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Immunoprobe development for detection of *Ralstonia solanacearum* in potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*)

MD RASHID KHAN[1], JAMEEL AKHTAR[2], V S PUNDHIR[3] and ANIL KUMAR[4]

G B Pant University of Agriculture and Technology, Pantnagar, Uttarakhand 263 145

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ABSTRACT

Anti-potato and anti-tomato polyclonal antibodies were developed for serodiagnosis of bacterial wilt. Optimum antigen concentration was standardized as 7.5×10^6 cells for potato (*Solanum tuberosum* L.) and 5×10^7 cells for tomato (*Solanum lycopersicum* L.) per well and dilution of antibacterial antibody was optimized as 1:250 for potato and 1:175 for tomato strains for indirect enzyme-linked immunosorbent assay (ELISA). As small as 1×10^4 bacterial cells were detected by indirect ELISA which showed the greater affinity of raised polyclonal anti-bacterial antibodies for its antigen and higher sensitivity for detection of bacterial pathogen in suspected plants. In the reactivity test of the bacterial isolates with antibodies, all the isolates of *R. solanacearum* identified through conventional methods, showed positive results. All the tomato isolates reacted with both the antibodies. The pathogenic isolates of chilli could not be identified by both the antibodies. The cross reactivity of anti-potato antibodies with only chilli strain of *R. solanacearum* suggests the specificity of these polyclonal antibodies against these strains. While, other strains, except chilli strain, indicates non-homology of antigenic epitopes of bacterial wall proteins with virulent potato strain..

Key words: Bacterial wilt, Immunodiagnosis, Indirect ELISA, Potato, Tomato

Of the various vegetables cultivated in India, potato (*Solanum tuberosum* L.), a poor man's food, is a versatile solanaceous vegetable crop with capacity to be grown from mean sea level to the snowline. This crop produces more food and nutrition per unit area per unit time than any other crops (Shekhawat *et al.* 2000, Sharma *et al.* 2005). It ranks fourth in production, after rice, wheat and maize and provides wholesome food worldwide. Annually about 300 million tonnes of potatoes are produced and consumed by over one billion people world over (FAO 2010). Potato crop is susceptible to many pests including pathogens.

Bacterial wilt caused by *Ralstonia solanacearum* (Smith) Yabuuchi *et al* is one of the most important and destructive bacterial disease, widely distributed in tropical, subtropical and some warm temperate regions of the world (Zhou *et al.* 2011). It is first bacterial disease recorded in India from Pune district of Maharashtra and also first record of its occurrence on potato (Shekhawat *et al.* 2000). In India, losses caused by bacterial wilt vary from 20-100%. Since pathogen is mainly transmitted through seed tubers as latent infection in vascular tissue, which is responsible for introduction of the pathogen in disease free area. Therefore, reliable diagnostic techniques are urgently needed to detect latent infection in seed tubers and for its certification.

[1]Department of Plant Pathology, (e mail: rashid_patho@rediffmail.com), [2]Senior Scientist, National Bureau of Plant Genetic Resources, New Delhi 110012 (e mail: jameelbau@rediffmail.com), [3]Professor, Department of Plant Pathology, (e mail: pundhirvs@gmail.com), [4]Professor, Department of Molecular Biology and Genetic Engineering, (e mail: ak_gupta2k@rediffmail.com)

MATERIALS AND METHODS

The experiment was conducted during the year 2004 to 2008 at Centre of Advances Studies in Plant Pathology and Department of Molecular Biology and Genetic Engineering, G B Pant University of Agriculture & Technology, Pantnagar, Uttarakhand. Antigen for production of antiserum in animal was prepared as described by Chakrabarti *et al*. (1995). The bacterium was cultured on nutrient glucose agar plates for 48 hr at 30°C and the growth was collected by washing with minimum amount of' sterile normal saline (0.855 NaCl). The suspension was centrifuged at 5000 rpm for 20 minutes and the pellet was washed four times with normal saline by resuspending and centrifuging every time at 5000 rpm for 20 minutes. The pellet from the final centrifugation was suspended in 0.3 per cent formalinised saline and the bacterial density was adjusted to 2×10^9 cells/ml. The suspension was stored at 4°C as stock and used as whole cell antigen. Antigen was prepared for two bacterial isolates collected from CPRS,

Modipuram, isolated from potato and tomato (*Solanum lycopersicum* L.) crops. Pathogenicity was proved and virulent colonies were used for antigen preparation.

To develop immunoprobe against bacterial strains (potato and tomato), New Zealand white rabbits were injected with intact bacterial cells. The immunogen was prepared as follows: For first dose, 0.5 ml antigen + 0.5 ml CFA + 1.0 ml phosphate buffer saline (PBS) and for booster dosages, 0.5 ml antigen + 1.0 ml FIA + 1.5 ml PBS were used as per immunization schedule mentioned below:

Days	Injection	Antigen	Adjuvant	Type	Mode
0	I	0.5 ml antigen 1.0 ml PBS	0.5 ml	CFA	SC, IP, foot pad
10	II	0.5 ml antigen 1.5 ml PBS	1.0 ml	FIA	SC, IP, foot pad
40	III	0.5 ml antigen 1.5 ml PBS	1.0 ml	FIA	SC, IP, foot pad
70	IV	0.5 ml antigen 1.5 ml PBS	1.0 ml	FIA	SC, IP, foot pad

After three booster doses, antisera were collected from rabbits hyper-immunized with both the strains of *R. solanacearum* and processed for testing antibody reactivity with collected isolates using indirect ELISA.

The blood was first clotted at room temperature by keeping it for 2 hr and then kept overnight at 4°C. The clear serum was decanted into eppendorf tubes, spinned at 10 000 rpm for 10 minutes at 4°C to get rid-off any remaining red blood cells. The straw coloured clear supernatant was transferred into capped cryovials and stored at –20°C.

The ELISA was developed with minor modification as described initially by Engrall and Perlmann (1971) and later by Clark and Adams (1977). Microtitre ELISA was developed to check the reactivity and specificity of polyclonal antibodies raised against intact bacterial cells (bacterial isolate from Modipuram) as an antigen for immunodetection of identified *R. solanacearum* strains and making differential diagnosis with other isolates made from collections. Microtitre plates were coated with different concentrations of bacterial antigen in coating buffer, i.e. 100 µl/well. Then the plates were incubated for 1 hr at room temperature, 1 hr at 37°C and kept overnight at 4°C. The plates were washed twice with PBS + 0.01 per cent Tween-20. The wells were filled with PBS containing 5% skimmed milk for 2 hr at room temperature. Binding was done to prevent adventitious binding.

Again washing was done 2-3 times with PBS + 0.5 % skimmed milk + Tween-20. Hundred µl of first antibody dilution was incubated for 2 hr at room temperature. The antibody dilution was done with PBS along with 0.5% skimmed milk. Washing was done thrice with PBS + 0.5% skimmed milk and 100 µl of 1:1000 diluted alkaline phosphatase. Conjugated second antibody was incubated for 2 hr at room temperature. Microtitre plates were washed thrice with PBS + 0.5% skimmed milk + Tween-20 (0.01%). Alkaline phosphatase activity was assayed with the substrate solution p-nitrophenyl phosphate sodium salt dissolved in substrate buffer (1 mg/ml) and the plates were incubated for 30 minutes in dark and the reaction was stopped with 100 µl of 1.5 M NaOH solution and finally ELISA reading was taken at optical density $(OD)_{405\ nm}$ by ELISA reader.

To determine optimal antigen concentration, two types of colony (virulent and avirulent) from each of the strains (potato and tomato) were used as antigens in the form of intact bacterial cells. The wells of microtitre plate were coated with different number of bacterial cells (stock suspension) as antigen ranging from 1 to 5×10^4, 1 to 5×10^5, 1 to 5×10^6, 1 to 5×10^7 and 1 to 5×10^8 bacterial cells/well in triplicate. For studying antigen concentration kinetics, primary and secondary antibodies were diluted to 1:250 and 1: 1000 times, respectively.

For determining optimum dilution for antibodies, bacterial concentrations of 7.5×10^6 and 5×10^7 cells for potato and tomato strains, respectively as antigen were coated in each well and different sets of primary antibodies (both strains) dilutions such as 1:100, 1:250, 1:500, 1:750, 1:1000, 1:1250, 1:1500, 1:1750, 1:2000 and 1:2500 were used in triplicate.

Bacterial cells of virulent and avirulent colony of potato and tomato strains and virulent colony of chilli and brinjal strains collected from CPRS, Modipuram were used as antigen for raising antibodies of both strains of *R. solanacearum* to check the cross reactivity. The sample suspension (100 µl) of each strain used as antigen containing 7.5×10^6 cells was coated in the wells of microtitre plate. Primary antibodies developed from potato and tomato strains were diluted at optimum antibodies concentrations determined earlier, viz. 1:250 and 1:175 times, respectively while secondary antibodies (enzyme linked alkaline phosphatase) was diluted to 1:1000 times. Serological identification was confirmed on the basis of colour production after the reaction.

RESULTS AND DISCUSSION

There are marked differences in surface topology of cells of various bacterial pathogens which make the basis of their differential diagnosis. Hence, intact bacterial cells were taken as immunogens for raising polyclonal antibodies and development of immunoassay system in order to detect surface antigen (s) of bacterial cells. New Zealand white rabbits were injected with intact bacterial cells. After three booster doses, the rabbits were bled and antisera were collected from rabbits hyper-immunized with both strains and checked for antibody reactivity with collected isolates using indirect ELISA. The results obtained are described in following section.

Microtitre ELISA was developed to check the reactivity and specificity of polyclonal antibodies raised against intact bacterial cells (bacterial isolates from Modipuram) as an antigen for immunodetection of identified *R. solanacearum*

Table 1 Determination of optimum antigen concentration for antibacterial antibodies of *R. solanacearum* by indirect ELISA

Bacterial Cell Count	ELISA reading at OD_{405} (nm)			
	Potato		Tomato	
	Virulent	Avirulent	Virulent	Avirulent
1×10^4	0.533	0.569	0.424	0.447
5×10^4	0.733	0.672	0.464	0.513
1×10^5	0.753	0.801	0.565	0.614
5×10^5	0.976	0.853	0.999	1.095
1×10^6	0.976	1.149	1.291	1.326
5×10^6	1.827	1.970	1.949	2.265
1×10^7	2.371	2.476	2.972	3.445
5×10^7	3.829	3.899	3.954	3.997
1×10^8	3.911	3.901	3.996	3.999

strains and making differential diagnosis with other isolates made from collections.

As evident from Table 1 and Fig 1 and 2 the optical density (at 405 nm) showed a linear increase with increase in antigen concentration, from 1×10^6 to 1×10^8 and 3×10^6 to 1×10^8 bacterial cells/ml for potato and tomato strains, respectively. In the subsequent experiments carried out by indirect ELISA, the mid point value of a linear increasing up line from the semi log graph was recorded as 7.5×10^6 and 5×10^7 bacterial cells/well for potato and tomato strains, respectively and taken as optimum antigen concentration for indirect ELISA. As small as 1×10^4 bacterial cells were detected by ELISA to justify the greater affinity of raised anti-bacterial antibodies for its antigen and higher sensitivity for detection of bacterial pathogen in suspected plants.

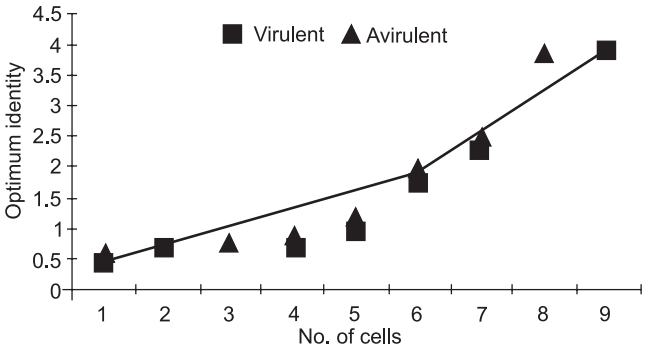

Fig 1 Determination of optimum concentration for anti-potato antibodies of *R. solanacearum* by indirect ELISA.

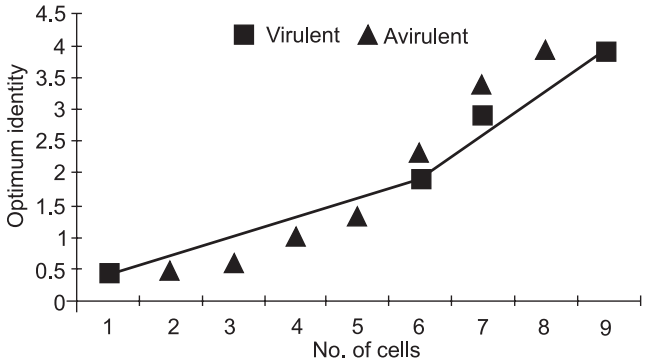

Fig 2 Determination of optimum number of bacterial cells by indirect ELISA using anti-potato antibodies of *R. solanacearum*.

It is evident from Table 2 and Fig 3 and 4 that optical density (at 405 nm) showed a linear decrease with increasing dilutions. A mid point from the antibody dilution curve was selected for potato strain (1:250, mid point of 1:100 and 1:500) and for tomato strain (1:175, mid point of 1:100 and 1:250). These selected antibody dilutions were taken as optimum dilution of antibacterial antibody for further study.

As evident from Table 3, the virulent colony of potato strain showed maximum reactivity with OD_{405} (1.34 nm) followed by chilli strain (1.08 nm) and both were found statistically significant with dark yellow colour reaction. Rest of the strains were found non-significant with no colour production. The OD_{405} recorded for strains except potato

Table 2 Determination of optimum dilution of antibodies for antibacterial antibodies of *R. solanacearum* by indirect ELISA.

Antibodies dilution	ELISA reading at OD_{405} (nm)			
	Potato		Tomato	
	Virulent	Avirulent	Virulent	Avirulent
1:100	3.946	3.873	0.934	0.733
1:250	2.701	2.864	0.623	0.299
1:500	0.397	0.567	0.182	0.136
1:750	0.228	0.253	0.172	0.115
1:1000	0.216	0.237	0.127	0.116
1:1250	0.206	0.221	0.127	0.116
1:1500	0.204	0.217	0.127	0.117
1:1750	0.191	0.217	0.123	0.116
1:2000	0.181	0.216	0.122	0.117
1:2500	0.126	0.210	0.122	0.114

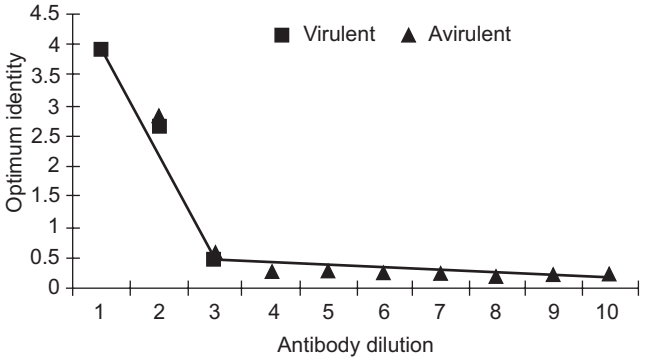

Fig. 3 Determination of optimum antibody dilution by indirect ELISA using anti-potato antibodies of *R. solanacearum*.

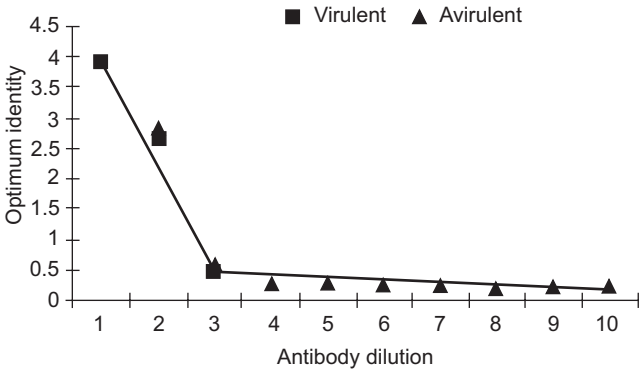

Fig. 4 Determination of optimum antibody dilution by indirect ELISA using anti-tomato antibodies of *R. solanacearum*.

(virulent) and chilli varied from 0.36 to 0.44 nm. The reactivity with virulent strain of potato was taken as 100% for comparison of cross reactivity of anti-potato antibodies with other strains from different solanaceous crops, which varied from 26.86 to 32.83%. On the basis of per cent cross reactivity results, the maximum reactivity was observed with chilli strain (80.59 %) followed by brinjal strain (32.83 %), virulent tomato strain (30.59%), avirulent potato strain (28.40%). Result showed lowest cross reactivity with avirulent tomato strain (26.86%). However, all the strains produced morphologically similar colony on Kelman's TZC medium.

The cross reactivity of anti-potato antibodies with only chilli strain of *R. solanacearum* suggests the specificity of these polyclonal antibodies against these strains. While, other strains except chilli strain, indicate non-homology of antigenic epitopes of bacterial wall proteins with virulent potato strain. The maximum cross reactivity of anti-tomato antibodies was observed with virulent tomato strain (OD_{405} = 1.53 nm), which was statistically significant from control. Rest of the strains gave non-significant result with no colour production.

Table 3 Cross reactivity of Modipuram strains with potato and tomato antibodies

Isolate	ELISA reading at OD_{405} (nm)		Colour Reaction			
			Potato		Tomato	
	P-Ab	T-Ab	P-Ab	T-Ab	P-Ab	T-Ab
Pm-1	1.340	0.380	D			D
Pm-3 (2)	0.380	0.300				
Cm	1.080	0.370	D			D
Bm	0.440	0.400				
Tm-1	0.360	0.320				
Tm-2	0.410	1.530		D	D	
Without Ag	0.055	0.049				
Without Ab	0.052	0.049				
Blank	0.032	0.048				
CD (P = 0.05)	0.412	0.103				
CV	52.037	15.639				

D, Dark yellow

The per cent cross reactivity of virulent tomato strain was assumed 100 per cent for comparison with other strain which varies from 19.60 per cent (with avirulent potato strain) to 26.10% (with brinjal strain). The results revealed that no other strain except virulent tomato strain showed specificity with anti-tomato antibodies.

Anti-bacterial antibodies were tested for their cross reactivity with antigens from different isolates collected from wilted potato fields. Absorbance recorded at OD_{405} could showed the highest reactivity of 1.234 and 1.104 of isolate P-24 with anti-potato and anti-tomato antibodies, respectively. It was considered as 100 per cent reactivity for comparison with other isolates (Table 4). Based on per cent cross reactivity patterns, isolates were divided into two groups, Group A and B. Group A gave more than 50% cross reactivity and colour reaction also in the form of light, dull light and dark yellow. Whereas, Group B did not produce significant colour reaction, with less that 50% cross reactivity with both anti-bacterial antibodies.

On the basis of colour reaction of isolates with both the anti-bacterial antibodies, isolates were divided into 3 groups named as Group 1, Group 2 and Group 3.

The isolates belonging to Group 1 produced colour after reaction with both anti-bacterial antibodies. All potato isolates, which produced colour with anti-tomato antibodies, also gave colour with anti-potato antibodies. Those isolates, which reacted with only anti-potato antibodies resulted colour production either light or dull light colour were placed in Group 2. The isolates of Group 3 did not give any colour reaction with either of the two anti-bacterial antibodies.

The results obtained revealed that only those isolates gave reactivity with anti-bacterial antibodies, which were characterized as *R. solanacearum* by preliminary conventional method of identification.

The maximum OD_{405} was recorded (0.848 and 0.921 nm) in case of isolate with potato and tomato antibodies, respectively. On the basis of per cent cross reactivity, the tomato isolates were classified into two major groups A and B. Group A consisted of 12 isolates where reactivity was more than 75%, except isolates T-19 (2) that gave 47.3% cross reactivity with potato antibodies. Group B showed less that 50% reactivity with 7 isolates. All the isolates of Group A produced colour reaction with both of the anti-bacterial antibodies (Table 5).

Maximum OD_{405} was recorded (1.051 and 0.801 nm) for isolate M-2 against both bacterial (potato and tomato) antibodies (Table 6). The isolates were classified into different groups on the basis of per cent cross reactivity and colour production. On the basis of per cent cross reactivity, isolates were grouped into two groups, i.e. Group A and Group B. Cross reactivity in group A ranged from 68.58-100%. The isolates were found to produce colour reaction with cross reactivity between reaction with cross reactivity between 68.58-100% viz. B-1, M-2, M-5, M-7 and M-8 (2). In Group

Table 4 Cross reactivity of potato isolates with antibacterial antibodies

Isolate	ELISA reading at OD_{405} (nm)		Cross reactivity (%)		Colour reaction	
	P-Ab	T-Ab	P-Ab	T-Ab	P-Ab	T-Ab
P-1	0.144	0.132	11.67	11.96		
P-1	0.187	0.232	15.15	21.01		
P-3	0.138	0.052	11.18	4.71		
P-4 (1)	0.067	0.234	5.43	21.2		
P-5	0.184	0.197	14.91	17.84		
P-11	0.151	0.214	12.24	19.38		
P-13	0.744	0.189	60.29	17.12	L	
P-14	0.704	0.663	57.05	60.05	L	L
P-15 (1)	0.780	0.643	63.21	58.24	D	DL
P-15 (2)	0.726	0.489	58.83	44.29	DL	
P-16	0.116	0.104	9.4	9.42		
P-17	0.800	0.741	64.82	67.12	D	L
P-19	0.090	0.099	7.29	8.97		
P-22	0.721	0.309	58.43	27.99	L	
P-23	0.118	0.117	9.56	10.6		
P-24	1.234	1.104	100	100	D	D
P-25	0.130	0.101	10.53	9.15		
P-26	0.124	0.170	10.05	5.4		
P-30	0.106	0.106	8.59	9.6		
P-31	0.190	0.341	15.4	30.89		
P-32	0.718	0.695	58.18	62.95	L	L
P-34	0.120	0.114	9.72	10.33		
P-35	0.704	0.178	57.05	16.12	DL	
P-42 (1)	0.921	0.867	74.64	78.53	D	D
P-42 (2)	0.729	0.136	59.08	12.32	DL	
P-43	0.763	0.129	61.83	11.68		
P-44 (1)	0.749	0.662	60.7	59.96	L	DL
P-44 (2)	0.703	0.186	56.97	16.85	L	
P-49	0.754	0.693	61.1	62.77	L	DL
P-50	0.824	0.808	66.77	73.19	D	D
P-51	0.393	0.236	31.82	21.38		
P-52	0.219	0.317	17.75	28.71		
P-53	0.689	0.780	55.83	70.65	D	D
CD *(P = 0.05)* for isolates			= 0.053			
Cross reactivity			= 0.013			
Interaction			= 0.075			
CV			= 11.376			

D, Dark yellow; DL, dull light yellow; L, light yellow

B, per cent cross reactivity ranged from 7.23 to 51.38. These isolates, viz. C-2, C-5 and M-8 (1) did not give colour reaction. Whereas, on the basis of colour reaction isolates were clustered into three groups, i.e. Group 1, Group 2 and Group 3. The isolates of Group 1 were found to react with both antibodies and produced colour. Isolates of Group 2 produced dull yellow colour reaction with only tomato antibodies. Isolates of Group 3 did not produce any colour reaction with both of the antibodies.

The pathogenicity of 13 tomato isolates was established

Table 5 Cross reactivity of tomato isolates with antibacterial antibodies

Isolate	ELISA reading at OD405 (nm)		Cross reactivity (%)		Colour reaction	
	P-Ab	T-Ab	P-Ab	T-Ab	P-Ab	T-Ab
T-3	0.814	0.795	95.99	86.32	D	D
T-4 (1)	0.848	0.921	100.0	100.0	D	D
T-4 (2)	0.718	0.762	84.67	82.74	DL	DL
T-5	0.784	0.751	92.45	81.54	L	DL
T-6	0.720	0.742	84.91	80.56	DL	DL
T-7	0.355	0.334	41.86	36.26	-	-
T-8	0.773	0.890	91.16	96.63	D	D
T-9	0.931	0.774	98.00	84.04	D	D
T- 10 (1)	0.291	0.347	34.32	37.68	-	-
T- 10 (2)	0.162	0.258	19.10	28.01	-	-
T- 11 (1)	0.109	0.083	12.85	09.01	--	-
T- 11 (2)	0.294	0.150	34.67	16.29	-	-
T- 11 (3)	0.744	0.772	87.74	83.82	L	L
T- 13	0.667	0.724	78.66	78.61	L	L
T- 14	0.739	0.758	87.15	82.30	D	D
T- 15	0.712	0.708	83.96	7687	DL	DL
T- 16	0.440	0.296	51.89	32.14	-	-
T- 19 (1)	0.257	0.166	30.31	18.02	-	-
T- 19 (2)	0.755	0.772	47.34	83.82	L	L
CD *(P=0.05)* For isolates			= 0.070			
Cross reactivity			= 0.023			
Interaction			= 0.099			
CV			= 10.498			

D, Dark yellow; DL, dull light yellow; L, light yellow

which gave colour reaction with both antibodies except T-10 and T-11 isolates collected from Sangudi and Puccinikera regions of Uttarakhand that were not identified by the serodiagnosis. Five isolates found non-pathogenic, also gave colour reaction with antibodies except T-16 (from Uppal Nagni). While, strain of two variants (virulent and avirulent) from Uppal Nagni was tested and only avirulent variant gave reaction with both antibodies.

The antibodies against tomato and potato strains also recognized brinjal isolate (B-1) and some chilli isolates except virulent variant of M-8 isolate. The pathogenic isolates of capsicum could not be identified by both of antibodies.

Several methods in the past have been used to identify plant pathogenic bacteria, viz. biochemical (Shekahwat *et al*. 2000), physiological (Schaad *et al*. 2001, Williamson *et al*. 2002) and few specific media for selective isolation of bacterium, but these are not entirely satisfactory.

In recent years research has been directed towards developing rapid, sensitive and specific diagnostic assays to detect the presence or absence of *R. solanacearum* in plants as well as in soil. Various immunological techniques till date have been employed successfully for detection and identification of *R. solanacearum* among population and to

Table 6 Cross reactivity of brinjal, chilli and capsicum isolates with antibacterial antibodies

Isolate	ELISA reading at OD_{405} (nm)		Cross reactivity (%)		Colour reaction	
	P-Ab	T-Ab	P-Ab	T-Ab	P-Ab	T-Ab
B-1	0.728	0.649	69.27	81.02	DL	DL
C-2	0.076	0.292	07.23	36.45		
C-5	0.245	0.276	23.31	34.46		
M-2	1.051	0.801	100.0	100.0	D	D
M-4	0.782	0.733	74.41	91.51	L	DL
M-5	0.720	0.705	68.51	88.01	DL	DL
M-7	0.540	0.706	51.38	88.14		DL
M-8 (1)	0.160	0.150	15.22	18.73		
M-8 (2)	0.109	0.734	10.37	91.64		DL
CD *(P = 0.05)* for isolates			= 0.070			
Cross reactivity			= 0.033			
Interaction			= 0.099			
CV			= 12.05			

D, Dark yellow;DL, dull light yellow; L, light yellow

other closely related pathogens through polyclonal or monoclonal antibodies by ELISA (Rajeshwari *et al*. 1998, Kumar *et al*. 2002, Caruso *et al.* 2002, Machmud and Yadi 2008, Mwangi *et al*. 2008, Khan *et al.* 2012 and Kumar *et al*. 2012).

It is important to note that a plant disease should be properly identified before any control is attempted, as some treatments designed to destroy the pathogen may encourage others or may not be cost effective. Diseases that are seed-borne and plants affected by wilts are often very difficult to diagnose as the pathogen is deep seated in host tissues/soil. Using this immunoprobe, indirect ELISA could be one of the most efficient, rapid and sensitive tool for the detection and characterization of *R. solanacearum* causing wilt of potato/ tomato.

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