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# A portable fluorescence-based recombinase polymerase amplification assay for the detection of mal secco disease by *Plenodomus tracheiphilus*

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ABSTRACT

In this study, a new diagnostic assay to detect *Plenodomus tracheiphilus*, the causative agent of mal secco of citrus, was developed based on the recombinase polymerase amplification (RPA) technology. Mal secco is a well-known and damaging vascular disease, affecting primarily lemon (*Citrus limon*) and, to a lesser extent, other citrus species, including those in the genera *Citrus, Fortunella, Poncirus* and *Severina*. The disease poses a considerable threat to lemon production in most of the citrus-producing countries of the Mediterranean region and in the Black Sea area. RPA primers and probes were designed to amplify a 142 bp amplicon from the ITS regions of *P. tracheiphilus*. The inclusivity and specificity of the RPA assay were tested on gDNA isolated from a panel including 29 strains of various origin of *P. tracheiphilus* and 18 non-target fungal and oomycete plant pathogens typically isolated from citrus trees. The assay was specific to *P. tracheiphilus* and had a detection threshold of 1.0 pg of gDNA. Preliminary tests carried out on plant crude extract highlighted RPA's potential for the rapid, user-friendly, and cost-effective field diagnosis of mal secco.

## 1. Introduction

Mal secco of citrus, caused by the mitosporic fungus *Plenodomus tracheiphilus* (Petri) Gruyter, Aveskamp, and (de Gruyter et al., 2013), is one of the most relevant diseases of cultivated citrus (Migheli et al., 2009). It is a vascular disease occurring primarily in lemon (*Citrus limon* (L.) Burm. f.) and, to a lesser extent, in citron, bergamot, lime, sour orange, and rough lemon (Migheli et al., 2009; Nigro et al., 2015; Rovetto et al., 2024). Mal secco of lemon is present throughout most of the Mediterranean region, including the Black Sea area, with the exception of a few countries (Abbate et al., 2019; Demontis et al., 2008).

The European and Mediterranean Plant Protection Organization (EPPO) has classified *P. tracheiphilus* as an A2 quarantine pest. The pathogen is also considered a quarantine concern by several regional plant protection agencies worldwide, including the Asia and Pacific Plant Protection Commission, the Caribbean Plant Protection Commission, the Comité Regional de Sanidad Vegetal para el Cono Sur, and the North American Plant Protection Organization (Migheli et al., 2009).

The most effective strategies for the management of mal secco include the adoption of preventive measures, the planning of phytosanitary programs, and the early diagnosis of the disease (Demontis et al., 2008; El boumlasy et al., 2022; Leonardi et al., 2023). According to the EPPO diagnostic protocol PM 7/048 (EPPO Standards, 2015), a positive diagnosis of mal secco is obtained when the pathogen is identified in potentially infected plants either by direct isolation or by molecular detection from plant tissues. The molecular methods currently approved by EPPO for the diagnosis of mal secco are a polymerase chain reaction (PCR)-based *P. tracheiphilus*-specific assay (Balmas et al., 2005) and a quantitative TaqMan®/SYBR® Green I Real Time-PCR protocol (Demontis et al., 2008). In spite of the well-known advantages of PCR-based technologies, such as their reliability resulting from years of application, a formidable sensitivity, high specificity, and increased sample throughput, such technologies have limitations in terms of cost, complexity and can be unsuitable for disease diagnosis in the field

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(Demontis et al., 2008; Ray et al., 2017). In the last decade, several new molecular methods have been developed. Among these new methods, isothermal techniques are particularly noteworthy, given they do not require thermal cycling equipment for the amplification of nucleic acids (Crannell et al., 2014). The recombinase polymerase amplification (RPA) is an isothermal technology that stands out for its simplicity, specificity, high sensitivity, and rapidity (Lobato and O'Sullivan, 2018; Piepenburg et al., 2006). The RPA technology relies on a recombinase-mediated hybridization of primer pairs with a double-stranded DNA target fragment. Core enzymes, including recombinase, single-stranded DNA-binding protein (SSB), and a strand-displacing polymerase, drive the amplification process. Recombinase binds to primers, forming a complex chimera that pairs with homologous sequences in the target fragment. SSB stabilizes this complex, allowing the strand-displacing polymerase to initiate DNA synthesis. Amplification proceeds exponentially through cyclic repetition of this process at low and constant temperatures ranging between 25 and 42 °C (Piepenburg et al., 2006). RPA's simplicity and cost-effectiveness are facilitated by its minimal thermal requirements, compatible with portable devices (Lobato and O'Sullivan, 2018). Primers are 28–35 nucleotides long, have a GC content between 30% and 70% and can ensure highly specific amplification of target DNA. Probes, incorporating a tetrahydrofuran (THF) abasic-site flanked by fluorophore-modified nucleotides and a quencher, further confer specificity by generating measurable fluorescence upon target binding (Piepenburg et al., 2006). Lyophilized reagents enhance practicality, while RPA's tolerance for impure samples enables efficient detection in crude extracts, making it well-suited for field applications (Daher et al.,

**Table 1**
Isolate code, species, source of isolation, location, year of recovery, reference work, and RPA outcome of fungal and oomycete isolates used in this study.

| Isolate code | Species | Source of isolation | Location[a] | Year | Reference | RPA outcome (+[1]/-[2]) |
|---|---|---|---|---|---|---|
| Pt2 | *P. tracheiphilus* | *Citrus limon* 'Femminello' | Syracuse (Sicily) | 2021 | El boumlasy et al. (2021) | + |
| Pt22 | *P. tracheiphilus* | *C. limon* 'Femminello' | Syracuse (Sicily) | 2022 | This study | + |
| Pt3 | *P. tracheiphilus* | *C. limon* 'Femminello' | Catania (Sicily) | 2022 | This study | + |
| Pt6-Lim-Sic | *P. tracheiphilus* | *C. limon* 'Femminello' | Catania (Sicily) | 2022 | This study | + |
| Pt8 | *P. tracheiphilus* | *C. limon* 'Femminello' | Catania (Sicily) | 2022 | This study | + |
| Pt9-Lim-Sic | *P. tracheiphilus* | *C. limon* 'Monachello' | Catania (Sicily) | 2022 | This study | + |
| Pt10 | *P. tracheiphilus* | *C. limon* 'Monachello' | Catania (Sicily) | 2022 | This study | + |
| PVS Pt S1 | *P. tracheiphilus* | *C. limon* 'Monachello' | Capoterra (CA) | 2004 | Demontis et al. (2008) | + |
| ISPaVe ER 1139 | *P. tracheiphilus* | *C. limon* 'Femminello' | Cisterna (LT) | 2000 | Demontis et al. (2008) | + |
| Pt Ad4b | *P. tracheiphilus* | *Citrus aurantium* | Mazzarrà Sant'Andrea (ME) | – | Demontis et al. (2008) | + |
| Pt Ad4a | *P. tracheiphilus* | *C. aurantium* | Mazzarrà Sant'Andrea (ME) | – | Demontis et al. (2008) | + |
| Pt Ad3 | *P. tracheiphilus* | *C. limon* | Parco d'Orleans (PA) | – | Demontis et al. (2008) | + |
| Pt Ad2 | *P. tracheiphilus* | *C. aurantium* | Altofonte (PA) | – | Demontis et al. (2008) | + |
| Pt Ad1 | *P. tracheiphilus* | *C. limon* | Altofonte (PA) | – | Demontis et al. (2008) | + |
| Pt 20 | *P. tracheiphilus* | Unknown | – | – | Demontis et al. (2008) | + |
| Pt V | *P. tracheiphilus* | *Citrus volkameriana* | – | 1992 | Demontis et al. (2008) | + |
| Pt C | *P. tracheiphilus* | *Citrus microcarpa* | – | 1983 | Demontis et al. (2008) | + |
| ITEM 2338 | *P. tracheiphilus* | *C. limon* fruit | – | – | Demontis et al. (2008) | + |
| Pt 87 | *P. tracheiphilus* | Air sampling | Ognina (CT) | 1985 | Demontis et al. (2008) | + |
| Pt 86 | *P. tracheiphilus* | Air sampling | Ognina (CT) | 1985 | Demontis et al. (2008) | + |
| Pt 84 | *P. tracheiphilus* | *C. limon* 'Monachello' | C.da Scorsonello Savoca (ME) | 1988 | Demontis et al. (2008) | + |
| Pt 83 | *P. tracheiphilus* | Air sampling | Giardini (ME) | 1985 | Demontis et al. (2008) | + |
| Pt 81 | *P. tracheiphilus* | Air sampling | Giardini (ME) | 1985 | Demontis et al. (2008) | + |
| Pt 80 | *P. tracheiphilus* | Unknown | Giardini (ME) | 1985 | Demontis et al. (2008) | + |
| Pt 79 | *P. tracheiphilus* | Air sampling | Ognina (CT) | 1985 | Demontis et al. (2008) | + |
| Pt 77 | *P. tracheiphilus* | *C. limon* 'Monachello' | Giardini (ME) | 1985 | Demontis et al. (2008) | + |
| Pt 75 | *P. tracheiphilus* | *C. limon* 'Monachello' | Acireale (CT) | 1985 | Demontis et al. (2008) | + |
| Pt 73 | *P. tracheiphilus* | *C. limon* 'Monachello' | Balatelle Acireale (CT) | 1985 | Demontis et al. (2008) | + |
| Pt 71 | *P. tracheiphilus* | *C. limon* 'Monachello' | Balatelle Acireale (CT) | 1985 | Demontis et al. (2008) | + |
| ITEM 201 | *Didymella glomerata*[b] | *Laurus nobilis* | Italy | 1981 | Demontis et al. (2008) | – |
| ITEM 203 | *Heterospora chenopodii*[c] | *Vitis vinifera* | Italy | 1981 | Demontis et al. (2008) | – |
| ITEM 243 | *Pleospora betae*[d] | *Beta vulgaris* | The Netherlands | 1966 | Demontis et al. (2008) | – |
| ITEM 244 | *Coniothyrium carteri*[e] | *Castanea sativa* | The Netherlands | 1966 | Demontis et al. (2008) | – |
| ITEM 246 | *Paraphoma fimeti*[f] | Greenhouse soil | The Netherlands | 1970 | Demontis et al. (2008) | – |
| ITEM 2077 | *Plenodomus lingam*[g] | *Brassica napus* | Italy | 1990 | Demontis et al. (2008) | – |
| ISPaVe ER 693 | *Ascochyta medicaginicola*[h] | *Medicago sativa* | Foggia (FG) | 1991 | Demontis et al. (2008) | – |
| 646 | *Alternaria alternate* | *C. clementina* | Catania (Sicily) | 2021 | El boumlasy et al. (2021) | – |
| AaMDC1 | *Alternaria arborescens* | *Citrus sinensis* | Catania (Sicily) | 2020 | El boumlasy et al. (2021) | – |
| CAM | *Colletotrichum karsti* | *Camellia* sp. | Catania (Sicily) | 2020 | Riolo et al. (2021) | – |
| C2 | *Colletotrichum gloeosporioides* | *Citrus limon* | Calabria (Italy) | 2020 | Riolo et al. (2021) | – |
| UWS14 | *Colletotrichum acutatum* | *Citrus limon* | Syracuse (Sicily) | 2020 | El boumlasy et al. (2021) | – |
| 1G | *Phyllosticta citricarpa* | *Citrus limon* | Tunisia | 2022 | This study | – |
| T3-B-K1A | *Phytophthora nicotianae* | *Citrus limon* | Syracuse (Sicily) | 2021 | El boumlasy et al. (2021) | – |
| T2-C-M1A | *P. nicotianae* | *Citrus limon* | Syracuse (Sicily) | 2020 | El boumlasy et al. (2021) | – |
| Ax1Ar | *Phytophthora citrophthora* | *Citrus limon* | Syracuse (Sicily) | 2021 | El boumlasy et al. (2021) | – |
| P1PP0 | *Penicillium digitatum* | *Citrus sinensis* | Catania (Sicily) | 2021 | El boumlasy et al. (2021) | – |
| T4N0 | *Penicillium italicum* | *Citrus sinensis* | Catania (Sicily) | 2022 | This study | – |

[1] + = positive; [2] - = negative.
[a] Codes in brackets indicates Italian administrative provinces: PA, Palermo; CT, Catania; ME, Messina; LT, Latina; CA, Cagliari.
[b] ≡ *Phoma glomerata*.
[c] ≡ *Phoma exigua*.
[d] ≡ *Phoma betae*.
[e] Previously syn. of *Phoma cava*.
[f] ≡ *Phoma fimeti*.
[g] ≡ *Phoma lingam*.
[h] ≡ *Phoma medicaginis*.

2016). In light of these advantages, RPA has been successfully used for the development of assays deployed in the early detection of several plant pathogens, including viruses (Babu et al., 2018; Kapoor et al., 2017) bacteria (Buddhachat et al., 2022; Cesbron et al., 2023), oomycetes (Miles et al., 2015) and fungi (Changtor et al., 2023; Ju et al., 2020; Zhao et al., 2021).

Due to the importance of the mal secco disease, this study's aim was the development of an alternative and novel *P. tracheiphilus*-RPA assay to be used as a rapid, user-friendly, and cost-effective diagnostic tool. The sensitivity of the RPA assay, as well as its performance on rapidly extracted non-purified target DNA, were evaluated in comparison to the EPPO standard SYBR® Green I Real Time-PCR assay of Demontis et al. (2008). Finally, to preliminary asses the suitability of the *P. tracheiphilus*-RPA assay for field applications, tests were carried out on plant samples collected from both mal secco-symptomatic and non-symptomatic lemon plants.

## 2. Materials and methods

### 2.1. Fungal isolates

Fungal and oomycete isolates employed in this study came from the collection of the Laboratory of Molecular Plant Pathology of the Department of Agriculture, Food and Environment of the University of Catania (Catania, Italy). Test isolates included a wide collection of 29 virulent strains of *P. tracheiphilus*, seven *Pleosporales* isolates from species taxonomically close to *P. tracheiphilus*, and 11 specimens of major fungal and oomycete species commonly associated with citrus, including *Phytophthora*, *Alternaria*, *Colletotrichum*, *Penicillium* species, and *Phyllosticta citricarpa* (Table 1). Most of them were characterized at the species level in previous studies (Demontis et al., 2008; El boumlasy et al., 2021; Riolo et al., 2021; Rovetto et al., 2023). Specimens of fungal organisms obtained in this study (Table 1) were molecularly characterized by PCR amplification, analysis and sequencing of specific barcode regions.

For species identification, the chosen *P. tracheiphilus* barcode was the internal transcribed spacer (ITS) region amplified using primer pairs ITS1/ITS4 (White et al., 1990). For *Penicillium* isolates, the barcodes were the ITS region, amplified as above, and the β-tubulin gene (*tub2*), amplified by using primer pairs Bt2a/Bt2b (Glass and Donaldson, 1995). For *Phyllosticta citricarpa,* the barcode was part of the translation elongation factor 1-α gene (*tef1*), amplified by using primer pairs EF1-728F/EF2 (Carbone and Kohn, 1999; O'Donnell et al., 1998). PCR reactions were performed using the *Taq* DNA polymerase recombinant (Invitrogen™, Carlsbad, 254 CA, USA) following the manufacturer's instructions. Obtained PCR products 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. Barcode sequences of isolates obtained in this study were submitted to the GenBank database under the following accession numbers: *P. tracheiphilus* isolate Pt22 (OR656741), Pt3 (OR656742), Pt6-Lim-Sic (OR656743), Pt8 (OR656744), Pt9-Lim-Sic (OR656745), Pt10 (OR656746); *P. citricarpa* isolate 1G (OR665395); *P. italicum* isolate T4N0 (OR652459, OR665396).

### 2.2. Selection of Plenodomus tracheiphilus target region and development of RPA primers and probe

A 142 bp ITS barcode of *P. tracheiphilus* (Supplementary Fig. S1) was selected by alignment in MEGAX (MEGA - Molecular Evolutionary Genetics Analysis) of several ITS sequences deposited in NCBI's GenBank (GB accession numbers: AY531665–AY531682 and AY531689) all belonging to isolates of *P. tracheiphilus* that have been formally identified at the species level (Balmas et al., 2005). Barcode specificity was confirmed by BLAST searches on the NCBI nucleotide database. Primers for the RPA amplification of the 142 bp selected barcode (Supplementary Table S1) were designed using the Primer BLAST NCBI tool. *P. tracheiphilus*-specificity of designed primers was preliminarily confirmed by *in silico* amplification using the Primer BLAST NCBI tool followed by conventional PCR amplification, performed as described above on 1 ng of genomic DNA (gDNA) of each organism listed in Table 1. PCR products were visualized by electrophoresis in TAE 1X Agarose gel and sequenced as described above. The sequences obtained were aligned on MEGAX and all were found to perfectly match the 142 bp of the selected barcode.

The RPA probe, designed on a 50 bp fragment within the selected barcode (Supplementary Fig. S), was labeled by a THF abasic–site, flanked by a FAM and a corresponding dt-Q group. Finally, the probe was blocked at the end 3′ by a C3-Spacer (C3-S) modification group.

### 2.3. RPA amplifications

Each RPA reaction performed in this study was conducted in a 0.25 mL tube by using the Rapid DNA/RNA Amplification Test Kit (AmplifyRP® - Discovery, Agdia Emea, France) following the manufacture's instructions. Specifically, the kit consisted of RPA mastermix pelleted reagents, a rehydration solution (rehydration buffer - 68.6% v/v, forward and reverse primers - 0.40 μM, XRT probe - 0.11 μM, and nuclease free water till the volume of 21.5 μl), MgOAc (1.25 μL – final concentration 14.70 mM) and gDNA (1.0 μL). Amplifications were run in the AmpliFire® Isothermal Fluorometer (Agdia Emea, France). Conditions consisted of a 20-min heating at the constant temperature of 39 °C. Each RPA run included negative (nuclease free water) and positive (1 ng of gDNA of the isolate Pt2) controls. Each reaction was carried out in triplicate. All RPA experiments carried out in this study were repeated three times, and the observed variations in results are presented in this paper. Amplification curve files were used for constructing linear regression curves and for the statistical analyses.

### 2.4. Demontis's Real Time-PCR reactions

Each Real Time-PCR reaction carried out in this study consisted of 10 μl of PowerUp™ SYBR™ Green Master Mix (2 × ) (Applied Biosystems™, Foster City, CA, United States), 0.5 μM of each primer (Phomafor: 5′-GCTGCGTCTGTCTCTTCTGA-3′; Phomarev: 5′-GTGTCCTACAGGCAGGCAA-3′), 1 μl of DNA template and nuclease free water till the final volume of 20 μL. Amplification runs were carried out in a QuantGene 9600 Fluorescent Quantitative Detection System (Bioer Technology, Hangzhou, Zhejiang, China); amplification conditions were 2 min at 50 °C (UDG activation) and 2 min at 95 °C (Dual-Lock™ DNA polymerase) followed by 40 cycles of 95 °C for 15 s (denaturation), 59.5 °C for 15 s (annealing) and 72 °C (extension) for 1 min. Each run included negative (nuclease free water) and positive (1 ng of gDNA of the isolate Pt2) controls. All Real Time-PCR reactions were carried out in triplicate. All Real Time-PCR experiments carried out in this study were repeated three times, and observed variations in the results are presented in this paper.

### 2.5. Evaluation of P. tracheiphilus-specificity and -inclusivity of RPA assay

Specificity of the RPA assay was checked against the plant host, *Citrus limon* and against a panel of selected fungal and oomycete isolates, Inclusivity was checked by analyzing results of the assay on DNA isolated from 29 specimens of *P. tracheiphilus* (Table 1). Plant samples were represented by stem fragments of *Citrus limon* cv. Femminello Siracusano and, for fungal and oomycete pathogens, by fresh mycelium collected from the edges of 7-days-old cultures grown on Potato Dextrose Agar at 25 °C. Genomic DNA (gDNA) of all samples was extracted by using the PowerPlant® Pro DNA Isolation Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. For each sample, the gDNA concentration was checked using a Qubit fluorimeter (Invitrogen™,

Waltham, MA, United States) and adjusted at 1 ng/μL.

### 2.6. Evaluation of sensitivity of the RPA assay

The sensitivity of the newly developed RPA assay was compared with that of the SYBR® Green I Real Time-PCR test of Demontis et al. (2008).

#### 2.6.1. Evaluation of analytical sensitivity

The analytical sensitivity of the RPA assay was evaluated on gDNA of *P. tracheiphilus* isolate Pt2 extracted from $10^9$ conidia by using the PowerPlant® Pro DNA Isolation Kit as described above, further subjected to multiple 10-fold dilutions from 1.0 to 0.0001 ng/μL using 1X TE buffer (10 mM Tris–HCl and 1 mM EDTA; pH 8.0).

#### 2.6.2. Evaluation of performance of the RPA assay toward rapidly extracted non-purified target DNA

To assess the sensitivity of RPA assay on rapid crude extractions of non-purified target DNA, tests were conducted on the gDNA of *P. tracheiphilus* isolate Pt2, obtained using three distinct procedures.

Procedure i.: $10^9$ conidia of isolate Pt2 were suspended in 1 mL GEB lysis buffer (Agdia Emea, France) and the suspension was then ground using a Kontes pestle (Fisher Scientific, Waltham, MA, US) until a satisfactory liquefaction was observed. DNA was extracted using rapid DNA extraction protocol of Edwards et al. (1991) following a slightly modified protocol below. After liquefaction, 1 mL of isopropanol was added. The sample was then vortexed for 5 s and centrifuged at 13,000 rpm for 5 min. After centrifugation, the supernatant was discharged and the obtained pellet was air-dried for 5 min. Then, the pellet was dissolved in 100 μL of 1X TE buffer (10 mM Tris–HCl and 1 mM EDTA; pH 8.0) and the obtained solution was centrifuged at 13,000 rpm for 1 min. After centrifugation, the supernatant was transferred into a new 1.5 ml tube. Then, 10-folds dilutions (from 1.0 to 0.0001 ng/μL) in 1X TE buffer (10 mM Tris–HCl and 1 mM EDTA; pH 8.0) were realized and stored at −20 °C until molecular analyses (Fig. S2a).

Procedure ii.: $2.0 \times 10^8$ conidia of the isolate Pt2 were suspended in 2 mL of GEB lysis buffer and 10-fold dilutions (from $10^8$ to 10 conidia/mL) were obtained using the same buffer. One mL of each dilution was first ground using a Kontes pestle until a satisfactory liquefaction was observed and, then, DNA was extracted from each sample as described above in Procedure i (Fig. S2b).

Procedure iii.: in order to verify the influence of plant matrix (twig cuttings and petioles) on the RPA sensitivity, a two steps specific, rapid gDNA extraction procedure was accomplished (Fig. S2c). STEP 1 – Preparation of a crude plant extract: mal secco-free twig cuttings (or petioles) were finely ground to a powder by using a cheese grater; then, 1 g of the obtained powder from each sample was added to 10 mL GEB lysis buffer). The mixture was then vortexed for 5 s and centrifuged at13,000 rpm for 5 min, and the obtained supernatant was transferred to new 1.5 ml tubes and employed instead of the lysis buffer in the gDNA extraction below. STEP 2 – gDNA extraction: $2.0 \times 10^8$ conidia of the isolate Pt2 were suspended in 2 mL of crude plant extract. The extract was used to generate several 1 mL-dilutions using a 10-fold dilution series starting from the $10^8$ conidia/mL concentration and ending with a 10 conidia/mL dilution. Each 1 mL sample of conidia was ground by a Kontes pestle until a satisfactory liquefaction was observed; then, for each sample, the extraction of the DNA proceeded in accordance with the rapid DNA extraction protocol described in Procedure ii.

#### 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) of both RPA and Real Time PCR assays at 95% of probability and confidence interval was calculated by Probit regression "Dose-Response" analysis performed with eight replicates by using MedCalc Software (Ostend, Flanders, 8400, Belgium). The genome copy number was calculated following Cesbron et al. (2023) by using the estimated genome size of the reference isolate *P. tracheiphilus* strain IPT5 (34,242,632 bp; GenBank accession number: GCA_010093695.1), knowing that the mean weight of one nucleotide pair is $1.023 \times 10^{-9}$ pg (Dolezel et al., 2003).

### 2.7. Efficiency of the Plenodomus tracheiphilus RPA assay in symptomatic and asymptomatic plant material

In order to evaluate the practical application of the RPA assay, tests were carried out on mal secco-symptomatic and -asymptomatic plant material (lemon twigs and petioles). For this purpose, plant samples were collected in October 2022 from commercial lemon orchards of the area of Catania and Syracuse (Italy) (Table 2). For each sample, total DNA was extracted from 0.1 g of powdered plant material (lemon twigs or petioles) as described at paragraph 2.6.2., procedure 1.

Then, obtained DNA samples were tested using both the RPA assay and Demontis's Real Time-PCR assay (Demontis et al., 2008). Positivity to mal secco of the samples was additionally verified by isolation and molecular identification of cultures by conventional PCR, according to the PM 7/048 *P. tracheiphilus* EPPO diagnostic protocol (EPPO Standards, 2015).

## 3. Results

### 3.1. Identifying a suitable barcode for the development of the RPA assay

First, a 142 bp RPA technology-compatible barcode within the Internal Transcriber Spacer (ITS) of *P. tracheiphilus* was selected based on a consensus alignment of *P. tracheiphilus* ITS sequences deposited in NCBI (Supplementary Fig. S1). BLAST searches of the NCBI nucleotide database confirmed *in silico* that only *P. tracheiphilus* sequences had a 100% homology with the chosen barcode, while the second closest match was that of *Plenodomus collinsoniae* with a 93.33% homology.

Second, a primer pair was designed that amplified the selected barcode and met all of the RPA amplification requirements (Supplementary Fig. S1, Supplementary Table S1). Third, the specificity of the designed primer pair to *P. tracheiphilus* was preliminarily confirmed by both *in silico* and conventional PCR amplifications. Finally, an RPA probe was designed to complete the assay (Supplementary Fig. S1, Supplementary Table S1).

### 3.2. Plenodomus tracheiphilus-specificity and -inclusivity of RPA assay

Specificity tests evidenced that neither gDNA from plant material nor gDNA of non-target organisms cross reacted and produced RPA amplifications, thus confirming the complete specificity of the assay. Additionally, all of the *P. tracheiphilus* strains (Table 1) were detected by the RPA assay, confirming its within-species inclusivity.

### 3.3. Sensitivity of the RPA assay

Results reported in this section show the sensitivity of the RPA assay and compare it to the sensitivity of the SYBR® Green I Real Time-PCR test of Demontis et al. (2008).

#### 3.3.1. Analytical sensitivity

Analytical sensitivity was evaluated on 10-fold dilution series of a 1.0 ng/μl concentrated (corresponding to about $2.85 \times 10^3$ genome copies/μl) solution of *P. tracheiphilus* isolate Pt2 purified gDNA. Both RPA and Demontis's Real Time-PCR gave amplifications up to 0.001 ng of gDNA (*i.e.* 1.0 pg of gDNA, corresponding to ~29 genome copies/reaction) (Fig. 1, a and c). The RPA LoD was about 0.048 ng of gDNA (confidence interval – CI – range: $1.02 \times 10^{-2}$ – $8.049 \times 10^3$ ng of gDNA), while the Demontis's Real Time-PCR LoD was about 0.02 ng of gDNA (CI range: $5.27 \times 10^{-3}$ – $1.602 \times 10^7$ ng of gDNA) (Fig. 1, b and d).

**Table 2**
Results from detection tests carried out on plant matrices (twigs/petioles) collected from mal secco-symptomatic and -non symptomatic lemon plants.

| Sample ID | Source of sample | signs/symptoms on the plant (P[a]/A[b]) | Sample (lemon twig/petiole) | Location | *P. tracheiphilus* isolation[f] outcome (+[c]/-[d]) | Demontis's Real Time-PCR (Cycle threshold) | RPA outcome (+[c]/-[d]) |
|---|---|---|---|---|---|---|---|
| ID01 | *Citrus limon* | P | Twig | Syracuse (Sicily) | + | 28.81 | + |
| ID02 | *C. limon* | P | Petiole | Syracuse (Sicily) | – | nd[e] | + |
| ID03 | *C. limon* | A | Twig | Avola (Syracuse, Sicily) | – | 31.49 | + |
| ID04 | *C. limon* | P | Twig | Avola (Syracuse, Sicily) | + | 26.46 | + |
| ID05 | *C. limon* | A | Petiole | Mineo (Catania, Sicily) | – | nd | – |
| ID06 | *C. limon* | P | Petiole | Mineo (Catania, Sicily) | + | 25.51 | + |
| ID07 | *C. limon* | P | Twig | Mineo (Catania, Sicily) | + | 27.11 | + |
| ID08 | *C. limon* | A | Twig | Mineo (Catania, Sicily) | – | nd | – |

[a] P = presence.
[b] A = absence.
[c] + = positive.
[d] - = negative.
[e] nd = no-detection by Real Time-PCR.
[f] Isolates were identified as specimens of *P. tracheiphilus* by conventional PCR analysis performed according to the PM 7/048 *P. tracheiphilus* EPPO diagnostic protocol (EPPO Standards, 2015).

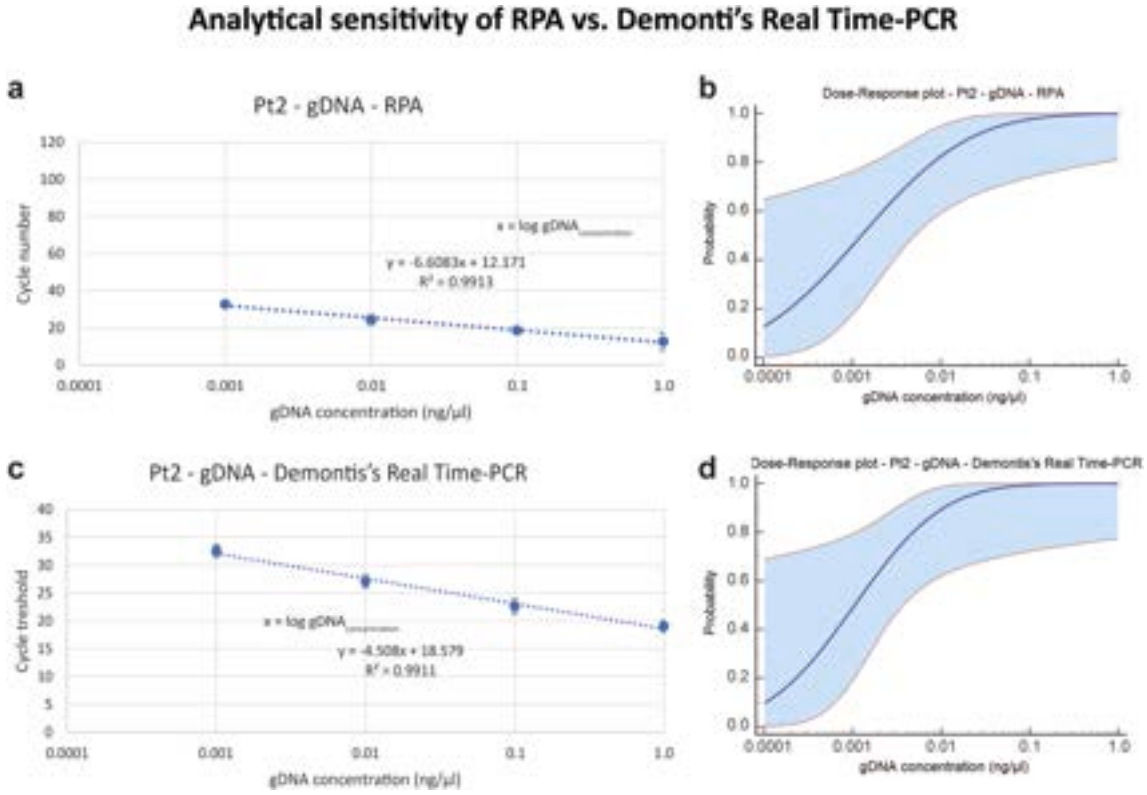


**Fig. 1.** Analytical sensitivity of RPA vs. Demonti's Real Time-PCR. On the left, graphs showing the linear regression analysis of RPA (a) and Demontis's Real Time-PCR (c) assays for the evaluation of their sensitivity toward independent serial dilutions of purified gDNA of the *P. tracheiphilus* isolate Pt2; bars indicate standard deviation (SD); blue dashed lines are the linear regression curves (linear equations and $R^2$ values are reported). On the right, Dose-Response probit regression analyses showing the probability of detection with RPA (b) and Demontis's Real Time-PCR (d) assays.

### *3.3.2. Performances of the RPA assay in rapidly extracted and non-purified target DNA*

Results on the sensitivity of the RPA assay in rapidly extracted non-purified DNA of *P. tracheiphilus* isolate Pt2, obtained using three distinct procedures, are presented below. For each extraction procedure, sensitivity of RPA assay toward the extracted DNA was compared to that achievable by Demontis's Real Time-PCR.

In 'procedure i' (Fig. S2a), both RPA and Demontis's Real Time-PCR gave amplifications up to 0.001 ng of gDNA (*i.e.* 1.0 pg of gDNA, corresponding to ~29 genome copies/reaction) (Fig. 2, a and c).With this procedure the LoD was about 0.20 ng of gDNA (CI range: $3.26 \times 10^{-2}$ – $2.42 \times 10^{3}$ ng of gDNA) for both RPA and Demontis's Real Time-PCR (Fig. 2, b and d).

In 'procedure ii' (Fig. S2b) a similar sensitivity was measured for

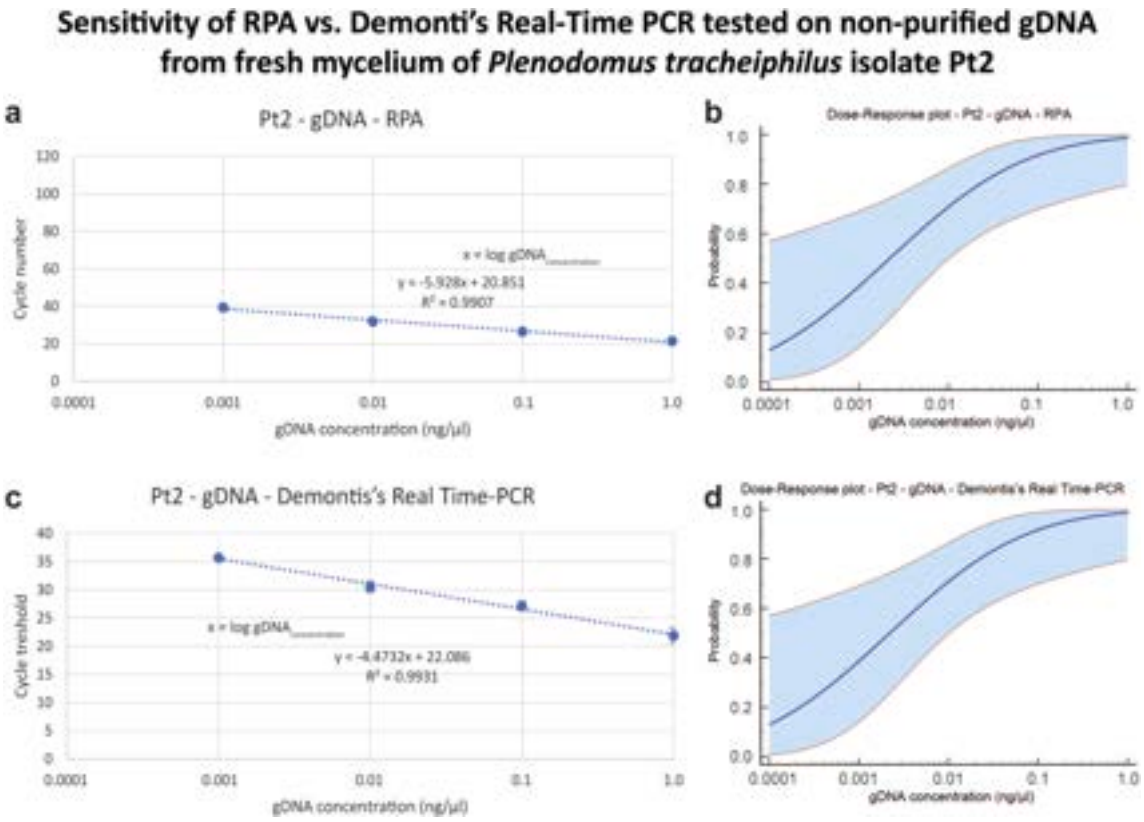


**Fig. 2.** Sensitivity of RPA vs. Demonti's Real-Time PCR tested on non-purified gDNA from fresh mycelium of *Plenodomus tracheiphilus* isolate Pt2. On the left, graphs showing the linear regression analysis of RPA (a) and Demontis's Real Time-PCR (c) assays for the evaluation of their sensitivity toward independent serial dilutions of non-purified gDNA of the *P. tracheiphilus* isolate Pt2; bars indicate SD; blue dashed lines are the linear regression curves (linear equations and $R^2$ values are reported). On the right, Dose-Response probit regression analyses showing the probability of detection with RPA (b) and Demontis's Real Time-PCR (d) assays.

both RPA and Demontis's Real Time-PCR, with amplifications, for both techniques, in samples that contained $10^4$ conidia/ml (Fig. 3, a and c) and above. For RPA the LoD was about $5.49 \times 10^6$ conidia/ml (CI range: $9.22 \times 10^5$–$1.99 \times 10^9$ conidia/ml), while for Demontis's Real Time-PCR the LoD was about $6.7 \times 10^6$ conidia/ml (CI range: $1.16 \times 10^6$–$1.59 \times 10^9$ conidia/ml) (Fig. 3, b and d).

In 'procedure iii' (Fig. S2c) Results from samples suspended in plant crude extract from twig cuttings highlighted that both RPA and Demontis's Real Time-PCR could amplify suspensions containing $10^4$ conidia/ml (Fig. 4, a and c) and above. In this experimental condition RPA LoD was about $3.46 \times 10^6$ conidia/ml (CI range: $4.72 \times 10^5$–$4.03 \times 10^{10}$ conidia/ml), while the Demontis's Real Time-PCR LoD was about $4.97 \times 10^7$ conidia/ml (CI range: $3.21 \times 10^6$–$3.68 \times 10^{10}$ conidia/ml) (Fig. 4, b and d).

With reference to samples obtained by the suspension of conidia in plant crude extract from petioles, both RPA and Demontis's Real Time-PCR had positive amplifications for suspensions of $10^4$ conidia/ml and above (Fig. 5, a and c); in this experiment RPA LoD was about $4.30 \times 10^6$ conidia/ml (CI range: $1.07 \times 10^6$–$9.48 \times 10^9$ conidia/ml), while the Demontis's Real Time-PCR LoD reached $2.29 \times 10^7$ conidia/ml (CI range: $4.49 \times 10^6$–$1.01 \times 10^{10}$ conidia/ml) (Fig. 5, b and d).

### *3.4. Efficiency of RPA assay in detecting P. tracheiphilus DNA in symptomatic and asymptomatic plant material*

The efficiency of the RPA assay in detecting *P. tracheiphilus* DNA in symptomatic and asymptomatic plant material (twigs and petioles) was tested in comparison with Demontis's Real Time-PCR (Demontis et al., 2008). Positivity to mal secco of collected samples was additionally verified through isolations followed by molecular detection of cultures by conventional PCR, according to the PM 7/048 *P. tracheiphilus* EPPO diagnostic protocol (EPPO Standards, 2015). Detection of *P tracheiphilus* was successful on all symptomatic twig samples (ID01, ID04, ID07; Table 2) by pathogen isolation following the EPPO diagnostic protocol (EPPO Standards, 2015), by the RPA assay described here and by Demontis's Real Time-PCR. In this last assay, cycle thresholds were in the range 27.11–28.81 (Table 2).

Twig samples collected from non-symptomatic plants (ID03 and ID08), ID03 were positive based on the RPA and Demonti's Real Time-PCR assays, but were negative based on isolations. Twig sample ID08 was negative for *P. tracheiphilus* with all three methods. Symptomatic petiole sample ID02 (Table 2), was *P. tracheiphilus*-positive just by RPA, while symptomatic petiole ID06 (Table 2) was positive for the presence of *P. tracheiphilus* by all the employed detection methods (Demontis's Real Time-PCR cycle threshold, 25.51). Finally, no positive detection by any method was reported from the petiole sample ID05 collected from a non-symptomatic host (Table 2).

## 4. Discussion

Phytosanitary emergencies require tools capable of reducing the time necessary for the selection of the most effective management strategies (Cacciola and Gullino, 2019). This is particularly relevant for quarantine plant pathogens, such as *P. tracheiphilus*, whose management often involves the adoption of strict measures that limit the trade of plant products and propagation material (Migheli et al., 2009). For this reason, nowadays, these emergencies must rely on technologically advanced means, such as molecular techniques able to satisfy the major requirements of the detection, *i.e.* specificity, sensitivity and rapidity (Aslam et al., 2017; Boonham et al., 2016; Donoso and Valenzuela, 2018; Hariharan and Prasannath, 2021; Marcolungo et al., 2022; Patel et al., 2022).

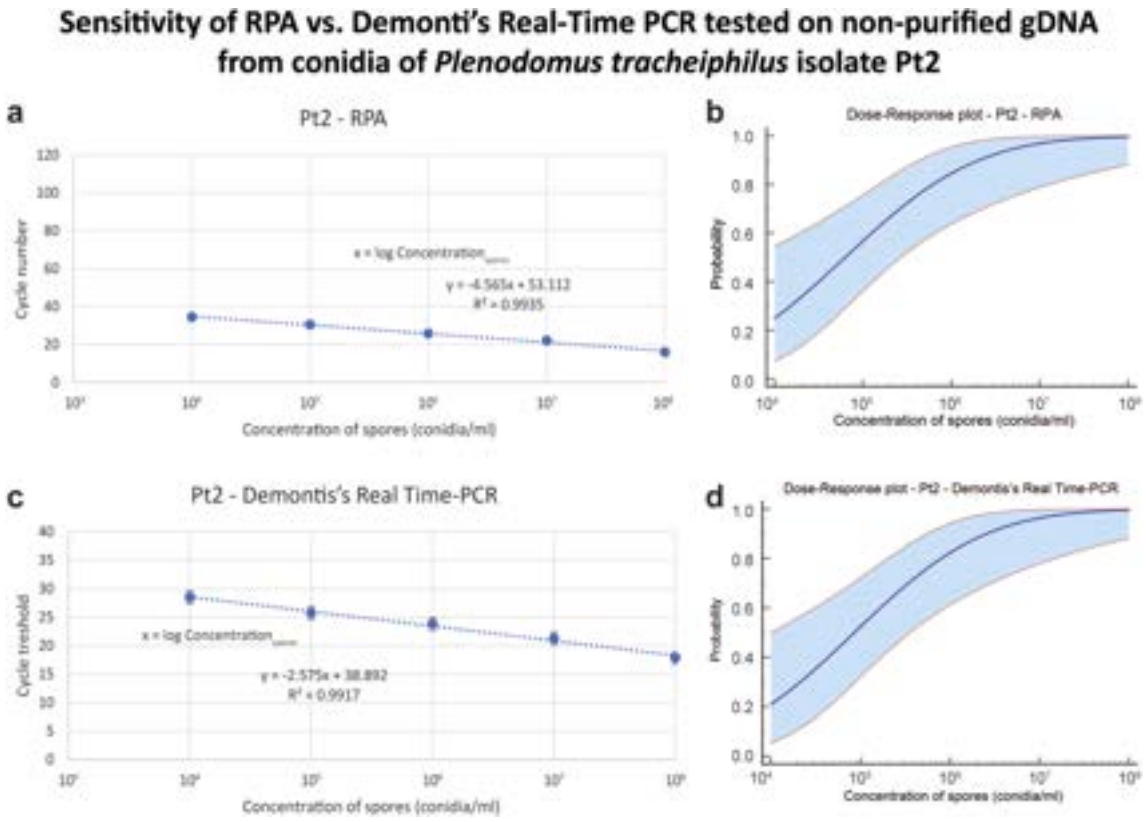


**Fig. 3.** Sensitivity of RPA vs. Demonti's Real-Time PCR tested on non-purified gDNA from conidia of *Plenodomus tracheiphilus* isolate Pt2. On the left, graphs showing the linear regression analysis of RPA (a) and Demontis's Real Time-PCR (c) assays for the evaluation of their sensitivity toward non-purified gDNA of the *P. tracheiphilus* isolate Pt2 obtained from 10-folds conidia suspensions (from $10^8$ to 10 conidia/mL) in GEB lysis buffer; bars indicate SD; blue dashed lines are the linear regression curves (linear equations and $R^2$ values are reported). On the right, Dose-Response probit regression analyses showing the probability of detection with RPA (b) and Demontis's Real Time-PCR (d) assays.

The most reliable diagnostic protocols so far developed for the early detection of *P. tracheiphilus* in potentially infected plant material are officially listed by EPPO and are based on different molecular approaches. The first molecular standard issued by EPPO for the diagnosis of *P. tracheiphilus* (EPPO Standards, 2005) was based on studies carried out by Rollo et al. (Rollo et al., 1987, 1990), and employed a dot blot approach in combination with the polymerase chain reaction (PCR). Advancements in DNA sequencing and bioinformatic tools have fostered the search for highly specific protocols for the detection of plant pathogens (Venbrux et al., 2023). The study by Balmas et al. (2005) achieved a very high diagnostic specificity resulting in a very reliable detection of *P. tracheiphilus* causing mal secco. Specifically, in Balmas et al. (2005), the phylogenetic characterization of a population of Italian isolates of *P. tracheiphilus* provided several ITS1-5.8S-ITS2 sequences. The alignment of such sequences highlighted a 544 bp highly specific consensus sequence that provided the basis for the design of the primer pair Pt-FOR2/Pt-REV2. The high *P. tracheiphilus*-specificity of these primers made them an obvious choice in the updated version of the 2015 of the EPPO diagnostic protocol (EPPO Standards, 2015). Other primers targeting the ITS region for the detection of *P. tracheiphilus* in infected tissues of the host plant by conventional PCR were subsequently developed by other authors (Ezra et al., 2007; Kalai et al., 2010). The performance of these primers was comparable to that of the primers designed by Balmas et al. (2005). Despite its high versatility, conventional PCR is often not enough sensitive to guarantee plant material as being free from the pathogen; additionally, it is not quantitative (Demontis et al., 2008). More recently, the development of real time PCR, also known as qPCR, has resulted in protocols with improved sensitivity for the detection of *P. tracheiphilus* (Demontis et al., 2008). The TaqMan®/SYBR® Green I real-time PCR protocol of Demontis et al. (2008) was included as quantitative method in the PM 7/048 (3) *Plenodomus tracheiphilus* EPPO diagnostic protocol (EPPO Standards, 2015), due to its reliability and high sensitivity (detecting as little as 15 pg of the target pathogen's DNA).

Although the above protocols all meet the required specificity and sensitivity, their major drawback remains the time necessary to complete the assay. In fact, in addition to the technical time needed to complete the PCR run (which usually ranges between 1,30 and 1,45 h) and to analyze results, DNA samples that will undergo amplification by *Taq* polymerase require high purity (Demeke and Adams, 1992; Demeke and Jenkins, 2010; Schrader et al., 2012; Wei et al., 2008). The need for pure DNA extracts extends the time necessary for pathogen detection by PCR techniques to about one working day (Demontis et al., 2008). Isothermal amplification technologies, such as loop-mediated amplification (LAMP), helicase-dependent amplification (HDA), nucleic acid sequence-based amplification (NASBA), rolling circle amplification (RCA) and recombinase polymerase amplification (RPA) have shortened the time necessary to achieve a diagnostic outcome (Ivanov et al., 2021). These methods differ from each other in terms of amplification temperatures, sensitivities, reaction times, and other advantages and drawbacks (Gill and Ghaemi, 2008; Ivanov et al., 2021). More recently, diagnostic assays based on RPA have become widely used for the molecular-based diagnosis of diseases (Tan et al., 2022). The outbreak of SARS-CoV-2 has further promoted the application of RPA in nucleic acid detection (Bai et al., 2022). Previous studies on RPA detection have shown several advantages of this technique, including its user-friendliness (in terms of simplicity of operations and low equipment requirements), high sensitivity, and great specificity (Tan et al., 2022). Additionally, RPA best matches the requirement of 'rapidity'. In this respect, with a reaction time of no more than 20 min, RPA is not only the

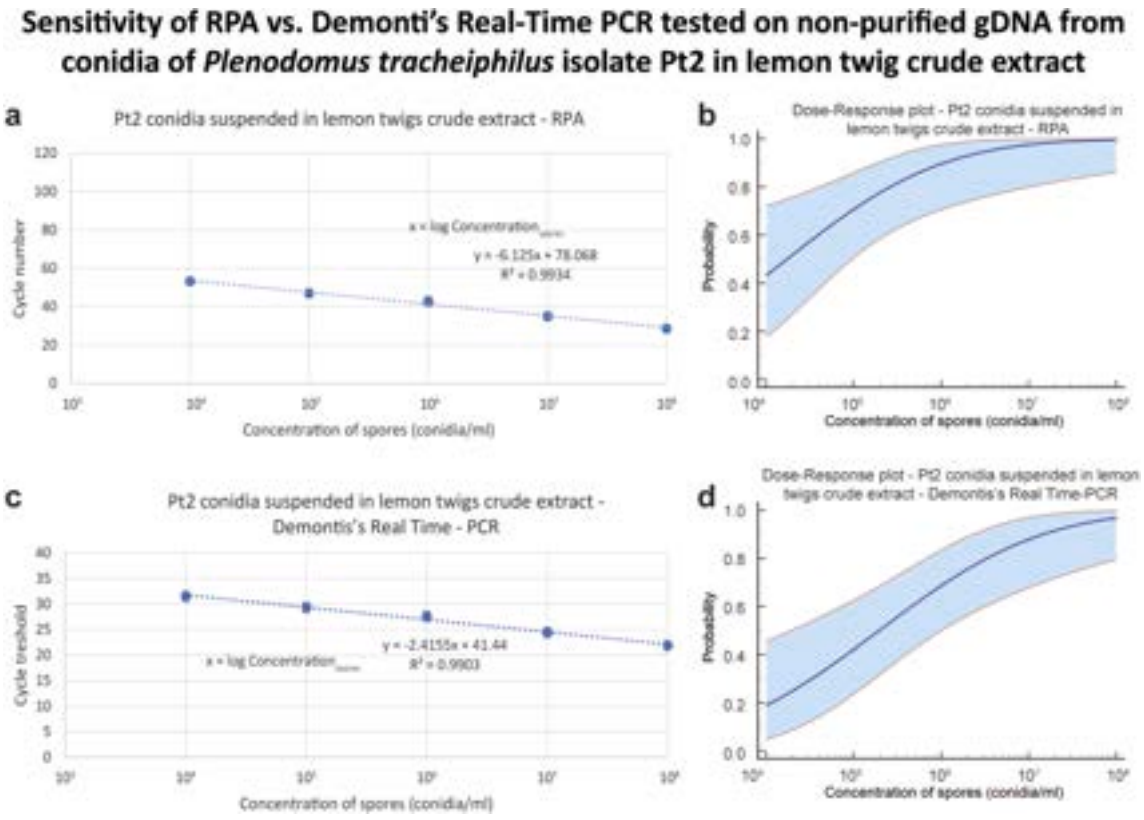


**Fig. 4.** Sensitivity of RPA vs. Demonti's Real-Time PCR tested on non-purified gDNA from conidia of *Plenodomus tracheiphilus* isolate Pt2 in lemon twig crude extract. On the left, graphs showing the linear regression analysis of RPA (a) and Demontis's Real Time-PCR (c) assays for the evaluation of their sensitivity toward non-purified gDNA of the *P. tracheiphilus* isolate Pt2 obtained from 10-folds conidia suspensions (from $10^8$ to 10 conidia/mL) in lemon twig crude extract; bars indicate SD; blue dashed lines are the linear regression curves (linear equations and $R^2$ values are reported). On the right, Dose-Response probit regression analyses showing the probability of detection with RPA (b) and Demontis's Real Time-PCR (d) assays.

fastest isothermal amplification technique, but it is also the most reliable technique in terms of tolerance to inhibitors of the molecular reaction (Boluk et al., 2020; Chandu et al., 2016; Kapoor et al., 2017). Inhibition of molecular reactions is one of the major concerns in PCR technologies (Sidstedt et al., 2020). A PCR inhibitor is defined as any substance that is capable of interfering with one or more of the molecular reactions involved in PCR (denaturation, primer annealing, binding of polymerase to primer-DNA complex, primer extension, and fluorescence - in real-time technologies) and that consequently determines an inhibition of the amplification (Hedman et al., 2013). PCR inhibitors comprise a wide array of substances, including inorganic ions, organic salts, organic acids, some dyes and other molecules (Hedman et al., 2013). The wide tolerance of RPA to common PCR inhibitors is a highly relevant strength of this technique, because DNA samples obtained through rapid extractions, although not purified from inhibitors, can be used in RPA amplifications with a high chance of success (Ivanov et al., 2021). This is the first study describing the development of a molecular diagnostic assay for the mal secco of lemon caused by *P. tracheiphilus* that is based on isothermal amplification of nucleic acids. The tests presented here confirmed the exclusive amplification of the selected barcode of the target organism and led to the design of the final element of this detection assay, the RPA probe. Then, RPA runs made it possible to validate the whole *P. tracheiphilus*-specificity and -inclusivity of the developed RPA assay. In this study, various experimental assays were assessed to test the sensitivity of the developed RPA assay and to highlight its strengths and limitations in comparison to the SYBR® Green I Real Time-PCR test by Demontis et al. (2008). Results of analytical sensitivity test carried out on purified DNA extracted from conidia of *P. tracheiphilus* highlighted that the RPA assay was extremely sensitive and as sensitive as Demonti's Real Time-PCR under the same conditions. However, under our experimental conditions, RPA was ten times more sensitive than Demonti's Real Time-PCR when testing conidial DNA extracts by a rapid procedure. A one order of magnitude higher sensitivity of the RPA compared to Demonti's assay was also confirmed when crude plant extracts, and supposedly inhibitors, were added to the substrate tested. This result is consistent with previous studies that have demonstrated the high reliability of RPA in amplification runs in the presence of plant macerates (Boluk et al., 2020; Chandu et al., 2016; Kapoor et al., 2017), as well as with other studies that highlighted a significant inhibition of PCR in the presence of plant metabolites such as pectin, polyphenols, polysaccharides, and xylan (Demeke and Adams, 1992; Monteiro et al., 1997; Schrader et al., 2012; Wei et al., 2008).

To preliminarily assess the applicability of the method for field applications, the effectiveness of the RPA assay was finally evaluated for detecting mal secco infections *in planta*. The reliability of the assay was compared to Demontis's Real Time-PCR and classical pathogen isolation followed by molecular detection of the isolates. Both RPA and Demontis's Real Time-PCR were superior to the isolation approach and comparable to each other.

In conclusion, the RPA assay's specificity and inclusivity were validated through various tests, and it was found that RPA was more sensitive than the Demonti's Real Time-PCR in samples containing plant crude extract. However, it is important to note that the accuracy and reproducibility of any diagnostic tool are critical for its effective use. Therefore, results obtained here deserve to be validated through ring tests, which involve multiple laboratories performing the same assay on the same samples. The RPA assay has several advantages over traditional detection methods, including its speed, ease of use, and potential for field applications. Based on the promising result presented in this study, further research is needed to optimize and validate this technique for use

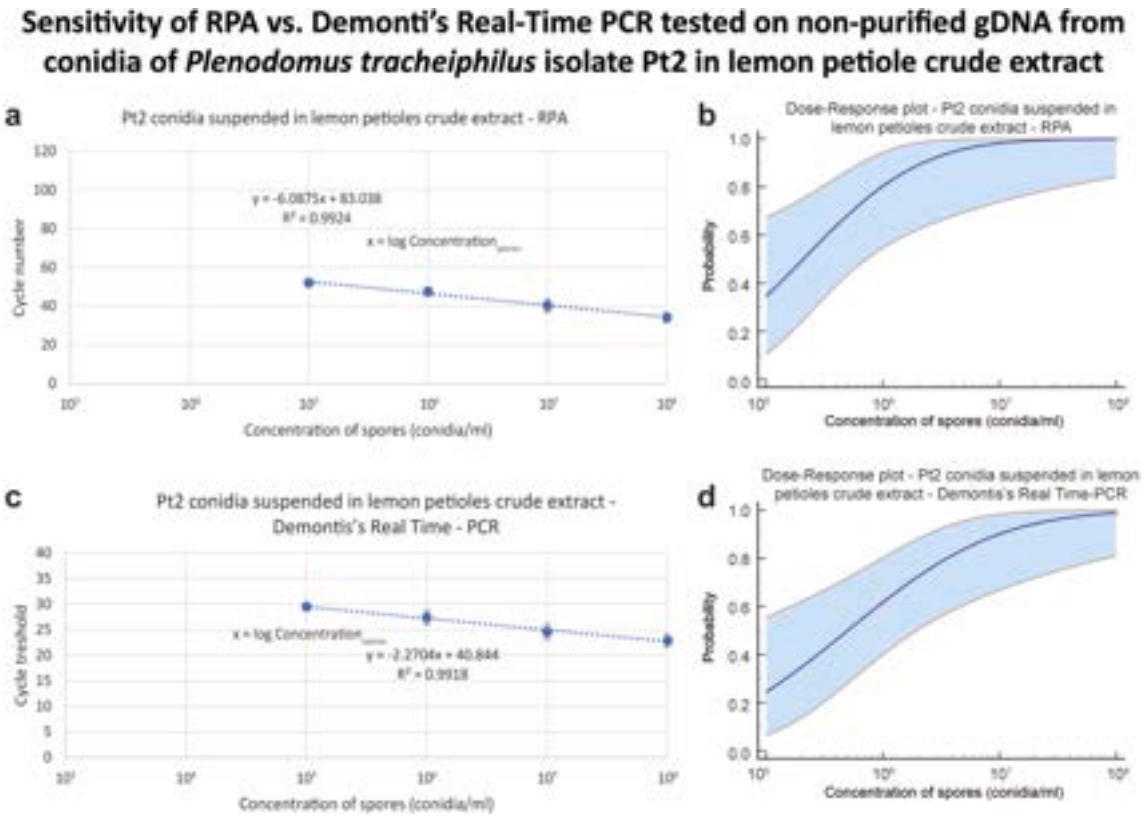


**Fig. 5.** Sensitivity of RPA vs. Demonti's Real-Time PCR tested on non-purified gDNA from conidia of *Plenodomus tracheiphilus* isolate Pt2 in lemon petiole crude extract. On the left, graphs showing the linear regression analysis of RPA (a) and Demontis's Real Time-PCR (c) assays for the evaluation of their sensitivity toward non-purified gDNA of the *P. tracheiphilus* isolate Pt2 obtained from 10-folds conidia suspensions (from $10^8$ to 10 conidia/mL) in lemon petiole crude extract; bars indicate SD; blue dashed lines are the linear regression curves (linear equations and $R^2$ values are reported). On the right, Dose-Response probit regression analyses showing the probability of detection with RPA (b) and Demontis's Real Time-PCR (d) assays.

in various conditions and to make it more accessible to farmers and researchers.

## CRediT authorship contribution statement

**Ermes Ivan Rovetto:** Writing – original draft, Validation, Investigation, Formal analysis, Data curation. **Matteo Garbelotto:** Writing – review & editing, Methodology, Conceptualization. **Salvatore Moricca:** Writing – review & editing, Methodology, Conceptualization. **Marcos Amato:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Federico La Spada:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Santa Olga Cacciola:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

## 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. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cropro.2024.106825.

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