Indian Journal of Agricultural Sciences **84** (3): 391–5, March 2014/Article
https://doi.org/10.56093/ijas.v84i3.38590

Genetic diversity of biovar 3 and 4 of *Ralstonia solanacearum* causing bacterial wilt of tomato using BOX- PCR, RAPD and *hrp* gene sequences

DINESH SINGH[1], SHWETA SINHA[2], GARIMA CHAUDHARY[3], D K YADAV[4] and K K MONDAL[5]

Indian Agricultural Research Institute, New Delhi 110 012

Received: 30 March 2013; Revised accepted: 17 February 2014

ABSTRACT

Genetic diversity of *Ralstonia solanacearum,* causal agent of bacterial wilt of tomato was assessed by using three different molecular methods such as random amplified polymorphism DNA (RAPD), BOX-PCR and *hrp* (hypersensitive response and pathogenicity) gene sequence analysis technique. Twelve isolates of *Ralstonia solanacearum* belonging to biovar 3 (9 isolates) and 4 (3 isolates) were collected from Northern parts of India including Himachal Pradesh and Uttarakhand states from infected tomato plants. Out of 16 primers used in RAPD fingerprinting, four primers (OPA-2, OPA-11, OPC-5, OPE-7) showed monomorphic bands and remaining 12 primers exhibited polymorphic amplified products of both the biovars of *R. solanacearum*. The primer OPE-10 showed the highest level of genetic diversity among the isolates. Ten isolates of *R. solanacearum* were classified into two clusters at 20 per cent similarity coefficient and cluster 1 represented all isolates of biovar 3 (UTT-23, UTT-10, UTT-26, HPT-11, UTT-9, UTT-32, HPC-3) and cluster 2 comprised the biovar 4 (UTT-22, HPT-3, UTT-24). BOX-PCR fingerprint of *R. solanacearum* clearly distinguished biovar 3 and 4 grouped into two distinct clusters at 40% similarity coefficient. Cluster 1 represented all isolates of *R. solanacearum* biovar 3 and cluster 2 comprised the biovar 4 isolates. The isolates of *R. solanacearum* have genetic diversity in *hrpB* gene, but it could not differentiate the isolates of biovar 3 and 4. However, the biovars 3 and 4 of *R. solanacearum* can be genetically distinguished by using BOX- PCR and specific primer of RAPD.

Key words: Biovar, Genetic diversity, *Ralstonia solanacearum*, Tomato

Ralstonia solanacearum is a soil borne bacterium, causes very serious bacterial wilt disease of solanaceous crops. It has a broad geographical distribution and wide host range, including more than 200 plant species in over 50 families (Hayward 2000, Sharma *et al.* 2005, Singh *et al.* 2010). *R. solanacearum* is considered a species complex, which is a heterogenous group of related but genetically distinct strains (Fegan and Prior 2005). They show great pathogenic and genetic diversity and divided into five races based loosely on host range. However, the race structure of *R. solanacearum* is not well defined taxonomically. The biovars of *R. solanacearum* are better defined than its races, which are mainly based on acidification of medium during metabolism of six carbohydrates (maltose, lactose, cellobiose, mannitol, sorbital and dulcitol). *R. solanacearum* isolates were generally grouped into different biovars (James *et al.* 2003). In India, biovars 2, 3 and 4 of *R. solanacearum* are found (Singh *et al.* 2010), however, biovar 3 is most dominated one across the country as compared to biovar 4 and both the biovars able to cause wilt disease in solanaceous and Zingiberaceae crops. Occurrence of biovar 2 is rare in India and mostly found in cold climate, which can infect and cause symptoms in potato at temperatures as low as 16^0C. Genetic diversity of plant pathogenic bacteria including *R. solanacearum* has been reported using RAPD (Grover *et al.* 2006), PCR-RFLP of *hrp* gene (Poussier *et al*. 2000) and BOX-PCR (Singh *et al.* 2011) to differentiate the pathogenic population into the haplotypes and biovars (Horita and Tsuchiya 2001). From many years, the use of RFLP allowed grouping of species into different clusters, correlated with the geographical origin of strains (Stevens and Elsas 2010). Poussier *et al*. (2000) had done PCR-RFLP analysis of the *hrp* gene region to discriminate *R. solanacearum* strains from *P. syzygii* and blood disease bacterium strains. A very little information is available to genetically differentiate biovar 3 and 4 of *R. solanacearum.* Therefore, the present study was taken up with the aim to determine the existing variation between biovar 3 and 4 of *R. solanacearum* by using RAPD, BOX- PCR and *hrp* gene sequence analysis.

MATERIALS AND METHODS

The *Ralstonia solanacearum* was isolated from infected

[1]Principal Scientist (e mail: dinesh_iari@rediffmail.com), [2]Senior Research Fellow (e mail: shwetaiari@gmail.com), [3]Senior Research Fellow (e mail: mahak2222chaudhary@gmail.com), [4]Senior Research Fellow (e mail: dhananjaymkp@gmail.com), [5]Senior Scientist (e mail: mondal_kk@rediffmail.com), Division of Plant Pathology

tomato plant from Himachal Pradesh and Uttarakhand by standard casamino acid peptone glucose (CPG) agar medium and tetrazolium chloride (TZC) medium (Schaad *et al.* 2001). Bacterial ooze was mostly collected from upper parts of the infected tomato plant in sterilized distilled water and diluted up to 10^{-8} by serial dilution method. The 100µl oozed suspension was taken from 10^{-6}, 10^{-7} and 10^{-8} and poured on to the media separately and spread uniformly by L-shaped glass rod using standard procedure (Singh *et al.* 2010). The inoculated plates were incubated at 28±1^{0}C for 72 hr and stored at -80^{0}C in 25% glycerol stock.

A fresh culture of 12 isolates of *R. solanacearum* containing 4.2×10^9 cfu/ml was used to pathogenicity test on tomato plant. The white-pinkish, extracellular polysaccharide (EPS) producing irregular colonies of *R. solanacearum* were grown on TZC medium after 48 hr at 28°C. Tomato plant cv. Pusa Ruby was grown in pots in the Phytotron, IARI, New Delhi at 26^{0}C and five plants of 40 days old were maintained in each pot with three replications. The bacterial culture containing 4.2×10^9 cfu/ml was inoculated at root zone by making slight injury to the root with disposable tip and 5.0 ml of inoculum was poured at the crown region of the plant. The wilt symptom was observed regularly.

Biovar of each isolate of *R. solanacearum* was determined by standard procedure (Schaad *et al.* 2001) following the basal medium used for biovar identification: 1.0g $NH_4H_2PO_4$, 0.2g KCL, 0.2 g $MgSO_4.7H_2O$, 1.0 g Difco Bacto peptone, 3.0 g agar, 80mg bromothymol blue in 1000 ml distilled water) and pH was adjusted at 7.0 (an olivaceous green colour) by addition of 40% (wv^{-10}) NaOH solution before addition of the agar. In autoclaved basal medium, 10% aqueous carbon sources (Dextrose, mannitol, sorbitol, dulcitol, trehlose, lactose, maltose and D (+) cellobiose) sterilized by membrane filtered (0.45 µm membrane filter) except Dulcitol, which was sterilized by autoclave at 110°C for 20 min was added in each separate flasks. Cultures of *R. solanacearum* isolates (20µl of 48 old) were added separately into each culture tube containing basal medium and carbohydrate solution with control (without carbohydrate) in three replicates. The culture tubes were incubated at 28°C for 10 days and examined at 2, 5, 7 and 10 days after inoculation for change of colour from olive green to yellow due to change of pH from neutral to acidic. Characterization of biovar was done as described by Schaad *et al.* (2001).

Total DNA of *R. solanacearum* was extracted from the cultures grown in nutrient broth (28g/1; Himedia) at 28°C on a shaking incubator (200 rpm) for 24 hr by using cetyltrimethyl ammonium bromide (CTAB) method (Murray and Thompson 1980). The total genomic DNA was dissolved in 1X TE buffer (10mM ×Tris-hydrochloric acid and 0.5mM sodium EDTA, pH 8) and stored at -20°C. The estimation of quantity and quality of DNA was done by Nanodrop and gel electrophoresis.

For genetic diversity, 10 isolates of *R. solanacearum* belonging to biovar 3 (HPT-11, UTT-12, UTT-11, UTT-27, UTT-32, UTT-9 and UTT-26) and biovar 4 (HPT–3, UTT-22 and UTT-24) were taken to do fingerprinting by using BOX-PCR primer (BOXA1R: 5'-ACGTGGTTTGAAGAGATTTTCG-3'). PCR amplification was done in a final reaction volume of 25µl consist of 10% DMSO, BSA, 10mM $MgCl_2$, 25mM dNTP, 10pmol of each primers, 1.25U Taq polymerase. The total genomic DNA (50ng) from isolates of *R. solanacearum* was used as template in 25µl of reaction mixture. BOX-PCR was performed in thermal cycler using PCR conditions as described by Schaad *et al.* (2001). The PCR product 15 µl was separated by electrophoresis on a 1.5% agarose gel in 1x TAE (Tri-borate EDTA) buffer for 6.5 hr at 100V. Gel was stained with ethidium bromide and photographed on gel documentation system (Gel Doc^{TM} XR$^+$ BIORAD). NTSYS (2.02e version) software was used to analyze the fingerprint and the similarity coefficient of BOX-PCR fingerprint was calculated with Pearson coefficient. Cluster analysis of the similarity matrix was performed by unweighted pair group method using arithmetic averages (UPGMA) algorithm.

The RAPD molecular marker was used for the determination of genetic diversity of biovar 3 and 4 of *R. solanacearum* collected from two different states representing Uttarakhand and Himachal Pradesh of India. A set of 16 RAPD primers sequence, viz. OPA-02 , OPA-03, OPA-11, OPA-18, OPC-05, OPC-09, OPE-07, OPE-10, OPF-07, OPF-08, OPH-03, OPH-15, OPH-19, OPH-20, OPM-1, and OPM-04 (Operon Technologies, USA) was tested for DNA amplification by RAPD to produce monomorphism and polymorphism pattern between biovar 3 and 4 of *R. solanacearum.* The genomic DNA (50ng) from isolates of *R. solanacearum* used as template in 25µl of reaction mixture consists of 10mM $MgCl_2$, 25mM dNTP, 10pmol of primer, 1.25U Taq polymerase (Biochem Lab). The amplification was performed in thermal cycler (Eppendorf thermocycler). The initial denaturation at 94^{0}C for 5 min was followed by 45 cycles of 94^{0}C for 1 min, 37^{0}C for 1 min and 72^{0}C for 2 min and final extension at 72^{0}C for 2 min. The RAPD amplified product (15µl) was separated by electrophoresis on a 1.5% agarose gel in 1× TAE (Tri-borate EDTA) buffer for 1.5 hr at 80V. Gel was stained with ethidium bromide and photographed on gel documentation system ultraviolet light using alfa imager Gel Doc system (Gel Doc^{TM} XR$^+$ BIORAD).

To compare the *hrp* gene regions, total 10 isolates of *R. solanacearum* were selected and of these seven isolates, viz. UTT-23, UTT-25, HPT-11, UTT-32, UTT-26, UTT-11 and HPT-2, belong to biovar 3, three isolates namely, HPT-3, UTT- 24 and UTT-22 belong to biovar 4 were amplified by the *hrp* gene specific primer with amplicons size at 323bp by using a pair of primer (Hrp_rs2F: 5'-AGAGGTCGACGCGATACAGT 3'and Hrp_rs2R: 5'-CATGAGCAAGGACGAAGTCA 3'), which was design from locus of *hrp* gene. PCR amplification of *hrp* gene, genomic DNA (50 ng) from each isolates of *R. solanacearum* was used as template in 25µl of reaction mixture consist of

10mM $MgCl_2$, 25mM dNTP, 10pmol of primer, 1.25U Taq polymerase (Biochem Lab). The amplification was performed in thermal cycler (Eppendorf thermocycler). The initial denaturation at 95^0C for 2 min was followed by 35 cycles of 95^0C for 30 sec, 51^0C for 30 sec and 72^0C for 1 min and extension at 72^0C for 10 min. The PCR product (15µl) was separated by electrophoresis on a 1.5% agarose gel in 1X TAE (Tri-borate EDTA) buffer for 1.5 hr at 80V. Gel was stained with ethidium bromide and photographed on gel documentation system (Gel Doc^{TM} XR$^+$ BIORAD), amplicon gels were purified by using PCR purification kit (Ganeaid). The amplified fragments were ligated with pGEMT vector (Promega) according to the manufacturer instructions. The ligated mixture incorporated into competent cell *Escherichia coli* (Dh5α) plated on Luria agar medium included ampicillin (50µg/ml), X-gal, IGPT (20 µg/ml) and plates were incubated at 37°C for overnight. The cloned products were sequenced and identified by using NCBI BLAST analysis. The evolutionary history was inferred using the unweighted pair group method using arithmetic averages UPGMA method.

RESULTS AND DISCUSSION

Out of 12 isolates of *R. solanacearum,* nine isolates were collected from Uttarakhand and three isolates from Himachal Pradesh (Table 1). Nine isolates of the pathogen were belonging to biovar 3, viz. UTT-9, UTT-11, UTT-12, UTT-23, UTT-25, UTT-26, UTT-32, HPT-2 and HPT-11, while three isolates UTT-22, UTT-24 and HPT-3 were found under biovar 4 based on utilization of carbon sources. Both the biovars of *R. solanacearum* were found under the same environmental conditions and location. They were characterized based on ability to oxidize the disaccharides cellobiose, lactose and maltose and utilize the sugar alcohols dulcitol, mannitol and sorbitol. Biovars 2, 3 and 5 can oxidize the disaccharides whereas biovars 1 and 4 cannot oxidize. Both the biovars were found pathogenic to tomato plants and produced wilt symptoms after 6 days of inoculation. All the isolates of *R. solanacearum* belong to race 1.

Table 1 Characterization of *Ralstonia solanacearum* isolates causing bacterial wilt of tomato collected from Uttarakhand and Himachal Pradesh used in the present study.

Isolate name	Geographical region in India	Biovar	Race
UTT-9	Bajonioya, Haldu, Nainital, Uttarakhand	3	1
UTT-11	Roodki, Nainital, Uttarakhand	3	1
UTT-12	Takpatiya, Nainital, Uttarakhand	3	1
UTT-22	Bajania, Haldu, Nainital, Uttarakhand	4	1
UTT-23	Bajania, Haldu,Nainital, Uttarakhand	3	1
UTT-24	Purbikhera, Nainital, Uttarakhand	4	1
UTT-26	Pijaya, Nainital, Uttarakhand	3	1
UTT-27	Devlatalla kuarpur, Nainital, Uttarakhand	3	1
UTT-32	Sitapur, Nainital, Uttarakhand	3	1
HPT-2	Sadhupul, Solan, Himanchal Pradesh	3	1
HPT-3	Kohari, Solan, Himanchal Pradesh	4	1
HPT- 11	Nagaon, Solan, Himanchal Pradesh	3	1

Genetic diversity

BOX-PCR analysis: The fingerprinting patterns generated by BOX-PCR consisted of 7-16 fragments ranging in size of about 250 bp to 3.5 kb (Fig 1). The variations in number of fragments, their size were found among isolates of *R. solanacearum*. Isolate HPT-3 had maximum 16 fragments and minimum in UTT-9 (7 fragments). Computer assisted analysis of the BOX-PCR fingerprint showed that the isolates were grouped into two distinct clusters at 40% similarity coefficient. Cluster 1 represented all isolates of *R. solanacearum* biovar 3 and cluster 2 comprised the biovar 4 isolates. Cluster 1 was further divided into distinct 4 subgroups A, B, C and D at 80% similarity coefficient (Fig 2). Subgroup A contained isolates HPT- 11, UTT- 12, UTT- 11 and UTT-27, subgroup B had UTT- 32, in subgroup C UTT- 9, while isolate UTT- 26 was clustered under

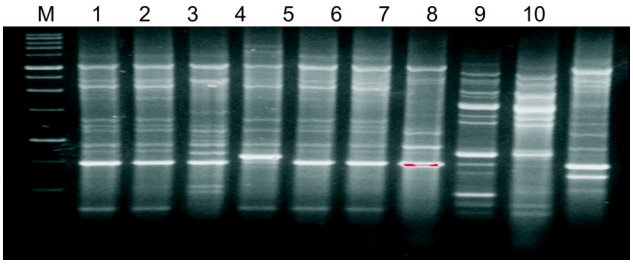

Fig 1 The amplified DNA fragments produced by Box –PCR primer for isolates of *R. solanacearum*. Lane name with respective isolates, lane M: 1kb DNA ladder, 1: HPT-11, 2: UTT-12, 3: UTT-26, 4: UTT- 32, 5: UTT-11, 6: UTT-27, 7: UTT- 9, 8: HPT-3, 9: UTT-22, UTT-24. Isolates in lanes 1-7 belong to biovar 3 and lanes 8-10 belong to biovar 4.

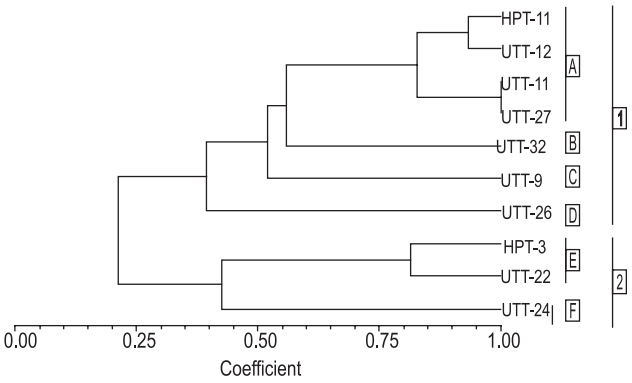

Fig 2 Cluster analysis of *Ralstonia solanacearum* biovar 3 & 4 based on Box-PCR fingerprinting and unweighted paired group mathematical average (UPGMA) dendrogram were generated using Pearson's correlation coefficient. The minimum similarity coefficient of all isolates of *R. solanacearum* was used to define distinct groups which are labeled numerically. Distinct groups of band based genotypes are labeled alphabetically.

Table 2 Analysis of RAPD primers with genomic DNA of 10 isolates of *R. solanacearum* belonging to biovar 3 and 4

RAPD primers	Sequences of primer	Isolates of *Ralstonia solanacearum*										No. of bands	No. of polymorphic bands	No. of monomorphic bands	Amplified bands
		1*	2	3	4	5	6	7	8	9	10				
OPA-2	5'-TGCCGAGCTG-3'	3	3	2	1	3	2	2	4	4	4	28	5	1	6
OPA-3	5'-AGTCAGCCAC-3'	2	1	4	4	2	2	2	5	5	5	32	10	0	10
OPA-11	5'-CAATCGCCGT-3'	4	2	1	2	4	3	3	3	2	2	26	11	1	12
OPA-18	5'-AGGTGACCTG-3'	1	2	2	2	2	3	2	2	2	3	21	7	0	7
OPC-5	5'-GATGACCGCC-3'	1	2	2	1	2	1	1	5	5	5	25	4	1	5
OPC-9	5'-CTCACCGTCC-3'	4	3	0	0	2	5	2	1	4	4	25	10	0	10
OPE-7	5'-AGATGCAGCC-3'	3	3	4	3	3	3	4	7	5	3	36	7	1	8
OPE-10	5'-CACCAGGTGA-3'	6	6	3	5	6	6	7	3	2	6	50	13	0	13
OPH-3	5'-AGACGTCCAC-3'	3	3	3	2	2	2	2	6	6	4	33	7	0	7
OPH-20	5'-GGGAGACATC-3'	3	3	2	1	3	3	2	4	4	4	29	7	0	7
OPM-4	5'-GGCAACGTG-3'	0	1	2	1	2	2	2	3	2	2	17	7	0	7
OPF-7	5'-CCGATATCCC -3'	2	2	2	1	1	3	1	2	0	1	14	7	0	7
OPF-8	5'-GGGATATCGG-3'	2	2	2	1	1	1	1	1	1	1	13	5	0	5
OPH -19	5'-CTGACCAGCC-3'	3	6	3	3	5	3	2	1	1	1	28	7	0	7
OPH-15	5'-AATGGCGCAG-3'	4	3	1	0	3	3	1	0	3	3	21	8	0	8
OPM-1	5'-GTTGGTGGCT-3'	2	3	4	4	6	3	4	3	4	3	35	13	0	13
Total		37	39	37	31	47	45	38	50	50	51				132

*1= UTT-23, 2= UTT-10, 3= UTT-26, 4= UTT-11, 5= UTT-9, 6= UTT-32, 7= HPT-2, 8= UTT-22, 9= HPT-3, 10= UTT-24

subgroup D. Cluster 2 had *R. solanacearum* biovar 4, which isolates were further divided in subgroup E (HPT–3 and UTT-22) and F (UTT-24). In many earlier reports, it has been mentioned that BOX-PCR analysis was used for differentiation of strains of *R. solanacearum* species by geographical region and biovar type they belong (Khakvar *et al*. 2008). According to the phylogenetic studies of the biovar 3 and 4 showed a separate genetic lineage distant from the other biovars like biovar 1, 2 and 6 (Timothy 2006). In our present study, BOX-PCR result helped to genetically discriminate between biovar 3 and biovar 4 isolates of *R. solanacearum*.

RAPD marker analysis: In PCR amplification with 16 RAPD primers, 13-50 bands were observed on agarose gel in the range of 0.25-1.0 Kb (Table 2). All the primers amplified DNA fragments of both the biovar 3 and 4 of *R. solanacearum* and total 132 polymorphic and monomorphic bands were obtained. Out of 16 primers, four primers (OPA-2, OPA-11, OPC-5, OPE-7) showed monomorphic bands and remaining 12 primers (OPA-3, OPA-18, OPC-9, OPE-10, OPH-3, OPH-20, OPM-4, OPF-7, OPF-8, OPH-19, OPH-15, OPM-1) exhibited polymorphic amplified products of both the biovars 3 and 4 of *R. solanacearum*. Maximum of 50 DNA fragments obtained in OPE-10 RAPD primer followed by OPE-7 (36 bands) (Table 2). These isolates of *R. solanacearum* were classified into two clusters at 20 per cent similarity coefficient. Cluster 1 represented all isolates of biovar 3 (UTT-23, UTT-10, UTT-26, HPT-11, UTT-9, UTT-32, HPC-3) and cluster 2 comprised the biovar 4 (UTT-22, HPT-3, UTT-24). A wide range of diversity was observed among the isolates of *R. solanacearum* collected from different geographical regions or hosts were also demonstrated by molecular marker analysis using RAPD primers (Poussier *et al.* 2000, Grover *et al.* 2006). James *et al.* (2003) stated that OPF-8 primer of RAPD showed unique banding pattern at 1.45 kb for isolates A1, A2 and A3 belongs to the race 3 in Kerala, which could be considered as a molecular marker for the identification of race 3 of *R. solanacearum*. The biovar 4 was more genetically homogeneous and has more number of bands (50-51 bands) than biovar 3 (37- 47 bands). The difference was found in cluster distribution between the two biovars. This result confirms the genetic variation between biovars 3 and 4 of *R. solanacearum*.

Hrp gene sequence analysis: The *hrp* gene sequences of total 10 isolates of *R. solanacearm* belonging to biovar 3 (UTT-23, UTT-25, HPT-11, UTT-32, UTT-26, UTT-11, HPT-2) and biovar 4 (HPT- 3, UTT- 24, UTT-22) were determined and compared with 8 reference isolates obtained from NCBI database. *Hrp*B gene is responsible for hypersensitive response and pathogenicity gene located at

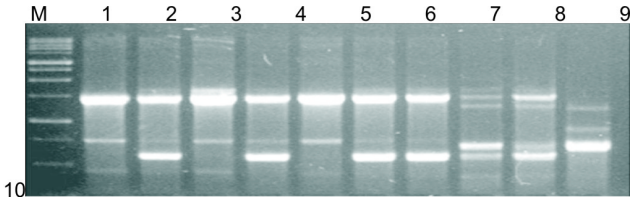

Fig 3 The amplified DNA fragments produced by RAPD primer OPE-7 to differentiate biovar 3 & 4 of *R.solanacearum.* Lane M:1 kb DNA ladder; Lanes 1 -7: Biovar 3 (UTT-23, UTT-10, UTT-26, HPT-11, UTT-9, UTT-32, HPC-3); lanes 8 - 10: Biovar 4 (UTT-22, HPT-3, UTT-24).

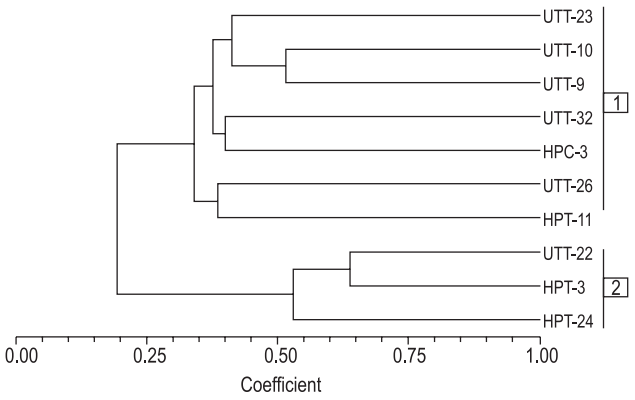

Fig 4 Cluster analysis of *R. solanacearum* biovar 3 and 4 based on 17 random amplified polymorphic DNA (RAPD) primers by unweighted paired group mathematical average (UPGMA) dendrogram were generated using Pearson's correlation coefficient. The significant of each branch is indicated by the bootstrap percentage calculated for 1000 subsets (only values greater than 50 % are shown). The minimum similarity coefficient of all isolates of *R. solanacearum* was used to define distinct groups which are labeled numerically. The figure shows distinct clusters of biovar 3 & 4 of *R. solanacearum.*

megaplasmid. Isolates UTT-23, UTT-25, HPT-3, HPT-11, UTT-32, UTT-22 and UTT-26 along with 7 isolates of other countries including one 1 isolate of *R. syzygii* strain R24 were clustered into cluster 1 and UTT-11 was formed separated into cluster II whereas, 2 isolates, viz. HPT-2 and UTT-24 were grouped into cluster III (Fig 3). It indicates that the isolates of *R. solanacearum* isolated from Northern parts of India have genetic diversity in *hrpB* gene. However, all isolates of *R. solanacearum* found in India were pathogenic to tomato plants to cause bacterial wilt. Hence, the biovar 3 and 4 could not be differentiated based on *hrp* gene sequence analysis. Poussier *et al*. (2000) analyzed PCR –RFLP *hrpB* gene and they found that *R. solanacearum* isolates grouped into three major clusters (clusters 1, 2, and 3), and the blood disease of banana isolate formed a single isolate cluster. Cluster 1 contains all isolates of biovars 3, 4 and 5), cluster 2 contains isolates of biovars 1, 2 and N2 from Africa, the Antilles, USA and Central and South America, cluster 3 contains isolates of biovars 1 and N2 from Africa and the islands of Reunion and Madagascar. They could not be able to differentiate biovars by using this gene, which is confirmatory to our results.

In this study, we assessed the genetic diversity of *R. solanacearum* biovars 3 and 4 strains obtained from northern parts of India, which causes a serious wilt disease of solanaceous crops using different techniques. The biovars could not be differentiated by colony, morphological character and pathogenicity test and *hrp* gene sequence analysis, as both the biovars are able to cause wilt disease in solanaceous crop. However, the biovars 3 & 4 of *R. solanacearum* can be genetically distinguished by using BOX- PCR and RAPD markers.

ACKNOWLEDGEMENTS

The authors are thankful to Dr M Anandraj, Director, Indian Institute of Spices Research, Calicut and Indian Council of Agricultural Research, New Delhi for financial support under PHYTOFURA project. They are also grateful to Dr R K Jain, Head, Division of Plant Pathology, IARI, New Delhi for his constant support and encouragement to conduct the experiments.

REFERENCES

Fegan M and Prior P. 2005. How complex is the *Ralstonia solanacearum* species complex? (*In*) Bacterial wilt disease and the *Ralstonia solanacearum* species complex, pp 449–61. Allen C, Prior P, Hayward A C (Eds). American Phytopathological Society Press, St. Paul.

Grover A, Azmi W, Gadewar A V, Pattanayak D, Naik P S, Shekhawat G S and Chakrabarti S K. 2006. Genotypic diversity in a localized population of *Ralstonia solanacearum* as revealed by random amplified polymorphic DNA markers. *Journal of Applied Microbiology* **101**(4): 798–806.

Hayward A C. 2000. *Ralstonia solanacearum*. (*In*) *Encyclopedia of Microbiology*, pp 32–42. Lederberg J (Ed.) Academic Press.

Horita M and Tsuchiya K. 2001. Genetic diversity of Japanese strains of *Ralstonia solanacearum*. *Phytopathology* **91**: 399–407.

Khakvar R, Sijan K, Wong M Y, Radu S and Thong K L. 2008. Genomic diversity of *Ralstonia solanacearum* strains isolated from banana in West Malaysia. *Plant Pathology Journal* **7** (2): 162–7.

James D, Girija D, Mathew Sally K, Nazeem P A, Babu T D and Varma S A. 2003. Detection of *Ralstonia solanacearum* race 3 causing Bacterial wilt of solanaceous vegetables in Kerala, using Random amplified polymorphic DNA (RAPD) analysis. *Journal of Tropical Agriculture* **41**: 33–7.

Murray and Thompson. 1980. Rapid islation of high molecular weight DNA. *Nucleic Acids Research* **8**: 4321–5.

Poussier S, Trigalet- Demery D, Vandewalle P, Goffiner, B, Luisetti J and Trigalet A. 2000. Genetic diversity of *Ralstonia solanacearum* as assed by PCR-RFLP of the Hrp region, AFLP and 16S rRNA sequence analysis, and identification of an African subdivision. *Microbiology* **146**: 1 679–92.

Schaad N W, Jones J B and Chun W. 2001. Laboratory guide for identification of plant pathogenic bacteria. *APS Press* 174.

Sharma J P, Jha A K, Singh A K, Pan R S, Rai M and Kumar S. 2005. Screening of parental lines and their F2 crosses of brinjal (*Solanum melongena*) to *Ralstonia* wilt. *Indian Journal of Agricultural Sciences* **75** (4): 197–9.

Singh D, Dhar, S and Yadava D K. 2011. Genetic and pathogenic variability of Indian strains of *Xanthomonas campestris* pv. *campestris* causing black rot disease in crucifers. *Current Microbiology* **63**: 551–60.

Singh D, Sinha S, Yadav D K, Sharma J P, Srivastava D K, Lal H C, Mondal, K K and Jaiswal R K. 2010. Characterization of biovar/races of *Ralstonia solanacearum,* the incitant of bacterial wilt in solanaceous crops. *Indian Phytopathology* **63**: 261–5.

Stevens P and Elsas J D. 2010. Genetic and phenotypic diversity of *Ralstonia solanacearum* biovar 2 strains obtained from Dutch waterways. *Antonie van Leeuwenhoek* **97:** 171–88.

Timothy T P. 2006. Plant Pathogenic *Ralstonia* sp. (*In*) *Plant-Associated Bacteria*, pp 573–644. Gnanamanickam S S (Ed). Springer, Netherlands.