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First Report of Bacterial Wilt Caused by *Ralstonia solanacearum* Biovar 2 Race 1 on Tomato in Egypt

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This study aims to isolate and identify the causal pathogen of tomato bacterial wilt in Egypt. In 2008, tomato plants showing typical symptoms of bacterial wilt disease with no foliar yellowing were observed in Minia, Assiut and Sohag governorates, Egypt. When cut stems of symptomatic plants were submerged in water, whitish ooze was evident and longitudinal sections showed a brown discoloration in the vascular tissues. Bacteria were isolated on triphenyl tetrazolium chloride medium and fifteen isolates shown typical morphological and cultural characteristics were confirmed as *Ralstonia solanacearum* biovar 2 race 1. Pathogenicity tests showed that all isolates proved to be pathogenic to tomato plants, varied from 52 to 97% wilting. This is the first report of *R. solanacearum* biovar 2 race 1 causing bacterial wilt in tomato crop in Egypt.

Keywords **:** bacterial wilt, *Ralstonia solanacearum*, tomato

In Egypt, tomato (*Solanum lycopersicum* Mill.) is considered one of the most important vegetable crops. The cultivated area is 216,385 ha producing 8,544,990 tones with a productivity of 394,898 kg/ha (FAO, 2010). Bacterial wilt of tomato caused by *Ralstonia solanacearum*, causes a considerable amount of damage to tomatoes and many other crops in tropical, subtropical and warm temperature regions of the world (Ji et al., 2005) and limits the production of many crops e.g. potato, tomato, eggplant and pepper (Williamson et al., 2002). Strains of *R. solanacearum* were classified into five races based loosely on host range, and into five biovars based on differential ability to produce acid from a panel of carbohydrates (Denny, 2006). There is no general correlation between races and biovars. The five races of *R. solanacearum* have different host ranges and geographic distributions. Race 1 is a poorly-defined group with a very wide host range and is endemic to the southern United States as well as Asia, Africa, and South America. Race 2 principally attacks bananas, and is found mainly in Central America and Southeast Asia. Race 3 is distributed worldwide and has primarily been associated with potato. Race 4 affects ginger in much of Asia and Hawaii, and race 5 affects mulberries in China (Denny, 2006). In this study, we aimed to isolate the causal pathogen of tomato bacterial wilt in Egypt and identified biovar 2 race 1 of *R. solanacearum.*

Tomato plants, showing typical symptoms of bacterial wilt disease were collected from different localities of Minia, Assiut and Sohag governorates during 2008 season. Sections of discolored tissue from the vascular strands in stems of tomato were removed, washed with tap water several times, surface sterilized for three minutes in 1% sodium hypochlorite solution and then rinsed in sterile water. Samples were homogenized in a sterile mortar and pestle with 5 ml of sterile 0.05 M potassium phosphate buffer and were streaked on Petri plates containing Kelman's triphenyl tetrazolium chloride (TTC) agar medium (Kelman, 1954). The single colony technique was used to obtain pure culture. Discrete colonies were purified by subculturing successively for four times on nutrient agar medium. From the last plate individual colonies were picked up and transferred to Nutrient agar media slants and maintained at 4°C for further studies. Also, The stock culture of the isolates were stored in sterile distilled water at 4°C.

Pathogenicity assay of fifteen bacterial isolates were carried out under greenhouse condition by inoculating tomato seedling G.S cultivar. Bacterial isolates were grown on nutrient agar medium for two days at 28±2°C, suspended in sterile distilled water and an optical density of 0.1 at 600 nm wavelength (using spectrophotometer model 6405UV/VIS), approximately 10^8 cfu/ml was adjusted. Inoculation was made at the three to four true leaf stage by punctur-

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ing the stem at the axils of the third fully expanded leaves from the apex with a needle dipped in the inoculum, and each plant stem was injected with 100 µl bacterial suspension (Winstead and Kleman, 1952). Eight tomato seedlings were used for each isolate. Inoculated seedlings with sterile water served as negative control. Inoculated seedlings were kept in a climate chamber with 30/27°C day/night temperature, 85% relative humidity. Plants were watered well, with avoided wetting the foliage for 24 h (Williamson et al., 2002). The experiment was undertaken with completely randomized design and repeated twice.

Wilt index has been calculated over 21 days of inoculation according to Winstead and Kelman, (1952), using the following formula:

$$\text{Disease index (\%)} = [\sum (ni \times v_i) \div (V \times N)] \times 100$$

Where the *ni*=number of plants with the respective disease rating; *vi*=disease rating; *V*=the highest disease rating (5); and *N*=the number of plants observed. Disease rating was calculated as following scale: 1=no symptoms, 2=one leaf wilted, 3=two to three leaves wilted, 4=four or more leaves wilted and 5=whole plant wilted.

Worldwide the most commonly used method for detection and identification of *R. solanacearum* has been isolated on TTC medium (Kelman, 1954). Tomato bioassay is also currently recommended by the European Plant Protection Organization (EPPO) for detection of *R. solanacearum* in soil and tomato plants and for pathogenicity testing (Elphinstone et al., 1996). The fifteen isolated bacteria proved to be pathogenic and cause bacterial wilt to tomato plants were identified according to their morphological, cultural and physiological characteristics recommended by Billing et al. (1961); Dye (1968); Schaad (1992); Bergey's Manual of systematic Bacteriology (Krieg and Holt, 1984) and Bergey's Manual of Determinative Bacteriology 9th edition (Holt et al., 1994).

To test the ability of isolates to induce a hypersensitivity reaction on tobacco leaves to determine the race of isolates, tobacco plants (*Nicotiana tabacum*) at 30 to 45 day stage were obtained from the plant pathology department, Agriculture faculty, Assiut University, Assiut, Egypt. For tobacco leaf infiltration, all of the isolates used for pathogenicity test were used. Two day cultures grown on nutrient agar medium at 28±2°C were used to prepare bacterial suspension of 10^8 cfu/ml for each isolate as described before. The fully expanded leaves were infiltrated by injecting water suspension of bacteria into the intercellular spaces with a hypodermic syringe fitted with a fine needle (Klement et al., 1964). From the lower side of the leaves, an area of 3–5 cm^2, in each of 8 to 12 intercostal areas of each leaf, was infiltrated with a suspension of bacteria. Four leaves were infiltrated by each isolate. Leaves infiltrated with sterile water served as a negative control. Leaf reactions were recorded at 24 and 48 h incubation under the greenhouse conditions similar to the pathogenicity test at 26 to 30°C temperature and 60 to 80% relative humidity (Horita and Tsuchiya, 2001).

Fifteen isolates were isolated from thirty-three of tomato tissues, water and soil samples which were collected from different locations, shown bacterial wilt symptoms or taken from location hit by the disease. These isolates shown typical morphological and cultural characteristics of *R. solanacearum*. The highest number of isolates was obtained from the Minia governorate (7 isolates) followed by Assiut governorate (6 isolates) while the lowest number was obtained from Sohag governorate (2 isolates).

Pathogenicity tests through stem inoculation of tomato was done during the 2009 season on tomato cultivar G.S. Wilt index percentage has been calculated after three and four weeks from inoculation; the results obtained are in Table 1. Fifteen isolates were tested for their pathogenicity to tomato plants under greenhouse conditions. All isolates proved to be pathogenic to tomato plants and produced typical symptoms of wilt. Isolates No. (2 and 1) exhibited

Table 1. Pathogenicity tests for *Ralstonia solanacearum* isolates on seedlings under greenhouse condition

No. of isolate	Sources of isolate		Disease index (%)	
	District	Crop	3 week	4 week
R1	Minia	Tomato	52^{gh}	81^{b}
R2		Tomato	76^{a}	97^{a}
R3		Tomato	65^{c}	74^{cd}
R4		Tomato	60^{d}	66^{ef}
R5		Water	48^{i}	52^{h}
R6		Water	53^{fg}	55^{h}
R7		Tomato	70^{b}	70^{cde}
R8	Assiut	Tomato	67^{c}	73^{cd}
R9		Tomato	74.5^{a}	76^{bc}
R10		Tomato	54.5^{ef}	55^{h}
R11		Tomato	56^{e}	68^{def}
R12		Tomato	56.75^{e}	72^{cde}
R13		Soil	53^{fg}	53^{h}
R14	Sohag	Tomato	50^{hi}	62^{fg}
R15		Tomato	53^{fg}	56.25^{gh}
Control			0^{j}	0^{i}
LSD (P = 0.05)			2.46	6.29

*Values in the column followed by the same letter within a column are not significantly different as determined by the LSD test (P = 0.05).

Table 2. Morphological and Biochemical characteristics of *Ralstonia solanacearum* isolates from different locations

	Tests		Isolates (R1-R5)*
Morphological	Shape of cells		Rod
	Gram stain		−
	Spore formation		−
	Motility		+
	Yellow pigmentation on KB		−
Biochemical	Oxidase test		+
	Catalase test		+
	Gelatin hydrolysis		−
	Arginine dihydrolase		−
	Tolerance of NaCl	0.5%	+
		1%	+
		1.5%	+
		2%	−
	H_2S production		+
	Indole formation		−
	Starch hydrolysis		−
	Aesculin hydrolysis		−
	Levan production		−
	Growth at	37°C	+
		41°C	−
	Acid from Hexose alcohols	Mannitol	−
		Sorbitol	−
		Dulcitol	−
		Cellobiose	+
	Disaccharides	Lactose	+
		Maltose	+

* all isolates gave the same reaction
+ Positive
− Negative

Table 3. Reaction of tobacco leaves to infiltration with isolates of *Ralstonia solanacearum*

No. of isolate	Hours after infiltration		Race
	48 h	72 h	
R1	HR	HR	1
R2	HR	HR	1
R3	HR	HR	1
R4	HR	HR	1
R5	C	C	3
R6	C	C	3
R7	C	C	1
R8	HR	HR	1
R9	HR	HR	1
R10	N	HR	1
R11	HR	HR	1
R12	HR	HR	1
R13	C	C	3
R14	C	C	3
R15	C	C	3

N = No reaction, HR = hypersensitivity reaction and C = collapse of infiltrating area.

the highest disease index with 97 and 81% wilting respectively, followed by isolates No. (3, 7, 8 and 12) which caused 74–70% wilting. The rest of isolates caused a moderate percentage of disease index (less than 60% wilting) after four weeks of inoculation.

Fifteen pure cultures of the pathogen isolate have typical cultural characteristics and proved to be pathogenic and causing wilt symptoms to tomato plants were identified. Table 2 showed that all tested isolates have a positive reaction for motility, oxidase, catalase, H_2S production, growth at 37°C and NaCl tolerance (0.5, 1, 1.5%) tests. In other hand, they have a negative reaction for Gram stain, spore formation, yellow pigmentation on KB, gelatin hydrolysis, arginine dihydrolase, NaCl tolerance (2%), indole formation, starch hydrolysis, aesculin hydrolysis, levan production and growth at 41°C tests. In addition, results of production acid tests showed that all tested isolates produced acid from cellobiose, lactose and maltose and did not produce acid from mannitol, sorbitol and dulcitol. Based on the morphological, cultural, physiological and pathological characteristics of the isolated bacteria and according to those reported by Hayward (1964) and Krieg and Holt (1984), it could be concluded that all the tested isolates identified as *Ralstonia solanacearum* biovar 2. The results of infiltrating tobacco leaves with different isolates showed that R1, R2, R3, R4, R7, R8, R9, R10, R11 and R12 isolates, caused necrosis that appears 48–72 h after infiltration, identified as race 1; isolates induced a distinct yellow zone at the spreading edge of the lesion. The lesion became progressively darker, and the yellow halo surrounding the dark central area became more noticeable by 48 h. While, the rest isolates, showed collapse of the infiltrating area, identified as race 3. Tobacco leaves infiltrated with sterile water showed no reaction (Table 3).

Bacterial wilt caused by *R. solanacearum* is one of the major diseases of tomato (Tahat and Kamaruzaman, 2010). The disease causes concern for tomato production because it can drastically reduce tomato up to 90% (Kishun, 1985). It is one of the most difficult tomato diseases to control; it has a wide host range; more than 200 plant species in over 50 families, including a wide range of crop plants, ornamentals and weeds. The pathogen can survive for long periods in other host crops and in infected tomato debris (Tahat and Kamaruzaman, 2010). Efforts to obtain

resistant tomato cultivars by breeding have so far not been satisfactory (Gartemann et al., 2003).There is no effective control measures for this disease. In the present study, out of tomato tissue, water and soil samples collected from different locations shown bacterial wilt symptoms or take from location hit by the disease, only fifteen isolates have typical morphological and cultural characteristics were obtained. The highest number of isolates was obtained from the Minia governorate (7 isolates) followed by Assiut governorate (6 isolates) while the lowest number was obtained from Sohag governorate (2 isolates). According to Kelman (1954), the TTC media has used to distinguish *R. solanacearum* among other bacteria during isolation. Also, when TTC media are used with *R. solanacearum* there is the difference between stable avirulent colonies with, dark red and the unstable fluidal virulent with white with pink center when *R. solanacearum* isolates were cultured on the TTC media is used as an identification test because of the specific characteristics of the colonies of a virulent strain (Klement, 1990). Fifteen isolates here were fluidal whitish with a pink center; proof of the presence of pathogenic bacteria as suggested by Klement (1990) and thirteen isolates were dark pink. Among fifteen pathogenic isolates, two of them isolated from water and one from soil. Detecting *R. solanacearum* in soil and water samples is more difficult, because the pathogen population is usually lower ($<10^4$ cfu/g soil or ml water) and saprophytic bacteria are present in equal or greater numbers. Such samples are best cultured on a semi-selective medium, such as modified SMSA (Denny and Hayward, 2001; Elphinstone et al., 1996), which usually suppresses contaminants well enough to permit detection of *R. solanacearum* down to 100 to 500 cfu/g soil or ml water, and about 10-fold lower in tissue extracts (Elphinstone et al., 1996; Pradhanang et al., 2000; Poussier et al., 2002). Other semi-selective media may work better in some locations or with particular soils (Denny and Hayward, 2001; Pradhanang et al., 2000).

The results reported herein indicate that the fifteen bacterial isolates obtained from different localities of Minia, Assiut and Sohag governorates have proved to be pathogenic and could infect tomato plants causing wilt symptoms and varied in their pathogenicity. Under greenhouse conditions, pathogenicity tests through stem inoculation of tomato were performed on tomato cultivar G.S where the stem inoculation is reliably for *R. solanacearum* when it is present at $>10^4$ cfu/ml in the sample (Elphinstone et al., 1996; Van der Wolf et al., 2000). *R. solanacearum* were detected in xylem vessels in the taproot and lower stem 2 or 3 days after inoculation and plants began to wilt after 4 days (Araud-Razou et al., 1998; McGarvey et al., 1999; Saile et al., 1997). Pathogenicity tests showed that isolates No. 2 exhibited the highest disease incidence with 97% wilting. Such results agree with previous reports; who mentioned that the different isolates of *R. solanacearum* were varied in their pathogenicity (Abo-Elyousr and Asran, 2009; El-Ariqi et al., 2005; Galal et al., 2003). A variety of pathogens can cause wilting, so symptoms alone are not definitive for bacterial wilt causing by *R. solanacearum*. Pure cultures of *R. solanacearum* are not difficult to identify. Cultural and physiological tests can quickly rule out related organisms (Anon, 2004). Cultural, physiological, and biochemical tests revealed that all isolates identified as *Ralstonia solanacearum* biovar 2. Our hypersensitivity reaction test results showed that almost tested belong to race 1. Race 1 strains were originally described as affecting tobacco, tomato, many solanaceous weeds, some other weeds, and diploid bananas (Buddenhagen et al., 1962). Over time, the acceptable host range was gradually expanded to include many other plants, including but not limited to groundnut, potato, pepper, eggplant, olive, ginger, strawberry, geranium, and eucalyptus. One concern about the definition of race 1 is that some strains are highly virulent on tomato and eggplant, but low in virulence on tobacco (Granada and Sequeira, 1975) and most of these induce a hypersensitive response when infiltrated into tobacco leaves (Robertson et al., 2004). In contrast, strains virulent on tobacco almost always cause necrosis that appears 48–72 h after infiltration. Race 1 strains are phenotypically diverse and not part of a natural taxonomic group (Hayward, 1994). To our knowledge, this is the first report of tomato bacterial wilt caused by *Ralstonia solanacearum* biovar 2 race 1 in Egypt.

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