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Antigenic and pathogenicity activities of *Ralstonia solanacearum* race 3 biovar 2 molecularly identified and detected by indirect ELISA using polyclonal antibodies generated in rabbits

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ABSTRACT

Eight molecular-characterized isolates of *Ralstonia solanacearum* from potato belonging to race 3 biovar 2, their virulence were evaluated on potato cv. Lady Rosette, tomato cv. Strain B, eggplant cv. Balady and pepper cv. Balady and showed high virulence on potato and tomato, and lower virulence on eggplant and pepper. A laboratory study conducted to produce polyclonal antibodies against the potato brown rot bacterium; *R. solanacearum* cells were generated in female New Zealand white rabbits. A modification were made on the technique of indirect enzyme-linked immunosorbent assay (ELISA) to improve the sensitivity of detection, including antigenic and sensitivity to *R. solanacearum* race 3 biovar 2 isolates. Determination of the optimum period to collect the antiserum (including, polyclonal antibodies) showed that the best collection dates were at 14, 3 and 7 days, in that order. The efficiency of the antiserum was compared among 42 isolates that cause potato brown rot disease; our polyclonal antiserum (14 days) reacted positively with all tested isolates at a dilution of $1{:}6.4 \times 10^3$. Data indicated the different reactions of eight *R. solanacearum* isolates at various dilutions ($1{:}1.6 \times 10^3$ to $1{:}5.12 \times 10^6$) at 14 days against polyclonal antiserumat a concentration of approximately 1×10^8 CFU/mL and we found the lowest detection level by the indirect ELISA technique was 10^6 CFU/mL. Finally we recommended the reasonable sensitivity results of the ELISA technique to detect the bacterial pathogen given than the cost of this technique if much lower than that of other expensive molecular techniques.

1. Introduction

Diseases such as potato brown rot and bacterial wilt in tomato, which are caused by *Ralstonia solanacearum* [1–3], are one of the most serious diseases of crops in the tropical, subtropical and warm temperate regions worldwide. In Egypt, the disease is widespread in solanaceous vegetables and cucurbits. The pathogen exhibits wide variability and diversity, allowing the infection of resistant plant varieties generated by extensive breeding programs and confounding breeding efforts. Isolates of *R. solanacearum* are generally grouped into races based on their host range and then into biovars based on their use of disaccharides and hexose alcohols [4,5]. Five races have been described so far, but they differ in host range, geographic distribution and their survival under different environmental conditions: race 1 infects many solanaceous plants, including tomato, tobacco, and pepper, as well as other plants, including some weed species; race 2 causes wilt of triploid banana (*Musa* spp.) and *Heliconia* spp.; race 3 affects potato and tomato but is weakly virulent on other solanaceous crops; race 4 infects ginger in the Philippines; and race 5 infects mulberry in China [6–9].

Researchers have improved the indirect enzyme-linked immunosorbent assay (ELISA) technique for *R. solanacearum* biovar 2 polyclonal antiserum to detect plant tissue homogenate or soil suspension infections at concentrations as low as 10^4 CFU/mL [10,11]; similar levels of sensitivity were achieved as those via detection in potato tissue using immunofluorescent antibody stains [12]. This indirect ELISA method used in conjunction with a tomato bioassay is currently recommended by the European Plant Protection Organization (EPPO) for the detection of *R. solanacearum* in potato tubers. The bioassay of tomato seedlings can reliably detect potato tuber extract infections at concentrations as low as 10^4 CFU/mL [12–14].

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Serological tests such as agglutination and precipitation tests as well as immunofluorescence and ELISA offer an important presumptive diagnosis that aids in the identification process of bacterial organisms [14–18]. The indirect ELISA technique using polyclonal antibodies was applied for the detection of *R. solanacearum* isolates representing race 1 biovar 3, race 2 biovar 1, and race 3 biovar 2, either from pure cultures or plant parts. The lowest detection level of the ELISA technique was 10^3 CFU/mL [11].

This research aimed to examine the virulence reactions of the tested *R. solanacerum* identified isolates against some solanaceae hosts, the production of polyclonal antibodies to the antigen *R. solanacearum* generated in the serum of New Zealand white rabbits, and the antigenic activities of *R. solanacearum* in its antiserum.

2. Materials and methods

2.1. Bacterial isolation

Isolation trials were conducted on infected potato tubers showing internal symptoms of brown rot disease. Samples were collected from Alexandria (Rs2), Menofia (RsMo2), Behera (RsBe2) and Ismailia (RsIs2) Governorates. Diseased potato samples were also obtained from the Plant Pathology Institute (Project of Potato Brown Rot, Rs48) in Egypt as well as European countries such as France (RsFr5), Scotland (RsSc1) and the Netherlands (RsNe1); samples were also obtained from the Central Administration of Plant Quarantine, Egypt (CAPQ). For standard isolation, the infected tubers were segmented into small pieces and placed in test tubes containing 5 mL of sterile distilled water [19]. The bacteria were allowed to flow from the vascular bundles for 5–10 min (Fig. 1). One loop full of the bacterial suspension was streaked onto 2, 3, 5-triphenyltetrazolium chloride (TZC) agar medium [20] (Fig. 2), after which the medium was incubated at 28 °C for 48 h. For selecting virulent colonies (fluidal aspect and white to pink coloration) for pathogenicity tests. A single colony was selected and maintained on casamino peptone glucose (CPG) medium [21] for further pathogenicity and ELISA tests [22].

2.2. Pathogenicity test and statically analysis

Plants of potato cv. Lady Rosetta, tomato cv. Strain B, eggplant cv. Baldy and pepper cv. Baldy with six to eight expanded true leaves grown at 25–30 °C in potted soil, were needle-inoculated at the insertion point of petioles of the third pair of true leaves, in the region where shortly before 30 µL were deposited of a bacterial suspension prepared from a 48 h culture in CPG (10^8 CFU/mL), calculated spectrophotometrically [23]. Control plants were inoculated with sterile distilled water only (Fig. 3). The experiments were carried out with five replicates for each inoculated isolate. Plants were kept in a greenhouse at the Plant Pathology Institute, Laboratory of Plant Pathology, Agriculture Research Center at Alexandria, Egypt at 25–30 °C. In the experiments, 8 *R. solanacearum* isolates, were tested (Table 1). Virulence of the isolates was evaluated at 28 days post inoculation (dpi) using of a scale adapted from Morgado et al. [24], ranging from 0 to 5 where: 0 = healthy plants, 1 = plants with one leaf with yellowing or wilted, 2 = plants with two leaves wilted, 3 = plants with three to five leaves wilted, 4 = plants with all leaves wilted except the apex, and 5 = plants with all leaves wilted or plant dead. The disease index (DI) was calculated according to Winstead and Kelman [25]:

$$DI = [\Sigma (i \times Ni)]/(N \times 5)$$

Where Ni = number of plants in each class of symptom; i = score given to the ranging wilt symptoms of inoculated plants (0–5); N = total number of inoculated plants; 5 (maximum score of the scale). The data were analyzed using the statistical analysis system SAS 9.3.1 software [26]. Means were compared with L.S.D. test at $P < .05$ levels.

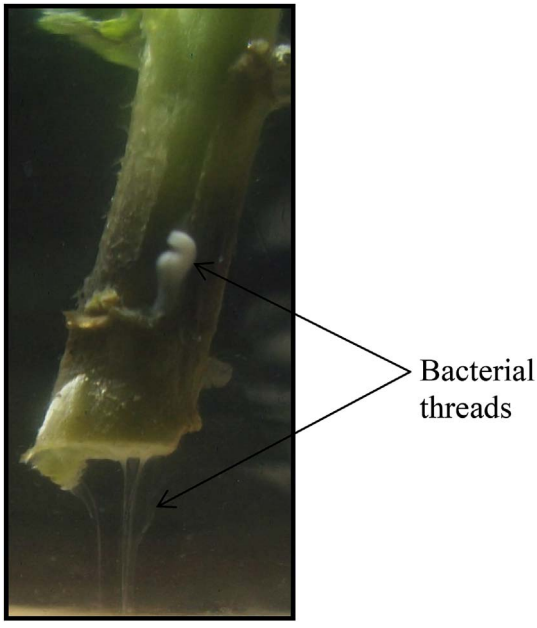

Fig. 1. Bacterial threads streaming from an infected potato stem cut in clean water.

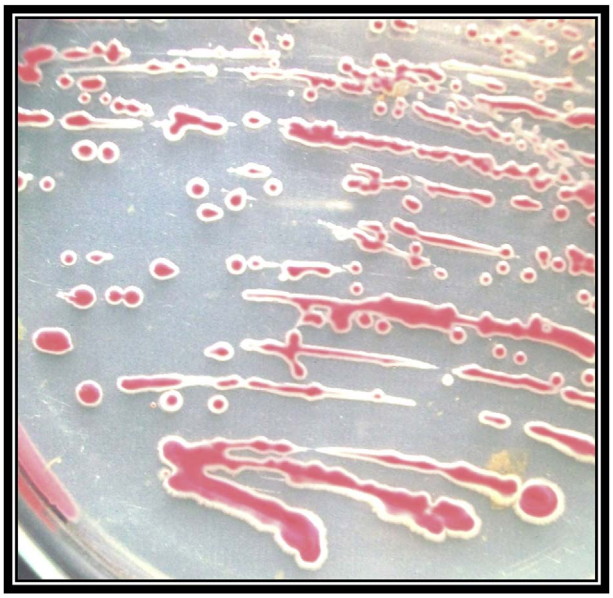

Fig. 2. Colonial morphology of *Ralstonia solanacearum* on TZC medium.

2.3. Antiserum production

Suspensions (approximately 10^8 CFU/mL in phosphate buffer saline, PBS) of live cells from the brown rot bacterial isolate *R. solanacearum* (RsMo2) were used to produce antiserum in female New Zealand white rabbits by a course of five injections (0.5, 1.0, 1.5, 2.0 and 2.5 mL). The first injection was subcutaneous, and the others occurred in muscle thigh tissue; the injections occurred at 7-day intervals [27]. Bleeding from the marginal ear vein was carried out after 3, 7 and 14 days from the last injection; the blood was left to clot for 2–3 h at room temperature, after which it was stored overnight at 4 °C. Antiserum was collected and clarified by centrifugation at 5000 rpm for 10 min, after which it was stored in the presence of 0.05% sodium azide at −20 °C until use [28,29]. Normal serum was obtained from the rabbits before immunization for use as a control in subsequent tests (Fig. 4).

Fig. 3. Artificially infection of seedlings in (a)potato; (b)tomato; (c) eggplant and (d) pepper showing wilting symptoms caused by *Ralstonia solanacearum* isolate (RsBe2) (I)inoculated; (H) healthy control seedlings.

Table 1
Disease Index (%) obtained in the virulence experiments with isolates of *Ralstonia solanacearum* on potato cv. Lady Rosetta, tomato cv. Strain B, eggplant cv. Baldy and pepper cv. Baldy.

Isolate code	Disease Index (%)[a]				
	Potato	Tomato	Eggplant	Pepper	Mean of isolates
Rs2	92	96	22	26	59
Rs48	26	76	20	20	35.5
RsMo2	100	96	40	80	79
RsBe2	100	96	80	100	94
RsIs2	30	74	24	22	37.5
RsFr4	90	74	40	74	69.5
RsSc1	94	32	70	70	66.5
RsNe1	90	80	38	80	72.75
Mean of plant cultivars	78.125	78	41.75	59	

L.S.D interaction = 1.84, L.S.D isolates = 0.92 and L.S.D cultivars = 0.652.
[a] Average of five plants at 28 days post inoculation (dpi).

2.4. Indirect ELISA

2.4.1. Determination of the optimum antiserum collection period

Indirect ELISAs were carried out as described [11,29]. Bacterial suspensions were prepared in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.2) at a concentration of approximately 10^8 CFU/mL. Wells were coated by adding 100 µL of the bacterial suspension to the bottom of the wells (2 wells per antiserum dilution) and incubation overnight at 4 °C. The plates were then rinsed three times with washing solution PBS-Tween 20 (Sigma Aldrich, St. Louis, MO, USA) for 3 min.

Eight dilutions ranging from twofold to 1:1.28 × 10^4 for normal serum (as a control) and the three antisera obtained after 3, 7 and 14 days after the last injection in serum buffer (PBS-Tween 20 containing 2% soluble polyvinylpyrrolidone, 0.2% bovine serum albumin (BSA)) were used. One hundred-microliter aliquots from the diluted normal serum and antisera were added to each well, after which the plates were incubated at 37 °C for 2 h and then washed as before.

Goat anti-rabbit gamma globulin conjugated to alkaline phosphatase (Sigma Aldrich, St. Louis, MO, USA) was diluted 1:2 × 10^4 in serum buffer, and 100 µL was added to each well. The plates were subsequently incubated at 37 °C for 1 h and then washed as before.

One hundred microliters of the enzyme substrate and 1 mg/mL pNPP (p-Nitrophenyl-phosphate) in 10% diethanolamine buffer (pH 9.8) were added to each well, after which the plates were incubated at room temperature (25 °C) for approximately 30 min. The enzyme activity was stopped by adding 50 µL of 3 M sodium hydroxide. The ELISA values were measured by an ELISA reader and were expressed as absorbency at 405 nm; absorbance values at least twofold greater than those of the healthy control (normal serum) were considered positive.

2.5. Antiserum titer

The titer of *R. solanacearum* antiserum was determined by using an indirect ELISA as described previously. Bacterial suspensions were prepared in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6) at a concentration of approximately 10^8 CFU/mL. Wells were coated by adding 100 µL of the bacterial suspension to the wells (2 wells per antiserum dilution), and serial dilutions up to 1:1.28 × 10^4 of antiserum in serum buffer were used. The absorbance values were measured at 405 nm [11,29].

2.5.1. Determination of antiserum efficiency

The indirect ELISA previously described was used to demonstrate the sensitivity of the produced antiserum to differentiate among all brown rot bacterial isolates.

2.5.2. Sensitivity of the indirect ELISA technique

Serial dilutions ranging from concentrations of 1 × 10^2 to 1 × 10^8 CFU/mL of *R. solanacearum* isolates (RsMo2) were prepared. The indirect ELISA was carried out to measure detection sensitivity as

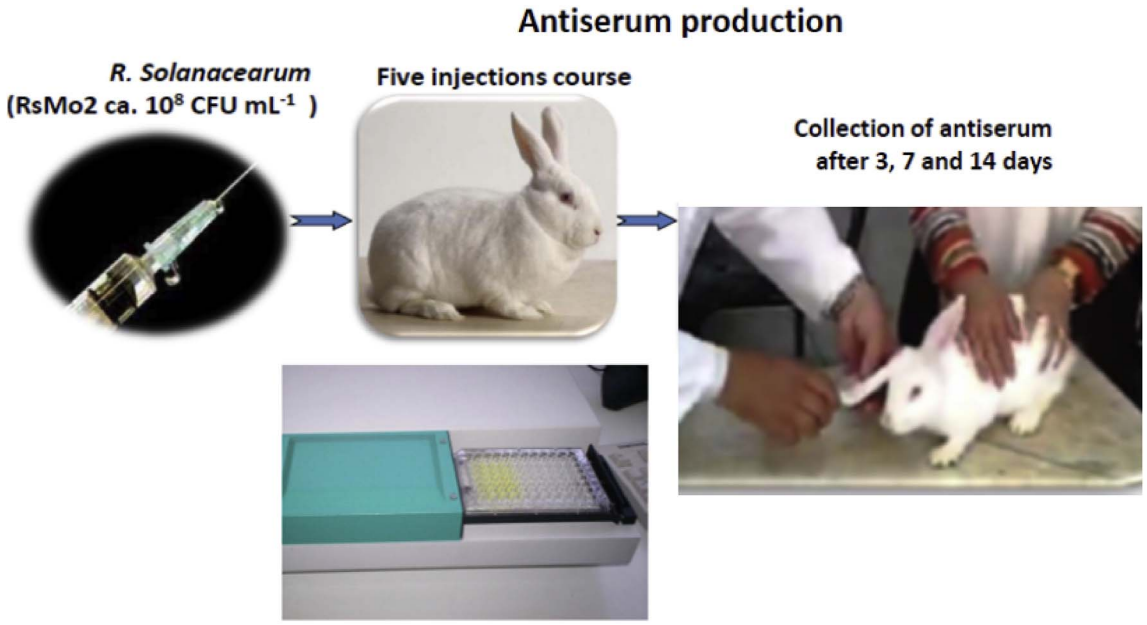

Fig. 4. Antiserum production collected after 3, 7 and 14 days of the five injections (0.5, 1.0, 1.5, 2.0 and 2.5 mL, *R. solanacearum* (RsMo2) in female New Zealand white rabbits.

described previously.

3. Results and discussion

3.1. Pathogenicity test

Virulence experiments showed that all *R. solanaceurm* isolates were pathogenic to potato, tomato, eggplant and pepper with a large variation in the disease index (DI) (Table 1 and Fig. 3) at 28 dpi. Of the four species of solanaceae tested, potato cv. Lady Rosetta was the most susceptible, with a DI equal to 79%, to 8 isolates only two isolates (Rs48 and RsIs2), showed DI less than 40% (Table 1). All strains used in the experiments were able to induce wilt symptoms on tomato seedlings cv. Strain B, but isolate RsSc1, was less virulent, with DI equal to 32%. The most aggressive isolates to tomato were Rs2, RsMo2 and RsBe2 with DI of 96% (Table 1). It should be noted that the isolates RsMo2 and RsBe2, belonging to Race3 biovar 2, isolated from potato, showed DI values higher than those afforded by most isolates when inoculated to the four species of solanaceae plants. These results indicate that isolates belonging to race 3 could be more aggressive to potato other than solanaceous hosts under natural conditions based on host range and other cited papers confirmed that race 3 had wide host range and could move from ornamental plants into potato fields, where it could cause both direct economic damage and quarantine problems [23,30].

3.2. Determination of the optimum antiserum collection period and antiserum titer

The indirect ELISA technique was used to determine the best collection period of the antiserum after the last injection. The results showed that the best collection dates were at 14, 3 and 7 days, in that order. The best positive ELISA values were obtained (14 days) with dilutions of 1:6.4 × 10^3 (Table 2).

3.3. Efficiency of the antiserum to identify R. solanacearum isolates

The indirect ELISA was used to identify 42 bacterial isolates that cause potato brown rot disease. The data presented in Table 3 revealed that our polyclonal antiserum (14 days) reacted positively with all tested isolates at a dilution of 1:6.4 × 10^3.

The data presented in Table 4 show the different reactions of the eight *R. solanacearum* isolates (Rs2, Rs48, RsMo2, RsBe2, RsIs2, RsFr5, RsSc1 and RsNe1) at various dilutions (1:1.6 × 10^3 to 1:5.12 × 10^6) at 14 days with an antiserum concentration of approximately 1 × 10^8 CFU/mL. Five isolates, i.e., Rs48, RsBe2, RsNe1, RsSc1 and RsFr5, positively reacted to dilutions ranging from 1:1.6 × 10^3 to 1:6.4 × 10^3 and negatively reacted to the dilution of 1:5.12 × 10^6, while 3 isolates i.e., Rs2, RsMo2 and RsIs2, reacted positively to dilutions up to 1:2.56 × 10^5.

Table 2
Indirect ELISA absorbance values (405 nm) of *Ralstonia solanacearum* (RsMo2) isolate at approximately 10^8 CFU/mL in various dilutions of its antiserum at different collection periods.

Antiserum dilution	Normal serum	Antiserum collection periods after the last injection		
		3 days	7 days	14 days
1:10^2	0.373	0.708	0.698	0.752
1:2 × 10^2	0.347	0.681	0.702	0.734
1:4 × 10^2	0.331	0.721	0.720	0.719
1:8 × 10^2	0.262	0.705	0.717	0.711
1:1.6 × 10^3	0.225	0.671	0.659	0.663
1:3.2 × 10^3	0.218	0.613	0.596	0.611
1:6.4 × 10^3	0.216	0.517	0.515	0.549
1:1.28 × 10^4	0.256	0.441	0.479	0.461

ELISA absorbance values at 405 nm are the average of two replicates.
Absorbance values at least twice those of the healthy control were considered positive.

3.4. Indirect ELISA sensitivity of detecting R. solanacearum

Sevenfold serial dilutions from concentrations of 1 × 10^8 to 1 × 10^2 CFU/mL of *R. solanacearum* isolates (RsMo2) were used to elucidate the sensitivity of the indirect ELISA to detect *R. solanacearum* dilution limits (Table 5). The results showed that three concentrations of *R. solanacearum* reacted positively with antiserum at dilutions ranging from 1 × 10^8 to 1 × 10^6 CFU/mL but negatively with the other dilutions. These results are in accordance with those obtained by several investigators [11,31,32] who found the use of monoclonal or polyclonal antibodies, can be applied in the case of massive contamination, but latent infections consisting of low concentrations of the pathogen (below 10^6 CFU/mL) require a more sensitive and reliable

Table 3
Reaction of 42 *Ralstonia solanacearum* isolates (approximately 10^8 CFU/mL) to the best collected antiserum (14 days) at a titer dilution of 1:6.4 × 10^3.

Isolate code	Accession number	ELISA	Isolate code	Accession number	ELISA	Isolate code	Accession number	ELISA	Isolate code	Accession number	ELISA
Egypt			Egypt			Egypt			Scotland		
Rs 1	HG425351	+[a]	Rs13		+	RsMo4		+	RsSc1	LN681203	+
Rs2	LN681198	+	Rs14		+	RsBe1	LN681200	+	RsSc2		+
Rs3		+	Rs15		+	RsBe2	LN681202	+	RsSc3		+
Rs4		+	Rs16		+	RsIs1		+	RsSc4		+
Rs5		+	Rs17		+	RsIs2	LN681201	+	RsSc5		+
Rs6		+	Rs18	HG425354	+	France			Netherlands		
Rs7		+	Rs19		+	RsFr1		+	RsNe1	LN681204	+
Rs8	GH4253532	+	Rs20		+	RsFr2		+	RsNe2		+
Rs9		+	Rs48	GH425353	+	RsFr3		+	RsNe3		+
Rs10		+	RsMo1		+	RsFr4		+			
Rs11	HG425355	+	RsMo2	LN681199	+	RsFr5	LN827661	+			
Rs12		+	RsMo3		+						

[a] +, positive reaction.

method to detect the bacterium in a field sample.

The bacterial strains we tested were all pathogenic to all tested solanaceae. They were more aggressive to potato than to tomato or eggplant or pepper [6,7,33]. Ji et al. [30] observed that *R. solanacearum* isolates from ornamentals, pepper, tomato, tobacco and potato showed significant differences in virulence on tobacco, but had similar virulence levels on tomato. In this study, eggplant and pepper were less susceptible to most of the tested isolates, since only two of them (RsMo1 and RsBe2) were aggressive, with DI greater than or equal to 80%.

Serological tests have been used to screen seed potatoes for latent populations of *R. solanacearum* but have generally been found to lack the required specificity and sensitivity. However, the results of the indirect ELISA to determine the optimum period for antiserum collection showed that the third collection date (14 days after the last injection) was the best, followed by the first and then the second (3 and 7 days, respectively), so it could be concluded that the efficiency of the antiserum decreased when the antiserum collection period began earlier. These results contrast with those of Mohamed et al. [34] and with those of Hamwieh et al. [35] and Kayali et al. [36]. The differences could be attributed to the different genera, species and types of pathogenic bacteria involved in the tests.

Positive ELISA antiserum titer values (14 days) were obtained with dilutions up to 1:6.4 × 10^3. These results are in agreement with those of Ashmawy et al. [22], who mentioned the importance of determining the antiserum titer to reduce consumption.

In general, serological assays do not require expensive laboratory equipment and therefore can be used anywhere where simple, inexpensive and relatively sensitive and specific detection on a large scale is desirable, especially under field conditions.

Data obtained in our experiments are of great importance since in the search for resistance of economic plant species to bacterial wilt the following traits should be considered: prevalence of strains in different hosts and regions, susceptibility of certain hosts, virulence of certain strains combined with other features, like the possibility of bacterial survival in association with nonhost plants, soil type and temperature, among other factors, which were not part of our study. The need of understanding the evolutionary relationship, virulence and genetic diversity of strains from different species as *R. solanacearum* hosts may lead to the development of further molecular tools for diagnosis which

Table 5
Sensitivity of the indirect ELISA to detect *Ralstonia solanacearum* (RsMo2) concentration limits*.

Normal serum	Seven serial dilutions of *Ralstonia solanacearum* (CFU/mL)						
	1 × 10^8	1 × 10^7	1 × 10^6	1 × 10^5	1 × 10^4	1 × 10^3	1 × 10^2
0.304	1.599 +++**	1.046 ++	0.690 ++	0.481 -	0.483 -	0.491 -	0.431 -

*, ELISA absorbance values at 405 nm are the average of two replicates.
*, Absorbance values at least twice those of the healthy control (normal serum) were considered positive.
*, One hundred microliters of the bacterial suspension was added to each well.
*, Antiserum titer dilution used was 1:6.4 × 10^3.
** +, positive reaction and -, negative reaction.

Table 4
Indirect ELISA readings for eight *Ralstonia solanacearum* isolates to various dilutions (14 days) of antiserum*.

Antiserum dilution	Normal serum	*Ralstonia solanacearum* isolates (approximately 10^8 CFU/mL)							
		Rs2	Rs48	RsMo2	RsBe2	RsIs2	RsFr5	RsSc1	RsNe1
1:1.6 × 10^3	0.231	0.99 +**	0.575 +	1.43 +	0.532 +	1.27 +	0.555 +	0.666 +	0.619 +
1:3.2 × 10^3	0.230	0.878 +	0.554 +	1.371 +	0.513 +	1.205 +	0.54 +	0.621 +	0.596 +
1:6.4 × 10^3	0.221	0.774 +	0.52 +	1.35 +	0.446 +	0.934 +	0.467 +	0.542 +	0.549 +
1:1.28 × 10^4	0.281	0.674 +	0.491 –	1.244 +	0.486 –	0.811 +	0.475 –	0.495 –	0.502 –
1:2.56 × 10^5	0.267	0.687 +	0.489 –	0.886 +	0.464 –	0.691 +	0.473 –	0.502 –	0.497 –
1:5.12 × 10^6	0.312	0.574 –	0.459 –	0.599 –	0.455 –	0.612 –	0.474 –	0.49 –	0.495 –

*, ELISA absorbance values at 405 nm are the average of two replicates.
*, Absorbance values at least twice those of the healthy control (normal serum) were considered positive.
*, One hundred microliters of the bacterial suspension at a concentration of 10^8 CFU/mL was added to each well.
** +, positive reaction and -, negative reaction.

may be useful for studying the ecology and epidemiology of this important bacterial species. The knowledge of the distribution of *R. solanacearum* in any given country could be very useful for plant breeding programs and/or for quarantine measures.

4. Conclusion

In this study, all molecular identified isolates of *R. solanacearum*, the causal agent of brown rot disease were high to moderate pathogenic on potato and tomato, whereas on the other solanaceae hosts such as eggplant and pepper were moderate to low pathogenic. The antiserum polyclonal antibodies were produced against *R. solanacearum* bacterium. The indirect ELISA technique provided decent detection of the pathogen but was less able to differentiate among the *R. solanacearum* isolates studied.

Conflicts of interest

The authors have declared that there are no competing interests.

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