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BIO-CONTROL OF POTATO BACTERIAL WILT CAUSED BY *Ralstonia solanacearum* ISOLATED FROM DIFFERENT SOURCES

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ABSTRACT: Seventeen pathogenic isolates of brown rot pathogen were collected from field soil, potato tubers, weeds and irrigation water at major potato districts in Egypt. Isolates recovered produced pink, or light red, colour with whitish margin on tetrazolium chloride medium (TZC) indicated possible virulent *Ralstonia solanacearum* pathogenic isolates. Pathogenicity test revealed the ability of the isolates to cause wilt symptoms in potted potato plant and three leaved tomato seedlings. Morphological, physiological and biochemical tests were used in identification. The biovar determination was made using for oxidization of disaccharides (maltose, lactose, and cellobiose) and confirmed the identity to *R. solanacearum*, race 3, biovar II equivalent to phylotype II, sequevar 1. A polymerase chain reaction (PCR) confirmed the identity of highest virulent isolates from tuber (T6) and from water (W11) to *R. solanacearum*. The application of biological agents *Pseudomonas fluorescens, Bacillus subtilis, Pseudomonas aeruginosa* and *Trichoderma* spp. decreased *R. solanacearum* infected plants and caused greater decrease in severity.

Key words: *Ralstonia solanacearum*, race 3, biovar II, phylotype II, sequevar 1, brown rot, potato, identification, PCR, biological control.

INTRODUCTION

Brown rot is the second most important potato disease in tropical and sub-tropical regions of the world after late blight (Champoiseau *et al*., 2010). The pathogen nomenclature was changed successively from time to time ended by *R. solanacearum* named by Yabuuchi *et al*. (1995). A soil-borne Gram-negative bacterium is a recognized parasite in over 200 families of plants, including potato, brinjal and tomato as well as many native plant species (Ahmed *et al*., 2013).

The brown rot disease was recorded on potato in Egypt and the bacterium was isolated for the first time in Egypt from potato tubers showing rot symptoms (Sabet, 1961) and extended to El-Habbaa *et al*. (2016) who reported the isolation from naturally infected potato tubers of three potato cultivars and bacteria showed typical morphology for *R. solanacearum* on SMSA medium (Balabel, 2006). Identification of the isolated bacteria were non-sporulating short rods with weak Gram negative reaction. Also, their developed colonies on nutrient agar (NA) medium were irregularly/round, convex, smooth surface, entire margin, translucent and yellowish brown in colour (Holt *et al*., 1994). The colonies were whitish-gray in colour on King's B (KB) medium forming brown pigments in most cases.

R. solanacearum has already been classified into five races based on differences in host range and six biovars on the basis of biochemical properties according to carbon source utilization (Hayward, 1991; Tohamy *et al*., 2007; Ahmed *et al*., 2013).

Pastrik *et al*. (2002) used PCR technique as one of rapid, highly specific and sensitive tests for detection and identification of *R. solanacearum* which isolated from different sources. In

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practice sensitivity of PCR reaction depends upon recovery of target DNA sequence and efficiency of the PCR reaction (Grover *et al*., 2012).

Biological control is supposed to preserves environmental quality by minimizing dependency on chemical inputs and maintaining sustainable management practices (Barea and Jeffries, 1995). Soesanto *et al*. (2013) reported the successful antagonistic *Bacillus* sp. and *Pseudomonas fluorescens* isolated from rhizosphere to control bactrial wilt on potato with delaying incubation period, suppressing disease intensity, decreasing final pathogenic population and inducing plant resistance. Atia *et al*. (2010) found that, the application of *Bacillus* sp*., B. subtilis* and *P. fluorescens* effectively controlled brown rot pathogen and increased potato yield (g/plant).

This research work aimed to survey *Ralstonia solanacearum* the causative agent of brown rot at different localities in Egypt as well as controlling the disease using some bioagents.

MATERIALS AND METHODS

Collection of Samples

One hundred and forty samples were collected from field soils, infected tubers, irrigation water and weeds (Table 1).

Isolation of the Pathogen

Potato tubers were washed in running tap water, surface sterilized by flaming. Then sections from stolen end were macerated in 10 ml sterile phosphate buffer (pH 7.0) in sterile plastic bags. The macerated tissues were allowed to stand for 30 minute**s** and the obtained suspension was used to inoculate plates of Semi Selective South Africa (SMSA) medium as described by Engelbrecht (1994) and modified by Elphinstone *et al.* (1996). Incubation was done at 28^{o}C and observed daily for developing fluidal, slightly raised, irregular white or white pinkish center colonies, typical for virulent colonies of *R. solanacearum.* Resulted virulent colonies were selected, picked up and inoculated on glucose nutrient agar medium and incubated for 48 hr., at 28^{o}C (Schaad, 1980).

Water samples (50 ml/ sample) were labeled, placed in an ice box and directly transferred to the laboratory. Samples collected from each site were centrifuged at 10,000 rpm for 15 minutes at 15^{o}C. The supernatant was discarded and pellet was re-suspended in one ml of phosphate buffer (pH 7.2), vortex for homogenization and plated on SMSA plates. Observation for the colonies growth was carried out as previously mentioned in isolation from potato tuber.

Soil samples (100 g) from each location as mentioned in collectio of sample were taken and mixed in the laboratory for homogenization. Isolation of the brown rot pathogen from soil was made on SMSA medium according to the method described by Van der Wolf *et al*. (1998).

Decimal dilutions of soil suspension obtained was made in 90 ml sterile phosphate buffer (pH 7.0) to 10^{-6}, shacked for two hours at 15^{o}C and inoculate SMSA plates incubated at 28^{o}C and observation for the colonies growth was carried out as previously mentioned.

Different weed plants associated with potato crop were collected. Roots were washed in running tap water and surface sterilized by flaming. Sterilized roots were aseptically cut into 5-10 mm sections, containing the main vascular and cortical tissues, were macerated in10 ml sterile phosphate buffer (pH 7.0) and were allowed to stand for 30 minutes before use.

Plating of the resulting supernatant and suspensions were streaked on Semi Selective South Africa (SMSA) medium selective for the pathogen as described by Wenneker *et al*. (1999).

Pathogenicity Test

Pathogenicity tests were conducted in greenhouse of Botany Department, Faculty of Science, Zagazig University. Seventeen *R. solanacearum* isolates recovered from different habitats and exhibited virulent morphology were confirmed by inoculation. Two weeks old healthy three leaved seedlings of GS12 tomato cultivars potted in 10 cm diameter plastic pots with sandy-clay soil (1/1, *V/V*) and Nicola potato plants grown in 30 cm diameter plastic pots containing sandy-clay soil. Inoculation with 10^{7}cfu/ml was done by stem puncture technique described by Janse (1988). Similarly, controls were carried out using sterile water instead of

bacteria. Three pots as replicates were used for each isolate. The inoculated plants were covered with polyethylene bags for three days, at 30°C to maintain high relative humidity, then the bags were removed and pots were irrigated regularly till the end of experiment (Algam *et al*., 2010). The disease progress was determined according to the key proposed for describing the wilt as follows: 1 = no symptoms; 2 = 1-25% of the plant was wilted; 3 = 26-50% of the plant was wilted; 4= 51-75 % of the plant was wilted and 5 = more than 75% of the plant was wilted. The disease severity was calculated by using the formula as disease index (%) (Kemp and Sequeira, 1983) as given below:

DI (disease index) =

$$\frac{\sum(\text{No. of wilted plants in treatment} \times \text{wilt grade})}{\text{Total No of plants} \times \text{highest grade}} \times 100$$

According to Koch's postulates, the inoculated bacteria were re-isolated from plants with typical wilt symptoms of the disease and the most pathogenic isolates were selected for the following tests. These isolates included soil isolates (S1, S2, S3 and S4), tuber isolates (T5, T6, T7, T8 and T9), water isolates (W10, W11, W12, W13 and W14) and weed isolates (We15, We16 and We17).

Identification of the Pathogen

Morphological and biochemical studies were carried out according to Bergy's Manual of Determinative Bacteriology (Holt *et al*., 1994; George and Garrity, 2012).

Morphological characteristics of isolated bacteria was done on King's B and TZC (Denny, 2006) and SMSA medium according to Engelbrecht (1994) and modified by Elphinstone *et al*. (1996).

The bacterial biochemical identification including pigmentation on KB medium, Gram stain, oxidase, catalase test, starch hydrolysis, gelatin liquefaction, lecithin hydrolysis, denitrification, casein hydrolysis, urea hydrolysis, growth in KCN, citrate utilization as C source, arginine dihydrolase, Levan formation, growth in KOH 3%, indol production and oxidation were carried out according to Koneman *et al*. (1983).

The isolates of *R. solanacearum* were differentiated into biovars based on their ability to utilize glucose and disaccharides (cellobiose, lactose, maltose) and sugar alcohols (manitol, sorbitol) as described by Hayward (1964).

The races of *R. solanacearum* were identified by pathogenicity test on host range. Identification of *R. solanacearum* isolates was confirmed using polymerase chain reaction (PCR) techniques according to Pastrik *et al*. (2002). The PCR assay was conducted at Potato Brown Rot Project, Agric. Res. Cent., Giza, Egypt.

A specific oligonucleotide primers are used, the forward oligonuclutide primer RS-1-F (5`-ACT AAC GAA GCA GAG ATG CAT TA -3`) and the reverse Primer RS-1-R, (5-`CCC AGT CAC GGC AGA GACT-3`). (Pastrik *et al*., 2002). The expected amplicon size from *Ralstonia solanacearum* template DNA is 718 bp. under the following reaction conditions:

Extraction of DNA

Crude DNA of *R. solanacearum* investigated in two isolates previously isolated from water and tuber (W11) and (T6) which proved to be virulent causing the highest disease severity, were extracted by heating 100 µl aliquots of cell suspension (10^6 CFU/ml) to 100°C for 5 minutes followed by cooled rapidly on ice.

DNA amplification

From each isolate 2 µl were added to 23 µl reaction mixtures [(16.025 µl, Sterile Ultra Pure Water (SUPW); 2.5 µl of 10X PCR buffer; 1.5 µl $MgCl_2$; 0.25 µl of BSA (fraction V) (10%); 0.125µl d-Ntp mix; 0.5µl primer Ps-1; .5µ primer Ps-2, and 0.1 µl Taq polymerase)]. Different PCR cycles were performed (1 cycle of 5 min at 95°C; 35 cycles of 30 sec at 68°C., 45 se. at 72°C and; final cycle at 72 of 5min (Pastrik *et al*., 2002).

Analysis of the PCR production

PCR fragments were detected by using agarose gel electrophoresis (AGE) and stained with ethidium bromide. Agarose gel was prepared by gently bringing agarose to the boiling 1X tris acetate EDTA (TAE) buffer (Seal *et al*., 1993). Agarose in TAE buffer was boiled for 5 min then cooled to 50-60°C.

Twenty wells were made in gel at 10-15 mm from the edge comb and the sealing tape was removed, the tray was placed in a large electrophoresis tank containing (1X) TAE buffer to a depth of at least 5 mm buffer above the gel. Three microleter droplets of loading buffer on parafilm were added to 12 µl of the PCR product from either sample. The positive control and distilled water as negative controls were mixed gently then loaded into the wells of the gel. An appropriate DNA marker was included as reference in at least one well. Gel was run by applying 80 voltages (V) at 400 mA (8 v/1 cm) until the front of tracking indicator being within one cm from the end then the power supply was switched off. Gel was removed carefully and soaked in the eithidium bromide solution (0.5 µg per L) for 30-45 min. A specific PCR product of 718 bp w as visualized under UV trans-illumination at 355 nm (Pastrik *et al*., 2002).

Effect of Bio-control Agents on Potato Bacterial Wilt

Isolates of *Bacillus subtilis, Pseudomonas fluorescensa* and *Pseudomonas aeruginosa* were kindly obtained from Plant Pathology Department, Faculty of Agriculture, Benha University, Egypt. *Tichoderma* sp. were kindly provided from Botany Department, Faculty of Science, Zagazig University.

Tubers

Tubers of three potato cultivars (Nicola, Mustang and Orchestra cvs.) were kindly provided by the Potato Brown Rot Project (PBRP), Dokki, Giza, Egypt.

Soil

Soil mixture was prepared at the ratio of 1:1 from clay and sandy soil collected from Abo-Hammad district, El-Sharkia Governorate, Egypt.

Preparation of Bio-control Organisms and Ralstonia Solanacearum Pathogen

The antagonists bacteria were prepared by culturing on King's B broth liquid at 150 rpm on rotary shaker, then incubated at 30°C for 48 hours and Czapek's broth at 150 rpm on rotary shaker for antagonistic fungi and incubated at 28°C for 5 days. The pathogen was prepared by culturing on King's B broth at 150 rpm on rotary shaker as well. Cultures were centrifuged at 10,000 rpm under cool 10°C for 10 min and the sediment was suspended in sterile buffer and adjusted to reach concentration of 10^8 cfu/ml at OD 600 for bacterial antagonists and 10^7 spore/ ml for fungal one. (Nurbaya *et al*., 2011).

Experiment

The experiment was conducted with completely randomized block design with three replications and 1 tuber per pot. Five kilogram of soil mixture was filled into pots, each 35 cm in diameter (Naser *et al*., 2008). The experiment conducted under greenhouse condition with:

1. The control; three control (s) were prepared

   Plant + bio-agent (+ve control),

   Plant (-ve control),

   Plant + *R. solanacearum* (control pathogen).

2. Three application tome *i.e.*, before, after and at the time of planting.
3. Two isolates of the pathogen; *R. solanacearum* W11 from water and T6 from tubers.
4. Biological control agents were tested individually with *P. fluorescens, B. subtilis, P. aeruginosa* and *Trichoderma* sp.

Experimental Infestation

Soil infestation was made at a rate of 150 ml of either adjusted suspention (s) *R. solanacearum* and adjusted antagonistic microbe through soil drenching (Sulistyo *et al*., 2012). The Pot treatments were divided into three groups:

In pot experiment I, the antagonists were introduced one week before planting and two weeks before pathogen inoculation.

In pot experiment II, the antagonists were applied one week after planting and two weeks after pathogen in this inoculation method, soils in pots were made to be fully infested with the *R. solanacearum.*

In pot experiment III, both antagonists and pathogen were introduced at the same time of planting. Each of the four antagonist suspensions was mixed separately with pathogen suspension in a screwed cap bottle equally, and after one hour, inoculated in the pots filled with planted soil.

Plants were kept in the greenhouse during the growing period at 24–28° and 75–90% relative humidity in 12 hr., light and 12 hr., dark conditions. All the pots were regularly irrigated throughout the growth periods.

Data Collection

Wilt incidence or disease index (%), percent survival or disease reduction (%) were recorded 85 days after planting.

The disease severity was calculated as mentioned in pathogenicity test.

Statistical Analysis

Data were subject to analyse of variance (ANOVA) according to Cochran and Cox (1957). ANOVA was carried out using IBM compatible computer using basic language. LSD test was used to compare treatments means at a 0.05 level of significance.

RESULTS AND DISCUSSION

Pathogen Isolation

A total of one hundred and forty isolates were isolated from the wilted potato tubers, irrigation waters, field's soil and weeds from different localities of Egypt during seasons of 2010 and 2011.

Total viable counts (CFU/ml) is shown in Table 1. The highest bacterial count (8.53×10^8 CFU/ml) was detected in tuber samples of El-Gharbia and the lowest count (3.53×10^3 CFU/ml) was recorded in soil samples collected from El-Dakahlia Governorate. Results demonstrated that Ismailia Governorate was almost free from the disease at the time of testing. The results showed that the selected isolates revealed the incidence of the disease in the territories surveyed and then underwent pathogenicity from the isolates. These results are similar to those reported by Saad (2011).

Pathogenicity Tests

Pathogenicity tests of the selected seventeen isolates were carried out under the greenhouse condition on tomato seedlings and potato plant (Table 2).

Results indicated that plants inoculated with the isolated pathogen were highly virulent and induced complete wilting at 20 to 23 days after inoculation. Tomato seedlings and potato plants showed disease symptoms within a short period after inoculation. After plant emergence, it took approximately two to three weeks for symptoms development (Jenkins *et al*., 1967). Disease severity was more in potato plants than tomato seedlings. The pathogen induced 36.4- 100.0% wilting in potato compared to 18.7- 87.3% wilt in tomato seedlings at the same period.

Soil isolates of *R. solanacearum* showed the lowest wilt symptoms and less virulence. On contrary, the plants inoculated with water isolates were the highest, causing 100.0- 72.3% and 84.1 - 48.2% wilt in potato and tomato, respectively. The highest severity of the disease was recorded with isolates coded T6 and W11, followed by T8, W12 and W14, and then T5, T9 and W10 isolates. Meanwhile, all isolates recorded positive results when reisolated on TZC medium. Similar results of isolated *R. solanacearum* from different sources were reported by Janse (1988) and Tohamy *et al*. (2007), Abd-Elrahim *et al*. (2015) and El-Habbaa *et al.* (2016).

Identification of the Isolated Pathogenic Bacteria

The macroscopic and the microscopic examination of the selected seventeen isolates, from different sources, were performed (Table 3 and Figs. 1 and 2). Microscopic studies revealed that bacterial isolates were gram-negative, rod-shaped, 0.5-0.7 × 1.5-2.0 µm in size non-capsulated and non-spore forming. Motility of each isolate was confirmed by performing hanging drop method (Table 3). Fig. 1 showed that florescent pigment is not produced on King's B medium. All isolates produced a brown pigment when grown on King's B medium after 72 hr., (Fig. 2) similar results were obtained by El-Habbaa *et al*. (2016). Typical colony characters of virulent *R. solanacearum* isolates were presented in Fig. 3. They produced typically fluid, irregular white colonies with reddish-pink centers on TZC medium. The same results were obtained by Engelbrecht (1994).

Table 1. The counts (CFU/ml) of *Ralstonia solanacearum* isolated from different sources and different localities in Egypt

Governorate	Region	No. of samples	Source of isolates			
			Soil	Tuber	Water	Weeds
Sharkia	El-Salhia city	16	-	3.82×10^4	-	-
	El-Molak village	9	-	-	-	-
Ismailia	Ismailia canal	12	-	-	-	-
	Ismailia city	11	-	-	-	-
	Ismailia canal	7	-	-	-	-
Gharbia	Kafr Yakoupe village	10	-	8.53×10^8 5.68×10^6	-	-
	Kafr El-Zayat	12	-	6.78×10^3 3.77×10^4	-	-
	Kafr Yakoupe water stream	14	-	-	6.78×10^7 4.18×10^5	-
Monufia	Tallia village	20	6.76×10^7 5.32×10^5	-	-	7.71×10^8 2.65×10^4 3.55×10^3
	Tallia village water stream and El-Basha	17	-	-	6.55×10^5 4.67×10^8 3.87×10^7	-
Dakahlia	Kotama village	12	3.59×10^4 3.53×10^3	-	-	-

CFU/ml = Number of colony forming units in ml - = Negative result.

Table 2. Pathogenicity tests of *Ralstonia solanacearum* isolates on tomato seedlings and potato plants

Isolate source		Severity of disease					
		Tomato seedlings			Potato plants		
		Severity group	Disease severity (%)	Re-isolation	Severity group	Disease severity (%)	Re-isolation
Soil	**S1**	2	27.8	+	2	36.4	+
	S2	2	30.6	+	3	42.3	+
	S3	2	18.7	+	2	36.7	+
	S4	2	30.1	+	3	49.8	+
Tuber	**T5**	3	51.4	+	3	67.6	+
	T6	5	87.3	+	5	100.0	+
	T7	2	33.4	+	3	72.7	+
	T8	4	79.4	+	5	89.8	+
	T9	3	57.4	+	4	79.6	+
Water	**W10**	3	55.7	+	4	80.4	+
	W11	5	84.1	+	5	100.0	+
	W12	4	73.8	+	4	85.9	+
	W13	2	48.2	+	3	72.3	+
	W14	4	74.0	+	5	89.7	+
Weed	**We15**	2	45.0	+	3	54.0	+
	We16	2	43.0	+	3	51.4	+
	We17	2	32.9	+	2	41.7	+

+ = Positive result.

Table 3. Macroscopic and examination of recovered isolates

Character	Source of bacterial isolates			
	Soil isolates 1, 2, 3, 4	**Tuber isolates 5, 6, 7, 8, 9**	**Water isolates 10, 11,12,13,14**	**Weeed isolates 15,16,17**
Shape of cell	Short rod shape	Short rod shape	Short rod shape	Short rod shape
Motility	+ + + +	+ + + + +	+ + + + +	+ + +
Gram reaction	G -ve	G -ve	G -ve	G -ve
Sporulation	no spore	no spore	no spore	no spore
Brown pigment	+ + + +	+ + + + +	+ + + + +	+ + +
Fluorescent pigment	not produced	not produced	not produced	Not produced

+= Positive reaction - = Negative reaction

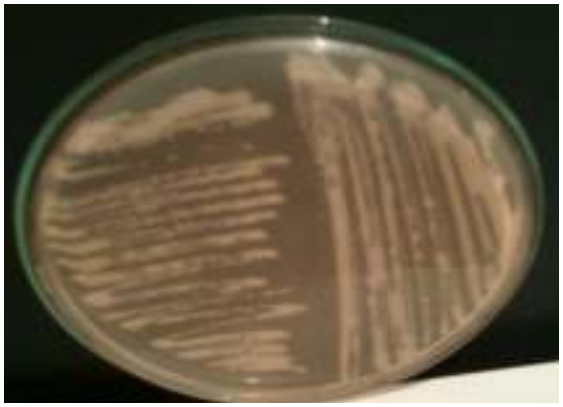
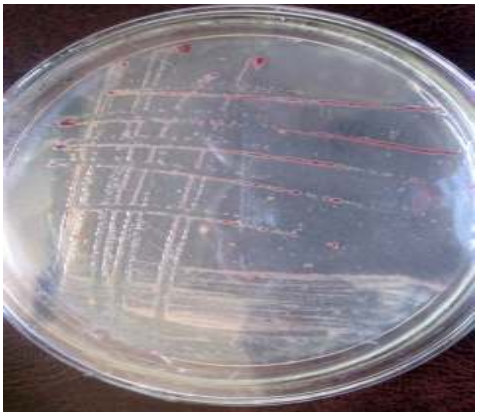


Fig. 1. Colony morphology of virulent isolate *Ralstonia solanacearum* on King's B medium

Fig. 2. Brown pigmentation of virulent *Ralstonia solanacearum* on King's B medium



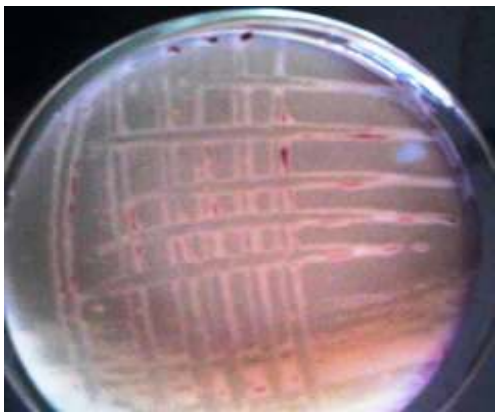


Fig. 3. Virulent colonies of *Ralstonia solanacearum* isolates on Tetrazolium chloride medium (TZC)

The isolates were characterized physiologically and biochemically and results tabulated as seen in Table 4. All isolates were grown at 37°C and failed at 41°C, strictly aerobic bacterium and positive for oxidase and catalase activities and Levan test (Schaad, 1980). They are denitrifying bacteria and were able to use citrate. They can hydrolyze of casein and urea but not starch. They do not show gelatin liquefaction, lecithin hydrolysis produced indol, arginine dihydrolase. These results are in accordance with those described by Holt *et al*. (1994). Like other gram negative bacteria, the isolates of *R. solanacearum* were able to develop blue color in Kovac's test by oxidizing the sugars.

Table 5 show that *R. solanacearum* isolates oxidized monosaccharides (glucose), disaccharides (maltose, lactose, and cellobiose) and did not oxidize sugar alcohols (sorbitol and mannitol) within 3-5 days. The oxidation reaction was indicating by the change of colour. The results revealed the change of colour blue to yellow indicating the oxidization of sugars by bacterial. The oxidation reaction was indicating by the change of colour. Therefore, all groups of *R. solanacearum* isolated have shown affiliation to race 3, biovar 2 as shown in Table 5. The differentiation of biovars of *R. solanacearum* based on the utilization of carbohydrates was reported previously by Hayward (1964), Tohamy *et al*. (2007) and Abd-Elrahim *et al*. (2015).

The races of *R.solanacearum* were identifyed by pathogenicity tests in host range such as potato and tomato. The result of the pathogenicity test in the greenhouse showed that, none of the group of *R. solanacearum* isolates was un able to cause wilt in inoculated tomato seedlings and potato plants indicating a narrow host range. Therefore, all isolates of *R. solanacearum* causing bacterial wilt of potato collected from three selected growing areas belong to race 3. These results have agreed with Graham and Lloyd (1978) who they reported that tomato seedlings and potato plants have commonly been used as indicator plants. Five races have been described so far, but they differ in host range, geographical distribution and ability to survive under different environmental conditions (French, 1986). Patrice (2008) reported that, *R. solanacearum* was initially subdivided into races and biovars based on variability in host range. He added that five races have been identified within the species.

Torres (2001) stated that strains of *R. solanacearum* are grouped into five races according to the host or according to the use of selected properties. Balabel (2006) stated that potato brown rot, caused by *R. solanacearum* race 3 biovar 2, the so- called potato race, is the only strain in potato on Egypt and is a serious endemic disease in the Nile Delta, race1, however, could not be detected. Molecular analysis have confirmed that the Egyptian isolate confirm with those of phylotype II, sequevar 1.

Identification of *Ralstonia solanacearum* by Polymerase Chain Reaction (PCR)

Identification of *R. solanacearum* isolates to strain level/reference from different sources was confirmed using PCR technique, as illustrated in Fig. 4. Results of PCR technique of the highly virulent tuber T6 and water W11, showed that the two samples visualized specific 718 bp PCR product under UV light. PCR results indicated very close similarity without any variation among the two tested isolates. The positive control using *R. solanacearum* identified by the Brown Rot Project in Egypt sponsored this investigation, has confirmed that the two tested bacterial isolates are *R. solanacearum.* Several workers confirmed the identification of *R. solanacearum* using PCR (Pastrik *et al*., 2002; Tohamy *et al*., 2007; Mahdy *et al*., 2012; Abd-Elrahim *et al*. 2015).

Effect of Adding Different Bio-control Agents on Potatoes Bacterial Wilt at Three Different Times

Biological control studies were carried out in greenhouse with *P. fluorescens, B. subtilis, P. aeruginosa* and *Trichoderma* sp. Results indicated that the tested cultivars were highly susceptible to *R. solanacearum* W11 and T6 with score 100% of disease index compared to control plants.

The application of the tested bi-control organisms to *R. solanacearum* infected plants caused higher decrease in the disease index compared with control. The potential of the bio-control agents in decreasing the disease in a descending order as *P. fluorescens, B. subtilis, P. aeruginosa* and *Trichoderma* sp. Potato cultivar Orchestra, Mustang and Nicola were less sensitive to disease progress with the isolates W11 than T6 strain under effect of such agents (Table 6).

Table 4. Physiological and biochemical characteristics of pathogenic bacteria recovered

Character	Source of bacterial isolates			
	Soil 1, 2, 3, 4	Tuber 5, 6, 7, 8, 9	Water 10, 11, 12, 13, 14	Weed 15, 16, 17
Growth at 41˚C	- - - -	- - - - -	- - - - -	- - -
Kovac's test (Oxidase reaction)	+ + + +	+ + + + +	+ + + + +	+ + +
Gelatin liquefaction	- - - -	- - - - -	- - - - -	- - -
Starch hydrolysis	- - - -	- - - - -	- - - - -	- - -
Lecithin hydrolysis	- - - -	- - - - -	- - - - -	- - -
Denitrifaction	+ + + +	+ + + + +	+ + + + +	+ + +
Casein hydrolysis	+ + + +	+ + + + +	+ + + + +	+ + +
Urea hydrolysis	+ + + +	+ + + + +	+ + + + +	+ + +
Growth in KCN	+ + + +	+ + + + +	+ + + + +	+ + +
Citrate utilization	+ + + +	+ + + + +	+ + + + +	+ + +
Catalase activity	+ + + +	+ + + + +	+ + + + +	+ + +
Indol production	- - - -	- - - - -	- - - - -	- - -
KOH 3%	+ + + +	+ + + + +	+ + + + +	+ + +
Arginine dihydrolase	- - - -	- - - - -	- - - - -	- - -
Levan formation	+ + + +	+ + + + +	+ + + + +	+ + +

+= Positive reaction - = Negative reaction

Table 5. Results of *Ralstonia solanacearum* biovar determination

Bacterial isolates	Sugar sources					
	Glucose	Maltose	Lactose	Cellobiose	Sorbitol	Mannitol
Soil 1,2,3,4	+ + + +	+ + + +	+ + + +	+ + + +	- - - -	- - - -
Tuber 5,6,7,8,9	+ + + + +	+ + + + +	+ + + + +	+ + + + +	- - - - -	- - - - -
Water 10,11,12,13,14	+ + + + +	+ + + + +	+ + + + +	+ + + + +	- - - - -	- - - - -
Weeds 15,16,17	+ + +	+ + +	+ + +	+ + +	- - -	- - -

+ = Positive reaction - = Negative reaction

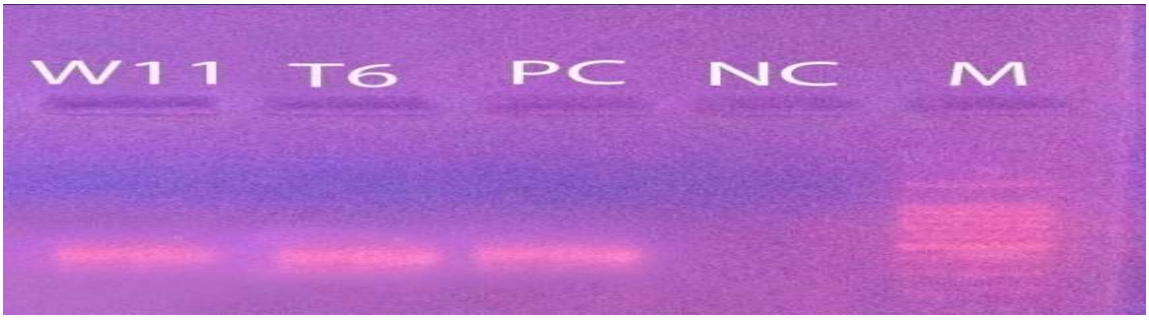


Fig. 4. PCR products amplified using (Oli-1) from genomic DNA of different two *R. solanacearum* isolates from different habitats. PC= positive control, NC = negative control, M = marker, T6 = tuber isolate, W11 = water isolate

Table 6. Effect of different bio-agents at different times using different cultivars of potato against. *Ralstonia solanacearum* W11 and T6 strains on disease index (%)

Treatment	Strain	Disease index (%)					
Plant (-ve control)		**Cultivars**					
		Orchestra		**Mustang**		**Nicola**	
		0.0		0.0		0.0	
Plant + pathogen		Pathogen strains					
		Water W11			Tuber T6		
		Cultivars					
		Orchestra	**Mustang**	**Nicola**	**Orchestra**	**Mustang**	**Nicola**
		100.0	100.0	100.0	100.0	100.0	100.0
Plant + bio-agents (+ve control)		**Cultivars**					
		Orchestra		**Mustang**		**Nicola**	
	Pseudomonas fluorescens	0.0		0.0		0.0	
	Bacillus subtilis	0.0		0.0		0.0	
	Pseudomonas aeruginosa	0.0		0.0		0.0	
	***Trichoderma* sp.**	0.0		0.0		0.0	
Plant + bio-agents + pathogen		**Pathogen strains**					
		Water strain W11			**Tuber strain T6**		
		Cultivars					
		Orchestra	**Mustang**	**Nicola**	**Orchestra**	**Mustang**	**Nicola**
Before	***Pseudomonas***	23.0	23.0	32.0	25.0	27.0	32.31
After	***fluorescens***	36.0	45.0	42.31	37.5	38.6	43.85
Zero		24.0	26.6	27.3	33.3	28.0	30.3
Before	***Bacillus***	20.63	26.6	28.1	22.27	28.0	32.0
After	***subtilis***	35.0	40.88	45.1	38.0	50.0	51.5
Zero		22.33	26.1	37.0	24.0	42.0	40.0
Before	***Pseudomonas***	27.1	32.0	29.0	37.8	44.0	49.0
After	***aeruginosa***	37.0	38.9	42.0	43.0	65.0	52.0
Zero		30.66	37.14	43.0	39.0	38.0	50.0
Before	***Trichoderma* sp.**	55.0	60.0	60.0	50.0	60.0	60.0
After		70.0	74.0	80.5	72.5	73.5	79.8
Zero		59.0	64.0	64.5	56.0	64.2	76.65

LSD 0.05 C	0.44	1.03
LSD 0.05 B	0.55	0.72
LSD 0.05 C × B	0.95	1.25
LSD 0.05 T	0.37	0.37
LSD 0.05 C × T	0.65	0.64
LSD 0.05 B × T	1.24	1.33
LSD 0.05 C × B × T	2.15	2.12

C = cultivars B=Bio-agents T = Treatments

Soil treated with the tested agents one week before planting or preplanting decreased disease index of the three potato cultivars compared to post planting or at zero time of experimentation. Nguyen *et al*. (2011) reported that disease severity was high when both antagonist and pathogen were introduced simultaneously compared to antagonist supplemented prior the pathogen. The increased disease severity may be attributed to the chemotactic effect of the rizosphere on the pathogen, hence greater inoculum density before reassemble establishment of the antagonist in the root zone appeared to be the opportunity available for the pathogen to easily invade the root cells in the logically, the suppression must occur before infection start in situ.

Potato treated with *Trichoderma* spp. one week after planting showed the highest wilt index recording 70.0, 74.0 and 80.5% in case of *R. solanacearum* W11and 72.5, 73.5 and 79.8.0% of disease index in case of *R. solanacearum* T6, on cv. Orchestra, Mustang and Nicola, respectively (Table 6). Similar results were obtained by, Atia *et al*. (2010). Rosyidah *et al*. (2013) reported that the single application of *P. fluorescens* or combined application of *Streptomyces* spp. *and Trichoderma viride* + *Streptomyces* spp. was capable reducing disease incidence, reducing disease intensity and reducing the population *R. solanacearum.* Nguyen *et al*. (2011) declared that disease severity varied with antagonist and time of application. The disease severity decreased when antagonists were applied one week prior to transplanting tomato seedlings. Suppression mechanisms are typically attributed to the antibacterial metabolites produced by biological control agents or those present in natural products; however, the number of studies related to host resistance to the pathogen is increasing (Yuliar and Toyota, 2015). It is worth mentioning that the tested fungal bioagent was less effective than the bacterial bioagent in the decreasing of wilt disease index.

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المكافحة الحيوية للذبول البكتيرى على البطاطس المتسبب عن رالستونيا سولاناسيرم المعزولة من مصادر مختلفة

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تم جمع سبعة عشرة عزلة مرضية من مصادر مختلفة من درنات البطاطس المصابة بمرض العفن البنى، مياه الرى، تربة الحقول وكذلك الحشائش من محافظات ومناطق مختلفة وأجرى اختبار إحداث القدرة المرضية على شتلات الطماطم والبطاطس وكشفت النتائج أن جميع العزلات المختبرة كانت ممرضة لنباتات الطماطم والبطاطس وقد تم اختبار تعريف العزلات تبعا للخصائص المورفولوجية والبيوكيميائية والفسيولوجية وأكدت الدراسة أن العزلات البكتيرية هي بالفعل تابعة لبكتيريا رالستونيا سولاناسيرم للسلالة ٣ والطراز البيولوجي ٢حيث أنها تستفيد من سكريات المالتوز واللاكتوز والسيليبيوز ولا تستفيد من سكريات المانيتول والسوربيتول مما يؤكد أنها تنتمي إلى الطراز البيولوجي ٢، وأظهرت النتائج أيضا عدم وجود اختلاف واضح بين العزلات البكتيرية وقد أوضحت نتائج تقنية تفاعل البلمرة المتسلسل لتعريف العزلتين المختبرتين (إحداهما معزولة من درنات البطاطس والأخرى من المياه) والتي أعطيتا أعلى شدة إصابة وجود تطابق محدد من أزواج الحمض النووى مما يدل على وجود تشابه وثيق بين العزلتين تحت الدراسة، أدى استخدام بعض عوامل المكافحة الحيوية مثل سيدوموناس فلورسنس وباسيلس ستلس وسيدوموناس اريجنوزا وترايكودرما ما قبل وأثناء وبعد العدوى إلى تقليل مؤشرات الإصابة بمرض الذبول البكتيرى على البطاطس.

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