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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; Carrisse 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 (Carrisse 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 strains 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 Spectrophotometer (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 MgCl₂, 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 Gladioux 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

103 performed using the CLUSTALW algorithm, through Molecular Evolutionary Genetics Analysis
104 (MEGA6) software, version 6.0. The alignment was used to design the primers and probe used in this
105 study.

106 Six sets of primers and two probes were designed using Primer Express™ software 3.0 (Applied
107 Biosystem, Foster City, USA) and are listed in **Table 2**. The TaqMan probes were labelled at the 5'-
108 end with the 6-carboxyfluorescein dye (6-FAM) reporter and Black Hole Quencher (BHQ1) or the
109 non-fluorescent quencher minor groove binder (NF-MGB) at the 3'-end. OligoCalc tool
110 (<http://biotools.nubic.northwestern.edu/OligoCalc.html>) was used to verify the presence of hairpins
111 and potential secondary structures, while *in silico* specificity was verified using the BLASTN tool of
112 the National Centre of Biotechnology Information (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).
113 Invitrogen (Carlsbad, USA) and Metabion (Steinkirchen, Germany) synthesized primers and probes,
114 respectively.

115

116 **Conventional end-point PCR and qPCR optimization and amplification**

117 The primer pair sets were first assessed by means of conventional end-point PCR using DNA from
118 *V. inaequalis* strains and other selected species. The PCR endpoints were performed in a 25 µL
119 reaction, composed of 2 µL of Buffer 10x, 0.8 µL of MgCl₂, 1 µL of dNTPs (10 mM), 1 µL of each
120 primer (10 mM), 0.2 µL of Taq Platinum Pfx DNA polymerase (Invitrogen, USA) and 20 ng of DNA.
121 The PCR thermal cycler conditions were 3 min at 95°C followed by 30 cycles of 95°C for 45 s, 54°C
122 for 45 s, 72°C for 1 min and a final extension of 5 min. The PCR products were run on 1% agarose
123 gel in a TBE buffer, and visualized under UV transilluminator using the Quantity One software
124 (BioRad Labs, Hercules, USA). After performing end-point PCR, the primer pairs that gave the best
125 results were selected and used in qPCR with SYBR Green in order to compare them with the
126 specificity and sensitivity of the TaqMan assay. Real-time reactions were performed using a
127 StepOnePlus qPCR system (Applied Biosystems) with 96 well-plates (Optical reaction plate, Applied
128 Biosystems) sealed with MicroAmp optical adhesive film (Applied Biosystems).

129 SYBR Green reactions were carried out using 10 µL of Power SYBR Green Mastermix 10x (Applied
130 Biosystems), 1 µL of each primer (3 µM) and 1 µL of template DNA. Amplification conditions were
131 95 °C for 10 min, followed by 40 cycles of 57 °C for 1 min and 95 °C for 15 s. The melting curves
132 were acquired after each run at the following conditions: 95 °C for 15 s, 60 °C for 15 s and 95 °C for
133 15 s. Sterile water was used as a negative control. In order to determine the sensitivity of the SYBR
134 Green assay, a standard curve was obtained with *V. inaequalis* DNA using 1b14 strain 8-fold serially
135 diluted (from 20 ng to 2 fg).

136 The TaqMan assay was performed using TaqMan Universal Mastermix 2X (Applied Biosystems).
137 Each 96-well plate was loaded with a negative control, standard DNA and a positive control in
138 triplicate. Different primer concentrations (from 3 μ M to 0.3 μ M) and different temperatures were
139 initially tested at the annealing stage (57 to 60 °C). Reactions were carried out in a final volume of
140 20 μ l, with 1 μ l of DNA, 0.4 μ l of each primer (3 μ M) and 0.2 μ l of probe (5 μ M). Sterile water was
141 used as a negative control. Amplification conditions were 95 °C for 10 min, 40 cycles of 57 °C for 1
142 min and 95 °C for 15 s. The newly developed TaqMan assay was compared, in terms of sensitivity
143 and specificity, with the TaqMan assay previously published by [Gusberti et al., \(2012\)](#).
144 The Ct values generated by qPCR were compared with the standard curve to quantify the DNA of *V.*
145 *inaequalis* in target samples. The approximate number of cells was calculated by dividing the DNA
146 quantity by the weight of the genome of *V. inaequalis* (0.0000597 ng; [Deng et al., 2017](#)), thereby
147 obtaining the number of target cells (*V. inaequalis*) per μ L of reaction.
148

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

150 The TaqMan assay was validated according to the international EPPO standard PM 7/98 ([EPPO](#)
151 [2014](#)). In order to evaluate the specificity of the assay for *V. inaequalis*, 20 different species, including
152 other pathogenic *Venturia* species that affect different hosts and *V. asperata*, which has recently been
153 reported in Italy on apples, were used ([Table 1](#)). The presence of aspecific amplification signals was
154 evaluated after 40 cycles in different experiments.

155 In order to determine the sensitivity of the TaqMan assay, a standard calibration curve was obtained
156 with *V. inaequalis* DNA using the 1b14 strain 8-fold serially diluted (from 20 ng to 2 fg) in sterile
157 deionized water. Furthermore, in order to verify the influence of the host DNA on *V. inaequalis*
158 amplification, the pathogen DNA was 8-fold diluted in *Malus x domestica* ‘Ambrosia’, ‘Mondial
159 Gala’ and ‘Golden Delicious’ DNA. The standard curve reaction was carried out in triplicate and used
160 as an internal control in order to quantify the target DNA in different samples. Repeatability was
161 checked by running three independent assays of the test. Two different operators tested the
162 reproducibility of the assay in two different laboratories and on different days.
163

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

165 During the 2015-2017 seasons, leaves from *Malus x domestica* plants showing symptoms of apple
166 scab were harvested and stored for DNA extraction. From ‘Ambrosia’, ‘Golden Delicious’ and
167 ‘Mondial Gala’ leaves, respectively 11, 11 and 5 samples were collected. Furthermore, four apples
168 that had been stored for 2 months and which showed apple scab symptoms were selected for DNA
169 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.

203 Repeatability of the assay on air samples was evaluated by performing 6 independent reactions using
204 the same set of samples.

205

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

209

210 **3. Results**

211

212 **TaqMan assay optimization**

213 The *EF1-α* gene was selected because of the presence of a conserved region and the deletion of 31
214 nucleotides in the *V. inaequalis* species, able to differentiate from the other *Venturia* species,
215 including *V. asperata*. The alignment of partial *EF1-α* gene sequences from different *V. inaequalis*
216 strains and 5 other *Venturia* species was used to design the primers and probes reported in **Table 2**.
217 The F1/R11 primer pair, which gave the best results with conventional end-point PCR, was selected
218 for the real-time assays. The optimization of the TaqMan qPCR assay was tested using different
219 primer and probe concentrations and considering different cycling conditions. The best conditions
220 were found for a primer concentration of 3 μM at 57 °C for 1:00 for the annealing stage. The Ven1
221 probe and F1/R11 primer pair were selected for the TaqMan assay as they showed the most suitable
222 amplification.

223

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

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

229 The DNA of *V. inaequalis*, serially diluted from 20 ng to 0.2 fg in sterile distilled water, was used to
230 build a standard curve in order to evaluate the limit of detection (LOD). The pathogen was
231 quantifiable from 20 ng to 20 fg (**Fig. 1**), and a LOD threshold cycle (Ct), ranging between 36 and
232 37, was obtained. The LOD of 20 fg is lower than a single genome of *V. inaequalis*, (0.0000597 ng,
233 **Deng et al., 2017**). The mean value of the regression slope was -3.28, and the mean relative efficiency
234 was between 99% and 110%. No influence was observed on the selectivity of the TaqMan assay when
235 *V. inaequalis* DNA, serially diluted in *Malus x domestica* 'Ambrosia', 'Golden Delicious' and
236 'Mondial Gala' DNA, was used (**Fig. 2**). The amplifications showed similar PCR efficiencies and a

237 reliable correlation between the Ct values and the amount of measured *V. inaequalis* DNA (Fig. 2).
238 No statistical differences ($p>0.05$) were found for the results of the student's t-test, which was used
239 to analyze the repeatability and reproducibility of the assay.

240

241 **Specificity and sensitivity comparison of the SYBR Green and TaqMan assays**

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

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

252

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

254 The TaqMan assay was used to quantify *V. inaequalis* in naturally infected leaves of three *Malus x*
255 *domestica* cultivars and apple fruit. No influence of the host DNA was detected, and all the analyzed
256 samples resulted positive for the target amplification (Fig. 3A and ST3). The average number of
257 cells/ μ L was 5.26×10^4 for the 'Ambrosia' samples, 4.28×10^4 for the 'Mondial Gala' samples and
258 3.76×10^4 for the 'Golden Delicious' samples. The assay was able to detect 2.83 *V. inaequalis*
259 cells/ μ L in the naturally infected leaves (mean Ct 34.35) and 182 cells/ μ L in the naturally infected
260 fruit (mean Ct 28.38) (Fig. 3A and ST3).

261

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

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

269

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

271 In order to test the developed TaqMan assay on environmental-like samples, *Malus x domestica*
272 leaves were artificially inoculated with a *V. inaequalis* conidial suspension. The results obtained for
273 the quantification of *V. inaequalis* are reported in Figure 4. All the samples amplified with a clear
274 amplification signal, with Ct values ranging from 28, for the samples inoculated with highest
275 concentration, to 36 for the lowest. No amplification was obtained for the negative controls. The
276 amplification showed linearity in the serial dilutions, and the assay allowed us to quantify from 1.65×10^2 cells/ μ L, for the initial sample, to 1.21 cells/ μ L, for the 100-fold diluted sample.

278

279 **Detection of *V. inaequalis* from spore trap samples**

280 The TaqMan assay was used to detect and quantify the presence of airborne inoculum of *V. inaequalis*
281 in the spore trap samples. The estimated conidial concentrations, based on microscope counts, were
282 higher than the estimated concentrations calculated from the amount of *V. inaequalis* DNA detected
283 by the TaqMan assay. Only one of the 14 analyzed samples showed no amplification (Table 3). The
284 estimated mean number of cells/ μ L obtained from the microscope count was 1.01×10^4 , while it was
285 5.26×10^3 for the TaqMan assay. The correlation coefficient between the two assays was positive,
286 with an R^2 value of 0.8186. The repeatability of the assay was variable, with at least one positive
287 amplification per sample over six reactions (Table 3).

288

289 **4. Discussion**

290 The molecular techniques currently applied for the detection of plant pathogens are often used to
291 specifically identify and quantify fungal species in crops and food commodities (Postollec et al.,
292 2001; Capote et al., 2012; Aslam et al., 2017). In this study, a highly sensitive TaqMan real-time
293 assay has been developed for the specific detection and quantification of *V. inaequalis*, and it has
294 successfully been used with symptomatic and asymptomatic leaves, fruit and air samples.

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

305 i.e. TaqMan chemistry, which includes specific fluorescent probes into the PCR (Amaral Carneiro et
 306 al., 2017).

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

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

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

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

336 When the sensitivity of the assays was compared, the TaqMan assay resulted in a lower detection
 337 limit than the SYBR Green assay. The advantage of using the TaqMan assay has been reported in a
 338 qPCR comparative study by Soltany-Rezaee-Rad et al. (2015). A qPCR with SYBR Green could

339 inhibit the Taq DNA polymerase, thereby reducing the sensitivity of the assay (Kermekchiev et al.,
340 2009). The LOD of our TaqMan assay (20 fg) proved to be more sensitive than that reported by
341 Gusberti et al. (2012) for which the LOD was 100 fg. The LOD of the TaqMan assay (20 fg) is similar
342 to those reported for other species i.e. *Botrytis cinerea* (Suarez et al., 2005), *Fusarium solani* (Bernal-
343 Martínez et al., 2012), *Aspergillus fumigatus* (Fernandez-Molina et al., 2014), *Lichtheimia*
344 *corymbifera* (Springer et al., 2016), *Fusarium fujikuroi* (Amaral Carneiro et al., 2017) and *Fusarium*
345 *culmorum* (Bilska et al., 2018).

346 The TaqMan assay was also tested to quantify the target DNA in the presence of the plant material.
347 The sensitivity was not affected by the presence of the DNA of *Malus x domestica* from different
348 cultivars, and the assay allowed to detect and quantify *V. inaequalis* in inoculated samples with
349 significant linearity. Positive amplifications were also obtained from leaf samples, collected both
350 from susceptible and resistant cultivars, that were, respectively, symptomatic or asymptomatic. In
351 addition, the assay proved to be useful for the detection of the pathogen in fruit samples.

352 When the assay was performed on DNA obtained from the spore trap, 13 samples out of 14 resulted
353 positive to *V. inaequalis*, even with a low conidial concentration. In our study, the overall
354 concentration estimated with a microscope count resulted higher than that estimated with the TaqMan
355 assay, but with a linear relationship (R^2 : 0.8186), as previously reported by Carisse et al. (2009),
356 pertaining to the quantification of *Botrytis squamosa*. However, the repeatability of the assay was
357 variable for air samples, as inhibition of PCR may occur when DNA is extracted from air samples,
358 thus false-negative plate readings and a reduced amplification efficiency may be obtained. The
359 reasons for these false negatives could be linked to competition in the amplification due to: high
360 amounts of non-target DNA, inoculum density, co-extraction of contaminants (PCR inhibitors) or
361 unequal distribution of conidia on tapes (McDevitt et al., 2007; Rogers et al., 2009; Williams et al.
362 2001; Bilodeau et al 2012; Klosterman et al., 2014; Dung et al., 2015).

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

371 This newly developed TaqMan assay could be a useful tool, combined with a weather-based data
372 model, to plan phytosanitary treatments in order to control apple scab. The here developed TaqMan

373 assay could also be used, together with nanobiosensor technology, to quantify airborne inoculum in
374 the field, without time consuming DNA extraction and processing, and could therefore be used to
375 implement a decision support system for apple scab management.

376

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383 assay.

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

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

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535 Table 2. Primer pairs and probes, designed on *EF1-α* gene used for the detection of *V. inaequalis* with
536 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 | CACTTCCCCGCTATTACAGT GCAATCGTTAGCATCGTCATAGTG |
| F2.3/R6 | 127 bp | TTGCCCCTCCAAAATTACAGTG GGCGGCTTCCTATTGCAATC |
| F1/R6 | 100 bp | CACTTCCCCGCTATTACAGT 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] |

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538 **Table 3** – Number of *V. inaequalis* cells estimated using the optical microscope compared with the
539 number of cells estimated with the TaqMan real-time assay for the analyses of spore trap samples,
540 and repeatability of the assay, expressed as the number of positive amplifications in six different
541 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 |

542
543 *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 \pm 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 \pm SD (n=9).

562 **Supplementary Tables**
563 **Supplementary Table 1** - Results of the q-PCR amplification (Ct values \pm standard deviation) with
564 the target DNA (\cong 20 ng) of different *V. inaequalis* strains used to evaluate the specificity of the
565 TaqMan real-time assay.

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

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567 * The values are expressed as the mean \pm SD (n=9)

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

| <i>V. inaequalis</i> 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 |

571

572 **Supplementary Table 3** – Ct mean values \pm standard deviation and the estimated number of *V.*
573 *inaequalis* cells/ μ L \pm standard deviation for the symptomatic *Malus x domestica* ‘Ambrosia’,
574 ‘Mondial Gala’ and ‘Golden Delicious’ leaves obtained using the TaqMan real-time PCR assay.

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

575 * Values are expressed as the mean \pm SD (n=9).

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

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

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580 * Values are expressed as the mean \pm SD (n=9).