

Development and validation of a Real-Time PCR for detection of *Ceratocystis ficicola*, the agent of canker, wood discolouration and wilt disease of common fig tree (*Ficus carica*)

*Massimo Pilotti*

Work by:

Valentina Lumia<sup>1</sup>, Lorenzo Sciarroni<sup>1</sup>, Giorgio Gusella<sup>2</sup>, Daniele Del Corpo<sup>1</sup>, Giuliano Manetti<sup>1</sup>, Erica Cesari<sup>1</sup>, Angela Brunetti<sup>1</sup>, Giancarlo Polizzi<sup>2</sup>, and Massimo Pilotti<sup>1</sup>

<sup>1</sup> Research Centre for Plant Protection and Certification (CREA-DC), Council for Agricultural Research and Economics of Italy (CREA), Rome, Italy

<sup>2</sup> Department of Agriculture, Food and Environment, University of Catania, Catania, Italy

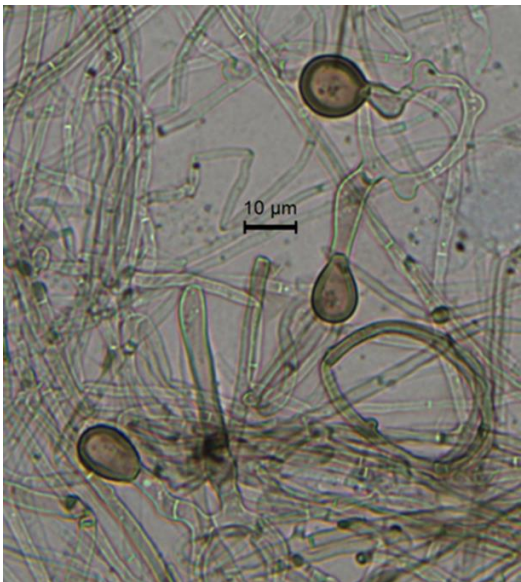


## *The host*

- **Fig is indigenous to the Middle East and Asia Minor** and one of the oldest domesticated tree crops globally
- **A rustic species, tolerates well drought and heat** (it grows well in poor marginal areas not suitable for a high-income agriculture)
- **Worldwide cultivation concentrated in the Mediterranean area** (Turkey leading producer)

## *The pathogen*

**CF was first reported in Japan** as *Ceratocystis fimbriata* (Kato et al. 1982) and then reclassified as a new species (*Ceratocystis ficicola*). Severe damage caused to the orchards



**In 2018-2020 CF was found on fig in different regions in Greece** (a considerable damage was ascertained in the orchards, Tsopeles et al. 2021).  
**Since 2021 it was found in Southern Italy** (Sicily and Apulia)

As a consequence **CF was included in the EPPO alert list in 2022** and subsequently in **EPPO A2 list in 2025**, thereby qualifying as a quarantine pest for which regulation is recommended

○ CF found in Southern EU – Greece (Attica region and Euboea island) and Italy (Sicily and Salento-Apulia)



Ceratocystis canker and wilt disease caused by CF:

- **Bark canker and necrosis of cambium**
- **Severe wood discolouration patterns** namely a parenchymatic radial necrotic progression and a longitudinal progression through the vascular elements which consequently are no longer able to support the water flow.
- **Canopy yellowing and wilting**
- **Death of the tree**

Short- and long-distance dispersal pathways  
(mainly human- and insect-mediated)



Figure is from: Tsopelas et al. 2021

## Isolation and detection of CF

Isolations on selective media has been reported as problematic as CF colonies can be suppressed by fast-growing saprophytic fungi (Tsopelas et al. 2021)

Baiting technique has been successfully used to isolate CF from soil and wood (Tsopelas et al. 2021). However, this method is labour and time-consuming and, at least for detection, a molecular method is preferable

## Identification of CF:

Microscopic morphological features are suitable for identification at the genus level

Sequencing of:

ITS, second largest subunit of the RNA polymerase II (*rpb2*),  $\beta$ -tubulin 1 (*bt1*), Translation Elongation Factor 1- $\alpha$  (*tefl*)

# Development and first validation of a real-time PCR test

*First choices and steps.....*

Main work for optimization, development and validation was based on the use of **strain C1355 from Japan** (kindly provided by **T.C. Harrington, Iowa State University, USA**) and **CPC 44213 (= CBS 149669) from Sicily, Italy** (kindly provided by **G. Polizzi, University of Catania, Italy**).

DNA extraction: DNeasy Plant Mini/Maxi Kit (Qiagen, Hilden, Germany)



Useful,  
if needed

DNeasy PowerClean Pro Cleanup Kit (Qiagen): potentially beneficial for strongly discoloured wood.

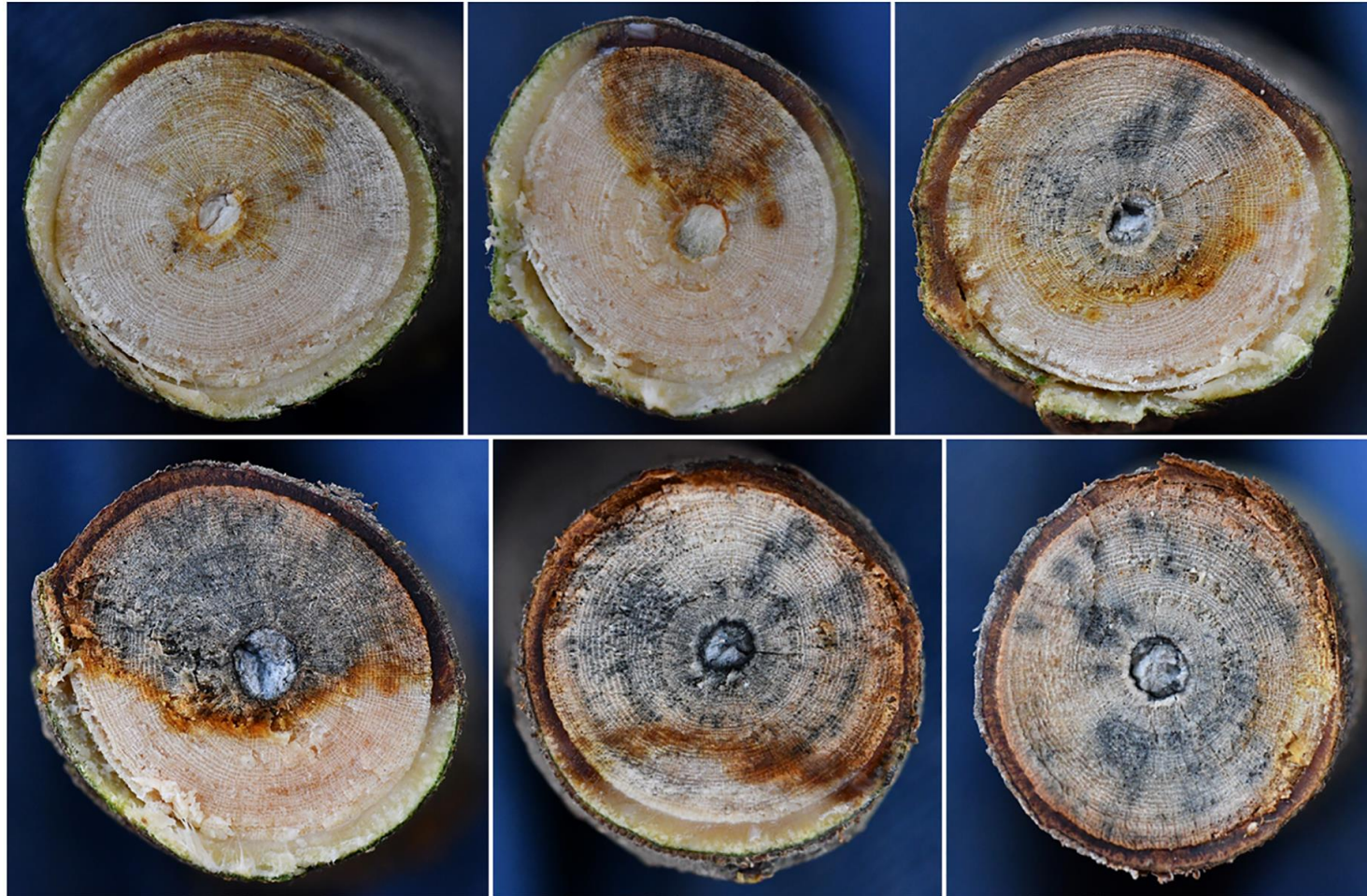
- Primers designed manually in the **ITS region** of *C. ficicola* (ITS1) – **62°C annealing temperature**
- Saturating dsDNA fluorescent dyes: **primarily EvaGreen and secondarily SYBR Green**

- **Agarose gel electrophoresis**: visualization of a **single product of the expected size (92 bp)**
- **Sequencing confirmed** the identity of the amplicon as the **CF target sequence**
- **Any tendency of primers to form dimers was ruled out** in the no-template reactions

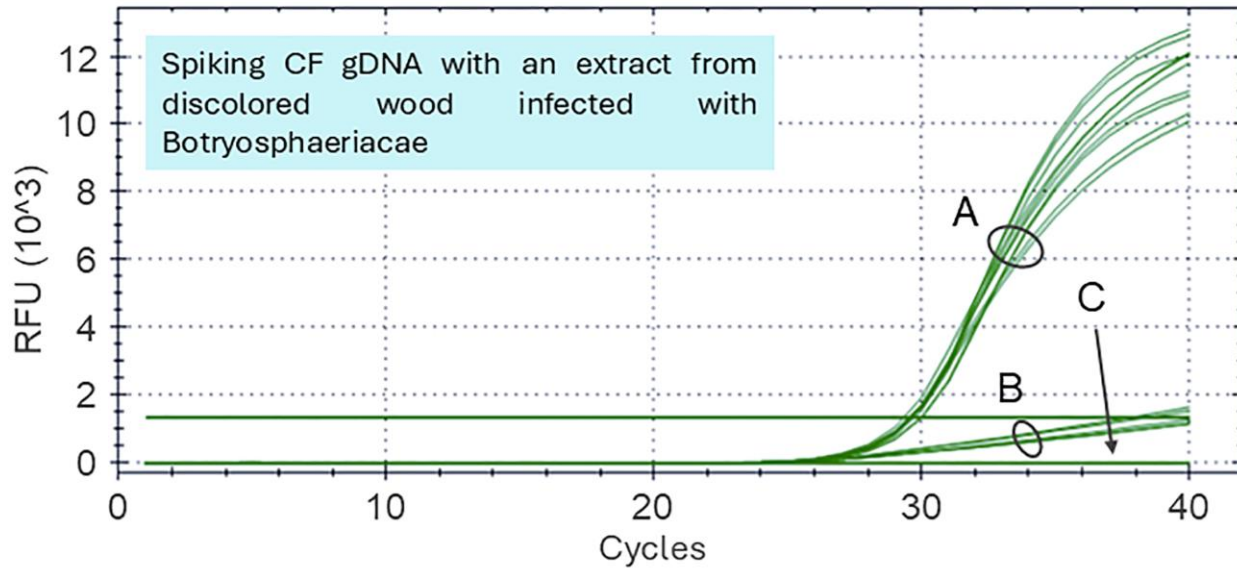
**Initial basic checks confirmed that the test was working properly**

## *Selectivity*

To assess the effect of the wood matrix on PCR amplification, we tested 500 fg and 25 fg of CF gDNA either alone or spiked with 1 or 3  $\mu$ l of a meta DNA extracted from discolored/necrotic wood of two fig trees from the Latium region (CF-negative and infected with other fungi)



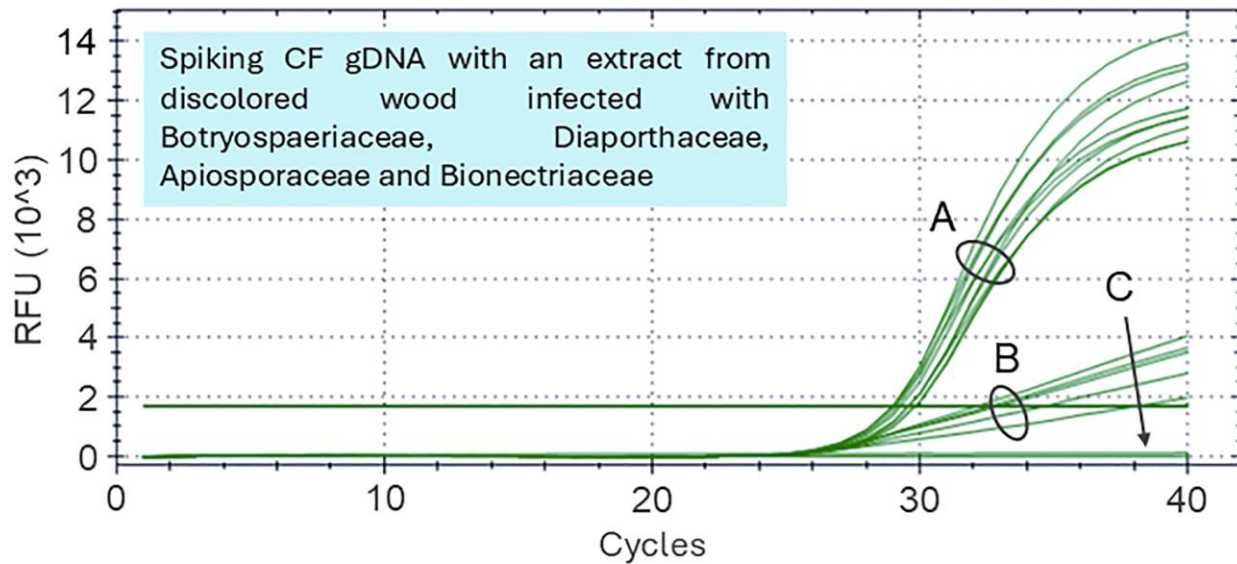
... a complex experiment... here most relevant treatments are presented



**Bundle A:** high-quality signals from CF gDNA spiked with 3  $\mu$ l of necrotic wood extract treated with the inhibitor removal Kit (...and other control treatments...).

**Bundle B:** collapsed signals from CF gDNA spiked with 3  $\mu$ l of untreated necrotic wood extract.

**Bundle C:** no-template controls



*The standard curves to evaluate: i) the efficiency of the amplification process, ii) a preliminary evaluation of the limit of detection (LoD), iii) the selectivity (matrix effect):*

5-fold serial dilutions (78.12 pg to 200 ag DNA), three technical replicates

Different standard curve experiment to evaluate the influence of the plant tissue on the amplification process

➤ Test gDNA extracted from an axenic culture of CF

➤ Spiking fungal gDNA with an extract from healthy-looking wood

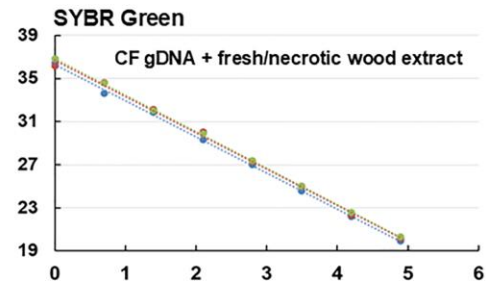
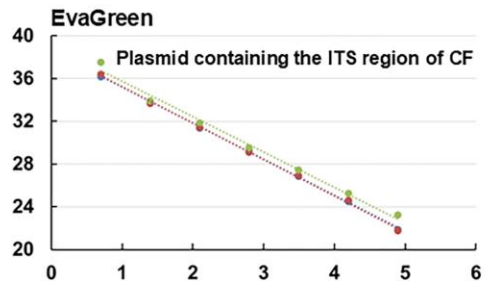
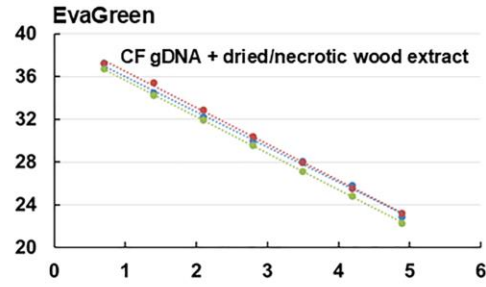
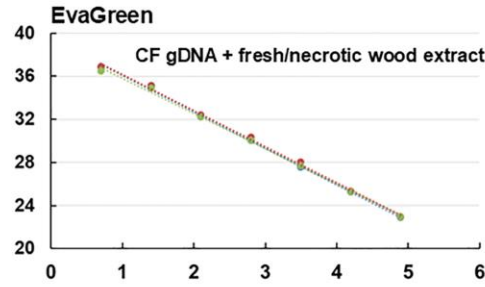
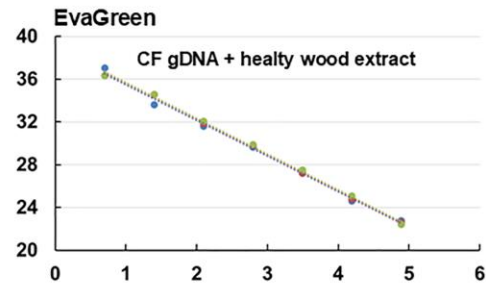
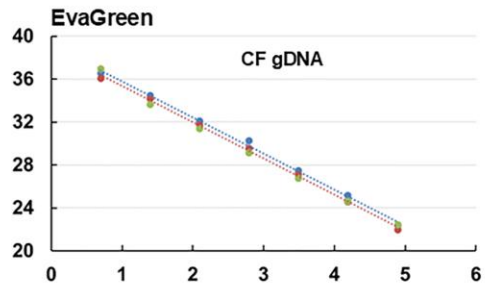


➤ Spiking fungal gDNA with an extract from fresh discoloured wood colonized with fungi other than CF (inhibitor removal kit)



➤ Spiking fungal gDNA with an extract from dead-dried wood colonized with fungi other than CF (inhibitor removal kit)

**Each experiment type performed three times**



Across all assay types, PCR efficiency was consistently high and reproducible, ranging from 95.7% to 102.6%

In all standard curves CF gDNA was detected repeatably till 5 fg gDNA with mean Ct values between 36.1 and 37.3

Dye	Standard curve	E <sup>1</sup> (%)	R <sup>2</sup>	Slope <sup>3</sup>	Intercept
Eva Green	I - CF gDNA	98.3	0.991	-3.36	39.2
	II - CF gDNA	97.8	0.995	-3.38	38.8
	III - CF gDNA	97.7	0.994	-3.38	38.7
Eva Green	I - CF gDNA + HW fig wood DNA	99.5	0.990	-3.33	38.8
	II - CF gDNA + HW fig wood DNA	99.3	0.994	-3.34	38.9
	III - CF gDNA + HW fig wood DNA	99.2	0.994	-3.34	39.0
Eva Green	I - CF gDNA + NW fig wood DNA	97.5	0.994	-3.38	39.5
	II - CF gDNA + NW fig wood DNA	98.7	0.994	-3.35	39.5
	III - CF gDNA + NW fig wood DNA	101.5	0.994	-3.29	39.1
Eva Green	I - CF gDNA + NDW fig wood DNA	102.6	0.990	-3.26	39.2
	II - CF gDNA + NDW fig wood DNA	96.7	0.985	-3.40	39.9
	III - CF gDNA + NDW fig wood DNA	95.7	0.990	-3.43	39.1
Eva Green	I - ITS target-containing plasmid (circular)	98.2	0.997	-3.37	43.9
	II - ITS target-containing plasmid (circular)	96.7	0.995	-3.40	44.1
	III - ITS target-containing plasmid (circular)	100.8	0.990	-3.30	44.3
Eva Green	I - ITS target-containing plasmid (linear)	89.5	0.995	-3.60	45.2
	II - ITS target-containing plasmid (linear)	94.8	0.997	-3.45	44.6
	III - ITS target-containing plasmid (linear)	90.7	0.996	-3.57	44.5
SYBR Green	I - CF gDNA + NW fig wood DNA	97.8	0.999	-3.38	36.3
	II - CF gDNA + NW fig wood DNA	98.3	0.992	-3.36	36.7
	III - CF gDNA + NW fig wood DNA	97.0	0.997	-3.39	36.9

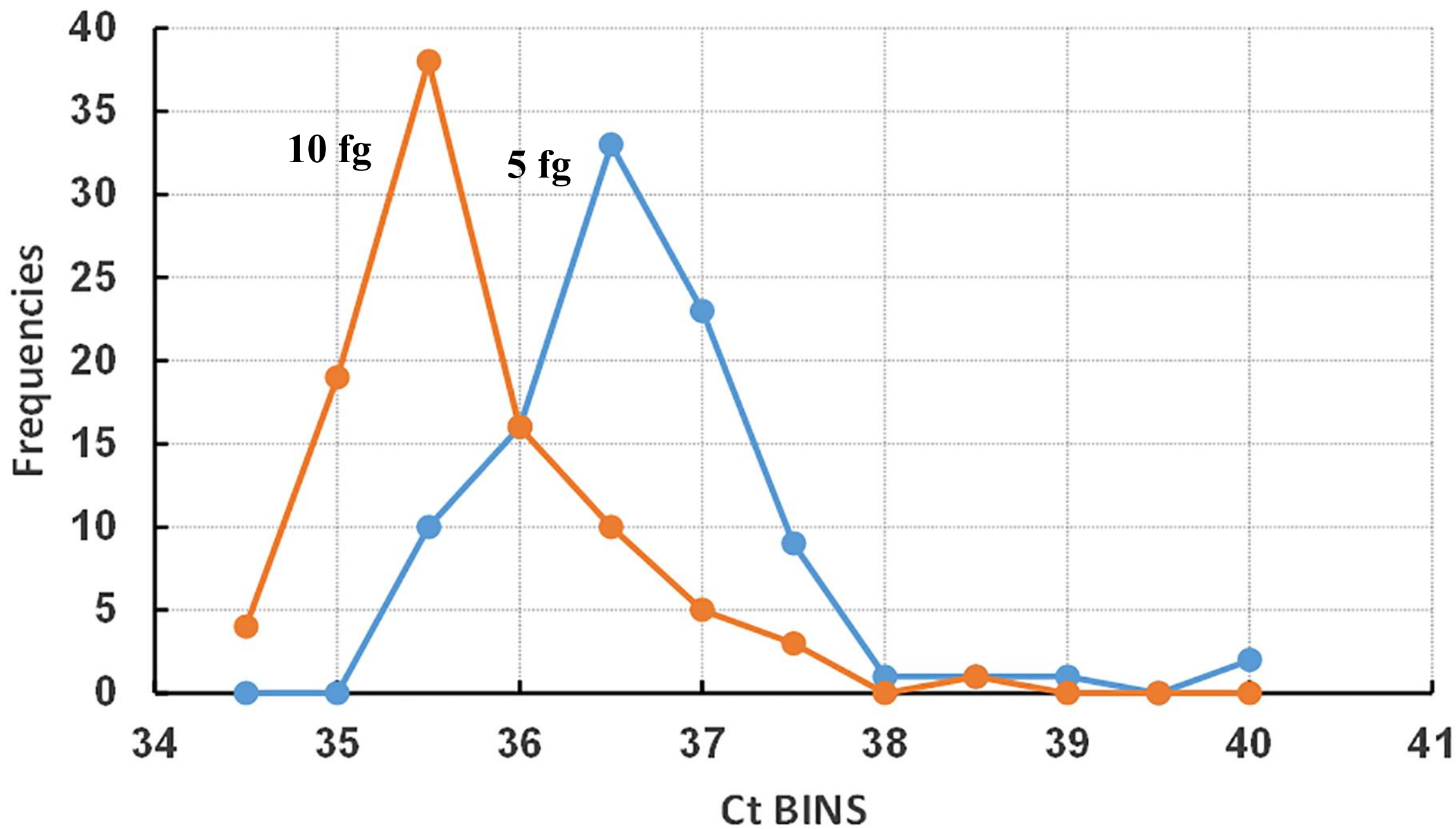
*Analytical sensitivity in EvaGreen  
(...and selectivity)*

Limit of Detection (LoD) = the lowest DNA quantity detected per PCR reaction

- Based on standard curve experiments: LoD = **5 fg of CF gDNA** (...but *Ct* in the range 36-37....increased risk of overlap with low-level contamination signals... )
- **Thus, an additional LoD was established at *Ct* ~35–36**

Consequently: **5 and 10 fg CF gDNA were tested** by alone or spiked with the three types of wood extracts.

**A total of eight treatments with 8 technical replicates, each treatment performed three times.  
Repeatability and reproducibility guaranteed by: 24 technical replicates for each treatment**



*Analytical sensitivity in SYBR Green (...and selectivity)*

- Based on standard curve experiments: LoD = 5 fg of CF gDNA (...but Ct in the range 34-35....a chance to increase the sensitivity... )
- **Thus, an additional LoD was established at Ct ~35–36.**

Treatment	gDNA CF + DNA fresh necrotic wood	
Limit of detections (LoD)	3fg	5fg
I exp.	35.5 (0.72)	34.9 (0.51)
II exp.	35.6 (0.69)	34.4 (0.32)
III exp.	35.4 (0.66)	34.1 (0.55)
<b>Overall</b>	<b>35.5 (0.66)</b>	<b>34.4 (0.56)</b>

**Operational LoD = 3 fg**

**(repeatably and reproducibly detected in the  
Ct ~35–36)**

# Exclusivity 50 non-target fungi tested

Fungal isolate code (FIC=fig, OL=olive)	Geographical origin	Molecular Identification (single or multi-locus)	Detection of 500pg gDNA
CREA-DC TPR FIC.3	Apulia, Italy	<i>Diaporthe</i> sp.	N.D.
CREA-DC TPR FIC.4	Apulia, Italy	<i>Diplodia seriata</i>	N.D.
CREA-DC TPR FIC.5	Apulia, Italy	<i>Neofusicoccum vitifusiforme</i>	N.D.
CREA-DC TPR FIC.6	Apulia, Italy	<i>Neofusicoccum parvum</i>	N.D.
CREA-DC TPR FIC.7	Apulia, Italy	<i>Botryosphaeria dothidea</i>	N.D.
CREA-DC TPR FIC.8	Apulia, Italy	<i>Fusarium</i> sp.	N.D.
CREA-DC TPR FIC.10	Latium, Italy	<i>Apiospora arundinis</i>	N.D.
CREA-DC TPR FIC.11	Latium, Italy	<i>Alternaria</i> sp.	N.D.
CREA-DC TPR FIC.12	Latium, Italy	<i>Epicoccum</i> sp.	N.D.
CREA-DC TPR FIC.14	Latium, Italy	<i>Pseudopithomyces</i> sp.	N.D.
CREA-DC TPR FIC.16	Latium, Italy	<i>Diplodia mutila</i>	N.D.
CREA-DC TPR FIC.17	Latium, Italy	<i>Biscogniauxia mediterranea</i>	N.D.
CREA-DC TPR FIC.20	Latium, Italy	<i>Fusarium</i> sp.	N.D.
CREA-DC TPR FIC.21	Latium, Italy	<i>Dothiorella viticola</i>	N.D.
CREA-DC TPR FIC.22	Latium, Italy	<i>Clonostachys</i> sp.	N.D.
CREA-DC TPR FIC.23	Latium, Italy	<i>Neodidymelliopsis</i> sp.	N.D.
CREA-DC TPR FIC.24	Latium, Italy	<i>Diaporthe</i> sp.	N.D.
CREA-DC TPR FIC.27	Latium, Italy	<i>Alternaria</i> sp.	N.D.
CREA-DC TPR FIC.31	Latium, Italy	<i>Sardiniella</i> sp.	N.D.
CREA-DC TPR FIC.35	Latium, Italy	<i>Neofusicoccum parvum</i>	N.D.
CREA-DC TPR FIC.36	Latium, Italy	<i>Diaporthe</i> sp.	N.D.
CREA-DC TPR FIC.37	Latium, Italy	<i>Apiospora</i> sp.	N.D.
CREA-DC TPR FIC.38	Latium, Italy	<i>Diaporthe</i> sp.	N.D.
CREA-DC TPR FIC.40	Latium, Italy	<i>Peniophora</i> sp.	N.D.
CREA-DC TPR FIC.44	Latium, Italy	<i>Apiospora marii</i>	N.D.
CREA-DC TPR FIC.45	Latium, Italy	<i>Diaporthe cinerascens</i>	N.D.

Fungal isolate code (FIC=fig, OL=olive)	Geographical origin	Molecular Identification (single or multi-locus)	Detection of 500pg gDNA
CREA-DC TPR FIC.46	Latium, Italy	<i>Geosmithia pallida</i>	N.D.
CREA-DC TPR FIC.49	Latium, Italy	<i>Lasiodiplodia</i> sp.	N.D.
CREA-DC TPR FIC.51	Latium, Italy	<i>Botryosphaeria dothidea</i>	N.D.
CREA-DC TPR FIC.52	Sicily, Italy	<i>Fusarium</i> sp.	N.D.
CREA-DC TPR FIC.56	Sicily, Italy	<i>Fusarium</i> sp.	N.D.
CREA-DC TPR FIC.62	Sicily, Italy	<i>Fusarium</i> sp.	N.D.
CREA-DC TPR FIC.63	Sicily, Italy	<i>Clonostachys</i> sp.	N.D.
CREA-DC TPR FIC.64	Sicily, Italy	<i>Fusarium</i> sp.	N.D.
CREA-DC TPR FIC.65	Sicily, Italy	<i>Fusarium</i> sp.	N.D.
CREA-DC TPR FIC.66	Sicily, Italy	<i>Neofusicoccum parvum</i>	N.D.
CREA-DC TPR FIC.70	Latium, Italy	<i>Dothiorella viticola</i>	N.D.
CREA-DC TPR FIC.71	Latium, Italy	<i>Botryosphaeria dothidea</i>	N.D.
FUS C8C = CPC 44200	Sicily, Italy	<i>Neocosmospora perseae</i>	N.D.
FUS.C11A= CPC 44202	Sicily, Italy	<i>Neocosmospora bostrycoides</i>	N.D.
CREA-DC TPR OL.193	Latium, Italy	<i>Neoscytalidium dimidiatum</i>	N.D.
CREA-DC TPR OL.297	Latium, Italy	<i>Apiospora rasikravindrae</i>	N.D.
CREA-DC TPR OL.345	Latium, Italy	<i>Apiospora marii</i>	N.D.
CREA-DC TPR OL.350	Latium, Italy	<i>Apiospora italica</i>	N.D.
CREA-DC TPR OL.427	Apulia, Italy	<i>Neofusicoccum mediterraneum</i>	N.D.
CREA-DC TPR OL.431	Apulia, Italy	<i>Neofusicoccum stellenboschiana</i>	N.D.
CREA-DC TPR OL.463	Apulia, Italy	<i>Diplodia olivarum</i>	N.D.
CREA-DC TPR OL.587	Apulia, Italy	<i>Diplodia africana</i>	N.D.
CREA-DC TPR OL.661	Apulia, Italy	<i>Apiospora aurea</i>	N.D.
CREA-DC TPR CER.23	Latium, Italy	<i>Ceratocystis platani</i>	N.D.

No detection signals or detectable melting curves observed

**N.D. = not detected**

## *Inclusivity*

- In addition to the Japanese isolate, seven isolates from Sicily

CF isolate code	<i>Cts</i> with 500fg (SD)	<i>Cts</i> with 10fg (SD)
CREA-DC TPR CPC 44183	29.2 (0.06)	34.8 (0.32)
CPC 44213	29.0 (0.07)	34.4 (0.61)
CREA-DC TPR CPC 44218	29.9 (0.10)	35.5 (0.77)
CREA-DC TPR CPC 44222	29.2 (0.07)	34.9 (0.48)
CREA-DC TPR CPC 44223	30.0 (0.04)	35.6 (1.17)
CREA-DC TPR CF Sic2	29.9 (0.08)	35.1 (0.04)
CREA-DC TPR CF Sic5	29.4 (0.10)	34.9 (0.61)

All isolates detected

**The LoD of 10 fg gDNA confirmed for all**

*Diagnostic specificity: testing samples from fig trees inoculated with Botryosphaeriaceae and Ceratocystis platani*



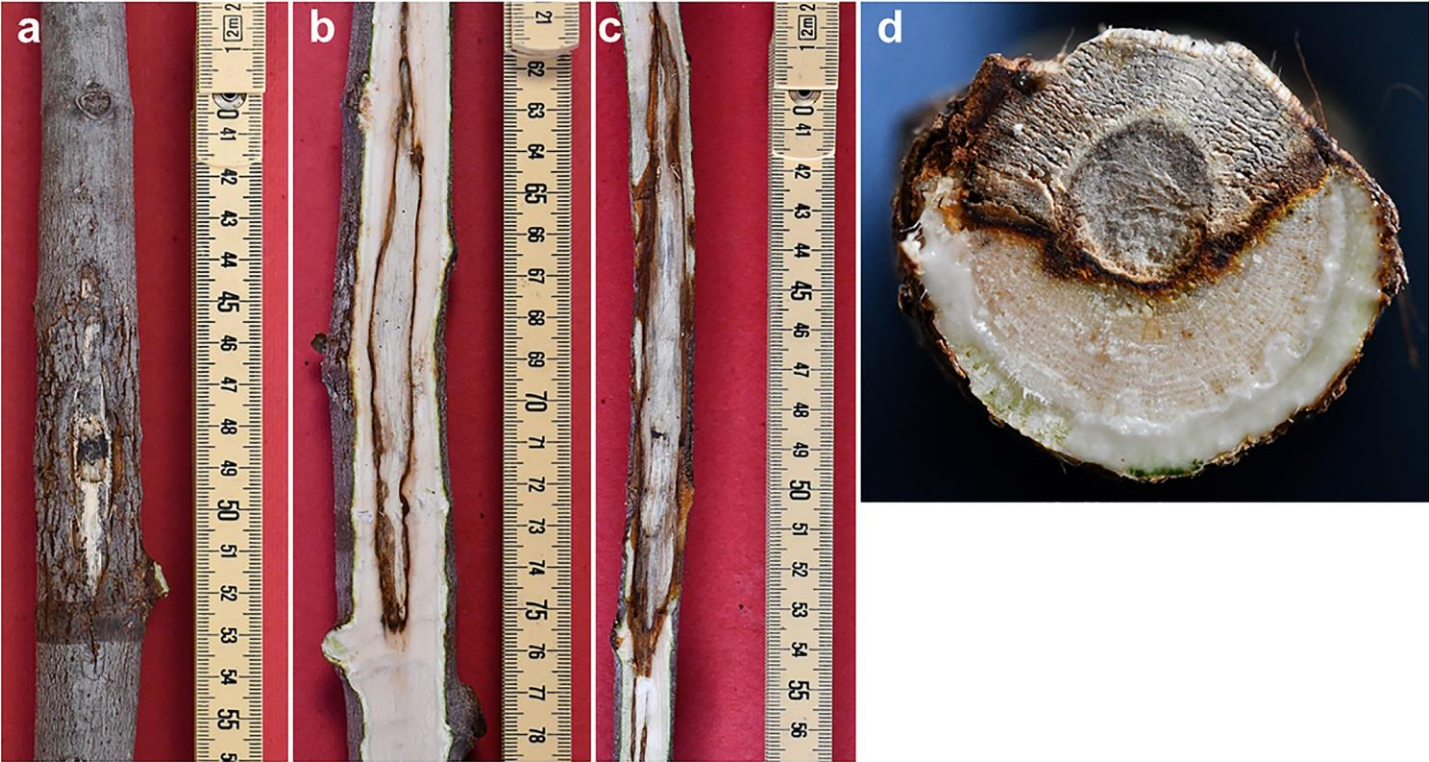
*Neof. vitifusiforme*

*Botryo. dothidea*



*Neof. parvum*

Negative control



**Outcome of *Ceratocystis platani* inoculation**  
**(A novelty: *Ceratocystis platani* can infect fig trees)**

All 16 samples tested negative by PCR, yielding a diagnostic specificity of  $DSP = NTN/N^- = 16/16 = 1$

## Diagnostic specificity: testing natural samples

Wood sample code	Fungal microbiota associated	Detection of 2µl wood extract
1-H	<i>Diplodia mutila</i> <i>Biscogniauxia mediterranea</i> <i>Diplodia</i> sp. <i>Diaporthe</i> sp.	N.D.
2-H	<i>Fusarium</i> sp. <i>Dothiorella viticola</i> <i>Neodidymelliopsis</i> sp.	N.D.
3-H	<i>Alternaria</i> sp.	N.D.
4-H	<i>Alternaria</i> sp.	N.D.
5-H	<i>Apiospora arundinis</i> <i>Alternaria</i> sp. <i>Epicoccum</i> sp. <i>Pseudopithomyces</i> sp.	N.D.
6-H	<i>Sardiniella</i> sp. <i>Alternaria</i> sp.	N.D.
7-H	<i>Diplodia</i> sp.	N.D.
8-H <sup>4</sup>	<i>Diaporthe</i> sp.	N.D.
9-H <sup>4</sup>	<i>Diaporthe</i> sp. <i>Alternaria</i> sp.	N.D.
10-H	N.A.	N.D.
11-H	N.A.	N.D.
12-H	N.A.	N.D.
13-H	N.A.	N.D.
14-S	<i>Diaporthe</i> sp. <i>Diplodia</i> sp.	N.D.
15-S	<i>Clonostachys</i> sp. <i>Botryosphaeria</i> sp.	N.D.
16-S	<i>Neofusicoccum</i> sp.	N.D.
17-S	<i>Diplodia</i> sp.	N.D.

Wood sample code	Fungal microbiota associated	Detection of 2µl wood extract
18-S	<i>Neofusicoccum parvum</i> <i>Diaporthe</i> sp. <i>Apiospora marii</i> <i>Diaporthe cinerascens</i> <i>Geosmithia pallida</i> <i>Diplodia</i> sp.	N.D.
19-S	<i>Peniophora</i> sp. <i>Neofusicoccum</i> sp.	N.D.
20-S	<i>Botryosphaeria dothidea</i>	N.D.

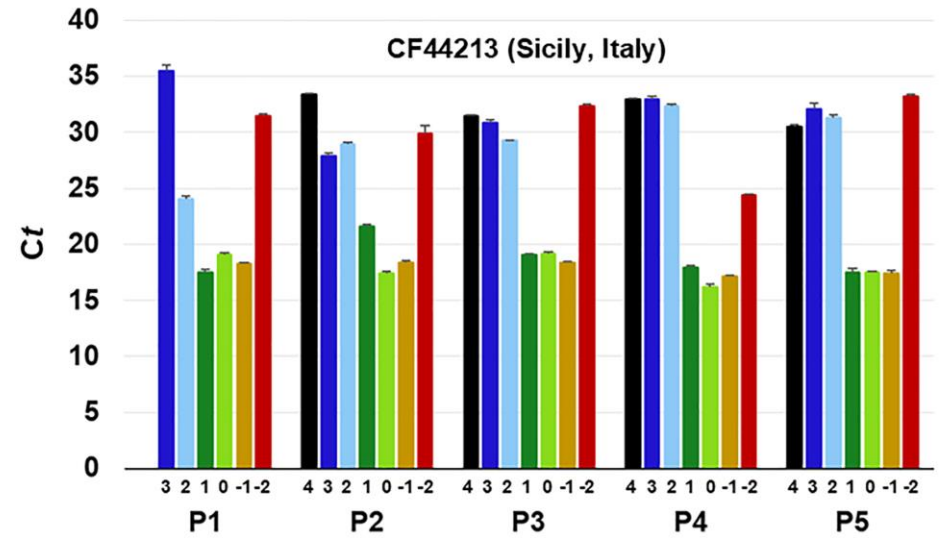
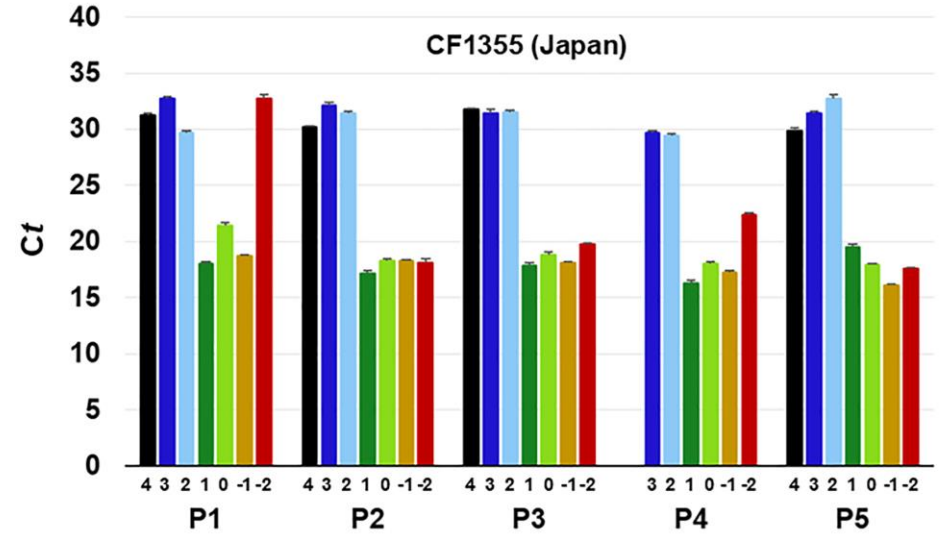
Fig samples collected in the Latium region (CF not present), from trees with healthy-looking wood or necrotic wood infected with fungi other than CF

All 20 samples tested negative by PCR, yielding a diagnostic specificity of  $DSP = NTN/N^- = 20/20 = 1$

**N.D. = not detected**

*Diagnostic sensitivity:  
testing samples from CF artificially-infected fig trees*

Sicilian and Japanese isolates inoculated (5 trees each); 68 wood samples collected after 3 months at increasing distances from the inoculation point



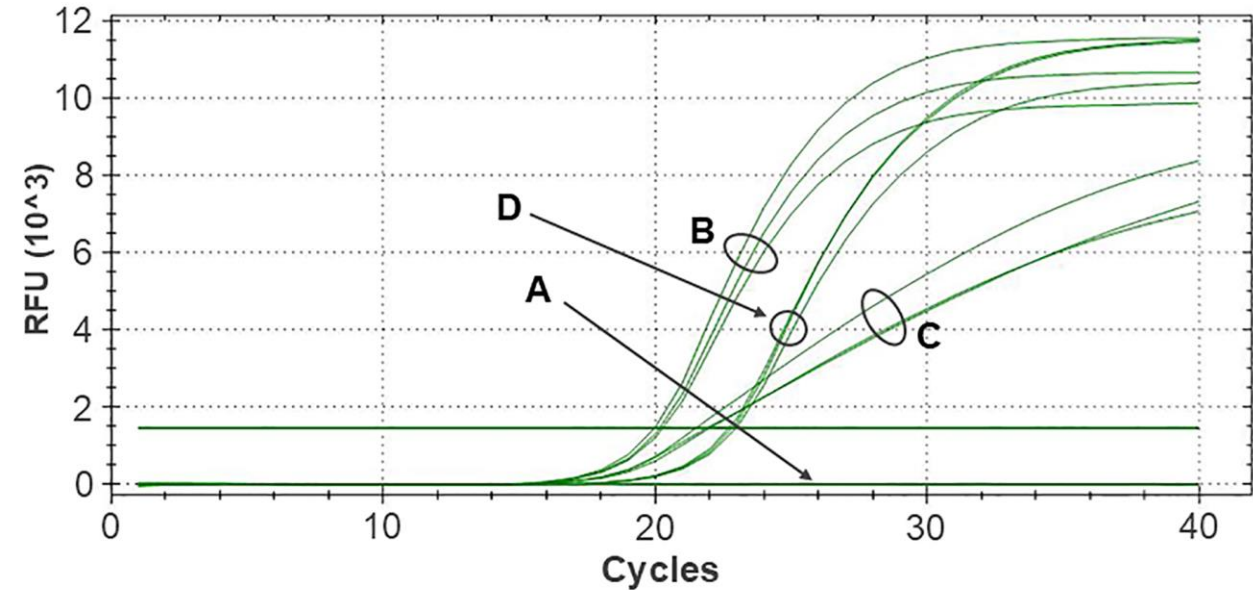
All 68 aliquots tested positive by PCR, yielding a diagnostic sensitivity of  $DSe = NTP/N^+ = 68/68 = 1.00$ .

*Diagnostic sensitivity:  
testing samples from CF naturally-infected fig trees*

Natural samples (Sicily)	2 $\mu$ l	1 $\mu$ l	2 $\mu$ l of 1:10 diluted	2 $\mu$ l + inhibitor removal Kit
1	21.8 (0.20)	22.6 (0.15)	25.1 (0.14)	22.3 (0.06)
2	<b>N.D.</b>	21.8 (0.26)	22.8 (0.14)	20.1 (0.15)
3	22.9 (0.34)	23.5 (0.21)	26.8 (0.53)	23.2 (0.10)
4	22.5 (0.17)	23.4 (0.18)	26.1 (0.29)	23.2 (0.32)
5	29.8 (0.20)	30.6 (0.18)	33.4 (0.25)	30.1 (0.13)
6	24.5 (0.25)	25.9 (0.14)	28.2 (0.22)	25.1 (0.34)
7	27.0 (0.10)	28.1 (0.21)	30.5 (0.37)	27.6 (0.20)
8	23.1 (0.24)	24.0 (0.12)	26.2 (0.19)	23.6 (0.15)
9	<b>N.D.</b>	35.2 (0.39)	37.2 (0.36)	34.1 (0.23)
10	22.8 (0.20)	23.9 (0.29)	26.3 (0.10)	23.3 (0.27)

- **N.D.** = not detected (false negative detection)
- Azure-shaded are samples positively detected (TP) (high quality with any option)
- Grey-shaded are collapsed fluorescent signals (low quality)

Reducing template volume, diluting the extract, and using the cleanup kit, all help overcome amplification inhibition; **however, only treatment with the inhibitor removal kit fully restores diagnostic signals at the earliest Ct.**



- **A** = 2 $\mu$ l extract, false negative detection
- **C** = 1 $\mu$ l extract, low quality detection
- **D** = 2 $\mu$ l 1:10 diluted extract, high quality detection
- **B** = 2 $\mu$ l extract treated with inhibitor removal kit, high quality detection

Using the inhibitor removal kit, all 10 aliquots tested positive by PCR, yielding a diagnostic sensitivity of  $DSe = NTP/N^+ = 10/10 = 1.00$ .

## Future work required:

- Test additional CF isolates as they become available
  - Evaluate assay exclusivity using additional *Ceratocystis* spp. (e.g., *C. manginecans* infecting fig in South America)
    - Validate the capacity of the test to detect CF in soil
- Validate the capacity of the test to detect CF on coleoptera body
  - **Evaluate interlaboratory reproducibility via test performance study (TPS)**



Thank you for the attention