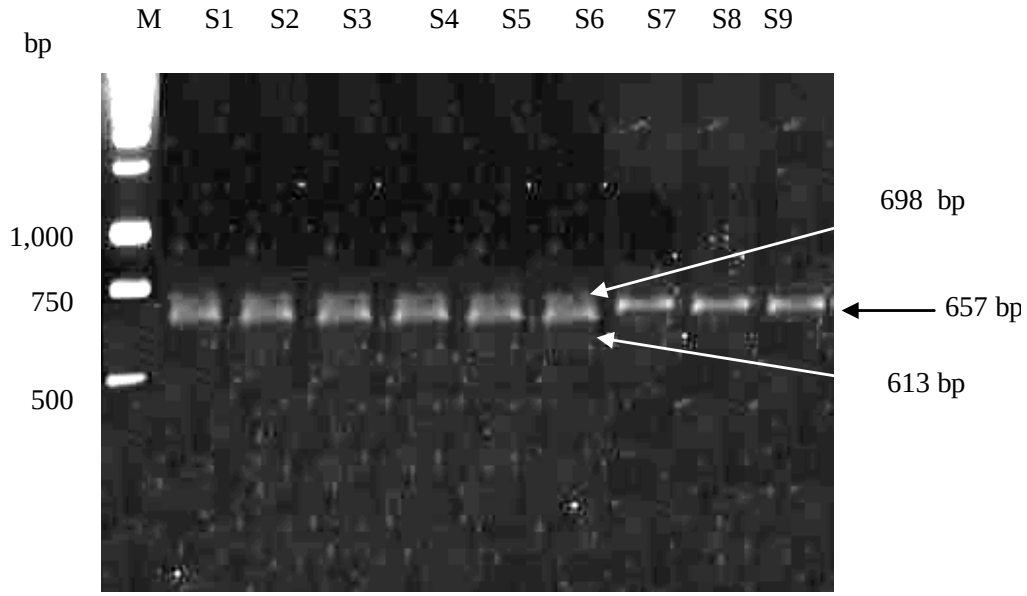

Figure 9. *egl* gene digested by DraI enzyme, Lane (M): DNA marker, Lane (S1, S2, S3, S4, S5 and S6): amplified products from the six virulent isolates, Lane (S7, S8 and S9): amplified products from the three avirulent isolates.

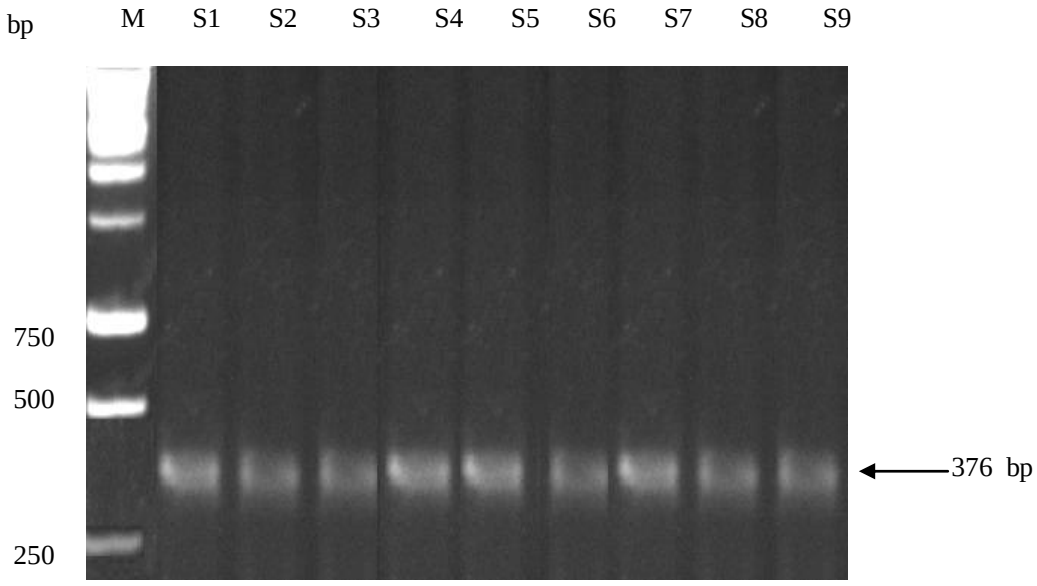

Figure 10. *fliC* gene digested by DraI enzyme, Lane (M): DNA marker, Lane (S1, S2, S3, S4, S5 and S6): amplified products from the six virulent isolates, Lane (S7, S8 and S9): amplified products from the three avirulent isolates.

different source was greatly different, all virulent isolates recovered showed pathogenic potential to the potato plants, the percentage of the disease index ranged from 19.80 to 100% when virulent isolates from different sources were used on different potato cultivars. It is important to note that the most pathogenic isolates were those recovered from potato tubers. While, the water isolate was the least one. Such differences between virulent isolates were also reported by Gerges (2007), the long distance dissemination from one continent to another was reported to be in diseased tubers, thus it is logic to score the tuber isolates with the most pathogenic

bacteria recovered. The intermediate pathogenic potential of the soil isolates may be attributed to the extrinsic factors, outside the plant, in the surrounding environment. The long term saprophytic soil phase may be considered as the most important extrinsic cause related to such intermediate pathogenic potential (Farag, 1976).

On the other hand, avirulent isolates didn't cause wilting symptoms on the plants. However, isolates were still pathogenic to the three different potato cultivars causing stunting, chlorosis and adventitious root formation. This result corresponds with those reported by Brumbley and Denny (1990) and also with those reported by Denny and Baek (1991) .Even so, this result contrasted with those of Ibrahim et al. (2005) who found that the weakly virulent or avirulent isolate produced symptoms comparable to those produced by the virulent isolate when getting an access to the host plant.

R. solanacearum is organized into two large circular replicons called the chromosome (the larger replicon) and the megaplasmid (Salanoubat et al., 2002). The two replicons in this bacterium have likely coevolved over a long time span. The megaplasmid “flexible” or “accessory” genome contains genes that differ among strains, are dispensable, and usually improve bacterial fitness. The flexible genome includes genes associated with virulence, antibiotic resistance, and mobile elements (Sarkar and Guttman, 2004). There is some degree of plasticity and instability for the *R. solanacearum* genome and a potential propensity to rapid evolution and adaptation. DNA rearrangements affecting virulence have already been reported to occur naturally in this pathogen (Brumbley et al., 1993).

This bacterium is characterized by a high level of phenotypic and genotypic diversity due to the horizontal gene transfer (HGTs) between strains (Gluidot et al., 2009) .Which mean that, the ability of bacterial species to acquire new genetic information from other strains or species by horizontal gene transfer (HGT) is a dominant force of variation in gene content (Koonin and Wolf, 2008).

Therefore, in a trial to investigate the genetic differences between the virulent and avirulent forms of *R. solanacearum*, losing the megaplasmid in the PC-type variant form was studied. Detection of three genes located on the megaplasmid showed the existence of the megaplasmid in the two forms of *R. solanacearum.* These results are in harmony with those reported by Gadewar et al. (1999) who demonstrated that there was no loss of the plasmid in both forms of *R. solanacearum* during the conversion.

The genetic variation of these three genes was also studied between both forms. In this work the three virulence-related genes which are located on the megaplasmid, are implicated directly as (*egl*) or indirectly as (*hrpB* and *fliC*) in disease-causing processes. The *egl* gene encodes an endoglucanase that likely acts at the front line of host invasion by partially degrading host cell walls (Liu et al., 2005). *hrpB* encodes an araC (1-ß-D-arabinofuranosylcytosine) type transcriptional regulatory protein that governs multiple virulence pathways (Occhialini et al., 2005). Flagellin, encoded by the *fliC* gene, is the essential subunit of the flagellar filament that is needed for invasive virulence (Tans-Kersten et al., 2001). In *R. solanacearum*, flagellin is not a major elicitor of host defenses (Pfund et al., 2004). DNA digestion with endonuclease restriction enzymes (EcoRI and DraI) showed no variation among the isolates either virulent or avirulent types in two genes *hrpB* and *fliC*. However, there was a variation in the *egl* gene between the virulent and avirulent isolates only when DraI enzyme was used, digested *egl* gene by DraI enzyme gave two bands with all virulent isolates the first band at 698 bp and the second one at 613 bp .While, it gave only one band with avirulent isolates at 657 bp .It could be noticed that the *egl* gene may partly play an important role in the different symptoms between the two forms of *R. solanacearum ,* as *egl* gene encodes an endoglucanase enzyme which is very important as one of the cell wall degrading enzymes (Denny et al., 1990). Besides, the *egl* gene may partly play a role in the shifting from mucoid to non-mucoid colony, whereby the avirulent bacteria lose their ability to produce exopolysaccharides and reduce their endoglucanase activity, also increase endo-polygalacturonase activity (Brumbley and Denny, 1990).

Generally, the results of the genetic variation of these three genes are in harmony with the results obtained by Castillo and Greenberg (2007) who found that Virulence-related genes have two evolutionary profiles: *hrpB* and *fliC* behave like essential genes, whereas *egl* is undergoing diversifying selection together with high recombination mainly phylotypes III and IV.

Finally, the genetic variations between the two different forms of *R. solanacearum* need further studies and more investigations to determine the genetic diversity of the natural phenomenon known as phenotypic conversion (PC).

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