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# Real-time loop-mediated isothermal amplification assay for rapid detection of *Fusarium circinatum*

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## ABSTRACT

*Fusarium circinatum* is the causal agent of pitch canker, a lethal disease of pine and other conifers. Since *F. circinatum* is a quarantine organism, its timely detection could efficiently prevent its introduction into new areas or facilitate spread management in already infected sites. In this study, we developed a sequence-specific probe loop-mediated isothermal amplification (LAMP) assay for *F. circinatum* using a field-deployable portable instrument. The assay was able to recognize the pathogen in host tissues in just 30 min, and the sensitivity of the assay made it possible to detect even small amounts of *F. circinatum* DNA (as low as 0.5 pg/μl). The high efficiency of this method suggests its use as a standard diagnostic tool during phytosanitary controls.

## METHOD SUMMARY

Here we present real-time loop-mediated isothermal amplification based on assimilating probe. This method is rapid, sensitive, specific and field-portable for *F. circinatum* detection.

**KEYWORDS:**

elongation factor gene • field-deployable • invasive species • isothermal amplification • LAMP • pine pitch canker

Pitch canker is a lethal disease of pine trees caused by *Fusarium circinatum* (= *Gibberella circinata*), a quarantine fungal pathogen native to Central America [1]. The pathogen is one of the most economically important diseases and is established in many parts of the world including North, Central and South America, east Asia, South Africa and southwest Europe [2–14].

Symptoms associated with pitch canker are large cankers on the stem and branches oozing a huge amount of resin [3]. The disease is particularly damaging in intensively managed plantations of nonnative pine species because it drastically reduces the wood yield and inhibits the use of pine species and Douglas fir in the area. All stages of tree development are susceptible and even seeds or plant parts could act as efficient pathways of the disease [4]. Because the eradication of nonnative organisms is generally difficult and very expensive, unless the presence of the pathogen is limited to a restricted number of plants the only reliable and economic means of containing the spread of nonnative plant pathogens is early detection.

DNA-based (usually PCR-based) detection tools are preferred for their higher sensitivity and specificity than classical tools, but they need a well-equipped lab and time-consuming data processing to produce a result. Significant advantages such as prompt response and rapid, sensitive detection can be achieved by using field-deployable portable loop-mediated isothermal amplification (LAMP)-based methods [5,6].

As a quarantine organism, *F. circinatum* is subjected to provisional emergency measures in the EU as in several other countries in the world [7]. A rapid and specific on-site identification method that can be used at points of entry such as ports and airports, as well as in plantations and nurseries, is of primary concern in order to discern as sharply as possible infected from noninfected material, thus preventing the introduction and spread of this harmful pathogen into a new area, or facilitating the rapid application of quarantine regulations as they are requested.

The aim of this study is to provide a rapid, simple, specific, and sensitive LAMP assay to detect *F. circinatum* in infected plant tissue.

## Materials & methods

### LAMP primers & fluorescent-assimilating probe design

Six LAMP primers and the fluorescent-assimilating probe strand were designed for *F. circinatum* using the software LAMP Designer (OptiGene Ltd, Horsham, UK) on the basis of the consensus sequence of *EF1α*, previously selected by Luchi *et al.* for a *F. circinatum* qPCR assay [8]. The theoretical specificity of the designed LAMP primers was assessed by analyzing the complete amplicon on

Table 1. cLAMP and qLAMP primers and probe for *Fusarium circinatum*.

| Primer name | Primer type | Length (bp) | Sequence (5′–3′) |
|---|---|---|---|
| Fctef F3 | F3 | 21 | CATTGAGAAGTTCGAGAAGGT |
| Fctef B3 | B3 | 21 | TGTCGAATGATTAGTGACTGC |
| Fctef FIP | FIP | 36 | TTGGTCTCGAGCGGGGTATTTGCCCATCGATTCTCC |
| Fctef BIP | BIP | 36 | GAGCGATGCGCGTTTCTGTTAACACGTGACGATGCG |
| Fctef LF | FLP | 18 | GGCACGTTTCGAGTCGTA |
| Fctef LB | BLP | 18 | CCTCCCATTGCCACAACT |
| Fctef LB probe | Fluorescent strand | 58 | FAM - ACGCTGAGGACCCGGATGCGAATGCGGATGCGGATGCCGACCTCCCATTGCCACAACT† |
| Fctef BHQ | Quencher strand | 40 | TCGGCATCCGCATCCGCATTCGCATCCGGGTCCTCAGCGT - BHQ |

† The underlined fragment acts as the backward loop primer.
BHQ: Black hole quencher-1; FAM: 6-carboxyfluorescein.

BLAST® (http://www.ncbi.nlm.nih.gov/BLAST) [9]. To increase the assay specificity, a sequence-specific assimilating LAMP probe was designed. The fluorescent-assimilating probe is incorporated into the amplicon and the fluorescence produced by the amplification of the selected specific loop is evident only when the backward loop primer is amplified, increasing the specificity of the assay [10]. Due to its high specificity (100% homology only with *F. circinatum*), the backward loop primer (BLP) was selected and used to design the fluorescent-assimilating probe that includes two distinct oligonucleotide strands. The first oligonucleotide strand was labeled with FAM (6-carboxyfluorescein) dye at the 5′ end, while the second oligonucleotide strand was modified with BHQ (Black Hole Quencher) at the 3′ end (Table 1). LAMP primers and the fluorescent-assimilating probe were synthetized by Eurofins Genomics (Ebersberg, Germany) (Table 1).

## LAMP assays

Two different *F. circinatum* assays were developed: a conventional LAMP (cLAMP) only using designed primers, and a quantitative LAMP (qLAMP) that also included the fluorescent-assimilating probe.

DNA samples for both assays were tested in Genie® Strips (OptiGene Ltd, Horsham, UK), each one comprising eight 0.2-ml isothermal reaction tubes with a locking cap providing a closed-tube system, using the portable instrument Genie® II (OptiGene Ltd).

Each isothermal reaction (both for cLAMP and qLAMP) was performed at 65°C for 30 min. The cLAMP assay was followed by a post-amplification analysis that allowed the generation of derivatives' melting curves and was performed by heating samples from 98 to 80°C with ramping of 0.05°C per second. When the fluorescent probe was used (qLAMP assay), reactions were terminated by heating amplification products at 85°C for 5 min.

DNA amplification was assayed in duplicate in a final volume of 25 µl. For each run, two tubes containing diethylpyrocarbonate water were included as no-template controls. The reaction mixture used for cLAMP was as described by Aglietti *et al.* [5]. The reaction mixture for qLAMP was composed of 15 µl of the Isothermal Master mix without intercalating dyes (ISO-001nd; OptiGene Ltd), 6 µl LAMP primer mixture (at a final concentration of 0.2 µM for each F3 and B3, 0.8 µM for each FIP and BIP, 0.4 µM for the forward loop primer), 0.75 µl diethylpyrocarbonate water, 0.25 µl LAMP probe mixture (fluorescent and quencher strands at a final concentration of respectively 0.04 and 0.06 µM). For each LAMP assay, 3 µl DNA was used as template for each reaction, at a final concentration of 2.5 ng/µl.

## Specificity & sensitivity of LAMP assays

The specificity of LAMP assays (both cLAMP and qLAMP) was tested by using aliquots of the gDNA *Fusarium* samples described in Ioos *et al.*, and other species [11]. These samples include 16 *F. circinatum* isolates collected from different geographical areas, as well as 28 phylogenetically related *Fusarium* species and 8 fungal species related to pine or other forest tree species (Table 2).

The sensitivity of both LAMP assays was assessed by testing a tenfold serial 1:5 dilution (ranging from 8.5 ng/µl to 4.4 fg/µl) of gDNA extracted from the target species (*F. circinatum* isolate FC096) (Figure 1). Each known concentration of *F. circinatum* DNA was analyzed in triplicate in five independent assays.

To further validate the LAMP assays, the same aliquots of each dilution were processed by a real-time PCR (qPCR) assay developed by Luchi *et al.* (Figure 1) [8].

## LAMP assay from pine tissues

To assess the effectiveness of LAMP assays in pine tissues, 10 *F. circinatum* infected bark samples and 10 seedling samples were collected from a symptomatic *Pinus radiata* tree in Cantabria (Spain). Infection of the challenged tissues was ensured by pathogen isolation using classical methods in the plant pathology lab in the University of Valladolid. An additional ten healthy pine bark and ten healthy pine seedling samples were included as negative controls. DNA was extracted from small pieces of woody tissues (~100 mg) with an Invisorb Spin Plant Mini Kit (Invitek Molecular GMBH, Berlin, Germany). To assess the effectiveness of DNA extraction, all DNA plant samples were tested using a previously developed LAMP assay with *COX* primers [5].

Table 2. Fungal species used in this study.

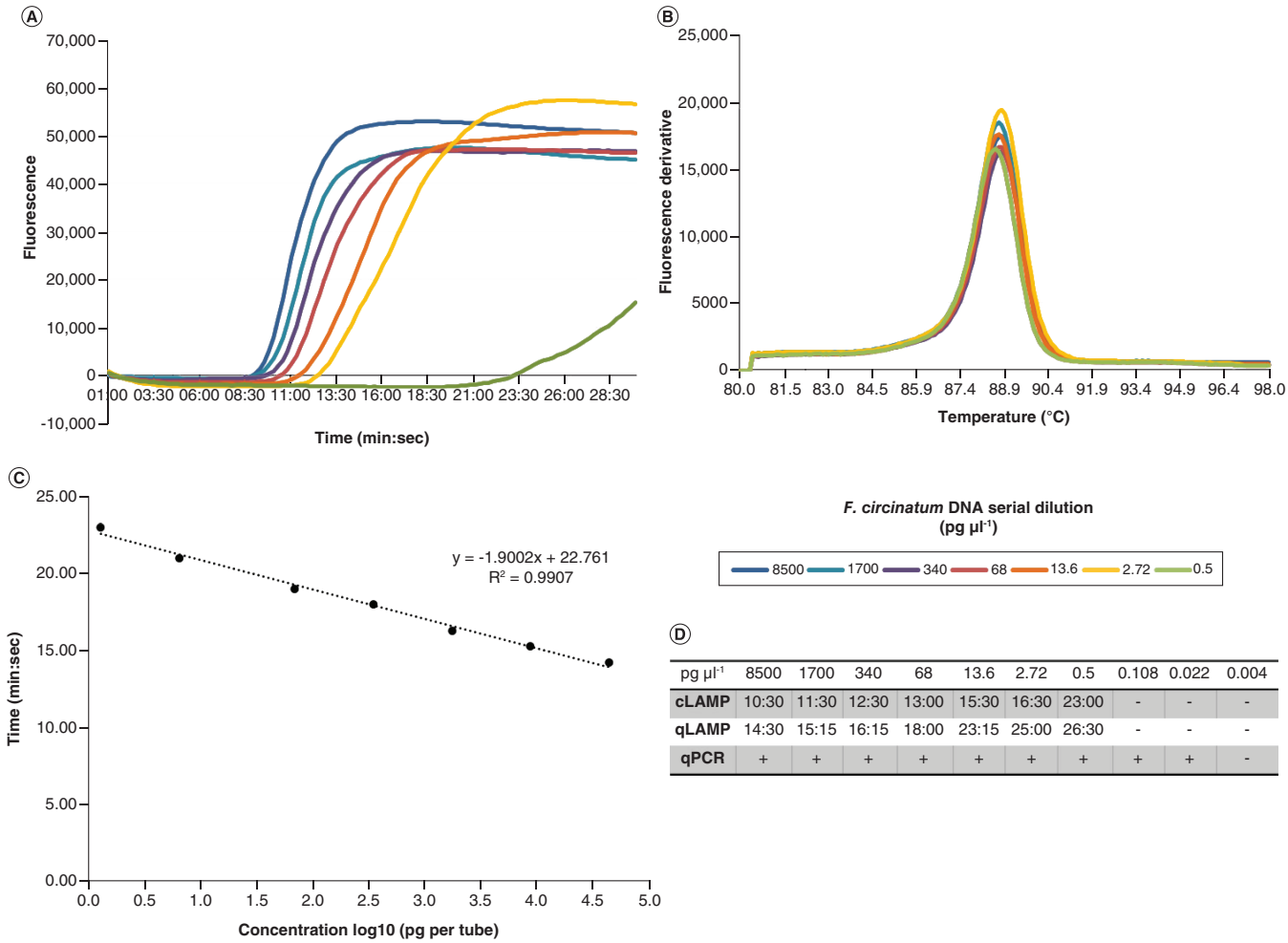
| Fungal species | Isolate number | Origin | Host | Collector | cLAMP [ $T_a$ ($t_{amp}$)] | qLAMP | qPCR |
|---|---|---|---|---|---|---|---|
| *Fusarium circinatum* | FcCa02† | Cantabria, Castrourdiales (Spain) | *Pinus radiata* | J. Diez | 88.93 (11:00) | + | + |
| F. circinatum | LSVM217† | Côtes d'Armor (France) | *P. radiata* | R. loos | 88.88 (9:15) | + | + |
| *F. circinatum* | 2738† | Chile | *P. radiata* | R. Ahumada | 88.83 (12:30) | + | + |
| *F. circinatum* | CSF-4† | León (Spain) | *P. radiata* | A. Sanz-Ros | 88.73 (10.45) | + | + |
| *F. circinatum* | CSF-8† | Palencia (Spain) | *Pinus nigra* | A. Sanz-Ros | 88.73 (11.00) | + | + |
| *F. circinatum* | CSF-11† | Valladolid (Spain) | *P. nigra* | A. Sanz-Ros | 88.73 (11.30) | + | + |
| *F. circinatum* | CSF-12† | Valladolid (Spain) | *Pinus sylvestris* | A. Sanz-Ros | 88.73 (11.00) | + | + |
| *F. circinatum* | CSF-13† | Valladolid (Spain) | *Pinus pinaster* | A. Sanz-Ros | 88.83 (10.45) | + | + |
| *F. circinatum* | 116† | Galicia (Spain) | *P. nigra* | M. Berbegal | 88.83 (10.30) | + | + |
| *F. circinatum* | 164† | Asturias (Spain) | *P. sylvestris* | M. Berbegal | 88.73 (12.45) | + | + |
| *F. circinatum* | 221† | Cantabria (Spain) | *P. radiata* | M. Berbegal | 88.73 (11.15) | + | + |
| *F. circinatum* | 253† | Galicia (Spain) | *P. nigra* | M. Berbegal | 88.83 (12.15) | + | + |
| *F. circinatum* | 822† | Galicia (Spain) | *P. pinaster* | M. Berbegal | 88.83 (11.30) | + | + |
| *F. circinatum* | 07/0649 1b† | Asturias (Spain) | *P. pinaster* | M. Berbegal | 88.83 (12.00) | + | + |
| *F. circinatum* | 310/061† | Asturias (Spain) | *Pinus palustris* | M. Berbegal | 88.83 (11.15) | + | + |
| *F. circinatum* | 2028† | Chile | *P. radiata* | R. Ahumada | 88.73 (12.15) | + | + |
| *Fusarium acuminatum* | Do_US_VC_49_1† | USA | Seed of *Pseudotsuga menziesii* | WSL – Phytopathology | - | - | - |
| *Fusarium avenaceum* | Do_US_Nat_2_1† | USA | Seed of *P. menziesii* | WSL – Phytopathology | - | - | - |
| *Fusarium begoniae* | LSV293† | France | *Begonia elatior* | R. loos | 88.53 (15:45) | - | - |
| *Fusarium concentricum* | NRRL 25181† | France | Unknown | K. O'Donnell | 88.33 (20:45) | - | - |
| *Fusarium culmorum* | CSF–14† | Palencia (Spain) | *Pinus pinea* | A. Sanz-Ros | - | - | - |
| *Fusarium fracticaudum* | CMW 25245† | Colombia | *Pinus maximinoi* | G. Fourie | 88.43 (18:15) | - | - |
| *Fusarium fractiflexum* | NRRL 28852† | Unknown | Unknown | K. O'Donnell | - | - | - |
| *Fusarium fujikuroi* | LSV667† | France | *Zea mays* | R. loos | 87.83 (17:30) | - | - |
| *Fusarium graminearum* | Do-Mur/17–1† | USA | Seed of *P. menziesii* | WSL – Phytopathology | - | - | - |
| *Fusarium incarnatum-equiseti* species complex | Do_US_Nat_3_1† | USA | Seed of *P. menziesii* | WSL – Phytopathology | - | - | - |
| *Fusarium mangiferae* | NRRL 25226† | Unknown | Unknown | K. O'Donnell | 88.43 (23:15) | - | - |

† Isolate provided and assessed in the framework of COST Action FP1406 PINESTRENGTH.
$T_a$: Annealing temperature (°C); $t_{amp}$: Time amplification (min:s).

Table 2. Fungal species used in this study (cont.).

| Fungal species | Isolate number | Origin | Host | Collector | cLAMP [ $T_a$ ($t_{amp}$)] | qLAMP | qPCR |
|---|---|---|---|---|---|---|---|
| *Fusarium marasasianum* | CMW 25261† | Colombia | *Pinus patula* | G. Fourie | 88.33 (14:00) | - | - |
| *Fusarium nygamai* | NRRL 13448† | Unknown | Unknown | K. O'Donnell | - | - | - |
| *Fusarium oxysporum* | CSF-16† | Spain (Palencia) | *P. pinea* | A. Sanz-Ros | - | - | - |
| *Fusarium parvisorum* | CMW 25267† | Colombia | *P. patula* | G. Fourie | 88.33 (16:00) | - | - |
| *Fusarium pininemorale* | CMW 25243† | Colombia | *Pinus tecunumanii* | G. Fourie | 88.53 (16:00) | - | - |
| *Fusarium proliferatum* | FGSC 7421† | Dominican Republic | *Musa* sp. | M Pasquali | - | - | - |
| *Fusarium redolens* | Do-D/11–1† | Switzerland | Seed of *P. menziesii* | WSL – Phytopathology | - | - | - |
| *Fusarium reticulatum negundis* | FI-BOS/14–1† | Switzerland | Seed of *Picea* sp. | WSL – Phytopathology | - | - | - |
| *Fusarium sacchari* | NRRL 13999† | Unknown | Unknown | K. O'Donnell | - | - | - |
| *Fusarium sororula* | CMW 25254† | Colombia | *Pinus* spp. | G. Fourie | 88.74(15:30) | - | - |
| *Fusarium sporotrichioides* | Do_US_Nat_32_1† | USA | Seed of *P. menziesii* | WSL – Phytopathology | - | - | - |
| *Fusarium subglutinans* | LSVM869† | France | *Z. mays* | R. Ioos | 88.13 (20:30) | - | - |
| *Fusarium temperatum* | LSVM870† | France | *Z. mays* | R. Ioos | 88.63 (16:45) | + | + |
| *Fusarium thapsinum* | NRRL 22045† | Unknown | Unknown | K. O'Donnell | - | - | - |
| *Fusarium torulosum* | Do_US_VC_5_1† | USA | Seed of *P. menziesii* | WSL – Phytopathology | - | - | - |
| *Fusarium tricinctum* species complex | Do_US_Sno_49_1† | USA | Seed of *P. menziesii* | WSL – Phytopathology | - | - | - |
| *Fusarium verticillioides* | LSVM873† | France | *Z. mays* | R. Ioos | - | - | - |
| **Other species** | | | | | | | |
| *Diplodia scrobiculata* | 124 | Wisconsin (USA) | *Pinus banksiana* | M. Palmer | - | - | - |
| *Diplodia pinea* | 411 | Minnesota (USA) | *Pinus resinosa* | M. Palmer | - | - | - |
| *Caliciopsis pinea* | US27 | New Hampshire (USA) | *Pinus strobus* | I. Munck | - | - | - |
| *Caliciopsis nigra* | I163 | Spain | *Quercus ilex* subsp. *rotundifolia* | I. Garrido-Benavent | - | - | - |
| *Phythophthora ramorum* | PramGr | Greece | Rhododendron | N. Soulioti | - | - | - |
| *Ophiostoma novo-ulmi* | 182E | Italy | *Ulmus minor* | F. Ferrini | - | - | - |
| *Ceratocystis platani* | CF0 | Italy | *Platanus x acerifolia* | A. Panconesi | - | - | - |
| *Ceratocystis fimbriata* | CBS114723 | North Carolina (USA) | *Ipomoea batatas* | D. McNew | - | - | - |

† Isolate provided and assessed in the framework of COST Action FP1406 PINESTRENGTH.
$T_a$: Annealing temperature (°C); $t_{amp}$: Time amplification (min:s).




| pg µl$^{-1}$ | 8500 | 1700 | 340 | 68 | 13.6 | 2.72 | 0.5 | 0.108 | 0.022 | 0.004 |
|---|---|---|---|---|---|---|---|---|---|---|
| **cLAMP** | 10:30 | 11:30 | 12:30 | 13:00 | 15:30 | 16:30 | 23:00 | - | - | - |
| **qLAMP** | 14:30 | 15:15 | 16:15 | 18:00 | 23:15 | 25:00 | 26:30 | - | - | - |
| **qPCR** | + | + | + | + | + | + | + | + | + | - |

**Figure 1. Sensitivity results obtained by testing tenfold 1:5 serial dilution (8.5 ng/µl – 4.352 fg/µl) of standard DNA template *Fusarium circinatum* (isolate 096). (A)** cLAMP amplification plot. **(B)** cLAMP annealing temperature. **(C)** qLAMP standard curve. **(D)** Comparison of sensitivity between different assays. cLAMP and qLAMP results are based on amplification time ($t_{amp}$; min:s), while qPCR results are reported as positive (+) or negative (-).

## Results & discussion

*Fusarium* species show a high genetic similarity, sharing their *ITS* region, which is generally used as a barcode sequence for the identification of fungal species [12,13]. Here, a sequence-specific LAMP probe targeting *EF1α* has been developed to circumvent the risk of low specificity and has been implemented for use on a portable instrument.

To assess the theoretical specificity of the probe, the target amplicon of the LAMP primers was paired with other sequences present on GenBank database (NCBI) by the BLAST® software, revealing complete homology (100%) only with *F. circinatum* sequences.

A high homology, ranging from 97.14 to 97.89%, was found with other *Fusarium* species (*F. ananatum*, *F. anthophilum*, *F. bactridioides*, *F. begonia*, *F. bulbicola*, *F. fujikuroi*, *F. guttiforme*, *F. mexicanum*, *F. oxysporum*, *F. subglutinans*, *F. temperatum*).

All tested *F. circinatum* strains were amplified with the cLAMP assay and showed melting curves with a specific peak ($T_a$ = 88.83°C), despite other *Fusarium* species also being detected (Table 2).

The qLAMP assays had higher specificity than cLAMP for the detection of *F. circinatum*; no other *Fusarium* species were amplified, with the exception of *F. temperatum* (Table 2). These results were consistent with other studies in which a TaqMan MGB probe showed a cross-reaction between *F. circinatum* and *F. temperatum* [8,11]. However, *F. temperatum* is only present on *Zea mays* and, to our knowledge, has never been reported on any coniferous host; therefore, it is very unlikely to cause false-positive results on pine tissue [14].

The detection limit of both the cLAMP and qLAMP assays was 0.5 pg/µl (Figure 1). The compared qPCR assay was more sensitive, allowing amplification of *F. circinatum* DNA at concentrations as low as 0.06 pg/µl [8].

LAMP analyses carried out on plant host DNA were further validated by *COX* gene amplification, showing a specific melting peak at the annealing temperature ($T_a$ = 85°C) for each analyzed plant sample (both healthy and infected pine tissues). *COX* gene amplification was a reliable internal positive control confirming that host DNA extractions had been successful, as reported in our previous study [5].

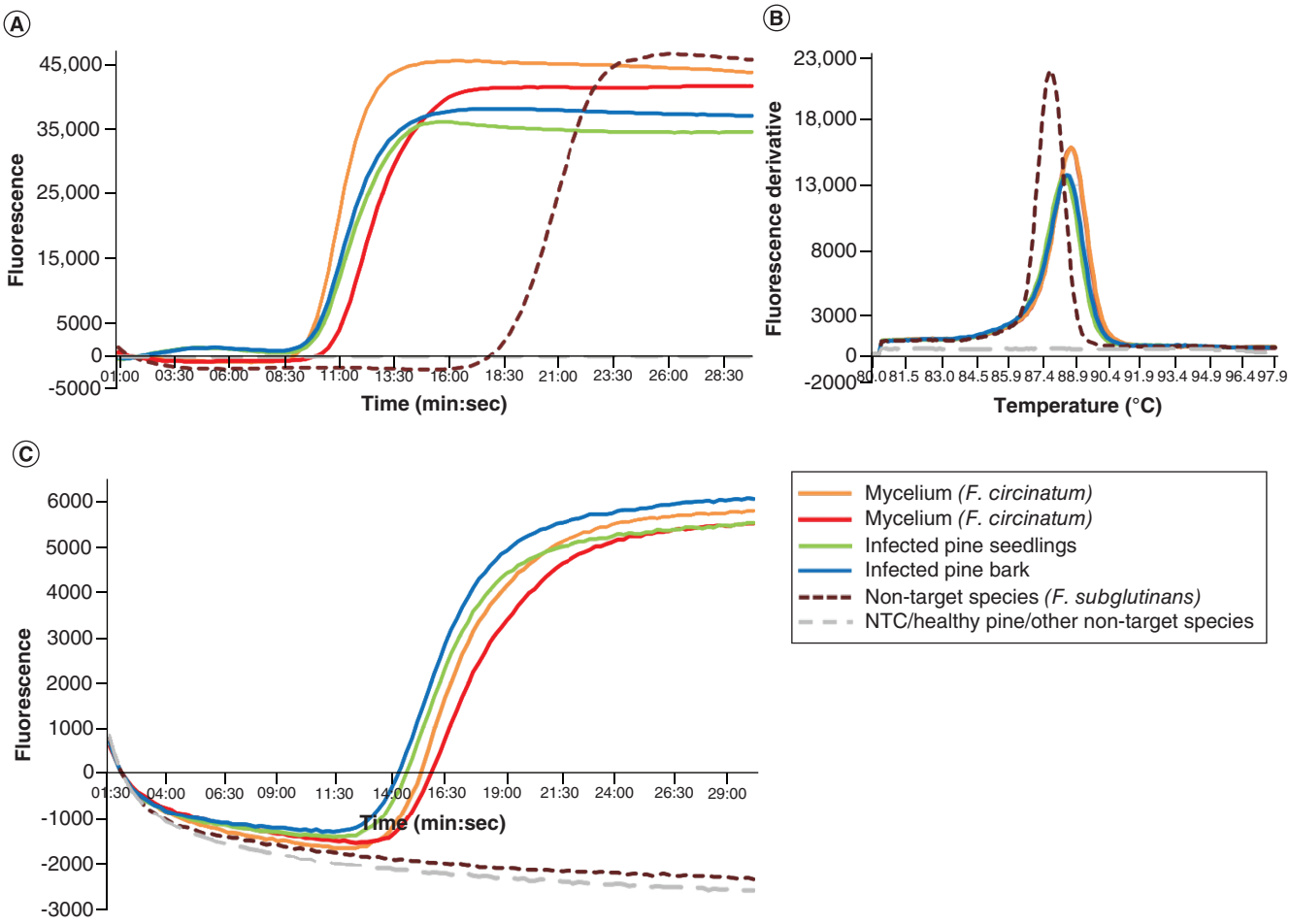



**Figure 2. Selection of kinetics of *Fusarium circinatum* detection on mycelium and pine samples. (A)** cLAMP amplification plot. **(B)** cLAMP annealing temperature. **(C)** qLAMP amplification plot.

All symptomatic plant samples were successfully amplified with the cLAMP (showing $T_a$ similar to those obtained with DNA extracted from axenic cultures of the target pathogen) and qLAMP assays, while no amplification was observed in healthy samples (Figure 2). These assays confirm the reliability of the LAMP method to detect *F. circinatum* in infected pine tissues.

The new challenges in molecular diagnostics research are concerned with the need to rapidly and accurately identify the causal agent of plant disease [15]. A rapid diagnostic technique is crucial to intercept a new pathogen before its introduction into new ecosystems and to correctly manage the disease, and plays a relevant role in the prevention of further spread. In this context, classical methods based on isolation and immunological assay, or on lab diagnostics, are time-consuming and show low sensitivity in comparison with a LAMP-based approach. The LAMP molecular assay developed here could become an efficient and user-friendly tool that could be used to prevent a further spread of *F. circinatum*.

## Author contributions

A Santini and N Luchi designed the study; D Stehlíková, C Aglietti, AL Pepori and JJ Diez performed the experiments and critically revised the data. D Stehlíková, A Santini and N Luchi wrote the manuscript.

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e arbustive ornamentali e forestali' (2017–2020). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

## Open access



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