Development of a Sensitive TaqMan qPCR Assay for Detection and Quantification of *Venturia inaequalis* in Apple Leaves and Fruit and in Air Samples

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Abstract

A TaqMan quantitative PCR (qPCR) assay based on the translation elongation factor 1- α gene was developed for the quantification of *Venturia inaequalis* in leaves and fruits of *Malus × domestica* and in spore trap samples. The designed primers and hydrolysis probe amplified a specific 86-bp fragment for *V. inaequalis*. The specificity of the assay was tested using 35 strains of *V. inaequalis* and 20 different fungal species, including common pathogens of apple and other species of *Venturia*. The limit of detection was 20 fg, which is lower than a single genome of *V. inaequalis*. The selectivity of the assay was tested using DNA from three cultivars of *Malus × domestica*, and no influence on pathogen amplification was found. The assay was also validated for repeatability and reproducibility. With this assay, it was possible to detect and quantify *V. inaequalis* in four cultivars (Ambrosia, Florina, Golden Delicious, and Mondial Gala) in both symptomatic and asymptomatic

leaves and in symptomatic Golden Delicious apple fruit stored for 2 months. Furthermore, the assay was successfully tested on spore trap samples originating from apple orchards. The quantification of the molecular assay when compared with the estimated number of *V. inaequalis* cells, using an optical microscope, showed a correlation coefficient of 0.8186. The developed technique could be used to detect *V. inaequalis* in asymptomatic samples without any cross-reaction with other fungal species. Furthermore, to improve the efficacy of disease management with a timely application of fungicides, this assay could be used for the analysis of spore trap samples by using an implemented extraction method.

Keywords: apple scab, *Malus × domestica*, real-time PCR, TaqMan, *Venturia inaequalis*

Apple scab is a worldwide disease of apple (*Malus × domestica* Bork H.) caused by the ascomycete *Venturia inaequalis* (Cooke) G. Winter (Sutton et al. 2014). The disease is particularly severe in temperate climate regions characterized by humid and cool springs (Bowen et al. 2011) and, if not appropriately managed, can cause huge economic losses (MacHardy 1996). Disease control requires an integrated strategy based on prophylactic practices, application of fungicides, and increasing the use of resistant cultivars (MacHardy et al. 2001).

V. inaequalis has a hemibiotrophic life cycle. Ascospores, which are released from pseudothecia in the fallen leaves, cause primary infections during the growing season (spring to early summer). If the weather conditions are favorable, asexual conidia are released and cause secondary infections during the growing season (Bowen et al. 2011; Carisse et al. 2000). The entire life cycle of *V. inaequalis*

is strongly influenced by humidity and temperature conditions (MacHardy 1996). In order to reduce scab infections, fungicide application programs are scheduled based on the local weather conditions, on disease prediction models, and on the level of infection in spy plants (Carisse et al. 2000; Gadoury and MacHardy 1986; Meitz-Hopkins et al. 2014; Mills and Laplante 1951). Spy plants are apple plants potted with a mulching of diseased leaves, which are exposed in orchard to a single rain until the end of leaf wetness. These plants permit an a posteriori evaluation to identify the first infection period for *V. inaequalis* (Rizzolli and Acler 2010).

In the field, the level of apple scab infection is normally estimated by visually assessing the symptoms and assigning a score (Bock et al. 2010; Gusberti et al. 2012). However, the observation of disease symptoms does not always reflect the real pathogen colonization (Gu et al. 2011; Shaw et al. 2010). Furthermore, the lesion development rate, related to leaf age and fungicide treatments, could underestimate the severity of the disease and lead to incorrect crop protection programs (Keitt and Jones 1926; Li and Xu 2002; Schwabe 1979). In addition, through the use of visual scoring as a diagnostic method, no information is acquired from asymptomatic leaves, important sources for both primary and secondary infections.

Molecular techniques have the advantages of being sensitive, specific, not biased by the operator and could be also used on asymptomatic tissues. Different studies have been reported for specific detection of *V. inaequalis* using conventional PCR (Koh et al. 2013; Schnabel et al. 1999; Stehmann et al. 2001) or, recently, LAMP (Prencipe et al. 2020). Daniëls et al. (2012) developed a quantitative real-time PCR (qPCR) assay targeting the internal transcribed spacer (ITS), while Gusberti et al. (2012) developed a qPCR assay based on primers and hydrolysis probe designed on the housekeeping gene *ABC2* (*ATP-binding cassette transporter 2*) to detect *V. inaequalis*. Nevertheless, in a study by Gusberti et al. (2012), the specificity of the assay was not tested against other fungal species to avoid cross-reactions, resulting in the possibility of false-positive results and pathogen overestimation. A blast analysis of the primers and hydrolysis probes used by Gusberti et al. (2012) was performed in our laboratory. The search

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*The e-Xtra logo stands for “electronic extra” and indicates there are supplementary materials published online.

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gave 100% homology and 100% coverage with *Alternaria solani* (accession number CPO22033.1) and 100% homology and 72% coverage with *A. alternata* (accession number XM_01852723.1), indicating a possible cross reaction to species commonly present in apple orchards. While, in the study by Daniëls et al. (2012), it is suggested to design new primers and a hydrolysis probe to analyze naturally infected samples because of the cross reaction with other *Venturia* species. Both methods needed some improvements to meet an adequate specificity.

An important factor for fungicide application in the field is the quantification of the inoculum of airborne plant pathogens. A tool to quantify the airborne inoculum is the spore trap (Ribbert et al. 2007; Sholberg et al. 2005; West and Kimber 2015), which is used to capture airborne fungal spores on adhesive tape, to be counted later under an optical microscope, to quantify the inoculum of *V. inaequalis*, and to plan phytosanitary treatments (MacHardy 1996; West and Kimber 2015). Current limitations with the use of spore traps are mainly related to the long time spent counting *V. inaequalis* ascospores under an optical microscope on the tapes and the need for a quick response to program fungicide management. Different studies have used spore traps in combination with qPCR to quantify the airborne inoculum of plant pathogens (Carisse et al. 2005; Huang et al. 2016; Klosterman et al. 2014), including *V. inaequalis* (Meitz-Hopkins et al. 2014). The application of qPCR on samples from spore traps could reduce the time to analyze the samples and provide faster results even on a larger number of samples.

To date, no studies used spore traps combined with TaqMan qPCR assays. The aim of the current work was to develop and validate a new specific TaqMan qPCR assay that could be used to detect and quantify *V. inaequalis* on different *Malus × domestica* cultivars, both in the leaves and fruit. *V. inaequalis* isolates from different fields and growing seasons were used to evaluate the assay specificity. Moreover, different plant materials (symptomatic and asymptomatic) and artificially inoculated leaves were tested. The assay was validated for specificity on 20 different species, including *Venturia asperata*, which has recently been reported as a pathogen on scab-resistant varieties of apple having the *Rvi6* gene (Caffier et al. 2012; Turan et al. 2019) and on the other species showing cross-reactions in previously published qPCR assays. Furthermore, the assay was tested on spore trap samples to detect and quantify the airborne inoculum of *V. inaequalis*.

Materials and Methods

Fungal strains. Strains of *V. inaequalis* were isolated from leaves of apple tree (*Malus × domestica*) cultivars Ambrosia, Golden Delicious, and Mondial Gala, cultivated in northern Italy (Piedmont) during 2015 and 2016. Thirty-five isolates were selected for this study. Fungi that are pathogenic or commonly present in apple orchards were isolated from symptomatic and asymptomatic apple leaves. All the isolates were identified through the amplification of the ribosomal DNA ITS region, following the protocol by White et al. (1990). Reference strains (*V. inaequalis* CBS 815.69, *V. asperata* IRHS 2345, *V. pirina* CBS 120.825, *V. nashicola* CBS 794.84, *V. cerasi* CBS 444.54, and *Fusicladium carpophilum* CBS 497.62) and strains from the Agrioinnova collection were used to verify the assay specificity. The strains used in this study are summarized in Table 1 and were maintained as monoconidial cultures in tubes of malt extract agar (MEA) (Sigma Aldrich, Germany) and were stored at 4°C.

Fungal DNA extraction and quantification. Strains of *Venturia* spp. were grown on MEA for 30 days at 20 ± 1°C in the dark, while the other fungal species were grown for 10 days at 25 ± 1°C. The fungal DNA was extracted from approximately 100 mg of fresh-weight mycelium, using an Omega E.Z.N.A. Fungal DNA Mini Kit (VWR, Radnor, PA, USA), according to manufacturer instructions. The DNA quality and the concentration were measured using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Sequence analysis of the translation elongation factor gene and design of primers and hydrolysis probe. The translation elongation

factor 1 alpha (*EF1-α*) sequences were amplified for the 35 *V. inaequalis* strains, using ELF1 (CGAGAAGTTCGAGAAGGT) and ELF2 (CCAATGACGGTGACATAG) primers (Gladieux et al. 2010). PCR was carried out using Taq DNA polymerase kit (Qiagen, Hilden, Germany) in a total volume of 25 µl, containing 2.5 µl of Qiagen PCR buffer 10×, 0.5 µl of MgCl₂, 0.75 µl of dNTPs (10 mM), 1 µl of each primer (10 µM), 0.2 µl of Taq DNA polymerase, and 20 ng of template DNA. The thermal cycling program was an initial denaturing step of 95°C for 5 min, followed by 95°C for 40 s, 58°C for 50 s, and 72°C for 1 min (for 35 cycles), and a final extension at 72°C for 5 min. After agarose gel electrophoresis, the PCR products were purified using a QIAquick PCR purification kit (Qiagen) and were sequenced in both directions by Macrogen, Inc. (Amsterdam, The Netherlands). The consensus sequences were obtained by assembling forward and reverse sequences, using DNA Baser (Heracle Biosoft, Romania). The consensus sequences obtained for the *V. inaequalis* strains were compared with those deposited in GenBank and a multialignment was performed, using the CLUSTALW algorithm, through Molecular Evolutionary Genetics Analysis (MEGA6) software, version 6.0 (Supplementary Fig. S1). The alignment was used to design the primers and hydrolysis probes used in this study.

Six sets of primers and two hydrolysis probes were designed using Primer Express software 3.0 (Applied Biosystems, Foster City, CA, USA) and are listed in Table 2. The hydrolysis probes were labeled at the 5' end with the 6-carboxyfluorescein dye reporter and Black Hole Quencher (BHQ1) or the nonfluorescent quencher minor groove binder (NF-MGB) at the 3'-end. OligoCalc tool (<http://biotools.nubc.northwestern.edu/OligoCalc.html>) was used to verify the presence of hairpins and potential secondary structures, while *in silico* specificity was verified using the BLASTN tool of the National Center of Biotechnology Information (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Invitrogen (Carlsbad, CA, U.S.A.) and Metabion (Steinkirchen, Germany) synthesized primers and hydrolysis probes, respectively.

Conventional end-point PCR and qPCR optimization and amplification. The primer pair sets were first assessed by means of conventional end-point PCR using DNA from *V. inaequalis* strains and other selected species. The PCR end-point assays were performed using a Taq DNA polymerase kit (Qiagen) in a 25-µl reaction, composed of 2 µl of Qiagen buffer 10×, 0.8 µl of MgCl₂, 1 µl of dNTPs (10 mM), 1 µl of each primer (10 µM), 0.2 µl of Taq Platinum Pfx DNA polymerase (Invitrogen, Waltham, MA, USA) and 20 ng of DNA. The PCR thermal cycler conditions were 3 min at 95°C followed by 30 cycles of 95°C for 45 s, 54°C for 45 s, 72°C for 1 min, and a final extension of 5 min. The PCR products were run on 1% agarose gel in a Tris-borate-EDTA buffer and were visualized under a UV transilluminator, using the Quantity One software (BioRad Labs, Hercules, CA, USA). After performing end-point PCR, the primer pairs that gave the best results were selected and were used in qPCR with SYBR Green in order to compare them with the specificity and sensitivity of the TaqMan qPCR assay. Real-time reactions were performed using a StepOnePlus qPCR system (Applied Biosystems), with 96 well-plates (Optical reaction plate; Applied Biosystems) sealed with MicroAmp optical adhesive film (Applied Biosystems).

SYBR Green reactions were carried out using 10 µl of Power SYBR Green Mastermix 10× (Applied Biosystems), 1 µl of each primer (3 µM), and 1 µl of template DNA. Amplification conditions were 95°C for 10 min, followed by 40 cycles of 57°C for 1 min and 95°C for 15 s. The melting curves were acquired after each run at the following conditions: 95°C for 15 s, 60°C for 15 s, and 95°C for 15 s. Sterile water was used as a negative control. To determine the sensitivity of the SYBR Green assay, a standard curve was obtained with *V. inaequalis* DNA using strain 1b14 eightfold serially diluted (from 20 ng to 2 fg).

The TaqMan qPCR assay was performed using TaqMan Universal Mastermix 2× (Applied Biosystems). Each 96-well plate was loaded with a negative control, standard DNA, and a positive control in triplicate. Different primer concentrations (from 3 to 0.3 µM) and

different temperatures were initially tested at the annealing stage (57 to 60°C). Reactions were carried out in a final volume of 20 µl, with 1 µl of DNA ($\cong$ 20 ng), 0.4 µl of each primer (3 µM), and 0.2 µl of hydrolysis probe (5 µM). Sterile water was used as a negative control. Amplification conditions were 95°C for 10 min, 40 cycles of 57°C for 1 min, and 95°C for 15 s. The newly developed TaqMan qPCR assay was compared, in terms of sensitivity and specificity, with the TaqMan qPCR assay previously published by Gusberti et al. (2012).

The quantification cycle (Cq) values (Bustin et al. 2009) generated by qPCR were compared with the standard curve to quantify the

DNA of *V. inaequalis* in target samples. The approximate number of cells was calculated by dividing the DNA quantity by the weight of the genome of *V. inaequalis* (0.0000597 ng) (Deng et al. 2017), thereby obtaining the number of target cells (*V. inaequalis*) per microliter of reaction, using TaqMan Universal Mastermix 2x (Applied Biosystems).

Specificity, sensitivity, selectivity, repeatability, and reproducibility of the TaqMan qPCR assay. The TaqMan qPCR assay was validated according to the international EPPO standard PM 7/98 (EPPO 2014). In order to evaluate the specificity of the assay for *V. inaequalis*, 20 different species, including other pathogenic

Table 1. Strain ID, host, source of isolation, and origin of the fungal species used in this study to develop the TaqMan quantitative PCR assay

Species	Strain ID	Host	Source	Origin
<i>Venturia inaequalis</i>	1b1	<i>Malus × domestica</i> ‘Ambrosia’	Leaf	Italy
<i>V. inaequalis</i>	1b5	<i>Malus × domestica</i> ‘Ambrosia’	Leaf	Italy
<i>V. inaequalis</i>	1b10	<i>Malus × domestica</i> ‘Ambrosia’	Leaf	Italy
<i>V. inaequalis</i>	1b7	<i>Malus × domestica</i> ‘Ambrosia’	Leaf	Italy
<i>V. inaequalis</i>	1b6	<i>Malus × domestica</i> ‘Ambrosia’	Leaf	Italy
<i>V. inaequalis</i>	116_c2	<i>Malus × domestica</i> ‘Ambrosia’	Leaf	Italy
<i>V. inaequalis</i>	216_b4	<i>Malus × domestica</i> ‘Golden Delicious’	Leaf	Italy
<i>V. inaequalis</i>	3b6	<i>Malus × domestica</i> ‘Ambrosia’	Leaf	Italy
<i>V. inaequalis</i>	3b2	<i>Malus × domestica</i> ‘Ambrosia’	Leaf	Italy
<i>V. inaequalis</i>	3b5	<i>Malus × domestica</i> ‘Ambrosia’	Leaf	Italy
<i>V. inaequalis</i>	516_6	<i>Malus × domestica</i> ‘Ambrosia’	Leaf	Italy
<i>V. inaequalis</i>	516_2	<i>Malus × domestica</i> ‘Ambrosia’	Leaf	Italy
<i>V. inaequalis</i>	516_3	<i>Malus × domestica</i> ‘Ambrosia’	Leaf	Italy
<i>V. inaequalis</i>	3aa	<i>Malus × domestica</i> ‘Golden Delicious’	Leaf	Italy
<i>V. inaequalis</i>	3a1	<i>Malus × domestica</i> ‘Golden Delicious’	Leaf	Italy
<i>V. inaequalis</i>	3ac	<i>Malus × domestica</i> ‘Golden Delicious’	Leaf	Italy
<i>V. inaequalis</i>	416_a12	<i>Malus × domestica</i> ‘Mondial Gala’	Leaf	Italy
<i>V. inaequalis</i>	416_a5	<i>Malus × domestica</i> ‘Mondial Gala’	Leaf	Italy
<i>V. inaequalis</i>	416_a9	<i>Malus × domestica</i> ‘Mondial Gala’	Leaf	Italy
<i>V. inaequalis</i>	516_h	<i>Malus × domestica</i> ‘Ambrosia’	Leaf	Italy
<i>V. inaequalis</i>	516_4	<i>Malus × domestica</i> ‘Ambrosia’	Leaf	Italy
<i>V. inaequalis</i>	516_5	<i>Malus × domestica</i> ‘Ambrosia’	Leaf	Italy
<i>V. inaequalis</i>	3af	<i>Malus × domestica</i> ‘Golden Delicious’	Leaf	Italy
<i>V. inaequalis</i>	416_b2	<i>Malus × domestica</i> ‘Golden Delicious’	Leaf	Italy
<i>V. inaequalis</i>	416_a1	<i>Malus × domestica</i> ‘Mondial Gala’	Leaf	Italy
<i>V. inaequalis</i>	516_10	<i>Malus × domestica</i> ‘Ambrosia’	Leaf	Italy
<i>V. inaequalis</i>	1b2	<i>Malus × domestica</i> ‘Ambrosia’	Leaf	Italy
<i>V. inaequalis</i>	416_a13	<i>Malus × domestica</i> ‘Mondial Gala’	Leaf	Italy
<i>V. inaequalis</i>	1b9	<i>Malus × domestica</i> ‘Ambrosia’	Leaf	Italy
<i>V. inaequalis</i>	3b3	<i>Malus × domestica</i> ‘Ambrosia’	Leaf	Italy
<i>V. inaequalis</i>	3b4	<i>Malus × domestica</i> ‘Ambrosia’	Leaf	Italy
<i>V. inaequalis</i>	3b10	<i>Malus × domestica</i> ‘Ambrosia’	Leaf	Italy
<i>V. inaequalis</i>	1b14	<i>Malus × domestica</i> ‘Ambrosia’	Leaf	Italy
<i>V. inaequalis</i>	416_a3	<i>Malus × domestica</i> ‘Mondial Gala’	Leaf	Italy
<i>V. inaequalis</i>	1b13	<i>Malus × domestica</i> ‘Ambrosia’	Leaf	Italy
<i>V. inaequalis</i>	CBS 815.69	<i>Malus sylvestris</i>	Fruit	The Netherlands
<i>V. cerasi</i>	CBS 444.54	<i>Prunus cerasus</i>	Unknown	Germany
<i>V. pirina</i>	CBS 120.825	<i>Pyrus communis</i>	Unknown	Brazil
<i>V. nashicola</i>	CBS 794.84	<i>Pyrus serotina</i> var. <i>Culta</i>	Unknown	Japan
<i>V. asperata</i>	IRHS 2345	<i>Malus × domestica</i>	Fruit	France
<i>Monilia fructicola</i>	MON1	<i>Malus × domestica</i>	Leaf	Italy
<i>Monilia laxa</i>	LAXA3	<i>Prunus persica</i>	Fruit	Italy
<i>Botryosphaeria dothidea</i>	BOTRYO1	<i>Malus × domestica</i>	Leaf	Italy
<i>Sclerotinia sclerotiorum</i>	SCLA2	Unknown	Unknown	Italy
<i>Colletotrichum fioriniae</i>	COLLRIV	<i>Malus × domestica</i>	Fruit	Italy
<i>Epicoccum nigrum</i>	EPI2	<i>Malus × domestica</i>	Leaf	Italy
<i>Stemphylium</i> sp.	STEM	<i>Pyrus communis</i>	Fruit	Italy
<i>Cladosporium cladosporioides</i>	CLAD1	<i>Malus × domestica</i>	Leaf	Italy
<i>Alternaria mali</i>	CBS 106.24	<i>Malus sylvestris</i>	Unknown	U.S.A.
<i>Alternaria</i> sp.	MALT1	<i>Malus × domestica</i>	Leaf	Italy
<i>Botrytis cinerea</i>	BOT1	<i>Malus × domestica</i>	Leaf	Italy
<i>Phoma</i> sp.	PH5	<i>Malus × domestica</i>	Leaf	Italy
<i>Fusarium equiseti</i>	3FEQS	Unknown	Unknown	Italy
<i>Penicillium expansum</i>	PEX16	<i>Malus × domestica</i>	Leaf	Italy
<i>Ramularia mali</i>	RM2	<i>Malus × domestica</i>	Fruit	Italy

Venturia species that affect different hosts and *V. asperata*, which has recently been reported in Italy on apples, were used (Table 1). The presence of aspecific amplification signals was evaluated after 40 cycles in different experiments.

To determine the sensitivity of the TaqMan qPCR assay, a standard calibration curve was obtained with *V. inaequalis* DNA using the 1b14 strain eightfold serially diluted (from 20 ng to 2 fg) in sterile deionized water. Furthermore, to verify the influence of the host DNA on *V. inaequalis* amplification, the pathogen DNA was eightfold diluted in *Malus × domestica* cultivars Ambrosia, Mondial Gala, and Golden Delicious DNA (host DNA concentration of 10 ng/μl). The standard curve reaction was carried out in triplicate and was used as an internal control in order to quantify the target DNA in different samples. Repeatability was checked by running three independent assays of the test. Two different operators tested the reproducibility of the assay in two different laboratories and on different days.

Detection of *V. inaequalis* in naturally infected *Malus × domestica* leaves and in apple fruit. During the 2015 through 2017 seasons, leaves from *Malus × domestica* plants showing symptoms of apple scab were harvested and stored for DNA extraction. A total of 29 samples were collected (11 from Ambrosia, 11 from Golden Delicious, and seven from Mondial Gala leaves). Furthermore, four Golden Delicious apples that had been stored for 2 months and which showed apple scab symptoms were selected for DNA extraction. DNA from the infected leaves and fruits were extracted from approximately 100 mg fresh weight of leaves or fruit. The samples were previously freeze-dried, were round with liquid nitrogen, and were then extracted with E.Z.N.A. Plant DNA kit (VWR), following manufacturer instructions.

Detection of *V. inaequalis* in asymptomatic *Malus × domestica* leaves. A total of 30 samples of asymptomatic Ambrosia, Florina, and Golden Delicious leaves were analyzed in May of 2018 to assess the presence of *V. inaequalis*, using the TaqMan qPCR assay. The leaves were divided into two equal parts, using sterile blades, to obtain the 100 mg needed for the DNA extraction. The DNA extraction was performed as previously described.

Detection of *V. inaequalis* in artificially inoculated *Malus × domestica* leaves. *V. inaequalis* conidia were obtained from a single spore strain (1b14), according to Parker et al. (1995). Leaves were obtained from *Malus × domestica* 'Florina' plants. The absence of *V. inaequalis* in the plant material was confirmed using a binocular microscope (Nikon Eclipse 55i; Tokyo). Leaf disks (1 cm diameter) were collected for the assay, were disinfected with a 10% solution of sodium hypochlorite, were washed by immersion in sterile deionized water, and were air-dried. The inoculation of the leaf disks was performed on the adaxial leaf surface with an initial concentration of 10² conidia per milliliter of *V. inaequalis* (three drops of 5 μl each),

which was subsequently serially diluted (1:2, 1:3, 1:5, 1:10, 1:15, 1:20, 1:50, and 1:100). Control leaves were prepared in a similar way with sterile deionized water. Two replicates were tested for each conidial concentration. DNA was extracted from artificially inoculated leaves 60 min after inoculation, following the procedures previously described in the paragraph about detection of *V. inaequalis* in naturally infected *Malus × domestica* leaves and apple fruits.

Detection of *V. inaequalis* from spore trap samples. A volumetric spore sampler (Burkard Manufacturing Co. Ltd., Rickmansworth, Hertfordshire, UK), placed in an apple orchard in Manta (Cuneo, Italy), was used to detect the airborne conidia of *V. inaequalis*. The tapes were collected at 24-h intervals for 14 days (from May 1 to May 14, 2018). Each daily tape was first visualized under an optical microscope to count the *V. inaequalis* cells at 40× magnification. The same segments were subsequently placed in 50-ml tubes and were stored at 4°C until processing. An aliquot of 25 ml of a polyethylene glycol (PEG) alkaline buffer (50 g of PEG average M_n 4600 per liter, 20 mM KOH, pH 13.5) was added to each tube and was vortexed for 20 min for the DNA extraction. After incubating for 1 h at 65°C, the samples were vortexed for 20 min and were centrifuged for 30 min at 8,000 × g. The supernatant was collected and the DNA was extracted using an E.Z.N.A. Fungal DNA Mini kit, according to manufacturer protocol, by adjusting the buffer volumes. Repeatability of the assay on spore trap samples was evaluated by performing six independent reactions using the same set of samples.

Data analysis. StepOne software was used to automatically generate the baseline range and the qPCR standard curves, as well as to determine the C_q values. Student's *t* test was used to analyze the reproducibility of the assay.

Results

TaqMan qPCR assay optimization. The *EF1-α* gene was selected because of the presence of a conserved region and the deletion of 31 nucleotides in the *V. inaequalis* species, enabling differentiation from the other *Venturia* species, including *V. asperata*. The alignment of partial *EF1-α* gene sequences from different *V. inaequalis* strains and five other *Venturia* species was used to design the primers and hydrolysis probes reported in Table 2. The F1/R11 primer pair, which gave the best results with conventional endpoint PCR, by amplifying only *V. inaequalis* strains without any non-specific bands on agarose gel electrophoresis, was selected for the real-time assays. The optimization of the TaqMan qPCR assays was tested using different primer and hydrolysis probe concentrations and considering different cycling conditions. The best conditions were found with a primer concentration of 3 μM and an annealing temperature of 57°C for 1 min. The Ven1 hydrolysis probe and F1/R11 primer pair were selected for the TaqMan qPCR assay, as

Table 2. Primer pairs and hydrolysis probes, designed on the *EF1-α* gene used for the detection of *Venturia inaequalis* with the TaqMan quantitative PCR assay

Primers and hydrolysis probes	Amplicon (bp)	Sequence (5'–3')
F 3.2/R11	191 bp	ACCCGGATTTCATTTTCGAAACT GCAATCGTTAGCATCGTCATAGTG
F 4.4/R11	154 bp	TTTTGCACTGTGGCAGCCC GCAATCGTTAGCATCGTCATAGTG
F1/R11	86 bp	CACTTCCCCGCTATTACAGT GCAATCGTTAGCATCGTCATAGTG
F 2.3/R6	127 bp	TTGCCCTCCAAAATTACAGT GGCGGCTTCCTATTGCAATC
F1/R6	100 bp	CACTTCCCCGCTATTACAGT GGCGGCTTCCTATTGCAATC
F 4.3/R6	171 bp	AAATTTTGCACTGTGGCAGC GGCGGCTTCCTATTGCAATC
F 2.2/R6	127 bp	TTGCCCTCCAAAATTACAGT GGCGGCTTCCTATTGCAATC
Vina	–	[FAM]–AGCCCAACTT TCTCCGGTCC–[NF-MGB]
Ven1	–	[FAM]–CTCAAGGCAGCCCAACTTTCTCCGGT–[BHQ1]

they showed the most suitable amplification, using TaqMan Universal Mastermix 2× (Applied Biosystems).

Specificity, sensitivity, selectivity, repeatability, and reproducibility of the TaqMan qPCR assay. The TaqMan qPCR assay, with

the selected primer pair and hydrolysis probe, was able to amplify the 86-bp fragment of the *EFL-α* gene in different experiments from 35 *V. inaequalis* strains (Supplementary Table S1). No amplification was detected after 40 cycles for *V. asperata*, *V. pirina*, *V. carpophila*,

Fig. 1. Standard curve obtained with genomic DNA of the *Venturia inaequalis* 1b14 strain showing the correlation between the *V. inaequalis* DNA amount (from 20 ng to 20 fg) and the quantification cycle values (the standard deviation values range from 0.04 to 0.22, thus the standard deviation bars are too small to display on the graph). The dilutions were run in triplicate.

Fig. 2. Standard curve obtained with genomic DNA of the *Venturia inaequalis* 1b14 strain diluted in 10 ng of DNA from *Malus × domestica* 'Ambrosia', 'Golden Delicious', and 'Mondial Gala'. The amount of *V. inaequalis* DNA is plotted against quantification cycle values (the standard deviation values range from 0.03 to 0.53, thus the standard deviation bars are too small to display on the graph). The dilutions were run in triplicate.

V. cerasi, *V. naschicola*, or for the other tested species listed in Table 1.

The DNA of *V. inaequalis*, serially diluted from 20 ng to 0.2 fg in sterile distilled water, was used to build a standard curve to evaluate the limit of detection (LOD). The pathogen was quantifiable from 20 ng to 20 fg (Fig. 1), and a LOD C_q, ranging between 36 and 37, was obtained. The LOD of 20 fg is lower than a single genome of *V. inaequalis* (0.0000597 ng [Deng et al. 2017]). The mean value of the regression slope was -3.28, and the mean relative efficiency was between 99 and 110%. No influence was observed on the selectivity of the TaqMan qPCR assay when *V. inaequalis* DNA serially diluted in Ambrosia, Golden Delicious, and Mondial Gala DNA was used (Fig. 2). The amplifications showed similar PCR efficiencies and a reliable correlation between the C_q values and the amount of measured *V. inaequalis* DNA (Fig. 2), showing, also, the lack of non-specific amplification of the DNA of three cultivars of apple. No statistical differences ($P > 0.05$) were found for the results of the student's *t* test, which was used to analyze the repeatability and reproducibility of the assay.

Specificity and sensitivity comparison of the SYBR Green and TaqMan qPCR assays. The SYBR Green assay using the selected F1/R11 primer pair provided positive results for the target DNA from different cultivars, and no amplification was observed with other *Venturia* species after 40 cycles. However, aspecific signals

were detected for some species used as negative controls, i.e., *Cladosporium cladosporioides*, *Colletotrichum fiorinae*, *Fusarium equiseti*, *Penicillium expansum*, and *Phoma* sp., after 34 to 36 cycles. These species often showed double peaks in the melt analysis, with one peak at 79.17°C, similar to *V. inaequalis* positive control, and one peak with lower melting temperature (67.87°C). The sensitivity of the assay with SYBR Green revealed a 10-fold higher LOD (200 fg) than the TaqMan qPCR assay designed on the *EF1-α* gene.

The TaqMan qPCR assay previously developed by Gusberti et al. (2012) gave a cross-reaction for the strain MALT1 (identified as *Alternaria arborescens* species complex, based on the ITS, OPA 10-2, and endopolygalacturonase sequences) isolated from apple leaves (C_q after 35 to 36 cycles), while no amplification was obtained with other tested species. Sensitivity, assessed using the method by Gusberti et al. (2012) on the *V. inaequalis* 1b14 strain, showed a LOD of 100 fg (Supplementary Table S2; Supplementary Fig. S2).

Detection of *V. inaequalis* in naturally infected *Malus × domestica* leaves and fruit. The TaqMan qPCR assay was used to quantify *V. inaequalis* in naturally infected leaves of three *Malus × domestica* cultivars and apple fruit. No influence of the host DNA was detected, and all the analyzed samples resulted positive for the target amplification (Fig. 3A; Supplementary Table S3). The average number of cells per microliter was 5.26×10^4 for the Ambrosia

Fig. 3. Detection of *Venturia inaequalis* with the TaqMan quantitative PCR assay on **A**, naturally infected leaves of *Malus × domestica* 'Ambrosia', 'Golden Delicious', and 'Mondial Gala' and Golden Delicious fruits, and **B**, asymptomatic leaves of *Malus × domestica* 'Ambrosia', 'Golden Delicious', and 'Florina'. The values are expressed as the mean ± standard deviation ($n = 9$). The sample ID represents leaves derived from different trees.

samples, 4.28×10^4 for the Mondial Gala samples, and 3.76×10^4 for the Golden Delicious samples. The assay was able to detect 2.83 *V. inaequalis* cells per microliter in the naturally infected leaves (mean Cq 34.35) and 182 cells per microliter in the naturally infected fruit (mean Cq 28.38) (Fig. 3A; Supplementary Table S3).

Detection of *V. inaequalis* in asymptomatic *Malus × domestica* leaves. The TaqMan qPCR assay was able to detect *V. inaequalis* in both the asymptomatic resistant cultivar Florina and susceptible cultivars Ambrosia and Golden Delicious (Fig. 3B; Supplementary Table S4). The lowest concentration of 0.02×10^2 cells per microliter was found in the As9 sample (resistant Florina), while the highest concentration of 4.50×10^3 cells per microliter was found in the susceptible Ambrosia sample (As5). The mean *V. inaequalis* concentration with Florina, Golden Delicious, and Ambrosia samples was 1.90×10^2 , 3.46×10^2 , and 1.91×10^3 cells per microliter, respectively.

Detection of *V. inaequalis* in artificially inoculated *Malus × domestica* leaves. The developed TaqMan qPCR assay was further tested on *Malus × domestica* leaves artificially inoculated with a *V. inaequalis* conidial suspension. The results obtained for the quantification of *V. inaequalis* are reported in Figure 4. All the samples amplified with a clear amplification signal, with Cq ranging from 28 to 36 for samples inoculated with the highest and lowest conidial concentrations, respectively. No amplification was obtained for the negative controls. The amplification showed linearity in the serial dilutions, and the assay allowed us to quantify from 1.65×10^2 cells per microliter, for the initial sample, to 1.21 cells per microliter, for the 100-fold diluted sample.

Detection of *V. inaequalis* from spore trap samples. The TaqMan qPCR assay was used to detect and quantify the presence of airborne inoculum of *V. inaequalis* in spore trap samples. The estimated conidial concentrations, based on microscope counts, were higher

Fig. 4. Detection of *Venturia inaequalis* with the TaqMan quantitative PCR assay on *Malus × domestica* 'Ambrosia' leaf disks artificially inoculated at different concentrations. The values are expressed as the mean ± standard deviation ($n = 9$).

Table 3. Number of *Venturia inaequalis* cells estimated using the optical microscope compared with the number of cells estimated with the TaqMan qPCR assay for the analyses of spore trap samples, and repeatability of the assay, expressed as the number of positive amplifications in six different reactions

Sample	Estimated number of cells (optical microscope)	Estimated number of cells (TaqMan qPCR assay)	Number of positive results
ST1	2.73×10^4	5.49×10^3	6/6
ST2	5.21×10^4	6.88×10^3	6/6
ST3	1.31×10^4	1.12×10^4	5/6
ST4	7.94×10^3	4.42×10^3	6/6
ST9	1.30×10^3	2.72×10^3	3/6
ST10	2.05×10^3	4.80×10^3	3/6
ST10/2 ^a	4.67×10^2	—	—
ST11	8.92×10^2	1.66×10^3	2/6
ST12	8.51×10^3	9.34×10^3	1/6
ST13	1.37×10^3	2.58×10^3	4/6
ST17	2.83×10^3	5.14×10^3	2/6
ST21	1.09×10^4	7.93×10^3	2/6
ST27	1.36×10^3	3.70×10^3	2/6
ST28	2.21×10^3	2.52×10^3	2/6

^a Extraction failed for the ST10/2 sample.

than the estimated concentrations calculated from the amount of *V. inaequalis* DNA detected by the TaqMan qPCR assay. Only one of the 14 analyzed samples showed no amplification (Table 3). The estimated mean number of cells per microliter obtained from the microscope count was 1.01×10^4 , while it was 5.26×10^3 for the TaqMan qPCR assay. The correlation coefficient between the two assays was positive, with an R^2 value of 0.8186. The repeatability of the assay was variable, with at least one positive amplification per sample over six reactions (Table 3).

Discussion

The molecular techniques currently applied for the detection of plant pathogens are often used to specifically identify and quantify fungal species in crops and food commodities (Aslam et al. 2017; Capote et al. 2012; Postollec et al. 2011). In this study, a highly sensitive TaqMan qPCR assay has been developed for the specific detection and quantification of *V. inaequalis*, and it has successfully been used with symptomatic and asymptomatic leaves, fruit, and spore trap samples.

End-point PCR permits specific detection of *Venturia* species (Koh et al. 2013; Schnabel et al. 1999) without sample quantification (Suarez et al. 2005), while qPCR assays are more specific, sensitive, and allow pathogen quantification (Baskarathevan et al. 2016; Kuzdraliński et al. 2017; Mirmajlessi et al. 2015; Selma et al. 2008). Real-time PCR was used in previous studies to detect and quantify *V. inaequalis* in different types of samples, using both SYBR Green I and the TaqMan chemistries.

In preliminary studies, different target sequences were explored for the development of the TaqMan qPCR assay, following multiple sequence alignments (data not shown). The sequences were obtained from strains isolated in Italy and from sequences available in the GenBank public database. Due to the high intraspecific variability, the ITS region and the beta-tubulin gene were discarded (data not shown), whereas the *EF1-α* gene proved a highly conserved species-specific region for *V. inaequalis*, and it was, therefore, selected to design primers and hydrolysis probes.

SYBR Green I technology, with the same primers used to develop our TaqMan qPCR assay, and the TaqMan qPCR assay previously published by Gusberti et al. (2012) were used to compare the developed assay. By using SYBR Green I, there was a lack of specificity of the designed primers with various tested species, as described by Martínez et al. (2011). Cross-reaction was also observed in the work by Meitz-Hopkins et al. (2014), where two qPCR assays were developed, based on the CYP51A1 gene and the ITS region, amplifying *A. alternata*, which was used as negative control, while, in the study by Daniëls et al. (2012), using a qPCR assay based on the ITS region, cross-reactions with different species (*V. asperata*, *V. nashicola*, *V. pirina*, *V. cerasi* and *V. carpophila*) are shown. With the use of SYBR Green, the sensitivity was 200 fg, five times lower than the detection limit obtained by Meitz-Hopkins et al. (2014) but two times higher than the sensitivity reported by Daniëls et al. (2012).

When the specificity was tested using hydrolysis probe technology, no cross-reaction was detected with other *Venturia* species, including *V. asperata*, or for other pathogens. On the contrary, when the specificity was tested with the hydrolysis probe developed by Gusberti et al. (2012), a positive amplification was obtained for *Alternaria arborescens* species complex from apple leaves, confirming the preliminary result of the blast search for the primers (100% homology with *A. solani* and *A. alternata*).

Because of the high intraspecific variability that exists within the species *V. inaequalis* (Ebrahimi et al. 2016; Tenzer and Gessler 1999; Tenzer et al. 1999), the specificity of the assay was confirmed on DNA extracted from a large number of pure cultures of *V. inaequalis* strains, isolated from different cultivars over different years, and positive amplifications were observed for all the strains. The assay showed a low variation in the Cq values obtained in independent experiments and results were highly reproducible.

The Taqman qPCR assay developed in this study was more sensitive than the SYBR Green assay. The advantage of using the TaqMan qPCR assay has been reported in a qPCR comparative study by Soltany-Rezaee-Rad et al. (2015). A qPCR with SYBR Green could inhibit

the Taq DNA polymerase, thereby reducing the sensitivity of the assay (Kermekchiev et al. 2009). The LOD of our TaqMan qPCR assay (20 fg) proved to be more sensitive than that reported by Gusberti et al. (2012), for which the LOD was 100 fg. The LOD of the TaqMan qPCR assay (20 fg) is similar to those reported for other species, i.e., *Botrytis cinerea* (Suarez et al. 2005), *Fusarium solani* (Bernal-Martínez et al. 2012), *Aspergillus fumigatus* (Fernandez-Molina et al. 2014), *Lichtheimia corymbifera* (Springer et al. 2016), *Fusarium fujikuroi* (Amaral Carneiro et al. 2017), and *Fusarium culmorum* (Bilska et al. 2018).

The TaqMan qPCR assay was also tested to quantify the target DNA in the presence of the plant material. The sensitivity was not affected by the presence of the DNA of *Malus × domestica* from different cultivars, and the assay allowed us to detect and quantify *V. inaequalis* in inoculated samples with significant linearity. In our study, positive amplifications were obtained from asymptomatic leaves collected from susceptible cultivars, though *V. inaequalis* was detected in the resistant Florina cultivar at a lower concentration. The pathogen occurrence on the leaf surface of both susceptible and resistant cultivars has been reported by Bus et al. (2011) and Sarkate et al. (2018). In addition, the assay proved to be useful for the detection of the pathogen in fruit samples showing scab symptoms.

When the assay was performed on DNA obtained from the spore trap samples, 13 of 14 samples resulted positive to *V. inaequalis*, even with a low conidial concentration. In our study, the overall concentration estimated with a microscope count was higher than that estimated with the TaqMan qPCR assay, but with a linear relationship ($R^2 = 0.8186$). Similar results were reported by Carisse et al. (2005), pertaining to the quantification of *Botrytis squamosa*. However, variability in repeatability of the assay with spore trap samples seemed to have affected both the presence of false-negatives and amplification efficiency. The reasons for the false negatives could be linked to competition in the amplification due to high amounts of nontarget DNA, inoculum density, or coextraction of contaminants (PCR inhibitors) (Bilodeau et al. 2012; Dung et al. 2015; Klosterman et al. 2014; McDevitt et al. 2007; Rogers et al. 2009; Williams et al. 2001).

In conclusion, the use of a TaqMan qPCR assay proved to be specific and highly sensitive for the detection of *V. inaequalis*, both in symptomatic and asymptomatic apple leaves and in spore trap samples. The developed assay, when combined with weather-based data models and spore traps, could be a useful tool to plan fungicide applications for apple scab management.

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