

BOX-PCR and ERIC-PCR Based Genomic Finger Printing Among *Ralstonia solanacearum* Isolates Associated with Wilt Disease of Solanaceous Vegetables



Agriculture

KEYWORDS : *Ralstonia solanacearum*, BOX-PCR, ERIC-PCR, taxonomic diversity

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ABSTRACT

The genetic diversity among Indian isolates of *Ralstonia solanacearum* was assessed by repetitive sequence based polymerase chain reaction (rep-PCR) method with BOX and ERIC primer sets. The study comprised of 27 isolates collected from wilted tomato, chilli and brinjal plants from different locations of India. Molecular analysis by BOX- and ERIC- PCR showed wide genetic variation among the isolates exhibiting unique fingerprint patterns forming seven and six main clusters respectively. This indicates that these genomic fingerprinting techniques can be used as a rapid means of determining taxonomic diversity and phylogenetic structure, especially of large collections of bacterial isolates.

INTRODUCTION

Bacterial wilt in *solanaceous, cucurbits and ginger is* caused by the soil-borne bacterium *R. solanacearum* (Smith) Yabuuchi (synonyms: *Pseudomonas solanacearum*, *Burkholderia solanacearum*), is one of the devastating and a widespread disease that can affect more than 450 plant species in 54 families and occurred in tropical, subtropical, and warm temperate regions of the world (Melanie *et al.*, 2007). The members of *R. solanacearum* comprise a relatively diverse group of isolates referred to as a species complex (Gillings and Fahy, 1994). In order to describe this intraspecific variability it has been classified into 5 races (Buddenhagen *et al.*, 1962) based on the host range: Race 1 (Solanaceous vegetables), Race 2 (banana), Race 3 (potato and tomato), Race 4 (ginger), Race 5 (mulberry) and 6 biovars (Xue *et al.*, 2011). Similarly based on the ITS region, *hrpB* gene and *egl* gene sequences it has been further divided into four phylotypes (Fegan and Prior, 2005).

Bacterial wilt affects mainly the solanaceous vegetables in India (Singh *et al.*, 1997). On the basis of comparative analysis, the isolates causes wilt disease of solanaceous vegetables in India is classified as Asiaticum group (Khan *et al.* 1974). For successful management programme a detailed study of the pathogen diversity is a must (Sanchez Perez *et al.*, 2008). Several molecular techniques have been used to identify subgroups within *R. solanacearum* are 16S rDNA (Horita and Tsuchiya, 2000), *egl* (Fegan and Prior, 2005), and *hrpB* (Poussier *et al.*, 2000) genes to separate isolates of *R. solanacearum* into genetic divisions (Villa *et al.*, 2005). But all these techniques have their own limitation in differentiation and identification of the isolates of *R. solanacearum*. The multi-copy target sequences allow greater sensitivity as compared to single or low-copy target sequences. BOX and ERIC-PCR utilize primers derived from consensus sequence of highly conserved repetitive DNA elements from gram-negative bacteria (Versalovic *et al.*, 1991). These primers have been useful in discriminating among species among pathovars within a single species (Louws *et al.* 1994, 1995). PCR- based fingerprinting has the advantage of being relatively simple and is faster for genotyping (Aye and Matsumoto, 2010). Therefore in the present study, we established BOX-PCR and ERIC-PCR methods for discriminating and genotyping *Ralstonia* isolates isolated from tomato, eggplant and chilli.

MATERIALS AND METHODS:

Bacterial isolates: Isolation and culture conditions

Twenty seven *R. solanacearum* isolates were isolated from wilted

tomato, chilli, and eggplant collected in different geographical location of India (Karnataka, Kerala, Bhuvaneshwar, Delhi and Goa) and cultured on TZC medium (Kelman, 1954) (Table 1). After incubation, the virulent *R. solanacearum* colonies were selected and pure cultures were maintained at -80°C in 30% glycerol stocks in Pathology laboratory at Indian Institute of Horticultural Research, Bengaluru.

Table1: Strains of *Ralstonia solanacearum* used in the study

Strain	Place	Host
DB9	India: Delhi	Brinjal
SNGBRS58	India: Hessaraghatta, Karnataka	Brinjal
SNGBRS57	India: Hessaraghatta, Karnataka	Brinjal
ICAP1	India: Hessaraghatta, Karnataka	Capsicum
BHUTOM	India: Bhubaneswar	Tomato
DEVTOM10	India: Devanahalli, Karnataka	Tomato
KTBR2	India: Kodagi Thirumalapura, Karnataka	Brinjal
MKCAP1	India: Muthkur, Karnataka	Capsicum
SKCAP2	India: Shivakote, Karnataka	Capsicum
BETHACAP	India: Betha, Karnataka	Capsicum
DBCAP4	India: Doddaballapur, Karnataka	Capsicum
RKCAP5	India: Rajanakunte, Karnataka	Capsicum
DEVTOM11	India: Devanahalli, Karnataka	Tomato
KTBR1	India: Kodagi Thirumalapura, Karnataka	Brinjal
MKTOM22	India: Muthkur, Karnataka	Tomato
MKTOM23	India: Muthkur, Karnataka	Tomato
MKBR24	India: Muthkur, Karnataka	Brinjal
RK25	India: Rajanakunte, Karnataka	Tomato
DBTOM26	India: Doddaballapur, Karnataka	Tomato
BETHATOM	India: Betha, Karnataka	Tomato
BHUHP1	India: Bhubaneswar	Chilli
BHUHP2	India: Bhubaneswar	Chilli
ITOM1	India: Hessaraghatta, Karnataka	Tomato
KTOM2	India: Kodagi Thirumalapura, Karnataka	Tomato
KTOM3	India: Kodagi Thirumalapura, Karnataka	Tomato
KERTRS1	India: Kannur, Kerala	Tomato
KERPRS	India: Kannur, Kerala	Pepper
SKTRS	India: Shivakote, Karnataka	Tomato

R. solanacearum identification by PCR

DNA isolation: Extraction of the total genomic DNA from the *Ralstonia* isolates by modification of Sambrook *et al.* (2009) method. Initially all the strains were identified by PCR using Rs-specific primer pair AU759f (GTCGCCGTCAACT-CACCTTCC) and AU760r (GTCGCCGTGACG AATCGGAACG (Opina *et al.*, 1997).

Diversity of *R. solanacearum* isolates:**BOX and ERIC-PCR analysis:**

R. solanacearum strains were fingerprinted using BOX and ERIC primers adapted from previous reports (Versalovic *et al.*, 1994, Louws *et al.*, 1994; Horita *et al.*, 2005). The amplification was performed using three primers: ERIC1R (5'-ATG TAA GCT CCTGGGGAT TCA C-3') and ERIC2 (5'-AAGTAAGTG ACTGGG GTG AGC G-3'); and BOX, BOXA1R (5'-CTA CGG CAA GGC GAC GCT GAC G-3') (28) corresponding to ERIC and BOX elements, respectively. The reaction mixture in each tube consisted of 25 µl (50ng of DNA template, 1 U Taq DNA polymerase (Sigma), 2.5µl of 10X Taq buffer, 2µl MgCl₂, 2.5µl of 0.2mM each dNTP mix (Sigma) and 25pmol of each primer. The PCR reactions were carried out in a Gene Amp PCR system 9700 (PE Applied Biosystems, Foster City, CA) thermocycler. With BOX and ERIC primers, cycling programmes started with an initial denaturation of 95°C for 5 min, followed by 35 cycles of 94°C for 3sec, 50°C (ERIC) and 52°C (BOX) for 1 min for primer annealing and 8 min at 65 °C for primer extension. After the extension step, the temperature was held at 65 °C for 15 min for all three primers. Amplified PCR products were separated by 1.5% agarose gel electrophoresis in 0.5X Tris-borate-EDTA buffer, pH 8.0. Gels were stained with ethidium bromide (10 mg/ml) and were visualized and documented by Alpha digi-doc1000 system (Alpha Innotech Corporation, USA). For confirmation of banding patterns PCR experiments were repeated at least two times.

Data Analysis: BOX and ERIC fingerprint profiles were used to measure genetic similarity among strains. Each band with a different electrophoretic mobility was assigned a position number and scored as either 1 or 0 based on the presence or absence of the band. Variations in intensity of bands among isolates were not considered as differences. The data generated with three primers were combined and Cluster analysis was performed on a similarity matrix, which was produced using the Jaccard coefficient and subjected to the unweighted pair group method with arithmetic average (UPGMA) clustering algorithm, using NTSYSpc ver. 2.1 software (Exeter Software, New York, USA).

RESULTS :

Isolation, identification and characterization: Pathogen was isolated from wilted plant samples on TZC medium and *R. solanacearum* colonies were selected and purified based on their fluidal nature with pink centres having whirling pattern after 48 h of incubation. The DNA isolated from the pure cultures when amplified through PCR with Rs specific primers resulted in amplification of ~ 0.3Kb DNA fragment which confirmed the identity of *R. solanacearum*.

Diversity of *R. solanacearum* isolates: BOX and ERIC PCR: Twenty seven *R. solanacearum* isolates were subjected to BOX and ERIC PCR using universal primers of BOX and ERIC to study the genetic diversity. ERIC and BOX primer set gave reproducible genomic PCR profiles consisting of approximately 0.15 to 5Kb bands. Polymorphic bands were clearly observed, thus helping in differentiation.

BOX PCR profile: BOX primer produced 2 to 13 bands per isolate. ERIC primer produced 3 to 14 bands per isolate. When BOX PCR fingerprints were analyzed, isolates from Karnataka showed as many as 20 distinct patterns and Kerala isolates showed 2 patterns. Delhi isolate also gave a very distinct pattern. 3 isolates from Karnataka (SKTRS, MKCAP1 RKCAP5) and Goa (GOABRS) didn't show any polymorphism. 3 distinct fingerprint patterns were produced by isolates from Bhuvaneshwar only (Fig. 1)

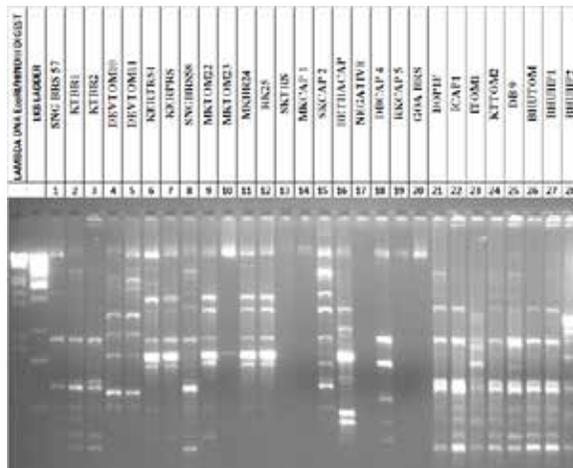


Fig 1: Agarose gel electrophoresis of polymerase chain reaction fingerprint patterns obtained from isolates representing *R. solanacearum* using BOXA-1R primer. 25µL of each PCR mixture is loaded on to a 2.5% agarose gel. Lane 1: Lambda DNA EcoRI/HindIII digest, Lane 2 1kb DNA ladder and Lanes 3–30: *Ralstonia* isolates.

ERIC PCR profile: In ERIC PCR analysis, isolates from Karnataka showed as many as 16 distinct patterns, Kerala isolates showed 2 patterns, Bhuvaneshwar isolates showed 2 patterns and Goa isolate showed a very distinct pattern. Thus PCR amplification showed distinct polymorphism from different places (Fig 2).

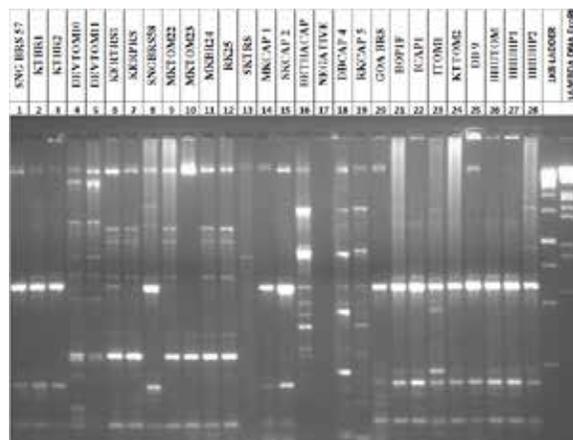


Fig 2: Agarose gel electrophoresis of polymerase chain reaction fingerprint patterns obtained from isolates representing *R. solanacearum* using ERIC primer set. 25µL of each PCR mixture is loaded on to a 2.5% agarose gel Lanes 1-28: *Ralstonia* isolates, Lane 29 1kb DNA ladder, Lane 30: Lambda DNA EcoRI/HindIII digest.

Cluster Analysis : In BOX PCR, totally seven clusters (Cluster I, II, III, IV, V, VI and VII) were identified (Fig.3). Cluster I comprised of mixed group of isolates from Karnataka, Bhuvaneshwar and Delhi split up into four subgroups (A1, A2, A3 and A4). Cluster II comprised of isolates from Kerala and Karnataka in two subgroups (B1 and B2). Cluster III belonged to chili isolates from Karnataka and Bhuvaneshwar (BHUPH2 and BETHACAP). Cluster IV belonged to tomato isolates from Karnataka only (DEVTOM10 and DEVTOM11). Cluster V belonged to isolates from Karnataka and Goa but formed two subgroups E1 and E2. (MKCAP1 and SKTRS, GOABRS and MKTOM23). RKCAP5 and DBCAP4 formed separate clusters.

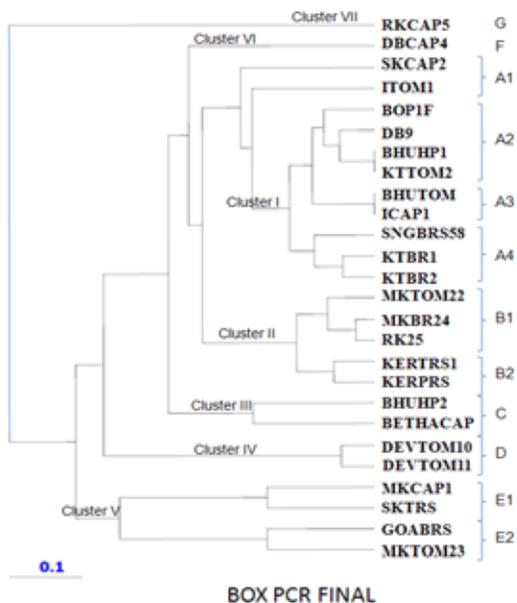


Figure 3: Dendrogram showing diversity among *Ralstonia* isolates generated by NTSYS-PC (Applied Biostatics, Inc., Setanket, NY) based on presence (1) or absence (0) and total number of fragments amplified by BOX-PCR

In ERIC PCR totally six clusters (Cluster I, II, III, IV, V and VI) were identified. (Fig.4). Cluster I comprised of mixed group of isolates from Karnataka, Bhuvaneshwar and Delhi split up into five subgroups (A1, A2, A3, A4 and A5). Cluster II comprised of isolates from Goa and Karnataka (MKCAP1 and GOABRS). Cluster III belonged to isolates from Karnataka only. Cluster IV comprised of isolates from Kerala and Karnataka in two subgroups (D1 and D2). SKCAP2 and SKTRS formed separate clusters.

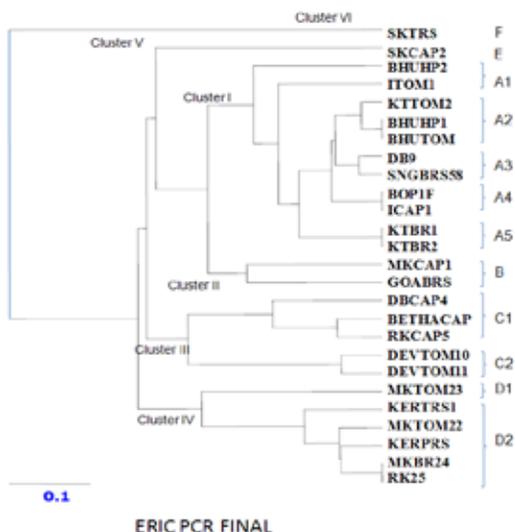


Figure 4: Dendrogram showing diversity among *Ralstonia* isolates generated by NTSYS-PC (Applied Biostatics, Inc., Setanket, NY) based on presence (1) or absence (0) and total number of fragments amplified by ERIC-PCR

DISCUSSION:

In the present study, isolates were identified as *R. solanacearum* on the basis of phenotypic characters as well as based on PCR amplification. All the virulent *R. solanacearum* from Karnataka morphologically resembled those from other regions of the

world (Williamson *et al.*, 2002) by producing typical white coloured fluidal colonies with pink centres and irregular in shape (Hayward, 1964). Furthermore, using species specific primers, ~0.3Kb band was obtained from all the isolates, which indicates that those isolates were of similar confirmation which was used for *Ralstonia* identification (Opina *et al.*,1997, Villa *et al.*, 2003; Chandrashekara *et al.*, 2012).

Rep PCR analysis has been used to differentiate a great number of species and strains of phytopathogenic bacteria (Horita and Tsuchya, 2005; Peters *et al.*,2004; Trindade *et al.*,2005; Horita *et al.*,2005; Poliakoff *et al.*,2005). Here we used rep-PCR (BOX and ERIC), analysis to detect the genetic diversity of *Ralstonia* isolates from different geographical locations and different hosts.

Earlier *Ralstonia solanacearum* genetic diversity studies were conducted by using BOX and ERIC fingerprinting pattern (Kumar *et al.*, 2004). Fingerprint analysis showed there is a considerable variation among isolates collected. However, the clustering pattern did not reveal either host based grouping or geographic grouping. This type of variation was also noticed earlier (Chandrashekara *et al.*,2012).

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