JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Nov 2015. Vol. **9**(Spl. Edn. 2), p. 259-264

# Specific and Quick Detection of *Ralstonia solancearum* (E.F Smith) Yabuuchi *et al*, from Infected Rhizomes Causing Bacterial Wilt of Ginger by using PCR based Amplification of *fliC* Gene Coding for the Flagella Subunit

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(Received: 11 April 2015; accepted: 26 June 2015)

**The wilt caused by bacteria (*Ralstonia solanacearum*) is the most devastating pathogen among all the rhizome rot pathogens. An effort was made for quick detection of bacterial wilt pathogen using PCR based molecular techniques. Phylogram results of four RS isolates Revealed that there are two major clusters, cluster A comprising all *Ralstonia solanacearum* species, A glance towards dendrogram reveals that there was not much diversity among the four isolates of RS. The *fliC* gene was amplified and an expected size of 400bp it was confirmed the *Ralstonia solanacearum* flagellin protein (*fliC*) gene. Fragment of *fliC* gene sequences of RS-1 (Haveri) and RS-8 (Shimoga) isolates showed 99 per cent similarity with NCBI published sequence of RS Strain MR11 plasmid mega plasmid flagellin protein (*fliC*) gene, partial cds (KF031064.1) and DSM9544 flagellin (*fliC*) gene, partial cds (AY192724.1) respectively. Dharwad isolate showed 98 per cent similarity with *Ralstonia solanacearum* strain RCR-226 flagellin protein (*fliC*) gene, partial cds (KC834785.1). While Uttara Kannada isolate showe 97 per cent similarity with RS flagellin (*fliC*) gene, complete cds (AF283285.1).**

**Key words:** *Ralstonia solanacearum*, Bacterial wilt, quick detection, *fliC* gene, Polymerase chain reaction.

Ginger (*Zingiber officinale* Rosc) is an important crop used both as spice and medicine also grown for its aromatic rhizomes. It is herbaceous tropical perennial plant belonging to the family Zingiberaceae. The whole plant is refreshingly aromatic, but it is the underground rhizome (raw or processed) which is valued as spice. Its medicinal value is increasingly being recognized now days. The refreshing pleasant aroma, biting taste and carminative property of ginger make it an indispensable ingredient of food processing throughout the world. Fresh ginger, dry ginger, oleoresin and oil are used for food processing.

The wilt caused by bacteria (*Ralstonia solanacearum*) is the most devastating pathogen among all the rhizome rot pathogens. The bacteria alone or in association with other pathogens can cause severe damage to the crop. The crop infected by the bacteria shows green sudden wilting and the spreading to the nearby fields is most rapid

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thus leading to more damage the affected rhizomes emit a foul smell of bacteria and ultimately get rotten leading to severe harvest loss to the farmer (Raghu, 2011). So there is an urgent need to manage the disease to avoid the losses. But the problem with the disease management is many times these measures are become unproductive if they face environmental conditions like high rainfall and humidity where no one can take up the management measures and goes beyond our limit to check the spread of this disease. So the only option left with us is to select healthy planting materials along with other management options including cultural methods. In this regard to select a healthy planting material we should confirm is it disease free? So answer lies in quick detection of bacterial using PCR based molecular techniques. In this paper we discussed the quick detection technique to confirm the seed borne nature of bacteria and variability among the isolates collected from various ginger growing regions of Karnataka.

*Ralstonia solanacearum* is the causal agent of bacterial wilt in many solanaceous crops also but has also been recorded to infect a large range of more than 200 species representing over 50 families of plants (Hayward,A.,1994). Traditionally, the pathogen has been classified in five biovars according to carbon source utilization (Hayward,A.,1994 & Hayward,1964) and in six races based on host range (Buddenhagen, I., and A. Kelman. 1964 & Poussier, *et al.*,2000). Thus, reliable methods to detect the pathogen from diseased plant samples and soil are required. Several PCR based methods for the detection of *R. solanacearum* have been described in the literature.

## MATERIALS AND METHODS

A rapid roving survey was conducted in major ginger growing areas of Karnataka. After recording the disease incidence, the samples were collected for isolation and characterization of the bacterium. Ten isolates of *Ralstonia solanacearun* were finally used for molecular diversity studies by isolating the bacterium from ginger rhizomes by using Tetrazolium chloride Agar (TZC) medium. Further biochemical tests were conducted to confirm the morphology, physiology and biochemical characters. Afterwards all the ten isolates of were grown on 30 ml TZC broth contained in 100 ml flasks for 48 h at $30^0$C and used for the DNA isolation.

**DNA isolation for the strains of *R. solanacearum***

The DNA was isolated from the bacteria by following the method given by Kumar *et al.* (2006). ( The stock solution used contains, Lysis buffer (40 mM Tris base, 20 mM sodium acetate, 1 mM EDTA, 1% SDS and pH 8.0), Phenol: Chloroform: isoamyl alcohol (25: 24: 1), 1x TE buffer, 95 per cent ethanol, 5 M NaCl.) From TZC broth , About 1.5 ml aliquots of broth culture were taken in 2.0 ml eppendorf tubes and centrifuged at 13,000 rpm for 5 min. The supernatant was poured off, 200 ml of lysis buffer was added to the tubes containing pellet and was mixed well, l66 ml of 5 M NaCl was added and mixed well and contents were centrifuged at 13,000 rpm for 10 min. Supernatant (250 ml) obtained was transferred to a new tube, to which l ml of RNase (10 mg/ml) was added, mixed well and incubated at $37^0$C for 30 min. An equal volume of chloroform: isoamyl alcohol was added, mixed gently by inverting the tubes, centrifuged at 13,000 rpm for 6 min. The upper aqueous phase was transferred to a clean tube; 1.0 ml of cold 95 per cent ethanol was added, mixed well but gently. The tubes were then kept in deep freezer at $-20^0$C for 1 h, centrifuged at 13,000 rpm for 6 min. Ethanol was poured off, DNA pellet was air dried using speed vacuum for five min. Pellet was resuspended in 50 ml of 1x TE buffer, kept in the refrigerator at $40^0$C, for overnight, stored in deep freezer at $-20^0$C. The quality of isolated DNA was estimated by Agarose gel electrophores containing 0.8% Agarose at 70 volts for 60 min.

**Primer used and Optimumization of PCR conditions**

The primer used in this experiment was RS-F- 5GTCGCCGTCAACTCACTTTCC-3 and RS-R 5GTCGCCGTCAGCAATGCGGAATC G -3 which codes for *flic* genes with an amplified product size of 400bp at an annealing temperature of 60 $^o$C. The PCR amplifications for analysis were performed according to Kumar *et al.* (2004) with certain modifications. The optimum specifications followed for DNA amplifications were as follows (Initial denaturation- 95 $^o$C for 5 min one cycle, Primer annealing -94 $^o$C for 1 sec, Denaturation 60 $^o$C for 1 min, Extension 72 $^o$C for 1 min these three steps repeated for 35 cycles, Final extension-72 $^o$C for 10 min and hold temperature 4 $^o$C for 30 min).

**Table 1.** Comparison and identity of fragment of the flagellin protein (fliC) gene of Ralstonia solanacearum from ginger growing districts of Karnataka (Haveri, Dharwad , Shimoga, and Uttara kannada) with that of referred gen bank available in NCBI

| Isolate code | District | Identified as | Gene Bank Accession Number | Reference | Per cent similarity |
|---|---|---|---|---|---|
| RS-1 | Haveri | Ralstonia solanacearum strain MR111 plasmid megaplasmid flagellin protein (fliC) gene, partial cds. | KF031064.1 | Kong and Yang (2013) | 99 |
| RS-3 | Dharwad | Ralstonia solanacearum isolate RCR-226 flagellin protein (fliC) gene, partial cds. | KC834785.1 | Dutta et al. (2013) | 98 |
| RS-8 | Shimoga | Ralstonia solanacearum strain DSM9544 flagellin (fliC) gene, partial cds. | AY192724.1 | Schonfeld et al. (2003) | 99 |
| RS-4 | Uttara Kannada | Ralstonia solanacearum flagellin (fliC) gene, complete cds. | AF283285.1 | Tans Kersten et al. (2001) | 97 |

Agarose gel electrophores containing 0.8% Agarose was carries out at 70 volts for 60 min and The desired bands of 400 bp specific to *Flic* genes were observed in a Gel Doc™ system under UV (302 nm). The fragments of the RS- gene of about 400 bp amplicon were sequenced by using forward and reverse primer and by commercial sequencing centre, Bangalore Genei (India) Pvt. Ltd., Bangalore using the ABI 3130 XL sequencer.

**Sequence analysis**

The sequences obtained using forward and reverse primers were assembled using Vector NTI software. The sequences were subjected to BLAST analysis at http://www.ncbi.nlm.nih.gov. Also, the DNA sequences were aligned using MEGA 5.1 for further comparisons.

**Phylogenetic analysis**

Dendrogram was constructed using unweighted pair-group arithmetic average (UPGMA) method available in MEGA 5.1 software for the nucleotide sequences of RS *fliC* gene region of all isolates. Published sequence of RS *fliC* gene (CP002819) was downloaded from NCBI and used in the phylogenetic analysis. *Xanthomonas campestries* pv *campestries (*AE008922.1) nucleotide sequence was used as out group in the construction of dendrogram.

## RESULTS AND DISCUSSION

All the ten isolates of total genomic DNA from *Ralstonia solanacearum* was isolated and subjected for PCR analysis using flagellin protein (*fliC*) gene specific primer. The *fliC* gene was amplified or expected size of 400bp (Fig:1) it was confirmed the *Ralstonia solanacearum* flagellin protein (*fliC*) gene. Latter four isolates of selected district (Haveri, Dharwad, Shimoga and Uttara Kannada) of its PCR product was sequence by outsourcing with Bangalore Genei Pvt Ltd. The sequence was obtained and confirmed though BLAST in a NCBI Blast programme. The nucleotide sequences obtained are presented bellow

**Rs-1 (Haveri)**

GAACGCCAACGGTGCGAACTCGT ACCTGCAAACGGCCGACTCGTACCTG GGC CAGGTTGAAAACAACCTGCAACGT ATG CGC CAA CTGGCTGTG GAATCC AACAA CGGC GG T CTG TCGGCAGCCGACCAGACCAACCTG GACAAGGAATACCAACAGCTGGCAACGGC

CAACAAGAACATCGAAACCAACGCCAACTA
CAACGGCAACAAGCTGTTTGACGGCT
CGGTGGCTTCGACGACCTTCCAATATGGCC
AGAACGCAGCCACGGACGTGACCACGG
TCACCAACGTCAACATGTCGACCTTCGGCA
CGCTGACCGGCACGAGCGTGACCAGCGCTG
CCAACGCGACCGCAGCCCAGGCCGCGAT
CGATACCGACCTGACCTCCCTGAAGGCCGCC
C

**Rs-3 (Dharwad)**

GAACGCCAACGGTGCGAACTCG
TACCTGCAAACGGCCGACTCGTACCTGG
GCCAGGTTGAAAACAACCTGCAACGTATG
CGCCAACTGGCTGTGGAATCCAACAAC
GGCGGTCTGTCGGCAGCCGACCAGACCAACC
TGGACAAGGAATACCAACAGCTGGCA
ACGGCCAACAAGAACATCGAAACCAACG
CCAACTACAACGGCAACAAGCTGTTCGAC
GGCTCGGTGGCTTCGACGACCTTCCAAT
ATGGCCAGAACGCAGCCACGGACGTGA
CCACGGTCACCAACGTCAACATGTCGACCT
TCGGCACGCTGACCGGCACGAGCGTGACC
AGCGCTGCCAACGCGACCGCAGCCCAGG
CCGCGATCGATACCGACCTGACCTCCCTG
AAGGC

**Rs-8 (Shimoga)**

GAACGCCAACGGTGCGAACTCGTA
CCTGCAAACGGCCGACTCGTACCTGGG
CCAGGTTGAAAACAACCTGCAGCGTATGCGC
CAGCTGGCCGTGGAAGCCAACAACGGC
GGTCTGTCGGCAGCCGACCAGACCAACCT
GGACAAGGAATACCAACAGTTGGCAAC
GGCCAACAAGAACATCGAAACCAACGCCA
ACTACAACGGCAACAAGCTGTTCGACGGTTC
GGTGGCTTCGACGACCTTCCAGTACGGC
CAGAACGCCGCCACGGACGTGGCCAC
GGTCACCAACGTCGACATGTCGGCCTA
CGGCACGCTGGCCGGTACGAGCGTGAC
CAGCGTTGCCAACGCGACCGCAGCCCAGGC
GGCGATCGATACCGATCTGACCTCCCT
GAAGGCCGCC.

**Rs-4 (Uttara Kannada)**

CCTCAGCCTCAATAGCAACATCTC
GTCCCTGCAAACGCAGCAAGCTCTGTCG
CAATCGCAGTCCGCTCTGCAGAAGTCGC
TGCAGCGCCTGTCGACCGGCATGCGCGT
GAACAGCGCGCAGGACGACGCAGCGG
CCTATGCGGCATCGAGCAGCCTGACCACGAC
CCTGAACTCGCAAACG CAAG GTATT CAG
AACGCCAACGGTGCGAACTCGTACCTG
CAAACGGCCGACTCGTACCTGGGCCAG
GTTGAAAACAACCT GCAGC GTAT GCGCCA
GCTGG CCGTGGAAGCCAA CAACGGCGG CTG
TCGG CAGC CG ACCA GACCAACC TGGAC
AAGGAATACCAACA GTTG GCAACG GCCAA
CA AGAACATCGA AACCAACGCCAACTA
CAACGGCAAC.

Nucleotide sequences of four RS isolates flagellin protein (*fliC*) gene region were analyzed using bioinformatics tools at National Centre for Biotechnology Information (NCBI) BLAST programme. Based on sequence comparison, all the fragment of (*fliC*) gene sequences of four isolates were confirmed as *Ralstonia solanacearum* strain (*fliC*) gene, partial sequence; ribosomal RNA. The list of four isolates, identified

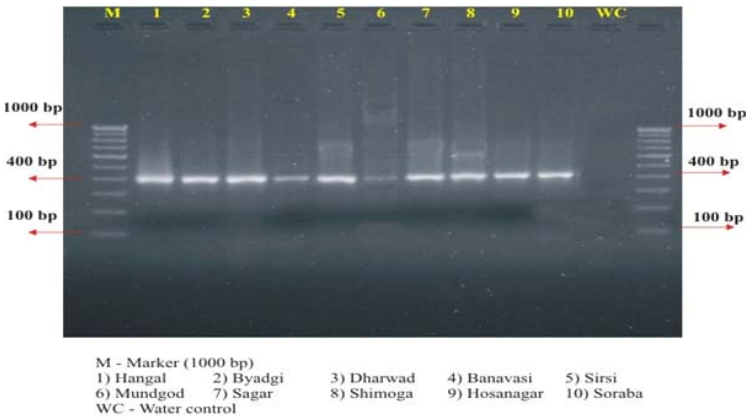


**Fig. 1.** PCR amplification of *fliC* gene of *Ralstonia solanacearum* by using primers targeting gene coding for Flagella

as, Gene Bank accession number and per cent similarity with reference are presented in (Table 1).

Fragment of *fliC* gene sequences of RS-1 (Haveri) and RS-8 (Shimoga) isolates showed 99 per cent similarity with NCBI published sequence of RS Strain MR11 plasmid mega plasmid flagellin protein (*fliC*) gene, partial cds (KF031064.1) and DSM9544 flagellin (*fliC*) gene, partial cds (AY192724.1) respectively. Dharwad isolate showed 98 per cent similarity with *Ralstonia solanacearum* strain RCR-226 flagellin protein (*fliC*) gene, partial cds (KC834785.1). While Uttara Kannada isolate showed 97 per cent similarity with RS flagellin (*fliC*) gene, complete cds (AF283285.1)

**Phylogenetic analysis**

A dendrogram was constructed using neighbour-joining tree method for the nucleotide sequences of flagellin protein (*fliC*) gene region of all four RS isolates

In the present study, *Xanthomonas campestries* pv *campestries (*AE008922.1) was used as out group to interpret the clustering of four RS isolates. Phylogram results of four RS isolates Revealed that there are two major clusters, cluster A comprising all *Ralstonia solanacearum* species, whereas cluster B comprises *Xanthomonas campestries* pv *campestries*. A glance towards dendrogram reveals that there was not much diversity among the four isolates of RS. As expected branching of *Xanthomonas campestries* pv *campestries* was altogether different having wide divergence with separate branch in dendrogram Published sequence of *Ralstonia solanacearum* CP002819.

The *fliC* gene coding for the flagellar subunit protein flagellin is highly specific and sensitive PCR-based detection system for *R. solanacearum*. The suitability of flagellin *fliC* genes for taxonomic applications has been shown in a number of studies for a large variety of bacterial species of several major bacterial groups: for á proteobacteria (Shah,*eta.l*,2000 ); for â proteobacteria, to which *R. solanacearum* belongs (Hales, et al. 1998); for low-G+C gram-positive bacteria (Tasteyre, *et al.*,200); for the genus *Pseudomonas*; and most notably for most enterobacterial species (Machado *et al.*,200).

Flagellin genes have been used for detection, studies of population genetics, and epidemiological analyses (Winstanley, C., and J. A. W. Morgan. 1997). Due to their structure, which is conserved in the terminal regions that flank a variable, central region, flagellin genes are regarded as good candidates for PCR-based detection (Winstanley, C., and J. A. W. Morgan. 1997). The main goal of the present study was to detect the bacterium in infected ginger rhizome samples by using primers for amplification of a flagellin gene fragment that target all subgroups of the *R. solanacearum* species complex and to investigate their application to the detection of *R. solanacearum* (Schonfeld *et al*,2003)Therefore, special attention was paid to achieving a sensitive amplifiability of the *fliC* gene fragments from DNA extracted from plant samples.

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