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Discrimination of *Ralstonia solanacearum* isolates by genetic signatures produced by single-strand conformation polymorphism and low-stringency single specific primer PCR analysis

Avinash Prakasha, S. Umesha*, Sri Raghava and Shirin Marahel

Department of Studies in Biotechnology, University of Mysore, Manasagangotri, Mysore-570006, Karnataka, India.

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Development of molecular biology based techniques have led to reliable characterization and determination of the genetic diversity among phytopathogens. Single-strand conformation polymorphism (SSCP) and Low-stringency single specific primer (LSSP)-PCR were assessed for genetic typing of *Ralstonia solanacearum* isolates from suspicious bacterial wilt fields. *R. solanacearum* isolates obtained were amplified by colony PCR analysis with *egl* specific primers which amplified a PCR product of 237 bp. These amplified products were denatured and separated in a polyacrylamide gel to develop PCR-SSCP fingerprints, which confirms *R. solanacearum* by producing similar four banding patterns. The amplified product of colony-PCR was subsequently used as a template for LSSP-PCR analysis. The individual genotyping of each *R. solanacearum* obtained by LSSP-PCR were able to discriminate solanaceae and ginger isolates into two different clusters along with pathogenic and non-pathogenic. The LSSP-PCR profile of *R. solanacearum* isolates were closely related and evolved by the genome of host origin and diverge in genomic stability which was further confirmed by sequence analysis. In conclusion, SSCP and LSSP-PCR techniques were most effective compared to biochemical and physiological assays for identification and genetic variability in *R. solanacearum,* which has high genetic divergence. The rapid identification of *R. solanacearum* plays a crucial role in prevention of bacterial wilt.

Key words: *Ralstonia solanacearum,* Bacterial wilt, SSCP-PCR, LSSP-PCR, Molecular detection.

INTRODUCTION

Ralstonia solanacearum (Smith) is a gram-negative, soil-borne bacterium belonging to the class β-proteobacteria, which causes a widespread disease known as bacterial wilt (Yabuuchi et al., 1994). Many economically important crops as well as weeds have been infected and they act as a carrier for this pathogen. The host range of *R.*

*Corresponding author. E-mail: su@appbot.uni-mysore.ac.in.

solanacearum is exceedingly wide, including 450 plant species representing more than 50 families of botanical flora (Ramesh et al., 2009).

The pathogen comprises diverse species such as *Ralstonia syzygii* and *Ralstonia pickettii* and blood disease bacterium (BDB) which differ in host range, pathogenicity, geographical distribution and physiological properties. The pathogen survives for years in wet soil, water ponds, on plant debris or in asymptomatic weed hosts, which act as inoculum reservoirs. *R. solanacearum* is endemic and made as quarantine important in many developed and developing countries in tropical, subtropical, and warm temperate regions of the world (Mansfield et al., 2012; Ramesh et al., 2014).

Conventionally the pathogen was characterized by its host range and divided into five races. race 1 infect solanaceae family and many other hosts; race 2 infect primarily banana; race 3 infect potato, tomato and a few other hosts, usually in more temperate conditions; race 4 infect ginger and race 5 infect mulberry (Hayward, 1991). In past few decades another classification was adapted; *R. solanacearum* was classified into six biovars along with the race. Biovars classification is based on the ability to produce acid from carbohydrates. The only association between races and biovars is that race 3 corresponds to biovar 2 (Hayward, 1991). These methods of characterization are of limited utility because, the host range overlaps especially after artificial inoculation (Santana et al., 2012).

Molecular-based approaches have been developed to enhance understanding of genetic diversity in *R. solanacearum.* There are seven extracellular enzymes secreted by type II secretion system during infection of bacterial wilt such as, β-1, 4-endoglucanase (*Egl*) (Ramesh et al., 2014) an exoglucanase (CbhA), an endopolygalacturonase (PehA or PglA), two exo-polygalacturonases (PehB and PehC), a pectin methylesterase (Pme) which have been extensively studied (Kang et al., 2002). The genetic variablity among the strains of *Ralstonia* spp (*R. syzygii* and *R. pickettii* and BDB) is due to recombination within the *egl* and *hrpB* genes, rather due to mutations (Castillo et al., 2007). Several genes were targeted such as *hrpB, egl* and *speI* genes for specific identification of *R. solanacearum* (Umesha and Avinash, 2014).

Single-strand conformation polymorphism (SSCP) and low-stringency single specific primer (LSSP-PCR) are the key alternative techniques which differentiate and identify the plant pathogens into species and isolate level. There are several reports that SSCP-PCR technique can be used for detection of most infectious infected virus and bacterial pathogens along with oncogenes and allelic variations in the human genome (Gadiou et al., 2009; Chandrasekhar et al., 2012; Delamare et al., 2012).

Five major pathogens *Escherichia coli, Clostridium perfringens, Campylobacter jejuni, Salmonella enterica* and *Bacillus cereus* were confirmed by SSCP using 16S rRNA gene (Oh et al., 2008). In our previous studies we have developed the species–specific DNA fingerprints for species identification and diagnosis of phytobacterium pathogens and their diseases (Umesha et al., 2012).

Low-stringency single specific primer technique represents a simple repetition of PCR process with one of the two primers used in the initial amplification by creating low-stringency. Serovars of *Leptospira* were analyzed by G1 and G2 primers that led to differentiation of *Leptospira* species (Oliveira et al., 2003). Discrimination of serogroups of *Leptospira* from different animal reservoirs and clinical suspicion of leptospirosis were studied using LSSP-PCR (Bomfim and Koury, 2006).

Low-stringency single specific primer PCR has been efficiently used to ascertain genetic variability in phytopathogenic virus and protozoal parasites such as *Entamoeba histotlytica, Trypansoma cruzi* and *Trypanosoma* (Marquez et al., 2007; Oh et al., 2008). Recently, kDNA genetic correlations among human and canine isolates of *Lesishmania infantum* were examined by LSSP-PCR (Alvarenga et al., 2012). Differentiation of Plum pox virus isolates was achieved by the SSCP and LSSP-PCR techniques with the help of HC-Pro genomic region (Gadiou et al., 2009).

Management of bacterial wilt using chemicals has faced constraints because of genetic diversity in *R. solanacearum* (Ramadasappa et al., 2012; Ramesh et al., 2014; Naik et al., 2015). Diversity in *R. solanacearum* strains is a major problem in India and Andaman Islands even today (Sakthivel et al., 2016). SSCP and LSSP-PCR techniques were efficiently employed to ascertain genetic diversity of many pathogens. Hence in this present study, our aim is to develop SSCP-PCR and LSSP-PCR techniques to study genomic variations of *R. solanacearum* isolated from the different host reservoirs. To our knowledge, this will be the first report to employ SSCP and LSSP-PCR to understand the polymorphic variations and discrimination of pathogenic and non pathogenic isolates among *R. solanacearum* with significant focus on *egl* specific gene.

MATERIAL AND METHODS

Collection and screening of plant material and soil samples for *R. solanacearum*

Bacterial wilt suspected plant material and soil samples from different hosts viz., tomato, chilli, potato, eggplant, ginger and black pepper from agricultural fields (Karnataka, India) were collected from during 2012 to 2014 (Table 1).

The samples were brought into the laboratory and subjected to laboratory assays such as direct plating and liquid assay methods for isolation of *R. solanacearum* by following the standard procedures of ISTA (ISTA, 2005). The suspected plant materials were cut into small pieces (5 mm) and the surface was disinfected with sodium hypochlorite solution (3%; v/v) followed by five repeated washings with sterile distilled water.

Samples were directly plated on semi-selective medium [Kelman's

Table 1. *Ralstonia solanacearum* isolates used in this study: source and year of isolation; pathogenicity test and biovars classification and sequencing with corresponding sequence accession number in NCBI database.

Host	Isolate	Year of isolation	Pathogenicity	Biovars	Accession no.
Tomato	DOB RS T1	2012	+	3	KP711278
Tomato	DOB RS T2	2012	+	3	KP658425
Tomato	DOB RS T3	2012	+	1	KP658424
Tomato	DOB RS TS4	2012	+	3	NS
Tomato	DOB RS TS5	2012	+	3	NS
Tomato	DOB RS TS6	2012	+	1	NS
Tomato	DOB RS T7	2012	+	1	NS
Tomato	DOB RS T8	2012	+	1	NS
Tomato	DOB RS TS9	2012	+	1	NS
Tomato	DOB RS T10	2013	+	3	KP658426
Tomato	DOB RS T11	2013	+	3	KP711279
Tomato	DOB RS TS12	2013	+	3	NS
Tomato	DOB RS TS13	2013	+	3	NS
Tomato	DOB RS TS14	2013	+	3	NS
Tomato	DOB RS TS15	2013	+	3	NS
Tomato	DOB RS TS16	2013	+	1	NS
Tomato	DOB RS TS17	2013	+	1	NS
Tomato	DOB RS TS18	2013	+	1	NS
Tomato	DOB RS TS19	2013	+	1	NS
Tomato	DOB RS T20	2013	+	1	NS
Tomato	DOB RS T21	2014	+	3	KP711280
Tomato	DOB RS T22	2014	+	1	KP711281
Tomato	DOB RS T23	2014	+	3	KP711282
Tomato	DOB RS T24	2014	+	3	NS
Tomato	DOB RS T25	2014	+	3	NS
Tomato	DOB RS T26	2012	-	1	NS
Tomato	DOB RS T27	2012	-	3	NS
Tomato	DOB RS T28	2012	-	3	NS
Tomato	DOB RS T29	2013	-	3	NS
Tomato	DOB RS T30	2013	-	3	NS
Chilli	DOB RS C1	2012	+	3	KP658422
Chilli	DOB RS CS2	2012	+	3	NS
Chilli	DOB RS CS3	2012	+	3	NS
Chilli	DOB RS CS4	2012	+	3	NS
Chilli	DOB RS CS5	2012	+	3	NS
Chilli	DOB RS CS6	2012	+	3	NS
Chilli	DOB RS CS7	2012	+	3	NS
Chilli	DOB RS C8	2012	+	3	NS
Chilli	DOB RS C9	2012	+	3	NS
Chilli	DOBRS C10	2014	+	3	NS
Chilli	DOB RS C11	2014	+	3	NS
Chilli	DOBRS C12	2014	+	3	KP658423
Chilli	DOB RS C13	2014	+	3	NS
Chilli	DOBRS C14	2014	+	3	NS
Chilli	DOB RS CS15	2014	+	3	NS
Chilli	DOBRS CS16	2014	+	3	NS
Chilli	DOBRS CS17	2014	+	3	NS
Chilli	DOB RS CS18	2014	+	3	NS
Chilli	DOB RS CS19	2014	+	3	NS

Table 1. Contd.

Potato	DOB RS P1	2012	+	2	NS
Potato	DOB RS P2	2012	+	2	NS
Potato	DOB RS PS3	2012	+	2	NS
Potato	DOB RS PS4	2012	+	2	NS
Potato	DOB RS PS5	2012	+	2	NS
Potato	DOB RS PS6	2012	+	2	NS
Potato	DOB RS PS7	2012	+	2	NS
Potato	DOB RS PS8	2013	+	2	KP701010
Potato	DOB RS P9	2013	+	2	KP701011
Potato	DOB RS P10	2014	+	2	KP701012
Potato	DOB RS P11	2014	+	2	KP701013
Potato	DOB RS PS12	2014	+	2	NS
Potato	DOB RS PS13	2014	+	2	NS
Potato	DOB RS PS14	2014	+	2	NS
Potato	DOB RS PS15	2014	+	2	NS
Potato	DOB RS P16	2014	+	2	NS
Potato	DOB RS P17	2014	+	2	NS
Potato	DOB RS P18	2014	+	2	NS
Potato	DOB RS P19	2012	-	2	NS
Potato	DOB RS P20	2012	-	2	NS
Potato	DOB RS P21	2012	-	2	NS
Potato	DOB RS P22	2014	-	2	NS
Eggplant	DOB RS E1	2012	+	1	NS
Eggplant	DOB RS E2	2012	+	1	NS
Eggplant	DOB RS E3	2012	+	1	NS
Eggplant	DOB RS E4	2012	+	1	KP148262
Eggplant	DOB RS E5	2012	+	3	NA
Eggplant	DOB RS E6	2012	+	3	KP221801
Eggplant	DOB RS E7	2012	+	1	KP221802
Eggplant	DOB RS E8	2012	+	1	KP221803
Eggplant	DOB RS E9	2012	+	3	NS
Eggplant	DOB RS ES10	2012	+	1	NS
Eggplant	DOB RS ES11	2012	+	1	NS
Eggplant	DOB RS ES12	2012	+	1	NS
Eggplant	DOB RS ES13	2012	+	1	NS
Eggplant	DOB RS ES14	2012	+	1	NS
Eggplant	DOB RS ES15	2012	+	3	NS
Eggplant	DOB RS ES16	2012	+	3	NS
Eggplant	DOB RS ES17	2012	+	3	NS
Eggplant	DOB RS ES18	2013	+	1	KP221804
Eggplant	DOB RS ES19	2013	+	1	KP221805
Eggplant	DOB RS ES20	2013	+	1	NS
Eggplant	DOB RS ES21	2013	+	1	NS
Eggplant	DOB RS E22	2013	+	1	NS
Eggplant	DOB RS E23	2013	+	1	NS
Eggplant	DOB RS E24	2013	+	3	NS
Eggplant	DOB RS E25	2013	+	3	NS
Eggplant	DOB RS E26	2013	+	3	NS
Eggplant	DOB RS E27	2014	+	1	KP711283
Eggplant	DOB RS E28	2014	+	1	KP711284
Eggplant	DOB RS E29	2014	+	1	NS
Eggplant	DOB RS E30	2014	+	1	NS

Table 1. Contd.

Eggplant	DOB RS E31	2014	+	1	NS
Eggplant	DOB RS E32	2014	+	1	NS
Eggplant	DOB RS E33	2014	+	1	NS
Eggplant	DOB RS E34	2014	+	3	NS
Eggplant	DOB RS E35	2014	+	3	NS
Eggplant	DOB RS E36	2014	+	3	NS
Eggplant	DOB RS E37	2012	-	3	NS
Eggplant	DOB RS E38	2012	-	1	NS
Eggplant	DOB RS E39	2012	-	1	NS
Eggplant	DOB RS E40	2012	-	1	NS
Eggplant	DOB RS E41	2013	-	1	NS
Eggplant	DOB RS E38	2014	-	3	NS
Black pepper	DOB RS BPS1	2014	-	4	KP658429
Black pepper	DOB RS BPS2	2014	-	4	KP658430
Black pepper	DOB RS BPS3	2014	-	4	NS
Black pepper	DOB RS BPS4	2014	-	4	NS
Black pepper	DOB RS BPS5	2014	-	4	NS
Black pepper	DOB RS BPS6	2014	-	4	NS
Ginger	DOB RS GS1	2014	-	4	NS
Ginger	DOBRS GS2	2014	-	4	NS
Ginger	DOBRS GS3	2014	-	4	NS
Ginger	DOBRS GS4	2014	-	4	NS
Ginger	DOBRS GS5	2014	-	4	NS
Ginger	DOB RS GS6	2014	-	4	NS
Ginger	DOBRS GS7	2014	-	4	NS
Ginger	DOBRS GS8	2014	-	4	NS
Ginger	DOBRS GS9	2014	-	4	NS
Ginger	DOB RS GS10	2014	-	4	NS
Ginger	DOBRS GS11	2014	-	4	NS
Ginger	DOBRS GS12	2014	-	4	NS
Ginger	DOBRS GS13	2014	-	4	NS
Ginger	DOB RS GS14	2014	-	4	NS
Ginger	DOBRS GS15	2014	-	4	NS
Ginger	DOBRS GS16	2014	-	4	NS
Ginger	DOBRS GS17	2014	-	4	NS
Ginger	DOB RS G18	2014	-	4	KP658428
Ginger	DOBRS G19	2014	-	4	NS
Ginger	DOBRS GS20	2014	-	4	NS
Ginger	DOBRS GS21	2014	-	4	KP658427
Potato	DOBCPR 12	2009	+	2	KP658421
Soil	NCIM 5331	2009	-	3	GQ17488

Ralstonia solanacearum isolates obtained from different host and agricultural fields of Karnataka. SI-Serial number, '+' indicates positive for pathogenicity test, '-' indicates negative for pathogenicity test, NS-Not Submitted to data base due to sequence similarity while annotation.

triphenyl tetrazolium chloride TZC medium; (glucose, 10 g; peptone, 10 g; casein hydrolysate, 1 g; agar, 18 g; distilled water 1,000 ml, 5 ml of TZC solution filter sterilized was added to the autoclaved medium to give final concentration of 0.005%)] (Kelman, 1954). Liquid assay was performed by macerating the plant material using sterile mortar and pestle in 10 ml of sterile distilled water.

The supernatant (1 ml) was mixed with 9 ml of sterile distilled water to obtain a dilution of 10^{-1} and further serial dilutions were prepared up to 10^{-5}. Fifty microliters of each dilution were spread on semi-selective media. In addition, the collected soil samples were also subjected to serial dilution technique up to 10^{-5} dilutions, and aliquots of 50 µl of each dilution were spread on semi-selective media using Drigalski's spreaders in triplicates. All plates were incubated at 28±°C for 24 to 48 h.

The bacterial culture supplemented with 20% glycerol stock and stored in Department Stock Collection Centre (Departmental studies

in Biotechnology, University of Mysore, Mysore, India) at -80°C. Bacterial isolates collected from agricultural fields from soil, and plant material were subjected to biochemical/physiological as well as hypersensitivity tests as reported earlier (Umesha and Avinash, 2014).

Pathogenicity tests were conducted in five week-old-highly susceptible cultivars of tomato (cv. PKM-I) and eggplant (cv. Chaman 363) by inoculating each isolates. The density of cell suspension was adjusted to 0.45 OD at 600 nm to obtain final concentration 1 x 10^7 cfu/ml using spectrophotometer (Beckman Coulter, CA. USA) (Avinash and Umesha, 2014). This bacterial suspension was poured near the roots of plants under green house condition. A reference culture of *R. solanacearum* was procured from NCIM (5331), Pune and Central Potato Research Institute (DOBCPR 12), Shimla, India and they are used as a standard for all these biochemical/physiological and pathogenicity tests. All experiments were carried out in three replicates and repeated thrice.

Molecular characterization of *R. solanacearum*

PCR reaction was performed with RS-Egl-F (5'-GACACCACGACCCTGAAGAC-3') and RS-Egl-R (5'-AAGGTATGCCAGGTGGCGCA-3') primers. The specific primers derived from sequence of *egl* gene were designed from primer 3 software and custom synthesized from Sigma, India. Detection of *R. solanacearum* was performed using sub cultures grown at 28±2°C in TZC medium. Colony PCR was performed by following the procedure of Umesha et al. (2012). Briefly, a loop full of colonies from each bacterial isolate was suspended in 100 *µl* sterile distilled water and serially diluted up to 10^{-6}. The suspensions were boiled for 10 min and for each isolate, an aliquot of 1.25 µl was used as template for PCR amplification. For identification of *R. solanacearum,* PCR was performed in a final volume of 25 µl prepared in 0.2 ml PCR tubes. The PCR reaction mixture consists of 1 µl of 100 mM dNTPs, 2.5 µl of 10 x buffer, 1 U of *Taq* DNA polymerase, 2.0 µl of each forward and reverse primers of 25 pmol. The PCR tubes were placed in a thermo cycler (Labnet, Multigene gradient, CA. USA). Programmed thermal cycle as initial denaturation at 95°C for 5 min, followed 25 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 30 s, elongation step at 72°C for 1 min and final extension at 72°C for 5 min. The amplification of the primers was checked by diluting single colony bacterial isolates from 10^{-1} up to 10^{-6} in sterile distilled water without isolating DNA. The PCR amplicons were stained with ethidium bromide and gels were documented using Geldoc 1000 System-PC (Bio-Rad, Gurgaon, India). Immediately, the amplified DNA products were excised from the agarose gel with a sterile scalpel and the gel was purified with a QIAquick® Gel Extraction Kit Quiaquick gel extraction Kit (Qiagen, Hilden, Germany) following manufacturer's instruction. DNA was further quantified with NanoDrop 2000, 10 µl eluted samples (20 ng/µl) were used as template for SSCP.

SSCP-PCR analysis

Ten µl of individual eluted (20 ng/µl) PCR products were mixed with 25 µl of denaturing buffer [95% formamide, 20 mM EDTA and 0.05% bromophenol blue]. The mixtures were heated at 96°C for 10 min and immediately chilled with ice (Chandrasekhar et al., 2012; Umesha et al., 2012). Denatured PCR products were loaded on to 8% acrylamide–bisacrylamide non-denaturing gel, containing 8 ml of acrylamide/bis (40%) and 4 ml of 10x TBE, 40 µl of tetramethylethylenediamine, 10% ammonium per sulfate and 28 ml water. Thirty five µl of each mixture was loaded and electrophoresed in pre-chilled 1x TBE buffer at 200 V for 2 h at room temperature. An aliquot of ssDNA ladder was also loaded into gel to facilitate comparison of SSCP fingerprinting patterns which were further stained with silver staining and gels were documented.

LSSP-PCR analysis

The LSSP-PCR analysis was carried out in 20 µl reaction mixture containing 3 µl of amplified DNA template 20 ng/µl, 100 mM dNTPs, 5 µl of 10 x buffer, 2 U of *Taq* DNA, and 50 pmol of RS-Egl-F primer. After denaturation step at 95°C for 10 min, annealing at 35°C for 1 min and extension at 72°C for 1 min, the same experimental conditions were repeated with respect to RS-Egl-R. LSSP-PCR profiles were visualized on silver staining in 8% polyacramide gels after electrophoresis in pre-chilled 1x TBE buffer at 200 V for 2 h at room temperature.

Genetic profiling of 126 *R. solanacearum* isolates which are confirmed by pathogenic test, along with 15 non-pathogenic was screened for inter-specific variability. The LSSP-PCR experiments were conducted using other phytopathogenic bacteria such as *Xanthomonas perforans* and *X. oryzae* pv. *oryzae* were analyzed to verify nonspecific banding patterns and all these experiments were repeated thrice for negative controls. Bands ranging from 100 to 600 bp were selected for phenotypic analysis. LSSP-PCR genetic profiles were scored based on the presence (1) or absence (0) of each amplified banding patterns and bands were compared using matching coefficient of similarity to determine the proportion of mismatched bands among isolates. Cluster analysis was carried out based on similarity of genetic profile from LSSP fingerprints. To assure the reproducibility and stability of genetic signature of LSSP-PCR in these *egl* primers, the experiments were conducted in triplicates with the same specific PCR template for two times. The most identical LSSP-PCR signatures were analyzed with the dendrogram. The obtained clusters from the dendrogram analyzed based on resemblance to original distance using Unweighted Pair Group Method with Arithmetic mean (UPGMA) software associated with arithmetic averages clustering algorithm and the randomization procedure as implemented in Tools for Population Genetic Analyses (TFPGA) (Bomfim and Koury, 2006).

Sequence analysis

To determine the nucleotide sequences of *R. solanacearum*, colony PCR amplified products (237 bp) (Figure 1) were sequenced (Eurofins, Bangalore, India). Prominent LSSP bands (Figure 3 and 4) were selected and used for excision and nucleotide sequence determination by a ''crush and soak'' method (Bharathkumar et al., 2008) and further sequenced. The alignment of nucleotide sequences from the isolates along with the reference isolate was analyzed in CLUSTALW software (http://www.ebi.ac.Uk/ Tools/msa/ clustalw2/). The homologies of the sequences were analyzed using BLAST-N (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analyses of sequences from different isolates were conducted using Mega 6.0 software. Phylogenetic tree was constructed using the neighbour-joining and maximum likelihood (p-distance) with bootstrap test of 1000 repetitions. Nucleotide sequences obtained from the current study have been deposited in the GenBank and obtained their accession numbers (Table 1).

RESULTS AND DISCUSSION

Collection and screening of plant material and soil samples for *R. solanacearum*

In the present study, we have isolated *R. solanacearum*

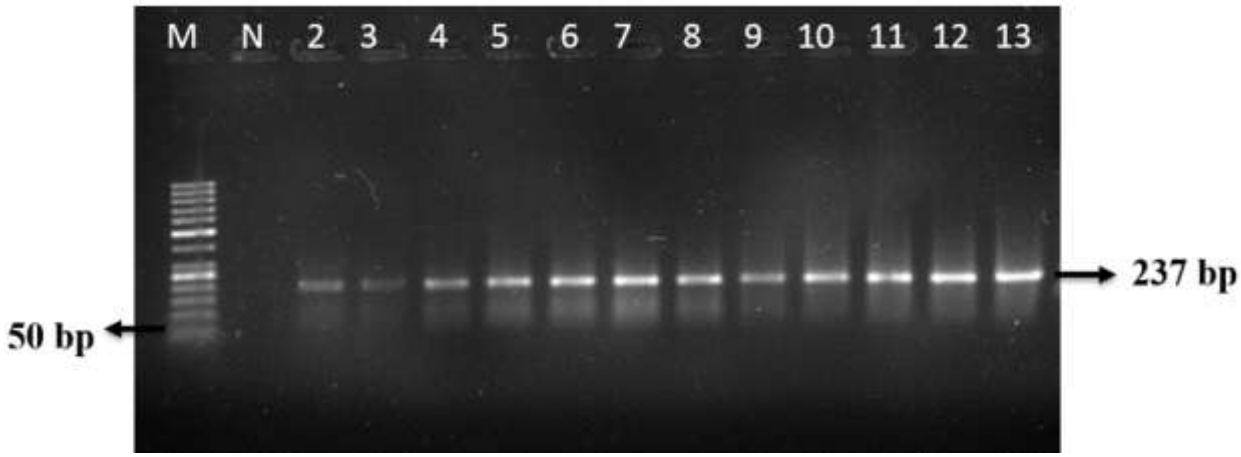

Figure 1. Colony PCR amplification of *Ralstonia solanacearum* with negative control. 1.5% Agarose gel electrophoresis of PCR products. Lane 2 to 7 indicates tenfold serial dilutions of *Ralstonia solanacearum* colony from 10^1 to 10^6. Lane 8-13 amplification of different isolates of *Ralstonia solanacearum*, N-Negative, M: 50-bp DNA ladder.

from agricultural fields and demonstrated the application of SSCP-PCR and LSSP-PCR techniques for molecular characterization. The present study indicated the presence of *R. solanacearum* in soil and plant material from the agricultural fields of India. The morphological identification of *R. solanacearum* showed typical creamy mucoid colony with pink centers which was observed in Lewis et al. (2007) and Avinash and Umesha (2014).

A total of 160 plant samples and 90 soil samples were collected during the field survey from 300 different suspected agricultural fields. Among these, 156 bacterial isolates showed the typical mucoid creamy colony with pink centers on TZC medium. Further *R. solanacearum* isolates were subjected to biochemical/physiological tests which exhibited typical characteristics of *R. solanacearum*. One hundred and fourty one out of 156 bacterial isolates proved to be *R. solanacearum* by biochemical as well as physiological characterization tests by differentiating into 4 different biovars biovras (1, 2, 3, 4) by utilization of carbohydrates (Table 1).

R. solanacearum isolates were preserved in 20% of glycerol stored at -80°C for long term preservation. Pathogenicity test conducted in highly susceptible cultivars of eggplant (cv. Chaman 363) and tomato (cv. PKM 1), exhibited typical wilt symptoms among 76% of isolates. Interestingly, *R. solanacearum* isolates which were isolated from ginger and black pepper did not induce any bacterial wilt symptoms either on tomato or eggplant. Control plants did not show any disease symptoms of bacterial wilt. The greenhouse experiments were performed in four replicates (16 plants each) and derived from three independent experiments with similar results.

Generally, tomato is being used as a biological indicator plant for conducting pathogenicity test of *R. solanacearum* (Hayward, 1991) and recently eggplant (cv. Agassaim) was used to assay pathogenicity of *R. solanacearum*. The present study has an evident that, eggplant cultivar (cv. Chaman 363) can be used for pathogenicity test along with tomato which confirm the earlier results of Ramesh et al. (2014).

R. solanacearum isolates from solanaceae species induce bacterial wilt diseases on eggplant and tomato cultivars, whereas the isolates obtained from the ginger and black pepper were non-pathogenic to these cultivars. Similar observations were reported earlier confirming that *R. solanacearum* isolates from ginger were non-pathogenic to solanaceous vegetables (Mondal et al., 2011).

Colony PCR and purification of amplified product

R. solanacearum subjected to specific PCR assay with developed primers RS-Egl-F and RS-Egl-R revealed specific amplification of a 237 bp product (Figure 1), thus confirming the pathogen as *R. solanacearum*. There are several reports with respect to *egl* specific region from different hosts, for instance ICMP 8229 from ginger, CIP 65 from chilli and E 152 from eggplant, Banana was identified in different geographical regions viz., Philippines, Cost Rica, Malaysia (Lewis et al., 2007).

Similarly, reports were also found with regard to highly virulent *R. solanacearum* strains UW120 and UW276 which are found in Mexico, USA and Kenya in solanaceous plant (Wicker et al., 2007). The results obtained from the present studies correlates previous reports in amplifying *R. solanacearum* isolates from Indian origin using endoglucanase region and confirms the presence of *R. solanacearum* in India (Ramesh et al., 2014; Sagar et al., 2014).

Diversity within the *R. solanacearum* isolates has

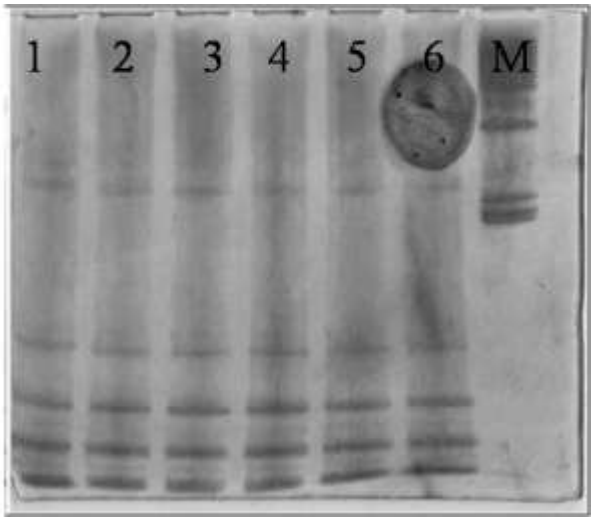

Figure 2. SSCP-PCR profile of *egl* region in *Ralstonia solanacearum* isolates. The purified DNA was denatured and SSCP banding patterns were visualized on non-denaturing polyacrylamide gels. Lane 1-6 indicates isolates of *Ralstonia solanacearum* from different hosts. ssDNA marker (M).

been studied from decades using techniques such as 16S rRNA, AFLP, RFLP, BOX-PCR, rep-PCR and multiplex PCR (Wicker et al., 2007; Parkinson et al., 2013). Even though these techniques are specific, sensitive and rapid but not free from difficulties such as additional enzymes, instrumentation and high cost, are difficult to adopt in developing countries when screening large number of isolates. However the problem can be overcome by simple repetition of PCR and denaturation of the amplified product by SSCP and LSSP-PCR.

SSCP-PCR analysis

In the present study, the SSCP was performed for 141 isolates according to the procedure by Orita et al. (1989) with slight modification as represented in Chandrasekhar et al., (2012). The samples were denaturated to get single stranded DNA followed by 3-dimentional folding of single strands. The motilities of the ssDNA of all tested isolates exhibited similar species–specific banding patterns.

Electrophoresis of denatured 237 bp purified products exhibited four major bands for *R. solanacearum* with respect to *egl* gene genome (Figure 2). There was no variation in the banding patterns of *R. solanacearum* among pathogenic and non-pathogenic isolates from different agricultural fields and variable hosts. These results indicated that the mobility of the molecules during SSCP analysis was similar for all isolates of *R. solanacearum,* along with reference isolate NCIM 5331 and DOBCPR12 (Figure 2). Whereas, SSCP-PCR profile of *R. syzygii*, *R. pickettii* and BDB were distinguish by variation of two to three bands (Supplementary 1).

Due to its robustness, SSCP-PCR can be used for rapid specific identification and differentiation of *R. solanacearum, R. syzygii*, *R. pickettii* and BDB up to species level. The developed technique was unable to show any genetic diversity among the isolates, but the SSCP-PCR profile has great advantage to distinguish the pathogen up to species level, viz., *R. solanacearum, R. syzygii*, BDB, and *R. pickettii*. Similarly, in our previous reports, 40 isolates of *R. solanacearum* were confirmed by 16S rRNA primers, which exhibited four major bands. The other phytopathogenic bacteria *Xanthomonas perforans* and *Xanthomonas oryzae* pv. *oryzae* exhibited two major bands differentiates phytopathogenic bacteria by SSCP-PCR (Chandrasekhar et al., 2012). Colony SSCP-PCR was developed for identification of phytopathogenic bacteria which exhibited SSCP banding patterns of *X. perforans* and *R. solanacearum* (Umesha et al., 2012).

LSSP- PCR analysis

Genetic profiling of *R. solanacearum* from different hosts and geographic origin was analyzed through LSSP-PCR, which showed variations in the genetic profiles with RS-Egl-F.*R.*

Solanacearum exhibited an interspecific variability, comprised of DNA fragments varying from 100 to 600 bp. However, LSSP-PCR profile was able to share similar DNA fragments of 200 and 300 bp in all 141 isolates of *R.*

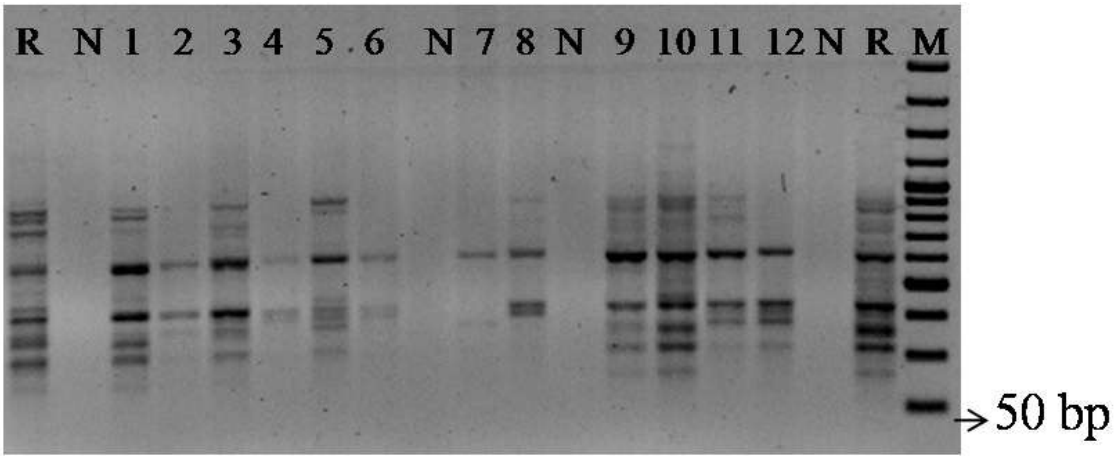

Figure 3. LSSP Signature of *Ralstonia solanacearum* isolated from different hosts in FR/F. Lane 1-6 indicates pathogenic isolated from solanaceae hosts, Lane 7-8 non-pathogenic isolated from solanaceae hosts. Lane 9-10 from ginger, 11-12 isolates are from black pepper, N-Negative control, R-NCIM 5331 reference strain and M-Marker.

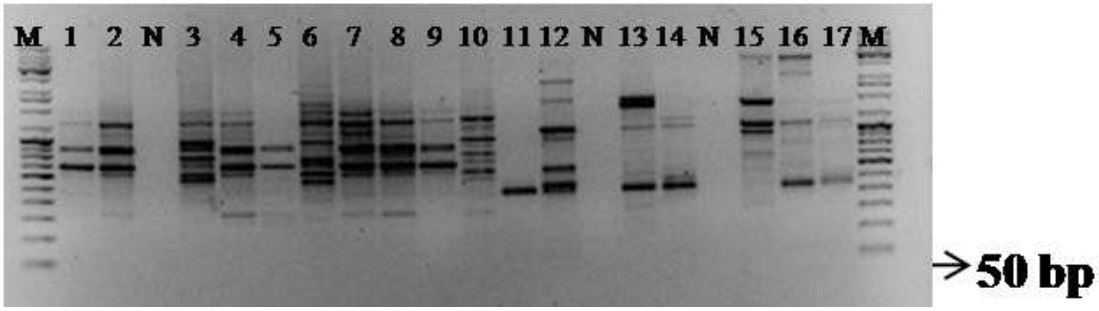

Figure 4. LSSP Signature of *Ralstonia solanacearum* isolated from different hosts in FR/R. Lane 1-2 indicates NCIM 5331 and DOBCPR 12 reference strains, N-Negative control; Lane 3-12 indicates isolates of solanaceous hosts, lane 13-14 from black pepper, 15-17 isolates are from ginger and M-Marker.

solanacearum. Similarly, the variation in the banding patterns at 100, 237, 290, 350, 430 and 500 bp and absence of some banding patterns also exhibited variations with different host range (Figure 3).

LSSP-PCR patterns exhibited four to six bands among *R. solanacearum* isolates which are pathogenic to solanaceous hosts. Whereas, LSSP patterns in 15 non-pathogenic isolates from solanaceous crops exhibited (one-four) decreased in the banding pattern (Supplementary 2) compared to pathogenic isolates. By comparing the size and number of DNA fragments, it was possible to determine the similarity existing among LSSP-PCR patterns obtained from *R. solanacearum* within solanaceous isolates. The developed technique is able to differentiate pathogenic and non-pathogenic isolates of *R. solanacearum* among solanaceous crops. Interestingly, the LSSP-PCR of *R. solanacearum* isolates from ginger and black pepper exhibited high level DNA polymorphism of seven to eight banding patterns (Figure 3). Out of 141 *R. solanacearum* isolates, all isolates showed variable genetic profile when examined by LSSP-PCR using *egl* forward (F) and *egl* reverse (R) primers. The LSSP-PCR analysis of RS-Egl-R primer in *R. solanacearum* isolates revealed diversified banding patterns compared to RS-Egl-F. The genetic profile of the obtained fragments varied with reference to different hosts. Genetic signature determined by Image3 software were made up of fragments ranging from 150 to 800 bp, exhibited an interspecific genetic variability. The similarities in the banding patterns were also observed with respect to reverse primer, amplified at 300, 400 and 600 bp in all 141 *R. solanacearum* isolates. Most similar genetic profiling of *R. solanacearum* was exhibited within the solanaceous host. The *R. solanacearum* isolated from ginger and black pepper exhibited different genetic profiles. There were no amplification from colony/template DNA of pathogens viz., *X. perforans* and *X. oryzae* pv. *oryzae* with respect to forward and reverse primers in LSSP-PCR analysis (Figure 4). The constitutive bands of DNA fragments of *R. solanacearum* were analyzed based

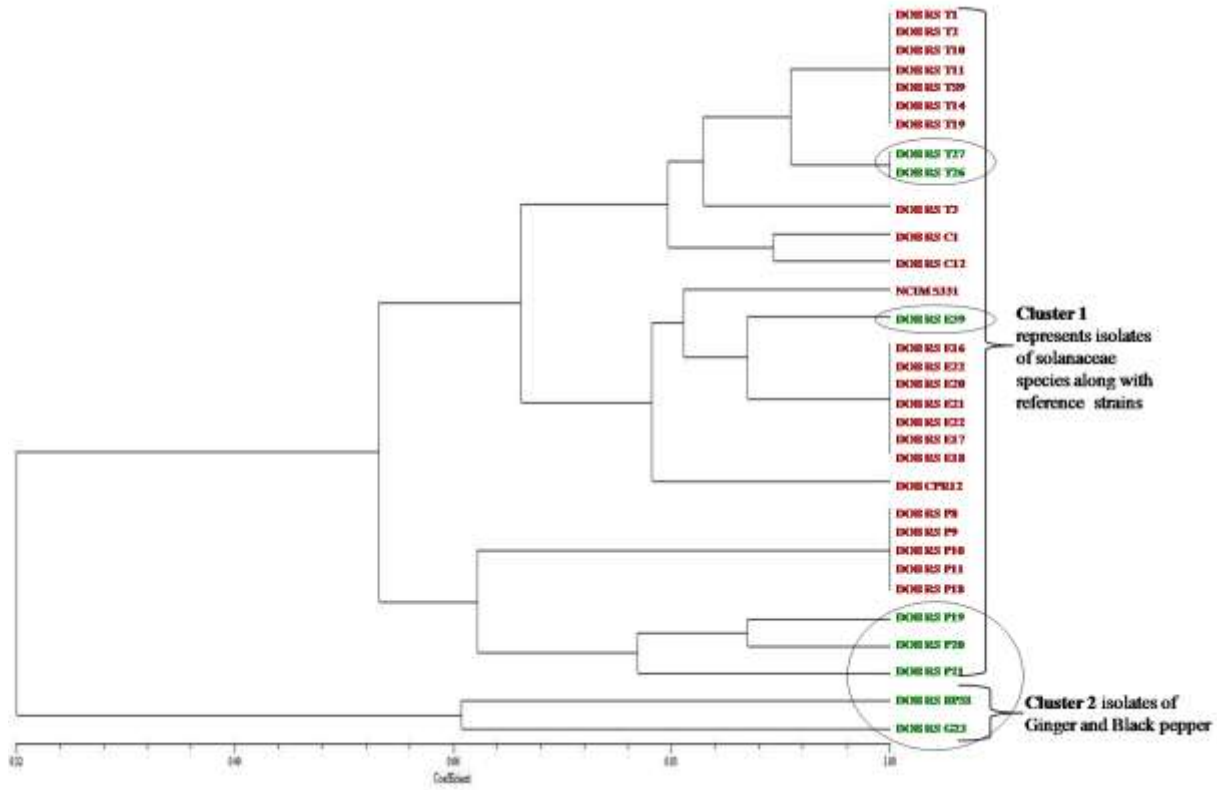

Figure 5. Dendrogram obtained by analysis of LSSP-PCR profile in 32 isolates of *Ralstonia solanacearum* from different hosts. Genetic distance obtained by LSSP-PCR banding profile in selected pathogenic (Red) and non pathogenic (Green, circular) isolates were used to build the phylogenetic tree among 141 isolates. Isolates belonged to the similar genotype of host specific indicated at right. Cluster 1 represents isolates of solanaceae, Cluster 2 represents isolates from ginger and black pepper.

on the data matrix represented by presence or absence of specific fragments using dendrogram. The isolates from distinct hosts were grouped in two main clusters with reference to fragments obtained by LSSP-PCR banding patterns. The *R. solanacearum* isolates obtained from the solanaceae species were grouped in the cluster I, and those obtained from the ginger grouped in the lower cluster in cluster II (Figure 5). The fragment distribution among solanaceae species were diversified among pathogenic and non-pathogenic isolates (Figure 5) when analyzed with UPGMA software along with their significant statistics (0.32-1.00). The polymorphism presumed that the heterogeneity of genetic profile obtained by LSSP-PCR analysis of *R. solanacearum* not only exhibited a frequent genetic diversity, but also exhibited the high interspecific variability existing among the *R. solanacearum* isolates infecting different hosts and pathogenicity. However, the genetic profile and the information of RFLP and AFLP mainly depend on the number of restriction enzymes employed.

In the present study, the *R. solanacearum* signatures obtained were informative and composed of genetic variations in a single LSSP-PCR without addition of multiple restriction enzymes and probes. Dendrogram obtained by the UPGMA cluster algorithm with FR/F and FR/R the distance allows the clustering in two main clusters which differentiates *R. solanacearum* infects solanaceous species and ginger. The polymorphism presumed that the heterogenecity of genetic profile obtained by LSSP-PCR analysis of *R. solanacearum* not only exhibited a frequent genetic diversity, but also showed the high inter-specific variability existing among the *R. solanacearum* infecting different hosts (Figure 5). Whereas similar results, dendrogram of UPGMA cluster analysis of *Leptospira* isolates differentiates the strains from urine and clinical samples of cattle. Similarly, dendrogram obtained by the analysis of LSSP-PCR profile of 40 isolates of *Leishmania infantum* differentiates human and canine samples correlate the present reports (Alvarenga et al., 2012).

Sequence analysis

The alignment of *R. solanacearum* sequence from different hosts of solanaceae members and ginger

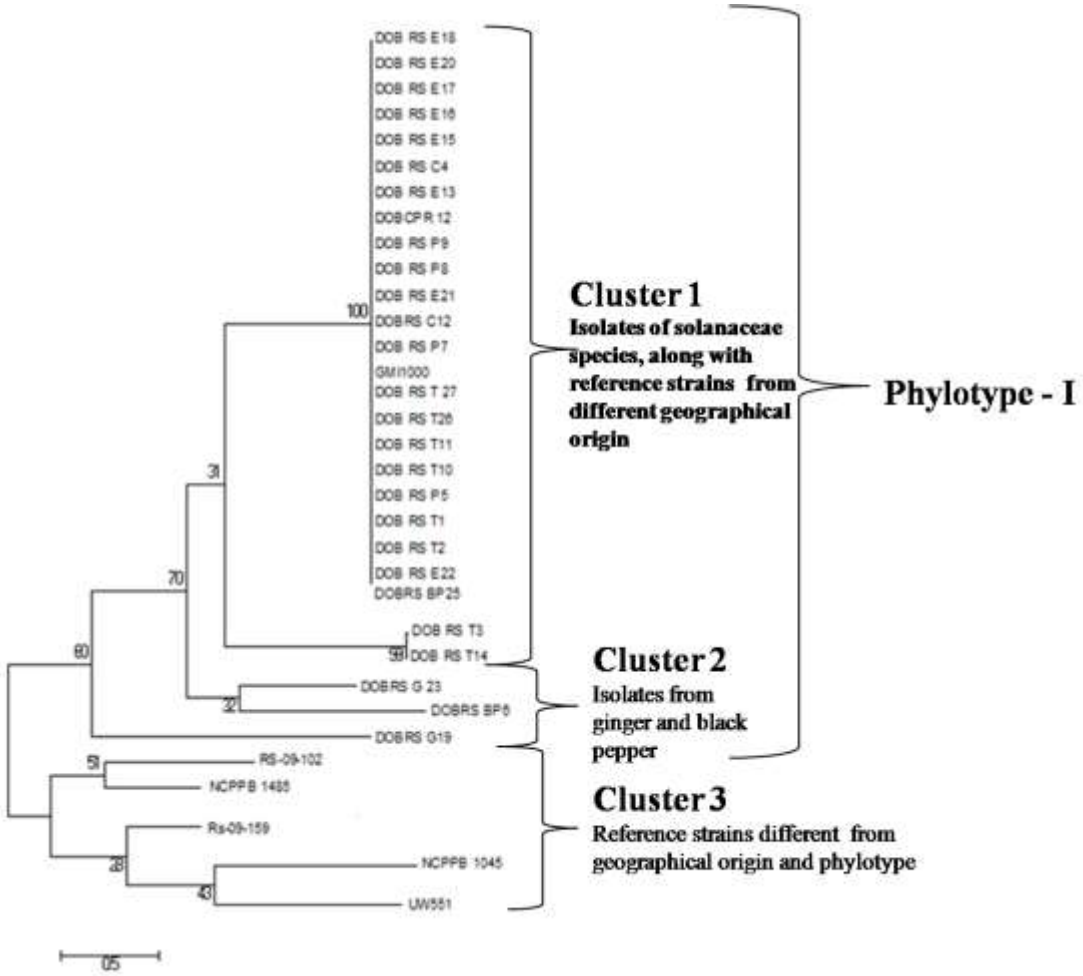

Figure 6. Phylogenetic analysis of *egl* sequences among *Ralstonia solanacearum* isolates. Sequences from NCBI of different isolates were analyzed in Mega 6.0. Phylogenetic tree was constructed using the neighbor-joining maximum likelihood (P-distance). Bootstrap test was for 1000 repetitions. Cluster 1 represents isolates of solanaceae species, Cluster 2 represents isolates from ginger and black pepper. Cluster 3 represents Reference strains different from geographical origin and phylotype.

revealed the presence of some nucleotide substitutions, which were shared within the geographical origin and host range. It can be hypothesised that, the distinct LSSP genetic profile of *R. solanacearum* obtained in this study is due to the presence of polymorphism in the target gene sequences. The sequenced data confirms solanaceae species in infecting *R. solanacearum* isolates which exhibited divergence from ginger isolates as reported earlier (Ramesh et al., 2014; Sagar et al*.,* 2014).

Nucleotide data base showed the presence of four phylotypes, subdivided into sequevars based on *egl* sequence similarities in which Indian isolates belongs to phylotype I and subdivided into two subgroups (Ramesh et al., 2014). Phylogenetic grouping by *egl* sequences of *R. solanacearum* isolates represented as phylotype I, whereas phylotype II, III and IV (Figure 6) were not recorded among isolates of Karnataka. Sequence information and diversity analysis of Indian isolates infecting solanaceous vegetables provides valuable information about the existence of major phylogenetic group, divergence with phylotype I by dividing two clusters (Ramesh et al., 2014; Sagar et al., 2014) which confirm in genetic profiles of LSSP-PCR among our isolates.

Conclusion

The study has demonstrated the application of SSCP and LSSP-PCR for the differentiation of *R. solanacearum* isolated from distinct hosts. The developed Egl-F and Egl-R primers are specific to identify *R. solanacearum* with direct colony PCR which avoids the extra step of DNA extraction. SSCP allowed specific patterns to

distinguish pathogen up to species level. The individual genetic profiles of *R. solanacearum* isolates were obtained by LSSP-PCR; the polymorphic variation discriminates pathogenic and non-pathogenic isolates by LSSP polymorphic banding patterns within and diverged host. The genetic variation of LSSP was confirmed by sequence analysis of isolated strains. The results correlated with the genetic variability of *R. solanacearum* discriminate from diverge host and represented as phylotype I, included strains originated primarily from Asia. Due to, low cost, sensitivity, specificity, simplicity of execution and high reproducible genetic profile, the use of LSSP-PCR technique could be extended to other similar plant pathogenic bacteria identification and can be considered as a valuable microbiological tool to study genetic diversity of quarantine pathogens along with their epidemiology in developing countries.

Conflicts of interest

The authors have not declared any conflicts of interest

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