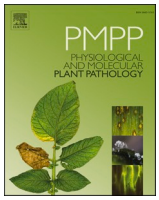
Physiological and Molecular Plant Pathology 144 (2026) 103304

Contents lists available at ScienceDirect

Physiological and Molecular Plant Pathology

journal homepage: www.elsevier.com/locate/pmpp



Development and analytical validation of a fluorescent recombinase polymerase amplification-assay for real-time detection of *Phyllosticta citricarpa*, the causative agent of Citrus Black Spot

Federico La Spada [a,*], Rossana Parlascino [a], Mario Riolo [a], Sebastiano Conti Taguali [a], Antonella Pane [a], Matteo Garbelotto [b], Bryant Davenport [c], Marcos Amato [d], Santa Olga Cacciola [a]

[a] Department of Agriculture, Food and Environment, University of Catania, Via Santa Sofia N. 100, Catania, 95123, Italy
[b] Department of Environmental Science, Policy and Management, University of California, Mulford Hall, 130 Hilgard Way, Berkeley, CA, 94720, United States
[c] Agdia Inc., 52642 County Road 1, Elkhart, IN, United States
[d] Agdia EMEA, 31 Rue de Seine, Soisy-sur-Seine, 91450, France

ARTICLE INFO

Keywords:
tef1
Isothermal diagnostics
Quarantine plant pathogen
Plant health surveillance
Crude plant macerate

ABSTRACT

Citrus Black Spot (CBS), caused by the fungus *Phyllosticta citricarpa*, is a disease of major diagnostic relevance in the citrus industry, as its causative agent is a regulated quarantine organism subjected to surveillance in the European and Mediterranean Plant Protection Organization (EPPO) Region. To develop a rapid molecular assay for pathogen detection, a real-time fluorescence recombinase polymerase amplification (RPA) assay targeting a 134 bp region of *tef1* was developed through sequence alignment and in-silico specificity analysis. The assay showed analytical specificity for *P. citricarpa*, with no amplification from diagnostically relevant non-target citrus-associated pathogens, including *Phyllosticta capitalensis* and *P. citriasiana*. Analytical sensitivity, evaluated on plasmid and purified genomic DNA, enabled detection down to 1.0×10^{-7} ng/μL of plasmid (~100 copies per reaction) and 3.5×10^{-3} ng of genomic DNA per reaction (~113 genome copies). The workflow was further validated on crude citrus peel macerates spiked with *P. citricarpa* mycelium, with reliable detection to 1.0 mg/mL across matrices from *Citrus sinensis*, *C. limon*, and *C. reticulata*. Performance benchmarking against the EPPO-recommended TaqMan qPCR showed comparable sensitivity together with operational simplicity and tolerance to amplification inhibitors. The ability to detect *P. citricarpa* directly in crude citrus peel macerates, combined with rapid real-time fluorescent readout at low temperature and minimal equipment requirements, lays the foundation for the use of this assay as a simple and rapid molecular detection approach for citrus-associated plant matrices.

1. Introduction

Citrus Black Spot (CBS) is a disease caused by the fungus *Phyllosticta citricarpa* (McAlpine) Van der Aa (formerly *Guignardia citricarpa*), in the family *Phyllostictaceae*, which is a major phytopathological concern for citrus production and disease diagnostics [1,2]. *Phyllosticta citricarpa* is a quarantine organism in several countries, including the members of the European and Mediterranean Plant Protection Organization [1]. It is not known to occur in European Union (EU) countries. Therefore, restrictions and surveillance measures are in place for the import of citrus fruits and host plants from third countries, to prevent the introduction and subsequent enlargement of the geographic range of the pathogen [3–5]. The symptoms of CBS include a variety of fruit and leaf blemishes, leading to losses in fruit quality and, in severe cases, premature fruit drop [2,6–8]. CBS primarily affects sweet orange (*Citrus sinensis*), lemon (*Citrus limon*), and tangerine (*Citrus reticulata*), which are considered the only confirmed hosts for *P. citricarpa* under natural infection conditions [9]. In addition, *P. citricarpa* has been reported to produce secondary metabolites with phytotoxic activity, further supporting the phytopathological relevance and metabolic potential of this pathogen [10,11]. Despite the wide host range generally attributed to species within the genus *Phyllosticta*, recent studies have confirmed a high degree of host

* Corresponding author.
E-mail address: federico.laspada@unict.it (F. La Spada).

https://doi.org/10.1016/j.pmpp.2026.103304
Received 14 April 2026; Received in revised form 25 May 2026; Accepted 31 May 2026
Available online 1 June 2026

Table 1
Isolate code, species, host, country of origin, year of recovery, reference study, and RPA outcome of fungal and oomycete isolates used in this study.

Isolate code	Species	Host	Country of Origin	Year	Reference	RPA outcome (+[a]/-[b])	van Gent-Pelzer TaqMan qPCR outcome (Cq)
1G	*Phyllosticta citricarpa*	*Citrus limon*	Tunisia	2022	[23]	+	21.3
PC3	*P. citricarpa*	*C. limon*	Tunisia	2022	This study	+	26.8
PC11	*P. citricarpa*	*C. limon*	Tunisia	2022	This study	+	21.2
PC12	*P. citricarpa*	*C. limon*	Tunisia	2022	This study	+	25.2
PC13	*P. citricarpa*	*C. limon*	Tunisia	2022	This study	+	23.4
PC14	*P. citricarpa*	*C. limon*	Tunisia	2022	This study	+	26.5
PC16	*P. citricarpa*	*C. limon*	Tunisia	2022	This study	+	24.3
PC18	*P. citricarpa*	*C. limon*	Tunisia	2022	This study	+	21.1
CBS 828.97	*P. citricarpa*	*Citrus aurantium*	Brazil	1997	[27]	+	23.2
CBS 102374	*P. citricarpa*	*C. aurantium*	Brazil	1999	[27]	+	21.7
CBS 127455	*P. citricarpa*	*Citrus sinensis*	Australia	2010	[28]	+	20.9
CBS 127451	*P. citricarpa*	*Citrus reticulata*	Australia	2010	[28]	+	21.4
CBS 127454	*P. citricarpa*	*C. limon*	Australia	2010	[6]	+	24.3
CBS 141357	*P. paracitricarpa*	*C. limon*	Greece	2016	[6]	+	21.3
CBS 141359	*P. paracitricarpa*	*C. limon*	Greece	2016	[6]	+	25.5
CBS 120426	*Phyllosticta citriasiana*	*Citrus maxima*	China	2006	[27]	-	26.8
CBS 120486	*P. citriasiana*	*C. maxima*	Thailand	2006	[6]	-	27.1
CBS 117118	*Phyllosticta capitalensis*	*Musa acuminata*	Indonesia	2005	[27]	-	-
Pt2	*P. tracheiphilus*	*C. limon*	Italy	2021	(El boumlasy et al., 2021)	-	-
Pt22	*P. tracheiphilus*	*C. limon*	Italy	2022	[29]	-	-
Pt3	*P. tracheiphilus*	*C. limon*	Italy	2022	[29]	-	-
646	*Alternaria alternata*	*Citrus clementina*	Italy	2021	[30]	-	-
ID8O10	*A. alternata*	*C. sinensis*	Italy	2022	[31]	-	-
ID3O16	*A. alternata*	*C. sinensis*	Italy	2022	[31]	-	-
AaMDC1	*Alternaria arborescens*	*C. sinensis*	Italy	2020	[30]	-	-
CAM	*Colletotrichum karsti*	*Camellia* sp.	Italy	2020	[32]	-	-
ALL2T	*C. karsti*	*C. sinensis*	Italy	2018	[32]	-	-
C2	*Colletotrichum gloeosporioides*	*C. limon*	Italy	2020	[32]	-	-
AC35	*C. gloeosporioides*	*C. sinensis*	Italy	2018	[32]	-	-
AC38	*C. gloeosporioides*	*C. sinensis*	Italy	2018	[32]	-	-
ID5O13	*C. gloeosporioides*	*C. sinensis*	Italy	2022	[31]	-	-
ID1O29	*C. gloeosporioides*	*C. sinensis*	Italy	2022	[31]	-	-
CBS 145950	*Fusarium proliferatum*	*Musa acuminata*	Costa Rica	2019	[30]	-	-
CBS 145949	*Fusarium sacchari*	*Musa acuminata*	Costa Rica	2019	[30]	-	-
UWS14	*Colletotrichum acutatum*	*C. limon*	Italy	2020	[30]	-	-
T3-B-K1A	*Phytophthora nicotianae*	*C. limon*	Italy	2021	[30]	-	-
T2-C-M1A	*P. nicotianae*	*C. limon*	Italy	2020	[30]	-	-
Ax1Ar	*Phytophthora citrophthora*	*C. limon*	Italy	2021	[30]	-	-
P1PP0	*Penicillium digitatum*	*C. sinensis*	Italy	2021	[30]	-	-
ID4O10	*P. digitatum*	*C. sinensis*	Italy	2022	[31]	-	-
ID10O4	*P. digitatum*	*C. sinensis*	Italy	2022	[31]	-	-
T4N0	*Penicillium italicum*	*C. sinensis*	Italy	2022	[23]	-	-

[a] + = positive.
[b] - = negative.

specificity: for instance, *P. citriasiana*, a morphologically similar species, has been detected exclusively on pomelo (*C. maxima*), while no evidence supports the natural occurrence of *P. citricarpa* on this host [9,12].

Accurate detection of *P. citricarpa* is essential for managing CBS, especially in regions where the pathogen has quarantine status [1]. The molecular diagnostic method currently recommended by EPPO for the detection of CBS is the TaqMan real-time PCR assay developed by Van Gent-Pelzer et al. [13] based on the presence of diagnostic SNPs in the DNA sequence of the transcribed spacer (ITS) regions of the ribosomal DNA (rDNA). As a multicopy genomic marker, the ITS provides strong amplification signals and confers high sensitivity to the assay. However, although highly effective under most diagnostic conditions, validation studies have occasionally revealed limitations in specificity. These include incidental amplification of *Phyllosticta capitalensis*, a ubiquitous endophytic and saprophytic species, and *P. citriasiana*, a phylogenetically close relative of *P. citricarpa that* is not relevant for CBS diagnostics, as it occurs only on *Citrus maxima*, a non-host for *P. citricarpa* [8,9,14–16]. In response to these limitations, several alternative qPCR assays have been developed using different genomic regions. Zajc et al. [8] introduced a *tef1*-based qPCR method with high specificity, which was later validated through a ring Test Performance Study (TPS) confirming its reliability in complex citrus matrices [17]. Similarly, Ioos et al. [18]

developed an assay leveraging comparative genomics, designed to exclude common non-targets such as *P. capitalensis* and *P. citriasiana*. Some of these efforts also aimed to distinguish *P. citricarpa* from *P. paracitricarpa*, a taxon originally considered separated [6]. However, recent phylogenomic analyses have resolved this ambiguity, demonstrating that *P. paracitricarpa* represents intraspecific variation within *P. citricarpa*, rendering the distinction unnecessary [19].

Beyond real-time PCR, isothermal amplification technologies are gaining momentum due to their portability and rapidity. Loop-mediated isothermal amplification (LAMP), as developed by Tomlinson et al. [20], offers a promising rapid tool. Like the qPCR method of van Gent-Pelzer et al. [13], this assay targets the multicopy ITS region, potentially conferring high sensitivity. However, as with other ITS-based methods, LAMP is subject to specificity challenges, especially in distinguishing *P. citricarpa* from closely related species. Moreover, it still requires purified DNA, which limits its utility in simplified diagnostic workflows and direct analysis of plant material [1].

Recombinase Polymerase Amplification (RPA) represents a more recent isothermal technique with unique advantages [21,22]. Its ability to amplify DNA at a constant low temperature within minutes, combined with compatibility with crude plant macerates, makes RPA particularly attractive for rapid molecular diagnostics [23]. Notably, RPA can also be adapted for real-time detection with fluorescence-based probes, thus combining the rapidity and portability of isothermal amplification with the quantitative capabilities of qPCR. This configuration allows for sensitive and rapid detection, while maintaining simplicity in terms of instrumentation and workflow. RPA has already proven to be a robust, user-friendly, and cost-effective alternative to PCR-based methods for the detection of various citrus pathogens [24–26]. Despite these advancements, CBS molecular diagnosis still lacks a rapid RPA assay tailored to *P. citricarpa* and suitable for pathogen detection in crude citrus plant matrices without DNA purification.

Therefore, the present study developed a novel real-time fluorescent RPA assay for the detection of *Phyllosticta citricarpa*. The specificity and analytical sensitivity of the RPA assay were evaluated in comparison with the official EPPO standard TaqMan qPCR assay [1]. The performance of the novel RPA assay in crude citrus matrices was further assessed using samples consisting of target *P. citricarpa* propagules mixed with crude macerates of orange, lemon, and tangerine peel.

2. Materials and methods

2.1. Fungal and oomycete material

The study included genomic DNA (gDNA) from target and non-target fungal and oomycete isolates listed in Table 1, as well as fresh mycelium of *Phyllosticta citricarpa* isolate 1G [23] for the assays performed in crude citrus peel macerates. The target panel comprised *Phyllosticta citricarpa* isolates, including reference strains and additional Tunisian isolates characterized at species level in the present study. The non-target panel included closely related *Phyllosticta* spp., such as *P. paracitricarpa* and *P. capitalensis*, as well as several specimens of fungal and oomycete species commonly associated with citrus (Table 1); these latter comprised *Phytophthora*, *Alternaria*, *Colletotrichum*, *Fusarium*, *Penicillium*, and *Plenodomus tracheiphilus*, which were characterized at species level in previous studies [23,30,32,33]. In the present study, species-level characterization of *Phyllosticta* specimens was performed by amplification, sequencing and analysis of the translation elongation factor 1-α gene (*tef1*), using primer pair EF1-728F/EF2 [34,35]. Amplification reactions were performed using the *Taq* DNA polymerase recombinant (Invitrogen™, Carlsbad, 254 CA, USA) following the manufacturer's instructions. In detail, each PCR reaction was carried out in a 25.0 μL reaction mix containing 2.50 μL of PCR buffer, 0.50 μL of dNTP mix, 0.75 μL of $MgCl_2$, 1.25 μL of each primer, 0.20 μL of *Taq* DNA polymerase, 1.00 μL of DNA and nuclease-free water to a final volume of 25.0 μL. The thermo-cycler conditions were as follows: 94 °C for 3 min; followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and then 72 °C for 10 min. Obtained PCR amplicons were sequenced by an external service (Macrogen, Seoul, South Korea) and species identifications were carried out by subjecting obtained sequences to BLAST searches on NCBI nucleotide database. The *tef1* sequences generated in this study have been deposited in GenBank under the following accession numbers: PZ373802, PZ373803, PZ373804, PZ373805, PZ373806, PZ373807, PZ373808.

2.2. Selection of Phyllosticta citricarpa target region and development of RPA primers and probe

A 134 bp target barcode belonging to the *tef1* gene of *P. citricarpa* was selected by alignment in MEGAX (MEGA - Molecular Evolutionary Genetics Analysis) of several NCBI deposited *tef1* sequences of officially identified isolates of the target species *P. citricarpa* from geographically distinct origins, including Brazil, Australia, Zimbabwe, and South Africa (Genbank accession numbers: FJ538376, FJ538371, JF343605, JF343601, FJ538375, JF343602, KF289221, KY855939, KY855940, KY855941), as well as sequences of sister species from the genus *Phyllosticta*, including *P. paracitricarpa*, *P. capitalensis*, *P. citriasiana*, and *P. paracapitalensis* [6]. Barcode specificity was preliminarily confirmed by BLAST searches on NCBI nucleotide database. Primers for the RPA amplification of the 134 bp selected barcode were designed by using the Primer BLAST NCBI tool on the basis of sequence differences among *P. citricarpa* and the above-mentioned closely related *Phyllosticta* spp.

Specificity for *P. citricarpa* of designed primers was preliminarily tested by conventional PCR, performed as described above on genomic DNA (gDNA) of each organism listed in Table 1. PCR products were revealed by electrophoresis in TAE 1X Agarose gel, sequenced as above described and aligned in MEGAX.

The RPA probe was designed on a 52 bp fragment within the selected barcode; the probe was labeled by a THF abasic–site, flanked by a FAM and a corresponding dt-Q group; additionally, the probe was blocked at the end 3′ by a C3-Spacer (C3-S) modification group. The specific sequences of the primers and probe used for the RPA assay have been deposited in Figshare (DOI: https://doi.org/10.6084/m9.figshare.31980219).

2.3. RPA amplifications

Each RPA reaction was conducted in a 0.25 mL tube (AmplifyRP Discovery kits Agdia Emea, France) containing RPA mastermix pelleted reagents, a rehydration buffer (61.5 % v/v), forward (RPA_PC_F) and reverse (RPA_PC_R) primers (0.44 μM each), the XRT probe (RPA_PC_Probe) (0.13 μM), MgOAc (14.59 mM), 1.0 μL of DNA template and nuclease free water to a final volume of 25.0 μL. Amplifications were carried out in the AmpliFire® Isothermal Fluorometer (Agdia Emea, France); each run consisted of a 20 min heating at the constant temperature of 39 °C. Each RPA run included positive and negative controls. Each reaction was carried out in triplicate and each run was repeated three times. The amplification curve files were used for constructing linear regression curves and statistical analyses.

2.4. van Gent-Pelzer TaqMan qPCR reactions

In this study, the QuantiNova™ Probe PCR Kit (Qiagen) was used for performing TaqMan qPCR reactions according to the primers and probe assay of Van Gent-Pelzer et al. [13]. In detail, each reaction consisted of 5.0 μL of 2x QuantiNova Probe PCR Master Mix, 0.4 μM of each primer (GcF1: 5′-GGTGATGGAAGGGAGGCCT-3'; GcR1: 5′-GCAACATGGTAGATA CACAAGGGT-3′), 0.2 μM of the TaqMan probe GcP1 (5′-AAAAAGCCGC CCGACCTACCTTCA-3′), 1.0 μL of DNA template, and nuclease-free water to a final volume of 10.0 μL. Amplifications were run on the QuantGene 9600 Fluorescent Quantitative Detection System (Bioer Technology, Hangzhou, Zhejiang, China). Amplification conditions were 2 min at 95 °C

(PCR initial activation step), followed by 40 cycles of 95 °C for 5 s (denaturation) and 60 °C for 5 s (combined annealing/extension). Each run included negative and positive controls. All Real-Time PCR reactions were carried out in triplicate, and each run was repeated three times. Amplification curves were considered valid (positive signal) when recorded Cq values were under 35. The amplification curve files were used for constructing linear regression curves and statistical analyses.

2.5. Assessment of RPA assay specificity and inclusivity

The specificity of the RPA assay was verified against purified gDNA from fruit peel of hosts of *P. citricarpa*, including *C. limon*, *C. sinensis*, and *C. reticulata*, as well as on gDNA from a panel of fungal and oomycete species commonly associated with citrus crops and citrus disease diagnostics. This included closely related *Phyllosticta* species relevant to CBS differential diagnosis and representatives of fungal and oomycete genera frequently associated with citrus diseases or citrus tissues, including *Alternaria*, *Colletotrichum*, *Fusarium*, *Penicillium*, *Plenodomus*, and *Phytophthora* (Table 1). For comparative purposes, the specificity of the developed RPA assay was compared to that of the van Gent-Pelzer TaqMan qPCR assay tested in all the organisms listed in Table 1 and performed as described in Section 2.4.

The inclusivity of the RPA assay was assessed using gDNA from *P. citricarpa* specimens from different geographic regions, including Tunisia (*i.e.*, 1G, PC3, PC11, PC12, PC13, PC14, PC16, PC18), Brazil (*i.e.*, CBS 828.97, CBS 102374), and Australia (*i.e.*, CBS 127455, CBS 127451, CBS 127454) (Table 1).

2.6. Sensitivity of the RPA assay

2.6.1. Standardization of RPA analytical sensitivity using a plasmid-inserted P. citricarpa target region

RPA assay sensitivity was standardized using a reference curve constructed from amplification values (cycles) obtained through serial dilution of a plasmid harboring the selected *P. citricarpa* target region, obtained by cloning with the TOPO® TA Cloning® Kit for Sequencing (Invitrogen™). First, the 134 bp *P. citricarpa* target of the *tef1* gene was amplified by using the same forward and reverse primers employed in the RPA assay (sequences available at https://doi.org/10.6084/m9.figshare.31980219). This PCR amplification was conducted in a reaction mix containing 5.0 µL of 10× PCR buffer, 0.5 µL of dNTPs at the concentration 50.0 mM, forward and reverse primers (1.0 µM each), 1 U of *Taq* DNA polymerase, 10.0 ng of gDNA of the *P. citricarpa* isolate 1G and nuclease-free water to the final volume of 50.0 µL. The thermocycler conditions were as follows: 94 °C for 3 min; followed by 35 cycles of 94 °C for 45 s, 68 °C for 30 s, and 72 °C for 30 s; and then 72 °C for 10 min. Then, the presence of the specific amplified band was confirmed by electrophoresis in 2 % agarose gel in 1× TAE buffer. The PCR product was then subjected to TOPO® Cloning, enabling the insertion of the amplified target into the pCR™4-TOPO® vector. The resulting pCR™4-TOPO® construct was subsequently transformed into DH5α™-T1R competent *Escherichia coli* using the Transform One Shot® TOP10 and DH5α™-T1R Competent Cells Protocol. Following transformation, plasmid DNA was isolated using the PureLink® Quick Plasmid Miniprep Kit (Invitrogen™) and analyzed by sequencing to confirm the successful cloning of the *P. citricarpa* target. Once verified, 10-fold serial dilutions of the plasmid DNA were established, ranging from 1.0 ng/µL (corresponding to 1×10^{8} plasmid copies/µL) to 1.0×10^{-8} ng/µL (corresponding to 1×10 plasmid copies/µL) and subjected to RPA runs as described at paragraph 2.3. The RPA standard curve was generated by plotting the amount of plasmid DNA (copies/µL) against the time to positivity (Tp).

2.6.2. Assessment of the analytical and matrix-based performance of the RPA assay

The analytical sensitivity of the RPA assay was evaluated using serial dilutions (at a concentration ranging from 1.0 to 1×10^{-6} ng/µL) in 1X TE buffer (10 mM Tris–HCl and 1 mM EDTA; pH 8.0) of gDNA from *P. citricarpa* isolate 1G, extracted from fresh mycelium using the PowerPlant® Pro DNA Isolation Kit following the manufacturer's instructions. This was compared to the sensitivity of the van Gent-Pelzer TaqMan qPCR assay. RPA and TaqMan qPCR runs were set up as described in Sections 2.3. and 2.4., respectively.

The matrix-based performance of the RPA assay was assessed separately in three crude citrus peel matrices, using samples consisting of fresh mycelium of *P. citricarpa* isolate 1G spiked with a crude macerate of fruit peel from *Citrus limon*, *Citrus sinensis* or *Citrus reticulata*. In detail, the crude macerate was prepared by homogenizing 100.0 mg of

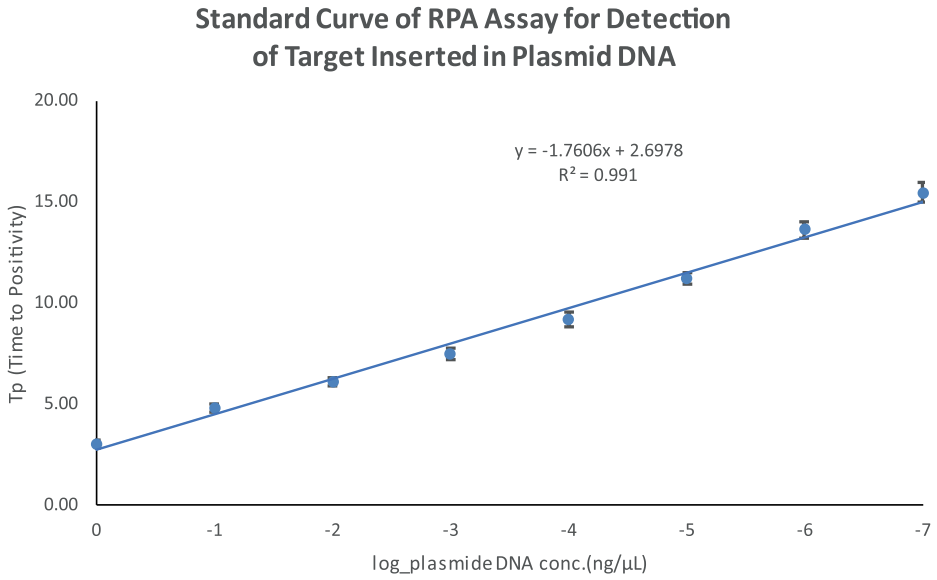


Fig. 1. Standard curve of the RPA assay for detection of the target inserted in plasmid DNA. The graph shows the time to positivity (Tp) plotted against the logarithm of plasmid DNA concentration (ng/µL). Each point represents the mean Tp of replicate reactions, with error bars indicating standard deviation. The blue line represents the linear regression fitted to the data (linear equation and R2 value is reported). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

pulverized plant material (fruit peel ground in liquid nitrogen) with 1 mL of AMP1 lysis buffer (Agdia Emea, France) in a 2 mL tube. After homogenization, the tube was centrifuged, the supernatant (crude macerate) recovered and added to fresh mycelium of *P. citricarpa* isolate 1G in a 1.0 mL: 100.0 mg ratio. The mycelium was mechanically ground for 1 min with a Kontes pestle (Fisher Scientific, Waltham, MA, US) and then serial dilutions (ranging from 100.0 to 0.1 mg/mL) in plant crude macerate were prepared and tested in RPA runs.

2.6.3. Data analysis

Linear regression curves of RPA and Real Time-PCR were calculated by using Excel software. The Limit of Detection (LoD) at 95 % probability and its confidence interval were estimated through Probit regression analysis using a generalized linear model in R (version 4.4.0), with $\log_{10}$-transformed doses and a Probit link function, based on ten replicates per DNA concentration. In this study the genome copy number was calculated using the estimated genome size of the reference isolate *P. citricarpa* CBS 122670 (34.43 Mbp; [19]), knowing that the mean weight of one nucleotide pair is 1.023×10^{-9} pg [36] and that the selected target (a portion of the *tef1* gene) occurs in the genome in a single copy [8].

3. Results

3.1. Assay design and preliminary specificity tests

BLAST searches of the NCBI nucleotide database confirmed *in silico* that the selected 134 bp *tef1* barcode was exclusive to *P. citricarpa* (100 % identity), with *P. paracitricarpa* the nearest non-target (97.76 %). Preliminary specificity tests showed amplification in *P. citricarpa* and cross-amplification in *P. paracitricarpa* by conventional PCR, with no signal in the other species tested. A real-time fluorescent probe was then incorporated to finalize the RPA assay.

3.2. Specificity of the RPA assay

Specificity tests of RPA assay gave positive results with gDNA of the target species *P. citricarpa* as well as with that of isolates designated as *P. paracitricarpa* (Table 1). No RPA amplifications were recorded with either gDNA from plant material or from non-target organisms. Concerning the van Gent-Pelzer TaqMan qPCR, this assay gave positives with gDNA from all tested *P. citricarpa* strains, as well as with that from strains of *P. paracitricarpa*, *P. citriasiana* and *P. capitalensis* (Table 1).

3.3. Sensitivity of the RPA assay

The analytical sensitivity of the RPA assay was standardized through amplification runs conducted on serial dilutions of plasmid DNA harboring the target insert. The related standard curve shows a statistically supported linear range of seven orders of magnitude, with amplifications up to 1.0×10^{-7} ng/μL of plasmid DNA (corresponding to ~ 100 plasmid copies/reaction) (Fig. 1).

Tests conducted on serial dilutions of purified gDNA of *P. citricarpa* isolate 1G at concentrations ranging from 1.0 to 1×10^{-6} ng/μL showed that the RPA assay produced positive amplifications up to 3.5×10^{-3} ng of gDNA (corresponding to ~ 113 genome copies/reaction) per reaction, with a 95 % LoD of about 7.52×10^{-3} ng of gDNA (CI: 4.58×10^{-3} – 1.23×10^{-2} ng of gDNA) (Fig. 2). qPCR tests on the same serial dilutions of gDNA conducted according to the van Gent-Pelzer TaqMan qPCR assay gave positive amplifications up to 1.0×10^{-4} ng of gDNA, with a 95 % LoD of about 2.28×10^{-3} ng of gDNA (CI: 6.93×10^{-4} – 7.49×10^{-3} ng of gDNA) (Fig. 2).

Tests to evaluate the matrix-based performance of the RPA assay were conducted on samples consisting of fresh mycelium from the *P. citricarpa* isolate 1G, spiked at various concentrations (ranging from 100.0 to 0.1 mg/mL) with crude macerate of fruit peel from *C. limon*, *C. sinensis*, or *C. reticulata*. Results showed that the RPA assay was able to detect *P. citricarpa* when the mycelium was dispersed in crude plant macerate from all three citrus species at concentrations as low as 1.0 mg/mL (Fig. 3).

The 95 % LoD in crude orange peel macerate was approximately 5.58 mg/mL of mycelium (CI: 1.22–25.35 mg/mL), in crude lemon peel macerate 8.79 mg/mL (CI: 1.95–39.59 mg/mL), and in crude tangerine peel macerate 7.12 mg/mL (CI: 1.56–32.40 mg/mL) (Fig. 3).

4. Discussion

The challenges posed by quarantine plant pathogens, heightened by globalization, climate change, and intensified international trade, underscore the urgent need for rapid, reliable, and deployable diagnostic tools in plant health surveillance systems [37–40]. Within this context, the development of molecular assays combining rapidity, analytical specificity, and robustness in complex biological matrices is particularly relevant for the diagnosis of regulated fungal pathogens such as *P. citricarpa*. Early detection is pivotal to prevent pathogen introduction and establishment and mitigate economic losses, particularly in the case of quarantine organisms such as *P. citricarpa*, the causative agent of CBS. This pathogen is listed as a quarantine pest in several regions, including the EU, and is subject to strict regulatory oversight due to its potential impact on citrus fruit production and restriction of international trade [1,2,5,9]. In recent years, molecular diagnostics have become the cornerstone of plant pathogen detection. However, conventional PCR and qPCR assays, while offering high sensitivity and specificity, often require laboratory infrastructure, extensive sample preparation, and technical expertise, thereby limiting their simplification for rapid direct testing of plant samples [20,22–24,26]. In contrast, isothermal amplification methods, such as the RPA, offer significant advantages in terms of operational simplicity, speed, and portability [23]. These characteristics make RPA particularly attractive for phytopathological diagnostics when rapid molecular detection must be achieved without extensive DNA purification [41,42]. To address these diagnostic needs, the present study developed and analytically validated a novel real-time fluorescent RPA assay for the detection of *P. citricarpa*. To the best of current knowledge, this represents the first report of an RPA-based molecular detection method capable of detecting this pathogen directly in crude citrus peel macerates. This feature constitutes a key innovation, as it allows pathogen detection without DNA purification, even in the presence of host-derived compounds that are typically inhibitory to molecular amplification [22,42]. Moreover, the assay was successfully applied to three major citrus hosts of *P. citricarpa*, *C. sinensis*, *C. limon*, and *C. reticulata*, demonstrating its robustness across a diversity of matrices. Overall, these findings support the value of this assay as a rapid molecular detection tool for CBS detection in citrus-associated plant matrices.

In order to develop and validate this assay, a series of sequential analyses were conducted. First, a 134 bp region of the *tef1* gene was identified through *in silico* analysis as a suitable diagnostic target, based on its conservation within *P. citricarpa* and divergence from closely related species. The identification of a diagnostic region within the *tef1* gene was guided by its established utility in resolving taxonomic boundaries within the *Phyllosticta* genus [6]. This gene has been consistently demonstrated to offer superior specificity compared to the internal transcribed spacer (ITS) regions in molecular diagnostic assays targeting *P. citricarpa* [8,17]. Its selection in the present study was therefore based on its diagnostic usefulness and taxonomic resolution within the genus *Phyllosticta*, particularly for the discrimination of *P. citricarpa* from closely related taxa.

The selected 134 bp fragment was conserved among multiple *P. citricarpa* isolates included in the sequence dataset used for assay design. This dataset comprised publicly available *tef1* sequences from geographically distinct origins, including Brazil, Australia, Zimbabwe, and South Africa. The same fragment showed critical sequence

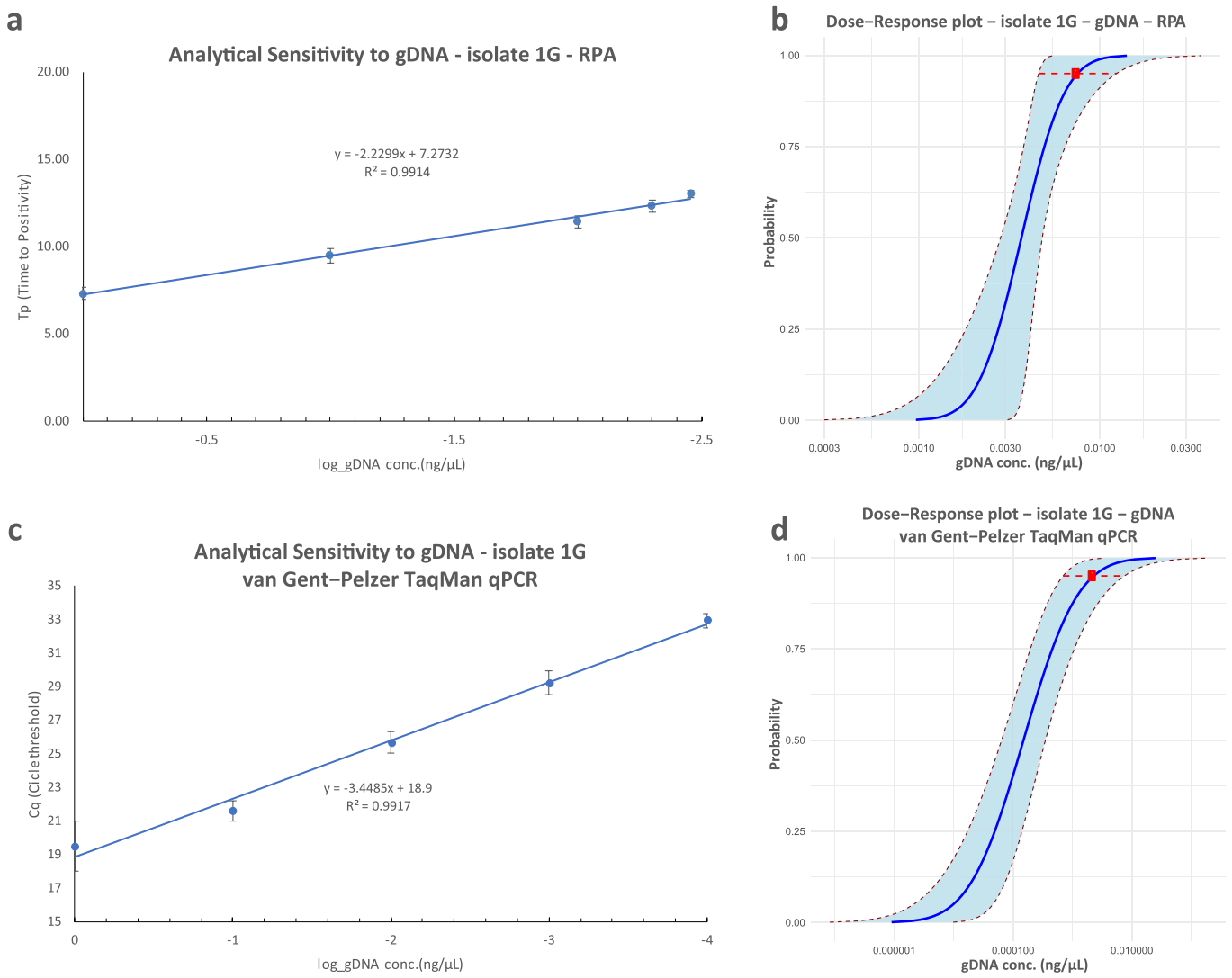


Fig. 2. Analytical sensitivity of RPA vs. van Gent-Pelzer TaqMan qPCR. On the left, graphs showing the linear regression analysis of RPA (a) and van Gent-Pelzer TaqMan qPCR (c) assays for the evaluation of their sensitivity toward independent serial dilutions of purified gDNA of the *P. citricarpa* isolate 1G; bars indicate standard deviation (SD); blue lines are the linear regression curves (linear equations and R2 values are reported). On the right, Dose-Response probit regression analyses showing the probability of detection with RPA (b) and van Gent-Pelzer TaqMan qPCR (d) assays. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

mismatches with non-target species, including *P. capitalensis* and *P. citriasiana*, as confirmed by multiple sequence alignment and BLAST analysis. Consistently, the experimental inclusivity panel included *P. citricarpa* isolates from different geographic regions, including Tunisia, Brazil, and Australia, all of which were successfully detected by the RPA assay. Previous studies employing the same locus in qPCR assays demonstrated high diagnostic accuracy and inter-laboratory consistency [8,17]. *In silico* analysis also revealed four single-nucleotide mismatches within the primer-binding region of *P. paracitricarpa*, indicating the potential for diagnostic distinction at the nucleotide level. Based on these results, the RPA primer pair was preliminarily validated through conventional PCR, a step consistent with established diagnostic workflows, in which primer specificity is initially assessed using purified DNA before implementation in isothermal formats [43,44]. In this study, PCR amplification was observed in *P. citricarpa* and also in *P. paracitricarpa*, despite the presence of four single-nucleotide mismatches in the primer-binding region predicted by the *in silico* analysis, whereas no amplification occurred in other *Phyllosticta* species. Consistent with the PCR results, the RPA assay also produced amplification signals for both *P. citricarpa* and *P. paracitricarpa*, but not for other closely related species, confirming that cross-reactivity is restricted to the taxon with the highest sequence similarity in the target region. These results are consistent with recent phylogenomic analyses suggesting that *P. paracitricarpa* and *P. citricarpa* represent intraspecific variation within a single taxonomic entity, based on extensive whole-genome comparisons [19]. Given the near-genomic identity between *P. citricarpa* and *P. paracitricarpa*, and recent phylogenomic evidence supporting their synonymy, the amplification of isolates originally designated as *P. paracitricarpa* should be interpreted within the current taxonomic boundaries of *P. citricarpa sensu lato*. Enforcing diagnostic separation between these two entities has limited practical value; accordingly, different approaches such as taxon-specific competitive-priming multiplex systems were not pursued here, as they are unnecessary and not applicable within an RPA-amenable window for these taxa [45]. It is also important to note that the official EPPO-recommended TaqMan qPCR assay developed by Van Gent-Pelzer et al. [13] amplifies both *P. citricarpa* and *P. paracitricarpa*, a limitation confirmed in this study and previously reported [8]. Additionally, the Van Gent-Pelzer qPCR

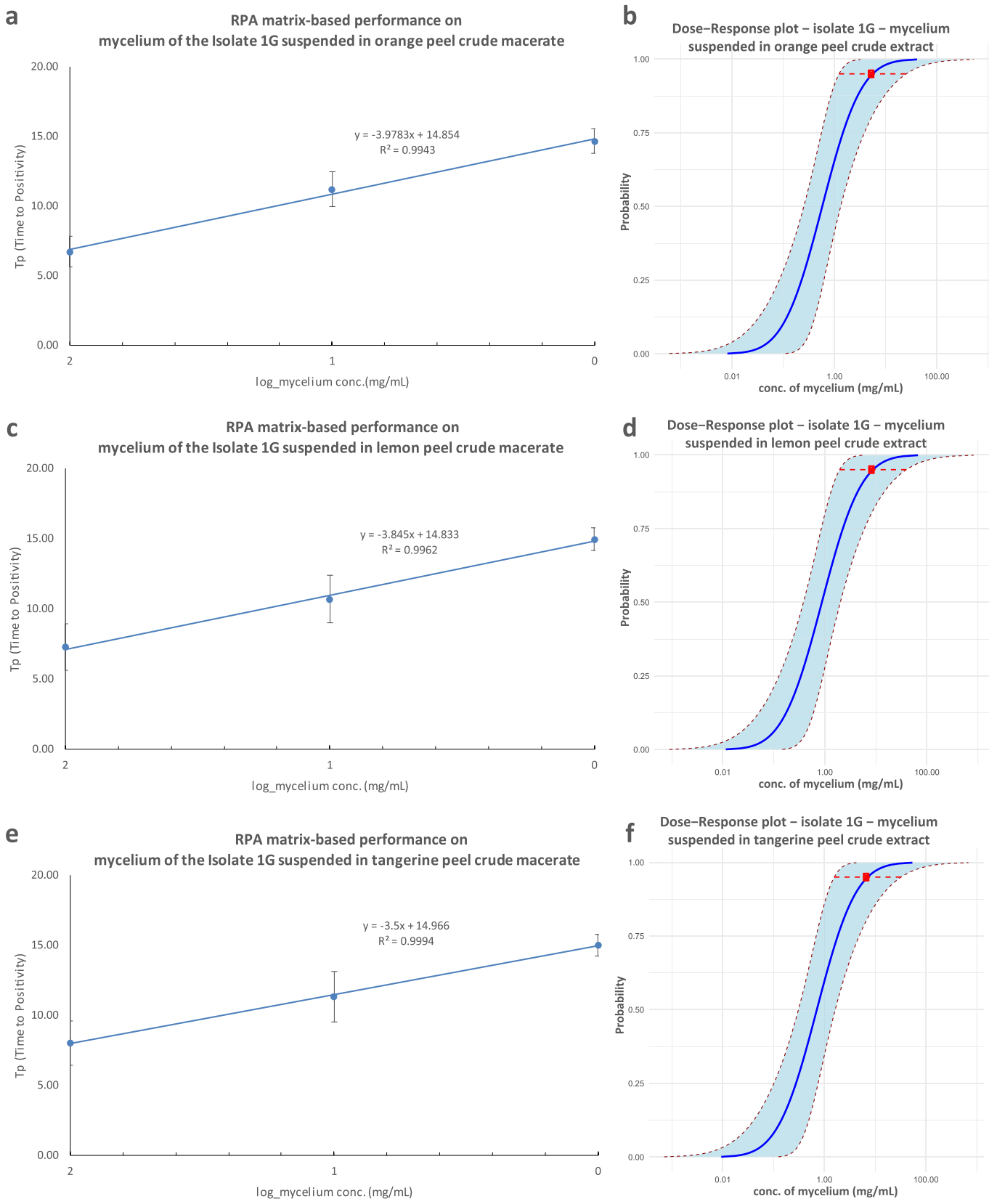


Fig. 3. Matrix-based performance of the RPA assay. On the left, graphs showing the linear regression analysis of RPA assay for the evaluation of the sensitivity toward independent serial dilutions of fresh mycelium of the *P. citricarpa* isolate 1G suspended in crude macerate of (a) orange, (c) lemon, or (e) tangerine peel. Bars indicate standard deviation (SD); blue lines are the linear regression curves (linear equations and R2 values are reported). On the right, Dose-Response probit regression analyses showing the RPA probability of detection of mycelium suspended in crude macerate of (b) orange, (d) lemon, or (f) tangerine. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

assay produced positive signals for *P. citriasiana* and *P. capitalensis*, consistent with findings reported previously [8]. While *P. citriasiana* is not a relevant concern for CBS diagnostics, being host-specific to pomelo (*Citrus maxima*) and absent from sweet orange, lemon, and tangerine [9], the amplification of *P. capitalensis* poses a more serious diagnostic issue. This species is a ubiquitous endophyte commonly found on citrus, including CBS host species, and is frequently recovered from asymptomatic fruit peel [15]. Its detection by a diagnostic assay may lead to false positives and unjustified diagnostic outcomes in citrus disease detection. In contrast, the RPA assay developed in this study did not yield amplification for either *P. citriasiana* or *P. capitalensis*, demonstrating a more selective and diagnostically appropriate specificity profile for the molecular detection of *P. citricarpa* in *C. sinensis*, *C. limon*, and *C. reticulata*. Considering the critical importance of diagnostic specificity in molecular assays for pathogen detection, it is important to consider the specificity trade-offs observed in other recently developed qPCR assays. While more recent assays have demonstrated the ability to distinguish *P. citricarpa* from *P. paracitricarpa* [8,18], they are not yet adopted in standard diagnostic practice. Moreover, despite their improved resolution in certain taxonomic distinctions, they may still exhibit cross-reactivity with non-targets of lesser diagnostic concern. For example, the assay developed by Ioos et al. [18] showed delayed amplification in *P. citriasiana* isolates, underscoring the broader complexity of achieving absolute specificity within the *Phyllosticta* genus. In this context, the RPA assay presented here offers a compelling balance between specificity, speed, and applicability to rapid molecular detection, particularly for citrus-associated matrices where the risk of non-target amplification by ubiquitous endophytes, like *P. capitalensis,* must be minimized. Nevertheless, these aspects should continue to be explicitly considered in assay validation and diagnostic interpretation.

Concerning the analytical sensitivity of the RPA assay, amplification was consistently observed down to 1.0×10^{-7} ng/μL, corresponding to approximately 100 plasmid copies per reaction. This detection threshold is consistent with previously reported performance levels for RPA assays, which reported detection limits ranging from 1 to 10 to 1000 plasmid copies per reaction in plasmid templates [41,46–48]. These results confirm that the assay developed in this study performs within the expected range for high-sensitivity RPA protocols based on recombinant standards. Subsequently, the assay's analytical sensitivity was evaluated using purified genomic DNA of *P. citricarpa*. Positive amplification was obtained down to 3.5×10^{-3} ng of DNA per reaction (corresponding to ~113 genome copies), and the 95 % limit of detection (LoD) was estimated at 7.52×10^{-3} ng (CI: 4.58×10^{-3} – 1.23×10^{-2} ng).

The performance was then compared with the qPCR assay by Van Gent-Pelzer et al. [13], which targets the multicopy ITS region and showed a lower endpoint detection of 1.0×10^{-4} ng and a 95 % LoD of 2.28×10^{-3} ng (CI: 6.93×10^{-4} – 7.49×10^{-3} ng). Despite the qPCR's higher endpoint sensitivity, consistent with its amplification of a multicopy target and use of thermal cycling, the two assays demonstrated overlapping LoD confidence intervals. This result supports the analytical performance of the RPA assay in relation to the EPPO-recommended qPCR assay, while sensitivity differences should be interpreted considering the different copy number of the targeted genomic regions. The reduced sensitivity of the *tef1*-based RPA is consistent with the findings of Zajc et al. [8], who reported analogous differences in LoD between ITS- and *tef1*-targeting assays on the same organism. Comparable results were observed by Rovetto et al. [23], who developed an RPA assay targeting the multicopy ITS1 of *P. tracheiphilus* and achieved a sensitivity of 29 genome copies per reaction. Taken together, these observations indicate that the slightly lower analytical sensitivity of the present assay reflects the expected trade-off associated with the use of a single-copy, highly specific target rather than a methodological weakness of the RPA format itself. The assay developed in this study, which targets a single-copy locus, showed analytical sensitivity compatible with rapid molecular detection while offering improved specificity.

An additional strength of the assay emerged from its performance in crude citrus peel macerates. For this purpose, the RPA assay was further evaluated through direct testing on crude citrus peel macerates spiked with varying concentrations of propagules of *P. citricarpa*, which in this test were represented by fresh mycelium fragments. The assay consistently produced positive amplification in all tested matrices (*C. sinensis*, *C. limon*, *C. reticulata*) at concentrations as low as 1.0 mg/mL. The 95 % LoD was estimated at 5.58 mg/mL in orange peel, 8.79 mg/mL in lemon, and 7.12 mg/mL in tangerine macerate. These results indicate that the assay retained detection capability in crude peel macerates from orange, lemon, and tangerine, with comparable performance across the three tested citrus matrices. This supports the suitability of the RPA format for use in unpurified citrus-derived matrices, where host-derived compounds may affect molecular amplification [42]. This property derives from the RPA reaction mechanism, which lacks thermal denaturation and employs recombinase and strand-displacing polymerases that are less prone to inhibition [22]. Similar observations were reported in previous studies that applied RPA directly to crude plant material. Rovetto et al. [23] showed that an RPA assay targeting *P. tracheiphilus* achieved reliable amplification in raw stem extracts, bypassing the need for DNA purification. Cesbron et al. [24] demonstrated the feasibility of the AmplifyRP XRT + system in detecting *Xylella fastidiosa* from crude grapevine tissues, showing comparable performance to qPCR. Likewise, Munguti et al. [49] validated the use of reverse transcription RPA on crude homogenates for virus detection in cassava, emphasizing its potential for deployment under field or low-resource settings. In the present study, successful amplification across the three citrus matrices indicated that the RPA assay can tolerate host-derived inhibitory compounds present in crude citrus peel macerates and retain amplification capability under matrix-based conditions. These findings support the suitability of RPA for rapid molecular detection in complex plant-derived matrices. While qPCR remains marginally more sensitive due to its target selection and amplification mechanism, the RPA assay developed here demonstrates a balance of specificity, inhibitor tolerance, and operational simplicity that makes it a promising tool for rapid molecular detection of *P. citricarpa* in phytopathological diagnostics. Further validation on naturally infected citrus material is required to complete the assessment of assay performance under real diagnostic conditions, including variable pathogen load and the biological complexity of naturally infected tissues, and to confirm its suitability for future routine diagnostic or field application.

5. Conclusion

Overall, the present study expands the molecular toolbox available for Citrus Black Spot diagnosis by introducing a real-time fluorescent RPA assay for the detection of *P. citricarpa*. The assay combines rapid amplification, selective target detection, and tolerance to complex citrus-derived matrices, while maintaining detection performance compatible with practical molecular detection. The use of a *tef1*-targeted approach improved discrimination against several closely related non-target *Phyllosticta* species, and the successful amplification from crude citrus peel macerates highlights the potential of this assay for rapid molecular detection of *P. citricarpa* in citrus-associated plant matrices.

Funding

This study was supported by the University of Catania, Italy, with the projects “Investigation of Phytopathological problems of the main Sicilian productive contexts and eco-sustainable defense strategies (MEDIT-ECO)”- PiaCeRi-PIAno di inCEntivi per la Ricerca di Ateneo

2020–22 linea 2" "5A722192155" and "Diagnosi di malattie poco note o emergenti e sviluppo di strategie di difesa innovative ed ecosostenibili (DIME-SIECO)" PIAno di inCEntivi per la RIcerca di Ateneo 2024/2026, Linea di Intervento 1''; by the European Union (NextGeneration EU), through the MUR-PNRR project SAMOTHRACE (ECS00000022); by the Italian Ministry of Agriculture, Food Sovereignty and Forests (MASAF), through the project "Difesa degli Agrumeti Italiani dal Malsecco – AGRIVITA (CUP: C83C23000650006)".

CRediT authorship contribution statement

Federico La Spada: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Rossana Parlascino:** Investigation, Methodology, Writing – review & editing. **Mario Riolo:** Visualization, Writing – review & editing. **Sebastiano Conti Taguali:** Visualization, Writing – review & editing. **Antonella Pane:** Visualization, Writing – review & editing. **Matteo Garbelotto:** Conceptualization, Methodology, Writing – review & editing. **Bryant Davenport:** Conceptualization, Methodology, Writing – review & editing. **Marcos Amato:** Conceptualization, Funding acquisition, Methodology, Resources, Writing – review & editing. **Santa Olga Cacciola:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Marcos Amato reports a relationship with Agdia EMEA that includes: employment. Bryant Davenport reports a relationship with Agdia Inc. that includes: employment. The remaining authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors wish to thank the projects EmergeNOW—Emergent detection technologies for plant health diagnostics at EU Import points (HORIZON- CL6–2024-FARM2FORK-02–3-two-stage, CUP: E63C24002790006), "Smart and innovative packaging, postharvest rot management, and shipping of organic citrus fruit (BiOrangePack)" under the Partnership for Research and Innovation in the Mediterranean Area (PRIMA)—H2020 (E69C20000130001), and "Piattaforma AvaNzata per la rilevAzione preCoce di patogEni in frutta e verdura frescA da banco (PANACEA)" funded by the Italian Ministry of Enterprises and Made in Italy (MIMIT) through the Fund for Sustainable Growth (Fondo per la Crescita Sostenibile), PN RIC 2021–2027 (CUP: B69J24000160005), for inspiring this study.

Data availability

The sequences of the primers and probe used for the RPA assay, together with the selected target barcode, have been deposited in Figshare under DOI: https://doi.org/10.6084/m9.figshare.31980219.

References

[1] EPPO, PM 7/017 (3) *Phyllosticta citricarpa* (formerly *Guignardia citricarpa*), EPPO Bull. 50 (2020) 440–461, https://doi.org/10.1111/epp.12700.
[2] E.I. Rovetto, F. La Spada, F. Aloi, M. Riolo, A. Pane, M. Garbelotto, S.O. Cacciola, Green solutions and new technologies for sustainable management of fungus and oomycete diseases in the citrus fruit supply chain, J. Plant Pathol. 2024 (2024) 1–27, https://doi.org/10.1007/S42161-023-01543-6.
[3] EFSA, Scientific opinion on the risk of *Phyllosticta citricarpa* (*Guignardia citricarpa*) for the EU territory with identification and evaluation of risk reduction options, EFSA J. 12 (2014), https://doi.org/10.2903/J.EFSA.2014.3557.
[4] European Food Safety Authority (EFSA), S. Tramontini, S. Parnell, D. Rzepecka, M. Scala, F. Baldassarre, B. Sánchez, A. Nougadère, S. Vos, A. Haegi, M.T. Valente, *Phyllosticta citricarpa* Pest Report to Support Ranking of EU Candidate Priority Pests, EFSA Supporting Publications. Wiley, 2025, https://doi.org/10.2903/sp.efsa.2025.EN-9322.
[5] S. Parnell, M. Schenk, G. Schrader, A. Vicent, A. Delbianco, S. Vos, Pest survey card on *Phyllosticta citricarpa*, EFSA Support. Publ. 17 (2020), https://doi.org/10.2903/SP.EFSA.2020.EN-1863.
[6] V. Guarnaccia, J.Z. Groenewald, H. Li, C. Glienke, E. Carstens, V. Hattingh, P. H. Fourie, P.W. Crous, First report of *Phyllosticta citricarpa* and description of two new species, *P. paracapitalensis* and *P. paracitricarpa*, from citrus in Europe, Stud. Mycol. 87 (2017) 161–185, https://doi.org/10.1016/j.simyco.2017.05.003.
[7] V. Guarnaccia, T. Gehrmann, G.J. Silva-Junior, P.H. Fourie, S. Haridas, D. Vu, J. Spatafora, F.M. Martin, V. Robert, I.V. Grigoriev, J.Z. Groenewald, P.W. Crous, *Phyllosticta citricarpa* and sister species of global importance to citrus, Mol. Plant Pathol. 20 (2019) 1619–1635, https://doi.org/10.1111/mpp.12861.
[8] J. Zajc, Z. Kogej Zwitter, S. Fišer, C. Gostinčar, A. Vicent, A.G. Domenech, L. Riccioni, N. Boonham, M. Ravnikar, P. Kogovšek, Highly specific qPCR and amplicon sequencing method for detection of quarantine citrus pathogen *Phyllosticta citricarpa* applicable for air samples, Plant Pathol. 72 (2023) 548–563, https://doi.org/10.1111/PPA.13679.
[9] M.M.J.P. van Raak, E.J. van Veen, B.T.L.H. van de Vossenberg, M. Westenberg, S. van der Linde, Occurrence of *Phyllosticta citricarpa* and *Phyllosticta citriasiana* in EU imported *citrus* spp. and its impact on diagnostics of citrus black spot on fruits, Eur. J. Plant Pathol. (2025) 1–17, https://doi.org/10.1007/S10658-025-03057-6/FIGURES/3.
[10] D.C. Savi, K.A. Shaaban, P. Mitra, L.V. Ponomareva, J.S. Thorson, C. Glienke, J. Rohr, Secondary metabolites produced by the citrus phytopathogen *Phyllosticta citricarpa*, J. Antibiot. (Tokyo). 72 (2019) 306, https://doi.org/10.1038/S41429-019-0154-3.
[11] M. Wang, B. Liu, R. Ruan, Y. Zeng, J. Luo, H. Li, Genomic sequencing of *Phyllosticta citriasiana* provides insight into its conservation and diversification with two closely related *Phyllosticta* species associated with citrus, Front. Microbiol. 10 (2020) 490818, https://doi.org/10.3389/FMICB.2019.02979/TEXT.
[12] W. Wang, T. Xiong, Y. Zeng, W. Li, C. Jiao, J. Xu, H. Li, Clonal expansion in multiple *Phyllosticta* species causing citrus black spot or similar symptoms in China, J. Fungi 9 (2023) 449, https://doi.org/10.3390/JOF9040449/S1.
[13] M.P.E. Van Gent-Pelzer, I.R. Van Brouwershaven, L.F.F. Kox, P.J.M. Bonants, A TaqMan PCR method for routine diagnosis of the quarantine fungus *Guignardia citricarpa* on citrus fruit, J. Phytopathol. 155 (2007) 357–363, https://doi.org/10.1111/j.1439-0434.2007.01244.x.
[14] E. Skreli, D. Imami, Citrus sector study. Albania, Tiranë, 2019.
[15] N.T. Tran, A.K. Miles, R.G. Dietzgen, A. Drenth, *Phyllosticta capitalensis* and *P. paracapitalensis* are endophytic fungi that show potential to inhibit pathogenic *P. citricarpa* on citrus, Australas. Plant Pathol. 48 (2019) 281–296, https://doi.org/10.1007/S13313-019-00628-0.
[16] S. Wikee, L. Lombard, P.W. Crous, C. Nakashima, K. Motohashi, E. Chukeatirote, S. A. Alias, E.H.C. McKenzie, K.D. Hyde, *Phyllosticta capitalensis*, a widespread endophyte of plants, Fungal Divers. 60 (2013) 91–105, https://doi.org/10.1007/s13225-013-0235-8.
[17] T. Jakomin, J.Z. Žunič, P. Kogovšek, Test performance study on qPCR assays for detection of *Phyllosticta citricarpa*, Pathogens 14 (2025) 413, https://doi.org/10.3390/PATHOGENS14050413/S1.
[18] R. Ioos, A. Puertolas, C. Renault, A. Ndiaye, I. Cerf-Wendling, J. Hubert, W. Wang, C. Jiao, H. Li, J. Armengol, J. Aguayo, Harnessing the power of comparative genomics to support the distinction of sister species within *Phyllosticta* and development of highly specific detection of *Phyllosticta citricarpa* causing citrus black spot by real-time PCR, PeerJ 11 (2023) e16354, https://doi.org/10.7717/PEERJ.16354/SUPP-8.
[19] V.A. van Ingen-Buijs, A.C. van Westerhoven, P. Skiadas, X.C.L. Zuijdgeest, S. Haridas, C. Daum, K. Duffy, J. Guo, H. Hundley, K. LaButti, A. Lipzen, J. Pangilinan, R. Riley, J. Wang, M. Yan, F. Martin, K. Barry, I.V. Grigoriev, J. Z. Groenewald, P.W. Crous, M.F. Seidl, *Phyllosticta paracitricarpa* is synonymous with the EU quarantine fungus *P. citricarpa* based on phylogenomic analyses, Fungal Genet. Biol. 175 (2024), https://doi.org/10.1016/J.FGB.2024.103925.
[20] J.A. Tomlinson, S. Ostoja-Starzewska, K. Webb, J. Cole, A. Barnes, M. Dickinson, N. Boonham, A loop-mediated isothermal amplification-based method for confirmation of *Guignardia citricarpa* in citrus black spot lesions, Eur. J. Plant Pathol. 136 (2013) 217–224, https://doi.org/10.1007/S10658-013-0168-9/FIGURES/3.
[21] R. Parlascino, S. Conti Taguali, M. Riolo, M. Salvatore, J. Bakonyi, D. Ezra, A. Benigno, F. La Spada, A. Pane, S.O. Cacciola, Unveiling the invisible threat: cutting-edge methods for detecting fungal and oomycete plant pathogens and mycotoxins, Physiol. Mol. Plant Pathol. 140 (2025) 102893, https://doi.org/10.1016/J.PMPP.2025.102893.
[22] O. Piepenburg, C.H. Williams, D.L. Stemple, N.A. Armes, DNA detection using recombination proteins, PLoS Biol. 4 (2006) 1115–1121, https://doi.org/10.1371/journal.pbio.0040204.
[23] E.I. Rovetto, M. Garbelotto, S. Moricca, M. Amato, F. La Spada, S.O. Cacciola, A portable fluorescence-based recombinase polymerase amplification assay for the detection of mal secco disease by *Plenodomus tracheiphilus*, Crop Prot. 184 (2024) 106825, https://doi.org/10.1016/j.cropro.2024.106825.
[24] S. Cesbron, E. Dupas, M.-A. Jacques, Evaluation of the AmplifyRP XRT+ kit for the detection of *Xylella fastidiosa* by recombinase polymerase amplification, PhytoFront. 3 (2023) 225–234, https://doi.org/10.1094/phytofr-03-22-0025-fi.

[25] H.Y. Lau, J.R. Botella, Advanced DNA-based point-of-care diagnostic methods for plant diseases detection, Front. Plant Sci. 8 (2017) 302813, https://doi.org/10.3389/FPLS.2017.02016/XML/NLM.
[26] F. Morán, M. Herrero-Cervera, S. Carvajal-Rojas, E. Marco-Noales, Real-time on-site detection of the three '*Candidatus Liberibacter*' species associated with HLB disease: a rapid and validated method, Front. Plant Sci. 14 (2023) 1176513, https://doi.org/10.3389/FPLS.2023.1176513/BIBTEX.
[27] N. Wulandari, C. To-anun, K. Hyde, L. Duong, J. de Gruyter, J. Meffert, J. Groenewald, P. Crous, *Phyllosticta citriasiana* sp. Nov., the cause of citrus tan spot of *Citrus maxima* in Asia, Fungal Divers. 34 (2009) 23–39.
[28] C. Glienke, O.L. Pereira, D. Stringari, J. Fabris, V. Kava-Cordeiro, L. Galli-Terasawa, J. Cunnington, R.G. Shivas, J.Z. Groenewald, P.W. Crous, Endophytic and pathogenic *Phyllosticta* species, with reference to those associated with citrus black spot, Persoonia Mol. Phylogeny Evol. Fungi 26 (2011) 47, https://doi.org/10.3767/003158511X569169.
[29] F. La Spada, M. Garbelotto, S. Moricca, M. Amato, D. Ezra, R. Parlascino, M. Riolo, G. Polizzi, A. Pane, S.O. Cacciola, Early detection of *Plenodomus tracheiphilus* by a fluorescence-portable RPA assay: a new smart solution in citriculture, Acta Hortic. 1448 (2026) 735–744, https://doi.org/10.17660/ACTAHORTIC.2026.1448.90.
[30] S. El boumlasy, F. La Spada, N. Tuccitto, G. Marletta, C.L. Mínguez, G. Meca, E. I. Rovetto, A. Pane, A. Debdoubi, S.O. Cacciola, Inhibitory activity of shrimp waste extracts on fungal and oomycete plant pathogens, Plants 10 (2021) 2452, https://doi.org/10.3390/plants10112452.
[31] E.I. Rovetto, C. Luz, F. La Spada, G. Meca, M. Riolo, S.O. Cacciola, Diversity of mycotoxins and other secondary metabolites recovered from blood oranges infected by *colletotrichum, Alternaria*, and *Penicillium* species, Toxins 15 (2023) 407, https://doi.org/10.3390/TOXINS15070407.
[32] M. Riolo, F. Aloi, A. Pane, M. Cara, S.O. Cacciola, Twig and shoot dieback of citrus, a new disease caused by *Colletotrichum* species, Cells 10 (2021), https://doi.org/10.3390/cells10020449.
[33] M.A. Demontis, S.O. Cacciola, M. Orrù, V. Balmas, V. Chessa, B.E. Maserti, L. Mascia, F. Raudino, G. Magnano Di San Lio, Q. Migheli, Development of real-time PCR systems based on SYBR® green I and TaqMan® technologies for specific quantitative detection of *Phoma tracheiphila* in infected citrus, Eur. J. Plant Pathol. 120 (2008) 339–351, https://doi.org/10.1007/s10658-007-9222-9.
[34] I. Carbone, L.M. Kohn, A method for designing primer sets for speciation studies in filamentous ascomycetes, Mycologia 91 (1999) 553–556, https://doi.org/10.2307/3761358.
[35] K. O'Donnell, H.C. Kistlerr, E. Cigelnik, R.C. Ploetz, Multiple evolutionary origins of the fungus causing Panama disease of banana: concordant evidence from nuclear and mitochondrial gene genealogies, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 2044–2049, https://doi.org/10.1073/pnas.95.5.2044.
[36] J. Dolezel, J. Bartos, H. Voglmayr, J. Greilhuber, Nuclear DNA content and genome size of trout and human, Cytometry A 51 (2003) 127–8; author reply 129, https://doi.org/10.1002/cyto.a.10013.
[37] I. Buja, E. Sabella, A.G. Monteduro, M.S. Chiriacò, L. De Bellis, A. Luvisi, G. Maruccio, Advances in plant disease detection and monitoring: from traditional assays to in-field diagnostics, Sensors 21 (2021) 2129, https://doi.org/10.3390/S21062129.
[38] J.B. Ristaino, P.K. Anderson, D.P. Bebber, K.A. Brauman, N.J. Cunniffe, N. V. Fedoroff, C. Finegold, K.A. Garrett, C.A. Gilligan, C.M. Jones, M.D. Martin, G. K. MacDonald, P. Neenan, A. Records, D.G. Schmale, L. Tateosian, Q. Wei, The persistent threat of emerging plant disease pandemics to global food security, Proc. Natl. Acad. Sci. U. S. A. 118 (2021) e2022239118, https://doi.org/10.1073/PNAS.2022239118/SUPPL_FILE/PNAS.2022239118.SAPP.PDF.
[39] M. Venbrux, S. Crauwels, H. Rediers, Current and emerging trends in techniques for plant pathogen detection, Front. Plant Sci. 14 (2023), https://doi.org/10.3389/FPLS.2023.1120968.
[40] Y. Zou, M.G. Mason, J.R. Botella, Evaluation and improvement of isothermal amplification methods for point-of-need plant disease diagnostics, PLoS One 15 (2020) e0235216, https://doi.org/10.1371/JOURNAL.PONE.0235216.
[41] I.M. Lobato, C.K. O'Sullivan, Recombinase polymerase amplification: basics, applications and recent advances, TrAC, Trends Anal. Chem. (2018), https://doi.org/10.1016/j.trac.2017.10.015.
[42] C. Schrader, A. Schielke, L. Ellerbroek, R. Johne, PCR inhibitors - occurrence, properties and removal, J. Appl. Microbiol. (2012), https://doi.org/10.1111/j.1365-2672.2012.05384.x.
[43] J. Dong, W. Feng, M. Lin, S. Chen, X. Liu, X. Wang, Q. Chen, Comparative evaluation of PCR-Based, LAMP and RPA-CRISPR/Cas12a assays for the rapid detection of *Diaporthe aspalathi*, Int. J. Mol. Sci. 25 (2024) 5773, https://doi.org/10.3390/IJMS25115773/S1.
[44] L. Meyer, R. Jacobs, J.M. Kotzé, M. Truter, L. Korsten, Detection and molecular identification protocols for *Phyllosticta citricarpa* from citrus matter, South Afr. J. Sci. 108 (2012) 1–6, https://doi.org/10.4102/sajs.
[45] M. Garbelotto, A. Ratcliff, T. Bruns, F. Cobb, W. Otrosina, Use of taxon-specific competitive-priming PCR to study host specificity, hybridization, and intergroup gene flow in intersterility groups of *Heterobasidion annosum*, Phytopathology 86 (1996) 543.
[46] Z.A. Crannell, B. Rohrman, R. Richards-Kortum, Equipment-free incubation of recombinase polymerase amplification reactions using body heat, PLoS One 9 (2014), https://doi.org/10.1371/journal.pone.0112146.
[47] B. Rohrman, R. Richards-Kortum, Inhibition of recombinase polymerase amplification by background DNA: a lateral flow-based method for enriching target DNA, Anal. Chem. 87 (2015) 1963–1967, https://doi.org/10.1021/AC504365V/SUPPL_FILE/AC504365V_SI_001.PDF.
[48] B.A. Rohrman, R.R. Richards-Kortum, A paper and plastic device for performing recombinase polymerase amplification of HIV DNA, Lab Chip 12 (2012) 3082–3088, https://doi.org/10.1039/C2LC40423K.
[49] F.M. Munguti, D.C. Kilalo, H.K. Yegon, I. Macharia, S.E. Seal, A.W. Mwango'mbe, E.N. Nyaboga, G. Silva, Real-time reverse transcription recombinase polymerase amplification (RT-RPA) assay for detection of cassava brown streak viruses, Sci. Rep. 14 (2024) 12438, https://doi.org/10.1038/S41598-024-62249-Y.