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RESEARCH NOTE

Development of real-time loop-mediated isothermal amplification for detection of *Ralstonia pseudosolanacearum* race 4 in rhizomes and soil

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Abstract Bacterial wilt in ginger caused by *Ralstonia pseudosolanacearum* race 4 causes significant economic loss in several Asian and Polynesian countries. Since the pathogen being soil and rhizome borne, an early detection would prevent the occurrence and spread of the disease. The present study aimed at developing a strain specific detection methodology for race 4 strain infecting ginger using Real Time Loop Mediated Isothermal Amplification (real-time LAMP). LAMP primers were designed from the *gyrB* gene, which can specifically detect race 4 *R. pseudosolanacearum* infecting *Zingiberaceae* plants. In real-time LAMP, a sigmoid amplification curve with a Ta value of 92 ± 1 °C was obtained only with race 4 with a detection limit of 10^3 CFU/g of soil or rhizomes. For on-farm diagnosis, the protocol was customized with soil supernatant as template instead of genomic DNA, the extraction of which is cumbersome under field conditions. The real-time LAMP thus developed can be used as an indexing tool for seed rhizomes and soil for latent infection.

Keywords Bacterial wilt · Detection · Ginger · *Ralstonia solanacearum* race 4 · Real-time LAMP · Rhizome

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Ralstonia solanacearum and *Ralstonia pseudosolanacearum* cause bacterial wilt disease in more than 250 plant species belonging to 54 botanical families, including several monocot and dicots like tomato, potato, tobacco, chilli, eggplant, banana, and ginger (Hayward 1994). The pathogen is reported to be transmitted through latently infected planting material such as rhizomes, tubers and suckers (Janse et al. 2004; Pradhanang et al. 2000; Kumar and Hayward 2005; Kumar and Abraham 2008). Race 4 strains of *Ralstonia pseudosolanacearum* are aggressive on ginger and also capable of infecting crops such as small cardamom (*Elettaria cardamomum* Maton.), large cardamom (*Ammomum subulatum* Rox.), turmeric (*Curcuma longa* L.) and many other *Zingiberaceae* plants. (Kumar and Sarma 2004; Kumar et al. 2004, 2014). Ginger is highly prone to bacterial wilt caused by race 4 strains and the quality of planting material is often compromised for want of seed rhizome. Therefore, the risk of planting latently infected rhizome is very high, as evinced from repeated outbreaks of bacterial wilt in many ginger growing tracts, especially Kerala and north eastern states of India. Hence, the objective of our study was to develop a strain specific detection method for race 4 strains of *R. pseudosolanacearum* based on real-time LAMP. For this, pathogenicity and cross-infectivity of *R. pseudosolanacearum* strains were tested by the soil inoculation technique (Kumar 2006). *R. pseudosolanacearum* strains representing race 1 [ORP1 (Potato), UTB1 (Brinjal), TRs-Klm (Tomato)] and race 4 [GRs-Spr, GRs-Mnt5, GRs-Idk1 (Ginger), and CaRs-Mep3 (Small cardamom)] (Supplementary Table 1) were used for the study. All seven *R. pseudosolanacearum* strains representing race 1 and race 4 infected tomato plants within 5 to 10 days. But none of the race 1strains infected ginger, where it was infected only by race 4 strains in 6 to 11 days of inoculation (Supplementary Table 1).

For designing LAMP primers specific for race 4 strains, 1444 bp *gyrB* gene (coding for DNA gyrase B) sequences of

Table 1 Primers designed for race 4 strain specific detection of *R. pseudosolanacearum*

Primer name	Sequence (5′-3′)	Tm	GC (%)
RsGyrB F3	CAGCAGCCAGGTAATCGC	63.3	61.1
RsGyrB B3	GATGCCTTCATGCAGTACGA	62.5	50.0
RsGyrB FIP	GCAACGCTGTCGCAGACTAT GGAAATCCGTAACACCC	–	54.0
RsGyrB BIP	CCACGAAGTCGGCATCGACG AGAAGCACGAGAAGTAC	–	57.0
RsGyrB LOOP F	GGATAAGCAGCGGGAGCA	64.3	61.1
RsGyrB LOOP B	TGCGATAGGCGCTGGTTG	65.3	61.1

R. solanacearum and *R. pseudosolanacearum* retrieved from NCBI database were used. A total of 48 *R. solanacearum* and *R. pseudosolanacearum* strains representing race 1, race 3 and race 4, and also *R. pickettii, R. celebensis* and *R. mannitolytica* were analysed using the DNAsp programme (http://www.ub.es/dnasp) (Librado and Rozas 2009). The sequences were further aligned and analysed using the muscle alignment algorithm in Molecular Evolutionary Genetics Analysis V6 (MEGA 6) software (Tamura et al. 2013). Thus a set of six primers, (RsGyrB F3, RsGyrB B3, RsGyrB FIP, RsGyrB BIP, RsGyrB LOOPF, RsGyrB LOOPB) were designed from the *gyrB* gene region (Table 1) of race 4 strain, GRs-Tms, using the software LAMP Designer 1.12 from http://www.premierbiosoft.com.

The primers were validated initially by the normal LAMP. The reaction mixture (25 μl volume) contained 0.2 μM F3, B3, FIP and BIP primers; 1 μM LOOPF and LOOPB primers; 1.4 mM deoxyribonucleoside triphosphates (dNTPs); 6 mM $MgCl_2$; 0.8 M betaine; 1X Thermopol buffer (New England Biolabs, Inc., Beverly, MA); 8 U of *Bst* DNA polymerase large fragment (New England Biolabs, Inc., Beverly, MA) and 50 ng of template DNA. The reactions were carried out in 0.2 ml microtubes, using a thermal cycler for temperature control. The tubes were incubated at 65 °C for 75 min and termination of the reaction was achieved by heating to 80 °C for 10 min to denature the *Bst* polymerase. The LAMP reaction products were electrophoresed in a 2% agarose gel and visualized under UV light after ethidium bromide staining. The specificity of the primers were validated in LAMP reactions using total genomic DNA of *R. pseudosolanacearum* race 1 and race 3 strains as well as by using genomic DNA of *Pseudomonas aeruginosa* and *Bacillus subtilis* as outgroups. The LAMP reactions showed the characteristic ladder-like amplification pattern with only race 4 strains of *R. pseudosolanacearum. R. solanacearum* race 3, *R. pseudosolanacearum* race 1 as well as out groups viz. *P. aeruginosa* and *B. subtilis* did not amplify with the primers (Supplementary Figs. 1 and 2a).

The same primers were used in real-time LAMP reaction, in 25 μl volume containing 15 μl of isothermal master mix (AmpliGene India Biotech Pvt. Ltd., Ahmadabad, India); 2.5 μl of primer mix (0.2 μM of F3, B3, FIP and BIP and 1 μM of LOOPF and LOOPB); and 50 ng template DNA. The reactions were carried out in a Genei II (Optigene Ltd., UK) at 65 °C for 45 min, with an annealing curve analysis from 98 °C to 80 °C at 0.05 °C/s ramp. In real-time LAMP assessment using genomic DNA of race 4/ biovar 3 and race 4/biovar 4 strains produced a sigmoid curve, showing positive amplification within 20 min (Supplementary Fig. 2b). Annealing curve analysis showed amplification at an annealing temperature of 92 ± 1 °C. The race 1 and race 3 strains, *P. aeruginosa* and *B. subtilis* did not produce a sigmoid curve as with race 4 strains. (Fig. 1 and Supplementary Figs. 2b and 3).

The sensitivity of LAMP was determined with serially diluted genomic DNA and serial dilutions of bacterial cells as template. For genomic DNA, serial dilutions ranging from 50 ng to 5 fg were prepared in sterile milli-Q water, where it showed amplification up to 5 pg of genomic DNA. For bacterial cells, a single colony was serially diluted up to 10^{-10} in milli-Q water.

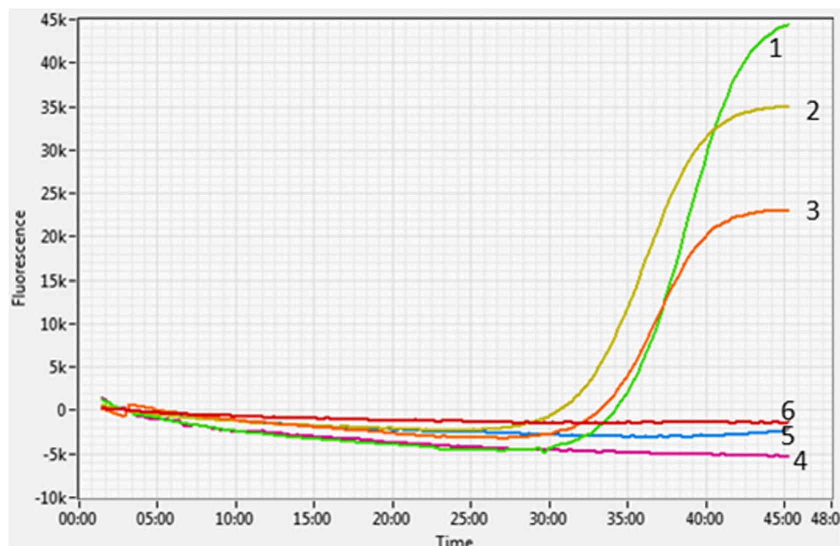

Fig. 1 Real-time LAMP showing primer specificity for race 4 strains of *R. pseudosolanacearum* in. Curve 1–3: race 4 strains from *Zingiberaceae* plants; Curve 4–6: race 1 strains from Solanaceaous vegetables

From each dilution, 100 µl were placed on selective medium SMSA to determine the number of viable cells of *R. pseudosolanacearum* in each dilution, (Engelbrecht 1994) and heated to 98 °C for 7 min to be used as template in real-time LAMP. Amplification was seen at 10^4 CFU ml^{-1} of *R. pseudosolanacearum* cells (Supplementary Figs. 4 and 5). A linear standard-curve was prepared by plotting amplification time with log cell concentration of *R. pseudosolanacearum,* as the amplification time depends on the population level (Supplementary Fig. 6). This standard curve can be used for deducing the number of *R. pseudosolanacearum* cells in the unknown samples.

Real-time LAMP protocol was standardized using purified DNA extracted from soil using a soil DNA Kit (HiPura, HiMedia Laboratories, Mumbai, India). However, while considering the cumbersome soil DNA isolation protocol and its impracticability under field situation, an attempt was made to use soil supernatant as template in a real-time LAMP detection. Briefly, one gram of infected soil was dispensed in 10 ml of sterile distilled water and vortexed at a maximum speed for 5 min. The tubes were allowed to stand still for about 30 min and one milliliter of supernatant was centrifuged at 6500×g for 1 min. at room temperature. From this one microliter was used as template for real-time LAMP. Positive amplification could be observed in soil supernatant obtained from soil artificially inoculated with bacterial cells. This experiment clearly indicated that the soil supernatant can be used for field level detection of *R. pseudosolanacearum* by real-time LAMP (Supplementary Fig. 7). Soil population counts of *R. pseudosolanacearum* on SMSA medium confirmed the presence of viable bacterial cells. The detection limit was found to be 3×10^3 CFU ml^{-1} of *R. pseudosolanacearum* from soil. Template preparation for ginger rhizomes was performed according to Weller et al. 2000, the central core of ginger rhizome was aseptically homogenized (0.1 g) in 1.0 ml of 50 mM phosphate buffer (pH 7.0). The resulting suspension (100 µl) was heated at 97 °C for 4 min and rapidly cooled on ice and diluted with 900 µl of sterile water. One microliter of this suspension served as template in the LAMP reaction. Positive amplification was observed from infected rhizomes up to 10^{-4} dilution which corresponds to 2×10^3 CFU ml^{-1} in SMSA plates (Supplementary Fig. 8). Real-time LAMP was also validated with soil and rhizomes collected from bacterial wilt endemic locations in Kerala. Among the seven samples tested, three field soil samples showed positive results, so also did the rhizomes collected from the endemic field (Supplementary Fig. 9).

So in conclusion the primers designed from *gyrB* sequence are specific for race 4/biovar 3 or race 4/biovar 4 strains of *Ralstonia pseudosolanacearum*. In order to simplify the detection protocol, soil extracts or rhizome extracts instead of soil or rhizome DNA was successfully used as template that not only circumvented the need for genomic DNA extraction, but also accelerated the detection assay where high throughput indexing of field samples is possible in an hour. Real-time LAMP-based detection developed in this study can be used for quality assessment of seed rhizomes for selection of disease free seed rhizomes for healthy crop. This protocol can be used as a quick on-farm diagnostic tool for pre-planting indexing of seed rhizomes.

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