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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Development and validation of a highly sensitive real-time PCR TaqMan® assay for specific detection and quantification of *Venturia inaequalis* in apple leaves and fruit and in air samples

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Abstract

A TaqMan real-time PCR assay, based on the translation elongation factor 1-α gene, was developed for the quantification of *Venturia inaequalis* in leaves and fruits of *Malus* x *domestica* and in spore trap samples. The designed primers and 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* x *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’, ‘Fiorina’, ‘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 air samples coming 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 and could be a promising tool for timely application of fungicides in orchards and to improve the efficacy of disease management.

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

1. Introduction

Apple scab is a worldwide disease affecting apple cultivation (*Malus* x *domestica*, Bork H.) and it is 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 prophylaxis practice, application of fungicides, and increasing use of resistant cultivars (MacHardy et al., 2001).

V. inaequalis has a hemibiotrophic life cycle. Ascospores, which are released from pseudothecia and form during winter in the fallen leaves, are the sexual reproductive structures of the pathogen, and cause primary infections during the growing season (spring - early summer). If the weather conditions are favorable, asexual conidia are released and cause secondary infections during the growing season (Carrisse et al., 2000, Bowen et al., 2011). The entire life cycle of *V. inaequalis* is strongly influenced by the 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 (Mills and Laplante 1951; Gadoury and MacHardy 1986; Meitz-Hopkins et al., 2014; Carisse et al., 2000). The evaluation of the level of infection is based on a visual scoring, which is an operator-dependent method used to attribute a disease severity index (Bock et al., 2010; Gusberti et al., 2012).

The detection and quantification of pathogens, even in asymptomatic tissues, currently rely on molecular techniques. Different studies have been reported for specific amplification of *V. inaequalis* using conventional PCR (Schnabel et al. 1999; Stehmann et al. 2001, Koh et al., 2013). Daniëls et al. (2012) developed a qPCR assay using the housekeeping genes ATP-binding cassette transporter 2 (ABC2) and the elongation factor (EF1), while Gusberti et al. (2012) developed a qPCR assay, based on primers and probe designed on the internal transcribed spacer (ITS) to detect *V. inaequalis*.

Spore traps were used in combination with qPCR in order to quantify the air-borne inoculum of different plant pathogens (Carisse et al., 2009; Klosterman et al., 2014; Huang et al., 2016), including *V. inaequalis* (Meitz-Hopkins et al., 2014).

To date, no studies have been carried out using the TaqMan technology combined with spore traps. The aim of the current work was to develop and validate a specific TaqMan quantitative PCR (qPCR) assay that could be used to detect and quantify *V. inaequalis* on different *Malus* x *domestica* cultivars, both in the leaves and in the fruits. *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). Furthermore, the assay was tested on spore trap samples in order to detect and quantify the airborne inoculum of *V. inaequalis*.

2. Materials and methods

Fungal strains

Strains of *V. inaequalis* were isolated from leaves of apple tree (*Malus* x *domestica*) 'Ambrosia', 'Golden Delicious' and 'Mondial Gala', cultivated in northern Italy (Piedmont) during 2015 and 2016. Thirty-five isolates were selected for this study. Other apple pathogenic or commonly present in orchard fungal species were isolated from apple leaves. All the isolates were identified through the amplification of the ribosomal DNA internal transcribed spacer (ITS) region, following the protocol of 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 Agroinnova collection were used to verify the assay specificity. The strains used in this study are summarized in Table 1. The stains were maintained as monoconidial cultures in tubes of malt extract agar (MEA) (Sigma Aldrich, Germany) and 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 200 mg of fresh-weight mycelium using an Omega E.Z.N.A. Fungal DNA Mini Kit (VWR, USA), according to the manufacturer's instructions. The DNA quality and the concentration were measured using a Nanodrop 2000 Spectrophotomer (Thermo Scientific, Wilmington, DE, USA).

Sequence analysis of the translation elongation factor gene and design of primers and TaqMan probe

The translation elongation factor 1 alpha (*EF1-α*) sequences were amplified, for the 35 *V. inaequalis* strains, using EF1 (CGAGAAGTTCGAGAAGGT) and EF2 (CCAATGACGGTGACATAG) primers. PCR was carried out in a total volume of 25 µL containing 2.5 µL of Buffer 10 X, 0.5 µL of $MgCl_2$, 0.75 µL of dNTPs (10 mM), 1 µL of each primer (10 mM), 0.2 µL of Taq DNA polymerase (Qiagen, Germany) and 20 ng of template DNA. The thermal cycling program was performed according to Gladieux et al. (2010). After agarose gel electrophoresis, the PCR products were purified using a QIAquick© PCR purification Kit (Qiagen), and sequenced in both directions by Macrogen, Inc. (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 multi alignment was performed using the CLUSTALW algorithm, through Molecular Evolutionary Genetics Analysis (MEGA6) software, version 6.0. The alignment was used to design the primers and probe used in this study.

Six sets of primers and two probes were designed using Primer Express™ software 3.0 (Applied Biosystem, Foster City, USA) and are listed in Table 2. The TaqMan probes were labelled at the 5’-end with the 6-carboxyfluorescein dye (6-FAM) reporter and Black Hole Quencher (BHQ1) or the non-fluorescent quencher minor groove binder (NF-MGB) at the 3’-end. OligoCalc tool (http://biotools.nubic.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 Centre of Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Invitrogen (Carlsbad, USA) and Metabion (Steinkirchen, Germany) synthesized primers and 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 endpoints were performed in a 25 µl reaction, composed of 2 µL of Buffer 10x, 0.8 µL of $MgCl_2$, 1 µL of dNTPs (10 mM), 1 µL of each primer (10 mM), 0.2 µL of Taq Platinum Pfx DNA polymerase (Invitrogen, 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 TBE buffer, and visualized under UV transilluminator using the Quantity One software (BioRad Labs, Hercules, USA). After performing end-point PCR, the primer pairs that gave the best results were selected and used in qPCR with SYBR Green in order to compare them with the specificity and sensitivity of the TaqMan 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 10x (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. In order to determine the sensitivity of the SYBR Green assay, a standard curve was obtained with *V. inaequalis* DNA using 1b14 strain 8-fold serially diluted (from 20 ng to 2 fg).

The TaqMan assay was performed using TaqMan Universal Mastermix 2X (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 µM 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, 0.4 µl of each primer (3 µM) and 0.2 µl of 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 assay was compared, in terms of sensitivity and specificity, with the TaqMan assay previously published by Gusberti et al., (2012).

The Ct values 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 µL of reaction.

Specificity, sensitivity, selectivity, repeatability and reproducibility of the TaqMan qPCR

The TaqMan 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 *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.

In order to determine the sensitivity of the TaqMan assay, a standard calibration curve was obtained with *V. inaequalis* DNA using the 1b14 strain 8-fold serially diluted (from 20 ng to 2 fg) in sterile deionized water. Furthermore, in order to verify the influence of the host DNA on *V. inaequalis* amplification, the pathogen DNA was 8-fold diluted in *Malus* x *domestica* 'Ambrosia', 'Mondial Gala' and 'Golden Delicious' DNA. The standard curve reaction was carried out in triplicate and 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* x *domestica* leaves and in apple fruit

During the 2015-2017 seasons, leaves from *Malus* x *domesica* plants showing symptoms of apple scab were harvested and stored for DNA extraction. From 'Ambrosia', 'Golden Delicious' and 'Mondial Gala' leaves, respectively 11, 11 and 5 samples were collected. Furthermore, four apples that had been stored for 2 months and which showed apple scab symptoms were selected for DNA extraction. DNA from the infected plants/fruit was extracted from approximately 100 mg of fresh-weight leaves or fruit. The samples were previously freeze-dried, ground with liquid nitrogen and then extracted with E.Z.N.A. Plant DNA kit (VWR, USA), following the manufacturer's instructions.

Detection of *V. inaequalis* in asymptomatic *Malus* x *domestica* leaves

A total of 30 samples of asymptomatic *Malus* x *domestica* 'Ambrosia', 'Fiorina' and 'Golden Delicious' leaves were analyzed in May 2018 to assess the presence of *V. inaequalis* using the TaqMan assay. The leaves were divided into two equal parts using sterile blades and two different extractions were performed (100 mg each). The DNA extraction was performed as previously described.

Detection of *V. inaequalis* in artificially inoculated *Malus* x *domestica* leaves

V. inaequalis conidia were obtained from a single spore strain (1b14), according to Parker et al. (2005). Leaves were obtained from *Malus* x *domestica* 'Fiorina' plants. The absence of *V. inaequalis* in the plant material was confirmed using a binocular microscope (Nikon Eclipse 55i, Tokyo, Japan). Leaf disks (1 cm diameter) were collected for the assay, disinfected with a 10% solution of sodium hypochlorite, washed by immersion in sterile deionized water and air-dried. The inoculation of the leaf disks was performed on the adaxial leaf surface with an initial concentration of 10^2 conidia/mL of *V. inaequalis*, 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, following previously described procedures.

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; 44.609217; 7.502627), was used to detect the airborne conidia of *V. inaequalis*. The tapes were collected at 24 h intervals for 14 days. 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 stored at 4 °C until processing. An aliquot of 25 mL of a polyethylene glycol (PEG) alkaline buffer (50 g/L PEG average Mn 4600; 20 mM KOH; pH 13.5) was added to each tube and vortexed for 20 minutes for the DNA extraction. After incubation of 1 h at 65 °C, the samples were vortexed for 20 min and 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 the manufacturer's protocol, by adjusting the buffer volumes.

Repeatability of the assay on air samples was evaluated by performing 6 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 Ct values. Student's t-test was used to analyze the reproducibility of the assay.

3. Results

TaqMan 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, able to differentiate from the other *Venturia* species, including *V. asperata*. The alignment of partial *EF1-α* gene sequences from different *V. inaequalis* strains and 5 other *Venturia* species was used to design the primers and probes reported in Table 2. The F1/R11 primer pair, which gave the best results with conventional end-point PCR, was selected for the real-time assays. The optimization of the TaqMan qPCR assay was tested using different primer and probe concentrations and considering different cycling conditions. The best conditions were found for a primer concentration of 3 µM at 57 °C for 1:00 for the annealing stage. The Ven1 probe and F1/R11 primer pair were selected for the TaqMan assay as they showed the most suitable amplification.

Specificity, sensitivity, selectivity, repeatability and reproducibility of the TaqMan qPCR

The TaqMan qPCR, with the designed primer pair and probe, was able to amplify the 86 bp fragment of the *EF1-α* gene in different experiments from 35 *V. inaequalis* strains (ST1). No amplification signal was detected after 40 cycles for *V. asperata, V. pirina, V. carpophila, V. cerasi, V. naschicola* or for the other tested species.

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 in order to evaluate the limit of detection (LOD). The pathogen was quantifiable from 20 ng to 20 fg (Fig. 1), and a LOD threshold cycle (Ct), 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 assay when *V. inaequalis* DNA, serially diluted in *Malus* x *domestica* 'Ambrosia', 'Golden Delicious' and 'Mondial Gala' DNA, was used (Fig. 2). The amplifications showed similar PCR efficiencies and a reliable correlation between the Ct values and the amount of measured *V. inaequalis* DNA (Fig. 2). 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 assays

The SYBR Green assay by using the selected F1/R11 primer pair provided positive results for the target DNA from different cultivars, and no amplification was observed for the other *Venturia* species after 40 cycles. However, aspecific signals were detected for some species used as negative controls, i.e. *Cladosporium cladosporioides*, *Colletotrichum fioriniae*, *Fusarium equiseti*, *Penicillium expansum* and *Phoma* sp., after 34 to 36 cycles. The sensitivity of the assay with SYBR Green revealed a 10-fold higher LOD (200 fg) than the TaqMan assay designed on the *EF1-α* gene.

The TaqMan assay previously developed by Gusberti et al. (2012) gave a cross-reaction for the strain MALT1 *Alternaria* sp. isolated from apple leaves, while no other amplification was obtained for the other tested species. Sensitivity, assessed using the method of Gusberti et al. (2012) on the *V. inaequalis* 1b14 strain, showed a LOD of 100 fg (ST2).

Detection of *V. inaequalis* in naturally infected *Malus* x *domestica* leaves and fruit

The TaqMan assay was used to quantify *V. inaequalis* in naturally infected leaves of three *Malus* x *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 and ST3). The average number of cells/µL was 5.26×10^4 for the 'Ambrosia' 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/µL in the naturally infected leaves (mean Ct 34.35) and 182 cells/µL in the naturally infected fruit (mean Ct 28.38) (Fig. 3A and ST3).

Detection of *V. inaequalis* in asymptomatic *Malus* x *domestica* leaves

The TaqMan assay was able to detect *V. inaequalis* in both the asymptomatic resistant and susceptible cultivars (Fig. 3B and ST4). The lowest concentration was 3.21 cells/µL and it was found in the As7 sample (resistant 'Fiorina'), while the highest concentration was found in the susceptible 'Ambrosia' samples, with 4.50×10^3 cells/µL. The mean *V. inaequalis* concentration was 1.90×10^2 cells/µL for 'Fiorina', while 3.46×10^2 cells/µL were found for 'Golden Delicious' and 1.91×10^3 cells/µL for 'Ambrosia'.

Detection of *V. inaequalis* in artificially inoculated *Malus* x *domestica* leaves

In order to test the developed TaqMan assay on environmental-like samples, *Malus* x *domestica* leaves were 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 Ct values ranging from 28, for the samples inoculated with highest concentration, to 36 for the lowest. 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 x 10^2 cells/µL, for the initial sample, to 1.21 cells/µL, for the 100-fold diluted sample.

Detection of *V. inaequalis* from spore trap samples

The TaqMan assay was used to detect and quantify the presence of airborne inoculum of *V. inaequalis* in the spore trap samples. The estimated conidial concentrations, based on microscope counts, were higher than the estimated concentrations calculated from the amount of *V. inaequalis* DNA detected by the TaqMan assay. Only one of the 14 analyzed samples showed no amplification (Table 3). The estimated mean number of cells/µL obtained from the microscope count was 1.01 x 10^4, while it was 5.26 x 10^3 for the TaqMan 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).

4. 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 (Postollec et al., 2001; Capote et al., 2012; Aslam et al., 2017). In this study, a highly sensitive TaqMan real-time 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 air samples.

Conventional molecular methods, based on PCR, showed specificity when used for the detection of *Venturia* species (Schnabel et al., 1999;, Koh et al., 2013), but there is a lack of effective quantitative results (Suarez et al., 2005). Real-time PCR gives more specific and sensitive results than conventional PCR and allows pathogens to be quantified (Selma et al., 2008; Mirmajlessi et al., 2015; Baskarathevan et al., 2016; Kuzdraliński et al., 2017). Real-time PCR was used in previous studies to detect and quantify *V. inaequalis* in different types of samples. Both SYBR Green I and the TaqMan assay were utilized. The first technique exploited a fluorescent measurement for DNA amplification through double strand DNA binding dyes (SYBR Green I®), although there were some limits pertaining to aspecific amplifications and difficulties in the interpretation of the results after the melting curve (Martinez et al., 2011). These issues could be solved by using the second technique, i.e. TaqMan chemistry, which includes specific fluorescent probes into the PCR (Amaral Carneiro et al., 2017).

In preliminary studies, different target sequences were explored for the development of the TaqMan real-time assay, following multiple sequence alignments (data not shown). The used 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 probes.

The developed assay was compared using SYBR Green I and with the TaqMan assay, previously published (Gusberti et al., 2012). The primers designed on the *EF1-α* gene cross-reacted with various tested species when used with SYBR Green I. Cross-reaction was also observed in the work of Meitz-Hopkins et al. (2014), where two qPCR assays, based on the CYP51A1 gene and the ITS region, were developed. The study of Daniëls et al. (2012), using a qPCR assay based on the ITS region, also showed cross-reaction. The main fungal species that showed cross-amplification with the aforementioned molecular tools were *Alternaria* sp. and *V. asperata*, *V. nascicola*, *V. pirina*, *V, cerasi* and *V. carpophila*. Our data demonstrated that the use of SYBR Green allowed to reach a sensitivity of 200 fg, which in turn results in a 5 times lower than the detection limit obtained by Meitz-Hopkins et al. (2014), but 2 times higher than that reported by Daniëls et al. (2012).

When the specificity was tested using the TaqMan probe technology, no cross-reaction was detected for the other *Venturia* species, including *V. asperata*, or for other pathogens. On the contrary, when the specificity was tested with the TaqMan probe developed by Gusberti et al. (2012), a positive amplification was obtained for a strain of *Alternaria* sp. from apple leaves. A blast search of the primers and probes used in the study of Gusberti et al. (2012) gave 100% homology and 100% coverage with *A. solani* (accession number CPO22033.1) and 100% homology and 72% coverage with *A. alternata* (accession number XM_01852723.1).

Because of the high intraspecific variability that exists within the species *V. inaequalis* (Tenzer and Gessler, 1999 ; Tenzer et al., 1999; Ebrahimi et al., 2016), 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 Ct values obtained in independent experiments and resulted to be highly reproducible.

When the sensitivity of the assays was compared, the TaqMan assay resulted in a lower detection limit than the SYBR Green assay. The advantage of using the TaqMan 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 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 assay (20 fg) is similar to those reported for other species i.e. *Botrytis cinerea* (Suarez et al., 2005), *Fusarium solani* (Bernal-Martìnez 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 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* x *domestica* from different cultivars, and the assay allowed to detect and quantify *V. inaequalis* in inoculated samples with significant linearity. Positive amplifications were also obtained from leaf samples, collected both from susceptible and resistant cultivars, that were, respectively, symptomatic or asymptomatic. In addition, the assay proved to be useful for the detection of the pathogen in fruit samples.

When the assay was performed on DNA obtained from the spore trap, 13 samples out of 14 resulted positive to *V. inaequalis*, even with a low conidial concentration. In our study, the overall concentration estimated with a microscope count resulted higher than that estimated with the TaqMan assay, but with a linear relationship (R^2: 0.8186), as previously reported by Carisse et al. (2009), pertaining to the quantification of *Botrytis squamosa*. However, the repeatability of the assay was variable for air samples, as inhibition of PCR may occur when DNA is extracted from air samples, thus false-negative plate readings and a reduced amplification efficiency may be obtained. The reasons for these false negatives could be linked to competition in the amplification due to: high amounts of non-target DNA, inoculum density, co-extraction of contaminants (PCR inhibitors) or unequal distribution of conidia on tapes (McDevitt et al., 2007; Rogers et al., 2009; Williams et al. 2001; Bilodeau et al 2012; Klosterman et al., 2014; Dung et al., 2015).

In conclusion, the development of a highly sensitive species-specific assay is important to detect pathogen at low concentrations, even during latent infection or in asymptomatic samples. In our study, the use of a TaqMan real-time assay increased the sensitivity of the molecular tool and led to the advantage of being able to detect less than a single target cell. The assay proved to be specific and highly sensitive for the detection of *V. inaequalis*, both in symptomatic and asymptomatic apple leaves. The developed qPCR could be used in apple scab risk management systems to quantify the inoculum in the field and to plan phytosanitary treatments. Furthermore, the technique proved to be sensitive with air samples, though assessed on a limited number of samples.

This newly developed TaqMan assay could be a useful tool, combined with a weather-based data model, to plan phytosanitary treatments in order to control apple scab. The here developed TaqMan assay could also be used, together with nanobiosensor technology, to quantify airborne inoculum in the field, without time consuming DNA extraction and processing, and could therefore be used to implement a decision support system for apple scab management.

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Tables

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

Species	Strain ID	Host	Source	Origin
Venturia inaequalis	1b1	*Malus* x *domestica* ‘Ambrosia’	Leaf	Italy
Venturia inaequalis	1b5	*Malus* x *domestica* ‘Ambrosia’	Leaf	Italy
Venturia inaequalis	1b10	*Malus* x *domestica* ‘Ambrosia’	Leaf	Italy
Venturia inaequalis	1b7	*Malus* x *domestica* ‘Ambrosia’	Leaf	Italy
Venturia inaequalis	1b6	*Malus* x *domestica* ‘Ambrosia’	Leaf	Italy
Venturia inaequalis	116_c2	*Malus* x *domestica* ‘Ambrosia’	Leaf	Italy
Venturia inaequalis	216_b4	*Malus* x *domestica* ‘Golden Delicious’	Leaf	Italy
Venturia inaequalis	3b6	*Malus* x *domestica* ‘Ambrosia’	Leaf	Italy
Venturia inaequalis	3b2	*Malus* x *domestica* ‘Ambrosia’	Leaf	Italy
Venturia inaequalis	3b5	*Malus* x *domestica* ‘Ambrosia’	Leaf	Italy
Venturia inaequalis	516_6	*Malus* x *domestica* ‘Ambrosia’	Leaf	Italy
Venturia inaequalis	516_2	*Malus* x *domestica* ‘Ambrosia’	Leaf	Italy
Venturia inaequalis	516_3	*Malus* x *domestica* ‘Ambrosia’	Leaf	Italy
Venturia inaequalis	3aa	*Malus* x *domestica* ‘Golden Delicious’	Leaf	Italy
Venturia inaequalis	3a1	*Malus* x *domestica* ‘Golden Delicious’	Leaf	Italy
Venturia inaequalis	3ac	*Malus* x *domestica* ‘Golden Delicious’	Leaf	Italy
Venturia inaequalis	416_a12	*Malus* x *domestica* ‘Mondial Gala’	Leaf	Italy
Venturia inaequalis	416_a5	*Malus* x *domestica* ‘Mondial Gala’	Leaf	Italy
Venturia inaequalis	416_a9	*Malus* x *domestica* ‘Mondial Gala’	Leaf	Italy
Venturia inaequalis	516_h	*Malus* x *domestica* ‘Ambrosia’	Leaf	Italy
Venturia inaequalis	516_4	*Malus* x *domestica* ‘Ambrosia’	Leaf	Italy
Venturia inaequalis	516_5	*Malus* x *domestica* ‘Ambrosia’	Leaf	Italy
Venturia inaequalis	3af	*Malus* x *domestica* ‘Golden Delicious’	Leaf	Italy
Venturia inaequalis	416_b2	*Malus* x *domestica* ‘Golden Delicious’	Leaf	Italy
Venturia inaequalis	416_a1	*Malus* x *domestica* ‘Mondial Gala’	Leaf	Italy
Venturia inaequalis	516_10	*Malus* x *domestica* ‘Ambrosia’	Leaf	Italy
Venturia inaequalis	1b2	*Malus* x *domestica* ‘Ambrosia’	Leaf	Italy
Venturia inaequalis	416_a13	*Malus* x *domestica* ‘Mondial Gala’	Leaf	Italy
Venturia inaequalis	1b9	*Malus* x *domestica* ‘Ambrosia’	Leaf	Italy
Venturia inaequalis	3b3	*Malus* x *domestica* ‘Ambrosia’	Leaf	Italy
Venturia inaequalis	3b4	*Malus* x *domestica* ‘Ambrosia’	Leaf	Italy
Venturia inaequalis	3b10	*Malus* x *domestica* ‘Ambrosia’	Leaf	Italy
Venturia inaequalis	1b14	*Malus* x *domestica* ‘Ambrosia’	Leaf	Italy
Venturia inaequalis	416_a3	*Malus* x *domestica* ‘Mondial Gala’	Leaf	Italy
Venturia inaequalis	1b13	*Malus* x *domestica* ‘Ambrosia’	Leaf	Italy
Venturia inaequalis	CBS 815.69	*Malus sylvestris*	Fruit	The Netherlands
Venturia cerasi	CBS 444.54	*Prunus cerasus*	Unknown	Germany
Venturia pirina	CBS120.825	*Pyrus communis*	Unknown	Brazil

Species	**Strain ID**	**Host**	**Source**	**Origin**
Venturia nashicola	CBS 794.84	*Pyrus serotina* var Culta	Unknown	Japan
Venturia asperata	IRHS 2345	*Malus* x *domestica*	Fruit	France
Venturia carpophila	CBS 497.62	*Prunus mirabelle*	Unknown	Switzerland
Monilia fructicola	MON1	*Malus* x *domestica*	Leaf	Italy
Monilia laxa	LAXA3	*Prunus persica*	Fruit	Italy
Botryosphaeria dothidea	BOTRYO1	*Malus* x *domestica*	Leaf	Italy
Sclerotinia sclerotiorum	SCLA2	Unknown	Unknown	Italy
Colletotrichum fioriniae	COLLRIV	*Malus* x *domestica*	Fruit	Italy
Epicoccum nigrum	EPI2	*Malus* x *domestica*	Leaf	Italy
Stemphylium sp.	STEM	*Pyrus communis*	Fruit	Italy
Cladosporium cladosporioides	CLAD1	*Malus* x *domestica*	Leaf	Italy
Alternaria mali	CBS 106.24	*Malus* sylvestris	Unknown	The USA
Alternaria sp.	MALT1	*Malus* x *domestica*	Leaf	Italy
Botrytis cinerea	BOT1	*Malus* x *domestica*	Leaf	Italy
Phoma sp.	PH5	*Malus* x *domestica*	Leaf	Italy
Fusarium equiseti	3FEQS	Unknown	Unknown	Italy
Penicillium expansum	PEX16	*Malus* x *domestica*	Leaf	Italy
Ramularia mali	RM2	*Malus* x *domestica*	Fruit	Italy

Table 2. Primer pairs and probes, designed on *EF1-α* gene used for the detection of *V. inaequalis* with the TaqMan real-time assay.

Primers and Probes	**Amplicon (bp)**	**Sequence (5'-3')**
F 3.2/R11	191 bp	ACCCGGATTTCATTTCGAAACT GCAATCGTTAGCATCGTCATAGTG
F 4.4/R11	154 bp	TTTTGCACTGTGGCAGCCC GCAATCGTTAGCATCGTCATAGTG
F 1/R11	86 bp	CACTTCCCCGCTATTCACGT GCAATCGTTAGCATCGTCATAGTG
F2.3/R6	127 bp	TTGCCCCTCCAAAATTACAGTG GGCGGCTTCCTATTGCAATC
F1/R6	100 bp	CACTTCCCCGCTATTCACGT GGCGGCTTCCTATTGCAATC
F4.3/R6	171 bp	AAATTTTGCACTGTGGCAGC GGCGGCTTCCTATTGCAATC
F 2.2/R6	127 bp	TTGCCCCTCCAAAATTACAGT GGCGGCTTCCTATTGCAATC
Vina	-	[FAM] - AGCCCAACTTTCTCCGGTCC - [NF-MGB]
Ven1	-	[FAM] - CTCAAGGCAGCCCAACTTTCTCCGGT - [BHQ1]

Table 3 – Number of *V. inaequalis* cells estimated using the optical microscope compared with the number of cells estimated with the TaqMan real-time 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 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	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

*the extraction failed for the ST10/2 sample.

Captions

Figure 1. Standard curve obtained with genomic DNA of the *V. inaequalis* 1b14 strain showing the correlation between the *V. inaequalis* DNA amount and the Ct 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).

Figure 2. Standard curve obtained with genomic DNA of the *V. inaequalis* 1b14 strain diluted in *Malus x domestica* DNA ‘Ambrosia’, ‘Golden Delicious’ and Mondial Gala. The *V. inaequalis* DNA amount is plotted against the Ct 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).

Figure 3. Detection of *V. inaequalis* with the TaqMan real-time PCR on: **A.** naturally infected leaves of *Malus x domestica* ‘Ambrosia’, ‘Golden Delicious’ and ‘Mondial Gala’; **B.** asymptomatic leaves of *Malus x domestica* ‘Ambrosia’, ‘Golden Delicious’ and ‘Fiorina’. The values are expressed as the mean ±SD (n=9).

Figure 4. Detection of *V. inaequalis* with the TaqMan real-time PCR on *Malus* x *domestica* ‘Ambrosia’ leaf disks artificially inoculated at different concentrations. The values are expressed as the mean ±SD (n=9).

Supplementary Tables

Supplementary Table 1 - Results of the q-PCR amplification (Ct values ± standard deviation) with the target DNA (≅ 20 ng) of different *V. inaequalis* strains used to evaluate the specificity of the TaqMan real-time assay.

Sample ID	Species	Cultivar	Ct Mean ±SD*	
1b1	*V. inaequalis*	Ambrosia	18.57	± 0.04
1b5	*V. inaequalis*	Ambrosia	18.23	± 0.24
1b10	*V. inaequalis*	Ambrosia	17.42	± 0.04
1b7	*V. inaequalis*	Ambrosia	17.13	± 0.23
1b6	*V. inaequalis*	Ambrosia	17.58	± 0.19
116_c2	*V. inaequalis*	Ambrosia	18.12	± 0.30
216_b4	*V. inaequalis*	Ambrosia	17.32	± 0.20
3b6	*V. inaequalis*	Ambrosia	17.14	± 0.20
3b2	*V. inaequalis*	Ambrosia	18.22	± 0.11
3b5	*V. inaequalis*	Ambrosia	17.20	± 0.13
516_6	*V. inaequalis*	Ambrosia	18.35	± 0.30
516_2	*V. inaequalis*	Ambrosia	17.41	± 0.13
516_3	*V. inaequalis*	Ambrosia	17.81	± 0.17
3aa	*V. inaequalis*	Golden Delicious	17.49	± 0.22
3a1	*V. inaequalis*	Golden Delicious	17.79	± 0.03
3ac	*V. inaequalis*	Golden Delicious	17.52	± 0.12
416_a12	*V. inaequalis*	Mondial Gala	18.49	± 0.10
416_a5	*V. inaequalis*	Mondial Gala	17.92	± 0.31
416_a9	*V. inaequalis*	Mondial Gala	18.29	± 0.30
516_h	*V. inaequalis*	Ambrosia	17.44	± 0.37
516_4	*V. inaequalis*	Ambrosia	18.14	± 0.37
516_5	*V. inaequalis*	Ambrosia	18.09	± 0.22
3af	*V. inaequalis*	Golden Delicious	17.75	± 0.31
416_b2	*V. inaequalis*	Golden Delicious	17.76	± 0.36
416_a1	*V. inaequalis*	Mondial Gala	17.76	± 0.39
516_10	*V. inaequalis*	Ambrosia	18.45	± 0.32
1b2	*V. inaequalis*	Ambrosia	17.66	± 0.23
416_a13	*V. inaequalis*	Mondial Gala	17.47	±0.24
1b9	*V. inaequalis*	Ambrosia	17.94	± 0.11
3b3	*V. inaequalis*	Ambrosia	17.11	± 0.24
3b4	*V. inaequalis*	Ambrosia	17.68	± 0.11
3b10	*V. inaequalis*	Ambrosia	17.13	± 0.25
1b14	*V. inaequalis*	Ambrosia	18.00	± 0.39
416_a3	*V. inaequalis*	Mondial Gala	17.85	± 0.28
1b13	*V. inaequalis*	Ambrosia	17.34	± 0.07

* The values are expressed as the mean ±SD (n=9)

Supplementary Table 2 – Standard curve obtained for the quantification of the *V. inaequalis* 1b14 strain using the TaqMan real-time PCR assay developed by Gusberti et al. (2012). Ct mean values ± standard deviation.

***V. inaequalis* DNA concentration (ng)**	**Ct values ± SD**
10	20.13 ± 0.11
1	23.24 ± 0.12
0.1	26.74 ± 0.10
0.01	30.10 ± 0.04
0.001	33.69 ± 0.71
0.0001	36.21 ± 0.86

Supplementary Table 3 – Ct mean values ± standard deviation and the estimated number of *V. inaequalis* cells/µL ± standard deviation for the symptomatic *Malus x domestica* 'Ambrosia', 'Mondial Gala' and 'Golden Delicious' leaves obtained using the TaqMan real-time PCR assay.

Sample ID	**Cultivar**	**Ct mean ± SD***	***V. inaequalis* cells/µL ± SD***
1b	Ambrosia	21.93 ± 0.10	1.67 x 1±0^{4} ± 1137.31
3b	Ambrosia	25.15 ± 0.20	1.59 x 10^{3} ± 209.73
Amba17	Ambrosia	19.89 ± 0.27	8.63 x 10^{4} ± 15945.86
316s2	Ambrosia	18.86 ± 0.18	1.25 x 10^{5} ± 15918.36
216	Ambrosia	25.38 ± 0.54	1.91 x 10^{3} ± 777.22
516	Ambrosia	22.35 ± 0.64	2.19 x 10^{4} ± 8523.70
Amb1	Ambrosia	23.89 ± 0.34	5.59 x 10^{3} ± 1262.95
Amb4	Ambrosia	25.29 ± 0.90	1.53 x 10^{3} ± 829.27
Col1	Ambrosia	18.71 ± 0.29	1.36 x 10^{5} ± 26719.38
Col2	Ambrosia	20.37 ± 0.38	5.90 x 10^{4} ± 1673.62
Col3	Ambrosia	19.12 ± 0.48	1.23 x 10^{5} ± 40605.49
416a1	Gala	27.96 ± 0.12	3.20 x 10^{3} ± 476.83
416a2	Gala	18.12 ± 0.05	2.48 x 10^{5} ± 8191.52
GalaA	Gala	20.76 ± 0.79	4.31 x 10^{4} ±17722.88
Gala1	Gala	25.81 ± 0.03	9.33 x 10^{2} ± 261.42
Gala2	Gala	24.85 ± 0.24	1.82 x 10^{3} ± 311.25
Gala3	Gala	24.98 ± 0.18	2.01 x 10^{3} ± 399.74
Gala4	Gala	27.79 ± 0.49	2.38 x 10^{2} ± 73.01
Gold11	Golden Delicious	21.04 ± 0.39	3.16 x 10^{4} ± 8540.33
116	Golden Delicious	18.32 ± 0.24	2.07 x 10^{5} ± 34735.35
1a	Golden Delicious	34.35 ± 0.37	0.28 x 10^{1} ± 0.67
416b2	Golden Delicious	22.58 ± 0.17	1.13 x 10^{4} ± 1400.69
416b3	Golden Delicious	23.53 ± 0.23	5.85 x 10^{3} ± 977.49
Gold22	Golden Delicious	21.26 ± 0.35	3.35 x 10^{4} ± 8582.51
3a	Golden Delicious	24.25 ± 0.23	3.46 x 10^{3} ± 582.48
Gold3a	Golden Delicious	20.17 ± 0.31	6.19 x 10^{4} ± 13818.83
GoldA	Golden Delicious	20.29 ± 0.14	5.79 x 10^{4} ± 5549.25
Gold4	Golden Delicious	31.65 ± 0.65	3.30 x 10^{1} ± 337.26
Gold1	Golden Delicious	26.12 ± 0.43	1.05 x 10^{3} ± 337.26
Mti2	Golden Delicious	36.36 ± 0.84	7.58 x 10^{2}± 359.66
MtiA	Golden Delicious	26.11 ± 0.44	3.21 x 10^{4} ± 9293.85
MtiB	Golden Delicious	21.51 ± 0.06	7.04 x 10^{3} ± 294.32
MtiC	Golden Delicious	28.38 ± 0.07	1.82 x 10^{2} ± 0.39

* Values are expressed as the mean ±SD (n=9).

Supplementary Table 4 – Ct mean values ± standard deviation and the estimated number of *V. inaequalis* cells/µL ± standard deviation for the asymptomatic *Malus x domestica* ‘Fiorina’, ‘Ambrosia’ and ‘Golden Delicious’ leaves obtained using the TaqMan real-time PCR assay.

Sample ID	**Cultivar**	**Ct mean ± SD***	***V. inaequalis* cells/µL ±SD***
As1	Fiorina	27.21 ± 0.42	4.23 x 10^{2} ± 56.27
As2	Fiorina	28.86 ± 0.11	1.30 x 10^{2} ± 10.25
As3	Fiorina	27.68 ± 0.12	2.98 x 10^{2} ± 26.45
As4	Ambrosia	25.56 ± 0.07	1.31 x 10^{3} ± 6.18
As5	Ambrosia	23.81 ± 0.21	4.50 x 10^{3} ± 175.73
As6	Fiorina	26.20 ± 0.18	8.40 x 10^{2} ± 10.92
As7	Fiorina	34.48 ± 0.07	0.03 x 10^{2} ± 0.22
As8	Fiorina	33.18 ± 0.12	0.06 x 10^{2} ± 0.55
As9	Fiorina	34.94 ± 0.13	0.02 x 10^{2} ± 0.29
As10	Ambrosia	24.32 ± 0.16	3.14 x 10^{3} ± 157.47
As11	Ambrosia	24.15 ± 0.16	3.53 x 10^{3} ± 7.29
As12	Ambrosia	24.16 ± 0.15	3.51 x 10^{3} ± 15.75
As13	Fiorina	33.54 ± 0.95	0.06 x 10^{2} ± 1.12
As14	Fiorina	28.89 ± 0.18	1.28 x 10^{2} ± 15.92
As15	Golden Delicious	28.11 ± 0.17	2.21 x 10^{2} ± 25.84
As16	Golden Delicious	29.87 ± 0.51	6.65 x 10^{1} ± 22.08
As17	Fiorina	29.91 ± 0.33	6.31 x 10^{1} ± 13.57
As18	Ambrosia	28.91 ± 0.05	1.26 x 10^{2} ± 33.85
As19	Ambrosia	27.57 ± 0.14	3.21 x 10^{2} ± 30.04
As20	Ambrosia	25.90 ± 0.20	1.03 x 10^{3} ± 43.97
As21	Golden Delicious	26.72 ± 0.13	5.82 x 10^{2} ± 39.72
As22	Golden Delicious	27.85 ± 0.07	2.64 x 10^{2} ± 13.54
As23	Golden Delicious	26.17 ± 0.25	8.61 x 10^{2} ± 15.32
As24	Golden Delicious	27.64 ± 0.07	3.04 x 10^{2} ± 15.71
As25	Golden Delicious	26.60 ± 0.19	6.34 x 10^{2} ± 89.10
As26	Ambrosia	25.55 ± 0.67	1.43 x 10^{3} ± 6.01
As27	Golden Delicious	27.31 ± 0.19	3.86 x 10^{2} ± 51.03
As28	Golden Delicious	28.00 ± 0.18	2.39 x 10^{2} ± 29.63
As29	Golden Delicious	30.81 ± 0.30	3.37 x 10^{1} ± 6.58
As30	Golden Delicious	29.47 ± 0.21	8.50 x 10^{1} ± 12.54

* Values are expressed as the mean ±SD (n=9).