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A combined recombinase polymerase amplification CRISPR/Cas12a assay for detection of *Fusarium oxysporum* f. sp. *cubense* tropical race 4

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The soilborne pathogen *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (Foc TR4) is currently devastating banana production worldwide. Once introduced, it is not possible to eradicate the pathogen from soils where it can survive for decades. The only management option available then is to replace Foc TR4-susceptible with -resistant varieties. Timely detection of the pathogen, however, is an important strategy to prevent the introduction of Foc TR4 into new areas and prevent its spread from infested sites. In this study, a single-tube detection technique was developed by combining recombinase polymerase amplification (RPA) and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas12a technology (RPA-Cas12a) for detection of Foc TR4. The RPA-Cas12a assay was conducted isothermally, had a sensitivity of up to 10 fg target DNA and did not cross react with any of the 76 non-target isolates included in the specificity testing. The RPA-Cas12a assay detected Foc TR4 from naturally infected banana samples collected in the field and visualization was possible with the naked eye under LED blue light transillumination. The method can be integrated with inexpensive fluorescent or electronic detection devices to accelerate Foc TR4 in-field detection and, thereby, fast-track disease containment strategies.

Keywords *Fusarium oxysporum* f. sp. *cubense* tropical race 4, CRISPR/Cas12a, Banana Fusarium wilt, Disease diagnosis, Rapid detection

Fusarium wilt, caused by the soilborne fungal pathogen *Fusarium oxysporum* f. sp. *cubense* (Foc), is globally regarded as one of the most devasting plant pathogens[1]. The current epidemic of the disease is caused by Foc tropical race 4 (TR4), which originated in Asia, and has since spread to 23 banana-growing countries[1]. Foc TR4 affects many banana varieties, but is particularly destructive to Cavendish bananas, which makes up almost half of all bananas grown worldwide and 99% of the international export market. If the fungus continues to spread unhindered, 1.65 million hectares of banana could be affected by 2040, with an estimated loss in production of over US$10 billion[2]. Foc produces chlamydospores that may survive in infested soils for decades. Due to its soilborne nature, the fungus cannot be eradicated, and spreads easily in soil attached to field equipment, shoes and vehicles, in water, and with infected plant material[3,4]. In the presence of a susceptible host, Foc inoculum can increase prolifically but may remain invisible, thereby allowing the fungus to establish and spread until symptomatic hosts indicate its presence[5]. The most effective way to manage banana Fusarium wilt is to prevent its

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introduction into disease-free areas with strict biosecurity measures and the use of clean planting material. Once introduced, the only viable strategy is to deploy resistant varieties[4]. Presently there is no fully resistant variety accepted by the market that can replace Cavendish, which accounts for most of commercial global production[6]. Therefeore, prevention and early detection of Foc TR4 is important to protect banana production worldwide.

The detection of Foc TR4 requires regular surveillance for symptomatic banana plants in high-risk areas. The presence of typical Fusarium wilt field symptoms, however, is not sufficient to confirm the causal agent, especially not the strain involved. Therefore, if symptomatic plants are found, samples must be collected from infected tissue and sent to diagnostic laboratories for phenotypic and molecular identification[7]. Molecular assays available for Foc TR4 identification include PCR[8,9], quantitative (q)PCR[10,11]and loop-mediated isothermal amplification (LAMP) assays[12]. In contrast to PCR and qPCR, LAMP or recombinase polymerase amplification (RPA) can be performed isothermally, negating the need for sophisticated and bulky equipment like thermocyclers[13].

Point-of-care (POC) diagnostic assays integrated into portable devices are now increasingly being used for on-site plant pathogen detection at affordable prices[14,15]. Positive results, however, still require validation in reference laboratories to confirm new disease outbreaks. POC molecular assays for plant pathogen detection require three steps. In the first, DNA of sufficient quantity and quality must be extracted from symptomatic plant tissue in the field[16]. In the second step, pathogen DNA is recognized and amplified using LAMP or RPA to improve sensitivity and accuracy[17]. In the final step, signal transduction is used to convert the detection of target DNA into a visible signal for the operator to interpret. Fluorescent reporter molecules released in response to DNA target detection and read with portable fluorometers are often used for this purpose[18–20]. Alternatively, electrochemical reporters generated in response to DNA target detection can be visualized with differential pulse voltammetry, amperometry, scanning wave voltammetry or electrochemical impedance spectrophotometry[21–23].

Detection and signal transduction of pathogen RNA or DNA with clustered regularly interspaced short palindromic repeats (CRISPR)-associated proteins and reporter molecules can also be used for POC target recognition and signal amplification[14,24,25]. This method can substitute RPA/LAMP but is often used in complement to achieve robust detection with up to zeptomolar (10^{-21}) sensitivity[25]. CRISPR-Cas12a systems are dependent on CRISPR RNA (crRNA), which guides Cas proteins to recognize and cleave nucleic acid targets[24]. The crRNA can be programmed towards a specific DNA or RNA region of interest through hybridization to a complementary sequence. Once bound, it results in Cas12a cleaving the target, followed by collateral cleavage of nearby reporter molecules with single nucleotide difference specificity[26]. To date, fungal plant pathogens detected with CRISPR/Cas include *Elsinoë*[27], *Magnaporthe*[23], *Verticillium*[28], *Diaporthe*[29] and several *Fusarium* species. The *Fusarium* species include *F. circinatum*[30], *F. graminarium*[31], *F. asiaticum*[32], *F. temperatum*[33], *F. verticillioides*[34].

The objective of this study was to optimize a RPA-Cas12a assay for Foc TR4 detection. A rapid crude DNA extraction method was employed, followed by a single-tube RPA-Cas12a assay conducted isothermally at 37 °C. The results were observed both in a portable fluorometer and with the naked eye as green fluorescence under a LED blue light transilluminator. The analytical sensitivity and specificity of this method, as well as its feasibility, was confirmed when detecting Foc TR4 from naturally infected samples.

Results

PCR and RPA amplification

When in silico PCRs were set to allow no mismatches with the Foc genome sequences in NCBI and the in-house genome database, it predicted that only target Foc TR4 (VCG 01213/16) genomes would amplify with the three primer sets tested (Supplementary Table S1). No amplification was predicted for any of the non-target isolates. When the same PCRs were set to allow three mismatches, the RPA primer set 2 (Foc TR4 F2 and R1) were predicted to amplify a closely related Foc VCG 0121 non-target isolate (CAV 2318). RPA primers Foc TR4 F1 and R1 were therefore selected for further testing. The expected amplification product sizes were predicted by the software including a 775 bp product for PCR primer set and for RPA primer sets, a 292 bp (F1xR1 combination) and 561 bp (F2xR1 combination) product size, respectively (Supplementary Table S1).

After gel electrophoresis, the PCR (Foc TR4 PCR F1 and R1) (Supplementary Figure S1) and RPA (Foc TR4 RPA F1 and R1) (Supplementary Figure S1) primer sets produced the expected amplicon sizes of 775 bp and 292 bp, respectively. Primers only amplified DNA from the positive Foc TR4 control (CAV 300) in the subset of isolates used for preliminary optimization and in vitro specificity testing, but not from any of the non-target isolates.

Cas12a assay optimization

The in vitro digestion assay confirmed that the synthesized crRNA could complex with Cas12a and mediate the recognition and cleavage of the 775 bp double stranded PCR amplicon. PCR amplified Foc TR4 DNA was cut into the expected sizes (Fig. 1). The negative control reaction showed no cleavage of the PCR amplicon.

ANOVA indicated that the type and concentration of the reporter, as well as the ratio of Cas12a to crRNA significantly influenced mean raw fluorescence measured by the Genie II fluorometer (Supplementary Table S3). The reporter published by Lv et al.[35]. showed significantly greater fluorescence than that published by Li et al.[36]. (Fig. 2a and b), and the Lv et al.[35]. reporter was thus selected for further experiments. The concentration of Lb Cas12a and crRNA had a significant effect on fluorescence measured (Fig. 2c and d). All concentrations significantly differed from each other, except the 200 nM and 300 nM concentrations which indicated the highest mean fluorescence. Therefore, the 200 nM concentration was selected for further experiments. The 1:1 ratio between Cas12a and crRNA resulted in significantly higher mean raw fluorescence than the other ratios tested (Fig. 2e and f) and was selected for further experiments.

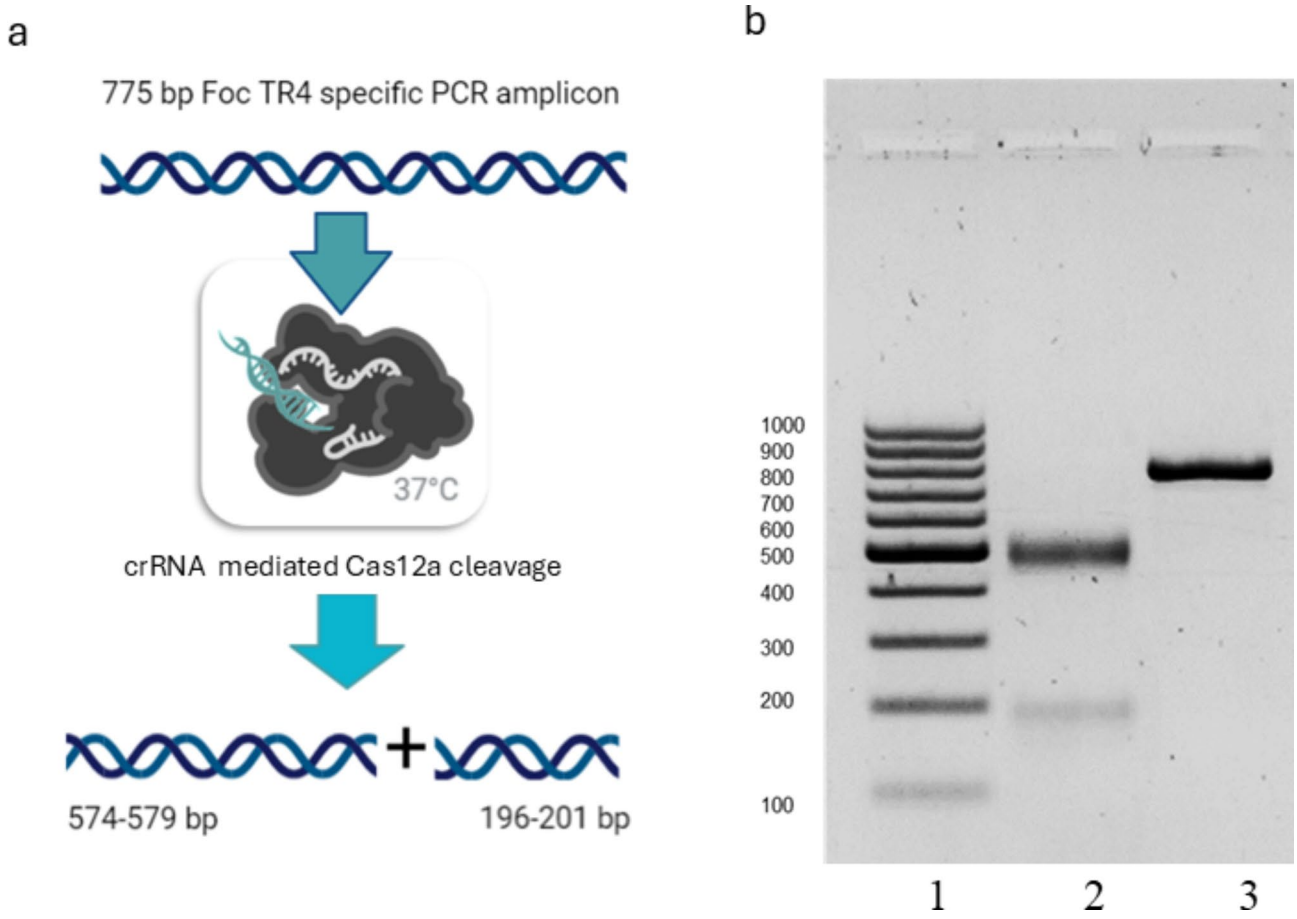

Fig. 1. Cas12a and crRNA complex recognizes and cleaves target region within the 775 bp *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (Foc TR4) PCR amplicon, resulting in a 5' overhang. (**a**) The cleavage products were shown using gel electrophoresis on a 1% agarose gel (**b**). Lane 1: GeneRuler 100-bp ladder (Thermo Fischer Scientific, Waltham, Massachusetts, USA), Lane 2: cleaved PCR products, Lane 3: intact Foc TR4 PCR product (775 bp).

RPA-Cas12a in vitro Foc TR4 detection

Foc TR4 was detected both when RPA and Cas12a assays occurred separately, and simultaneously in a one-tube reaction (Fig. 3). Peak fluorescence was reached slower when RPA and Cas12a occurred in single tube (Fig. 3b), but end point fluorescence did not differ significantly from when reactions were run in separate tubes (Fig. 3c). The RPA-Cas12 reaction improved fluorescence when single tube was mixed in between 30-min incubation periods. The one-tube RPA-Cas12a reaction with centrifugation was, therefore, selected for further experiments.

The RPA-Cas12a assay had a similar limit of detection to the qPCR assay (Fig. 4a) but was 10 times more sensitive compared to the LAMP assay, which was only able to detect 1×10^{-3} ng of DNA reliably as only one of the repeats at the 1×10^{-4} concentration amplified (Fig. 4b). The optimized RPA-Cas12a assay could successfully detect DNA as low as 1×10^{-4} ng of target DNA (Fig. 4c). Sensitivity was maintained when sample tubes were visualized under LED light (Fig. 4d).

The RPA-Cas12a assay proved specific with only Foc TR4 positive DNA ($n = 16$) resulting in high RFUs (Supplementary Table 2) and sample tubes showing visible fluorescence when visualized under blue LED light transilluminator (Fig. 5a and b). No fluorescent signal was present from samples containing non-target isolates ($n = 75$) or the non-template control (Fig. 5a; Supplementary Table 2). Likewise, non-target isolates displayed no visible fluorescence (Fig. 5b).

RPA-Cas12a *in planta* Foc TR4 detection

Foc TR4 could be successfully detected from naturally infected samples using the RPA-Cas12a-based assay (Fig. 6a; Table 1). When sample tubes were viewed under a LED blue light, the Foc TR4-infected samples also showed visible fluorescence (Fig. 6B). In contrast, DNA from plant tissue inoculated with non-Foc TR4 isolates did not yield any significant fluorescent signals (Table 1). This was confirmed when visualized with LED blue light transilluminator.

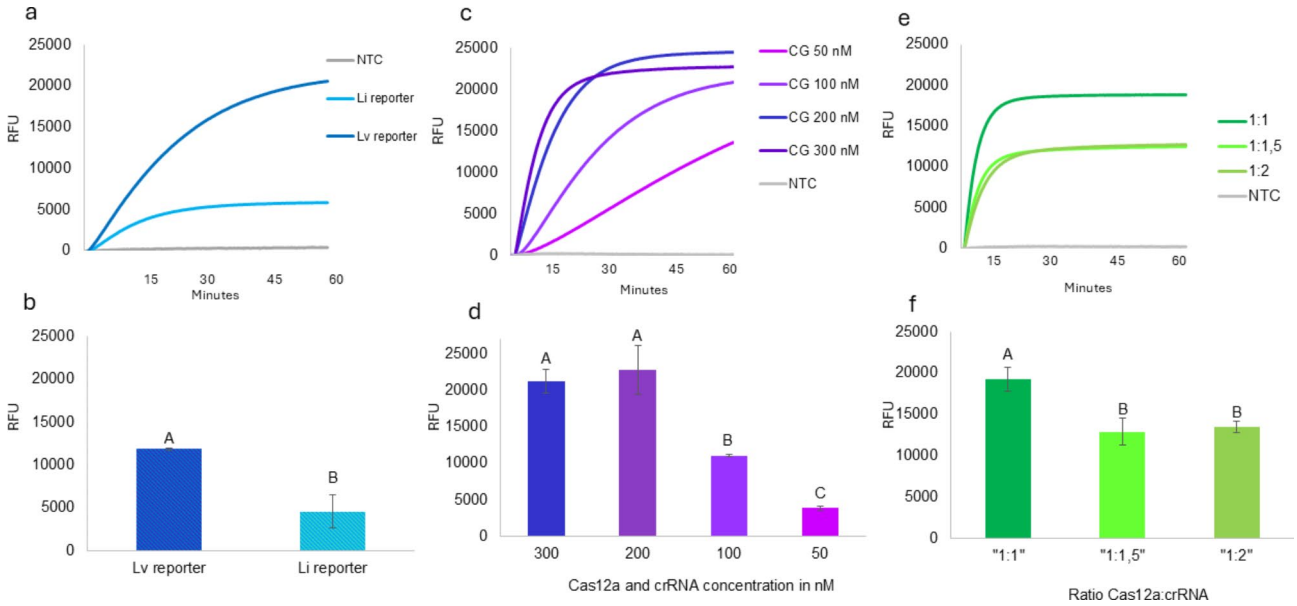

Fig. 2. Optimization of Cas12a assay. (**a**) Average raw fluorescence (Y-axis) when two reporters were compared. Reporters were based on the publications of Lv et al.[35]. and Li et al.[36], respectively. (**b**) Differences in end-point fluorescence. (**c**) Average raw fluorescence (Y-axis) measured when Cas12a and crRNA concentrations ranged from 50 to 300 nM. (**d**) Differences in end-point fluorescence. (**e**) Average raw fluorescence (Y axis) measured when Cas12a and crRNA ratios differed. Ratios of 1:1, 1:1.5 and 1:2 were tested. (**f**) Differences in end-point fluorescence. Fluorescence was measured in a Genie II fluorometer for 60 min. ANOVA and Tukey's HSD were used to evaluate if end-point fluorescence differed significantly after 30 min. Standard deviation is indicated with error bars and letters indicate groupings according to Tukey's HSD.

Discussion

Early and accurate detection of invasive plant pathogens, such as Foc TR4, is essential to protect crops and ensure effective containment strategies[1,8]. Current surveillance efforts are based on scouting for disease symptoms and the subsequent laboratory identification of the causal pathogen[1]. Banana Fusarium wilt symptoms alone, however, can easily be confused with symptoms caused by other biotic or abiotic factors[7]. Molecular assays based on nucleic acid amplification, such as PCR[8]and qPCR[10,11], are regarded as the gold standard for accurate and sensitive detection of Foc TR4. Both assays, as well as the LAMP assay published for Foc TR4[12], however, require either specialized skill or expensive infrastructure. There is thus a need for a simple, easy-to-use, and inexpensive detection systems that can be used in-field for surveillance. To this end we have designed a one-tube RPA-Cas12a assay that can rapidly and sensitively detect Foc TR4, and which has the potential to be developed into a POC tool.

This study is the first to utilize Cas12a for Foc TR4 detection. The RPA-Cas12a detection assay has several advantages over currently employed diagnostic methods for Foc TR4. Firstly, it has the potential to be adapted to detect Foc TR4 instrument-free. The assay was optimized with a portable fluorometer employed for quantitative detection of LAMP assays, but naked eye fluorescent detection was also possible by visualization under inexpensive blue light LED transilluminator. The RPA-Cas12a naked eye fluorescent detection protocol has also been optimized and employed for detection of various human pathogens including SARS-CoV-2[37] and *Shigella flexneri*[38], as well as for the detection a fungal plant pathogen *F. circinaturm*[30]. The assay can also be considered for the development of smartphone-based end-point fluorescence detection. Smartphone technology can be employed to take photos at the point of infection, convert images to fluorescent intensity, report the data, and transmit it to digital platforms and databases where skilled personnel can inform field technicians on how to proceed once positive results are recorded[37].

Secondly, the RPA-Cas12a assay for Foc TR4 detection is superior in sensitivity and specificity when compared to available protocols. Target DNA concentrations as low as 10 fg could be detected which was similar in limit of detection of the tested qPCR assay and 10 times lower than that of LAMP assays for Foc TR4[10–12]. RPA-Cas12a assays have the benefit of dual specificity, first in the amplification step with specific primers, and then in the Cas12a-mediated cleavage step that recognizes the specific target fragment that has illustrated single nucleotide specificity[26]. Some available detection assays for Foc TR4 have detected non-pathogenic endophytes that are ubiquitously associated with banana tissue[39]. The detection of false positives can have devastating effects, as was evident when Foc TR4 was incorrectly detected on a Mareeba farm in Northern Queensland[40,41]. It is, therefore, advisable to rely on multiple diagnostic assays when a new case of Foc TR4 is expected.

The time needed for an RPA-Cas12a-based detection assay is shorter than PCR and qPCR. Reaction times can potentially be reduced to as little as 10 min[30,42]. The concentration of the target DNA and reporter have a great effect on end-point fluorescence. By increasing reporter concentrations, fluorescence and sensitivity will

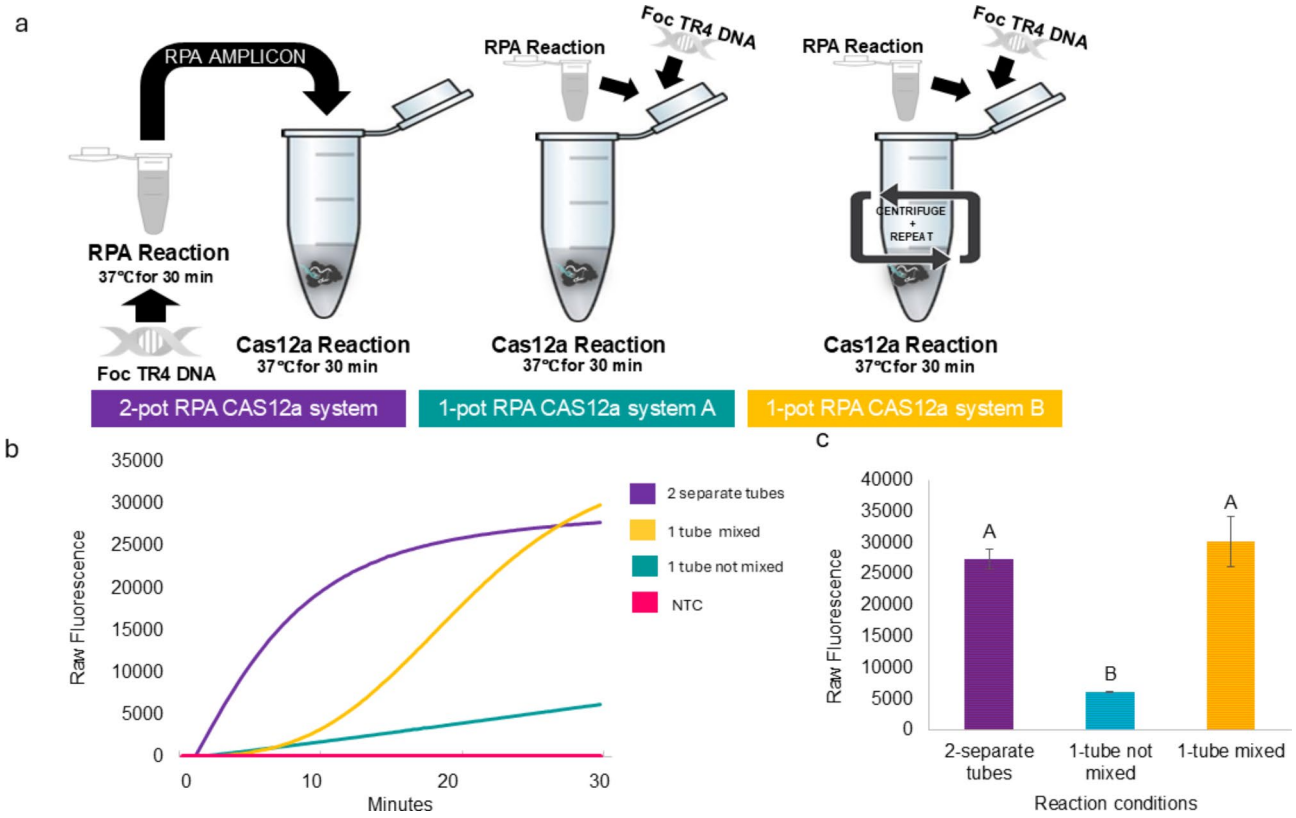

Fig. 3. Optimization of single tube RPA-Cas12a assay. (**a**) RPA and CRISPR/Cas12a were either conducted in separate tubes or in one-tube reactions, with or without mixing. Reactions were run for 60 min at 37 °C. In one-tube reaction that was mixed, a brief centrifugation occurred between two 30 min incubation times. (**b**) Average raw fluorescence (Y axis) per system was measured during the last 30 min incubation period. (**c**) Endpoint fluorescence compared with ANOVA. Standard deviation is indicated with error bars and letters indicate groupings according to Tukey's HSD.

be increased, but it will add cost. Currently, 200 nM of the reporter is required to detect Foc TR4 from crude DNA extracted from environmental samples. The amount of reporter can, therefore, be adjusted to cater for the required balance between sensitivity and cost. It has also been illustrated that combining multiple crRNAs can increase sensitivity, negating pre-amplification of target viral nucleic acids prior to Cas12 cleavage[43]. This approach can also be employed to lower the run time of the assay provided that more specific crRNAs with an adjacent PAM motif can be identified. Amplification-free detection, however, might be more challenging for Foc TR4 as fungal genomes are much larger and more complex than viral genomes.

The one-tube RPA-Cas12a assay designed in this study has distinct advantages when considering it for POC detection. Firstly, it does not require tubes to be opened to measure fluorescence, which reduces the risk of contamination. Secondly, the presence of amplified target DNA can be transduced into a variety of signals based on the reporters used, including fluorescent, colorimetric, electrochemical, or electromagnetic. These offer versatile options for development of different type of POC devices[18,32,44]. Thirdly, the assay can be integrated into a microfluidic platform and reagents lyophilized and prestored, which eliminates the need for cold chains and enables rapid detection outside of a laboratory setting[37]. Finally, it can be used for multiplexing different target pathogens of banana on the same microfluidic platform. This is a distinct advantage, as other banana pathogens such as Moko disease, caused by *Ralstonia solancearum*, may causes wilting symptoms like Fusarium wilt[45,46].

In conclusion, a novel RPA-CRISPR/Cas12a-based assay has been developed in this study for the accurate and sensitive detection of Foc TR4. The RPA-CRISPR/Cas12a-based assay has potential to be utilized in-field and in countries with limited resources.

Methods and materials

Fungal isolates and DNA extraction

A total of 91 fungal isolates were used in this study. These included Foc isolates representing all known vegetative compatibility groups (VCGs), lineages and races, non-pathogenic *F. oxysporum* strains and endophytic *Fusarium* spp. associated with banana pseudostems (Supplementary Table S1). All isolates are deposited at the *Fusarium* collection at the Department of Plant Pathology, Stellenbosch University, South Africa.

Isolates were plated onto PDA and grown for 7 days at 25 °C before DNA extraction. A subset of nine isolates was used for initial optimization assays. These include a Foc TR4 isolate (VCG 01213/16) and eight non-target

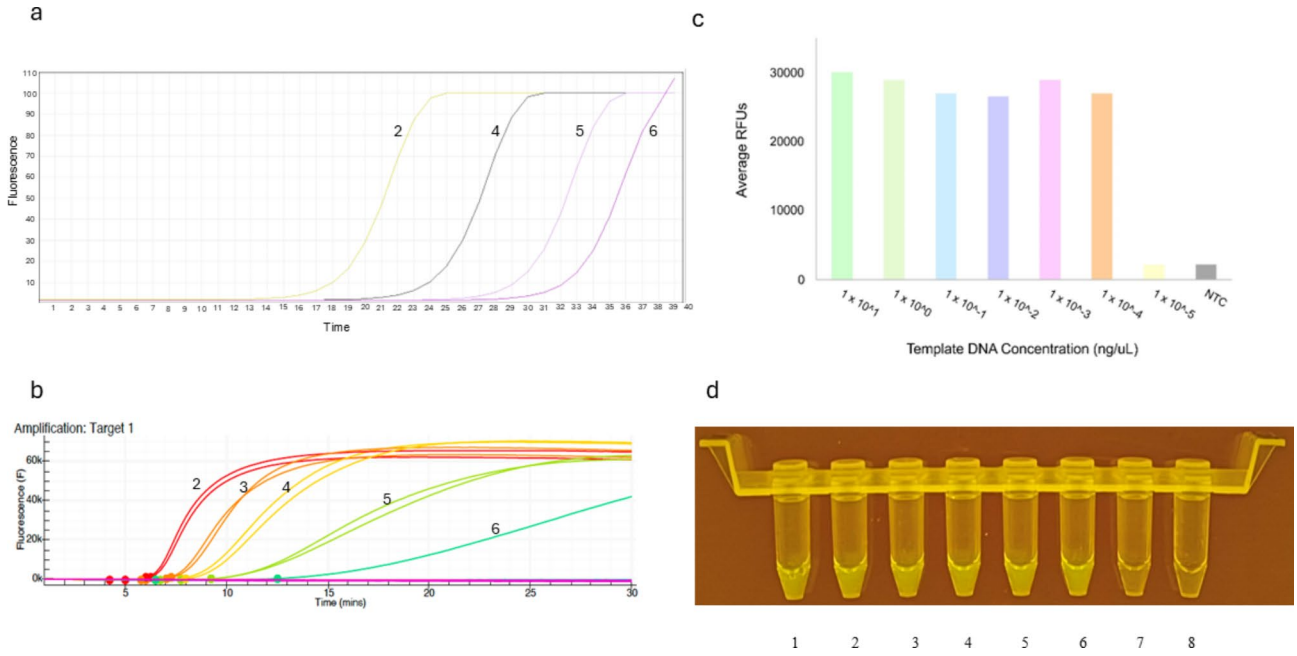

Fig. 4. Comparative sensitivity between *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (Foc TR4)-specific qPCR, LAMP and the optimized RPA-Cas12a assays. (**a**) Foc TR4-specific qPCR[11] (**b**) Foc TR4-specific LAMP[12]. (**c**) The average relative fluorescence units (RFUs) measured in a Genie II fluorometer for the RPA-Cas12a assay. (**d**) Visualization under LED blue light transilluminator. 1–7 show descending target DNA concentrations of 10^{1}–10^{-6} ng/uL, and 8 indicates the non-template control.

Foc isolates (VCGs 0120, 0120/15, 0121, 0122, 0125, 0126, 01210, 01226). The mycelia of the isolates were extracted using DNeasy® Plant Mini Kits (Qiagen, Hilden, Germany), according to manufacturer guidelines. The remaining isolates were extracted by using a simplified extraction method adapted from Osmundson et al.[47]. Mycelia of these isolates were scraped from the Petri dishes with a spatula and placed into a 2-ml micro-centrifuge tube. A 500-µl volume of 0.5 M NaOH and glass beads were then added, and cells lysed in a Tissue Lyser for 5 min. The suspension was then centrifuged for 1 min at 14 000 rpm to pellet the debris. A volume of 5 µl of the supernatant was then diluted in 195 µl of 100 mM Tris-HCl (pH 8). DNA was stored at −20 °C until it was used in downstream applications.

crRNA design, synthesis, and quality control

A non-coding region was selected for the design of a crRNA to overlap Foc TR4-specific primers previously validated for qPCR detection[11]. The qPCR primers proved specific were previously screened against 131 *Fusarium* and Foc isolates[11]. The crRNA was designed using CRISPR RGen tools[48](Supplementary Figure S3) to be complementary to the target DNA sequence, as well as a 5' TTTV protospacer adjacent motif (PAM) on the opposite DNA strand[49,50]. GC content and possible cross-specificity within the *Musa acuminata* genome (Assembly: GCA_904845865.1) was also evaluated. RNA folding version 2.3[51] was used to check if crRNA secondary structures would impede complexing with Cas12a (Supplementary Figure S4).

During initial optimization assays, the crRNA was produced based on the protocol used by Li et al.[36]. For specificity and sensitivity testing, the protocol was adapted. Instead of annealing the T7 forward and reverse primers with a temperature gradient, an already annealed T7 template synthesized by Macrogen Europe (Amsterdam, Netherlands) was used for transcription (Table 2). The transcribed crRNA underwent DNase treatments and was cleaned using RNA clean and concentrator (Zymo Research, Irvine, California, USA) according to the manufacturer's guidelines. The crRNA quality was evaluated using a NanoDrop™ spectrophotometer (Thermo Fischer Scientific, Waltham, Massachusetts, USA).

Primers and fluorescent reporters

PCR and RPA primers were designed up- and downstream of the selected crRNA target. Primer specificity was evaluated using in silico PCR with the iPCRess 2.2.0 package (part of the Exonerate package)[52]. iPCRess facilitates PCR simulations by allowing an input target DNA sequence and designed primers, and predicts the amplification results, including product size and binding position, based on specified parameters such as number of mismatches allowed between primer and target DNA. PCR simulation was conducted against a database constructed from 18 genomes downloaded from NCBI and 37 genomes available in-house. Genomes included different *Fusarium* species, non-pathogenic *F. oxysporum* isolates, *formae speciales* and Foc VCG groups (Supplementary Table S1). For the PCR primers, amplification results were tested when 0 and 3 mismatches were allowed, and for the RPA, 0 and 5 mismatches, respectively. Primers were selected based on their PCR simulation performance. Two previously designed ssDNA reporters[35,36] were used in this study. Both had a fluorescent FAM

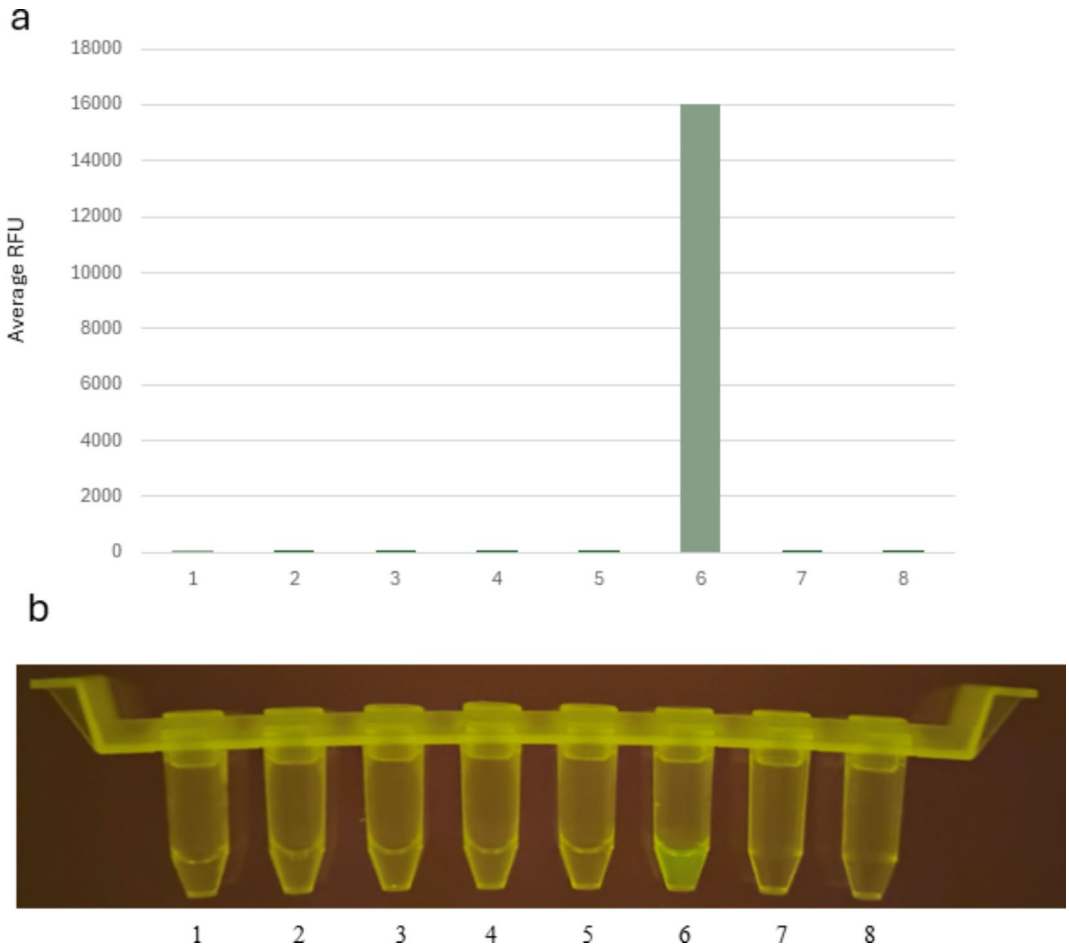

Fig. 5. Specificity test for optimized RPA-Cas12a assay. (**a**) Specificity testing when 1 ng/µL DNA from non-target *Fusarium oxysporum* f. sp. *cubense* DNA samples (1–5) and a Foc tropical race 4 (TR4) positive control (CAV 789) (6) were used. Well 7 and 8 were left blank. The average relative fluorescence units (RFUs) were measured in a Genie II fluorometer (**a**) and visualized under LED blue light transilluminator (**b**).

reporter on the 5′-end and BHQ-1 quencher on the 3′-end. The DNA templates, primers and reporters were synthesized by IDT (Coralville, Iowa, USA) and stored at −20 °C until use (Table 1).

PCR and RPA amplification

PCR amplification was performed using Velocity Taq polymerase (Meridian, Cincinnati, Ohio, USA) according to manufacturer guidelines. For each 50 µL PCR volume; 10 µL of 5x Hi-Fi Reaction Buffer, 0.5 µL of 100 mM dNTP Mix, 1 µL of each primer (20 µM), 1 µL of Velocity Taq, 1.5 µL of DMSO, 3 µL template DNA (10 ng/µL) and 27 µL dH_2O was added. The initial denaturing step at 98 °C was 2 min, followed by 25 cycles of 98 °C, 56 °C and 72 °C for 30 s each. The final extension step was 10 min at 72 °C. PCR products were used for initial optimization of the Cas12a assay.

RPA amplification was performed using TwistAmp Basic kits according to manufacturer guidelines (TwistDx, Maidenhead, UK). In a 50 µL RPA reaction mixture the following was present; 25 µL 2x Reaction Buffer, 9.5 µL dNTPs (final concentration 1.6 mM), 5 µL basic E-mix, 2.5 µL of each primer (10 µM), 2 µL core reaction mix, 2.5 µL MgOAc, and 1 µL of template DNA (10 ng/µL). The RPA was performed at 37 °C for 30 min for initial testing prior to use in a combined RPA-Cas12a assay.

PCR and RPA reactions were incubated in a Veriti 96 Thermal Cycler (Applied Biosystems, Foster City, California, USA). Amplicons were purified with MSB SpinPCRapace kit (Invitek Molecular, Berlin, Germany) and quantified with a NanoDrop™ spectrophotometer (Thermo Fischer Scientific, Waltham, Massachusetts, United States). DNA from Foc isolates mentioned previously (VCGs 0120, 0120/15, 0121, 0122, 0125, 0126, 01210, 01226) was used to evaluate PCR and RPA specificity. CAV 300 (Foc TR4) was used as a positive control and the non-template control (NTC) contained dH_2O rather than DNA. Amplified DNA products were visualized under UV filter with Gel DocTM XR + Imager and Image Lab software 5.2.1 after gel electrophoresis in a 1% agarose gel.

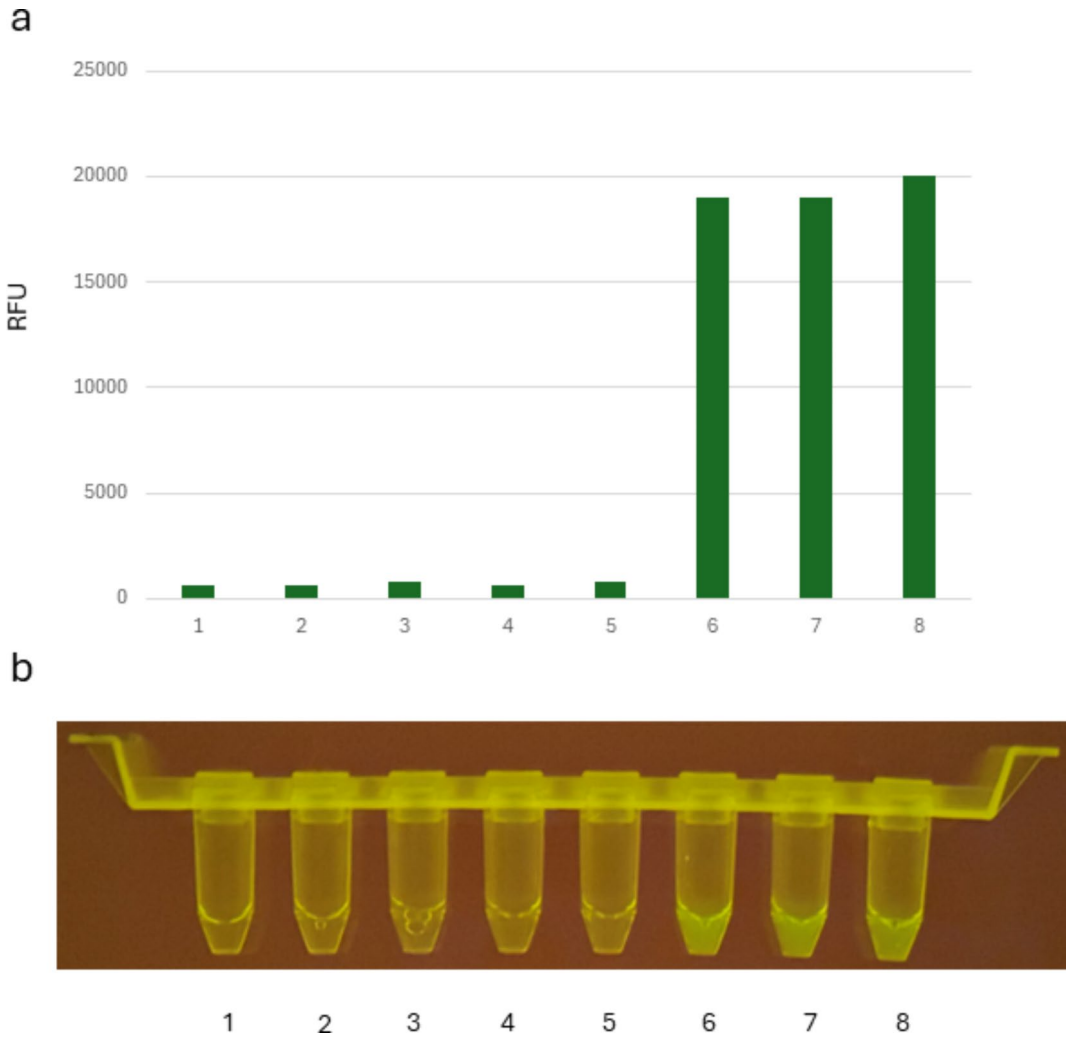

Fig. 6. RPA-Cas12a assay to detect Foc TR4 from naturally infected plant material. Non-infected (1–5) and Foc TR4-infected plant material (6–8) when tested in the optimized RPA-Cas12a reaction. The average relative fluorescence units (RFUs) were measured in a Genie II fluorometer (**a**) and visualized under LED blue light transilluminator (**b**).

Nr	CAV number	Alternative culture no.	Species	VCG	Variety	Country	RPA-Cas12a results
1	/	16	Not isolated	/	Pisang Awak	Vietnam	negative
2	/	17	Not isolated	/	Pisang Awak	Vietnam	negative
3	/	18	Not isolated	/	Pisang Awak	Vietnam	negative
4	/	19	Not isolated	/	Pisang Awak	Vietnam	negative
5	/	20	Not isolated	/	Pisang Awak	Vietnam	negative
6	5507	21	Foc	01213/16	Pisang Awak	Vietnam	positive
7	5508	22	Foc	01213/16	Cavendish	Vietnam	positive
8	5509	23	Foc	01213/16	Cavendish	Vietnam	positive
9	5510	24	Foc	01213/16	Cavendish	Vietnam	positive
10	5511	25	Foc	01213/16	Cavendish	Vietnam	positive
11	5512	26	Foc	01213/16	Cavendish	Vietnam	positive
12	5513	27	Foc	01213/16	Cavendish	Vietnam	positive
13	5515	29	Foc	01213/16	Cavendish	Vietnam	positive
14	5516	30	Foc	01213/16	Cavendish	Vietnam	positive

Table 1. Information on banana pseudostem samples collected during surveillance in Vietnam and tested in the Foc TR4-specific RPA-Cas12a assay.

Oligonucleotide name	Nucleotide Sequence 5'–3'
T7 forward primer	GAAATTAATACGACTCACTATAGGG
Foc TR4 T7 reverse primer	AGAGGAAGTAGCCGAGGAATCTACACTTAGTAG AAATTACCCTATAGTGAGTCGTATTAATTTC
Foc TR4 Guide RNA	UAAUUUCUACUAAGUGUAGAUUCCUCGGCTACUUCCUCU
Foc TR4 PCR F1	CAGTGAGGTCTATTGCTGGA
Foc TR4 PCR R1	ACTACTCATCCTGCTCTGCC
Foc TR4 RPA F1	GTGAAATCCGGCGTGTCTTATAATAGTTATGG
Foc TR4 RPA R1	GACCTCGCTGAATTATATCTAAACCC
Foc TR4 RPA F2	CTCCTCTCCCTTGGCCTTATCTTCGAGGCC
Reporter of Li et al. (2018)	6FAM/NNNNNNNNNNN/BHQ1
Reporter of Lv et al. (2021)	6FAM/CCCCCCCC/BHQ1

Table 2. Sequences of oligonucleotides used in this study.

Optimizing the Cas12a assay

To test if complexed crRNAs and Cas12a would cleave PCR amplified Foc TR4 DNA into expected sizes, digestion reactions were evaluated in vitro. The positive control reaction contained 1 µM LbCas12a protein (New England Biolabs, Ipswich, Massachusetts, USA), 1 µM crRNA, 2 µL 10x NEB 2.1 buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, 100 µg/ml BSA, pH 7.9), 100 nM Foc TR4 PCR amplicon and dH_2O in a 20 µL reaction volume. The negative control reaction did not contain crRNA. Reactions were incubated for 1 h at 37 °C in a Veriti 96 Thermal Cycler. To denature the Cas12a protein, 1 µL of proteinase K (New England Biolabs, Ipswich, Massachusetts, USA) was added per reaction and incubated for 10 min at 65 °C. Amplified DNA products were visualized as described previously.

Once it was confirmed that Cas12a could perform crRNA-mediated cleavage of target Foc TR4 DNA, conditions were optimized for efficient collateral cleavage of the reporter sequence. Optimum reporter sequences (Table 1), Cas12a-crRNA concentrations and Cas12a: crRNA ratios (1:1, 1:1.5 and 1:2) were investigated. Reactions were set up as follows: 50–300 nM LbCas12a protein, 50–300 nM crRNA, 100 nM reporter, 2 µL 10x NEB 2.1 buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, 100 µg/ml BSA, pH 7.9), 20 nM Foc TR4 PCR amplicon and dH_2O in a 20 µL reaction volume. Negative controls were void of target TR4 amplicons. Reactions were incubated in a Genie II (OptiGene, Horsham, United Kingdom) portable fluorometer for 1 h at 37 °C. Relative fluorescence units (RFUs) for each parameter were compared in triplicate and the whole experiment was repeated. Statistical analysis was performed in XLSTAT (2022 version) and considered the Shapiro Wilk's test for normality, analysis of variance (ANOVA) and Tukey's honest significant difference (HSD) test.

RPA-Cas12a in vitro Foc TR4 detection

To test if the RPA and Cas12a reactions could be combined, reaction mixtures for the two assays were combined in the same tube (Fig. 3). The volume of the RPA reaction described previously was reduced to 10 µL, and the RPA reaction mix placed in the reaction tube lid, while the optimized Cas12a reaction mix (20 µL) was placed in the tube base (Cas12a, crRNA and reporter concentrations were kept at 200 nM). Template DNA at 20 nM was added to the RPA reaction prior to closing the lid. In the first one-tube system, tubes were incubated in the Optigene Genie II machine for 60 min. In the second one-tube system, tubes were incubated for 30 min, removed from the machine and mixed by placing it in a benchtop MyFuge™ 12 micro centrifuge (Benchmark scientific, New Jersey USA) for 5 s and returning it to the Genie II machine for 30 min. Fluorescence was recorded for each system during the last 30 min incubation step. Average RFUs of the RPA and Cas12a reactions done in separate tubes, were compared to two different one-tube RPA-Cas12a systems when mixed or not. All reactions were done in triplicate, and the whole experiment was repeated. Statistical analysis was performed in XLSTAT (2022 version) and considered the Shapiro Wilk's test for normality, analysis of variance (ANOVA) and Tukey's honest significant difference (HSD) test.

Specificity and sensitivity testing

DNA of 91 *Fusarium* isolates were used for specificity testing of the combined RPA-Cas12a assay (Supplementary Table S1). The sensitivity of the assay was evaluated using a ten-fold serial dilution of Foc TR4 DNA (from 10 to10^{-5}ng/uL). The same DNA concentrations were also tested in qPCR[11]and LAMP[12] to make comparative evaluations of sensitivity. In all instances, non-template controls (NTCs) were included to ensure that cross-contamination did not occur.

To assess if visualization was possible with the naked eye, tubes for each reaction included in the specificity and sensitivity testing were visualized under LED blue light at a 465 nm excitation wavelength using a SmartBlue™ Transilluminator (Accuris Instruments, Edison, USA), and images captured with a phone camera. Detection of Foc target DNA was positive if the sample tube showed visible fluorescence but considered negative if not.

RPA-Cas12a Foc TR4 detection from naturally infected samples

To determine the ability of the RPA-Cas12a assay to detect Foc TR4 from naturally infected samples, pseudostem samples collected during a survey in Vietnam were tested. The necessary permission was granted to collect

these samples from collaborators at Plant Protection Research Institute, Hanoi, Vietnam. The tested samples included eight samples previously confirmed as Foc TR4 by VCG testing and five samples that were negative for Foc TR4 (Table 1). Pseudostem materials were transferred into BIOREBA extraction bags (BIOREBA, Reinac, Switzerland), 2 ml 0.5 M NAOH added, and ground using a pestle. The extracts were then diluted by adding 5 µl of extract in 195 µL 100mM Tris-HCl (pH 8.0). The DNA extracts were tested with the optimized RPA-Cas12a assay as previously described. Tubes for each reaction were also visualized under LED blue light at 465 nm excitation wavelength using a SmartBlue™ Transilluminator and images captured with a phone camera.

Data availability

The datasets generated and analyzed for this publication are available from the authors on reasonable request at diane@sun.ac.za where not provided within the manuscript and supplementary information.

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Author contributions

D.M., A.V. and I.R. acquired funding. D.M., M.M., J.B., A.V., I.R., W.P., U.O. and M.C. conceived experiments. M.M, J.v.d.L., U.A., N.M., S. R., B.C. and I.R. conducted experiments. M.M., J.v.d.L., S.C., B.C., S.R. and D.M. conducted data analysis. All authors reviewed the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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