Development of a quantitative PCR for improved detection of *Marssonina coronaria* in field samples

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Abstract

Since 2009, Marssonina coronaria *(Mc) leaf blotch disease has become a problem in low input apple production in Middle Europe resulting in early leaf fall and reduced yield and quality of the fruits. Due to difficulties in unambiguously assigning early leaf spots to Mc leaf blotch, a quantitative fluorogenic PCR (TaqMan) was developed for testing suspicious leaf and fruit samples for the presence of Mc.*

More than 100 symptomatic leaf and fruit samples originating from different organic and low input production sites were analysed. The majority of samples showing early symptoms without typical acervuli, but assumed to be Mc positive by growers and advisors, tested negative. Later in the season, the accordance between visual assessment and positive qPCR increased. This test can therefore be used for early diagnosis of unidentified leaf spots and to provide support for deciding the appropriate preventive and direct measures to control spread of the disease.

Keywords: TaqMan qPCR, apple, leaf blotch disease, *Marssonina coronaria* (Mc), *Malus* x*domestica* (Md)

Introduction

In recent years, *Marssonina coronaria* leaf blotch disease has become a significant problem in low input apple production in Middle Europe resulting in early leaf fall and reduced yield and quality of fruits (Lindner, 2012). The disease and its symptoms, initially yellowish spots which become darker and become surrounded by chlorotic areas, followed by the formation of pinhead-like asexual fruiting bodies (acervuli), become conspicuous in mid-summer (Lee *et al*. 2011). Under favourable conditions, the disease can then develop rapidly and cause early leaf fall in August. Particularly the early stages of the symptoms, however, are difficult to be clearly assigned by eye and furthermore, symptoms might differ significantly from one apple variety to another. Only when acervuli are built up, which contain the typical hyaline two-cell conidia, visual and microscopic examinations are unambiguous.

PCR and in particular the quantitative fluorogenic PCR (TaqMan) allow specific detection of disease stages early after primary infections. In addition, the possibility of using several fluorogenic probes emitting and excited by different wavelengths of light, allows the multiplex detection of several DNA targets simultaneously in the same reaction, such as the combination of the pathogen detection with an internal standard or host gene reaction. So, a clear and positive host-gene signal indicates the absence of inhibiting substances, which might have suppressed a specific Mc reaction, and allows false negative results to be excluded (Oberhänsli *et al*. 2011).

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Material and Methods

DNA was extracted from symptomatic apple leaves and fruits with 0.1-0.5 g of tissue ground in 5 ml of CTAB extraction buffer using the HOMEX 6 and Universal extraction bags (Oberhänsli *et al*. 2011). A 1 ml aliquot of the extract was treated with 0.8 ml chloroform to remove proteins and the DNA in the aqueous phase was then precipitated with isopropanol. This pellet was washed once with 70 % ethanol and the DNA finally resuspended in 0.1 ml of TE buffer (1mM Tris, 0.1 mM Na_2EDTA, pH 8.0). The yield and quality of DNA was assessed by UV absorption (Nanodrop, Thermo) resulting in 100-500 ng DNA/µl and 260/280 nm ratios in the range of 1.8-2.1.

DNA of fungal reference isolates (*Diplocarpon mali* strain no. 30405 from NITE Biological Resource Center (NBRC), Japan and a Mc isolate obtained from L. Lindner, Italy) grown on Potato Carrot Dextrose Agar (PCDA) was extracted first by heating 5 mm agar plugs in 0.5 ml of TE buffer for 10 min at 95°C and then followed by homogenization with 0.25 ml of 0.5 mm glass beads in a bead beater (Fast Prep 24) for 40 s and a speed of 6 m/s). A ten-fold dilution series of the supernatant was used directly as template for the qPCR and served as a calibrator between assays, assigning each dilution step a proportional amount of virtual units of target DNA.

Primer and Probe for Mc detection (Table 1) were designed using Beacon Designer (Version 7.2, Premier Biosoft International) based on a Mc specific region in the internal transcribed spacer 1 (ITS1) and 5.8S ribosomal RNA gene of Mc. Accession No. JN587494.1. This served as a representative for ten other almost identical Mc nucleotide database entries. The specificity of primer and probe was checked *in silico* for significant differences in the corresponding ITS1 sequences of other fungi occuring on apple tissue, such as *Venturia inaequalis*, *Monilinia mali*, *Alternaria mali*, *Glomerella cingulata*, *Cladosporium* sp., *Phoma* sp., *Trichothecium roseum* and *Penicillium expansum*. In addition, primer and probe sequences were both checked for homologies using NCBI-BLAST.

Table 1: Primers and Probes for duplex qPCR for detection of *Marssonina coronaria* (Mc, this work) and *Malus* x *domestica* (Md, Oberhänsli*et al*. 2011).

Primer/probe	Sequence 5'-3'	Final conc.
Mc_forward	GCCTACCCTACCTCTGTTGC	300 nM
Mc_reverse	CAGAACCAAGAGATCCGTTGTTG	300 nM
Mc_probe	FAM-CTCAGACATCACGTTATTCACACAAAGAGTTGGG-BHQ1	100 nM
Md_forward	AGAGGGAGCCTGAGAAACGG	50 nM
Md_reverse	CAGACTCATAGAGCCCGGTATTG	50 nM
Md_probe	ROX-CCACATCCAAGGAAGGCAGCAGGCG-BHQ2	50 nM

The specific primers and probes for both Mc and apple 18S rRNA gene as an internal control (Table 1) were run in duplex to talling 10 µl using 1 µl of DNA, 5 µl KAPA Probe Fast qPCR hotstart Mastermix 2x, and 4 µl of primers and probes at final concentrations as indicated in Table 1. FAM and ROX signals were recorded in real-time after each annealing/elongation step in a 72 well rotor (Rotorgene Q, Qiagen) during 40 cycles in a two-step protocol of 3 s at 95°C and 20 s at 60°C after an initial step of 3 min at 95°C for activation of the hotstart DNA polymerase.

Results

The fluorogenic real-time Mc PCR assay producing the FAM signal reacts specifically with the DNA of both Mc isolates grown on PCDA and field samples of infected leaf and fruit tissue of apple showing clear Mc leaf blotch symptoms. There was no cross-reactivity with either healthy apple tissue, or leaf and fruit tissue, or apple scab and powdery mildew, or with a variety of other, yet not identified fungal pathogens causing leaf blotch symptoms. Also no cross-reactions were observed with DNA extracts from leaf blotch symptoms on rose and quince caused by the closely related *Diplocarpon rosae* and *Diplocarpon mespili*, respectively.

More than 100 symptomatic leaf and fruit samples originating from different organic and low input production sites were analysed. The majority of samples showing early symptoms without typical acervuli, but assumed to be Mc positive by growers and advisors, tested negative. Later in the season, the correlationbetween visual assessment and positive qPCR increased.

Table 2: Representative field samples tested with multiplex qPCR on presence of *Marssonina coronaria* (Mc) and host gene *Malus* x*domestica* (Md). Results are indicated in No. of quantification cycles (Cq) or as relative quantity of target DNA. All apple samples without indication of variety originate from single trees >4m in height used for cider production. All other samples were collected in fruit production orchards.

Sample	Symptoms	Date	Mc (Cq)	Mc (log rel. DNA conc.)	Md (Cq)
Leaf, apple tree	sparse blotches	28.05.2013	>40	< 0.1	11.0
Leaf, apple tree	scab + unknown blotches	28.05.2013	>40	< 0.1	11.8
Leaf, apple tree	susp. blotches on chlorotic leaf	08.06.2013	>40	< 0.1	12.5
Leaf, apple tree	susp.blotches on green leaf	08.06.2013	>40	< 0.1	13.5
Leaf, Gala, orchard	suspicious blotches	20.06.2013	>40	< 0.1	9.1
Leaf, Topaz, orchard	blotches + acervuli	28.06.2013	26.7	2.9	16.4
Leaf, apple tree	suspicious blotches	12.07.2013	>40	< 0.1	10.9
Leaf, quince	*D. mespili*	12.07.2013	>40	< 0.1	13.7
Leaf, Ecolette, orchard	suspicious blotches	25.07.2013	36.0	1.0	11.8
Leaf, apple tree	dense blotches on chlorotic leaf	26.07.2013	17.7	5.5	11.9
Leaf, apple tree	suspicious blotches	02.08.2013	33.3	1.3	9.9
Rose leaf	*D. rosae*	02.08.2013	>40	< 0.1	12.7
Fruit, Otava	Mc symptom	24.10.2013	16.4	5.8	16.2
Fruit, Topaz	Mc symptom	24.10.2013	17.0	5.7	16.9
Fungal DNA extract	Mc NBRC ref. strain	12.06.2013	20.3	4.3	>40

In Table 2 some representative results are indicated in terms of quantification cycles (Cq) for illustration of the performance of the duplex assay. In some cases the DNA extracts were tested undiluted and diluted 10 or 100 times. There was always a linear correlation and the Cq values increased approx. 3.5-4.0 units for each dilution step and for both FAM and ROX signals. The efficiency of the assays ranged between 0.70 and 0.85 for both the Mc and Md assay in duplex format.

Discussion

This duplex fluorogenic real-time PCR assay for quantitative determination of Mc in apple tissues proved its usefulness for the confirmation of Mc in suspicious leaf and fruit samples. Particularly in early season, when diagnosis is very challenging, even for experienced advisors and experts, this test can be used for early diagnosis of unidentified leaf spots and to provide support for deciding the appropriate preventive and direct measures to control the spread of the disease. The simultaneously recorded ROX signal of the Md reaction (Table 2) indicated absence of inhibitory compounds in the DNA extracts of Mc negative samples.

This test was also very helpful when we tried to isolate this fungus from infected plant material. Slow growing fungal structures on agar media were tested early to confirm presence of the target which in fact is quite difficult to cultivate (Lee *et al*. 2011). Another application of the assay is the quantification of the pathogen in artificially inoculated seedlings or saplings to assess the proliferation of the fungus in the host in function of host variety or treatment efficiency, supplementary to visual scoring of symptoms as it was shown for studies with apple scab (Gusberti *et al*. 2012).

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