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PCR detection of *Ralstonia solanacearum*: A new approach for rapid detection of bacterium from infected plants

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ABSTRACT

Ralstonia solanacearum (Smith) is a soil-borne plant pathogen responsible for causing bacterial wilt and having wide host range which includes monocots, dicots, annual plants/trees and shrubs. It is a most destructive disease of solanceaous crops and ginger in north eastern region of India. The pathogen is primarily present in soils as saprophytic bacterium and it has ability to survive for long periods of time in various natural habitats. The bacterium causes sudden wilting in plants and difficult to detect at the initial level as similar symptom may also occur with many fungal organisms like *Fusarium* spp. and *Verticillium* spp. An attempt was made to develop a PCR-based rapid method for detection of this pathogen. This method requires only 3-5 hours against the conventional methods which generally require minimum 3 days to detect the pathogen. The PCR uses previously reported primer pairs for *fliC* gene (Rsol_*fliC*), which amplify 400bp region of *fliC* gene. The bacterial ooze from infected tissues was directly used as a source of DNA. The amplified product was cloned and sequenced for confirmation. The PCR based method developed in this report is very simple, robust and inexpensive and was successfully tested on four infected samples and further validated on over 50 samples of tomato which were infected by *R. solanacearum.*

Key words: Detective, PCR, *Ralstonia solanacearum*, Rapid Method

The symptomatological identification of bacterial wilt is difficult, as it is often confused with fungal wilt [*Ralstonia solanacearum* (Smith)] caused by *Fusarium* spp. (Anitha and Rabeeth 2009) and/or *Verticillium* spp. (Jabbnoun-Khiareddine *et al.* 2009). Moreover, the commonly used methods such as isolation of bacterium on semi-selective medium, serological detection (ELISA or immunofluorescence), or pathogenicity tests are often inadequate in terms of time, specificity, sensitivity (Khakvar *et al.* 2008). To optimize the efficiency of prophylactic measures the reliable rapid identification tools are required for early detection of the bacterial infection. Several PCR based methods for the detection of *R. solanacearum* have been described usually based on the amplification of ribosomal gene sequences, viz. 16S or 16S-23S intergenic spacer region of the ribosomal DNA (rDNA) (Seal *et al.* 1993, Fegan *et al.* 1998, Boudazin *et al.* 1999, Pastrik and Maiss 2000, van der Wolf *et al.* 2000, Weller *et al*. 2000). However, high degree of conserved nature of the ribosomal genes within the genus *Ralstonia* became main drawback for species identification (Schonfeld *et al.* 2003). The low level of resolution of 16S rDNA-based sequence analysis has been circumvented by using primers targeting functional genes such as endoglucanase and *hrpB* (Poussier *et al.* 2000) or a random fragment that was claimed to be *R. solanacearum* specific (Lee and Wang 2000). Later on, the suitability of *fliC* gene (coding for the flagellar subunit protein flagellin) in PCR-based detection system specific for *R. solanacearum* has been reported (Schonfeld *et al.* 2003). The PCR protocols have also been recommended for the detection of *R. solanacearum* in soil and water samples (Schonfeld *et al.* 2003, Khakvar *et al.* 2008). In addition, Weller *et al.* (2000) developed fluorogenic PCR-based (TaqMan) assays, which proved to be sensitive for the detection of the pathogen, yet too costly and labour-intensive, considering the number of samples to be tested when compared with general PCR-based assays. To best of our knowledge, no method has been proposed to reliably detect the pathogen from infected tissue at early stage of infection.

Therefore, the aim of this study was to develop a method for rapid detection of *R. solanacearum* at an early stage of infection and to propose a tool for reliable detection

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of *R. solanacearum* directly from plant samples. The PCR-based method developed and proposed in this study is robust, inexpensive and reliable for rapid detection of *R. solanacearum*. Special attention was paid to achieving a sensitive PCR amplification of desired fragments directly from ooze obtained from infected plant samples. The robustness and sensitivity of PCR was tested successfully on four infected samples at various ooze concentrations and further validated on over 50 samples of tomato which were collected from different location of north eastern India and were infected by *R. solanacearum*

MATERIALS AND METHODS

Field survey during 2012-13 identified wilting symptom in the tomato field situated at Pahamsyiem village, Nongpoh, Ri-Bhoi, Meghalaya, India. The infected plant samples were collected randomly from different fields. All field samples were properly maintained in separate zip lock polythene bags before testing for detection of *R. solanacearum*.

A presumptive ooze test was performed following standard protocol (Danks and Barker 2000) for initial identification of bacterial wilt infected plant materials. Four representative samples were randomly selected from the initial ooze-test positive samples for further studies.

All the four representative samples were cut into small pieces aseptically and were placed in 1-ml sterile nuclease and protease free water (Hi-media). Then those were kept for incubation at room temperature for 5 min or until the water became turbid followed by a final incubation at 96^0C for 6 min in thermal cycler (Eppendorf, Germany). Finally, the suspension was directly used for PCR amplification using previously reported Rsol_*fliC* primers (Forward: 5'GAA CGC CAA CGG TGC GAA CT 3'; Reverse: 5' GGC GGC CTT CAG GGA GGT C 3') designed to amplify a ~400bp fragment of *fliC* gene specific for *R. solanacearum* (Schonfeld *et al.* 2003). The PCR was carried out in 20µl reaction volume having 1X PCR buffer, 3 mM $MgCl_2$, 6% DMSO, 50µM of each dNTPs, 10 pmol of each primer, 1 U of *Taq* DNA polymerase and 2µl of bacterial ooze suspension. A known and identified bacterial strain of *R. solanacearum* was used as positive control and sterile nuclease free water as negative control. The PCR amplification was performed in a thermal cycler under cycling condition of initial denaturation at 96^0C for 9 min, followed by 34 cycles of 95^0C for 30 sec, 64^0C for 1 min, 72^0C for 2min and final extension of 72^0C for 10 min. Successful amplification of the expected fragment was confirmed by gel electrophoresis. A 10il aliquot of each amplified PCR product along with 100bp DNA ladder (Cat. # SM0241, Fermentas) were electrophoresed on a 1.5% (wt/vol) agarose gel, stained with ethidium bromide, and visualized on a UV trans-illuminator.

Out of four, one PCR product was ge purified using gel extraction kit (Qiagen, Germany) and ligated into pGEM-T cloning vector (Cat: A1360, Promega) following manufacturer's protocol. The ligated mixture was subsequently transformed in to *E. coli* (Top 10 F') competent cells. Individual positive colonies containing recombinant plasmid DNA were picked and grown in Luria–Bertani broth. Plasmid isolation and bidirectional sequencing of selected clone were performed commercially from Chromous Biotech Pvt Ltd., Bengalure, India.

The DNA sequences of cloned PCR product were analysed using Pregap4 and Gap4 programs within the Staden Molecular Biology Analysis software (Staden *et al*. 2000). The identity and homology of the derived sequence were first evaluated using the BLASTN program from the NCBI (www.ncbi.nlm.nih.gov/). The partial *fliC* gene of Meghalaya strain of *R. solanacearum* was compared with the available database on partial *fliC* gene of *R. solanacearum* from India. A single strain of a related species, *R. syzygii* (JF702411, protein id AEV52918) was also considered for comparison as an out group member. The pair-wise multiple alignments of nucleotide and corresponding amino acid sequence of partial *fliC* gene of all 44 strains including newly sequenced strain were performed using the CLUSTAL W algorithm of MEGA5 software (Tamura *et al.* 2011). The phylogenetic trees were constructed on matrices of aligned sequences with 1000 bootstrap replicates following the neighbor-joining method (Saitou and Nei 1987).

To determine the detection threshold of this method, bacterial ooze suspension of *R. solanacearum* infected tissue were further diluted following five dilution ratios, such as 1:2, 1:5, 1:10, 1:20 and 1:50 from an original suspension. A known and identified strain of *R. solanacearum* was used as positive control and sterile nuclease free water as negative control. The PCR mixture, cycles and gel running conditions were same as mentioned earlier.

RESULTS AND DISCUSSION

PCR detection and identification of R. solanacearum

The symptomatic field samples collected on the basis of wilt showed positive signal during initial ooze test. Then PCR assay was performed using bacterial ooze suspension from four randomly selected samples (ooze-test positive). PCR amplification using *R solanacearum* specific primers Rsol_*FliC* yielded an expected size products of 400bp in all four infected samples in spite of using bacterial ooze as a source of template DNA (Fig 1).

Characterization of amplified fragment

In order to confirm the identity of the PCR amplicons, obtained from the infected samples, one sample was cloned and sequenced bidirectionally using SP6 and T7 primers. The sequence was assembled and evaluated under BLASTN program of NCBI. The newly sequenced partial *fliC* gene (400 bp) showed 99% nucleotide identity with the previously reported GMI1000 strain of *R. solanacearum* at the genomic region spanning from 486414-486813 bp, a portion of complete *fliC* gene (486217-487038 bp). The 133 amino acid (aa) long protein, potentially encoded by newly sequenced *fliC* fragment, showed 100% identity with the protein encoded by similar isolate (Protein id CAD17533).

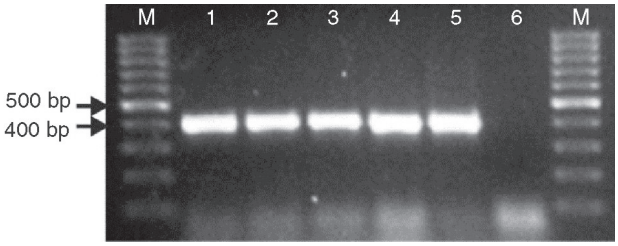

Fig 1 Specificity of PCR-based detection of *R. solancearum* using bacterial ooze extracted from infected plant materials as template. Presence of *R. solancearum* was confirmed by an amplification of 400 bp amplicon of *fliC* gene. M: 100bp DNA ladder; lanes 1-4: test samples from field of tomato; lane 5: positive control; lane 6: negative control.

Then the sequenced fragments of partial *fliC* gene of this new strain was deposited to NCBI database designating *Ralstonia solanacearum* strain RCR226 flagellin protein C gene, partial cds (KC834785).

To assess the relationship and diversity of RCR-226 strain of *R. solanacearum* from Meghalaya with previously reported Indian strain of *R. solanacearum*, the sequenced fragment was compared with earlier reported 42 partial *fliC* gene sequences of *R. solanacearum* from India and with a single strain of *R. syzygii* (available in NCBI databse). In pair-wise multiple alignments the present strain showed 99-100% identity with previously reported Indian starins of *R. solanacearum* at nucleotide level, whereas only 94% nucleotide identity was observed with *R. syzygii* strain. Similarly, the corresponding value at amino acid level was 98-100% with *R. solanacearum* and only 97% with *R. syzygii*. In nucleotide based bootstrap test of neighbor-joining phylogeny of partial *fliC* gene, two distinct groups of *R. solanacearum* and *R. syzygii* formed. The RCR-226 strain grouped with Indian strain of *R. solanacearum* (Fig 2a). At amino acid level, the RCR-226 strain under study clustered in a same manner along with Indian strains (Fig 2b) of *R. solanacearum*.

Evaluation of sensitivity of PCR

The sensitivity and detection threshold of the PCR method developed in this report for successful detection of *R. solanacearum* from infected plants was assessed up to five dilution ratios of 1:2, 1:5, 1:10, 1:20 and 1:50. All the dilutions prepared from original ooze suspension gave a reliable PCR amplification even at 1:50 dilution, with consistent intensity of the bands on 1.5% agarose gel (Fig 3). Therefore, the detection threshold for *R. solanacearum* by the newly developed rapid method could be beyond the dilution ratio of 1:20.

Bacterial wilt of is one of the most important plants diseases of bacterial origin in the world. The specific and rapid detection of causal pathogen at an early stage of infection is a crucial factor for management. The advancement in PCR method enhanced the possibility of specific and sensitive detection detection of *R. solanacearum* from bacterial cultures, soil, water etc. (Seal *et al.* 1993, Fegan *et al.* 1998, Boudazin *et al.* 1999, Pastrik and Maiss 2000, van der Wolf *et al.* 2000; Weller et al. 2000; Schönfeld *et al.* 2003; Khakvar *et al.* 2008; Poussier *et al.* 2000). However, present PCR-based identification and confirmation techniques are laborious and time-consuming (more than three days) as these require isolation and preparation of bacterial culture on selective media, extraction of DNA, PCR assay and gel electrophoresis. In this study, we have proposed a PCR-based detection of *R. solanacearum* within 24 hr. The symptomatic field samples of tomato were initially confirmed to be affected by bacterial wilt through ooze test (within 10 min). Then, the ooze suspension after initial incubation (<30 min) was directly used for PCR-based detection. The PCR programme continued for maximum 3 hr. and the gel electrophoresis for detection of successful amplification required additional one hour. Therefore, critically the time required for the whole process was less than five hours. Recently, Umesha *et al.* (2012) described colony PCR- single strand confirmation polymorphism technique to identify phytopathogenic bacteria by the PCR product without DNA isolation from selective media grown bacterial cultures. In this new method we have escaped both bacterial isolation and DNA extraction by using bacterial ooze as a source of template DNA resulting a time and money saving technique.

The PCR assay using the Rsol_*fliC* primers amplified the specific amplicon (400 bp) of *fliC* gene from all the four samples along with the positive control, but not from the negative control (Fig 1). The results indicated the successful applicability of this method, as the used primer pair was reported to be specific of *R. solanacearum* (Schönfeld *et al.* 2003). However, in earlier report (Schönfeld *et al.* 2003) successful detection of *R. solanacearum* was obtained from soil using Rsol_*FliC* primers, where total pure DNA was extracted and used for PCR reaction. The principle behind the feasibility of using bacterial ooze suspension (sterile) directly for PCR assay was same as of colony PCR (Umesha *et al.* 2012) where the initial incubation at high temperature (96 °C) facilitated the lysis of bacterial cell wall and removal of DNA. Further, to confirm the bacterium species genetically, the amplified fragment of a single sample was cloned, sequenced and analysed. The pair-wise multiple alignment showed 98-100% identity both at nucleotide and amino acid level with previously reported Indian strain of *R. solanacearum* at their partial *fliC* gene, while only 94% and 97% identity was observed with *R. syzygii* strain at nucleotide and amino acid level, respectively. Similarly, the phylogenetic analysis both at nucleotide and amino acid level confirmed the RCR226 as a member of *R. solanacearum* group (Fig 2).

The success of any PCR amplification is generally depends on the template of DNA used in PCR mixture. In present report, we used bacterial ooze as a source of DNA template for PCR amplification. Therefore, sensitivity and detection threshold of the PCR method developed in this report is very important for successful detection of *R. solanacearum* from infected plants. Different dilution

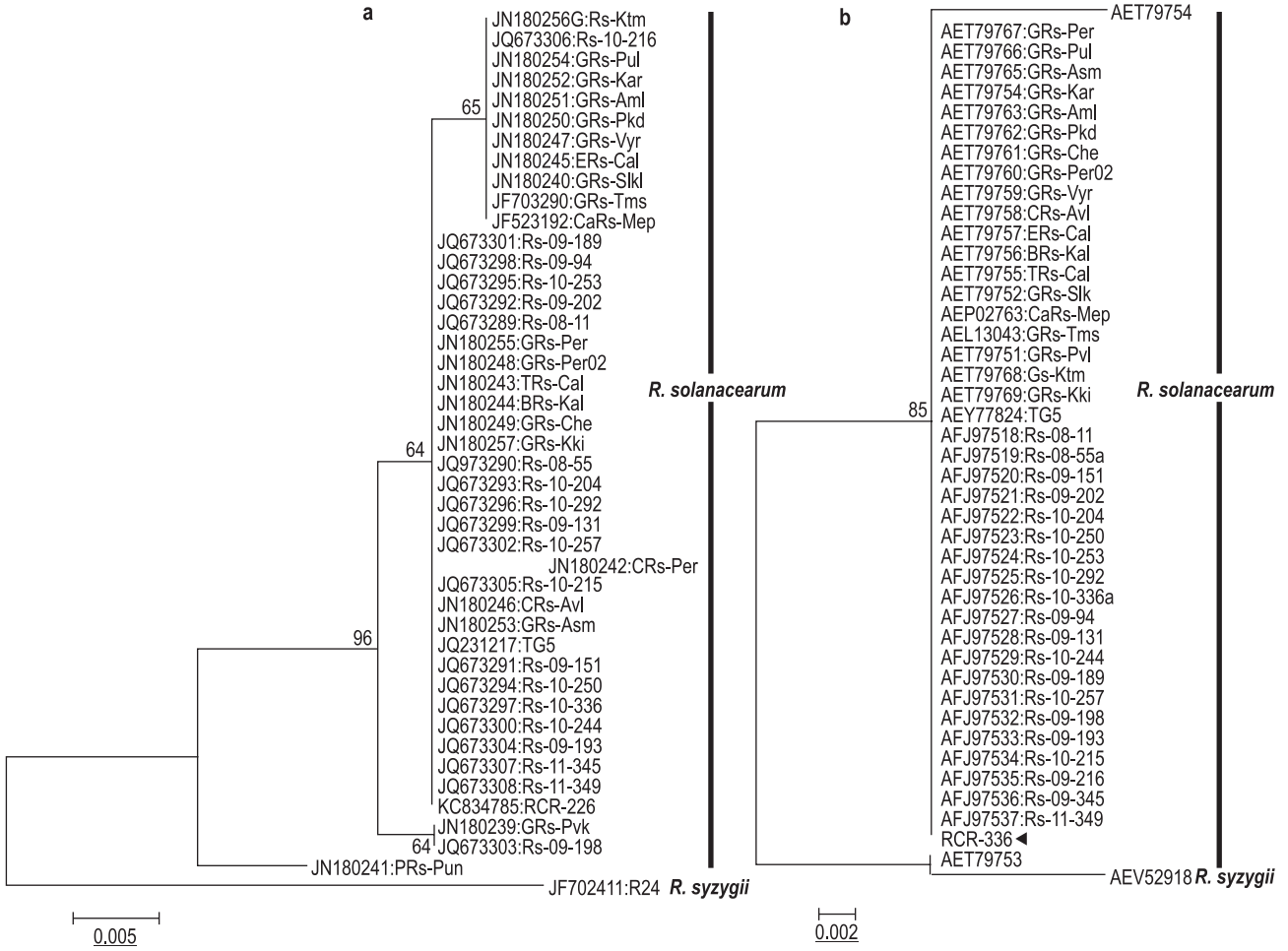

Fig 2 Phylogenetic relationships based on nucleotide and amino acid sequences of partial *fliC* gene (a, b) of the *R. solanacearum* strain (RCR-226) along with previously reported Indian strains. A single strain of a related species, *R. syzygii* was also considered for comparison as an out group member. In each case, the strain and accession number are given. Isolates under study have been marked by an arrow. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (shown only when >50%).

prepared from original ooze suspension and was used in PCR mixture gave a consistent PCR amplification even at 1:50 dilution (Fig 3), with consistent intensity of the bands on 1.5% gel. The successful PCR amplification obtained at various dilutions of crude DNA (ooze suspension), showed,

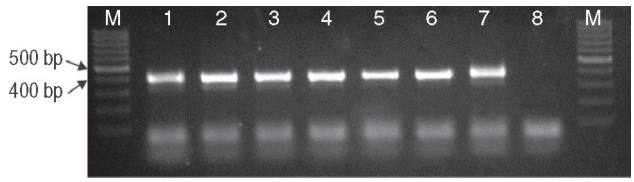

Fig 3 Sensitivity test of bacterial ooze as template for PCR detection of *R. solancearum* directly from wilt infected plant material by serial dilutions of ooze suspension. Presence of *R. solancearum* was confirmed by an amplification of 400 bp amplicon of *fliC* gene. M: 100bp DNA ladder; lanes 1: original ooze suspension; lane 2-6: 1:2-1:50 dilutions of ooze suspension; lane 7: positive control; lane 8: negative control.

the reliability and robustness of PCR method developed in this report.

The overall molecular assay confirmed the rapidity and specificity of the new method for successful detection of *R. solanacearum* directly from infected plant material and also reported successful identification of a north-eastern strain of *R. solanacearum* from wilt infected tomato plants. Moreover, the consistent amplicon pattern at a dilution ratio up to 1:50 showed the reliability and robustness of PCR method developed in this report. Therefore, it has been proposed that the PCR method developed in this report could be used as a diagnostic tool for rapid and reliable identification of *R. solanacearum* infestation at early stage and it would subsequently enhance the efficiency of prophylactic measures to be undertaken for management of this disease.

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