Title:

Interlaboratory performance of a Real-Time PCR method for detection of *Ceratocystis platani*, the agent of canker stain of *Platanus* spp.

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**Text/Table S1** - Basic information and conditions of the Real-Time PCR method for *Ceratocystis platani* detection used in the test performance study (TPS) by the organizing laboratory (OL) and the participating laboratories (PL): i) primers and probe sequences, ii) composition of master mixes, iii) reaction assembly, iv) thermal cycling conditions, v) establishing the best annealing time of Real-Time PCR based on master mixes other than Bio-Rad.

## *Primers and probe sequences*

The PCR target was the Internal Transcribed Spacer 1 region (ITS1) 95bp long

- Forward primer: C.P.Sn.For.I 5'-CGTACCTATCTTGTAGTGAGATGAATGC-3' (from position 89 to 116 of the *C. platani* reference sequence DQ399853).
- Reverse primer: C.P.Sn.Rev.I 5'-GAGTTTACAGTGGCGAGACTATACTG-3' (from position 158 to 183).
- Taqman probe: C.P.TM.Pr. 5'-CGGTGCCCTTCAGAAGGGCCCTACCACC-3', (from position 123 to 150). The probe was labeled with FAM (6- carboxy-fluorescein) at 5’ end, and contained Black Hole Quencher™ 1 (BHQ-1) at 3’ end.

# *List of master mixes used in the TPS:*

## BIO-RAD master mixes

- SsoFast™ EvaGreen® Supermix (for EvaGreen assay)
- SsoAdvanced™ Universal Probes Supermix (for Taqman assay)
- SsoAdvanced™ Universal SYBR® Green Supermix (for SYBR Green assay)

All three master mixes contain:

dNTPs, antibody-mediated hot-start Sso7d-fusion polymerase, $MgCl_2$, and stabilizers. In addition a blend of passive reference dyes, including ROX and fluorescein, is contained in the supermix for use in Taqman assay. The first and the third supermix contain respectively EvaGreen and SYBR Green I as the fluorescent dye

**Master mixes from other companies**

- qPCR MasterMix (Eurogentec, Seraing, Belgium). For Taqman assay.
  It contains: 2x reaction buffer contaning MgCl2, dNTP Mix, HotGoldStar DNA Polymerase, Uracyl-N-glycosilase, stabilizers and passive reference.

- qPCR MasterMix No ROX (Eurogentec, Seraing, Belgium). For Taqman assay.
  It contains: reaction buffer contaning dNTPs (including dUTP), HotGoldStar DNA Polymerase, MgCl2 (final concentration 5 mM), Uracil-N-Glycosilase, stabilizers.

- Maxima Probe/ROX qPCR Master Mix (Thermo Fisher Scientific, Waltham, Massachusetts). For Taqman assay.
  It contains: Maxima® Hot Start Taq DNA polymerase, dNTPs (including dUTP), Uracil-N-Glycosilase, KCl, (NH4)2SO4, and a ROX Passive reference dye.

- Maxima SYBR Green/ROX qPCR Master Mix. For SYBR Green assay.
  It contains: Maxima® Hot Start Taq DNA polymerase, dNTPs (including dUTP), Uracil-N-Glycosilase, KCl, (NH4)2SO4, SYBR Green I as the fluorescent intercalating dye, and a ROX Passive reference dye.

- Power SYBR Green PCR Master Mix (Applied Biosystem, Thermo Fisher Scientific, Waltham, Massachusetts). For SYBR Green assay.
  It contains: SYBR ® Green I Dye, AmpliTaq Gold® DNA Polymerase - UP, dNTPs, Passive reference, Optimized buffer components.

## *Reaction assembly*

**Reaction assembly with EvaGreen and SYBR Green dyes (any master mix)**

| Reagent | Working concentration | Volume per reaction (µl) | Final concentration |
|---|---|---|---|
| Molecular grade water * | N.A. | 6 | N.A. |
| Supermix | 2X | 10 | 1x |
| Forward Primer (C.P.Sn.For.I) | 10 µM | 1 | 0.5 µM |
| Reverse Primer (C.P.Sn.Rev.I) | 10µM | 1 | 0.5 µM |
| Subtotal | | 18 | |
| DNA sample | | 2 | |
| Total | | 20 | |

*Molecular grade and nuclease-free water should be preferably used.

**Reaction assembly with Taqman probe (any master mix)**

| Reagent | Working concentration | Volume per reaction (µl) | Final concentration |
|---|---|---|---|
| Molecular grade water * | N.A. | 5.7 | N.A. |
| Supermix | 2X | 10 | 1x |
| Forward Primer (C.P.Sn.For.I) | 10 µM | 1 | 0.5 µM |
| Reverse Primer (C.P.Sn.Rev.I) | 10 µM | 1 | 0.5 µM |
| Probe (C.P.TM.Pr.) | 20 µM | 0.3 | 0.3 µM |
| Subtotal | | 18 | |
| DNA sample | | 2 | |
| Total | | 20 | |

* Molecular grade and nuclease-free water should be preferably used.

# *Thermal cycling conditions*

**In bold and grey-shaded are the changes made accordingly to the canonical thermal cycling conditions.**

**Bio-Rad master mixes in CFX96**

Initial denaturation at 96°C for 3 min; 40 cycles at 95°C for 10 s (denaturation) and 66°C for 20 s (annealing/extension); final extension (for EvaGreen only) at 72 °C for 5 min. Only for EvaGreen and SYBR Green: dissociation run: increase of 0.5 °C, from 55 to 95 °C.

**Bio-Rad supermixes in Rotor-Gene™ 6000 and Roche Lightcycler 480**

Initial denaturation at 96°C for 3 min; 40 cycles at 95°C for 10 s (denaturation) and 66°C for **30 s** (annealing/extension).

**Eurogentec, Thermo Scientific and Applied Biosystem supermixes**

Initial denaturation at **95°C for 10 min**; 40 cycles at **95°C for 15 s** (denaturation) and 66°C for **a time to be experimentally determined**, (depending on the results of the preliminary test here below) (annealing/extension); final extension (for intercalating dye only) at 72 °C for 5 min. Only for SYBR Green: dissociation run: increase of 0.5 °C, from 55 to 95 °C.

## *Establish the best annealing time of Real-Time PCR based on master mixes other than Bio-Rad*

Results of testing different annealing time at different concentrations

| Chemistry<br>Master mix<br>(Company) (PL) | | Average Ct value (standard deviation) at each annealing time | | | | Chosen value |
|---|---|---|---|---|---|---|
| | DNA titer | 20s | 30s | 40s | 60s | |
| Taqman<br>qPCR MasterMix<br>(Eurogentec) (A) | undiluted | 19.7 (0.10) | | | 16.4 (0.10) | **60s** |
| | 1:1000 | 27.1 (0.09) | | | 26.1 (0.15) | |
| Taqman<br>Maxima Probe/ROX qPCR Master Mix<br>(Thermo Scientific) (B) | undiluted | 18.4 (0.20) | 17.8 (0.18) | 18.1 (0.18) | | **30s** |
| | 1:1000 | 28.7 (0.22) | 28.0 (0.11) | 28.3 (0.21) | | |
| Taqman<br>qPCR MasterMix No ROX<br>(Eurogentec) (C) | undiluted | 14.8 (0.05) | | | 13.6 (0.16) | **60s** |
| | 1:1000 | 23.8 (0.08) | | | 23.5 (0.13) | |
| SYBR Green<br>Power SYBR Green PCR Master Mix<br>(Applied Biosystem) (H) | undiluted | | 15.9 (0.06) | | 15.9 (0.06) | **30s** |
| | 1:1000 | | 26 (0.06) | | 26 (0.18) | |