Eco. Env. & Cons. 27 (May Suppl. Issue) : 2021; pp. (S390-S397)
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ISSN 0971–765X

Cloning, expression and specificity evaluation of type III effector, Rip4, from *Ralstonia solanacearum*

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(Received 14 November, 2020; Accepted 28 December, 2020)

ABSTRACT

Ralstonia solanacearum is a Gram-negative bacterial phytopathogen that belongs to the class of beta proteobacterium. It causes one of the most commercially devastating diseases in plants, known as bacterial wilt disease. The bacterium infects a variety of crops and to date the host range has reached to more than 450 species of plants globally. Various Gram-negative phytopathogens interact with their hosts using Type three secretion systems (TTSS) through which it can inject an array of protein known as effectors. These proteins play an important role in the pathogenicity as well as virulence of the bacterium and can be useful in early diagnosis of the pathogen. Although, several detection methods based on biochemical, molecular and immunological techniques are available for the pathogen identification, but most of them are expensive and time consuming. Hence, keeping above in mind this study was aimed with the cloning and expression of gene *RSc0321* encoding an important Type III effector (TTE) protein, *Ralstonia* injected protein 4 (Rip4) of *R. solanacearum* to produce *recombinant* Rip4 protein (*r*-Rip4). The recombinant protein was purified by affinity chromatography using nickel-nitrilotriacetic acid (Ni-NTA) column and further polyclonal antibodies were raised against the *r*-Rip4. Moreover, the raised antibodies were evaluated for its specificity using Indirect ELISA and Western Blotting. The results generated in the present study, would be helpful for the development of quick and easy immunological detection system for *R. solanacearum*.

Key words : *Ralstonia solanacearum, Type III effector, Cloning, ELISA, Western blotting*

Introduction

Ralstonia solanacearum is a Gram-negative soil-borne bacterial phytopathogen that belongs to the beta proteobacterium class and causes bacterial wilt disease in plants (Genin and Denny, 2012). The bacteria have an exceptionally wide host range of over 450 species of plants belonging to 54 botanical families, including some important commercial crops such as tobacco, tomato, potato and banana (Wicker *et al*., 2007). Various pathogenicity determinants play crucial roles in the virulence of *R. solanacearum*, since the invasion of pathogen to the development of symptoms in the host plant (Peeters *et al*., 2013b).

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These virulence factors include pili (Liu *et al*., 2001) and flagellin (Tans-Kersten *et al*., 2001) involved in root adherence and motility; exopolysaccharides (McGarvey *et al*., 1999) secreting cell wall degrading enzymes; type III secretory system (TTSS) (VanGijsegem *et al*., 1995), known to serve as molecular syringes for the injection into host cells of virulence factors, called effector proteins, where a variety of host cellular functions are suppressed or stimulated to promote pathogen development. The TTSS is known as the Hrp (hypersensitive response and pathogenicity) in plant pathogens because defects in the TTSS lead to the loss of ability to induce hypersensitive responses as well as pathogenicity in plants (Alfano and Collmer, 1997). Unlike the highly conserved TTSS, Type III Effectors (TTE) show variation in their distribution among strains and into the eukaryotic hosts and are prone to diversifying selection (Peeters *et al*., 2013a). Among, various TTE, Ralstonia injected proteins (Rips) are known as crucial for the pathogenicity of the bacterium (Peeters *et al*., 2013a). A broad range of Rips has been identified in *R. solanacearum* strains as 72 reported in the model strain GMI1000 (Mukaihara *et al*., 2010), while 94 *Rip* genes have been identified in other strains of *R. solanacearum* species complex (Peeters *et al*., 2013a). Owing to the vast devastation caused to various agriculturally important crops, there is a need for quick and early detection of the pathogen. Although, several techniques, including semi selective culture techniques (Elphinstone *et al*., 1996) and nucleic acid amplification-based assays using polymerase chain reaction (PCR) techniques (Stulberg *et al*. 2015) as well as serological assays such as enzyme-linked immunosorbent assays (ELISA) (Priou *et al*., 2006), have been developed to detect *R. solanacearum*. But, these studies are either costly due to thermocyclers and quantitative PCR (qPCR) or not efficient to diagnose the pathogen. ELISA assays involving polyclonal antibodies against the whole cell give a minimum detection limit in the range of $10^{4\text{-}5}$ cells/ml and can be useful for early diagnosis of the pathogen (Behiry *et al*., 2018). Hence, keeping above in mind, the present study was aimed with cloning and expression of the gene *RSc0321* encoding one of the important TTE protein, Rip4. The cloned gene was expressed in *E. coli* to produce *recombinant* Rip4 protein (*r*-Rip4). Further, polyclonal antibodies were raised against the purified *r*-Rip4 protein in rabbit and specificity was evaluated by Indirect Enzyme-linked immunosorbent assay (ELISA) and Western blotting against various strain of *R. solanacearum* and other non *R. solanacearum* species.

Materials and Methods

Bacterial strains and media

All the isolates were recovered from the diseased tomato plants, cultivated in the state Uttarakhand and confirmed by 16S rDNA sequencing. *R. solanacearum* strains namely DIBER115, DIBER116, DIBER117, DIBER118, DIBER119 and DIBER120 were submitted in the National Center for Biotechnology Information (NCBI) gene bank and assigned the accession number MG266193 (DIBER115), MG266202 (DIBER116), MG266199 (DIBER117), MG266203 (DIBER118), MG266201 (DIBER119) and MG266200 (DIBER120). *R. solanacearum* strain Rs0418 was procured from National Agriculturally Important Microbial Culture Collection (NAIMCC) India. However, non *R. solanacearum* strains such as *R. insidiosa* (ATCC® 49129™), *R. pickettii* (ATCC® 648™), *R. mannitolilytica* (MTCC 8774), *Bacillus subtilis* (ATCC® 11774™), *Escherichia coli* (ATCC® 10536™) used in this study were procured from American Type Culture Collection (ATCC), USA and Microbial Type Culture Collection (MTCC), Chandigarh, India. *R. solanacearum* strains were grown aerobically on modified semi-selective medium South Africa (mSMSA) composed of casamino acid, peptone, and glycerol supplemented with antibiotics (Elphinstone, *et al*., 1996) at 28°C for 24 to 72 h. Other non *R. solanacearum* strains were grown in SMSA medium. In addition, *B. subtilis* and *E. coli* strain were grown in nutrient broth with constant shaking at 37°C for 24h. However, *E. coli* strain M15, used for cloning and protein expression was grown in Luria-Bertani (LB) medium containing kanamycin (100µg/mL) (LB + kan) with shaking incubator 200 rpm at 37°C.

Protein selection and Primer designing

PSORTb v.2.0 (https://www.psort.org/psortb/) (Gardy *et al*., 2005), an online tool was used to identify different effectors proteins of *R. solanacearum*. Further, each of the protein encoded by genes of main chromosome and mega plasmid were individually blasted for its specificity using the NCBI-protein BLAST. Gene sequence *RSc0321* (Accession no. NC003295) encoding for the selected protein i.e.

Rip4 was retrieved from NCBI database. Based on the results, primers were designed with appropriate restriction enzyme sites incorporated on them, to facilitate the further cloning process. Importantly, selection of the restriction enzyme sites to be added in the primers depends upon the fact that particular restriction site should not be present in the gene to be amplified. Restriction sites present in the gene were identified using the online restriction enzyme analysis tool, WebCutter ver. 2.0 (http://heimanlab.com/cut2.html) (Maarek *et al*., 1997).

Gene inserts preparation

Cloning was carried out using synthetic DNA of *RSc0321* gene. Gene encoding the selected protein was PCR amplified with cloning primers i.e. forward 0321 FP-5'TGCGAGCTCGATAGGAACTTC3' and reverse 0321 RP-5'TTCAAGCTTCAGGTGCTGGGA3', and synthesized DNA as a template as per the method described by Mullis and Faloona (1987). A standardized PCR procedure was performed with initial denaturation temperature at 95°C for 5 min, 35 cycle of 95°C for 30 sec, 60°C for 20 sec, 72°C for 1.5 min followed by 72°C for 10 min using Q-Cycler 96+ (Hain Lifesciences, UK). Amplified PCR product was purified using Qiagen PCR product purification kit (Qiagen, Germany) according to the manufacturer's instructions. Further, double digestion of the purified PCR product and the vector pQE-30 (Qiagen, Germany) was done using suitable restriction enzyme at 37ºC to make the sticky ends. The restriction digested product was run in 1.2% agarose gel and extracted using Qiagen Gel purification kit (Qiagen, Germany) as per the manufacturer's instructions.

Ligation and transformation

Ligation of the gel purified double digested product of insert and vector was done using T4 Ligase Enzyme (ThermoFisher Scientific, Massachusetts, USA) under standard conditions as described by Sambrook and Russell (2001) to make vector-insert construct. The construct was transformed into competent *E. coli* strain M15 cells as per the standard protocol (Sambrook and Russell, 2001). Selection of transformed cells was done in LB agar plates supplemented with ampicillin (100 µg/mL), however, the *E. coli* strain M15 cells without pQE-30 vector were used as the control. Further, the transformed cells were grown overnight at 37ºC and few colonies were randomly selected; the presence of the insert in the transformed cells was confirmed by PCR using the specific cloning primers (0321 FP and 0321 RP).

Expression of recombinant proteins in *E. coli* strain M15 cells

Clones which tested positive in the PCR were selected and recombinant protein expression was carried out with final IPTG (Sigma, USA) concentration of 1 mM along with induced (M15 cells without insert) and non-induced (transformants) control at 37ºC for 4 h. Then, 1 mL of the samples were collected and pelleted before induction (0 h non-induced) and 4 h after induction (induced). Expression was confirmed by performing SDS PAGE - Western blotting according to the protocol by Sambrook and Russell (2001). The membrane was probed with anti-His-HRP conjugate antibodies at a dilution of 1:5000 and the Ag-Ab interaction was revealed using water-soluble chromogenic substrate 3,3'- Diaminobenzidine (DAB), (Sigma, USA) and H_2O_2, to visualize the immunolabelled bands.

Purification of recombinant protein and production of polyclonal antibodies

Recombinant proteins expressed in *E. coli* can be produced either in soluble form or they aggregate and form insoluble inclusion bodies, so before the bulk purification, solubility of the expressed recombinant protein was determined using sonicator with a microtip probe (Vibra-Cell™ Sonics, USA). Briefly, cells of IPTG induced culture of positive clones were harvested and washed three times with sterile 1X phosphate buffer saline (1X PBS), subsequently cells pellet was re-suspended in lysis buffer and sonicated at 6× 10 s with 10 s pause at 40 amplitudes. Sonicated cell lysate was centrifuged at 4ºC and both supernatant and pelleted cells were then individually analyzed by SDS-PAGE. Thereafter, bulk expression and purification of the r-Rs0321 protein was performed using Qiagen Nickel-Nitrilotriacetic acid (Ni-NTA) affinity purification column (Qiagen, Germany) as per the manufacturer's instructions. Further polyclonal antibodies against *r*-Rip4 were raised in rabbit by outsourcing from Abgenex Private Limited, Bhubaneswar, Odisha.

Indirect ELISA optimization

Indirect ELISA was performed as described previously by Rajeshwari *et al*., (1998) with slight modifi-

cations. Briefly, antigenic suspensions of recombinant protein were prepared in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6) at a concentration ~ 20 ng/mL. For each antiserum dilution, two replicates wells were coated with 100 µL antigenic suspension and incubation at 37°C for 1 h. Then, the plates were rinsed three times with 1X PBS-Tween 20 (PBS-T) washing solution for 3 min, followed by blocking the unbound sites in the wells by adding 200 µL of 1% BSA solution (in 1X PBS) and incubation overnight at 4°C. Doubling dilutions of polyclonal antisera ranging from $1:1.5\times10^3$ to $1:2.86\times10^5$ in 1X PBS were used and 100 µL from each dilution was added in respective wells and the plates were incubated at 37°C for 1 h and then washed as described previously. Goat anti rabbit IgG conjugated to horse raddish peroxidase (HRP) diluted to 1:5000 in 1X PBS, 100 µL of the diluted conjugate was added to each well and the plates were incubated at 37°C for 1 h and then washed as before. One hundred microliters of the development buffer [5 mg o-phenylenediamine (OPD) and 20 µL H_2O_2 in 20 mL of sodium phosphate buffer] was added to each well and subsequently plates were incubated at room temperature for 10 min. Thereafter, to stop enzyme activity 50 µL of 5M H_2SO_4 was added to each well. Absorbance was measured at 405 nm using microplate reader (Synergy HT Multi-Detection Microplate Reader, Bio Tek Instruments, Inc., USA). Absorbance values greater than the cut off value were considered as positive. Cut off value was calculated as described by Classen *et al*., (1987) using following formula:

Cut-off value = mean of negatives + 5 times the standard deviation

Further, checker board titration was done for ELISA optimization to determine the optimum working concentrations of antigen and antisera. Indirect ELISA was performed similarly as described previously except, different dilutions of purified protein (antigen) in the range of 320 - 5 ng/well and the immunized sera in the range $1:0.5\times10^3$ - $1:1.6\times10^4$ were tested.

Evaluation of antisera specificity

After determining the optimum working concentrations of antigen and its antisera, specificity evaluation of raised antiserum was carried out by ELISA and Western blotting. Indirect ELISA was also performed similarly as described above. Overnight grown bacterial cells of *R. solanacearum* and non *R. solanacearum* strains, having approximately 10^6 CFU/ml were harvested and washed three time in 1X PBS and approximately 1 mL of cell suspension was used in sample preparation for SDS-PAGE. Gel was electrotransferred onto nitrocellulose membrane following standard protocol. After consecutive incubation with the polyclonal antiserum (1:1000) and HRP conjugated anti-rabbit secondary antibody (1:5000), water soluble chromogenic substrate DAB and H_2O_2 was used to visualize the immunolabelled bands.

Results

Gene insert preparation, ligation and transformation

Designed forward and reverse cloning primers for the gene *RSc0321*: forward 0321 FP-5′TGC**GAGCTC**GATAGGAACTTC3′ and reverse 0321 RP- 5′TTC**AAGCTT**CAGGTGCTGGGA3′, have inserted *Sac* I and *Hind* III restriction sites (bold and underlined). PCR product was produced as a DNA fragment of 933 bp; both purified PCR product and vector were digested as confirmed by agarose gel electrophoresis of digested products.

Expression of recombinant proteins in *E. coli* strain M15 cells

Colonies observed in LB + kanamycin plates with ampicillin as selective agent, indicates the successful transformation. However, non transformed cells lacking pQE-30 vector did not grow in media supplemented with ampicillin. The presence of insert was primarily confirmed by PCR of the extracted plasmids from the selected clones, clones showing amplification in PCR were named as clones T1-T9 (Fig. 1).

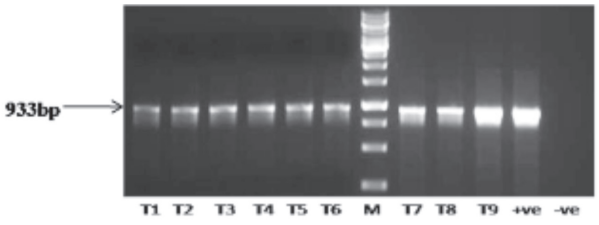

Fig. 1. Detection of positive clones by PCR; Positive clones showing DNA bands corresponding to 933bp, Agarose gel electrophoresis showing transformants (T1 to T9); positive and negative control; Marker (M) 1 kb DNA Ladder (Thermo Scientific).

Further upon IPTG induction three clones showed band corresponding to the molecular weight of protein i.e. 34 kDa in SDS-PAGE and western blotting (Fig. 2).

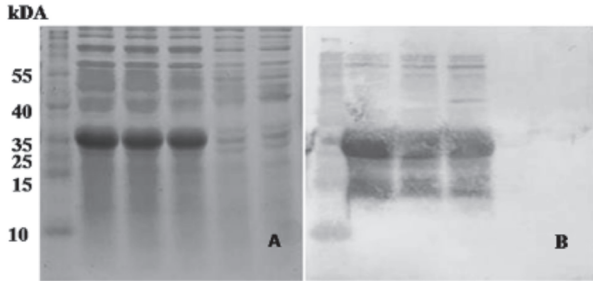

Fig. 2. A) SDS PAGE and B) Western Blot analysis of induced and non induced clones; Lane 1-3: 4 h induced cells of selected clones showing bands of protein corresponding to approximately 31 kDa; Lane 4, 5: 0 h cells of clones (non induced control); M - Pre-stained Protein Ladder (ThermolFisher Scientific; 10-180 kDa)

Purification of the r- protein

During elution eluates showing band corresponding to the molecular weight of the protein, were pooled (Fig. 3). Molecular weight of the recombinant protein was found to be approximately 34 kDa.

Optimum antibody titer and ELISA optimization

Mean absorbance of different dilutions summarized in Table 1.

Highest dilutions that showed absorbance greater than the *cut off* value were selected as the optimum antibody titer to detect its corresponding antigen. *Cut off* value of the absorbance for Indirect ELISA of r- Rs0321 protein (200 ng/100 µL) in various dilution of normal serum was 0.158. Here we observed that dilution $1{:}9.6\times10^4$ showed absorbance greater than the *cut off* value.

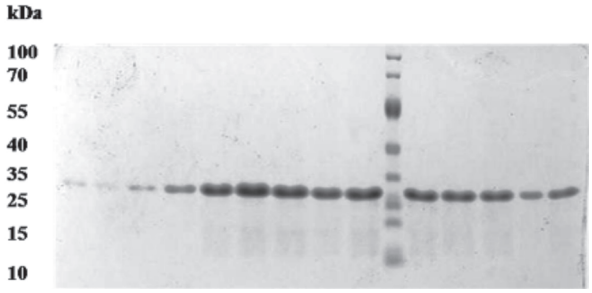

Fig. 3. SDS – PAGE analysis of the collected fractions FT: Flow through; W1, W2: Wash fraction; 1-11: Eluates, M-Pre-stained Protein Ladder (ThermoFisher Scientific; 10-180 kDa)

Table 1. Indirect ELISA absorbance values of purified r-RS0321 protein at 200 ng/100mL in various dilutions of its antiserum *cut off value* = 0.158 + 5(0.008) = 0.158. Dilution giving absorbance value greater than the *cut off value* of 0.158 was set as optimum antibody concentration. Absorbance given is the mean of the replicates.

Antiserum Dilution	Normal serum Absorbance (405nm)	Immunized serum Absorbance (405nm)
$1{:}1.5\times10^3$	0.120	0.434
$1{:}3\times10^3$	0.119	0.339
$1{:}6\times10^3$	0.140	0.270
$1{:}1.2\times10^4$	0.122	0.232
$1{:}2.4\times10^4$	0.119	0.193
$1{:}4.8\times10^4$	0.117	0.165
$1{:}9.6\times10^4$	0.114	0.160
$1{:}1.92\times10^5$	0.116	0.150

Mean absorbance of different antigenic dilution against various dilutions of antiserum is shown in Table 2. Dilutions having absorbance above the *cut off* value were selected as the optimum working dilutions of antigen and antisera, here we observed that antigen dilution of 5 ng/100µL and antiserum dilution of $1{:}8.0\times10^3$ showed absorbance greater than the *cut off* value of 0.317.

Evaluation of antisera specificity

Antiserum dilution of $1{:}8.0\times10^3$ showed reactivity with *R. solanacearum* strains DIBER115, DIBER116, DIBER117, DIBER118, DIBER119, DIBER120 and Rs0418, however it did not showed any reactivity with non *R. solanacearum* and other bacterial strain including *B. subtilis*, *E. coli* and *Ochrobactrum* sp. except for *R. pickettii*. This shows that the raised polyclonal antibodies could react specifically with the expressed Rip4 protein in DIBER's *R. solanacearum* strains along with non *R. solanacearum* strain *R. pickettii*. Mean absorbance of replicates of different strains was outlined in Table 3.

Also, similar results were observed in Western blotting as *R. solanacearum* strains DIBER115, DIBER116, DIBER117, DIBER118, DIBER119, DIBER120 and Rs0418 showed band corresponding to the molecular weight of protein. Results of the Western blotting were depicted in Fig. 4.

Discussion

Bacterial Type Three Secretion (TTS) system are the

Table 2. Absorbance of checkerboard titration involving serially diluted antigenic and antiserum suspension; *Cut off value* = 0.242 + 5 (0.015) = 0.317 Absorbance greater than 0.317 was selected as positive and the corresponding antigenic and antibody dilution was selected for further ELISA procedure. Absorbance shown here is the mean of the replicates used.

Antigen Dilution (ng/100 µL)	Antiserum dilution					
	1:0.5 × 10^3	1:1.0×10^3	1:2.0 × 10^3	1:4.0×10^3	1:8.0× 10^3	1:1.6 × 10^3
320	1.180	1.123	1.140	1.05	0.911	0.781
160	1.017	0.968	0.927	0.832	0.749	0.658
80	0.808	0.766	0.731	0.464	0.554	0.535
40	0.650	0.588	0.618	0.525	0.462	0.463
20	0.549	0.504	0.473	0.463	0.429	0.426
10	0.429	0.4	0.419	0.393	0.367	0.516
5	0.354	0.324	0.327	0.341	0.327	0.526
0	0.222	0.231	0.255	-0.263	0.240	0.240

Table 3. Table highlights the mean absorban ce values of ELISA at 405 nm of different stains. Reaction of *R. solanacearum* and non *R. solanacearum* strains (approximately 10^4 CFU/mL) with antiserum dilution of 1.8.0×10^3 *Cut off value* = 0.166 + 5(0.01)=0.216. Strains giving absorbance greater than the cutoff value were considered positive

S.No.	Strain	Abs	ELISA
	R. solanacearum		
1.	DIB115	0.217	+
2.	DIB116	0.220	+
3.	DIB117	0.219	+
4.	DIB118	0.216	+
5.	DIB119	0.218	+
6.	DIB120	0.217	+
7.	DIB418	0.218	+
	Non *R. solanacearum* strains		
8.	*E. coli*	0.185	–
9.	*R. subtilis*	0.170	–
10.	*R. pickettii*	*0.235*	+
11.	*R. insidiosa*	*0.165*	–
12.	*R. mannitolilytica*	*0.214*	–
13.	*Ochrobactrum* sp.	0.210	–

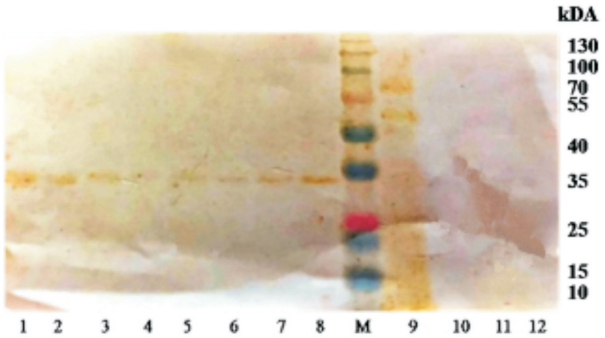

Fig. 4. Western Blotting results of washed cell suspensions (10^6 CFU/ml) of *R. solanacearum* and non *R. solanacearum* strains; Lane 1–DIBER 115, Lane 2-DIBER 116, Lane 3–DIBER117, Lane 4–DIBER118, Lane 5–119, Lane 6–120, Lane 7–Rs0418, Lane 8–Positive Control (recombinant protein rRip4), Lane 9–*R. pickettii*, Lane 10–*R. insidiosa*, Lane 11–*B. subtilis*, Lane 12 – *E. coli*, M – Thermo Scientific™ Page Ruler™ Plus Prestained Protein Ladder, 10 to 250 kDa

key components and plays important role in host-pathogen interaction as they can subvert as well as trigger the host plant immune system (Büttner, 2016). *R. solanacearum* have broad TTE repertoires among 72-94 Rips already identified in several strain (Peeters *et al*., 2013a). This wide range of TTEs might be one of the reasons behind its extensively broad host range and global distribution (Tan *et al*., 2019). Also *R. solanacearum* TTEs are conserved in all four phylotypes, spreads over all continents and infect a variable range of host plants (Peeters *et al*., 2013a). Thus, there is a need to identify the viulent proteins so that these can further be targeted with the main aim of the immunological based detection of the bacterium. In the present study results of ELISA as well as Western blotting analysis indicates that the raised polyclonal antibodies showed specificity against *R. solanacearum* strains DIBER115, DIBER116, DIBER117, DIBER118, DIBER119, DIBER120 and Rs0418, however it did not showed any reactivity with non *R. solanacearum* and other bacterial strains including *B. subtilis*, *E. coli* and *Ochrobactrum* sp. except for *R. pickettii*. Among, the *R. pickettii* lineage comprises *R. pickettii*, *R. mannitolilytica*, *R. solanacearum* and *R. insidiosa*, so could share the similar epitope. Earlier, Safni *et al*.,

(2014) also proposed that *R. solanacearum* should be renamed as RSSC and reclassified into three different species: *R. solanacearum* (phylotypes IIA and IIB), *R. pseudosolanacearum* (phylotypes I and III) and *R. syzygii* (phylotype IV and BDB). Importantly, ELISAs developed earlier for *R. solanacearum* based on polyclonal antibodies against the whole cell were of little use in the detection of the pathogen, as they could discriminate only Pseudomonads and non-Pseudomonads and not able to discriminate between various races, biovars, genera and species of *R. solanacearum* (Seal and Elphinstone, 1994).

Acknowledgement

The authors extend their heartfelt thanks to the Director, DIBER-DRDO for continuous support and providing working infrastructure. Research fellowship provided by DST-INSPIRE for conducting research work is deeply accredited. Author is also thankful to Dr. Ravindra Chhimwal for moral support and time to time guidance.

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to emend the descriptions of *Ralstonia solanacearum* and *Ralstonia syzygii* and reclassify current *R. syzygii* strains as *Ralstonia syzygii* subsp. *syzygii* subsp. nov., *R. solanacearum* phylotype IV strains as *Ralstonia syzygii* subsp. indonesiensis subsp. nov., banana blood disease bacterium strains as *Ralstonia syzygii* subsp. celebesensis subsp. nov. and *R. solanacearum* phylotype I and III strains as *Ralstonia pseudosolanacearum* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*. 64(9) : 3087-3103.

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