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Fatty Acid Polymorphism of *Ralstonia solanacearum* in Different Egyptian Governorates and other European Countries

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ABSTRACT
Eight isolates of *Ralstonia solanacearum*, the causal organism of potato brown rot (bacterial wilt) from Egypt and other European countries were characterized according to their biochemical and physiological properties. The selected bacterial isolates were classified as race 3 based on their virulence on different host plants. Virulence degree of all isolates was high on potato cultivar "Lady Rosetta" except isolates RsIs2 and Rs48. Virulence varied from high to low on potato cultivar "Cara", tomato, eggplant and pepper. Analysis of tested *R. solanacearum* fatty acid profiles showed two groups; group I contains European isolates with long chains-hydroxylated fatty acids while, the Egyptian isolates presented in group II with short chains. A comparative analysis of fatty acid composition and virulence of tested bacteria gives a possibility to suppose that fatty acids may be play an important role in pathogenicity of *R. solanacearum*.

Key words: Brown rot, *Ralstonia solanacearum*, potato, fatty acids, pathogenicity

INTRODUCTION

Bacterial wilt caused by *Ralstonia solanacearum* is one of the most serious diseases in tropics, subtropics and warm temperate regions of the world. In Egypt, the disease is widespread in Solanaceous vegetables. The pathogen exhibits wide variability and diversity which often confuses plant breeders by breaking down of resistance varieties evolved through extensive breeding programs. Isolates of *R. solanacearum* are generally grouped, based on utilization of disaccharides and hexose alcohols into biovars and based on host range into races (James *et al*., 2003; Kabeil *et al*., 2008). Five races have been described so far, but they differ in host range, geographical distribution and ability to survive under different environmental conditions; race 1 infects many Solanaceous plants such as tomato, tobacco, pepper and other plants including some weeds. However, race 2 causes wilt of triploid banana (*Musa* spp.) and *Heliconia* spp. and race 3 affects potato and tomato but is weakly virulent on other Solanaceous crops, race 4 infected ginger in Philippines and race 5 from mulberry in China (Buddenhagen *et al*., 1962; He *et al*., 1983; French, 1986).

Fatty acids profiles established well for classification and identification of plant associated bacteria; based on standardized culture, simple chemical extraction and gas chromatographic separation of fatty acids methyl esters. Previous reports have indicated that fatty acid methyl ester profiling corresponded well, to be the best phenotypic marker for *R. solanacearum* isolates classification (Weller *et al*., 2000; Varbanets *et al*., 2003, 2004; Dawyndt *et al*., 2006; Tohamy *et al*., 2007; Khakvar *et al*., 2011). The objective of this study was to characterize the potato bacterial wilt *R. solanacea*rum isolates collected from Egypt and European countries based on their pathogenicity and the polymorphism of fatty acid profiles.

MATERIALS AND METHODS

Isolation, phenotypic and biochemical characterization of the pathogen: Infected potato tubers showing internal symptoms of brown rot disease were collected from five locations in Egypt (Alexandria, El-Monofia, El-Nobaria, Ismailia and Plant Pathology Research Institute) and European countries (France, Scotland and Netherlands). Infected tubers

were segmented into small pieces and placed in test tubes containing 5 mL of sterile distilled water for standard isolation (Hayward, 1991). Bacteria were allowed to flow from the vascular bundles for 5-10 min. One loop full of the bacterial suspension was streaked onto 2, 3, 5 triphenyl tetrazolium chloride (TZC) agar medium (Kelman, 1954) and incubated at 28°C for 48 h. A virulent single colony showing fluidal, irregular and creamy white with pink at the center was selected and maintained on Casamino Peptone Glucose (CPG) medium (Khakvar *et al*., 2011) for further identifying by biochemical and physiological characteristics (Kelman, 1954).

Pathogenicity test: Pathogenicity tests of eight isolates of *R. solanacearum* were carried out on four different hosts; tomato (*Lycopersicon esculentum* L. cv. Strain B), eggplant (*Solanum melongena* L. cv. Baldy), pepper (*Capsicum annuum* L. cv. Baldy) and two cultivars of potato (*Solanum tuberosum* L. cvs. Lady Rosetta and Cara) (Klement *et al.*, 1990). All plantlets were planted in sterilized pots (20 cm diameter) containing sterilized peat-moss/sand (v/v) and allowed to grow for 6-8 weeks or until they were 15-20 cm high. Roots of each host plants were wounded and dipped in bacterial suspension (10^8 CFU mL^{-1}) of each isolate alone for 20 min and three replicates were used (Dhital *et al*., 2001). Degree of wilt was calculated according to He *et al*. (1983), as average of three plants at four weeks after inoculation and rating scales were as followed: H, High (disease index 4.1-5.0); M, Moderate (2.6-4.0) and L, Low (1.1-2.5).

Fatty acid profiling: Total lipids were extracted from *R. solanacearum* isolates grown on Nuterant Broth (NB) media (Kates, 1972). Fatty Acids (FAs) were prepared from total lipid as described by Radwan (1978) and analyzed using Shimadzu-8A, GLC equipped with Flam Ionization Detector (FID) and ordinary glass column (ID 30 m×0.32 mm) of 5% diphenyl and 95% dimethyl polysiloxane on chromosorb Q 80/100 mesh. The following conditions were used for GLC analysis: column temperature 150°C for 2 min, detector temperature 250°C, flow rates of nitrogen 1 mL min^{-1}. Standard FAs and their retention times were used for identification. The area under each peak was measured by the triangulation methods and expressed as percentage of each fatty acid with regard to the total area. Cluster analysis of long and short chains of fatty acid profiles of Egyptian and European *R. solanacearum* isolates determined by PAST program (Hammer *et al*., 2001) according to Jaccard index.

RESULTS AND DISCUSSION

Isolation and characterization of bacterial isolates: Eight bacterial isolates were isolated from infected tubers. Typical morphological characteristics of virulent *R. solanacearum* were purified (Swanson *et al*., 2007). Results of biochemical and physiological characteristics identified all isolates as *R. solanacearum* (Table 1).

Pathogenicity test and race determination: Pathogenicity test on potato and other Solanaceous host plants were classified all isolates as race 3. The Egyptian isolates Rs2 and RsMo2 were high virulent on potato cv. "Lady Rosetta" and tomato plants but were moderate to low virulent on potato cv. "Cara", eggplant and pepper except isolate RsBe2 which was high virulent on all host plants but was moderate virulent on eggplant. Similarly, the degree of virulence of the isolates RsFr4, RsSc1 and RsNe1 were high virulent on potato cv. "Lady Rosetta" and moderate to low virulent on tomato, eggplant and pepper. The other isolates RsIs2 and Rs48 were moderate virulent on tomato and low on the two potato cvs. ,eggplant and pepper. These results showed that potato cv. "Lady Rosetta" was more susceptible than "Cara" cv.

Table 1: Characteristics of *Ralstonia solanacearum* isolates

	Reaction of isolates collected from different origins								
Characteristics	Reference	France RsFr4	Netherlands RsNe1	Scotland RsSc1	Plant pathology research institute Rs48	Ismailia RsIs2	El-Nobaria RsBe2	El-Monofia RsMo2	Alexandria Rs2
Shape of cell	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Motility	+	+	+	+	+	+	+	+	+
Gram staining	-	-	-	-	-	-	-	-	-
Growth at 4°C	-	-	-	-	-	-	-	-	-
Growth at 44°C	-	-	-	-	-	-	-	-	-
Production of fluorescent pigment on K'B	-	-	-	-	-	-	-	-	-
Diffusible non fluorescent pigment	Brown	Brown	Brown	Brown	Brown	Brown	Brown	Brown	Brown
Arginine dihydrolase	-	-	-	-	-	-	-	-	-
Salt tolerance at 1%	+	+	+	+	+	+	+	+	+
Salt tolerance at 7%	-	-	-	-	-	-	-	-	-
Oxidase reaction	+	+	+	+	+	+	+	+	+
Levan production	-	-	-	-	-	-	-	-	-
Starch hydrolysis	-	-	-	-	-	-	-	-	-
Gelatin liquefaction	-	-	-	-	-	-	-	-	-
Catalase reduction	+	+	+	+	+	+	+	+	+
Potassium hydrolysis test (KOH)	+	+	+	+	+	+	+	+	+

+: Positive reaction (color of medium was changed from purple to yellow), -: Negative reaction (color of medium was not changed)

Table 2: Response of potato cultivars and other Solanaceous plants to Egyptian and European *Ralstonia solanacearum* isolates

Isolates	Accession No.	Pathogenicity reaction*				
		Potato cv. Lady Rosetta	Potato cv. Cara	Tomato cv. Strain B	Eggplant cv. Baldy	Pepper cv. Baldy
Egyptian isolates						
Alexandria Rs2	LN681198	H	M	H	L	L
Plant Pathology Research Institute Rs48	HG425353	L	L	M	L	L
El-Monofia RsMo2	LN681199	H	L	H	L	M
El-Nobaria RsBe2	LN681202	H	H	H	M	H
Ismailia RsIs2	LN681201	L	L	M	L	L
European isolates						
France RsFr4	LN827661	H	M	M	L	M
Scotland RsSc1	LN681203	H	M	L	M	M
Netherlands RsNe1	LN681204	H	H	M	L	M

*Average of three plants at four weeks after inoculation and rating scales (He *et al*., 1983) were as followed: H: High (disease index 4.1-5.0); M: Moderate (2.6-4.0) and L: Low (1.1-2.5)

Table 3: Percentage of total fatty acids composition of Egyptian and European *Ralstonia solanacearum* isolates

Fatty acids		Bacterial isolates							
		Egyptian isolates					European isolates		
Shorthand designation	Systematic name	Alexandria (Rs2)	Plant pathology research Institute (Rs48)	El-Minofia (RsMo2)	El-Nobaria (RsBe2)	Ismailia (RsIs2)	France (RsFr5)	Scotland (RsSc1)	Netherlands (RsNe1)
C6:0	Hexanoic	-	1.95	0.35	0.29	0.38	-	-	-
C8:0	Octanoic	1.05	14.91	5.69	9.38	15.37	0.06	0.14	0.18
C10:0	Decanoic	1.80	1.60	2.55	1.73	0.41	-	-	-
C11:0	Undecanoic	0.97	0.59	0.65	0.71	0.45	-	-	-
C12:0	Dodecanoic	4.10	1.09	1.07	1.75	1.19	3.08	2.47	5.90
C13:0	Tridecanoic	7.11	1.49	1.13	5.75	3.32	2.27	3.61	4.56
C14:1	Tetradecanoic	4.49	8.42	7.38	3.28	5.00	4.81	6.56	24.90
C14:0	Tetradecanoic	10.95	4.26	3.55	5.53	2.34	8.94	16.14	20.36
C15:1	Pentadecanoic	4.96	3.71	3.12	3.80	2.12	4.10	24.63	28.87
C15:0	Pentadecanoic	3.97	2.09	2.37	3.16	1.16	11.95	13.05	48.04
C16:1	Hexadecenoic	2.54	1.60	0.82	1.64	1.00	1.28	12.55	17.42
C16:0	Palmitic	7.65	5.00	3.31	3.21	2.44	1.28	18.40	22.34
C17:1	Heptadecenoic	1.43	0.73	0.59	0.72	0.44	1.82	2.75	3.38
C18:2c	Linoleic	1.87	1.20	0.83	0.66	0.59	1.13	2.15	4.11
C18:2t	Linolelaidic	1.57	1.43	0.98	0.44	1.48	1.55	1.10	4.23
C18:0	Octadecanoic	1.92	1.76	1.10	1.32	1.27	2.22	5.62	4.34
C20:0	Eicosanoic	-	-	-	-	-	1.44	0.86	0.40
C22:1	Docosanoic	-	-	-	-	-	3.17	2.40	1.13

-: Not detected

(Table 2). In a host range study, all isolates were high to moderate pathogenic on potato and tomato, whereas on the other Solanaceous hosts such as eggplant and pepper were moderate to low pathogenic. The obtained results were in agreement with earlier reports stating that, characteristic of race 3 of *R. solanacearum* had limited host range (Buddenhagen *et al*., 1962; He *et al*., 1983; French, 1986; Dhital *et al*., 2001; Ibrahim *et al*., 2005).

Determination and clustering of fatty acids profiles of *R. solanacearum* isolates: The cluster analysis of fatty acid profiles of tested *R. solanacearum* isolates divided them into two groups. The first group was represented by *R. solanacearum* isolates from European countries RsFr4, RsSc1 and RsNe1 which contained the hydroxylated fatty acids with long chains including: 3-hydroxytetradecanoic with C14 length chain, 2-hydroxyhexadecanoic (C16) and 2-hydroxyoctadecanoic from C18 to C26 while, the second group went to the Egyptian isolates (Table 3) which contained the hydroxylated fatty acids with short chains vary from C10; 2- hydroxydodecanoic ,C11 and C12; 3-hydroxydodecanoic (Varbanets *et al*., 2004).

Total fatty acid profiles have been used as a common phenotypic marker for identification subspecies level (Cother *et al*., 1992; Stead *et al*., 1992; Tohamy *et al*., 2007). In agreement with these results, a significant differences were found in C16:0 contents among all isolates as the scientists Clough *et al*. (1997) and Khakvar *et al*. (2011) previously pointed to the importance of palmitic acid (C16:0) in the physiology of *R. solanacearum*. Moreover, Weller *et al*. (2000) previously reported that the family of Pseudomonaceae species can be divided into eight fatty acid groups that parallels RNA homology grouping the fatty acids such as C14:0 and C16:0 and unsaturated fatty acid have been used for classification of many *Pseudomonas* at different levels (species and subspecies). Previous literatures confirmed that the specific auto inducer fatty acid (3-hydroxypalmitic acid ester) playing a role in virulence of *R. solanacearum*

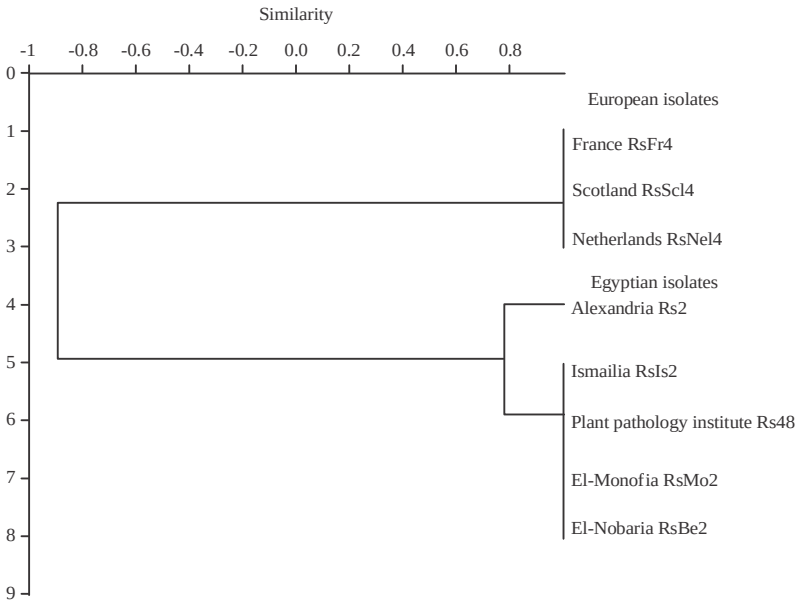

Fig. 1: Cluster analysis of long and short chains of fatty acid profiles of Egyptian and European *Ralstonia solanacearum* isolates determined by PAST program according to Jaccard index

(Clough *et al*., 1997; Flavier *et al*., 1997). Thus it has been shown the heterogeneity of *R. solanacearum* isolates tested in the composition of their fatty acids profiles. In the present study, fatty acid composition in all isolates differ in presence of short or long chains of hydroxylated fatty acids therefore, it clustered in two groups; group I contains European isolates with long chains-hydroxylated fatty acids while, group II contains Egyptian isolates with short chains of fatty acids (Fig. 1) (Varbanets *et al*., 2004).

CONCLUSION

A comparative analysis of fatty acid composition and virulence of tested bacteria gives a possibility to assume that fatty acids may be play an important role in pathogenicity of *R. solanacearum*.

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