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Detection of *Ralstonia solanacearum* from Asymptomatic Tomato Plants, Irrigation Water, and Soil Through Non-selective Enrichment Medium with *hrp* Gene-Based Bio-PCR

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Abstract Bacterial wilt of tomato caused by *Ralstonia solanacearum* (Smith) Yabuuchi et al. (Microbiol Immunol 39:897–904, 1995) is a serious disease, which causes losses up to 60 % depending on environmental conditions, soil property, and cultivars. In present investigation, nucleotide sequences of virulence, hypersensitive response and pathogenicity (*hrp*) gene were used to design a pair of primer (*Hrp*_rs 2F: 5′-AGAGGTCGACGCGATACAGT-3′ and Hrp_rs 2R: 5′-CATGAGCAAGGACGAAGTCA-3′) for amplification of bacterial genome. The genomic DNA of 27 isolates of *R. solanacearum* race 1 biovar 3 & 4 was amplified at 323 bp. The specificity of primer was tested on 13 strains of *R. solanacearum* with other group of bacteria such as *Xanthomonas oryzae* pv. *oryzae*, *X. campestris* pv. *campestris,* and *X. citri* subsp. *citri.* Primer amplified DNA fragment of *R. solanacearum* at 323 bp. The sensitivity of the primer was 200 cfu/ml and improved further detection level by using non-specific enrichment medium casamino acids-pepton-glucose broth followed by PCR (BIO-PCR). Out of 130 samples of asymptomatic tomato plants, irrigation water, and soil collected from bacterial wilt infested field in different agro-climatic regions of India, *R. solanacearum* was detected from 86.9, 88.5, and 90.9 per cents samples using BIO-PCR, respectively. The primer was found specific for detecting viable and virulent strains of *R. solanacearum* and useful for the diagnosis of *R. solanacearum* in tomato seedlings and monitoring of pathogen in irrigation water and soil.

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Introduction

Bacterial wilt of tomato caused by *Ralstonia solanacearum* (Smith) Yabuuchi et al. [19] is widespread in tropical, subtropical, and warm temperate regions of the world and causes serious losses to the crops (5, 16). This pathogen, responsible for bacterial wilt, can infect over 450 plant species belonging to over 54 botanical families including tomato, potato, eggplant, chili, and capsicum [6]. *R. solanacearum* can persist at low populations in soil latent infection in nursery plants and irrigation water. An efficient, highly sensitivity and specificity detection method is required to facilitate the elimination of pathogenic bacteria from possible source of primary inoculums, which is likely to reduces the risk of crop losses. Various detection methods such as isolation on selective medium [4], pathogenicity test, and serological methods [18] have been reported to detect the bacteria from different sources. However, these methods are lengthy, time consuming and also not specific. PCR-based techniques are currently used to detect phytopathogenic bacteria and different conserved genes of bacteria including 16S rDNA (16), *flic* C [13], cytochrome c1 signal peptide [8], and *hrp* genes [2] have been used to design primers. Among them, a *hrp* gene (hypersensitive response and pathogenicity gene) is used to detect bacteria from different sources and crops [2, 15] with advantage to discriminate virulent and avirulent strains of bacteria [7]. The plant pathogenic bacterial population on/in the seeds, soil and crop residues is declined in the absence of host and difficult to detect even through common PCR technique. To improve the sensitivity of PCR enrichment of targeted bacteria has been done by allowing samples containing bacteria to multiply in general or selective media followed by PCR with specific primers [6, 9]. The media used for enrichment of bacteria are selective, which containing

different group of antibiotics that hampers the growth of bacteria. However, until now there are samples including asymptomatic tomato plants at seedling stage, irrigation water, and soil [9] using non-selective medium. Therefore, it is essential to standardize protocol for detection of bacteria using non–selective enrichment medium like casamino acids-pepton-glucose (CPG) medium to allow growth of bacteria, which can be further used for PCR.

Therefore, present investigation was undertaken to design a *hrp* gene-based PCR primer and standardize protocol of BIO-PCR with non-selective medium such as CPG medium to detect viable and virulent strains of *R. solancearum* from asymptomatic tomato plants, irrigation water, and soil samples.

Materials and Methods

Isolation and Characterization of Bacteria

27 isolates of *R. solanacearum* were isolated from wilted tomato plants. They were collected from different states of India such as Goa, Himachal Pradesh, Jammu & Kashmir, Jharkhand, Karnataka, Orissa, Uttarakhand, and West Bengal isolated on CPG medium, triphenyl tetrazolium chloride (TTC) medium, and semi-selective modified SMSA media by serial dilution plate technique during 2009–2012 [12, 16]. The cultures were routinely grown on CPG medium and maintained at −80 °C with 20 % glycerol. Cultures of *Xanthomonas campestris* pv. *campestris* strain Xcc-C1, *X. oryzae* pv. *oryzae* strain BB-1, and *X. citri* subsp. *citri* were obtained from Plant Bacteriology Lab, Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi, India. Pathogenicity test of all the isolates of *R. solanacearum* performed on 30 days old tomato cv. Pusa Ruby grown in pots at National Phytotron Facility, IARI, New Delhi, India [16]. Biovar of each isolates of *R. solanacearum* was determined by standard procedure [1]. For DNA extraction, the bacterial culture was grown in nutrient broth for 48 h for 28 °C at 150 rpm. The bacterial cells were harvested and centrifuge for 5 min at 15,000 rpm to pellet them. Total DNA of bacteria was extracted by CTAB method [10]. Molecular characterization of 27 isolates of *R. solanacearum* was done by using a set of primer Y2 and OLI-1 amplify at 288 bp [14, 16].

Primer Design, PCR Amplification and Sequencing

A set of primer (Hrp_rs 2F and Hrp_rs 2R) was designed from locus of *hrp* gene between 7,629 bp to 7,952 bp having product size 323 bp of total 23,401 bp nucleotide sequences of the strain of *R. solanacearum* (Accession No. AJ245811.1) using Primer 3 program (www.frodo.wi.nit.edu) and specificity was checked by in silico using website (www.insilico.ehu.es). The developed primers were validated for their universality across *R. solanacearum* and other related group of bacteria by primer blasting in NCBI website (www.ncbi.nlm.nih.gov). For sequence analysis of *hrp* gene of *R. solanacearum* strain UTT-25 race 1 biovar 3 isolated from Uttarakhand was taken for amplification as described above. Sequencing of these isolates was performed using ABI3730XL sequencer and reaction was analyzed on a capillary sequencer. *Hrp* gene sequences of strain were compared with available database having high sequence matching >96 % using GenBank BLASTN to determine approximate ($\approx$ 323 bp) phylogenetic affiliation. The phylogenetic relationships were inferred by MEGA 5 [17]. Sequence data were submitted in NCBI and accession numbers of *R. solanacearum* strain UTT-25 were obtained (JM113816).

To test the specificity of primer, 1 µl DNA of *R. solanacearum*, *X. campestris* pv. *campestris*, X. *oryzae* pv. *oryzae,* and *X. citri* subsp. *citri* was used as template for PCR. To determine the detection threshold of primer, 48 h old culture of *R. solanacearum* strain UTT-25 was prepared by making a 10 fold dilution series up to 10^{-8} from a liquid culture. 2 and 5 µl bacterial suspensions were taken from culture 10^{9} cfu/ml and 5 µl from dilution 10^{-1} to 10^{-8} used as template for PCR. To determine the population of bacteria, 100 µl of bacterial suspension from dilution 10^{-6} to 10^{-8} was inoculated on the TTC medium into the Petri plates and incubated for 72 h at 28 °C. The reaction was performed in a final volume of 20 µl amplification reaction mixture containing Buffer 2.0 µl (5X), Primer F (20 pmol/µl) 0.4 µl, Primer R (20 pmol/µl) 0.4 µl, $MgCl_2$ (25 mM) 1.0 µl, dNTPs (10 mM) 0.4 µl, Taq polymerase 0.25 µl. Amplification conditions included, denaturation step at 95 °C for 2 min followed by 35 cycles at 95 °C for 30 s, 51 °C for 30 s, and 72 °C for 30 s, and then one cycle of 72 °C for 10 min in Thermal cycler gradient PCR (BIO-RAD; model: C1000^{TM} Thermal Cycler). Amplified PCR products were separated by electrophoresis on 1.2 % agarose gel (80 V) for 1 h and visualized UV light (300 nm) after staining with ethidium bromide under gel documentation system (BIO-RAD).

Detection of *R. solanacearum* from Asymptomatic Plants, Irrigation Water and Soil

Total 130 samples of asymptomatic tomato plants, irrigation water, and soil were collected from bacterial wilt infested farmer's field of different states of India (Table 2). Irrigation water was collected in 30 ml of sterilized vial from irrigation channel of fields. About 200 g of soil was collected from

rhizosphere of three wilted tomato plants from each field and mixed together uniformly. 10 g of soil was added into 100 ml of sterilized distilled water and put on orbital shaker for 30 min at 150 rpm at room temperature and allowed to settle down soil particles at the bottom of the flask. 1.0 ml of soil suspension and irrigation water were inoculated into 9.0 ml of CPG broth medium in culture tube separately and incubated at 28 ± 1 °C. 1.0 g of tissue of asymptomatic plant was macerated in the sterilized pastel-mortar containing 6 ml of 0.85 % of brine solution (NaCl). 100 µl of macerated plant tissue was added into 1.5 ml of CPG broth in 2.0 ml of eppendorf tube and allowed to grow for 24 h at 28 ± 1 °C. The reaction was performed by using a set of primer (Hrp_rs 2F and Hrp_rs 2R) for amplification. 5.0 µl of irrigation water, asymptomatic plants tissue, soil suspension were directly used for conventional PCR. In bio-PCR, DNA from broth culture of bacteria was extracted as described by George et al. [5] and 2.0 µl DNA of bacteria was taken for PCR. Preparation of master mix, PCR conditions and gel electrophoresis was done as described earlier. 100 µl of plant suspension from macerated tissue, soil suspension, and irrigation water were inoculated on to Petri plates containing TTC medium and incubated at 28 ± 1 °C for 72 h by using serial dilution method [16]. Statistical analyses of data were done using paired *t* test comparing the detection methods used in this study.

Results

Bacterial wilt of tomato was recorded in the states of Himachal Pradesh, Jammu & Kashmir, Uttarakhand, Jharkhand, Karnataka, Goa and West Bengal, India. Out of 27 isolates of *R. solanacearum*, 24 were biovar 3 and only three isolates belong to biovar 4 (Table 1). The biovar 3 was most frequent to all five states, whereas biovar 4 was isolated from Himachal Pradesh and Uttarakhand states of India. All the isolates of *R. solanacearum* were pathogenic to tomato cultivar Pusa Ruby and they produced symptoms after 4–7 days of inoculation. However, UTT-25 and UTT-23 isolates were most virulent among other isolates of *R. solanacearum* to produce wilt symptoms within 4 days of inoculation. For further confirmation, a set of primer Y2 and OLI-1 specific to *R. solanacearum* was used for molecular characterization. All 27 isolates of *R. solanacearum* amplified at 288 bp (Table 1).

Specificity and Sensitivity of Primer

Out of five pair of *hrp* gene-based primer Hrp_rs2F and Hrp_rs2R was found best in specificity and sensitivity for the detection of *R. solanacearum*. To check the specificity of the primers by using in silico-PCR, 323 bp nucleotide sequences were obtained. The nucleotide sequences of *hrp* gene of our isolate UTT-25 of *R. solanacearum* were matched only with the strains of *R. solanacearum* with having maximum identity in *hrp* gene cluster and as this part of the gene showed lower homology to sequences from *hrp* gene of *R. syzygii* R24 and other group of bacteria *Acidovorax avenae* subsp. *avenae, Acidovorax avenae* subsp. *avenae* str. N1141, *Acidovorax avenae* subsp. *avenae* ATCC 19860, *Burkholderia* sp. YI23 having 92.0 % similarity index (Fig. 1). The *hrp* based primer gave a single product at 323 bp having DNA as a template of 13 isolates of *R. solanacearum* (Fig. 2). To test the specificity of the primers, three different groups of bacteria such as *X. campestris* pv. *campestris, X. oryzae* pv. *oryzae,* and *X. citri* subsp. *citri* were tested along with 13 isolates of *R. solanacearum* using the primers. However, none of the other bacteria used in this study amplified with this primer (Fig. 2). The primer was found specific for detection for all strains of *R. solanacearum,* race 1 biovar 3 & 4. To determine the detection level of this primer was high and detected 2.0×10^2 cfu/ml (Fig. 3).

Detection of *R. solanacearum* from Asymptomatic Tomato Plants, Irrigation Water and Soil

Comparative study among TTC culture medium, conventional PCR and BIO-PCR was done for detection of *R. solanacearum* from asymptomatic plants, irrigation water, and rhizospheric soil. Out of 130 samples including 55 samples of asymptomatic plants, 23 of irrigation water and 52 of soil collected from bacterial wilt infested fields, 89.2 per cent samples showed positive in BIO-PCR, whereas *R. solanacearum* was detected from 66.2 percent samples using conventional PCR and 36.9 percent in TTC medium (Table 2). *R. solanacearum* was detected from 86.9, 88.5, and 90.9 per cent samples of soil, irrigation water, and asymptomatic tomato plants collected from bacterial wilt infested fields by using BIO-PCR, respectively. Figure 4a, b, c reveals that *R. solanacearum* was detected from asymptomatic plants, soil, and irrigation water collected from Jharkhand, Odisha, West Bengal and Uttarakhand states of India by using non- selective CPG broth medium in BIO-PCR. No amplification was found in healthy tomato plants, non infested soil, and sterilized distilled water. Significant variation was found among the detection methods used such as TTC medium, conventional PCR, and BIO-PCR (t- critical two tail: 2.20).

Table 1 Origin and characterization of *Ralstonia solanacearum* isolated from tomato and other bacteria used in this study

States of India/ Countries	Strains of *R. solanacearum*	Biovar	Race	Phylotype	PCR reaction based on 16S rRNA and *hrp* gene
Himachal Pradesh	HPT-3a	4	1	I	+
	HPT-5	3	1	I	+
	HPT-19a	3	1	I	+
	HPT-11a	3	1	I	+
Jammu and Kashmir	JKT-1	3	1	I	+
	JKT-2	3	1	I	+
Uttarakhand	UTT-1	3	1	I	+
	UTT-6	3	1	I	+
	UTT-23	3	1	I	+
	UTT-26	3	1	I	+
	UTT-25	3	1	I	+
	UTT-32	3	1	I	+
	UTT-24	4	1	I	+
	UTT-22	4	1	I	+
Jharkhand	JHT-2	3	1	I	+
	JHT-01	3	1	I	+
	JHT-2P	3	1	I	+
	JHT-15	3	1	I	+
West Bengal	WBT-28	3	1	I	+
	WBT-5	3	1	I	+
	WBT-20	3	1	I	+
Odisha	ORT-1	3	1	I	+
	ORT-2	3	1	I	+
Karnataka	BRS-57	3	1	I	+
	BRS-58	3	1	I	+
Goa	GT-1	3	1	I	+
	GT-2	3	1	I	+
China	DNA for *hrp* gene locus	–	–	–	nd
France	GMI1000	1	3	I	nd
France	CMR15	–	–	III	nd
France	*R. syzygii* R24	3	2	IV	nd
Europe	IPO1609 Genome	2	3	IIB1	nd
China	Po82 megaplasmid	3	1	IIB	nd
France	CFBP2957	1	1	IIA	nd
Japan	*Acidovorax avenae* subsp. *avenae* strain: N1141	–	–	–	nd
Japan	*A. avenae* subsp. *avenae gene*	–	–	–	nd
USA	*A. avenae* subsp. *avenae* ATCC 19860	–	–	–	nd
South Korea	*Burkholderia* sp. YI23	–	–	–	nd
Delhi, India	*Xanthomonas oryzae* pv. *oryzae* strain BB01	–	–	–	nd
Delhi, India	*X. campestris* pv. *campestris* strain Xcc-C1	–	–	–	nd
Delhi, India	*X. citri* subsp. *citri*	–	–	–	nd

– no information, *nd* not determined

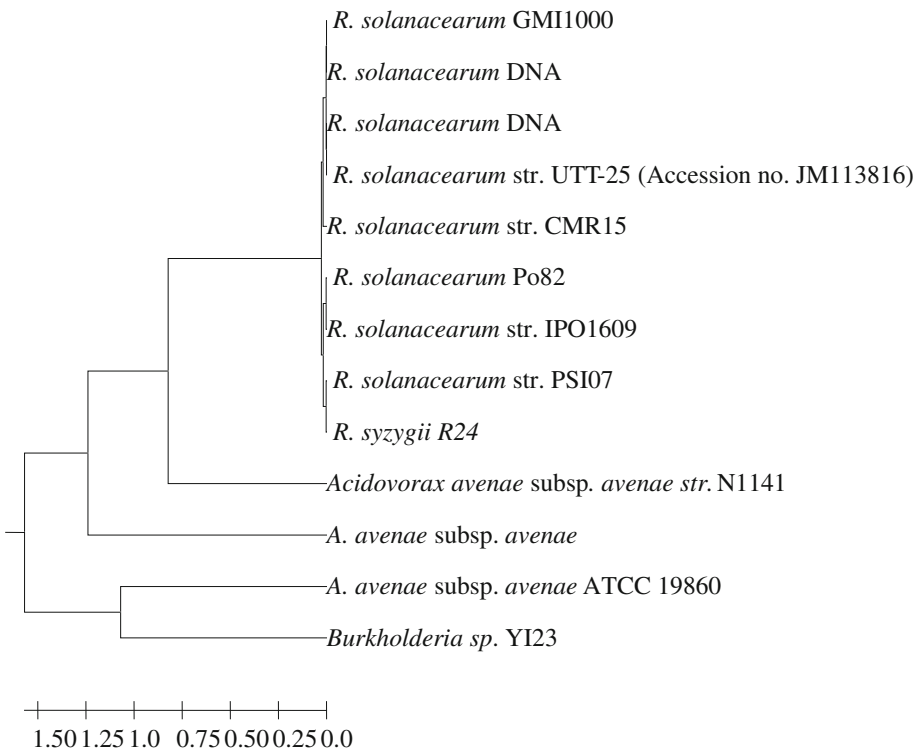

Fig. 1 Phylogenetic analysis based on *hrp* gene sequences of eight strains of *Ralstonia solanacearum* and one strain of *R. syzygii,* 3 strains of *Acidovorax avenae* ssp. *avenae,* and one strain of *Burkholderia* sp. showing the position with respect to related species available from NCBI database. There were a total of 306 positions in the final dataset. Evolutionary analyses were conducted in MEGA5

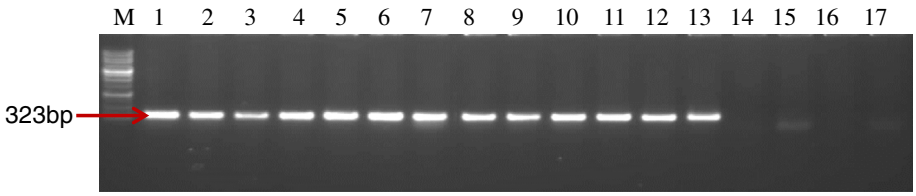

Fig. 2 Amplification of fragments of the *hrp* gene from *R. solanacearum* separated on an agarose gel showing the 323 bp. *Lane M*: 1 Kb ladder, lanes *1–13*: strains of *R. solanacearum* collected from different states (lanes *1–5*: Jharkhand, *6–7*: Jammu & Kashmir, *8–10*: Himachal Pradesh and *11–13*: Uttarakhand) *14*: *X. oryzae* pv. *oryzae*, *15*: *X. campestris* pv. *campestris*, *16*: *X. citri* subsp. *citri* and *17*: −ve control

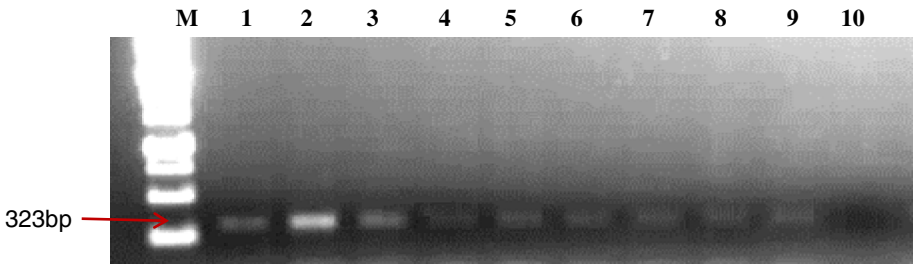

Fig. 3 Detection of *R. solanacearum* from different dilution by *hrp* gene-based primer amplified at 323 bp. *Lane M*: 1 Kb ladder, lanes *1*: 2 µl of bacterial suspension containing 20 × 108 cfu/ml, *2*: 5 µl of 20 × 108 cfu/ml, *3*: 20 × 107 cfu/ml, *4*:20 × 106 cfu/ml, *5*: 20 × 105 cfu/ml, *6*: 20 × 104 cfu/ml, *7*: 20 × 103 cfu/ml, *8*: 20 × 102 cfu/ml, *9*: 20 × 101 cfu/ml, *10*: 20 cfu/ml

Discussion

Bacterial wilt of tomato caused by *R. solanacearum,* race 1 biovar 3 & 4 is a serious problem in foot hills and coastal areas of India [16]. *R. solanacearum* survives in the soil, asymptomatic host, plant residue, which are sources of primary inoculum to cause the disease. For biochemical, colony characters, serological, and pathogenicity test have

Table 2 Detection of *Ralstonia solanacearum* from asymptomatic tomato plants, irrigation water, and soil using culture medium, conventional and BIO- PCR

States of India	Types of samples used	No. of samples tested	Detection of *R. solanacearum*		
			Samples showed +ve on TTC medium	Samples showed +ve in Conventional PCR	Samples showed +ve in BIO-PCR
Jharkhand	Asymptomatic plants	12	4 (33.3*)	7 (58.3)	11 (91.6)
	Irrigation water	6	2 (33.3)	4 (66.7)	5 (83.3)
	Rhizospheric soil	14	4 (28.6)	9 (64.2)	12 (85.7)
West Bengal	Asymptomatic plants	13	5 (38.5)	8 (61.5)	12 (92.3)
	Irrigation water	5	2 (38.5)	3 (60.0)	5 (100.0)
	Rhizospheric soil	11	5 (45.4)	8 (72.7)	11 (100.0)
Odisha	Asymptomatic plants	9	4 (44.4)	6 (66.7)	8 (88.9)
	Irrigation water	5	3 (60.0)	4 (80.0)	4 (80.0)
	Rhizospheric soil	9	4 (44.4)	6 (66.7)	8 (88.9)
Uttarakhand	Asymptomatic plants	21	6 (28.6)	16 (76.2)	19 (90.5)
	Irrigation water	7	3 (42.9)	4 (57.1)	6 (85.7)
	Rhizospheric soil	18	6 (33.3)	13 (77.7)	15 (83.3)
	Total	130	48 (36.9)	86 (66.2)	116 (89.2)
	Mean *t* test value*	39.26	67.28	89.18	

(t- critical two tail: 2.20)

Data showed in parenthesis are percent detection of *Ralstonia solanacearum* from samples

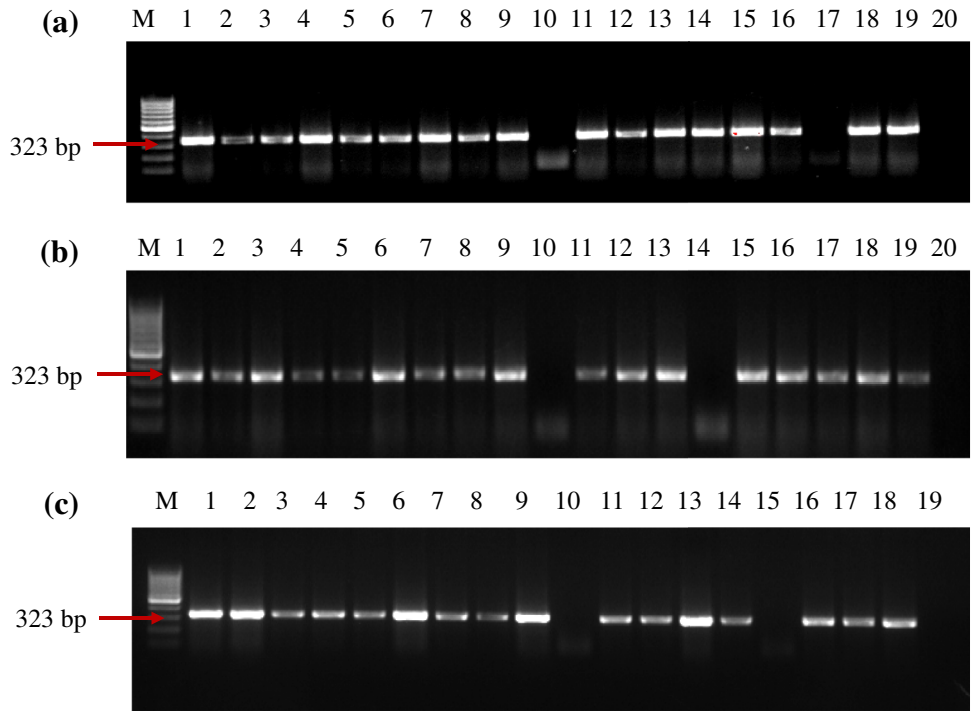

Fig. 4 Detection of *R. solanacearum* from asymptomatic plants, soil, and irrigation water coolecte from different states of India using *hrp* gene-based primer in BIO- PCR amplified at 323 bp. **a** Asymptomatic tomato plants, *Lane M*: 100 bp DNA ladder, lanes *1*: +ve control (DNA of *R. solanacearum*), *2–5*: Samples from Uttarakhand, *6–9*: Samples from West Bengal, *10*: −ve control, *11–15*: Samples from Odisha, *16–19*: Jharkhand, *20*: Healthy tomato plants. **b** Rhizopheric soil, *Lane M*: 100 bp DNA ladder, lanes *1*: +ve control (DNA of *R. solanacearum*), *2–5*: Soil samples from Uttarakhand, *6–9*: Soil samples from West Bengal, *10*: −ve control, *11–15*: Soil samples from Odisha, *16–19*: Soil samples from Jharkhand, *20*: rhizopheric soil of healthy plants. **c** Irrigation water, *Lane M*: 100 bp DNA ladder, lanes *1*: +ve control (DNA of *R. solanacearum*), *2–5*: Water samples from Uttarakhand, *6–9*: Water samples from West Bengal, *10*: −ve control, *11–15*: Water samples from Odisha, *16–18*: Water samples from Jharkhand, *19*: Sterilized water

been used earlier [13]. But these methods are more laborious and time consuming than nucleic acid-based PCR methods, which are now available for detection phytopathogenic bacteria including *R. solanacearum* from soil planting materials and seeds [2, 9, 13, 18]. A PCR method enhances sensitivity by virtue of the amplification reaction, enabling relatively low numbers of bacteria.

In this study, nucleotide sequences of *hrp* gene are used to design primer for PCR as earlier reported for detection of *X. campestris* pathovar in crucifers [2, 15]. The developed primers (Hrp_rs 2F and Hrp_rs 2R) are found specific to *R. solanacearum,* race 1 biovar 3 & 4. However, it could not be tested other races and biovars of *R. solanacearum*, however, it is tested in silico to analyze nucleotide sequences, that shows maximum similarity index with other races of *R. solanacearum* (Fig. 2). Moreover, this primer could not able to differentiate races and biovars of *R. solanacearum*. The primer is specific to only *R. solanacearum* strains may be due to the unique sequence of this pathogen. It is highly sensitive to detect 200 cfu/ml of *R. solanacearum*, it can be assumed that pathogen may be detectable by *hrp* gene-based primer and even before expression of wilt symptoms in tomato plants, because it has been reported that the regulatory network of *R. solanacearum* uses for virulence is activated only at cell densities of 10^7 cfu/ml [3].

In this study, three methods such cultural method using TTC medium, conventional PCR, and BIO-PCR were employed to detect the *R. solanacearum* from soil, irrigation water, and asymptomatic tomato plants. Detection level has been improved by increasing the population of *R. solanacearum* in CPG broth followed by PCR (BIO-PCR) to detect bacteria from soil, irrigation water, and asymptomatic plants and also to demonstrate the viable cells of bacteria followed by PCR detection [7] with *hrp* gene base specific primer. The method has been reported earlier for detection of *X. campestris* pv. *campestris* [15] and *R. solanacearum* [11] from various sources. In this study, CPG broth is used as enrichment medium for multiplication of *R. solanacearum,* while Pradhanang et al. [11] used SMSA broth from growth of the bacteria. CPG medium has advantages over SMSA medium that it is not inhibited the growth of bacteria due to the absence of antibiotics. The methods, are able to detect *R. solanacearum* from primary source inoculum like soil, water, and plants with enrichment steps definitely would be faster and have less PCR inhibitors particularly when bacterial DNA template is taken from soil suspension, bacterial ooze, and broth culture without DNA extraction. The Method of DNA extraction has affected the amplification of PCR products. The extraction of DNA of bacteria using 95 % ethanol is a simple and rapid method [5] and it can be combined with BIO- PCR to find better results.

In present study, the results clearly indicated the sensitivity of the primer pair Hrp_rs2F and Hrp_rs2R is improved in BIO-PCR using non-specific CPG broth medium for detection of *R. solanacearum* from asymptomatic tomato plants, soil, and irrigation water within 2 days with great accuracy. Non-specific bands are sometime observed in especially plant and soil samples and it may sometimes mislead the results.

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