Reports

BioTechniques

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

Dagmar Stehlíková[1,2], Nicola Luchi*[,2], Chiara Aglietti[2,3], Alessia Lucia Pepori[2], Julio Javier Diez[4] & Alberto Santini[2]

*[1]University of South Bohemia Faculty of Agriculture, Biotechnological Centre Na Sadkach 1780 CZ-37005 Ceske Budejovice, Czech Republic; [2]Institute for Sustainable Plant Protection, National Research Council (IPSP-CNR), Via Madonna del Piano 10, 50019, Sesto Fiorentino (Firenze), Italy; [3]Department of Agriculture, Food, Environment & Forestry (DAGRI), University of Florence, Piazzale delle Cascine 28, 50144, Firenze, Italy; [4]Universidad de Valladolid Escuela Técnica Superior de Ingenierías Agrarias, Campus Yutera Edificio E, despacho 204. 34071, Palencia, Spain; *Author for correspondence: nicola.luchi@ipsp.cnr.it*

BioTechniques 69: 11–17 (July 2020) 10.2144/btn-2019-0168

First draft submitted: 16 December 2019; Accepted for publication: 16 March 2020; Published online: 27 April 2020

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.

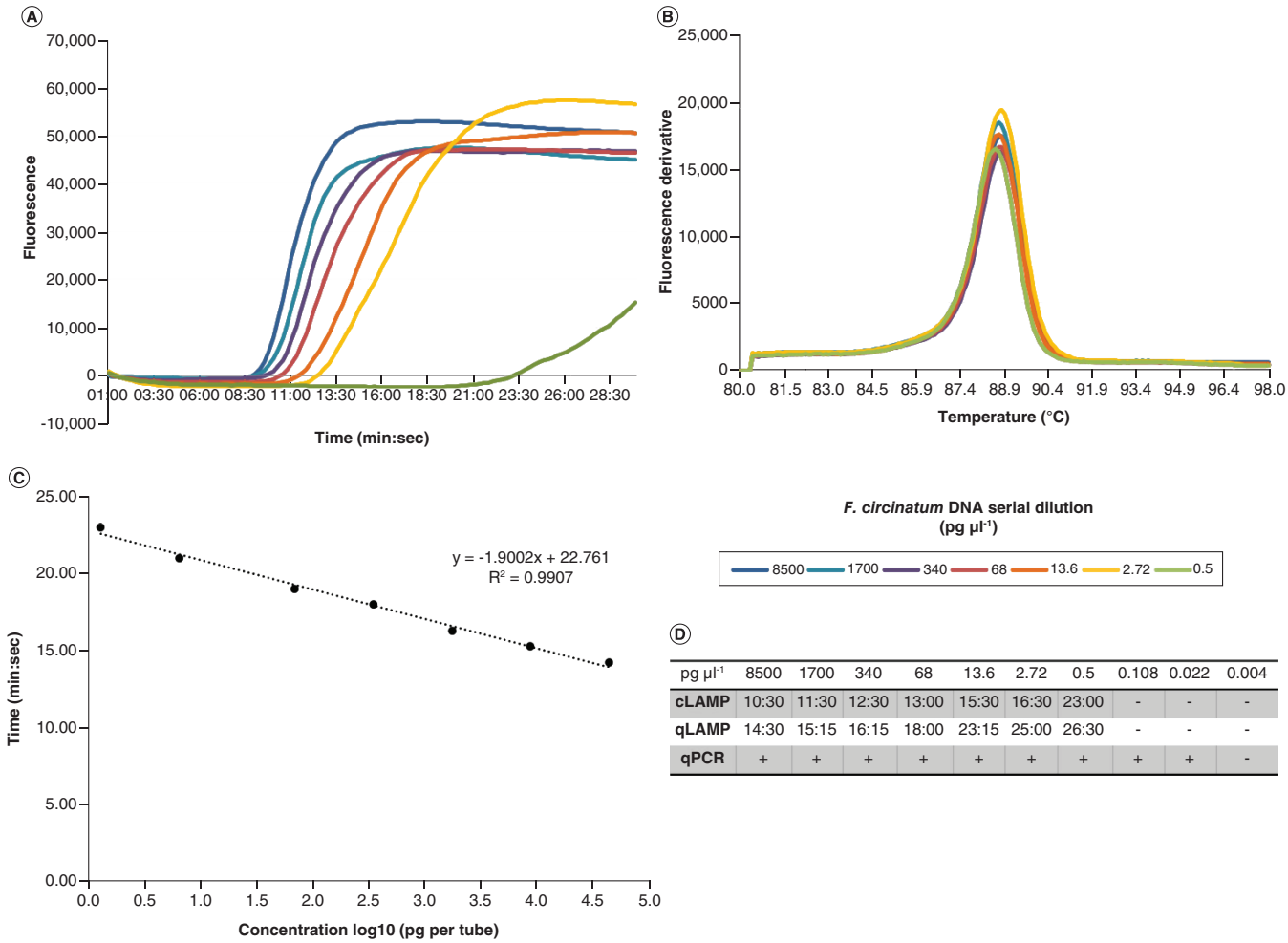
Fungal species	Isolate number	Origin	Host	Collector	cLAMP [T_a (t_{amp})]	qLAMP	qPCR
Fusarium circinatum	FcCa02†	Cantabria, Castrourdiales (Spain)	*Pinus radiata*	J. Diez	88.93 (11:00)	+	+
F. circinatum	LSVM217†	Côtes d'Armor (France)	*P. radiata*	R. loos	88.88 (9:15)	+	+
F. circinatum	2738†	Chile	*P. 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].

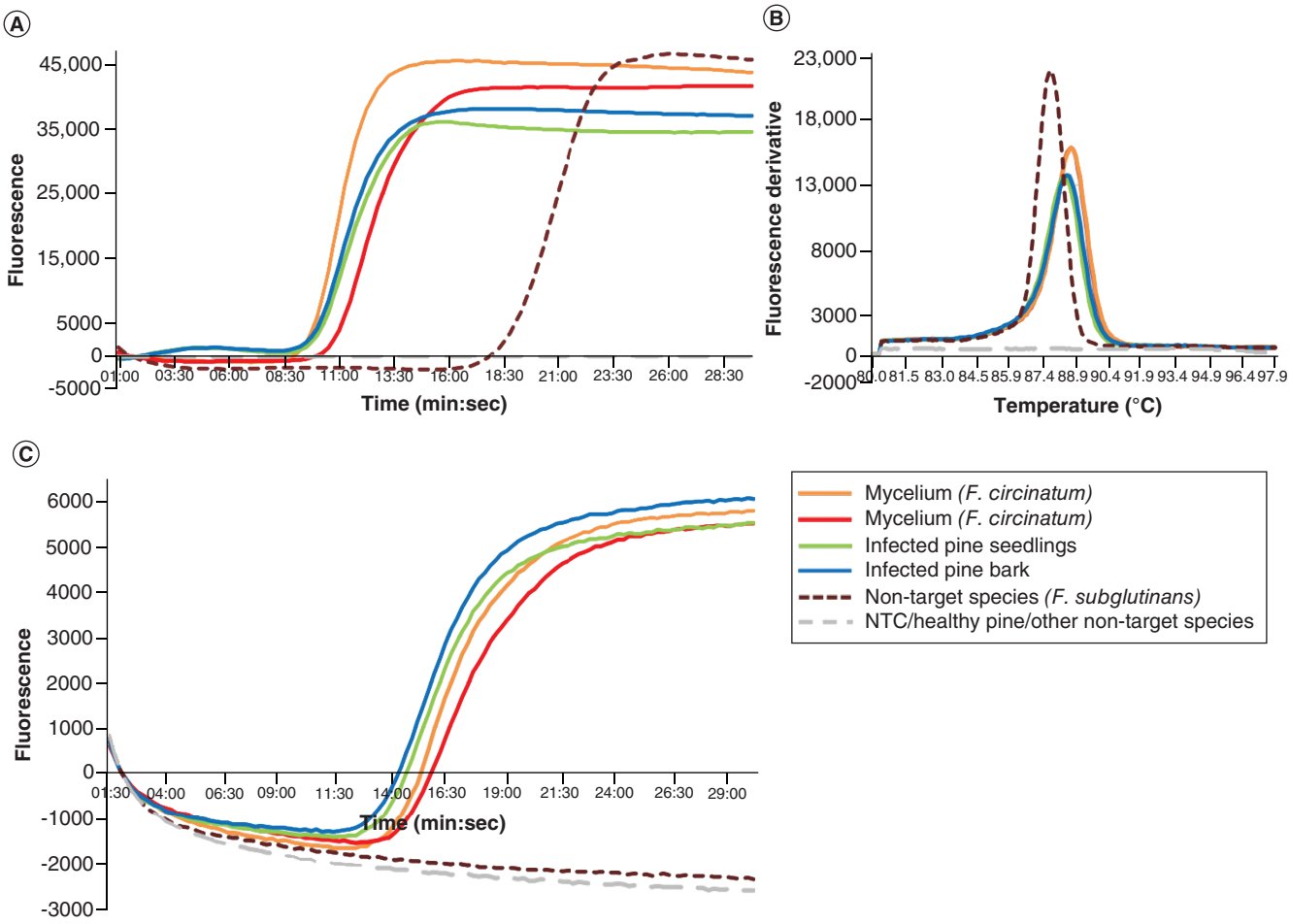


Figure 2. Selection of kinetics of *Fusarium circinatum* detection on mycelium and pine samples. (A) cLAMP amplification plot. **(B)** cLAMP annealing temperature. **(C)** qLAMP amplification plot.

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.

Acknowledgments

The authors wish to thank to R Ioos (ANSES, France) for providing most of the DNA samples of *Fusarium* spp. used in this work.

Financial & competing interests disclosure

This study was funded by the EU's Horizon 2020 Research and Innovation Programme (grant no. 771271) HOMED project and was supported by COST action FP1406 "Strategies for management of *Gibberella circinata* in greenhouses and forests" (PINESTRENGTH). Part of this work was funded by the agreement between IPSP-CNR and Regional Phytosanitary Service of Tuscany 'Accordo di collaborazione scientifica per la realizzazione di indagini, studi e ricerche di interesse comune nei settori della patologia e avversità delle piante arboree

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

References

1. EPPO. EPPO standards: EPPO A1 and A2 lists of pests recommended for regulation as quarantine pests (PM 1/2 (17)) (2008). www.eppo.int
2. Hepting GH, Roth ER. Host relations and spread of the pine pitch canker disease. *Phytopathology* 43, 475 (1953).
3. Barnard EL, Blakeslee GM. Pitch canker of slash pine seedlings: a new disease in forest tree nurseries. *Plant Dis.* 64(7), 695–696 (1980).
4. Wingfield MJ, Hammerbacher A, Ganley RJ *et al.* Pitch canker caused by *Fusarium circinatum* – a growing threat to pine plantations and forests worldwide. *Australas. Plant Pathol.* 37(4), 319–334 (2008).
5. Aglietti C, Luchi N, Pepori AL *et al.* Real-time loop-mediated isothermal amplification: an early-warning tool for quarantine plant pathogen detection. *AMB Express* 9(1), 50 (2019).
6. Blaser S, Diem H, Felten AV *et al.* From laboratory to point of entry: development and implementation of a loop-mediated isothermal amplification (LAMP)-based genetic identification system to prevent introduction of quarantine insect species. *Pest Manag. Sci.* 74(6), 1504–1512 (2018).
7. EU Regulation 2016/2031 on protective measures against plant pests (Plant Health Law). https://ec.europa.eu/food/plant/plant_health_biosecurity/legislation/new_eu_rules_en
8. Luchi N, Pepori AL, Bartolini P, Ioos R, Santini A. Duplex real-time PCR assay for the simultaneous detection of *Caliciopsis pinea* and *Fusarium circinatum* in pine samples. *Appl. Microbiol. Biotechnol.* 102(16), 7135–7146 (2018).
9. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J. Mol. Biol.* 215(3), 403–410 (1990).
10. Kubota R, Alvarez AM, Su WW, Jenkins DM. FRET-based assimilating probe for sequence-specific real-time monitoring of loop-mediated isothermal amplification (LAMP). *Biol. Eng. Trans.* 4(2), 81–100 (2011).
11. Ioos R, Aloi F, Piškur B *et al.* Transferability of PCR-based diagnostic protocols: an international collaborative case study assessing protocols targeting the quarantine pine pathogen *Fusarium circinatum*. *Sci. Rep.* 9(1), 8195 (2019).
12. Schoch CL, Robbertse B, Robert V *et al.* Finding needles in haystacks: linking scientific names, reference specimens and molecular data for Fungi. *Database (Oxford),* 1–21 (2014).
13. Schoch CL, Seifert KA, Huhndorf S *et al.* Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proc. Natl Acad. Sci. USA* 109(16), 6241–6246 (2012).
14. Boutigny AL, Scauflaire J, Ballois N, Ioos R. *Fusarium temperatum* isolated from maize in France. *Eur. J. Plant Path.* 148(4), 997–1001 (2017).
15. Luchi N, Ioos R, Santini A. Fast and reliable molecular methods to detect fungal pathogens in woody plants. *Appl. Microbiol. Biotechnol.* 104(6), 2453–2468 (2020).
16. Hepting GH, Roth ER. Pitch canker, a new disease of some Southern Pines. *J. For.* 44(10), 742–744 (1946).
17. McCain A, Koehler C, Tjosvold S. Pitch canker threatens California pines. *Calif. Agric.* 41(11), 22–23 (1987).
18. Kobayashi T. Pitch canker of *Pinus luchuensis*, a new disease in Japanese forest. *For. Pests.* 38(10), 169–173 (1989).
19. Britz H, Coutinho TA, Gordon TR, Wingfield MJ. Characterisation of the pitch canker fungus, *Fusarium circinatum*, from Mexico. *South Afr. J. Bot.* 67(4), 609–614 (2001).
20. Wingfield MJ, Jacobs A, Coutinho TA, Ahumada R, Wingfield BD. First report of the pitch canker fungus, *Fusarium circinatum*, on pines in Chile. *Plant Pathol.* 51(3), 397–397 (2002).
21. Cho WD, Shin HD. List of plant diseases in Korea (4th Edition). Korean Society of Plant Pathology, Seoul, Korea, 779 (2004).
22. Pérez-Sierra A, Landeras E, León M, Berbegal M, García-Jiménez J, Armengol J. Characterization of *Fusarium circinatum* from *Pinus* spp. in northern Spain. *Mycol. Res.* 111(7), 832–839 (2007).
23. Bragança H, Diogo E, Moniz F, Amaro P. First report of pitch canker on pines caused by *Fusarium circinatum* in Portugal. *Plant Dis.* 93(10), 1079 (2009).
24. Carlucci A, Colatruglio L, Frisullo S. First report of pitch canker caused by *Fusarium circinatum* on *Pinus halepensis* and *P. pinea* in Apulia (Southern Italy). *Plant Dis.* 91(12), 1683–1683 (2007).
25. Alonso R, Bettucci L. First report of the pitch canker fungus *Fusarium circinatum* affecting *Pinus taeda* seedlings in Uruguay. *Australas. Plant Dis. Notes* 4(1), 91–92 (2009).
26. Steenkamp ET, Rodas CA, Kvas M, Wingfield MJ. *Fusarium circinatum* and pitch canker of *Pinus* in Colombia. *Australas. Plant Pathol.* 41(5), 483–491 (2012).
27. Pfenning LH, Costa SDS, Melo MPD *et al.* First report and characterization of *Fusarium circinatum*, the causal agent of pitch canker in Brazil. *Trop. Plant Pathol.* 39(3), 210–216 (2014).