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# Molecular Characteristics of Ten *Ralstonia solanacearum* Strains of Brown Rot Disease in Potato from three Governorates in Egypt

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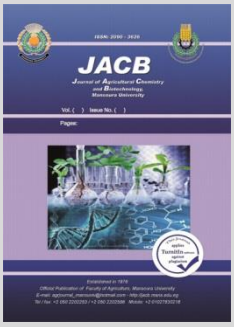


## ABSTRACT

Using tests based on serological, chemical and pathological aspects *Ralstonia solanacearum* strains (of brown rot disease) were identified in 10 strains were isolated from infected potato tubers grown in Egypt with biovar II pathogenicity and hypersensivity on leaves of tobacco and sugar utilization. , ISSR markers were used to differentiate the strains .Total number of amplified fragments was 68 bands and polymorphic bands were 38 representing 56% polymorphism. Twenty one bands were monomorphic and 9 were uniquely amplified DNA fragments of DNA which ranged between 7 to 16. The number of polymorphic fragments ranged between 1 to 13 for each primer. Values of genetic similarity among genotypes ranged from 63 to 92%. A dendrogram separated the strains into two major clusters. Pathogenicity tests showed QB.4, BK.5, BK.7 and Gk.8 isolates being highly virulent on tobacco in hypersensitivity test (HR) study. Virulence degree were between low and high on seedlings of tomato and potato and tomato seedlings. R. *solanacearum* (QB.4, QB.5, BK.7 and BK.8) showed highest disease incidence of 25.0, 24.6, 25.3 and 28.5%, respectively. Recorded wilt disease severity were 6.5, 6.2, 6.1 and 7.5 %, respectively at 42 days. Only the four isolates QB.4, QB.5, BK.7 and BK.8 were highly pathogenic to all of the studied seven potato cultivars of Accent, Alpha, Kara, Spunta, Draga, Sntana and Monalisa.

***Keywords:*** Brown rot disease, *Ralstonia solanacearum*, Genetic parameter, ISSR-PCR.

## INTRODUCTION

Following the three crops of wheat, maize and rice, potato (*Solanum tuberosum* L.) is the fourth largest food/feed crop in the world and is of utmost importance for mankind (Kaguongo *et al.* 2008 and Hamideldin and Hussien 2013). According to FAOSTAT data, the world production of potatoes reached about 376.453 million ton and the production of Egypt reached 4.8 million ton (http://faostat3.fao.org). Egypt is one of 15 countries which export 6.6% of unprocessed raw potatoes with shipped value of about 273 million US dollar during 2017 (Workman, 2018). About half of the daily requirements of vitamins C, tenth of vitamin $B_6$ and niacin for an adult person can be provided by about 150 g of potato ((Patil *et al.* 2016).

Potato is subjected to attack by many numerous fungal diseases such as Powdery scab, Powdery mildew, early blight, late blight, dry rot, wilt, silver scurf stem canker and black scurf. (Wilson *et al*., 2008 and Rubayet *et al*., 2018). Bacterial plant diseases are the most critical factors limiting production of the agricultural sector in most countries and often difficult to control. Brown rot caused by *Ralstonia solanacearum* predominates in tropical, subtropical and temperate regions and is one of the major constraints to growing many of the solanacea crops in these regions (Hayward 1991). It was first recorded in Egypt in a Gemmiza arable field in 1925 by Briton-Jones (1925). The pathogen is a heterogeneous and soil-borne.Five races were described according to the hosts (Hayward, 1991). The dominant race in Egypt is race 3, biovar 2 which widely spreads in Europe, suggesting it a possible origin of introducing to Egypt with seed tubers (Farag, 2000).

Currently, the species *R. solanacearum* is divided into 4 phylotypes, characterized by high genetic distances and genomic rearrangements, on basis of the following geographic regions: Asia (phylotype I) , America (IIA and IIB phylotypes), Africa (phylotype III ) (Fegan and Prior, 2005).

The range of host crops is spreading worldwide, aided by intensive agriculture systems and with the species being aerobic non-spore forming (Yabuuchi *et al.*, 1995). *R. solanacearum* belongs to β-Proteobacteria, Gram-negative, motile and soil borne limits the growth of many crops multiplies in the vascular plant parts causing wilting and death of the plant, with no change to the plant green colour (Momol *et al*., 2002). It is considered an infraspecific into 5 races because of its wide adaptation and variability (He *et al.*, 1983), and being in six biovars (Hayward, 1991). six biovars (Hayward, 1991). Molecular techniques enable assessing its evolution and phylogenecetic relationships (Silveira *et al*., 2005).

Inter Simple Sequence Repeats (ISSR) markers are derived from polymorphic genomic segment which are flanked by inversely oriented, closely spaced identical micro satellite sequences (Lenka *et al.*, 2015). Such markers are linked with some certain growth and yield traits of some crops used as Sequence Tagged Sites (STS) in the marker aided selection of these sites (Alam *et al.*, 2015). Therefore, these markers are suitable to identify and purify genotypes.

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Amplification of the ISSR was described as a quick stable by Ng and Tan (2015), Yadav *et al*. (2015) and Gautam *et a*, (2016).

The pathogen is extremely destructive (Yuliar *et al*. 2015) and causes extreme loss in great regions and areas in the (Champoiseau *et al*. 2009). It invades plant roots via openings and wounds, and eventually enters the xylem and spreads all over the plant (Yuliar *et al*. 2015). And eventually leads to death of the pant within three to four days (Sitaramaiah and Sinha, 1984). It resides in the vascular tissues, and in the lenticels as tolerant or latent carrier (Ghosh and Mandal, 2009).

The present work was aimed at the followings: 1. Isolating and identifying the pathogen from potato parts using different techniques. 2. Conducting a comprehensive study of the pathogen in different habitats and crops using ISSR-PCR. 3. Studying susceptibility of potato cultivars infection by it and 4. Conducting molecular studies using ISSR-PCR and the technique of Principals Co-ordinate Analysis (PCoA).

## MATRIALS AND METHODS

**Isolation and identification of *R. solanacearum* isolates:**

Potato stems and tubers infected with the disease were collected from the three Egyptian Governorates of Qalubiya (Moshtohor and Beltan), Beheira (Kom Hamada) and Gharbia (Kafr elnasria) during season 2017. The collected plant parts were washed thoroughly; air dried ready for laboratory work. Surface disinfection using 70 % ethanol was done followed by peeling and subsamples were taken and macerated in sterile distilled water; then streaked on TZC agar medium (Kelman,1954) and incubated for 48 to 72 h at 28±2 °C. Colonies of developed bacteria irregular mucoid were streaked onto fresh TZC medium for more purification. Then 2 loops of bacterial culture were transferred in 2 mL sterile double distilled water and stored at 20±2 °C. Identification of isolates was based on their morphological, physiological and biochemical properties i.e. cell shape, sporulation, Gram staining, motility, aerobic growth, potato soft rot, gelatin liquefaction, growth at 41°C, casein hydrolysis, H2S production, starch hydrolysis, nitrate reduction, catalase activity, o/f test and ability isolates to produce acid and gas from; glucose, mannose, fructose, maltose, lactose, mannitol and dulicitol, as described by Schaad, (1980), Lelliott and Stead, (1987) and Adhikari, (1993). Identification of R. solanacearum isolates was confirmed using polymerase chain reaction (PCR) ISSR techniques according to Lenka *et al.* (2015).

**Pathogenicity test and hypersensitive reaction:**

Isolates were subjected to hypersensitivity reaction (HR) tests using leaves of tobacco. Using spectrophotometer (SPECTRONIC 20-D), suspensions of bacteria were adjusted to 0.2 optical density at 600 nm ; about 108 colony forming units (cfu) per mL (Dhital et al. , 2001). One mL of the bacterial suspension was used to infiltrate one side of expanded tobacco leaf while the opposite side was treated with water as a control. Observation was carried out daily for the HR and lasted for 5 days following infiltration of the bacteria. Three host plants i.e. potato (*Solanum tuberosum* L. cv Spunta), tomato (*Solanum lycopersicum.* cv super strain b) and tobacco (*Nicotiana tabacum* L. cv Local) were planted in pots (25-cm Ø) containing 6 kg of sterilized sandy loam soil, and placed in the greenhouse at the Faculty of Agriculture Moshtohor, Benha University, Egypt and grew for 6 to 8 weeks or till reaching 15 to 20 cm high. From each of the three hosts , 3 plants were inoculated with the bacteria strains through insertion of sterile micropipettes containing 100uL at the axil of the fully-expanded leaf taThree plants of each host were inoculated with each strain of the bacterium by inserting a sterile micropipette tip containing 100 µL from the top of the axil of a fully expanded leaf. The tips of the pipette tips stayed in position till absorption of the inocula Inoculated plants were put under daily observation to detect any evaluation of pathogenicity and severity. Assessment of disease severity was done weekly for 4 weeks using the following scale and scoring system: 1: no symptoms; 2: two leaves wilted; 3: three leaves wilted; 4: four or more leaves wilted and 5: plant death; according to He *et al*. (1983)

**Virulence of isolates:**

The virulence test was done in the greenhouse where Potato sprouted tuber seeds were sown in the pots "one seed per pot". Pots were kept at 26 to 30°C and 60 to 80% relative humidity and seedlings were watered daily. For inoculation, the bacteria were grown on glycerol nutrient agar for 2 to 3 days at 28°C, suspended in sterile distilled water and adjusted at 109 cfu mL-1. Plants were inoculated at the stem base with bacterial suspension of the tested R. solanacearum isolates (Kelman, 1954). Plants inoculated with sterile water served as negative controls. Percentage of infection as well as severity of wilting was recorded 14, 28 and 42 days after inoculation.

Disease incidence percent (DI%) was determined considering the infected seedlings in relation to control according to the following equation:

**DI% = (No. of infected plants ÷Total No. of plants) x 100**

Area under disease progress curve AUDPC was determined to assess the response of inoculation (Pandey *et al.*, 1989)

**Evaluation of potato cultivar *in vitro*:**

Tubers of seven different potato cultivars, i.e. Accent, Alpha, Kara, Spunta, Draga, Sntana and Monalisa were inoculated with any of the isolated ten bacterial isolates to assess their pathogenicity. Bacterial dilution (0.5 ml at the rate of 1.3 × 108 cfu/ml) of each isolate was pipette individually at center of slice. Three slices were used for each tested isolate as well as the control. All treatments were incubated at 30±°C for 4 days then examined. Disease readings were expressed using the description of Lelliott and Dickey (1984) as follows:

**(-): Negative infection (+): Low positive infection (++): Moderate infection (+++): High infection.**

**Statistical analysis:**

Data were analyzed using analysis of variance (ANOVA) and means were compared using the least significant differences (LSD) at $p \leq 0.05$ (Song and Keane, 2006).

**ISSR-PCR Reactions:**

Six primers ISSR (Table 1) were used to detect polymorphism. Amplification reaction was done in 25 µL containing 1X PCR buffer, 1.5 mM MgCl2, 0.2 mM dNTPs, 1 µM primer, 1 U Taq DNA polymerase and 30 ng template DNA.

**Table 1.Sequence and names of ISSR primers.**

| Name Primer | Sequence 5'-3' |
|---|---|
| ISSR- 1 | 5'-AGAGAGAGAGAGAGAGYC-3' |
| ISSR- 2 | 5'-AGAGAGAGAGAGAGAGYG-3' |
| ISSR- 3 | 5'-ACACACACACACACACYC-3' |
| ISSR- 4 | 5'-AGAGAGAGAGAGAGAGYT-3' |
| ISSR- 5 | 5'-CTCCTCCTCCTCCTCTT-3' |
| ISSR- 6 | 5'-TCTCTCTCTCTCTCTCA-3' |

**A: Adenine; T: Thymine; G: Guanine; C: Cytosine and Y: (C or T).**

**Thermocyling Profile and Detection of the PCR Products**

PCR amplification was done using a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems) to fulfill 35 cycles following initial denaturation for 5 min at 94ºC. Each cycle was a denaturation step at 94ºC for 1 min, with annealing step at 50ºC for 1 min, and elongation for 1.5 min. at 72ºC. The primer extension segment extended for 7 min at 72ºC in the final cycle.

Amplification products were resolved by electrophoresis in a 1.5% agarose gel with ethidium bromide (0.5ug $mL^{-1}$) in 1X TBE buffer at 95 volts. A 1 kp DNA ladder was used as a molecular size standard. PCR products were visualized on UV light and photographed using a Gel Documentation System (BIO-RAD 2000).

**Data Analysis:**

The banding patterns generated by ISSR-PCR marker analyses were compared to determine the genetic relatedness of samples. Clear and distinct amplification products were scored as '1' for presence and '0' for absence of bands. Bands of the same mobility were scored as identical.

Genetic similarity coefficient (GS) between two genotypes was determined according to Dice coefficient of Sneath and Sokal (1973) as follows: -.

**Dice formula: GSij = 2a/(2a+b+c)**

**Where**

**GSij: is the measure of genetic similarity between individuals i and j; a is the number of bands shared by i and j; b is the number of bands present in i and absent in j, and c is the number of bands present in j and absent in i.**

The similarity matrix was used in the cluster analysis which was employed to organize the observed data for taxonomies. At the first step, when each accession represents its own cluster, distances between accessions are defined by the chosen distance measure (Dice coefficient). After everal accessions were linked, the distance between two clusters is calculated as an average between pairs of accessions. This method is called Unweighted Pair Group Method using Arithmetic Average (UPGMA) (Sneath and Sokal, 1973).

## RESULTS AND DISCUSSION

**Isolation of causal organism:**

Data in Table 2 showed that ten bacterial isolates were isolated from different parts of potato plants (stem and tuber) which were collected from different localities of Egypt. In this respect, four different isolates *i.e.,* QM.1, QM.2, QB.3 and QB.4 were isolated from Qalubiya Governorate (Moshtohor and Beltan). While, three isolates from Behera Governorate (Kom Hamada) were isolated from stem and tuber which coded as BK.5, BK.6 and BK.7. As for, three different isolates from Gharbia Governorate (Kafr Elnasria) were coded as GK.8, GK.9 and GK.10. In this study, ten bacterial isolates were isolated from potato parts tubers and stem. Using the traditional identification methods based on morphological, physiological and biochemical characteristics of bacteria, all isolates of bacteria were identified as *R. solanacearum*. Sumithra *et al.*, (2000) isolated *R. solanacearum* bacterium on tetrazolium chloride medium (TZC) from infected plants, symptom less plants, seeds extracted from fruits of wilted and stem inoculated plants developed with symptoms.

**Table 2. Source of bacterial isolates which collected from potato at different Governorates.**

| Governorate | Locality | Infected Sample | Isolate code |
|---|---|---|---|
| Qalubiya | Moshtohor | Stem | QM.1 |
| | | Tuber | QM.2 |
| | Beltan | Stem | QB.3 |
| | | Tuber | QB.4 |
| Behera | Kom Hamada | Tuber | BK.5 |
| | | Stem | BK.6 |
| | | Tuber | BK.7 |
| Gharbia | Kafr elnasria | Tuber | GK.8 |
| | | Tuber | GK.9 |
| | | Stem | GK.10 |

**Identification of bacterial isolates from potato plant:**

Results in Tables 3 showed that, all isolates showed short rod cells, gram negative, no sporulating, motile, aerobes, and gave positive reaction with tests of catalase activity, nitrate reduction, oxidative metabolism in O/F test and growth at 1% NaCl. However, all isolates gave negative reactions when tested for production of fluorescent on KB, gelatin liquefaction, starch hydrolysis, levan production, potato soft rot, $H_2S$ production, growth at 41º C and growth at 2% NaCl. Meanwhile, the tested isolates utilized glucose, mannose, Fructose, Lactose and Maltose but not Mannitol, Sorbitol and Dulicitol. Finally, the aforementioned tests and their result revealed that the all isolates *i.e.* QM.1, QM.2, QB.3, QB.4, BK.5, BK.6, BK.7, GK.8, GK.9 and GK.10 identified as *R. solanacearum.* Characterization of the causal agent was performed based on pathogenicity, biochemical, physiological and serological tests (Al-Ani *et al*., 2004 and Mahdy *et al*., 2012).

**Pathogenicity test and hypersensitive reaction:**

In this experiment, ten bacterial isolates were examined for their reaction on different hosts tobacco, tomato and potato. In this respect, data in Table 4 reveal that strain QB.4, BK.5, BK.7 and Gk.8 were virulent on tomato plants. The degree of virulence of the two strains QM.2 and GK.9 were moderate in both tomato and potato. The rest of the strains showed low virulence on potato and tomato. The QB.4, BK.5, BK.7 and Gk.8 isolates were highly virulent on tobacco in hypersensitivity test (HR) study. Symptoms on the tested hosts were recorded on the tobacco seedlings, appeared as water-soaking of inoculated tissue with 48 h. then dryness, light-brown localized necrosis with 3 days. All strains of *R. solanacearum* were pathogenic (low to high) on potato and tomato seedlings under greenhouse condition. In Pathogenicity test study, all strains were pathogenic (low to high) on potato and tomato seedlings. But other hosts such as tobacco show hypersensitive reaction. Hanafy, 2018 found that all tested 66 isolates of *R. solanacearum* infect potato plants (cv. Spunta) and tomato plants (cv. Ponto). El-

Ariqi *et al.* (2005) showed that isolates of *R. solanacearum* displayed varying levels of virulence on potato Spunta and Diamont cultivars. Matter (2008) stated that using indicator plant such as tomato had high accuracy for detecting *R. solanacearum*. Therefore, tomato seedlings were selected to detect pathogenicity for bacterial isolates causing wilt symptoms. Youssef (2013) found that potato cv. Draga-isolate of *R. solanacearum* infected many hosts with different degrees of wilt severity% where it was virulent to tomato, mallow, datura and little hogweed. While, it was not able to exhibit any visual wilt symptoms on pepper, eggplant, bean, maize, faba bean and onion plant hosts. Mikhail *et al.* (2017) found that virulent isolates of *R. solanacearum* showed high infection and disease severity on tomato plants after 5 days; and that virulent isolates showed low infection and disease severity with stunting and chlorosis.

**Table 3. Identification of bacterial isolates which isolated from potato plants.**

| Test | Reaction | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Qalubiya | | | | Behera | | | Gharbia | | |
| | QM.1 | QM.2 | QB.3 | QB.4 | BK.5 | BK.6 | BK.7 | GK.8 | GK.9 | GK.10 |
| Cell shape | Short rods | Short rods | Short rods | Short rods | Short rods | Short rods | Short rods | Short rods | Short rods | Short rods |
| Gram reaction | - | - | - | - | - | - | - | - | - | - |
| Spore production | - | - | - | - | - | - | - | - | - | - |
| Production of pigments | - | - | - | - | - | - | - | - | - | - |
| Catalase | + | + | + | + | + | + | + | + | + | + |
| Gelatin liquefaction | - | - | - | - | - | - | - | - | - | - |
| Starch hydrolysis | - | - | - | - | - | - | - | - | - | - |
| Nitrate reduction | + | + | + | + | + | + | + | + | + | + |
| Levan test | - | - | - | - | - | - | - | - | - | - |
| Production of $H_2S$ | - | - | - | - | - | - | - | - | - | - |
| Potato rot test | - | - | - | - | - | - | - | - | - | - |
| Growth at 41°C | - | - | - | - | - | - | - | - | - | - |
| Growth in 1% NaCl | + | + | + | + | + | + | + | + | + | + |
| Growth in 2% NaCl | - | - | - | - | - | - | - | - | - | - |
| O/F test | O | O | O | O | o | O | o | o | O | o |
| Utilization from: | | | | | | | | | | |
| Glucose | + | + | + | + | + | + | + | + | + | + |
| Mannose | + | + | + | + | + | + | + | + | + | + |
| Fructose | + | + | + | + | + | + | + | + | + | + |
| Lactose | + | + | + | + | + | + | + | + | + | + |
| Maltose | + | + | + | + | + | + | + | + | + | + |
| Mannitol | - | - | - | - | - | - | - | - | - | - |
| Sorbitol | - | - | - | - | - | - | - | - | - | - |
| Dulicitol | - | - | - | - | - | - | - | - | - | - |
| Biovar classification | II | II | II | II | II | II | II | II | II | II |
| Proposed identification bacterial isolates | *Ralstonia solanacearum* biovar 2 | | | | | | | | | |

**Table 4. Determination of *R. solanacearum* isolates based on hypersensitivity test (HR) and pathogenicity test on different hosts.**

| Isolates | Reaction | | |
|---|---|---|---|
| | Tobaco (HR) | Tomato | Potato |
| QM.1 | + | L | L |
| QM.2 | + | M | M |
| QB.3 | + | L | L |
| QB.4 | ++ | H | H |
| BK.5 | ++ | H | H |
| BK.6 | + | L | L |
| BK.7 | ++ | H | H |
| GK.8 | ++ | H | H |
| GK.9 | + | M | M |
| GK.10 | + | L | L |

**H= High virulent; M= moderate virulent; L= Low virulent; (+) = infiltrated area become necrosis and (-) = No reaction.**

**Virulence of *R. solanacearum* isolates on potato plants under greenhouse condition:**

Data in Table 5 reveal the virulence of ten *R. solanacearum* isolates on potato plants (cv. Spunta) in sterilized soil. A0ll tested *R. solanacearum* isolates caused bacterial wilt disease symptoms on potato plants compared with the un-inoculated control. In this respect, *R. solanacearum* the four isolates QB.4, QB.5, BK.7 and BK.8 recorded the highest disease incidence 25.0, 24.6, 25.3 and 28.5%. Meanwhile, recorded wilt disease severity 6.5, 6.2, 6.1 and 7.5 %, respectively at 42 day. The determined disease incidence and disease severities % were increased as time increased after inoculation from 14 to 42 days compared with the un-inoculated control under greenhouse conditions. Virulence of ten *R. solanacearum* isolates were showed the symptoms of brown rot and wilt disease caused by *R. solanacearum* on infected potato plants (cv. Spunta) grown under greenhouse conditions appeared in form of yellowing or sudden leaf wilting with down bending of these leaves before appearance the whole death onto potato plants. The recorded symptoms of brown rot disease are similar to those recorded by Mikhail *et al.* (2012) stated that *R. solanacearum* (Six virulent and three avirulent) recovered from the natural habitats (potato tubers, weeds, soil and water) were pathogenic to potato plants .El-Haj Saleh (2014) found that when potato plants were injected with *R. solanacearum* isolates, wilt symptoms appeared compared to non-injected potato plants this result confirms that all the tested isolates are virulent and belong to race 3 biovar 2. Hagag (2015) found that number of positive *R. solanacearum* isolates retrieved from infected tubers was higher than isolates retrieved from soil followed by weed isolates also, positive samples were highly recorded in Beheria governorate followed by Menofia governorate and the lowest recorded was in Ben-Seweif governorate.

**Table 5. Virulence of *R. solanacearum* isolates on potato plants (cv. Spunta) under greenhouse condition.**

| Tested isolates | 14 days | | 28 days | | 42 days | |
|---|---|---|---|---|---|---|
| | DI% | DS% | DI% | DS% | DI% | DS% |
| QM.1 | 7.8 | 1.0 | 10.0 | 2.0 | 12.4 | 3.3 |
| QM.2 | 10.4 | 2.4 | 11.0 | 2.8 | 13.6 | 3.9 |
| QB.3 | 7.5 | 1.0 | 10.2 | 2.1 | 13.1 | 3.2 |
| QB.4 | 20.0 | 5.0 | 20.4 | 5.3 | 25.0 | 6.5 |
| BK.5 | 15.8 | 4.3 | 18.0 | 4.8 | 24.6 | 6.2 |
| BK.6 | 7.3 | 1.0 | 9.8 | 2.2 | 13.0 | 3.7 |
| BK.7 | 15.2 | 4.2 | 17.5 | 4.6 | 25.3 | 6.1 |
| GK.8 | 20.1 | 5.0 | 21.4 | 5.4 | 28.5 | 7.5 |
| GK.9 | 10.6 | 2.6 | 13.3 | 3.9 | 18.0 | 4.5 |
| GK.10 | 7.0 | 1.0 | 9.9 | 2.0 | 14.5 | 3.9 |
| Control | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| LSD at 5% | | | Isolate | 0.152 | | |
| | | | Period | 0.041 | | |
| | | | Interaction | 0.455 | | |

**DI= %Disease incidence, DS=%Disease severity.**

**Susceptibility of seven potato cultivars infection by *R. solanacearum* isolates:**

In this experiment, ten isolates of *R. solanacearum* isolated from three governorates of Egypt were examined for their pathogenic capability to seven potato cultivars, *i.e.* Accent, Alpha, Kara, Spunta, Draga, Sntana and Monalisa. As shown in Table 6 only four isolates *i.e.* QB.4, QB.5, BK.7 and BK.8 were the highly pathogenic to all the potato cultivars used. On the other hand, the tested isolates QM.2 and GK.9 were moderately pathogenic to all tested potato cultivars. Moreover, four isolates QM.1, QB.3, BK.6 and GK.10 were low pathogenic to all potato cultivars. Meanwhile, cvs. Alpha and Spunta was susceptible to ten tested isolates of *R. solanacearum*. No infection was observed on the un-inoculated potato tubers of all cultivars. The evaluation test of the seven potato cultivars is in agreement with *in vitro* assays and approved their reliability. Dean *et al.*, (2006) tested that *R. solanacearum* isolates caused bacterial wilt disease symptoms on potato plants compared with the un-inoculated control. Also, all tested potato cultivars were susceptible to different extents and responded differently against infection with most tested isolates of *R. solanacearum*. Badr (2006) who found that all tested cultivars *i.e.*, Diamont, Braka, Picasso and Fabula were susceptible against potato wilt disease. Also, Hajhamed (2011) evaluated six potato cultivars against bacterial wilt disease using two different inoculation methods *i.e.* stem injection method and soil drench method. The cvs. *i.e.*, Diamont and Lady balfor were resistance to bacterial wilt disease. While, Nicola and Lady rosetta cvs., were highly susceptible to bacterial wilt disease. Mahdy, *et al;* 2012 noted that potato cultivars were susceptible to infect with *R. solanacearum* when cultivated in sterilized and un-sterilized soils. In the sterilized soil, isolate R6 recorded the highest percentage of infection and disease severity on cvs. Gelabica and kara whereas, the isolates R2 and R1 were the most virulent on cvs. Barren and Spunta, respectively. However, isolate R6 on cvs. Gelabica and isolate R4 on Cara, and R3 on Barren and R4 on Spunta recorded the highest disease severity in the un-sterilized soil. Hanafy, (2018) evaluated potato cultivars Bellini and Hermes and found them susceptible R. *solanacearum* infection.

**Table 6. Evaluation of seven potato cultivar by infection of *R. solanacearum* isolates *in vitro*.**

| Tested isolates | Potato cvs. | | | | | | |
|---|---|---|---|---|---|---|---|
| | Accent | Alpha | Kara | Spunta | Draga | Sntana | Monalisa |
| QM.1 | + | ++ | + | ++ | + | + | + |
| QM.2 | ++ | ++ | + | ++ | ++ | ++ | ++ |
| QB.3 | + | ++ | + | ++ | + | + | + |
| QB.4 | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| BK.5 | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| BK.6 | + | ++ | + | ++ | + | + | + |
| BK.7 | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| GK.8 | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| GK.9 | ++ | ++ | + | ++ | ++ | ++ | ++ |
| GK.10 | + | ++ | + | ++ | + | + | + |
| Control | - | - | - | - | - | - | - |

**(-): Negative infection; (+): Low infection; (++): Moderate infection and (+++): Highly infection.**

**Identification of ISSR primers:**

Ten genotypes of *Ralstonia solanacearum* strains of brown rot disease were selected which represented three governorates in Egypt.

The six primers which generated reproducible and scorable polymorphic marker were selected for this study. They produced multiple bands with a number of amplified DNA fragments ranging from 7 to 16, while the number of polymorphic fragments ranged from 1 to 13 (Table 7 and Fig. 1).

A maximum number of 16 fragments were amplified with the primers ISSR-1 and a minimum number of 7 fragments were amplified with the primers ISSR-5. The total number of fragments amplified was reached 68 bands. The polymorphic bands were 38, which represented a level of polymorphism of 56%. Moreover, 21 bands were monomorphic and 9 bands were unique. The size of amplifies fragments ranged from 100 to 1500 bp (Figure 1).

The ISSR analysis revealed a high level of polymorphism among genotypes. These results agree with Jackson *et al.* (2009), Gorji *et al.* (2011) and Moulin *et al.* (2012) who demonstrated that primers produced reliable and reproducible banding pattern and that the number, size of amplified DNA fragments and the percentage of generated polymorphic bands varied among primers in Potato. Hong *et al.* (2013) studied expression of the MSI-99m gene in transgenic potato plants which confers resistance to Phytophthora infectants and *Ralstonia solanacearum.*

**Table 7. Levels of polymorphism, total number of bands, monomorphic bands, polymorphic bands and percentage of polymorphism as revealed by ISSR markers among the ten *Ralstonia solanacearum* strains.**

| Code | Total number of bands | Monomorphic Bands | Polymorphic bands | Unique Band | Polymorphism (%) |
|---|---|---|---|---|---|
| ISSR- 1 | 16 | 2 | 13 | 1 | 81 |
| ISSR- 2 | 8 | 1 | 5 | 2 | 63 |
| ISSR- 3 | 13 | 1 | 12 | 0 | 92 |
| ISSR-4 | 15 | 8 | 1 | 6 | 7 |
| ISSR-5 | 7 | 6 | 1 | 0 | 14 |
| ISSR-6 | 9 | 3 | 6 | 0 | 67 |
| Total | 68 | 21 | 38 | 9 | 56 |

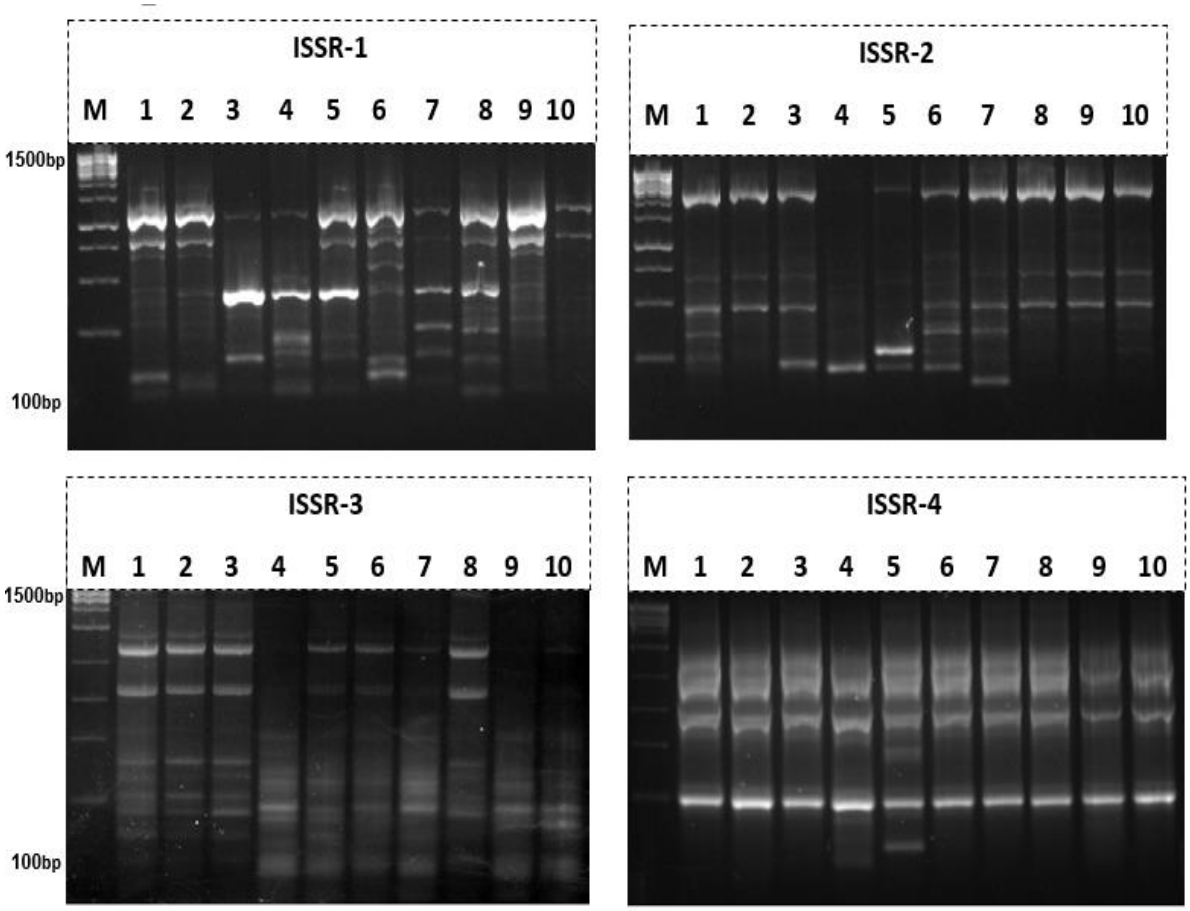


**figure 1. Amplification of the ten *Ralstonia solanacearum* strains of brown rot disease with ISSR-PCR primers No. 1, 2, 3 and 4.**

**M: marker; (1): QM.1; (2): QM.2 (3): QB.3; (4): QB.4; (5): BK.5; (6): BK.6; (7): BK.7; (8): GK.8; (9): GK.9 and (10): GK.10.**

**Genetic similarity:**

Genetic similarity among the ten *Ralstonia solanacearum* strains was determined on basis of scored ISSR data matrix. This similarity matrix was used to generate a dendrogram using the UPGMA method. The ISSR data analysis (Table 8) show that the genetic similarity ranged from 63 to 92 %. In addition to ISSR analysis the highest similarity level (92%) was detected between 1 (QM.1) and 2 (QM.2) which are closely related. However, lowest genetic similarity (63%) was between 1(QM.1) and 10 (QB.4).

A dendrogram separated the ten *Ralstonia solanacearum* strains into two major clusters. The first cluster could be divided into two sub-cluster; one sub-cluster divided into two groups which contained BK.6, BK.7 and GK.9, GK.10; respectively. Other sub-cluster contained QB.3, GK.8 and QM.2, QM.1. The second cluster contained QB.4 and BK.5 (Fig. 3).

The current ISSR analysis was successfully able to determine the phylogenetic between the genotypes under this study. These results agree with Bornet *et al.* (2002), Torabi *et al.* (2015) and Onamu *et al.* (2016). Gorji *et al.* (2011) assessed the genetic diversity and relationship of dominant Markers (SCOT, ISSR and RAPD) for Diagnostic Fingerprinting in Tetraploid Potato.

**Table 8. Genetic similarity among the ten Ralstonia *solanacearum* strains of brown rot disease as estimated using ISSR-PCR data using 6 primers.**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1.00 | | | | | | | | | |
| 2 | 0.92 | 1.00 | | | | | | | | |
| 3 | 0.85 | 0.90 | 1.00 | | | | | | | |
| 4 | 0.63 | 0.68 | 0.73 | 1.00 | | | | | | |
| 5 | 0.71 | 0.74 | 0.74 | 0.78 | 1.00 | | | | | |
| 6 | 0.81 | 0.80 | 0.80 | 0.72 | 0.82 | 1.00 | | | | |
| 7 | 0.73 | 0.76 | 0.76 | 0.70 | 0.71 | 0.83 | 1.00 | | | |
| 8 | 0.85 | 0.91 | 0.88 | 0.69 | 0.73 | 0.80 | 0.79 | 1.00 | | |
| 9 | 0.72 | 0.78 | 0.73 | 0.77 | 0.71 | 0.84 | 0.80 | 0.76 | 1.00 | |
| 10 | 0.73 | 0.77 | 0.77 | 0.76 | 0.72 | 0.78 | 0.82 | 0.78 | 0.89 | 1.00 |

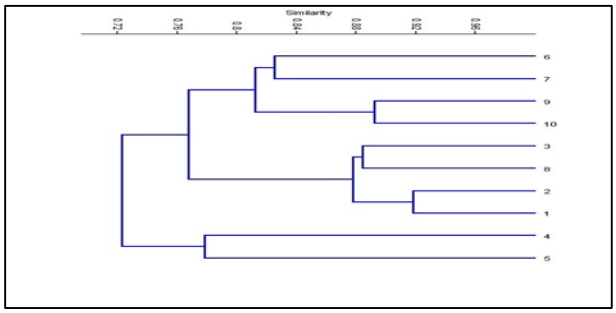

**Figure 2. Dendrogram generated based on UPGMA clustering method and Jacquared`s coefficient using ISSR analysis among the ten *Ralstonia solanacearum* strains.**

**(1): QM.1; (2): QM.2; (3): QB.3; (4): QB.4; (5): BK.5; (6): BK.6; (7): BK.7; (8): GK.8; (9): GK.9 and (10): GK.10.**

Genetic similarity matrix on basis of Jaccard's similarity coefficient was subjected to PCoA, for clearer visualization of genotypes relationships. The PCoA showed clustering in four quadrants variables (Fig. 3).

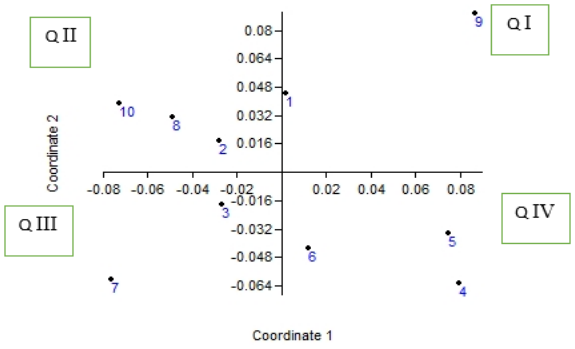


**Figure3.PCA plot for explained by two axes (Coordinates). Four quadrants were defined. M: marker; (1): QM.1; (2): QM.2; (3): QB.3; (4): QB.4; (5): BK.5; (6): BK.6; (7): BK.7; (8): GK.8; (9): GK.9 and (10): GK.10.**

PCoA analysis showed that 1 (QM.1) and 9 (GK.9) which on a distance from the other studied genotypes set in quadrant I. 2 (QM.2), 8 (GK.8) and 10 (GK.10) set in quadrant II, while 3 (QB.3) and 7 (BK.7) set in quadrant III. While 4 (QB.4), 5 (BK.5) and 6 (BK.6) genotypes located in quadrant IV (Fig.3). Differences of some results between PCoA and UPGMA may due to the difference of source or PCoA was more efficiency. These results agree with Dann, A. L., and Wilson, C. R. (2011), Gorji *et. al.,* (2011) and Ghebreslassie *et. al.,* (2016) who evaluated that genetic distance analysis generated three clusters correlating with the PCoA findings in Potato.

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## التوصيف الجزيئي لعشر سلالات *Ralstonia solanacearum* مسببة لمرض العفن البني في البطاطس من ثلاث محافظات في مصر

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تم إجراء توصيف سلالات *Ralstonia solanacearum* وهو العامل المسبب لمرض العفن البني في البطاطس من ثلاث محافظات في مصر ، وذلك بناءً على الاختبارات المرضية والإختبارات الكيميائية الحيوية / الفسيولوجية والمصلية. تميزت عشر سلالات من البكتريا المعزولة من درنات البطاطس المصابة التي تزرع في مصر بالبيوفار الثاني على أساس الإمراضية على عوائل النباتات المختلفة ورد فعل فرط الحساسية على أوراق الدخان واستخدام بعض اختبارات اختزال السكريات المختلفة. على المستوى الجزيئي تم استخدام تقنية ISSR-PCR للتمييز بين السلالات محل الدراسة , حيث أظهرت النتائج أن العدد الكلى للحزم الناتجة وصل إلى 68 حزمة منها 38 حزمة متعددة polymorphic bands و هى المسئولة عن الوصول إلى نسبة 56% تعدد مظهرى polymorphism . 21 حزمة من النوع monomorphic و 9حزم unique bands. أما على مستوى البوادئ فقد اختلف عدد الحزم باختلاف ال primer المستخدم حيث تراوح عددها من 7 الى 16 كما أن عدد polymorphic fragments تراوح من 1 الى 13 لكل primer. ومن خلال عمل تحليل لدرجة التشابه والقرابة بين العشرة تراكيب وراثية تم تقسيمهم إلى مجموعتين رئيسيتين حيث تراوحت درجة التشابه بينهم من 63-92% بين هذه التراكيب. كما أظهرت الإختبارات المرضية أن عزلات QB.4 و BK.5 و BK.7 و Gk.8 كانت شديدة العدائية على الدخان في دراسة اختبار فرط الحساسية كما تباينت درجة ضراوة جميع العزلات من منخفضة إلى عالية في شتلات البطاطس والطماطم. في هذا الصدد، سجلت QB.4 و QB.5 و BK.7 و BK.8 أعلى نسبة إصابة بالمرض هي 25.0 و 24.6 و 25.3 و 28.5 ٪. وفي الوقت نفسه، كانت شدة مرض الذبول المسجلة 6.5 ، 6.2 ، 6.1 و 7.5 ٪ ، على التوالي في 42 يوما. كانت أربعة عزلات فقط QB.4 و QB.5 و BK.7 و BK.8 مسببة للأمراض بشكل كبير لجميع أصناف البطاطس السبعة تحت الدراسة، وهي Accent :و Alpha و Kara و Spunta و Draga و Sntana وMonalisa.