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Research

Loop-Mediated Isothermal Amplification Assays for Rapid Detection of *Ralstonia solanacearum* Species Complex and Phylotype I in Solanaceous Crops

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Abstract

The *Ralstonia solanacearum* species complex (RSSC) causes bacterial wilt diseases, which affect a wide range of plant hosts, including Solanaceae, and is of major economic importance. Point-of-care (POC) techniques such as loop-mediated isothermal amplification (LAMP) enable the rapid and sensitive detection of plant pathogens and can be deployed directly in the field. For fast and reliable diagnosis on site, we optimized an RSSC-LAMP assay and developed a real-time LAMP assay targeting the phylotype I. Both LAMPs were highly specific, yielding negative results for a wide collection of nontarget strains and positive results for all target strains, except for two particular mulberry strains for the phylotype I assay. These results were supported by an extensive in silico analysis performed on 6,105 genomes of *Burkholderiaceae*. The two LAMP assays displayed high sensitivity on pure suspensions, with a detection limit at 10^3 and 10^4 CFU/ml for phylotype I and the RSSC, respectively. These thresholds correspond to theoretical quantities of as low as 5 and 50 CFU per reaction. Two simplified extraction methods were successfully used to detect the pathogen from different Solanaceae samples. We demonstrated that LAMP assays were operable on solanaceous crops during field surveys and varietal evaluation trials as rapid POC diagnostics tools, which can improve the disease management and control of the RSSC.

Keywords: detection, diagnosis, genomes, LAMP, phylotype I, point-of-care testing, *Ralstonia solanacearum* species complex, simplified extraction method

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Timely detection and accurate identification of plant pathogens are key for sustainable disease management in agro-ecosystems in our globalized world (Bull and Koike 2015; Miller et al. 2009; Paini et al. 2016). This is crucial for achieving food security (Rizzo et al. 2021; Savary et al. 2019) and tackling the environmental consequences of pest

introductions (Hulme 2009). Early and efficient diagnostics will be even more critical in pesticide-free agricultural systems in the future (Jacquet et al. 2022). Over the last decade or so, the significant increase in the availability of complete genome sequencing for pathogens, including plant pathogens, has greatly improved their characterization. This is the first step toward a genomics-informed global surveillance of pathogens (Gardy and Loman 2018). In the field of molecular detection of pathogens, the availability of genomic resource data has facilitated the design of highly specific PCR diagnostic assays, which are also very sensitive when used in real-time PCR format. However, samples must be transported to a suitable laboratory for analysis. New isothermal DNA polymerases have allowed for the development of point-of-care (POC) diagnosis assays, such as loop-mediated isothermal amplification (LAMP), which do not require sophisticated equipment and can be used on site. These techniques are developing rapidly in human, veterinary, and plant health (Becherer et al. 2020; Donoso and Valenzuela 2018; Le and Vu 2017). The LAMP technique, first described by Notomi (2000), amplifies DNA under isothermal conditions using a strand-displacing polymerase, typically *Bst* polymerase. The LAMP assay requires a set of four primers (regular primers), which recognize six distinct regions of the target DNA. Two supplementary primers (Loop primers) can be designed to speed up the reaction. The LAMP assay's major advantages over the gold-standard PCR method are described in the literature (Soroka et al. 2021). Its sensitivity is equivalent to that of real-time PCR, it has greater tolerance to PCR inhibitors, and, therefore, it can be used after a very simplified nucleic acid extraction suitable for on-site detection (Hassan and Than 2020).

The *Ralstonia solanacearum* species complex (RSSC) includes highly diverse strains that are globally widespread. They cause bacterial wilt (BW) disease on an unusually broad plant host range, which has major social and economic effects (Mansfield et al. 2012). The RSSC was classified into four major phylogenetic groups from different geographical origins (phylotypes) based on phylogenetic analyses from the 16S-23S internal transcribed spacer (ITS) region. Phylotype I originated from Asia, II from the Americas, III from Africa and the Indian Ocean, and IV from Australia, Indonesia, and Japan (Fegan and Prior 2005). Phylotype II has two clearly distinctive branches, A and B. A taxonomic revision (Prior et al. 2016; Safni et al. 2014) structured this group into three species: *Ralstonia pseudosolanacearum* (including phylotypes I and III), *Ralstonia solanacearum* (phylotype II), and *Ralstonia syzygii* (phylotype IV). Sequevars are subdivisions of phylotypes based on the sequence variation of the endoglucanase gene *egl* (Fegan and Prior 2005). Several major Solanaceae crops, such as potato, eggplant, tomato, and pepper, are severely impacted by BW disease worldwide. Phylotype I strains have a wide host range, including herbaceous and woody plants, and can be found all over the world (Hayward 1994). They are the most prevalent strains in the Southwest Indian Ocean region (Yahiaoui et al. 2017). Phylotype I has broad intraspecific diversity and comprises 21 of the 71 sequevars that have been identified so far. Recently, exchanges of infected *Rosa* cuttings were probably responsible for the introduction of phylotype I strains in *Rosa* cut flower production units in the Netherlands, Austria, Belgium, Germany, Poland, Portugal, and Switzerland (Tjou-Tam-Sin et al. 2017). Effective biosecurity surveillance and disease control depends on the use of appropriate and reliable diagnostic methods. Numerous diagnostic tools are already available for detecting RSSC. These include DNA-based techniques, such as conventional PCR or real-time quantitative PCR. The latter can identify the RSSC at the species level (Glick et al. 2002; Lee and Wang 2000; Opina et al. 1997; Schönfeld et al. 2003; Seal et al. 1993; Weller et al. 2000) and phylotype level (Fegan and Prior 2005), as well as target some important groups of strains, such as brown rot or Moko strains (Cellier et al. 2015; Ozakman and Schaad 2003; Prior and Fegan 2005). A further technique involves a diagnostic microarray (Cellier et al. 2017), which characterizes 17 major groups of interest in the RSSC in a single multiplex reaction.

The objective of this study was to develop LAMP assays to diagnose the presence of the RSSC on site and identify phylotype I, which is the most prevalent lineage in BW epidemics in tropical and subtropical environments and is sometimes associated with severe outbreaks in temperate regions (Fegan and Prior 2005; Tjou-Tam-Sin et al. 2017). Several LAMP protocols detecting the RSSC have been reported (Kubota et al. 2008; Lenarčič et al. 2014; Okiro et al. 2019). However, the LAMP assay developed in Kubota et al. (2008), targeting *fliC*, failed to detect some economically important RSSC strains. The LAMP in Lenarčič et al. (2014) based on *egl* reacted positively with healthy potatoes. In contrast, the LAMP assay in Okiro et al. (2019) targeting a putative UDP-3-O-acyl-GlcNAc deacetylase (*lpxC*) gene was demonstrated to be very specific to the RSSC and sensitive. It detected RSSC DNA at low concentrations (2.5 pg/µl). In this study, we evaluated and adapted this LAMP technique for real-time visualization using a portable device. We compared it with the published RSSC LAMP protocols (Kubota et al. 2008; Lenarčič et al. 2014). Furthermore, we developed a new LAMP assay specifically targeting phylotype I strains, which could be useful for the surveillance of this major lineage worldwide. Lastly, we assessed these two assays for in-field diagnostics.

MATERIALS AND METHODS

Bacterial strains

RSSC strains belonging to different phylotypes ($n = 82$), including 25 phylotype I strains and strains belonging to other species or genera ($n = 35$), were used in this study (Supplementary Table S1). The strains were conserved at CIRAD (French Agricultural Research Centre for International Development) at −80°C on cryobeads (Microbank, Prolabs Diagnostics, Toronto, Canada). Bacteria were cultured in nutrient broth (Condalab, Madrid, Spain) overnight at 28°C under constant agitation at 150 rpm. The RSSC strains were streaked onto semi-selective modified Granada and Sequeira medium (Poussier et al. 1999) and then incubated for 48 h at 28°C. The non-RSSC strains were streaked on YPGA medium containing agar (18 g/liter), yeast extract (7 g/liter), peptone (7 g/liter), and glucose (7 g/liter) and then incubated for 48 h at 28°C.

LAMP primer design

LAMP primers were designed using the Primer explorer V5 software (primerexplorer.jp/lampv5e/index.html) and its advanced primer design function for common primers in the multi alignment of DNA sequences showing polymorphism. The target DNA selected for the detection of phylotype I strains was a gene encoding for an NAD(P)-dependent oxidoreductase (locus_tag = RS_RS15835 for GMI1000 reference sequence), selected by Cellier et al. (2017) to characterize phylotype I strains in a diagnostic microarray. LAMP primers common to all the sequences were designed from an alignment of 20 genomic sequences of *Ralstonia pseudosolanacearum* phylotype I strains available in NCBI data (Supplementary Table S2). The selected phylotype I LAMP primer set is listed in Table 1.

LAMP reactions

Endpoint visualization. The lpxC LAMP assay was tested using hydroxy naphthol blue according to the published protocol (Okiro et al. 2019). The LAMP primers are listed in Table 1.

Real-time LAMP assays. LAMP reactions were performed with a portable device (Genie II, OptiGene, Horsham, U.K.), in a 25 µl total reaction volume, containing 15 µl of ISO-DR004 Isothermal Mastermix (OptiGene) or Lyse-n-LAMP (LNL) Mastermix (OptiGene), with 2.5 µl of pre-primer mix, giving final concentrations of 0.2 µM each of F3 and B3 primers, 0.8 µM each of FIP and BIP primers, 0.4 µM of each Loop primer, and 5 µl of template DNA. LAMP reactions were run at 65°C for a period of 30 min, followed by an anneal step with temperatures varying from 98 to 80°C at a speed of 0.05°C/s. Amplification curves (associated with a time-to-result [TTR] value) and annealing temperature (Ta) peaks were generated during the LAMP reaction. A sample was considered positive if TTR < 30 and if Ta peaks reached a threshold value of fluorescence (1,500) and displayed a specific temperature: 92.5°C < Ta < 94°C for Okiro and 89°C < Ta < 91°C for the Phyl-I LAMP assay.

Simplified DNA extraction protocols

A quick alkaline extraction method (NaOH method) modified from Wang et al. (1993) and the LNL protocol (Optigene), which includes both a lysis step and the LAMP reaction, were compared on several types of material: (i) healthy plant tissues spiked with dilution series of the RSSC and (ii) artificially infected stems from 5-week-old eggplants (*Solanum melongena*) inoculated with a 10^8 CFU/ml suspension of the RUN3012 strain by root scarification, as described in Salgon et al. (2017), and analyzed 35 days after inoculation. For (ii), detection of the RSSC was performed for each plant by soaking a part of the stem for 10 min in 0.01 M Tris buffer (pH 7.2) and plating 50 µl on Kelman's tetrazolium chloride agar medium (Kelman 1954). To compare extraction methods, the adjacent infected portions of stems were previously homogenized by dilaceration and separated in two to test the different simplified extractions.

NaOH method. About 100 mg of healthy stem spiked with the appropriate bacterial concentration (see the *S*ensitivity section below) (i) or 100 mg of infected stem (ii) was macerated for 10 min in 1 ml of freshly prepared 0.5 M NaOH. Five microliters of the homogenate was then removed and diluted with 195 µl of 100 mM Tris (pH 8.0). This homogenate was then used immediately for the LAMP assay with the ISO-DR004 Isothermal Mastermix.

LNL method. About 100 mg of healthy stem spiked with the appropriate bacterial concentration (see the *S*ensitivity section below) (i) or 100 mg of infected stem (ii) was macerated for 10 min in 1 ml of 100 mM Tris buffer (pH 7.2); an aliquot (100 µl) of the macerate was then added to an equal volume of 0.6 M KOH, as recommended by the supplier, and 5 µl was used for the LAMP using the Mastermix LNL.

Specificity

All publicly available genome assemblies belonging to *Burkholderiaceae* were obtained from the NCBI GeneBank database on June 20, 2022. An additional 119 unpublished genome assemblies generated by our laboratory were added for the in silico analysis. All these assemblies were filtered using BUSCO (version 5.2.2) for their completeness (>95%) and duplication level (<10%). We finally retained 6,105 genomes (Supplementary Table S3). Homologous sequences were identified by BLASTN (version 2.10.1+) and extracted using BEDTools getfasta (version 2-2.29.0). For each of the two genomic regions targeted by the two LAMP systems, a list of unique allelic variants (UDP and NAD variants for lpxC and Phyl-I targets, respectively) was defined by removing redundant sequences using SeqKit rmdup (version 0.16.0) and aligned using MUSCLE (version 3.8.425) (Supplementary Figs. S1 and S2; Supplementary Table S3). Full targets and LAMP primer specificity were defined from this alignment. For the Phyl-I LAMP targeted sequence and primer set, the analysis was performed on 160 genomes identified as phylotype I; 213 *Ralstonia* spp. genomes belonging to the three other phylotypes; 5,732 nontarget strains, including strains belonging to other species of *Ralstonia* ($n = 102$); and other genera belonging to the *Burkholderiaceae* family. For the lpxC LAMP primer set, the analysis was conducted on 593 RSSC target genomes and 5,512 nontarget genomes.

Analytical specificity was evaluated on bacterial suspensions of target and nontarget strains following the guidelines in the EPPO PM 7/98 standard and using two criteria: inclusivity (i.e., the ability of the LAMP systems to detect all strains of the target organism) and exclusivity (i.e., the capacity to generate negative results from nontarget strains) (Anonymous 2019).

LpxC LAMP primers were tested in duplicate in a colorimetric assay as described in Okiro et al. (2019) on several target strains

TABLE 1

LAMP primers used in this study

Primer	Specificity	Sequence 5′-3′	DNA target	Reference
	Phylotype I		NAD(P)-dependent oxidoreductase	This study
Phyl-I_F3		TGTCAAGCATGTCAGGATGG		
Phyl-I_B3		CAACGCTTGTCCACCGTG		
Phyl-I_FIP		TCTTGTTGAATGGCCGACGACTGCAGTTGTCGATGACGTTTG		
Phyl-I_BIP		AACGCCTGGCTCTTTCAGCCTTCGAAAGGCCGGCAAAG		
Phyl-I_LB		CATCGCGATCGTTCGTCAG		
Phyl-I_ST		CGGTTTGAAAGCTCTCCAGCA		
	Ralstonia solanacearum species complex		UDP-3- O-acyl-GlcNAc deacetylase	Okiro et al. 2019
lpxC_F3		GCTACACCCGCGAAATCG		
lpxC_B3		AGCGGATAGCCGACCAC		
lpxC_FIP		ACGATCGCGTTGTCCAGGCTGCACCTTCGGCTTTGCCCA		
lpxC_BIP		ACGAGCACCGCATGCTGAACGCGTCCAGAATCTTGTGG		
lpxC_LF		TCCCGCAGCATCTCGACCTC		
lpxC_LB		CGATGAACTGCGCTATGGC		

belonging to different RSSC phylotypes ($n = 14$) and nontarget strains ($n = 8$) (DNA adjusted to 10 ng/µl) (Supplementary Table S1). LpxC LAMP primers were also evaluated in a real-time LAMP assay on bacterial suspensions of target strains belonging to the different RSSC phylotypes ($n = 28$) and non-RSSC strains ($n = 35$). (Supplementary Table S1). The LAMP assay was performed in duplicate or in triplicate for closely related nontarget strains. Bacterial suspensions were prepared from 24-h-old cultures at 28°C. Suspensions were spectrophotometrically adjusted to 10^7 CFU/ml in 0.01 M Tris buffer (pH 7.2). The suspensions were heated at 100°C for 2 min in a dry bath and chilled on ice for 2 min. DNA at 1 ng/µl was used for the biohazard group 2 strains LMG21510, LMG3244, and LMG16656 instead of bacterial suspensions. The PhylI-specific LAMP assay was evaluated on 16 outgroup strains, in addition to 25 phylotype I strains belonging to different sequevars, and strains from other phylotypes ($n = 53$).

Sensitivity

The sensitivity of the real-time LAMP assays was assayed for (i) pure bacterial suspensions and (ii) bacteria diluted in plant matrices. For (i), bacterial suspensions from overnight cultures on modified Granada and Sequeira plates were adjusted spectrophotometrically to a concentration of 1×10^8 CFU/ml and serially diluted in 0.01 M Tris buffer (pH 7.2). The negative control received only Tris buffer. The different suspensions were heated as described above. Before boiling, bacterial cell concentration was checked by plating 50 µl of the 10^4 dilution on modified Granada and Sequeira. Sensitivity was verified on dilution series of both RUN312 (phylotype I) and UW 551 (IIB-1) for the colorimetric and real-time lpxC LAMP and on dilution series of three phylotype I strains (RUN312, RUN320, and RUN4267) for the Phyl-I specific LAMP. For (ii), dilution series of the three phylotype I strains were mixed with eggplant (*Solanum melongena*) or tomato (*Solanum lycopersicum*) material, extracted as described in the "Simplified DNA extraction protocols" section, and tested with the Phyl-I specific LAMP assay. The same plant extracts spiked with the RUN312 strain were also tested with the real-time lpxC LAMP. Extracts obtained in the same way from healthy potato material (*Solanum tuberosum*) spiked with the UW551 strain (IIB-1) were also tested using the same technique. Bacterial concentrations ranging from 1×10^6 to 1×10^2 CFU/ml were tested in triplicate using real-time LAMP assays for both pure suspensions and plant mixtures. Higher concentrations were tested when using the colorimetric lpxC LAMP, starting from 1×10^8 CFU/ml (RUN312) or 1×10^7 CFU/ml (RUN449) because it was less sensitive than real-time LAMP assays.

Comparison of the real-time lpxC LAMP assay to other RSSC-specific real-time LAMP assays

The specificity and sensitivity of the real-time lpxC LAMP assay were compared with the previous LAMP assay targeting *fli*C developed by Kubota et al. (2008) and optimized for real-time performance by Lenarčič et al. (2014), as well as the LAMP assay targeting *egl* developed by Lenarčič et al. (2014). Specificity was evaluated on a set of target ($n = 28$) and nontarget strain ($n = 35$) suspensions adjusted to 10^7 CFU/ml or 1 ng/µl (cf. § Specificity). The sensitivity of the three assays was compared on tenfold dilutions of total DNA (from 1 ng/µl to 1fg/µl) extracted from 24-h-old cultures grown on semi-selective modified Granada and Sequeira medium (Poussier et al. 1999) using the DNeasy Blood & Tissue kit (Qiagen, Courtaboeuf, France), according to the supplier's instructions.

Tests on infected plant material

The Phyl-I LAMP assay was also tested on site on naturally infected tomato plants (*Solanum lycopersicum*) in Réunion ($n = 21$) and on eggplants (*Solanum melonga* and *S. linneanum*), tomato (*Solanum lycopersicum*), and pepper (*Capsicum* spp.) cultivated varieties (the Core-TEP population) (Lebeau et al. 2011) in different plots in Mayotte. In this latter assay, the behavior of 11 accessions for each of the three plants was evaluated in plots naturally infected by phylotype I strains, and the Phyl-I LAMP assay was tested for its feasibility and reliability for rapid, in-field phenotyping of BW resistance. Initially, 99 plants (nine plants per accession) were grown for each plant species, but finally, 96, 87, and 76 plants could be analyzed for eggplant, tomato, and pepper, respectively, because of the premature death of 34 plants and two missing data for plating. Disease development was visually assessed weekly by scoring each plant as asymptomatic (no symptoms), wilted (at least one leaf wilted), or dead (all leaves wilted). The Phyl-I LAMP assay, plating technique, and multiplex PCR (Fegan and Prior 2005) were compared in their ability to detect phylotype I strains in the different accessions on symptomatic or asymptomatic plants. In both surveys, LAMP assays were performed using a portable device (Genie II, OptiGene) directly set up at the edge of the plot. Sections were cut from the bottom of the stem. One section was used for the LAMP and PCR tests after simplified extraction with NaOH, and the other was used for plating. Both sequevar determination and verification of doubtful colonies were performed with the *egl* typing method (Fegan and Prior 2005).

The two LAMP assays were also evaluated on macerates from naturally infected plants sampled from 34 plots with different Solanaceae species during a survey in the south of Madagascar. The samples for analysis were received as macerates in water (about 100 mg of stem portion soaked in water for 10 min). The macerates were plated on Kelman's tetrazolium chloride agar medium, and the plates were screened for typical RSSC colonies after 48 h of growth. Multiplex PCR (Fegan and Prior 2005) was used to type suspected colonies. First, the real-time lpxC LAMP was performed on 46 samples chosen from the different plots. The macerates were extracted and tested using the KOH/LNL LAMP method. When a plot tested positive using the lpxC LAMP, all samples from that plot were tested using the real-time lpxC LAMP, the Phyl-I LAMP, and the multiplex PCR (a total of 75 samples). The latter was directly applied on the macerates (preliminary PCR tests performed on KOH extracts showed less sensitivity; data not shown).

Statistics

All statistical analyses, including ANOVA, Fisher test, previous verification of data normality (histogram, normal QQ plot), Spearman's rank correlation, and the McNemar test on paired binary values, were performed using the R statistical software (version 4.1.1 [2021-08-10]; R Development Core Team, Vienna, Austria) with the packages stats, multcomp, emmeans, and ggplot2.

RESULTS

RSSC endpoint colorimetric LAMP assay (Okiro et al. 2019) and its real-time version

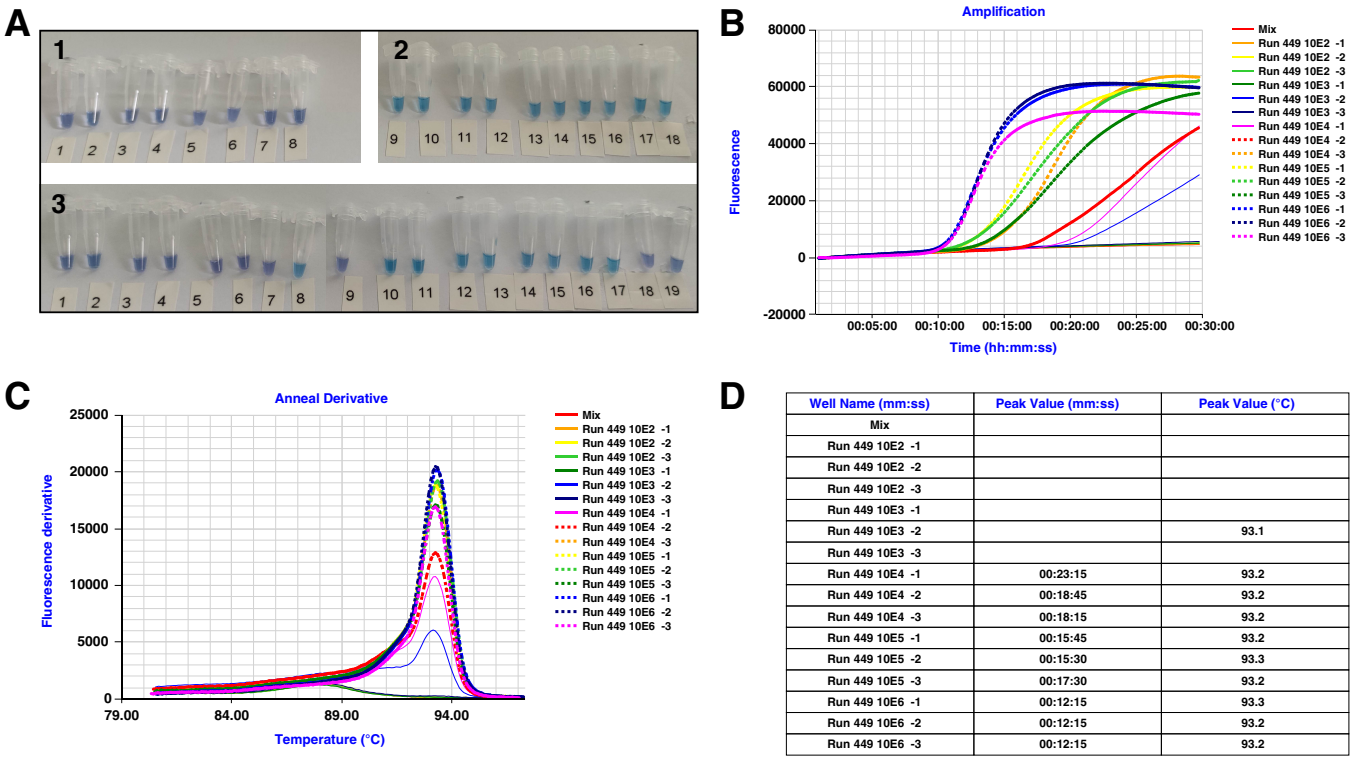
The colorimetric lpxC LAMP assay tested positive for the 14 target strains from different phylotypes and negative for the eight nontarget strains, including bacteria from the same genus (*Ral-*

stonia) or closely related genera. These results are consistent with those shown by the authors (Fig. 1A1-2; Supplementary Table S1).

The sensitivity evaluated on pure suspensions of RUN449 (phylotype IIB-1) and RUN312 (phylotype I), serially diluted in Tris buffer, was quite low, with a limit of detection (LOD, which corresponds here to obtaining 100% positive signals) of 10^6 and 10^7 CFU/ml, respectively (Fig. 1A3; Supplementary Fig. S3). The protocol was then adapted to a real-time visualization for on-site detection with the portable device Genie II (OptiGene) (Fig. 1B to D).

The in silico specificity was verified on a wide collection of target and nontarget genomes. Regions homologous to the lpxC primers, with percent identity ranging from 94.1 to 100%, were detected from all available 593 RSSC genomes. They were distributed in 34 allelic variants (UDP_V01 to UDP_V34) (Supplementary Table S3 and Fig. S1). The highest homology was found for the phylotype IIB-1 strains, which is consistent because the primers were designed from a phylotype IIB-1 strain (Okiro et al. 2019). One genome of *R. solanacearum* downloaded from NCBI (GCA_001065525.1) showed a low match score (86.36%). This was a clinical strain isolated from human material, which might have been misclassified. Among the 5,512 nontarget genomes, 341 did not match with the primers, 5,145 displayed identity <90% (from 68.64 to 89.55%, with a median value of 83.64%), and 26 showed identity ranging from 90 to 91.37%. The latter genomes belonged to *Cupriavidus* spp., *Ralstonia mannitolilytica*, and *Ralstonia pickettii* (UDP_V035 to UDP_V046). For these strains, some mutations accumulated in all the different primers, of which some were located in regions that are important for the LAMP reaction to operate (e.g., in the 3′ terminal of FIP primer's F2) (Supplementary Fig. S1). The specificity was experimentally verified on different target (n = 28) and nontarget strains (n = 35), especially including strains representative of the UDP_V035 to UDP_V046 and other strains belonging to the genera *Cupriavidus*, *Ralstonia*, and *Burkholderia* (Supplementary Table S1). All target strains were detected, with TTR ranging from 9.75 to 15.50 (median of 11.50) and a Ta range of 92.8 to 93.4 (100% inclusivity) (Supplementary Table S1). High specificity was confirmed when assaying the nontarget strains. Some very late amplification signals (TTR ranging from 23.75 to 29.75, median of 29.75) were obtained for LMG31391 (*Cupriavidus lacunae*), LMG21510 (*C. respiraculi*), LMG3244 (*C. pauculus*), LMG21421, and LMG18321 (*Ralstonia insidiosa*) (Supplementary Table S1). Nevertheless, these low signals were not reproducible for *Ralstonia insidiosa* strains (one out of three replicates) and were associated with Ta values out of the expected range (91.6 to 92.1) for *C. respiraculi*, *C. pauculus*, and *Ralstonia insidiosa* strains. For *C. lacunae*, no Ta value was associated with amplification for one out of three replicates.

Regarding the analytical sensitivity of the real-time lpxC LAMP assay, 100% positive results were obtained for pure suspensions of the two strains in Tris buffer with concentrations $\geq 10^4$ CFU/ml. This is a significant improvement compared with the colorimetric lamp assay (Fig. 1B to D; Table 2; Supplementary Fig. S3).



D

Well Name (mm:ss)	Peak Value (mm:ss)	Peak Value (°C)
Mix		
Run 449 10E2 -1		
Run 449 10E2 -2		
Run 449 10E2 -3		
Run 449 10E3 -1		
Run 449 10E3 -2		93.1
Run 449 10E3 -3		
Run 449 10E4 -1	00:23:15	93.2
Run 449 10E4 -2	00:18:45	93.2
Run 449 10E4 -3	00:18:15	93.2
Run 449 10E5 -1	00:15:45	93.2
Run 449 10E5 -2	00:15:30	93.3
Run 449 10E5 -3	00:17:30	93.2
Run 449 10E6 -1	00:12:15	93.3
Run 449 10E6 -2	00:12:15	93.2
Run 449 10E6 -3	00:12:15	93.2

FIGURE 1

Colorimetric and real-time lpxC LAMP assays. **A,** Colorimetric lpxC assay. Negative tests are purple, positive are blue. **1,** Tests on nontarget DNA (10 ng/µl): tubes 1-2: LMG5942; 3-4: LMG1199; 5-6: LMG5093; 7-8: LMG1368. **2,** Tests on target DNA (10 ng/µl): tubes 9-10: RUN17; 11-12: RUN1312; 13-14: RUN24; 15-16: RUN9; 17-18: RUN62. **3,** Serial dilutions of RUN449: tubes 1-3: 10^3 CFU/ml; 4-6: 10^4 CFU/ml; 7-9: 10^5 CFU/ml; 10-12: 10^6 CFU/ml; 13-15: 10^7 CFU/ml; 16-17: RUN60 (10 ng/µl); 18-19: mix (negative control). **B, C, and D,** Real-time lpxC assay. B, Amplification plots of tenfold dilutions of RUN449 from 10^2 to 10^6 CFU/ml, three replicates per dilution level, and a negative control (mix). C, Annealing peaks. D, Numeric results: Peak values (mm:ss) = time to result; Peak values (°C) = annealing (or Ta) temperatures.

Comparison of the real-time lpxC LAMP assay to other RSSC real-time LAMP assays

Tables 3 and 4 summarize the comparative analysis of the specificity and sensitivity of the real-time lpxC LAMP, fliC LAMP, and egl LAMP assays. Only the lpxC LAMP assay showed perfect inclusivity, with a positive signal for all the 28 RSSC strains. Conversely, the fliC LAMP and egl LAMP did not test positive for some RSSC strains, mainly those belonging to phylotype IV (Table 3; Supplementary Table S1). In addition, very late signals were obtained for some strains tested with these alternative real-time LAMP assays, especially the egl LAMP. Moreover, for this latter test, the Ta values were heterogeneous with a range higher than 2°C, supporting the results found in Lenarčič et al. (2014). Regarding the sensitivity, lpxC LAMP and egl LAMP showed the same LOD of 0.001 ng/µl when testing the phylotype I strain RUN312, compared with an LOD of 0.01 ng/µl for fliC LAMP. An LOD as low as 0.0001 ng/µl was obtained with the lpxC LAMP assay tested on strain IIB-1 RUN 449, which was 10 and 1,000 times lower than for the fliC and egl LAMP assays, respectively.

Design of phyl-I-specific primer set, in silico analysis, and performance on pure bacterial cultures

Several LAMP primer sets were designed from an alignment of 20 genomic sequences of phylotype I strains (Supplementary Table S2). They were screened on a few strains of phylotype I and nontarget strains (RUN4266, RUN304, LMG2129, LMG1222, RUN1367, RUN1368, and RUN1369). The primer set that combined the best signal precocity and specificity was selected for further analyses (data not shown).

The selected Phyl-I-specific LAMP primer set is listed in Table 1. It includes a Stem primer, located in the "Stem" part of the system, between the F1 and B1 regions, rather than the Loop primer LF as described in Gandelman et al. (2011) (Supplementary Fig. S4).

The in silico analysis showed that the Phyl-I-specific LAMP primer set matched with all the sequence genomes of phylotype I except one, with percent identity ranging from 98.80 to 100% (n = 159 positive results, distributed in 11 allelic variants NAD_V1-11) (Supplementary Fig. S2 and Table S3). The only genome sequence displaying a negative signal (GCA_021229115.1) belongs to sequevar 12 from China (Supplementary Table S3). No significant similarity was found when blasting the Phyl-I-specific LAMP primer set on the nontarget genome collection (213 other RSSC phylotypes, 102 other *Ralstonia* species, and 5,410 other *Burkholderiaceae* bacteria).

With regard to the analytical specificity, all but two of the target strains tested positive ($n = 23$), with specific annealing temperature peaks ranging between 89.9 and 90.6 (Supplementary Table S1). The two strains that responded negatively belong to sequevar 12 from China. All nontarget strains tested negative ($n = 69$) with the Phyl-I LAMP set (Supplementary Table S1).

Sensitivity was evaluated on tenfold dilution series of three phylotype I strains (RUN312, RUN320, and RUN4267). One hundred percent of the results obtained were positive for bacterial concentration $\geq 10^3$ CFU/ml for the three strains (Table 2).

FIGURE 2
The LAMP being implemented in the field (photo credit: Adrien Rieux).

Evaluation of simplified DNA extraction protocols and sensitivity of the LAMP assays in plant matrices

The two simplified extraction methods yielded amplification on the different plant matrices artificially spiked with phylotype IIB-1 and phylotype I strains. One hundred percent of the results were positive for bacterial concentrations ranging from 10^3 to 10^5 CFU/ml, depending on the strain and the matrix used (tomato, eggplant, or potato) (Table 2). The LOD values obtained with the KOH/LNL extraction were equal to or better than the results with NaOH extraction. For the specific Phyl-I LAMP assay, the detection limit was 10^4 CFU/ml or even 10^3 CFU/ml with the KOH/LNL extraction method, whereas it could reach 10^5 CFU/ml with the NaOH extraction method (Supplementary Fig. S5). However, a variance analysis followed by a Fisher test performed on the whole data set showed no significant differences between TTR values obtained for KOH/LNL and NaOH extraction but revealed a significant difference between TTR values for NaOH extraction compared with pure suspensions (P = 0.01783). Another significant result was found between the Phyl-I LAMP assay and the lpxC LAMP assay ($P = 00.0002819$): The TTR values were generated earlier with the Phyl-I LAMP assay. No significant effect of the plant matrix was evidenced on these data ($P = 0.06995$), although plant matrices, particularly tomato, showed slightly higher TTR values compared with the suspensions from pure cultures.

The two simplified methods were further evaluated on artificially inoculated eggplants, 35 days after inoculation (Table 5; Supplementary Table S4). Healthy plants tested negative irrespective of the extraction method used. All the symptomatic eggplants tested positive using the Phyl-I-specific LAMP assay with both extraction methods. Among the 14 asymptomatic plants from which typical RSSC colonies were isolated, 13 tested positive with the Phyl-I-specific LAMP assay (eight samples extracted with both methods, three samples extracted with the KOH/LNL method, and two samples extracted with the NaOH

method). The Phyl-I-specific LAMP assay tested positive on some plants that did not yield any *Ralstonia*-like colonies on plating (particularly using the KOH/LNL method). Moreover, the TTR values were generated earlier overall when using the KOH/LNL method. The two methods yielded concordant results for 79% of the samples. We found no significant difference between the two extraction methods ($P = 0.289$, McNemar test on paired binary values). Some of the samples ($n = 26$) were also tested with the real-time lpxC LAMP assay. The results showed 100% concordance with the Phyl-I-specific LAMP assay (Supplementary Table S4).

Applying the LAMP assays to detect RSSC strains from field samples

Several experiments were performed to demonstrate the pertinence of developing POC tools to detect and type RSSC strains.

Testing plant samples in the field (Réunion). Two tomato plots with some plants showing wilt symptoms were sampled and tested for the presence of RSSC in the field using the lpxC real-time LAMP (Fig. 2). Among the 21 symptomatic infected samples, 10 tested positive (TTR ranging from 11 to 24.75 and Ta between 92.8 and 93.4°C). These samples also tested positive

TABLE 2

Time-to-result values obtained for real-time lpxC and Phyl-I LAMP assays evaluated on tenfold dilutions of several *Ralstonia solanacearum* species complex strains in Tris buffer or spiked in different plant matrices

					Potato				Tomato				Eggplant			
Strain	LAMP assay	Concentration (log CFU/ml)	Pure suspension		NaOH		KOH/LNL		NaOH		KOH/LNL		NaOH		KOH/LNL	
RUN449	lpxC	6	3[a]	12.25[b] (0.00)	3	13.90 (0.44)	3	11.71 (0.30)								
RUN449	lpxC	5	3	16.25 (1.09)	**3**	**18.97 (3.59)**[c]	3	13.20 (0.35)								
RUN449	lpxC	4	**3**	**20.08 (2.75)**	1	28.43 (4.45)	**3**	**14.58 (0.59)**								
RUN449	lpxC	3	1	28.42 (4.47)	1	28.50 (4.33)	3	23.76 (6.39)								
RUN449	lpxC	2	0	31.00 (0.00)	1	26.83 (7.22)	1	28.3 (4.66)								
RUN312	lpxC	6	3	14.33 (0.38)					3	16.05 (0.89)	3	18.97 (1.38)	3	14.40 (1.22)	3	14.17 (0.14)
RUN312	lpxC	5	3	16.17 (0.14)					**3**	**21.09 (2.78)**	**3**	**23.92 (1.51)**	3	**18.33 (2.31)**	3	15.33 (0.38)
RUN312	lpxC	4	**3**	**20.00 (1.89)**					1	29.41 (2.75)	2	27.84 (3.39)	2	24.40 (6.98)	3	**21.50 (0.43)**
RUN312	lpxC	3	1	29.46 (2.67)					0	31.00 (0.00)	1	28.67 (4.04)	0	31.00 (0.00)	2	23.75 (6.31)
RUN312	lpxC	2	0	31.00 (0.00)					0	31.00 (0.00)	1	29.92 (1.88)	0	31.00 (0.00)	0	31.00 (0.00)
RUN312	PHYL-I	6	3	8.61 (0.19)					3	10.61 (1.04)	3	11.56 (1.64)	3	7.83 (0.20)	3	13.20 (1.49)
RUN312	PHYL-I	5	3	11.72 (1.66)					**3**	**14.77 (3.48)**	3	13.73 (0.41)	3	11.36 (0.94)	3	14.12 (1.96)
RUN312	PHYL-I	4	3	17.81 (3.53)					1	29.86 (1.97)	3	**15.87 (1.50)**	**3**	**25.01 (4.46)**	3	15.64 (0.86)
RUN312	PHYL-I	3	**3**	**21.82 (7.02)**					1	28.99 (5.75)	2	26.20 (4.27)	2	26.52 (5.36)	**3**	**23.91 (2.04)**
RUN312	PHYL-I	2	1	25.60 (9.36)					2	28.08 (2.88)	1	28.50 (4.33)	0	31.00 (0.00)	1	26.83 (7.22)
RUN320	PHYL-I	6	3	8.51 (0.42)					3	9.90 (1.49)	3	12.35 (0.66)	3	9.25 (0.64)	3	16.94 (0.43)
RUN320	PHYL-I	5	3	10.20 (0.72)					3	**14.41 (1.10)**	3	14.14 (2.65)	3	13.58 (3.28)	3	14.91 (0.40)
RUN320	PHYL-I	4	3	11.08 (1.90)					2	25.00 (5.19)	**3**	**15.23 (1.61)**	3	**15.64 (2.60)**	3	**17.41 (2.18)**
RUN320	PHYL-I	3	**3**	**17.50 (1.75)**					2	27.86 (5.43)	2	25.17 (6.10)	0	31.00 (0.00)	1	30.21 (1.36)
RUN320	PHYL-I	2	0	31.00 (0.00)					0	31.00 (0.00)	0	31.00 (0.00)	0	31.00 (0.00)	0	31.00 (0.00)
RUN4267	PHYL-I	6	3	8.64 (0.25)					3	13.42 (0.54)	3	14.76 (1.13)	3	8.19 (0.40)	3	12.03 (0.66)
RUN4267	PHYL-I	5	3	10.32 (0.96)					3	14.97 (1.75)	3	12.30 (1.79)	3	**12.33 (0.60)**	3	13.43 (0.44)
RUN4267	PHYL-I	4	3	15.64 (2.96)					3	**18.94 (4.81)**	**3**	**16.64 (0.78)**	2	17.94 (11.32)	3	**13.64 (2.43)**
RUN4267	PHYL-I	3	**3**	**19.61 (2.24)**					1	28.04 (3.25)	2	23.64 (6.38)	1	27.39 (6.24)	2	22.79 (5.52)
RUN4267	PHYL-I	2	1	31.00 (0.00)					2	27.92 (3.41)	0	31.00 (0.00)	0	31.00 (0.00)	0	31.00 (0.00)

[a] Numbers of positive samples out of three.
[b] Time-to-result means (SD).
[c] Bold print corresponds to the detection limit (i.e., lowest concentration for which 100% positive signals were obtained). By convention, a value of 31 (just above the time limit) was attributed for negative signals.

TABLE 3

Comparison of the analytical specificity of the real-time lpxC, fliC, and egl LAMP assays

Measurement	Phylotype	Total strains	lpxC LAMP	fliC LAMP	egl LAMP
Number of positive strains in the *Ralstonia solanacearum* species complex					
R. pseudosolanacearum	Phyl I	7	7	7	7
R. pseudosolanacearum	Phyl III	3	3	2	3
R. solanacearum	Phyl IIA	3	3	2	3
R. solanacearum	Phyl IIB	9	9	9	7
R. syzygii	Phyl IV	6	6	4	0
Total		28	28	24	20
Time-to-result range (median) (min)			9.75–19.25 (11.62)	13–29.75 (16)	15.25–29.75 (21.87)
Temperature range (°C)			92.6–93.4	91.6–92.7	91.3–93.5
Number of positive strains in nontarget taxa					
Ralstonia spp.		4	2[a]	0	0
Cupriavidus spp.		16	3[a]	2[a]	0
Burkholderia spp.		5	0	0	0
Other plant-pathogenic strains		10	0	0	0
Total		35	5[a]	2[a]	0

[a] Very late amplification signals, not always reproducible and not always associated with the right annealing temperature. These samples are considered negative.

with the Phyl-I-specific LAMP assay, as well as two other samples that had tested negative with lpxC (TTR ranging from 8.25 to 21 and Ta from 90.3 to 90.9°C). Typical colonies were isolated for 12 of the 21 samples and were identified as sequevar 31 using the *egl* typing method (Fegan and Prior 2005). A concordance of 89% was obtained between Phyl-I-specific LAMP and plating results, with two samples detected positive by plating and negative using LAMP and two samples showing the opposite results. The multiplex PCR performed on the different samples showed 90.5% concordance with LAMP results and supported the LAMP results for three of these four discordant results between LAMP and plating.

Testing plant samples in the field (Mayotte). Eleven accessions for each of the three cultivated Solanaceae of eggplant, tomato, and pepper were evaluated for their resistance to phylotype I in plots known to be naturally infected with this phylotype. Unfortunately, the LNL assay could not be used in the field despite its good performance on artificially inoculated plants because it requires refrigerated transport with dry ice, unlike the classic LAMP mix, which is available in lyophilized form. For each plant, LAMP detection, multiplex PCR, and bacterial isolation were compared (Table 6). Congruent results were obtained between the three detection methods, with 82% concordance obtained for all the data (256 plants analyzed). The LAMP assay yielded more positive samples than the other methods, irrespective of the plant sampled, tomato, pepper, or eggplant (McNemar test performed on all the data, P values of 5.199×10^{-8} and 0.000512, when compared with plating and PCR, respectively) (Table 6). Nevertheless, PCR results were mostly similar to the LAMP ones, with 95% concordance obtained for all the data. Moreover, six of the 13 discordant results (LAMP positive, PCR multiplex negative) displayed very late LAMP signals in the field (TTR > 20 min). The relationship between disease incidence obtained for each accession (i.e., the number of symptomatic plants out of the total number of plants) and the percentage of plants that tested positive for *R. pseudosolanacearum* by each method is provided using a color scale in Supplementary Table S5. The proportion of positive plants determined by LAMP and PCR overall fit better with the incidence than the plating method. A positive correlation (Spearman coefficient r_s) was found between disease incidence and the percentage of plants detected positive by LAMP or PCR for the three plant species (LAMP: $r_s = 0.73$ $P = 0.01001$, $r_s = 0.67$ $P = 0.02448$, $r_s = 0.61$ $P = 0.04743$; PCR: $r_s = 0.80$ $P = 0.00304$, $r_s = 0.67$ $P = 0.02267$, $r_s = 0.83$ $P = 3 \times 10^{-5}$, for eggplant, tomato, and pepper, respectively), whereas for plating results, a positive correlation was found only for the pepper accessions ($r_s = 0.67$ $P = 0.02439$). On the other hand, all three methods, and especially the LAMP assay, were able to detect *R. pseudosolanacearum* from asymptomatic material. Interestingly, the accessions T11, E7, and E9 did not express any symptoms, whereas *R. pseudosolanacearum* was detected in plants by the three methods. The tomato T11 accession, for instance, is a local variety traditionally cultivated in Mayotte but known to be partially susceptible to BW in the Mayotte conditions (M. Seguin, *personal communication*). This suggests that the plants of the three accessions were indeed contaminated but yet asymptomatic at the time the samples were collected.

Testing degraded plant samples from the field (Madagascar). The difficulty of collecting infected samples in the field (harsh conditions) can make plating assays impossible because of the absence of living bacteria. We used the two LAMP assays to test field samples received from the FOFIFA research center in Madagascar for diagnostic purposes. No typical RSSC colonies could be recovered from the different samples. This is probably because the macerates had undergone a 5-week journey at ambient temperature. A first screening was carried out with the real-time lpxC LAMP test on one or two plant samples for each plot using the KOH/LNL technique. When a sample was declared positive, all the samples from each plot were tested with lpxC and Phyl-I LAMP assays and the multiplex PCR assay. The first screening using lpxC LAMP on the set of 46 samples representing the geographical diversity of the survey gave six positive results, all from different plots. The results of the tests carried out on all the samples collected in the different plots ($n = 75$) are shown in Table 7. At least one further sample tested positive using the lpxC LAMP in each of the six plots. The lpxC LAMP yielded significantly more positive results than the multiplex PCR (McNemar test, $P = 1.275 \times 10^{-5}$). Although several discordant results were found at the sample scale, some interesting results were found at the plot level. Plots 2 and 3 tested positive with both lpxC and Phyl-I assays, and the presence of phylotype I was confirmed using the multiplex PCR. Plot 5 tested positive with lpxC but not with the Phyl-I LAMP assay. This is consistent with the multiplex PCR, which revealed the presence of phylotype II in plot 5. This result was confirmed by the use of a LAMP assay specific to IIB-I strains (data not shown). Plots 1 and 6 tested positive using the lpxC LAMP assay but negative with the Phyl-I LAMP assay and the multiplex PCR (and also with the LAMP assay specific to IIB-I strains). Another phylotype may be present in the plots, but the multiplex PCR failed to detect it because this technique is less sensitive than LAMP.

TABLE 4

Comparison of the analytical sensitivity of the real-time lpxC, fliC, and egl LAMP assays

Strain and concentration	No. rep.	No. positive		
		lpxC LAMP	fliC LAMP	egl LAMP
RUN 312 0.1 ng/µl	3	3	3	3
RUN 312 0.01 ng/µl	3	3	3	3
RUN 312 0.001 ng/µl	3	3	1	3
RUN 312 0.0001 ng/µl	3	0	0	0
RUN 312 0.00001 ng/µl	3	0	0	0
RUN 449 0.1 ng/µl	3	3	3	3
RUN 449 0.01 ng/µl	3	3	3	0
RUN 449 0.001 ng/µl	3	3	3	0
RUN 449 0.0001 ng/µl	3	3	1	0
RUN 449 0.00001 ng/µl	3	0	0	0

TABLE 5

Comparison of simplified extraction methods for LAMP detection of phylotype I in eggplants

Status	No. samples[a]	Phyl-I-specific LAMP		
		Plating	NAOH extraction	KOH/LNL
Symptomatic	6	+	+	+
Asymptomatic	8	+	+	+
Asymptomatic	6	−	−	−
Asymptomatic	3	−	+	+
Asymptomatic	3	−	−	+
Asymptomatic	1	+	−	−
Asymptomatic	3	+	−	+
Asymptomatic	2	+	+	−
Healthy	6	−	−	−
Total	38			
Percent positive samples		53	50	61

[a] Number of samples with the specified combination of positive (+) or negative (−) responses obtained with the three detection methods: plating and phylI-LAMP after NaOH extraction or KOH/LNL extraction.

DISCUSSION

Preventing the entry and spread of pathogens in agroecosystems is essential. Different measures include early pathogen detection and identification in a given location; monitoring the evolution of diseases in the field; controlling plant material before planting; and controlling the exchange of plant material at borders. Early warning systems enable the implementation of timely containment measures and enhance control. Obviously, the sooner information is available, the better. POC diagnostic methods can be used in the field and are effective for early detection, particularly LAMP in association with different readouts for real-time monitoring or endpoint detection (Becherer et al. 2020). Many LAMP assays have already been developed to meet diag-

TABLE 6

Comparison of plating, LAMP, and PCR methods for the detection of *Ralstonia pseudosolanacearum* phylotype I in eggplant, tomato, or pepper plants (Mayotte trial)

					Concordance (%)			
Plant	No. samples[a]	Plating	LAMP	PCR	LAMP/plating	Plating/PCR	LAMP/PCR	3 methods
Eggplant								
	12	+	+	+	82	85	97	82
	14	−	+	+				
	3	−	+	−				
	66	−	−	−				
Total	95	12	29	26				
% positive samples		13	31	27				
Tomato								
	15	+	+	+	87	88	96	86
	1	+	+	−				
	2	+	−	−				
	7	−	+	+				
	2	−	+	−				
	58	−	−	−				
Total	85	18	25	22				
% positive samples		21	29	26				
Pepper								
	3	+	+	+	86	93	89	84
	1	+	+	−				
	4	−	+	+				
	7	−	+	−				
	61	−	−	−				
Total	76	4	15	7				
% positive samples		5	20	9				
Combined results								
	30	+	+	+	85	89	95	84
	2	+	+	−				
	2	+	−	−				
	25	−	+	+				
	12	−	+	−				
	185	−	−	−				
Total	256	34	69	55				
% positive samples		13	27	21				

[a] Number of samples with the specified combination of positive (+) or negative (−) responses obtained with the three detection methods: plating, phylI-LAMP, and multiplex PCR. The combinations with 0 positive samples have been omitted.

TABLE 7

Degraded samples from the field (Madagascar) tested with LAMP and PCR assays

			LAMP		
Plot	Host	No. samples	lpxC (real-time)	Phyl-1	PCR
Plot 1 Sakaraha	*Solanum lycopersicum*	3	+	−	−
		9	−	−	−
Plot 2 Farafangana	*Solanum lycopersicum*	5	+	+	+ (Phyl−I)
		1	+	+	+ (Phyl−I)
		1	−	−	−
Plot 3 Fianarantsoa	*Capsicum annuum* (pepper)	3	+	+	+ (Phyl−I)
		2	+	+	−
		1	−	−	−
Plot 4 Ambatolahy	*Capsicum annuum* (pepper)	2	+	−	−
		5	−	−	−
Plot 5 Ilakaka Be	*Solanum tuberosum*	17	+	−	+ (Phyl−II)
		12	+	−	−
		8	−	−	−
Plot 6 Antanambao	*Capsicum annuum* (bell pepper)	2	+	−	−
		4	−	−	−
	Total	75	47	11	26

nostic requirements in animal, human, and plant health. However, there is still little evidence of their use as routine diagnostics on site. Nevertheless, the growing popularity of LAMP in a wide range of fields suggests that it could soon become a gold standard, alongside PCR (Soroka et al. 2021). Several LAMP assays have been reported for the detection of bacterial plant pathogens of agricultural importance (Jun-hai et al. 2015; Langlois et al. 2017; Palacio-Bielsa et al. 2015; Rigano et al. 2014; Stehlíková et al. 2020; Stulberg et al. 2020). In plant health, a large number of assays are supposed to be set up in the field. However, LAMP assays are generally tested on DNA that is extracted from plant samples using classical commercial kits, but their reliability is uncertain for diagnostics in the field (Aglietti et al. 2021; Enicks et al. 2020; Mahas et al. 2021; Stehlíková et al. 2020; Zhang et al. 2018).

In this work, we developed a specific and sensitive LAMP assay targeting the RSSC phylotype I, which causes BW on all the continents and affects the largest number of hosts (Hayward 1994). Moreover, emerging phylotype I strains have recently been identified infecting new hosts (Bocsanczy et al. 2022). We optimized a previously published RSSC-LAMP assay (Okiro et al. 2019). We also assessed and compared the application of these different tools for in-field diagnostics.

The Phyl-I LAMP assay included a Stem primer (instead of the Loop LF primer), as recommended by Gandelman et al. (2011), because it was not possible to design LF primers using the Primer explorer software. According to the authors, Stem primers used in LAMP in combination with loop-generating and displacement primers gave significant benefits: Their speed and sensitivity are similar to that offered by Loop primers; they also have additional options of forward and reverse orientations. This choice was the most effective compared with the other systems tested. This LAMP assay tested positive for 23 phylotype I strains, representing a large range of geographical and genetic diversity (13 countries and 14 sequevars). However, two phylotype I strains responsible for BW of mulberry were not detected by our LAMP assay. These mulberry strains belong to the sequevar 12 and were assigned to a particular race (previously race 5 biovar 5) because they do not occur anywhere else in the world. They are well adapted to mulberry, have a low pathogenicity on eggplant and potato (Xu et al. 2009), and are locally restricted to several provinces in China (He 1983; Pan et al. 2013). Specific diagnostics are already available for detecting these mulberry strains, involving a PCR method and a LAMP assay (Huang et al. 2017; Pan et al. 2013).

Interestingly, the Phyl-I LAMP assay was very specific and tested negative for all the nontarget strains, including closely related species and ubiquitous strains likely to be present in the same environment. These analytical specificity tests were supported with a thorough in silico analysis performed on 6,105 genomes of *Burkholderiaceae.* The only phylotype I genome sequence that did not match with the primers was also a mulberry strain belonging to sequevar 12. No significant match was found for any of the nontarget genomes analyzed. The sensitivity obtained on pure bacterial suspensions was very high, with 100% positive LAMP signals at 10^3 CFU/ml and above, corresponding to a theoretical value of 5 CFU per reaction. This approaches the sensitivity of optimized real-time PCR assays, as already reported (Panno et al. 2020; Tomlinson 2008).

We also improved a previously published LAMP method based on a robust and specific DNA marker of the RSSC, involving a putative gene, the UDP-(3-O-acyl)-N-acetylglucosamine deacetylase gene, which was already used to design species-specific molecular assays, such as a multiplex PCR (Fegan and Prior 2005), and a specific RSSC diagnostic microarray (Cellier et al. 2017). In our study, this colorimetric test was shown to be highly specific but not very sensitive. It performed extremely well when tested on extracted DNA using a commercial kit. Late signals were obtained from pure bacterial suspensions at 10^7 to 10^8 CFU/ml. We tested this colorimetric method on simplified extracts (NaOH and KOH/LNL) of plant material mixed with suspensions of the bacteria. The results could not be interpreted, probably because the NaOH and KOH/LNL procedures alter the colorimetric reading (data not shown). This colorimetric test is useful for identifying bacteria after their isolation on agar media. The real-time version was much more sensitive, with a 100% positive LAMP signal for pure bacterial concentrations at 10^4 CFU/ml and above. It showed later LAMP signals compared with the Phyl-I assay and seems a little less performant. However, the LOD (theoretical value of 50 CFU per reaction) is still sufficient. Another advantage of the real-time version is the post-amplification step that displays a specific temperature signature peak, which makes it possible to validate the assay.

A comprehensive in silico analysis showed some identity with nontarget strains ranging from 68.6 to 91.4%. As the lpxC LAMP assay covers the full spectrum of the RSSC complex, it is not surprising to find some homology with genetically related but nontarget strains. This in silico study has guided the selection of nontarget strains to be experimentally analyzed, especially 22 closely related (id $\geq$ 85.9%) nontarget strains representative of different *Ralstonia* species and the closely related genera *Cupriavidus* and *Burkholderia*. Only very late LAMP signals were detected for five strains belonging to different species of *Cupriavidus* and *Ralstonia insidiosa*, which were isolated from diverse environmental habitats, such as soil, water, and human clinical sources. These bacterial species are not pathogenic to plants and are unlikely to be found in solanaceous hosts. Moreover, these very weak amplifications were not associated with the expected Ta range for all strains, except for *C. lacunae* (isolated from an artificial pond). These LAMP background signals, probably due to mispriming, were obtained from high concentrations of pure nontarget cultures. They were easily distinguishable from early signals expected from pure target bacterial suspensions and should not interfere with the diagnostics of RSSC strains from plants.

The best results in terms of inclusivity were obtained for the real-time lpxC LAMP when compared with the other published real-time LAMP protocols targeting RSSC strains. Particularly, some strains belonging to phylotype IV were not amplified by the egl and fliC LAMP assays, supporting previous published results (Kubota et al. 2008; Lenarčič et al. 2014). Regarding the sensitivity, the best results were obtained with the real-time lpxC LAMP when compiling the results obtained for the two strains belonging to phylotype I and IIB-1.

Preliminary tests on crude plant extracts infected with the RSSC showed that an artifactual signal was produced on the LAMP equipment. Thus, we tested and selected some simplified DNA preparations (data not shown). Both simplified preparations (NaOH and KOH/LNL) allowed for the detection of the bacterium from the plant with a satisfactory threshold of detection: LOD values ranged between 10^3 and 10^5 CFU/ml (but most were 10^4 CFU/ml) using the KOH/LNL preparation and between 10^4 and 10^5 CFU/ml using the NaOH preparation. These sensitivity results are equal to or less efficient than those obtained on pure cultures of the bacteria. This suggests that the matrix has a slight negative effect in some cases, particularly for tomato (but results were not statistically significant in our study). The LAMP is supposed to be more tolerant than the PCR to inhibitory compounds (Soroka et al. 2021; Wong et al. 2018), but not totally insensitive. The effect depends on the nature and quantity of the biological substances (Kaneko et al. 2007; Soroka et al. 2021).

No significant results were found between the KOH/LNL preparation and the NaOH method, whereas a trend toward slightly earlier TTR values for the KOH/LNL preparation was observed on spiked samples and inoculated eggplant plants. The two simplified extraction techniques tested have advantages and disadvantages. The NaOH extraction seems to be a little less efficient, with slightly worse results for sensitivity overall, when evaluated on different types of spiked or infected plant material. On the other hand, it is an inexpensive method that can be used during surveys around the world because the generic mix used is freeze-dried and can be transported at ambient temperature. The KOH/LNL method is very easy to use, inexpensive, and yields good repeatable results in terms of sensitivity. Nevertheless, the constraint of keeping the mix frozen for transport restricts its use to the laboratory or to field experiments in the vicinity of a laboratory.

Lastly, we evidenced the usefulness of LAMP assays on different types of field samples after a simplified DNA sample preparation. We showed that real-time LAMP assays can be used to screen different field samples for the presence of the RSSC, even in the case of degraded samples. The real-time lpxC LAMP can test for the presence or absence of the RSSC in a plot for further characterization using other diagnostic tools. Information gathered from degraded samples can be used to identify the location of the RSSC for further investigation. Field experiments conducted in Mayotte and Réunion Island demonstrate the feasibility of using LAMP in the field. In Mayotte, it was not possible to keep the reagents at 4°C during the survey, and all experiments were successfully performed for several hours at ambient temperature (28°C). There was a good correlation between the LAMP and plating data for the field experiments (89 and 85% for Réunion and Mayotte samples, respectively). Nevertheless, different parameters can explain the few discordant results. The sampling could be one reason, as the two stem sections used for LAMP and isolation are adjacent but not the same for practical reasons. This could be critical when bacteria are in low concentration in plant samples. Masking or inhibition of *R. pseudosolanacearum* growth by nontarget strains on semi-selective media, or even entry into the viable but noncultivable state (Kong et al. 2014), could explain the difficulty of recovering *R. pseudosolanacearum* colonies on agar plates compared with direct detection by LAMP from plant samples. On the other hand, a cross-contamination of samples with DNA could explain the best scores for LAMP tests as well. Nevertheless, no cross-contamination was detected in the field using appropriate negative controls. Moreover, the multiplex PCR assay amplifying different DNA targets than those amplified in the LAMP assays showed 91% (Réunion Island) and 95% (Mayotte) concordance with LAMP results, which mostly excludes a LAMP amplicon carryover contamination of the samples. The absence of amplification by PCR for some samples amplified by the LAMP can be explained by the lower tolerance of PCR to plant inhibitors compared with LAMP. Another possibility to explain the better results of LAMP (and PCR) compared with plating results is the ability of molecular methods to amplify DNA from dead bacteria. It would be interesting in the future to test propidium monoazide before the LAMP to amplify only the living fraction of bacteria (Telli and Doğruer 2019).

The phylotype I LAMP assay was able to detect in a relevant way bacterial populations of *R. pseudosolanacearum* associated with plant material evaluated for their resistance in Mayotte. It was able to detect latent infections in asymptomatic plants, as did the other detection methods. Associated with symptom scoring, it could lead to a reliable characterization of the disease resistance status (susceptible, tolerant, or resistant) of the varieties. In this context, the in-field LAMP method could be an efficient tool for rapid and efficient varietal selection.

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