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A real time loop-mediated isothermal amplification (RealAmp) assay for rapid detection of *Pleurostoma richardsiae* in declining olive plants

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Summary. *Pleurostoma richardsiae* is associated with host trunk diseases, known to cause dieback, cankers and wilting of woody trees, and human infections. This fungus was isolated from wood tissues of declining olive trees and grapevines showing esca disease symptoms, in the Apulia region of Italy. Fungus detection has been based on morphological and molecular features, which are time-consuming to identify and require well-trained personnel. Improvement of *Pl. richardsiae* detection in olive was achieved through development of real time loop-mediated isothermal amplification targeting the intergenic spacer (IGS) region of the fungus. Specificity of the assay was confirmed using ten *Pl. richardsiae* strains and 36 other fungus strains of species usually isolated from declining olive trees. The achieved limit of detection was 7.5×10^{-2} ng μL^{-1} of *Pl. richardsiae* genomic DNA. A preliminary validation of RealAmp was also performed using material from infected olive plants artificially inoculated in a greenhouse.

Keywords. IGS, molecular assay, olive decline, fungi.

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INTRODUCTION

General decline of olive trees, similar to olive quick decline caused by *Xylella fastidiosa* (Saponari *et al.*, 2013), was observed in olive orchards in Barletta-Andria-Trani (BAT) and Foggia provinces of the Apulia region of Southern Italy, which is not in the demarcated area infected by *X. fastidiosa* (Commission Implementing Decision EU 2017/2352 https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32017D2352&from=IT). Typical symptoms were wilting of apical shoots, dieback of twigs and branches, and

brown streaking under the bark of the trunks of affected trees. According to Carlucci *et al.* (2013), three fungal species, *Pleurostoma richardsiae, Neofusicoccum parvum* and *Phaeoacremonium minimum*, were strongly associated with these symptoms, and were diagnosed through morphological, molecular and pathogenicity tests. *Phaeoacremonium* spp. and *Botryosphaeriaceae* spp. are known to cause dieback, cankers and wilting on olive and grapevine plants (Carlucci *et al*., 2015c; 2015b). *Pleurostoma richardsiae* was isolated and associated for the first time in Italy from brown wood streaking and canker of olive (Carlucci *et al.,* 2013) and grapevine plants (Carlucci *et al.,* 2015a), confirming its pathogenicity. Further reports have associated this pathogen with olive and grapevine plants. Pintos Varela *et al.* (2016), Özben *et al.* (2017) and Canale *et al.* (2019) confirmed *Pl. richardsiae* as a grapevine pathogen in Spain, Turkey and Brazil, while Ivic *et al.* (2018), Canale *et al.* (2019), Agustì-Brisiach *et al.* (2021), Lawrence *et al.* (2021) and Van Dyk *et al.* (2021) confirmed this fungus as an olive pathogen in Croatia, Brazil, Spain, California and South Africa.

Pleurostoma richardsiae is also known as an uncommon cause of human infections, usually through traumatic skin inoculations with contaminated vegetable matter, causing subcutaneous Phaeohyphomycosis (Pitrak *et al.,* 1988; Vijaykrishna *et al*., 2004).

Identification of *Pl. richardsiae* has been based on micromorphological and cultural features (Schol-Schwarz, 1970; Domsch *et al*., 1980; De Hoog *et al*., 2000; Vijaykrishna *et al*., 2004). Molecular tools targeting the 5.8S rDNA gene flanking internal transcribed spacers 1 and 2 (ITS) have more recently been used (Carlucci *et al*., 2015c; Vijaykrishna *et al.,* 2015; Spies *et al*., 2020). The polymerase chain reaction is an efficient technique for the detection of *Pl. richardsiae*, although the DNA extraction phase and the thermal cycle requirements for the Taq polymerase function are constraints in terms of costs and time for the application of this technique at large scale. These limitations can be overcome by the loop-mediated isothermal amplification (LAMP) assay, which relies on partial DNA extraction and isothermal polymerase to amplify the DNA (Notomi *et al.,* 2000). LAMP is a technique that uses six primers that are complementary to eight regions of genomic DNA, and therefore ensures high specificity and allows results to be obtained more rapidly than PCR or qPCR. In addition, the isothermal character of the reaction requires only a heat block or water bath at the target temperature to successfully amplify DNA. Bst polymerase used in LAMP assays allows a full reaction to take place in less than 1 h in an isothermal environment (ranging from 60 to 70°C) depending on the type of enzyme and the optimal temperature for the primer characteristics (Notomi *et al*., 2000).

LAMP reaction can be detected in end point, as resolving amplified products on agarose gel electrophoresis (Notomi *et al*., 2000), by eye observation of precipitate (Mori *et al*., 2001), or employing DNA-binding dyes (Le *et al*., 2012) or colorimetric indicators (Parida *et al*., 2008; Tomita *et al*., 2008). End point detection strategies do not allow real-time diagnoses. They may result in false positives detections due to contamination of samples (e.g., reaction tube opening procedure). In addition, determination of the LAMP reaction by visual observation is subjective, because it is based on individual perception of colour (Bista *et al*., 2007). These problems can be overcome by real-time LAMP reactions (RealAmp), that can also be performed *in situ* using Smart-DARTTM (Diagenetix), a portable device equipped with D&A software that can analyze eight samples each time. This is a major advantage of RealAmp for the rapid detection of pathogens in plant material, in the field and at entry points and nurseries, avoiding risks of pathogen spread through the movement of infected material (Yaseen *et al*., 2015). This technique has also been applied in the field and laboratory for diagnoses of human and animal diseases and food safety control (Abdulmawjood *et al*., 2014).

LAMP assays have been extensively developed for the detection of several plant pathogens, including *Candidatus Liberibacter solanacearum* (Ravindran *et al.,* 2012), *Pythium aphanidermatum* (Fukuta *et al.,* 2013), *Sclerotinia sclerotiorum* (Duan *et al.,* 2014), *Xylella fastidiosa* (Yaseen *et al*., 2015), *Erysiphe necator* (Thiessen *et al.,* 2016), and *Xanthomonas fragariae* (Gètaz *et al.,* 2017).

Considering the importance of *Pl. richardsiae* as a vascular pathogen of economically important woody plant species, and the high performance offered by the RealAmp assay, the present study aimed to develop a RealAmp assay specific for *Pl. richardsiae*.

MATERIALS AND METHODS

Fungus strains

All fungus strains used in this study are reported in Table 1. The *Pl. richardsiae* strains were obtained from olive or grapevine plants showing symptoms of decline. Eight of the strains were from the north of Apulia region (Southern Italy), and two were of Algerian origin. One strain each of *Pl. ootheca* and *Pl. repens* from Algeria, were also included to assess the specificity of the RealAmp. Another 36 fungal strains of *Phaeoacremonium* and *Neofusicoccum* spp. from different countries and hosts were included in this study. All fungal strains were

Table 1. List of fungal strains isolated from different sources used in this study.

Fungus species	Isolate code/collection[a]	Location; Source
Pleurostoma richardsiae	Pl31/ Dept. DAFNE	Italy; olive tree
	Pl4/ Dept. DAFNE	Italy; olive tree
	Pl_M 1/ CIHEAM of Bari	Italy; olive tree
	Pl_M 2/ CIHEAM of Bari	Italy; olive tree
	Pl_M 3/ CIHEAM of Bari	Italy; grapevine
	Pl_M 4/ CIHEAM of Bari	Italy; grapevine
	Pl_M 5/ CIHEAM of Bari	Italy; grapevine
	Pl_M 6/ CIHEAM of Bari	Italy; olive tree
	Pl_G1/ ENSA	Algeria; grapevine
	Pl_G2/ ENSA	Algeria; olive tree
Pl. ootheca	CBS 115329/ ENSA	British Columbia; degrading wood
Pl. repens	CBS 294.39/ ENSA	USA; pine lumber
Phaeoacremonium alvesii	CBS 408.78/ Dept. DAFNE	USA; humans
	CBS 729.97/ Dept. DAFNE	USA; humans
P. amygdalinum	Pm10/ Dept. DAFNE	Italy; almond
P. griseorubrum	CBS 111657/ Dept. DAFNE	South Africa; humans
P. hispanicum	CBS 123910 Dept. DAFNE	Spain; grapevine
P. iranianum	Pm121/ Dept. DAFNE	Italy; grapevine
P. italicum	Pm17/ Dept. DAFNE	Italy; olive tree
	Pm31M/ Dept. DAFNE	Italy; almond
	CBS 137763/ Dept. DAFNE	Italy; grapevine
	Pm45/ Dept. DAFNE	Italy; olive tree
	Pm50M/ Dept. DAFNE	Italy; olive tree
P. minimum	Pm41/ Dept. DAFNE	Italy; olive tree
	Pm67/ Dept. DAFNE	Italy; olive tree
	Pm68/ Dept. DAFNE	Italy; grapevine
P. oleae	Pm14/ Dept. DAFNE	Italy; olive tree
P. parasiticum	Pm88/ Dept. DAFNE	Italy; olive tree
P. rubrigenum	CBS 498.94/ Dept. DAFNE	USA; humans
	CBS 112046/ Dept. DAFNE	USA; humans
P. scolyti	Pm92A/ Dept. DAFNE	Italy; olive tree
	Pm73M/ Dept. DAFNE	Italy; olive tree
	P.sc1/ Dept. DAFNE	Spain; unknown
	PMM2242 / Dept. DAFNE	South Africa; pomegranate
	CSN 1081/ Dept. DAFNE	South Africa; quince
	CSN55 / Dept. DAFNE	South Africa; plum
	CBS 121755/ Dept. DAFNE	South Africa; peach
	PMM2442/ Dept. DAFNE	South Africa; loquat
	PMM2270 / Dept. DAFNE	South Africa; rose
	CSN 1471/ Dept. DAFNE	South Africa; guava
	CBS 121439/ Dept. DAFNE	South Africa; japanese plum
	CBS 121756/ Dept. DAFNE	South Africa; peach
P. sicilianum	Pm65/ Dept. DAFNE	Italy; olive tree
Neofusicoccum luteum	B531/ Dept. DAFNE	Italy; olive tree
N. mediterraneum	B604/ Dept. DAFNE	Italy; olive tree
N. parvum	B651/ Dept. DAFNE	Italy; olive tree

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Table 2. LAMP primers used for the detection of *Pleurostoma richardsiae*.

Primer	Primer sequences 5'→3'	Length	Tm°
F3	TGCCCTGCCGTCGTTG	16	60.87
B3	GGAACCGATTCGCCTCGA	18	60.99
FIP	TCACACACCTCCCGCAGCCTTTTCCCACTAGAGGTGCTGTCG	42	> 75
BIP	TGGGCTGTCCTGCTTCGCTTTTTAGGCGGCGCATCCC	38	> 75
LoopF	ACCGCTCCAGGTGCTGA	17	60.40
LoopB	GGCACCTTAGGGTCGCTCA	19	63.44

previously identified using morphological and molecular tools.

DNA extraction and IGS amplification

Genomic DNA of each strain reported in Table 1 was extracted from 200 mg of fresh mycelium, that was grown on potato dextrose agar plates (PDA, Oxoid Ltd) at 25°C for 14 d. according to the protocol optimized for vascular fungi by Carlucci *et al.* (2013). DNA quality and concentrations were determined with a Beckman Coulter DU-800 UV/Vis spectrophotometer.

PCR reactions to amplify IGS regions of *Pl. richardsiae* and *Phaeoacremonium* strains were carried out using the universal primers LR12R (5'-GAACGCCTCTAAGTCAGAATCC-3'; anchored in the 3' of the LSU gene) and invSR1R (5'-ACTGGCAGAATCAACCAGGTA-3'; anchored in the 5' of SSU of the RNA gene), according to Laidani *et al.* (2021).

Design of LAMP primers

As for the design of LAMP primers for *Pl. richardsiae* detection, the IGS regions of *Pl. richardsiae* and *Phaeoacremonium* sequences were aligned using BioEdit version 7.0.9 (http://www.mbio.ncsu.edu/BioEdit) to find conserved regions in the target fungi. Primers were designed using PrimerExplorer v. 5 (http://primerexplorer.jp) and were checked by BLAST in the GenBank to ensure their specificity. Three sets of LAMP primers were generated, with each set made of six LAMP primers (external primers F3 and B3, internal primers FIP and BIP, and loop primers LoopF and LoopB). All primers were analyzed to check for hairpin, self-dimer, and heterodimer production, using Integrated DNA Technologies (Coralville) OligoAnalyzer, software v. 3.1 (Owczarzy *et al.*, 2008). The best performing LAMP primer set was selected, and primer sequences (Table 2) were synthesized by Eurofins MWG Operon.

The designed primers were the outer primers F3 and B3, the loop primers LoopF and LoopB, and the inner primers BIP and FIP that are complementary, respectively, to the sequences of the regions B1C and B2, F1C and F2 (Figure 1).

RealAmp assay

RealAmp reactions were each carried out in a final volume of 25 µL, containing; 15 µL of OptiGene master mix, 2.5 µL of primer mix (final concentrations of 1.6 µM FIP and BIP, 0.2 µM F3, B3 and 0.4 µM LoopF, LoopB), 2 µL of DNA template (30 ng) and 5.5 µL of water. RealAmp fluorescence signal (RFU) was plotted automatically using the CFX96™ real-time PCR detection system (Bio-Rad) collecting fluorescence signals at

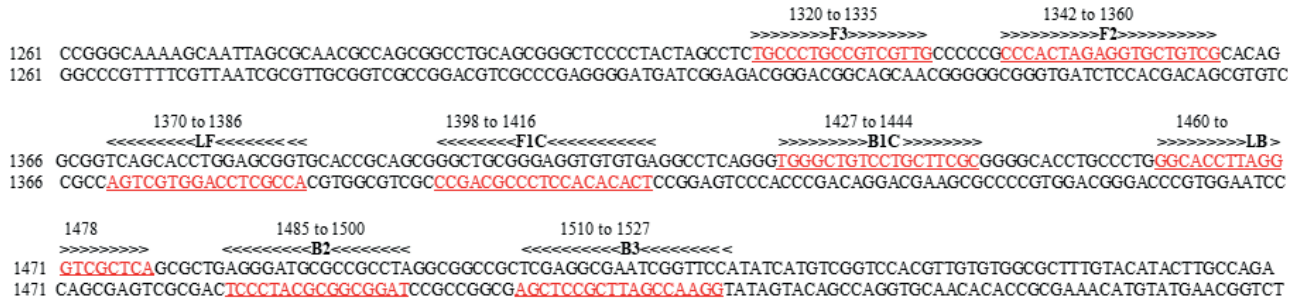

Figure 1. Positioning and orientation of LAMP primers within the nucleotide sequence of the IGS region of *Pleurostoma richardsiae*.

30 sec intervals at 70°C for 45 min. For all reactions, a result was considered positive when an amplification curve was obtained, while a linear or slightly oblique amplification curve indicated a negative result. All reactions were carried out three times, and the negative controls contained nuclease-free water.

Specificity and sensitivity of RealAmp

To assess the specificity of the LAMP primer dataset, assays were carried out using DNA extracted from ten strains of *Pl. richardsiae*, two strains phylogenetically related to *Pleurostoma* species, and other strains of *Phaeoacremonium* and *Neofusicoccum* commonly associated with olive trunk disease (Table 1).

The limit of detection (LoD) of the RealAmp assay was determined through two successive experiments using pure genomic DNA obtained from *Pl. richardsiae* cultures. In the first experiment, a ten-fold dilution series was prepared, from 3 ng μL^{-1} to 3×10^{-6} ng μL^{-1} (3 fg μL^{-1}). Each dilution was analyzed in ten replicates to obtain an initial estimate of LoD. Results of the first experiment were used to narrow the range of target concentrations and make a more precise estimate. In the second experiment, a two-fold dilution series was prepared, from 3×10^{-1} ng μL^{-1} to 7.5×10^{-2} ng μL^{-1}. Each dilution was tested in 20 replicates. In both experiments, nuclease-free water was used as negative controls (NTC).

RealAmp detection of Pleurostoma richardsiae *in inoculated olive plants*

As part of an ongoing experiment on olive plants ('Ogliarola salentina') artificially inoculated in a greenhouse with the *Pl. richardsiae* strain Pl31 (Table 1), six symptomatic plants were used for a preliminary validation of the *in vivo* RealAmp assay. The tested plants showed clear symptoms of necrotic wood lesions and vascular discolourations after bark removal. To ascertain the presence of the fungus, isolations were made from the stem portions at the inoculation points, according to Carlucci *et al.* (2013), and each sample was also used for DNA extraction using the DNA secure Plant Kit (Tiangen), according to the manufacturer's protocol. DNA used as negative controls was extracted from three uninoculated olive plants, three olive plants inoculated with *Neofusicoccum parvum* and three olive plants inoculated with *Phaeoacremonium italicum*. Each assay was carried out using 30 ng of genomic DNA from olive plants, and reactions were carried out using RealAmp.

RESULTS AND DISCUSSION

This is the first reported application of RealAmp as a rapid, specific and sensitive method for detection of *Pl. richardsiae* using specific primers. To screen for optimal LAMP primers, the intergenic spacer (IGS) region was employed as targets for primer design. All tested *Pl. richardsiae* strains showed positive reactions in the RealAmp assays, as reported in Figure 2. Optimized conditions in this study, at 70°C for 45 min, enabled amplification of the target sequence giving clear curves. The rapidity of this technique in pathogen detection was also reported by Besuschio *et al.* (2017). Unlike other nucleic acid amplification techniques, RealAmp can be carried out in 30 min, rather than at least 90 min (e.g. for PCR). For specificity of the RealAmp assay using DNAs of other relative fungi, the reaction curves indicated that the *Pl. richardsiae* primer dataset specifically amplified the target DNA sequence of this fungus (Figure 2), the DNA isolated from inoculated olive plants (Figure 3), but not DNA of the other fungi assayed (Figure 2; Table 1). The RealAmp products of *Pleurostoma*

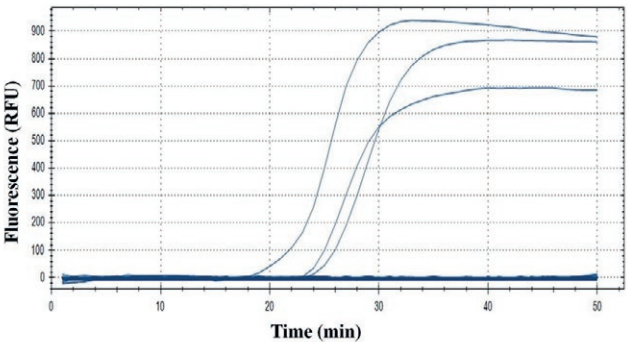

Figure 2. The specificity of the *Pleurostoma richardsiae* RealAmp method. The amplification plots (RFU) of three representative isolates of *Pl. richardsiae* and other fungus strains. Negative samples showed linear curves.

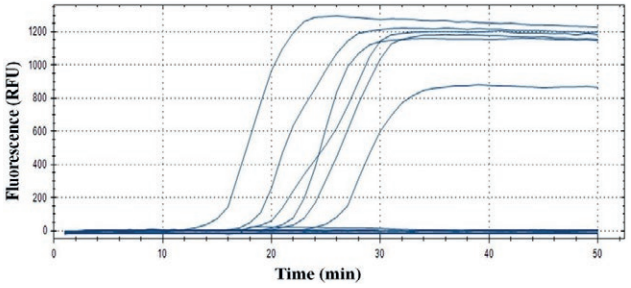

Figure 3. Detection of *Pleurostoma richardsiae* in inoculated olive plants, using RealAmp assay. Samples used as negative controls showed linear curves.

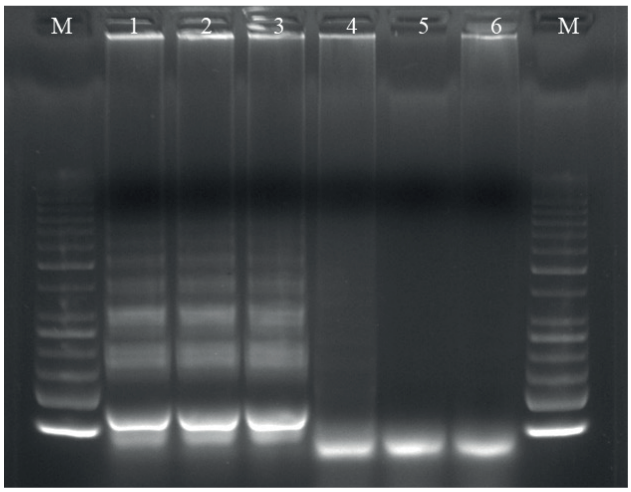

Figure 4. Detection of *Pleurostoma richardsiae* using the RealAmp products, which were electrophoresed in a 2% agarose gel and inspected under UV light. M: marker; 1, 2, and 3: *Pl. richardsiae*; 4: *Pl. repens*; 5: *Pl. ootheca*; 6: water control.

were also observed in the agarose gel electrophoresis, which confirmed the specificity of the RealAmp assay (Figure 4).

Figure 5 shows the results of experiments conducted to determine the LoD assay. The analysis carried out on the ten-fold dilution series of *Pl. richardsiae* genomic DNA resulted in an initial estimate of the LoD. Positive reactions were obtained for target concentrations ranging from 3 ng μL^{-1} to 3×10^{-2} ng μL^{-1}, with frequencies, respectively, of 100% and 50% (ten replicates for each dilution were analysed). No amplification signal was observed in subsequent dilutions from 3×10^{-3} ng μL^{-1} to 3×10^{-6} ng μL^{-1}, or in the NTC (Figure 5A). To define the target concentration that produced at least 95% positive replicates (LoD at 95%) (Forootan *et al.*, 2017), the assay was repeated over a narrower range of concentrations. On the basis of the results obtained, a two-fold dilution series was prepared starting from 3×10^{-1} ng μL^{-1}, corresponding to the lowest concentration of targets with 100% positives replicates, to 7.5×10^{-2} ng μL^{-1}.

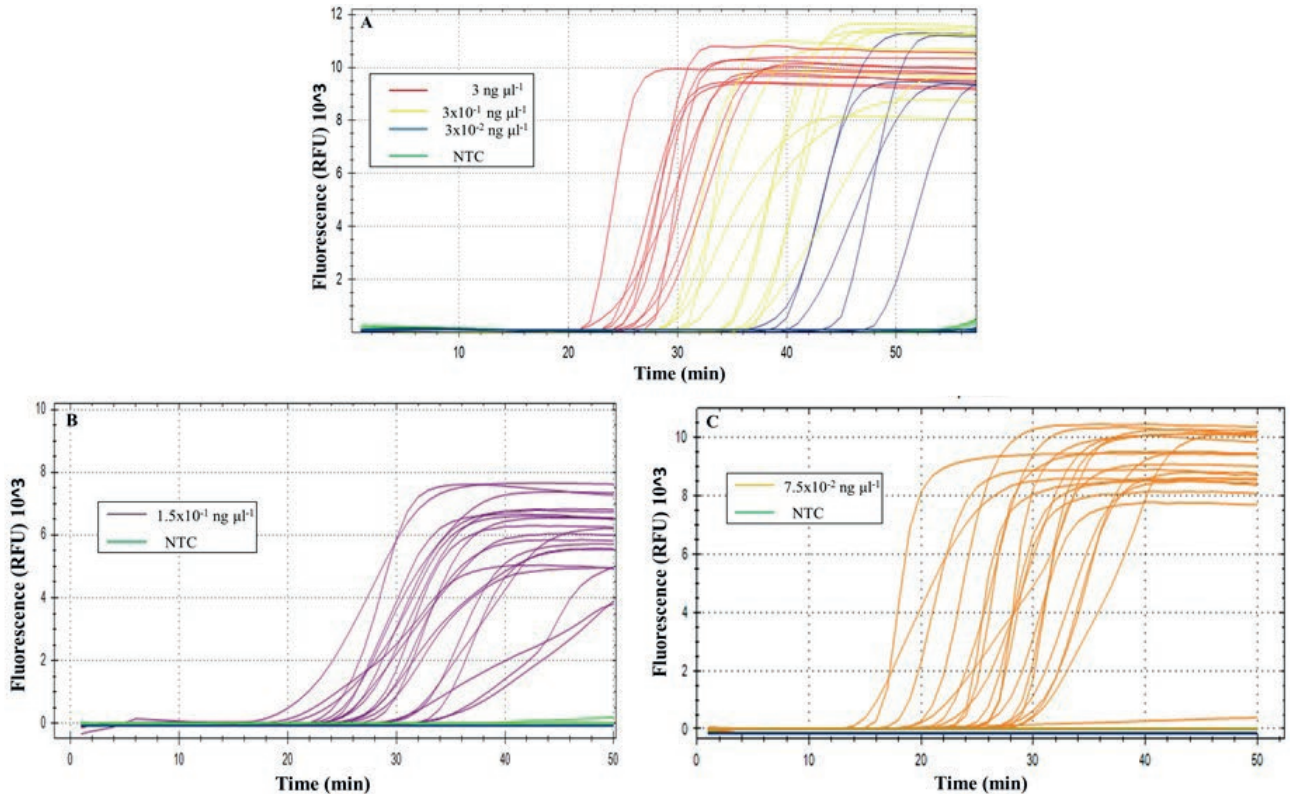

Figure 5. Sensitivity of the RealAmp assay for *Pleurostoma richardsiae* detection. (A) Results for the preliminary estimate of the LoD: 100% of positive reactions were obtained for target concentrations of 3 ng μL^{-1} (red curves) and 3×10^{-1} ng μL^{-1} (yellow curves); 50% of positive reactions for 3×10^{-2} ng μL^{-1} (blue curves); no reaction was observed from 3×10^{-3} ng μL^{-1} to 3×10^{-6} ng μL^{-1} or in the NTC (green curves). (B and C). Results for the estimate of LoD at 95%: positive reactions with frequency of 100% were obtained analysing target concentration of 1.5×10^{-1} ng μL^{-1} (purple curves), and 95% (orange curves) analysing 7.5×10^{-2} ng μL^{-1} (C).

Twenty replicates for each dilution were analysed for accurate estimation. Positive reactions with 100% frequency were obtained analysing target concentration of 1.5×10^{-1} ng μL^{-1} (Figure 5B), and 95% frequency analysing 7.5×10^{-2} ng μL^{-1} (Figure 5C). No amplification was observed in the NTC. Results of these analyses indicated 7.5×10^{-2} ng μL^{-1} as the LoD at 95% of the assay.

For the artificially inoculated olive plants, results from isolations indicated that all the plants were infected by *Pl. richardsiae*. These results were confirmed by RealAmp, which showed amplification for all inoculated olive plants and no amplification from the negative controls (Figure 3). Similar RealAmp results were obtained by Lee *et al.* (2020) for detection of Peach latent mosaic viroid in peach pollen.

The developed RealAmp assay is specific, sensitive, and rapid, for detecting *Pl. richardsiae* from DNA extracted from pure strains of the fungus and from infected olive plants.

Three of the six species of *Pleurostoma* were used to validate the developed assay in this study: *Pl. richardsiae* (ten strains), the ex-type of *Pl. repens*, and the ex-type of *Pl. ootheca*. However, full RealAmp validation with the remaining three *Pleurostoma* species could not be accomplished, due to lack of data (for *Pl. candollei*) and funding limitations (for *Pl. ochraceum* and *Pl. hongkongense*). In addition, further studies are required to evaluate the field performance of this test for grapevine and other hosts of this pathogen. These promising results are in accordance with the successful application of this technique for reliable and rapid detection of several plant pathogens, including *X. fastidiosa* in olive trees (Yaseen *et al*., 2015). Among the several features that make RealAmp an excellent diagnostic tool, its use, both with a real time instrument for processing numerous samples and with portable devices for few samples, are strengths compared to other PCR techniques.

In the last two decades, infections caused by *Pl. richardsiae* have been widespread on olive groves, often with severe symptoms leading to general tree decline, such as browning and leaf drop, wilting and decay of twigs and branches, and dark streaks and necroses in the inner tissues of host wood (Hallen *et al*., 2007; Carlucci *et al*., 2013; Carlucci *et al*., 2015a; Canale *et al*., 2019). RealAmp could become a useful tool in large-scale monitoring programmes for this pathogen, and in the phytosanitary controls with certified pathogen-free propagating material of olive. In addition, as *Pl. richardsiae* is an uncommon agent of human infection, it may also be of interest to further investigate the application of RealAmp assay in the medical field.

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