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Method for isolation of soil DNA and PCR based detection of ginger wilt pathogen, *Ralstonia solanacearum*

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ABSTRACT: An efficient DNA isolation protocol and PCR based detection of bacterial pathogen in soil are described here. The use of this DNA isolation protocol and PCR based method using universal *Ralstonia solanacearum* specific primer offer a rapid method for unambiguous detection of this pathogen in soil which can be employed for monitoring soil. The PCR based assay could detect the pathogen at a concentration of 10^3-10^4cells per gram of soil.

Key words: PCR, specific primers, *Ralstonia solanacearum*, ginger

Bacterial wilt caused by *Ralstonia solanacearum* (Smith) Yabuuchi is one of the important production constraints in ginger in many small and marginal farm holdings. This bacterial pathogen survives in soil and makes it unsuitable for ginger cultivation for long period once introduced through infected planting material. Pre-plant detection of the bacterium in seed rhizomes and soil assumes significance to avoid the disease epidemic. Serological methods such as indirect ELISA have reported for its detection in soil (Priou *et al.*, 1999), besides the conventional methods, like isolation on semi selective medium (Englebrecht, 1995) or bioassays using indicator host plants (Graham and Lloyd, 1978). Conventional methods are unsuitable to detect the pathogen as it survives at very low population in soil. However these methods, particularly the serological ones are not universal, as they are known to yield false positive or false negative results when adopted in new host-pathogen systems. Another potential alternative approach would be DNA based methods such as PCR using pathogen specific probes or oligo primers to detect the pathogens (Louws *et al.*, 1999). Recently molecular tools have become valuable for analyzing microbial populations and communities. Specific taxonomic groups can be identified and detected by using nucleic acid probes without having to isolate and culture them. The extraction and analysis of total soil microbial community DNA from soil is useful for several purposes (Stokes *et al.,* 2001). Application of microbial community DNA extraction methodologies allows investigations on the nature of non-culturable cells, which are known to abound in soil. After pioneering attempt by Trosvik in 1980, extraction of microbial DNA from soil has been primarily carried out using two different approaches (i) separation of microbial cells from soil particles followed by subsequent cell lysis and extraction (Holben *et al.,* 1988) and (ii) direct cell lysis and DNA extraction from soil in the presence of other soil components (Ogram *et al.,* 1987). In the present investigation an attempt was made to isolate bacterial DNA from soil by adopting the former approach using soil bacteria *R. solanacearum* as a model organism which causes lethal bacterial wilt in plants belong to 44 families encomposing around 450 plant species including tomato, potato, banana, ginger etc. We further report here the detection of ginger wilt pathogen *Ralstonia solanacearum* in soil using universal *Ralstonia solanacearum* specific primers (Rs specific primers).

MATERIALS AND METHODS

Soil DNA extraction

Soil inoculated with different concentration of *Ralstonia* cells (strain GRS-Vy, Biovar 3) (10^7, 10^6,

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10^5, 10^4, 10^3 and 0 cfu g^{-1} soil) were subjected to differential centrifugation in order to isolate the bacterial fraction from soil for extraction of bacterial DNA using SDS-CTAB method (Zhou *et al.,* 1996). Briefly, 10 g of soil was homogenized and extracted thrice with 20 ml of PBS (gL^{-1} Sodium chloride 0.8; potassium chloride 0.2; disodium hydrogen phosphate 1.44; potassium dihydrogen phosphate 0.24, 1L distilled water pH 7.4). Thus pooled 60 ml of soil suspension was centrifuged (Avanti J25; Beckman, USA) at 1000 g for 10 minutes at 4°C, followed by recovery of supernatant consisting of bacterial cells. The supernatant was centrifuged at 10000 g for 10 minutes at 4°C. The pellet containing bacterial fraction was suspended in sterile distilled water (2 ml) and was aliquoted into two microfuge tubes of 1 ml each before proceeding with DNA extraction.

Similarly DNA was isolated from soil inoculated with both *R. solanacearum* and *Pseudomonas fluorescens* in order to detect *R. solanacearum* in a mixture of soil bacterial community. Soil inoculated with two strains of *P. fluorescens* (IISR6 and IISR51) either individually or in combination was used in the extraction of bacterial community from soil using the protocol described above.

Thus collected soil bacterial fraction was pelleted at 10000g for 10 minutes at 4°C and the brown pellet was mixed with 675 µl of DNA extraction buffer [100 mM Tris-Cl (pH 8.0); 100 mM EDTA (pH 8.0); 100 mM Na_2HPO4; 1.4M NaCl; 2% Cetyl Trimethyl Ammonium Bromide; Proteinase K: 20mg, Lysozyme: 100 mg]. After making homogenous suspension the lysate was incubated at 37 °C for 30 minutes in a shaking water bath at 150 strokes per minutes with intermittent end over end inversion. Then the lysate was gently mixed with 75 µl of SDS (20%) and homogenous suspension was made. The lysate was incubated at 65 °C for two hours in a shaking water bath at 150 strokes per minute with intermittent end over end inversion in order to make a homogenous suspension. The suspension was centrifuged at 6000 g for 20 minutes at 28 °C. The clear lysate was transferred to new tube, twice extracted with equal volume of chloroform: isoamyl alcohol mixture (24:1). The DNA was precipitated with 0.6 volume of isopropanol and once washed with 70% alcohol, dissolved in TE buffer (10 mM Tris-Cl+0.1 mM EDTA, pH 8.0), purity checked, quantified and concentration was adjusted to get 100 ng of DNA per ml of sterile distilled water. The ratio of absorbance at 230/260 nm as well as 260/280 nm was calculated in order to assess the contaminating humic acid and protein, respectively. Similarly DNA was isolated from soil inoculated with both *R. solanacearum* and *P. fluorescens* using the protocol described above.

Restriction digestion of soil DNA: 200 ng of bacterial DNA isolated from soil was digested with *Msp* I or *Taq* I following manufacturer's instructions (Promega Corporation, USA) for 12 h. The digested and undigested DNA was resolved in 0.7% agarose gel in 1X Tris Acetate EDTA buffer at 4°C for 6 h at 4V/cm. The gel was stained with Ethidium bromide and photographed on an UV transilluminator (Alpha Innotech, USA) and the results were documented in Alpha imager 2002 for analysis.

PCR amplification of soil DNA using Internally Transcribed Spacer primer (ITS) and pathogen specific primers

ITS PCR: PCR amplification for 16S-23S rRNA gene intergenic spacer regions was done in 20 µl of reaction mixture containing PCR buffer (Fegan *et al.,* 1997): 1x (Genei, Bangalore), $MgCl_2$: 3 mM, dNTP mix: 0.2 mM, *Taq* DNA polymerase: 0.5U, Primer (ITS ALL F: 5'-TAggCgTCCACACTTATCggT-3'), 20 pmoles, soil DNA: 100 ng.

Detection of Ralstonia solanacearum using Rs specific primer: PCR amplification for detection of *R. solanacearum* in soil was performed using the DNA isolated from soil as template. Reaction volume (25 µl) contained PCR buffer (Mo Bio, USA), $MgCl_2$: 1.5 mM (Promega Corporation, USA), dNTP mix: 0.05 mM (Mol Bio, USA), DNA polymerase enzyme: 0.5U, Template DNA: 100 ng, BSA: 10 mg, Primers: 20pmoles each (Forward primer: 5'-gTC gCC gTC AAC TCA CTT TCC-3'; Reverse primer: 5'-gTC gCC gTC AgC AAT gCg gAA TCg-3') (Opina *et al,* 1997). PCR was performed in Eppendorf master cycler gradient thermal cycler at the following PCR conditions (Kumar *et al.,* 2004, Opina *et al.,* 1997) and the final PCR products were resolved in 1.5 or 2.0% agarose in 1x Tris Acetate EDTA buffer at 4°C for 6 hours at 4V/cm. The gel was stained with Ethidium bromide and photographed on an UV transilluminator and

the results were documented in Alpha imager for analysis.

RESULTS AND DISCUSSION

One of the prerequisites for the successful isolation of DNA from soil is that the isolated DNA should be amenable for DNA manipulations such as polymerase enzyme mediated amplification, restriction digestion and cloning. Particularly the DNA should be pure enough to be amplified by PCR for detection of target microorganisms or detection of gene (or bacterium) deployed in the environment intentionally or unintentionally. Soil DNA when isolated directly would accumulate impurities from soil that are potential inhibitors of restriction enzymes or polymerase enzyme (Tsai and Olson, 1992). Over the years the main objective of soil DNA isolation is that the soil DNA should be free from those PCR inhibitors or the concentration of those inhibitors must be low enough so that they do not interfere in the activity of DNA polymerase used in PCR. In order to achieve these objectives various protocols have been published in the past for successful isolation of PCR amplifiable DNA from soil (Yeates and Gillings, 1998). All published methods on extraction of DNA from soil and other complex environmental samples aim at isolating a high molecular weight and high quality DNA. High molecular weight DNA could be isolated from all the samples used in the present investigation (Fig. 1). Impurities as contamination in DNA preparation from soil were analyzed by calculating the ratio of A 230 and 260 nm as well as the ratio of A260 and 280 nm. The protocol adopted in the present work yielded DNA with A260/280 ratio ranging from 1.51 to 2.09 and A260/230 ratio ranging from 0.53 to 2.14 (Table 1). Zhou *et al.* (1996) has reported 0.91 and 1.35 for A260/230 and A 260/280, respectively. When restriction digested with *Msp* I or *Taq* I, the DNA could be digested completely (Fig 2). The yield of soil DNA was ranging from 0.095-1.840 µg g^{-1} soil. More *et al.* (1994) reported that soil DNA yield as high as 11.8 and 5.2 µg g^{-1} in bead beating and freeze thawing method, respectively. The bead beating direct lysis method described by Yeates *et al.* (1998) yielded DNA between 15-23.5µg g^{-1}. Extraction methods using small soil samples ranging from 5 mg to 100 mg of soil have extracted 9-25 µg g^{-1} (Porteous and Armstrong, 1991), 12 µg g^{-1} (Tsai and Olson, 1992), 1-100 µg g^{-1} (Porteous *et al.,* 1994), and 2.5-26.9 µg g^{-1} (Zhou *et al.,* 1996).

Purity of DNA as indicated by the ratio of absorbance at 260nm and 280nm (A 260/280) was comparable to that of other DNA isolation protocols (Zhou *et al.,* 1996, Yeates *et al.,* 1998). Bacterial DNA isolated from soil is known to coprecipitate with humic and fulvic acid, which are potential inhibitors of DNA polymerase even at nanogram quantities. Tebbe and Vahjen (1993) reported that restriction enzymes were inhibited at a humic acid concentration of 0.8-51.7 µg ml^{-1} whereas polymerase enzyme activity was inhibited at 0.24-0.48 µg ml^{-1}. The major part of soil material contains heterogeneous organic and inorganic particles, colloids and amorphous organic matter. The present attempt to isolate PCR amplifiable DNA from *R. solanacearum* inoculated soil resulted in high quality DNA that was amenable for restriction digestion as well as polymerase activity. The DNA could be

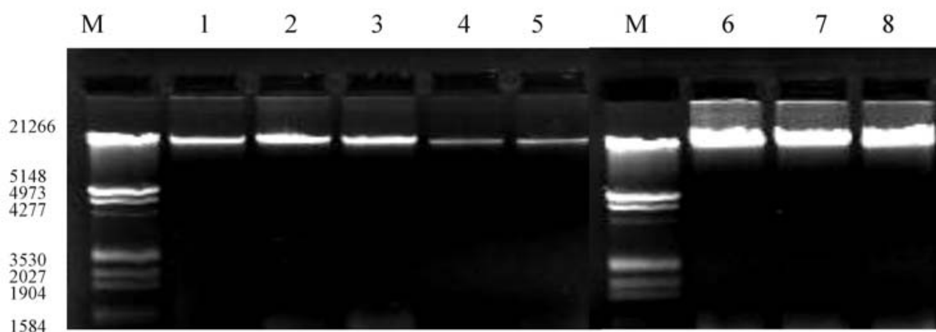

Fig. 1. DNA isolated from soil using CTAB+SDS method

M: DNA size marker (bp)-λ DNA digested with EcoR1 and Hind III, Lane 1-5: Concentration of *R. solanacearum* (Rs) in soil (cfu g^{-1}) 1: 10^7, 2: 10^6, 3: 10^5, 4:10^4, 5: Uninoculated soil, Lane 6-8: Concentration of *R. solanacearum* and *Pseudomonas fluorescens* in soil (cfu g^{-1}) 6: Rs+IISR6 each 10^7, 7: Rs+IISR51 each 10^7, 8: Rs+IISR6+IISR51 each 10^7

Table 1. Quantitative and qualitative analysis of DNA isolated from soil seeded with bacteria

Concentration of bacteria in soil* (Cells $gram^{-1}$)	Yield ($\mu g\ g^{-1}$)	A** 260/280	A** 260/230
Soil + *Ralstonia solanacearum* (GRS Vy) 10^7	4.10	1.56	0.53
Soil + *R. solanacearum* (GRS Vy) 10^6	0.80	1.89	1.50
Soil + *R. solanacearum* (GRS Vy) 10^5	0.78	2.00	1.80
Soil + *R. solanacearum* (GRS Vy) 10^4	0.28	1.96	1.05
Soil + *R. solanacearum* (GRS Vy) 10^3	0.21	1.80	0.80
Soil + *R. solanacearum* (GRS Vy) + *Pseudomonas fluorescens* (IISR 6)	1.95	1.97	1.96
Soil + *R. solanacearum* (GRS Vy) + *P. fluorescens* (IISR 51)	2.00	1.98	2.04
Soil + *R. solanacearum* (GRS Vy) + *P. fluorescens* (IISR 6)+ *P. fluorescens* (IISR 51)	2.15	2.00	2.14
GRS Vy as pure culture (10^9 cells per ml)***	80.0	1.99	2.24

*Soil consist of 2:1:1 mixture of forest soil, farmyard manure and river sand; **Ratio of absorbance of DNA at two wave lengths, *** Concentration of bacteria per ml of pure culture of *R. solanacearum* GRS-Vy

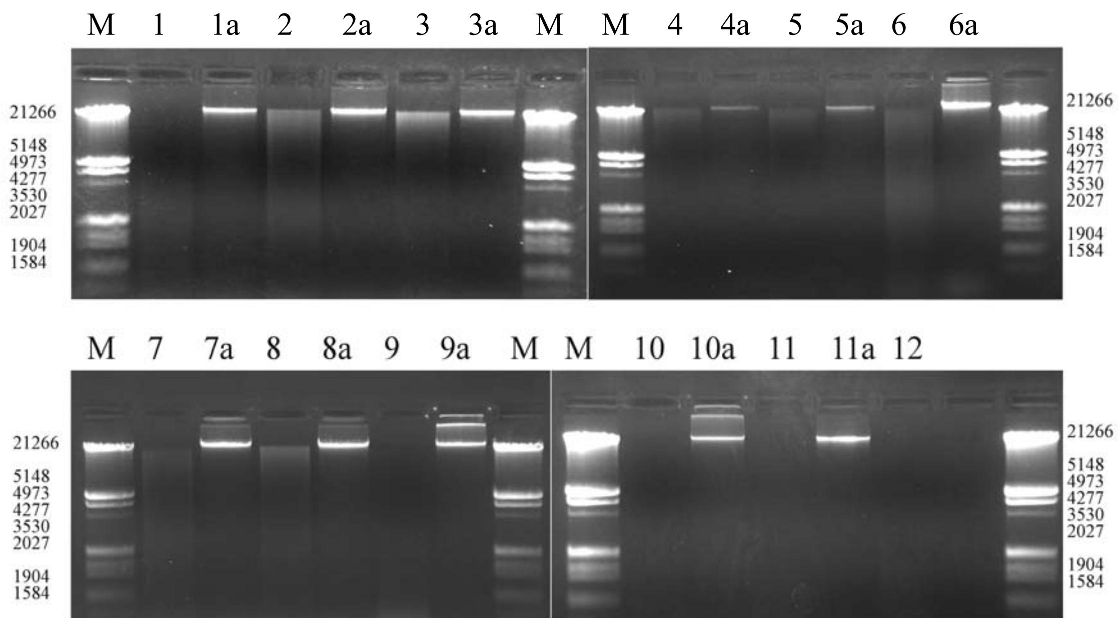

Fig. 2. Restriction digestion of bacterial genomic DNA isolated from soil
M: DNA size marker (bp)-λ DNA digested with EcoR1 and Hind III, Lanes 1, 2, 3, 4, 5, 6, *Msp* I Digested soil DNA, Lanes 1a, 2a, 3a, 4a, 5a, 6a, Undigested soil DNA
M: λ DNA digested with EcoR1 and Hind III, Lane 7, 8, 9, 10, 11, Soil DNA digested with *Taq* 1, Lane 7a, 8a, 9a, 10a, 11a, Undigested soil DNA, Lane 12: Negative control

digested with two restriction enzymes, *Msp* I and *Taq* I, which clearly indicated its purity and amenability for DNA manipulation (Fig. 2). Besides the DNA could be amplified in PCR using two kinds of primers viz., ITS all F as well as Rs specific primers (Fig. 3,4,5,6). Both the primers could amplify the target DNA sequences in the genomic DNA of *R. solanacearum* isolated from the soil.

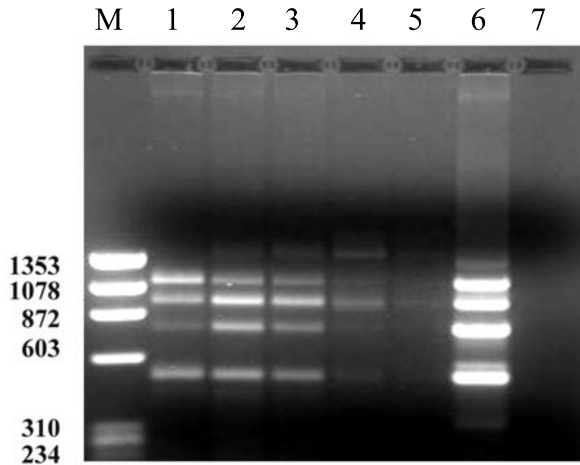

Fig. 3. Sensitivity of PCR based assay for detection of *R. solanacearum* (Amplification of soil DNA with ITS ALL F primer)
M: DNA size marker (bp) Phi X 174 Hae III, Lane 1-5: Soil inoculated with varying concentration of cells of *R. solanacearum* (cfu g^{-1}), Lane 1: 10^7, 2. 10^6, 3. 10^5, 4. 10^4, 5. 10^3, Lane 6: Positive control (pure DNA), Lane 7: Negative control (Uninoculated soil)

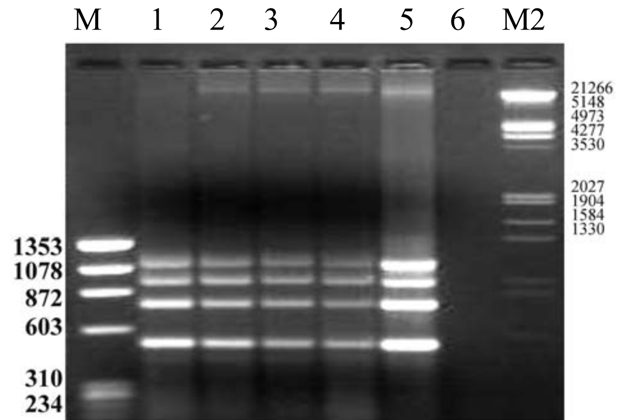

Fig. 4. Specificity of PCR based assay for the detection of *R. solanacearum* in soil
M1: DNA size marker (bp) Phi X 174 Hae III, Lane 1: *R.solanacearum* alone, Lane 2: *R. solanacearum* + *Pseudomonas fluorescens*-IISR 6, Lane 3: *R. solanacearum* + *P. fluorescens*-IISR 51, Lane 4: *R.solanacearum* +*P. fluorescens*-IISR 6 +IISR51, Lane 5: Positive control (pure DNA of *R.solanacearum*), Lane 6: Negative control, M2: λ DNA digested with EcoR1and Hind III

The sensitivity of the present assay using random and specific primer was 10^4 cells per gram of soil as very feeble amplicon was seen at a concentration of 10^3 cells g^{-1} (Fig.5). Low sensitivity of PCR assay using soil DNA was attributed mainly to the strong binding of bacterial cells in soil colloids and DNA polymerase inhibiting compounds present in soil (Tsai and Olson, 1992). The sensitivity of PCR based method using Rs specific primer was found to be 10^3 cells per gram of soil in earlier reports (Ito *et al.,* 1998; Lee and Wang, 2000). Primer sequence (Opina *et al.,* 1997) mentioned above is known to amplify 281bp sequence in the genomic DNA of *R. solanacearum*, which has been exploited in the detection assay for *R. solanacearum* using PCR (Ito *et al.*, 1998). The intensity of amplified product using Rs specific primer seems to have been influenced by the initial population level of target bacterium (Fig. 6). The sensitivity can be further improved by enrichment of soil suspension in selective medium for *R. solanacearum* as selective medium has already been developed for this bacterium (Ito *et al.,* 1998).

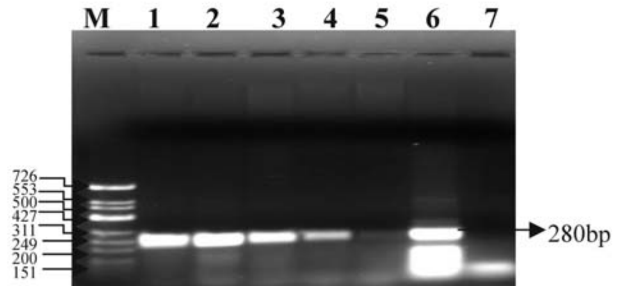

Fig. 5. PCR based assay for detection of *R. solanacearum* using Rs specific primers
M: DNA size marker (bp) Phi X 174 Hinf digest, Lane 1-4: DNA extracted from soil inoculated with varying concentration of cells of *R. solanacearum* (cfu g-1), Lane 1: 10^7, Lane 2: 10^6, Lane 3: 10^5, Lane 4: 10^4, Lane 5: 10^3, Lane 6: Positive control (pure DNA of *R. solanacearum* Biovar III), Lane 7: Negative control (Uninoculated soil)

In conclusion, an efficient DNA isolation protocol and detection of bacterial pathogen in soil are described here. The use of this DNA isolation method and PCR based method for detection of *R. solanacearum* in soil offer a rapid method for unambiguous detection of this bacterium in soil which can be employed for monitoring soil samples for this globally important plant pathogen.

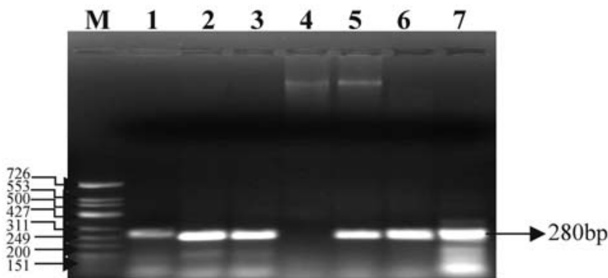

Fig. 6. Detection of *Ralstonia solanacearum* in contaminated soil using PCR based method M: DNA size marker (bp) Phi X 174 Hinf digest, Lane 1-3: DNA extracted from field soil contaminated with *R. solanacearum,* Lane 4: Impure DNA, Lane 5: Soil contaminated with *R. solanacearum* and *P. fluorescens* IISR 51, Lane 6: Soil contaminated with *R. solanacearum* and *P. fluorescens* IISR 51+ IISR 6, Lane 7: Positive control (pure DNA of *R. solanacearum* Biovar III)

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