A quantitative real-time PCR assay for early detection and quantification of *Ramularia mali*, an emerging pathogen of apple causing dry lenticel rot

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Abstract

Ramularia mali is an emerging pathogen of apple (*Malus domestica*) causing dry lenticel rot. No preventive measures have been adopted due to the lack of knowledge about the life cycle and epidemiology of this pathogen. In a preliminary survey to identify the agents of dry lenticel rot of apple, *R. mali* was constantly associated to the disease. Using isolates from this survey, a SYBR Green qPCR assay was developed, using calmodulin as target gene, for the detection and quantification of *R. mali* in apple fruits. The qPCR assay was validated in terms of specificity, sensitivity, repeatability and reproducibility following the international EPPO standard PM 7/98. The primers amplified a region of 237 bp specific to *R. mali*. The specificity was validated with 20 fungal species commonly found on apple, 36 strains of *R. mali* and closely related species of the *R. eucalypti* species complex. Positive amplifications were obtained only with DNA of *R. mali* and no cross-reaction was detected with the other fungal species. Sensitivity was assessed with serial dilutions of target DNA and the limit of detection was 100 fg. No influence of host DNA was observed when target DNA was diluted on the DNA of 'Ambrosia' and 'Golden Delicious' apple. The assay permitted to detect and quantify *R. mali* in symptomatic and asymptomatic fruits. The presence of *R. mali* on asymptomatic 'Ambrosia' and 'Golden Delicious' apples was demonstrated, and the presence of the pathogen was reported for the first time on 'Jeromine', 'Gala', 'Opal' and 'Story Inored' fruits. This assay could be useful to clarify the life cycle of this pathogen in order to build up an effective disease management strategy. Furthermore, the early detection of the pathogen on asymptomatic

apples could be used to forecast the development of dry lenticel rot, supporting the packinghouse operators in deciding the storage length of apple lots.

Keywords: apple, qPCR, *Ramularia mali*, dry lenticel rot

1. Introduction

Plant diseases associated with the genus *Ramularia* are worldwide reported: *R. collo-cygni*, *R. beticola* and *R. grevilleana* are able to cause relevant economic losses to barley, sugar beet and strawberry crops, respectively (Bakhshi and Arzanlou, 2017). The genus includes species with different lifestyle, from saprobic, to endophytic or hyperparasitic (Videira, et al. 2015a). High air humidity (≥ 90%) and relative low temperatures (ranging from 18°C to 21°C) favor the symptoms development, resulting in leaf and fruits spots, leaf necrosis or leaf chlorosis (Videira et al. 2016; Videira, et al. 2015b).

Italy is the second apple producer in Europe, with 55,000 hectares and a total production of 2.4 million tons in 2020 (FAO, 2022). The pathogen *Ramularia mali* Videira & Crous was first reported in north-western Italy (Piedmont) on cold-stored (*Malus domestica*, Bork H.) 'Ambrosia' apples and 'Conference' pears, causing dry lenticel rot on fruit (Gianetti et al. 2012; Giordani et al. 2012). Typical symptoms consisted in depressed brown to black spots with irregular margins on lenticels. The pathogenicity was confirmed on apple 'Ambrosia' by demonstrating the Koch's postulates (Giordani et al. 2012). Later, *R. mali* was reported in South Tyrol, north-eastern Italy, from different apple cultivars (*Malus domestica* 'Golden Delicious', 'Braeburn' and 'Rosy Glow'), but artificial inoculation on apple 'Golden Delicious' failed to confirm the pathogenicity of the strains (Lindner 2013). Gianetti et al. (2016) reported the pathogen on stored apples 'Golden Delicious', 'Red Delicious' and 'Red Delicious Scarlet Spur' in Piedmont. Infected fruits were mainly coming from orchards characterized by high relative humidity conditions and a high incidence of white haze (Spadaro et al., 2019). More recently, *R. mali* was isolated from different hosts, including *Prunus cerasus* and *Vitis vinifera* in Asia (Bakhshi and Arzanlou, 2017).

The knowledge about the pathogen life cycle and epidemiology is still limited. Gianetti et al. (2012) reported that asymptomatic apple fruit, harvested from trees showing *R. mali* leaf spots, developed dry lenticel rot after four months of cold-storage. Furthermore, the constant presence of infections in the same orchards on different years suggests that the pathogen could infect the fruit in orchard rather than during the postharvest phase (Gianetti et al., 2016). The presence of pseudothecia and ascospores on leaves was verified by Gianetti et al. (2016), suggesting that *R. mali* could probably have a

hemibiotrophic life cycle, as reported for other species of the genus (Videira et al. 2016), with ascospores released from pseudothecia causing primary infections during the growing season.

Traditional identification of *Ramularia* species is based on observation of micro- and macro-morphological characteristics, such as colony morphology on different media, conidial shape, and size, presence of septa and conidiogenous type (Videira et al. 2015a). A correct identification based on morphological features is difficult and time-consuming (Videira et al. 2015b). The advent of molecular techniques revolutionized the identification and detection of plant pathogens. Molecular approaches provide reliable species identification, and are considered more specific and sensitive than traditional identification (Spadaro et al., 2020). Among the molecular methods, quantitative PCR (qPCR) is one of the most used methods, as it lacks post amplification processing steps (e.g. gel electrophoresis) and allows a specific quantification of the target DNA in different environmental samples (Schena et al., 2004; Prencipe et al., 2020). Since the symptoms caused by *R. mali* are visible after 2-4 months of storage, an early detection and quantification of the pathogen is required in order to plan a proper disease management strategy.

To date, no studies have been carried out to detect and quantify *R. mali* in fruits by using a molecular approach. One aim of the current work was to identify the agents of dry lenticel rot of apple on a collection of fruit samples, to verify if the disease is caused only by *R. mali* or also by other species of the genus *Ramularia*. Nevertheless, the main goal was to develop and validate a specific and sensitive qPCR assay to detect and quantify *R. mali* on different apple cultivars.

2. Materials and methods

Fungal strains

Strains of *R. mali* were isolated during 2018/2019 and 2019/2020 seasons (January - March) from 'Ambrosia' apples cultivated in northern Italy (Piedmont region), harvested in October-November, and cold-stored (1±1°C) for approximately 2-4 months. The samples were surface disinfected with 1% sodium hypochlorite, washed in sterile deionized water and air-dried. Four fragments were cut at the margin of depressed brown to black spots with irregular margins on lenticels, from each symptomatic apple fruit (120 fruits), and plated onto Malt Extract Agar (MEA, Sigma Aldrich, Germany). After 15 days of incubation at 25±1 °C, 32 isolates showing typical colony morphology of *Ramularia* spp. (Videira et al. 2015b) were selected and identified as described later.

Eight reference strains were provided by the Westerdijk Institute (*R. mali* CBS 129581, *R. eucalypti* CBS 120726, *R. glennii* CBS 120727, *R. malicola* CBS 119227, *R. vizellae* CBS 115981, *R. grevilleana* CBS 719.84, *Venturia inaequalis* CBS 815.69 and *V. asperata* IRHS 2345), 17 strains

were provided by the University of Torino (species present in apple orchard or causing postharvest diseases on apples) and 3 strains were provided by Regione Piemonte - Settore Fitosanitario (*R. mali* strains MC, AC, A1). The strains used in this study are summarized in Table 1. The strains were maintained as monoconidial cultures in tubes of MEA and stored at 4° C.

Molecular identification of *R. mali* isolates

Thirty-two strains of *Ramularia* spp., isolated from cold-stored apples in this study and three provided by Regione Piemonte, were grown on MEA at 25±1 °C in the dark for 15 days, whereas the other fungal species were grown at the same conditions for 10 days. According to the manufacturer's instructions, Omega E.Z.N.A. Fungal DNA Mini Kit (VWR, USA) was used to extract the fungal DNA, from approximately 100 mg of fresh-weight mycelium. Nanodrop 2000 Spectrophotometer (Thermo Scientific, USA) was used to verify the concentration and the quality of DNA. *Ramularia* spp. isolates were identified through the amplification of RNA polymerase II second largest subunit (*rpb2*) and calmodulin (*cmdA*) genes. These genes were selected because they allow discriminating *R. mali* from the closely related species in the *R. eucalypti* species complex (Videira et al., 2015b). PCRs were carried out using Taq DNA polymerase kit (Qiagen, Germany) following protocols reported in Videira et al. (2016). The PCR products were purified, after agarose gel electrophoresis, using a QIAquick© PCR purification Kit (Qiagen), and sequenced in both directions by Macrogen, Inc. (The Netherlands). DNA Baser program (Heracle Biosoft, Romania) was used to assembly forward and reverse sequences to obtain the consensus sequences. The resulting 70 sequences, obtained from the sequencing of *rpb2* and *cmdA* genes, were compared with those deposited in GenBank and Molecular Evolutionary Genetics Analysis (MEGA6) software version 6.0 was used to perform a multi alignment, using the CLUSTALW algorithm. For the concatenated dataset, MEGA version 6 was used to determine the best-fit nucleotide and to perform the phylogenetic analysis with the Maximum Likelihood algorithm. After cutting the trimmed regions and manual correction, a dataset of 1051 bp and 411 bp was obtained for *rpb2* and *cmdA* genes respectively. The sequences were deposited in GenBank and the accession numbers of all the sequences used for the phylogenetic analysis are listed in Supplementary Table 1.

Sequence analysis and primers design

Actin (*act*), translation elongation factor 1-α (*tef1-α*), histone H3 (*his3*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), RNA polymerase II second largest subunit (*rpb2*), calmodulin (*cmdA*), β-tubulin (*tub2*) and chitin synthase I (*chs-1*) gene sequences from validated strains of the *R. eucalypti* species complex (Videira et al., 2015b) were used to perform a multi alignment. For each gene, the

alignments were done using the CLUSTALW algorithm through Molecular Evolutionary Genetics Analysis (MEGA6) software version 6.0. The alignments were used to assess the presence of polymorphisms useful to design specific primers for the qPCR assay. Nine sets of primers (Table 2) were manually designed using SnapGene software (from Insightful Science; available at snapgene.com) keeping whenever possible the polymorphic base, able to differentiate *R. mali* from other closely related species, close to the 3' end of the sequence. The primers were synthesized by Invitrogen (USA). The BLASTN tool of the National Centre of Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to verify *in silico* specificity, while the presence of hairpins and potential secondary structures was verified using OligoCalc (http://biotools.nubic.northwestern.edu/OligoCalc.html) and OligoAnalyzer (https://eu.idtdna.com/calc/analyzer) tools.

Amplifications with end-point PCR and qPCR

Conventional end-point PCR was used to preliminarily check the best promising primer pairs using DNA from *R. mali* strains and other species listed in Table 1. The end-point PCRs were performed using Taq DNA polymerase kit (Qiagen) in a total volume of 20 µL, composed of 2 µL of Qiagen Buffer 10x, 1 µL of $MgCl_2$, 0.5 µL of dNTPs (10 mM), 0.4 µL of each primer (10 µM), 0.2 µL of Taq DNA polymerase and 10 ng of DNA. The PCR thermal cycler conditions were 3 min at 95 °C followed by 34 cycles of 95 °C for 30 s, 60 or 65 °C for 30 s (Table 2), 72 °C for 30 s and a final extension of 5 min. To check the specificity of tested primers, the PCR products were run on 1% agarose gel in a TBE buffer and visualized using the Quantity One software (Bio-Rad, CA, USA). The primer pairs amplifying specifically *R. mali* were selected to accurately evaluate their specificity in qPCR reactions with SYBR Green. StepOnePlus qPCR system (Applied Biosystems, USA) with 96 well-plates (Optical reaction plate, Applied Biosystems) sealed with MicroAmp optical adhesive film (Applied Biosystems) were used to perform real-time reactions. SYBR Green reactions were carried out using SsoAdvanced Universal SYBR Green Supermix 2x (Bio-Rad, USA). Different annealing temperatures (60, 63 and 65 °C) and different primer concentrations (from 5 µM to 1 µM) were initially tested following the protocols for amplification and reactions described below. Amplification conditions were 95 °C for 10 min, followed by 40 cycles of annealing temperature for 1 min and 95 °C for 15 s. After each run, one cycle of melting curve step was conducted by ramping the temperature from 60 °C to 90 °C. Each 96-well plate was loaded with a negative control (RNAse free water), standard DNA and a positive control, in triplicate. Reactions were carried out in a final volume of 10 µL, with 1 µL of DNA (~10 ng), 0.25 µL of each primer (1 µM) and 5 µL of SsoAdvanced Universal SYBR Green Supermix 2x (Bio-Rad). After each run, data were collected

from the instrument and the Cq values obtained by qPCR were interpolated with the generated standard curve to quantify the DNA of *R. mali* in target samples. As the genome size of *R. mali* is not available, the approximate number of cells was calculated (Schena et al., 2017) by dividing the DNA quantity by the mean weight of the genome of other sequenced *Ramularia* species (0.0000351 ng; McGrann et al., 2016; Stam et al., 2018; Huang et al., 2021), in order to obtain the number of *R. mali* target cells per µL of reaction, using SsoAdvanced Universal SYBR Green Supermix 2x (Bio-Rad).

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

The international EPPO standard PM 7/98 (EPPO 2019) was followed to perform the validation of the qPCR assay.

A total of 36 *R. mali* strains (32 strains isolated in this study from symptomatic fruits, 3 provided by Regione Piemonte and the *R. mali* CBS 129581 reference strain) and 20 non-target species (Table 1) were used to evaluate the specificity of the assay. Non-target species included other *Ramularia* species reported on apple (*R. malicola*, *R. eucalypti* and *R. vizellae*), together with *R. glennii* and *R. grevilleana* reported on *Corymbia grandifolia* and *Fragaria* sp., respectively.

To further validate the specificity of the qPCR assay, amplicons of three samples resulting positive were sequenced in both direction by Macrogen Inc. (The Netherlands), with primers used for the amplification, and the resulting consensus sequences were compared to those deposited in GenBank using the BLASTN tool of the National Centre of Biotechnology Information.

A standard calibration curve was obtained using 8 serial dilutions in sterile deionized RNAse free water (from 10 ng to 1 fg) of the DNA of *R. mali* strain CBS 129581. To quantify the target DNA in different samples, a standard curve was used as internal control and carried out in triplicate. The sensitivity was determined both on fungal DNA and on inoculated apple tissues.

In order to determine the influence of the host DNA (selectivity), the same DNA was diluted in the DNA of *Malus domestica* 'Ambrosia' and 'Golden Delicious', used at a concentration of 10 ng/µL. Two different operators tested the reproducibility of the assay in two different laboratories and on different days, whereas the repeatability was checked by an operator running two independent assays in the same laboratory. The assay was further validated on symptomatic and asymptomatic fruits.

Validation of qPCR in artificially inoculated apple tissues

To verify the sensitivity of the assay on true negative and true positive apple samples, *R. mali* CBS 129581 strain was plated onto MEA and incubated at 25±1 °C. After 20 days, the conidia were gently scraped using a Drigalski spatula by adding 5 mL of sterile deionized water with 0.01 % (w/v) Tween-20 and counted using a Burker chamber. Peel disks (1 cm diameter) were prepared from 'Golden

Delicious' apples using a core drill. Fruit were washed by immersion in sterile deionize water for 5 min, disinfected with a 20% sodium hypochlorite solution, washed again with sterile deionized water and air-dried. Apple disks were tested with qPCR to verify the absence of *R. mali* and resulted free from the pathogen. Three drops of 5 µL each were inoculated on the peel fruit surface with an initial concentration of 5×10^3 conidia/mL subsequently serially diluted (1:2, 1:5, 1:10, 1:50, 1:100 and 1:1000). Controls were inoculated with sterile deionized water with Tween-20. For each conidial concentration tested, three replicates were prepared. The samples were dried at 70 °C for 3 hours prior to DNA extraction with E.Z.N.A. Plant DNA kit (VWR, USA) using procedures described previously.

Detection of *R. mali* in symptomatic apples

During the 2018-2020 seasons, symptomatic 'Ambrosia' apples cultivated in northern Italy (Piedmont region), harvested in October-November from orchards where dry lenticel rot was previously reported, and cold-stored for approximately 2-4 months, were sampled and selected for DNA extraction. From 29 samples (11 collected during 2018, 9 collected during 2019 and 9 during 2020), 100 mg of fresh-weight fruit peel were collected, freeze-dried and ground with liquid nitrogen, then DNA was extracted with E.Z.N.A. Plant DNA kit (VWR) following the manufacturer's instructions. To validate the positive amplifications obtained with qPCR assay, resulting amplicons of two samples were sequenced and analyzed as described before.

Detection of *R. mali* in asymptomatic apples

In order to evaluate the presence of *R. mali* in asymptomatic fruits, apples from different cultivars cultivated in northern Italy (Piedmont region) from orchards where no symptoms were previously reported, were analyzed. Thirty-six samples (12 from 'Ambrosia', 7 from 'Jeromine', 7 from 'Gala', 3 from 'Opal', 3 from 'Golden Delicious' and 4 from 'Story Inored') were harvested in September-October 2020 and stored for 1 month at 1±1 °C before sampling, whereas 7 samples from 'Ambrosia' were harvested during October 2018 and stored for 2 months at 1±1 °C before sampling. Fruit sampling was destructive. Fresh-weight fruit peel was collected, freeze-dried and ground with liquid nitrogen. Then 100 mg was used to extract DNA with E.Z.N.A. Plant DNA kit (VWR) following the manufacturer's instructions. To validate the positive amplifications obtained with qPCR assay, six positive amplicons (one from each cultivar) were sequenced and analyzed as described before.

Data analysis. The baseline range, the qPCR standard curves and the Cq values were automatically generated using StepOne™ software. Student's t-test was used to analyze the repeatability and the reproducibility of the assay.

3. Results

Sampling of apples with dry lenticel rot and molecular identification of the isolates

Starting from a collection of 'Ambrosia' apples showing dry lenticel rot, several fungal strains were isolated from 2018 to 2020. Thirty-two isolates showing typical colony morphology of *Ramularia* genus were selected for molecular identification. Three strains of *R. mali*, provided by Regione Piemonte and previously identified, were included. The phylogenetic analysis, based on the concatenation of *rpb2* and *cmdA* genes (Figure 1), assigned all the isolates to the species *R. mali*. The strains clustered into two main groups with a strong statistical support, 26 strains clustered in group I with the CBS reference strains (bootstrap 75%), whereas the remaining 9 strains clustered in group II (bootstrap 74%). Each group was further divided into different clusters with different statistical support, showing a high intraspecific variability between strains.

Optimization of SYBR Green qPCR assay

RNA polymerase II second largest subunit (*rpb2*) and calmodulin (*cmdA*) genes were selected because of the presence of conserved SNPs able to differentiate *R. mali* from other *R. eucalypti* species complex, whereas the other genes analyzed were discarded due to the lack of useful conserved polymorphisms to design the primers. The alignment of *rpb2* gene was used to design seven primer pairs (Table 2), whose 503FN/736R and 503FN/772R showed preliminary specific amplification with end-point PCR and were further tested with qPCR. The alignment of *cmdA* gene was used to design two primer pairs (Table 2), which showed specific amplification in end-point PCR and were preliminary selected for the qPCR assay. Table 2 reports in bold, for each primer set, the single base able to differentiate *R. mali* from the other most closely related species.

Among the primers tested in qPCR, only two pairs, one designed on *rpb2* gene (503FN/736R) and one designed on *cmdA* gene ((cmdFL/cmdRL) showed specificity for *R. mali.* The assay using either primer set and 10 ng of reference strain *R. mali* CBS 129581 showed that the couple cmdFL/cmdRL had higher sensitivity with lower Cq values (18.57± 0.11) compared to the other primer set (29.11± 0.10). For this reason, cmdFL/cmdRL primer pair was chosen for the qPCR optimization. The primer concentration of 1 µM and the annealing temperature of 63 °C for 1 min were the most suitable

conditions for qPCR amplification with SsoAdvanced Universal SYBR Green Supermix 2x (Bio-Rad).

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

All the *R. mali* strains, used to test the specificity of the assay, amplified the 237 bp by using cmdFL/cmdRL primer pairs with a mean Cq value of 18.49 ± 0.27 (Supplementary Table 2). To validate the specificity of the qPCR results, the amplicons obtained using genomic DNA of 3 strains (4/6, 5/2 and CBS 129581) were sequenced and the resulting consensus sequences were compared to those deposited in GenBank, using the BLASTN tool, obtaining 99% to 100% similarity and 100% coverage to the reference strain *R. mali* CBS 129581 (accession number KJ504506). No amplification of the other species tested (Table 1) was detected until 37 amplification cycles, a part from *R. mali*. After 37 cycles, amplification of the species *R. glennii*, *R. malicola* and *R. eucalypti* occasionally occurred.

The limit of detection (LOD) was evaluated building a standard curve using the DNA from *R. mali* CBS 129581 strain serially diluted from 10 ng to 1 fg in RNAse free water. The developed assay was able to quantify from 10 ng to 100 fg of *R. mali* (Fig. 2), with a LOD quantification cycle (Cq) of 34.40 ± 0.32. The mean relative efficiency was between 95% and 96% and the mean value of the regression slope was -3.43.

The qPCR assay showed similar amplifications, PCR efficiencies and Cq values when the pathogen DNA was diluted on the DNA of *Malus domestica* 'Ambrosia' and 'Golden Delicious', showing no influence of host DNA (Fig. 3).

The Student's t-test showed no statistical differences ($p>0.05$) in the samples used to assess the repeatability and reproducibility of the assay, meeting the requirements of EPPO standard 7/98.

Validation of qPCR in artificially inoculated apple tissues

Conidial suspension obtained from *R. mali* strain CBS 129581 was used to artificially inoculate apple peel disks to test the developed qPCR assay on true negative and true positive apple samples. All the samples amplified with a good correlation coefficient ($R^2= 0.9998$), between inoculated and estimated concentration, (Figure 4) and no amplification was obtained for negative controls. The Cq ranged from 23 for the highest inoculated concentration (5×10^3 conidia/mL) to 35 for the lowest (0.5×10 conidia/mL).

Detection of *R. mali* in symptomatic apples

The developed qPCR assay was used to detect and quantify *R. mali* in symptomatic 'Ambrosia' apples. All the analyzed samples amplified and resulted positive for the presence of the pathogen DNA (Fig. 5, Supplementary Table 3), and no influence of the host DNA was observed. The mean *R. mali* concentration found was 2.18×10^3 cells/µL, the lowest concentration found was 6.28×10^1 cells/µL in sample RM6B collected in 2018, and the highest concentration was 1.31×10^4 cells/µL in sample AR5 collected in 2020. To validate the results, among 29 samples amplified with qPCR, two (AR2 and RM1B) were sequenced and Blast-searched obtaining 100% similarity and 100% coverage with the reference strain *R. mali* CBS 129581 (accession number KJ504506).

Detection of *R. mali* in asymptomatic apples

Considering all the apple cultivars tested, 67% of the asymptomatic samples resulted positive for the presence of *R. mali* (Table 3). In detail, 3 out of 7 'Jeromine' samples were positive, 4/7 'Gala' samples, 2/3 'Golden Delicious' samples, 3/3 'Opal' samples, 15/19 'Ambrosia' samples, and 2/4 'Story Inored' samples. The lowest pathogen concentration (0.77 cells/µL) was found in sample A11, whereas the highest concentration (63.88 cells/µL) was found in sample AMBC, both from 'Ambrosia' apples. The mean *R. mali* concentration was 15.20 cells/µL, 11.03 cells/µL, 1.33 cells/µL, 2.20 cells/µL, 6.25 cells/µL and 1.10 cells/µL for 'Jeromine', 'Gala', 'Golden Delicious', 'Opal' 'Ambrosia' and 'Story Inored' apples, respectively (Table 3). To validate the results, two samples for each cultivar, amplified with qPCR (samples JERA, JERB, GALB, GALC, GOB, GOC, OP1, OP2, A2, AMBB, ST3 and ST5), were sequenced and Blast-searched obtaining 99% to 100% similarity and 100% coverage with the reference strain *R. mali* CBS 129581 (accession number KJ504506).

4. Discussion

In this study for the first time, a monitoring of the fungal species isolated from stored apples showing dry lenticel rot was carried out. The strains were identified through the amplification of *rpb2* and *cmdA,* genes selected because they allow discriminating *R. mali* from closely related species in the *R. eucalypti* species complex (Videira et al., 2015b). ITS region was used to identify the first strains isolated from stored 'Ambrosia' apples affected by dry lenticel rot, as *R. eucalypti* (Gianetti et al., 2012). Later Videira et al. (2015b), using a multilocus molecular approach, renamed these strains as *R. mali*, highlighting the low resolution power of ITS region to discriminate species in the *R. eucalypti* species complex.

Starting from a collection of apples showing dry lenticel rot, several fungal strains were isolated. A selection of 32 strains showing typical colony morphology of *Ramularia* genus were all identified as

335 *R. mali.* This result confirmed that the only causal agent of dry lenticel rot on apple is *R. mali*. The
336 phylogenetic tree highlighted a great intraspecific variability inside the species complex, with strains
337 clustering into two main groups supported by high bootstrap values. Similarly, a high genetic
338 variability was previously observed for other plant pathogens (Kumari et al., 2014; Fisher et al., 2017;
339 Prencipe et al., 2018; Turzhanova et al., 2020).
340 Among the molecular diagnostic techniques, qPCR is more specific, sensitive and less time-
341 consuming compared to conventional end-point PCR (McCartney et al., 2003; Zijlstra et al., 2011).
342 This technique allows pathogen quantification and has been used for many fungal plant pathogens
343 (Schena et al. 2004; Sanzani et al., 2014; Hariharan and Prasannath 2020), including *R. collo-cygni*
344 (Taylor at al., 2010) and *R. beticola* (Wieczorek et al., 2014), but not for *R. mali*. In this study, a
345 specific and sensitive qPCR assay was developed for the detection and quantification of *R. mali* in
346 symptomatic and asymptomatic samples.
347 The single copy *rpb2* and *cmdA* genes were selected for preliminary end-point PCR assays. As
348 reported by Schena et al. (2013), the use of a qPCR detection methods based on single copy genes
349 permits a higher accuracy for quantitative analyses. Furthermore, the primers were designed using
350 single nucleotide polymorphism, the most common and stable form of genetic variation: a single base
351 was able to differentiate sequences of the most closely related species of *R. eucalypti* species complex
352 (Liu et al., 2012; Rahman et al., 2022).
353 The assay was later developed using SYBR Green I technology and the primers designed on *cmdA*
354 gene. As reported in Adams (2006), a good qPCR efficiency ranges from 90 to 110%, and in our
355 qPCR assay the efficiency was between 95% and 96%. The obtained LOD (100 fg) is similar to those
356 reported for assays targeting other fungal pathogens (Daniëls et al., 2012; Wang et al. 2015; Xu et al.,
357 2020), but lower compared to the LOD reported for other *Ramularia* species (Taylor at al., 2010;
358 Wieczorek et al., 2014).
359 No cross-reaction was detected when the assay was carried out using DNA of species commonly
360 present in the field and during storage. No amplification was obtained before 37 cycles using DNA
361 from other *Ramularia* species tested, including species already reported on apple, such as *R. malicola*
362 and *R. eucalypti* on fruits, and *R. vizellae* on *Malus* death leaf litter (Videira et al. 2015a). As already
363 reported, the number of cycles influenced the reliability of late Cq values, as observed with our assay
364 with non-specific amplification of other *Ramularia* species after 37 cycles (Pfaff, 2004). For this
365 reason, this value was set up as cut-off to identify false positive results, meeting the number of qPCR
366 cycles (between 35 and 40 cycles) accepted for the development of a diagnostic assay (Bustin &
367 Nolan, 2004; Bustin et al., 2009; McMullen & Petter, 2014). Positive amplifications were obtained
368 using DNA of 35 *R. mali* strains isolated from stored apples 'Ambrosia' and 'Golden Delicious'

showing symptoms of dry lenticel rot, confirming the specificity of the assay. Results obtained in independent experiments were reproducible and showed low variation in Cq values ($P \geq 0.05$). To verify the sensitivity of the assay on true negative and true positive apple samples, the assay was tested using inoculated samples showing a good linearity and no influence on the selectivity was observed. Furthermore, a positive amplification and quantification was obtained from symptomatic 'Ambrosia' apples.

When the assay was used on asymptomatic fruits, positive amplifications were obtained for all the cultivars analyzed and the presence was further confirmed through sequencing of the amplicons obtained. The presence of the pathogen in a latent form was demonstrated in approximately two thirds (29/43) of the asymptomatic apples analyzed. Asymptomatic fruits of 'Ambrosia' and 'Golden Delicious' resulted positive confirming the previous reports of *R. mali* on these apple cultivars (Gianetti et al. 2012; Lindner, 2013; Gianetti et al., 2016). The cultivar with the highest presence of the pathogen DNA was 'Ambrosia', which is also the most susceptible cultivar to lenticel rot.

The presence of *R. mali* was reported for the first time on 'Jeromine', 'Gala', 'Opal' and 'Story Inored' apples, indicating a broader occurrence of the fungus as endophyte in several apple cultivars, though data are not available on the development of symptoms in these cultivars. Anyway, in a separate experiment some fruits from one batch of 'Gala' apples were sampled 30 days after cold-storage and tested with qPCR assay, showing the presence of *R. mali*. Eighteen percent of fruits from the same batch developed dry lenticel rot after 4 months storage and *R. mali* was isolated from the symptoms (data not shown). This experience shows the potential application of the assay, which could be used to early detect the pathogen presence and the potential development of dry lenticel rot, providing a support to the operator decision to reduce the storage length of particularly susceptible apple batches.

In conclusion, we demonstrated that the qPCR assay developed is highly specific and sensitive for the detection of *R. mali* from pure culture and symptomatic samples. Furthermore, the assay gave a reliable quantification of the pathogen at low concentration in different asymptomatic apple cultivars. Currently, no measures are implemented to control this emerging pathogen, recently reported also on *Prunus cerasus* and *Vitis vinifera*. This assay could be used in practical conditions, by following a working protocol (Supplementary Appendix I), for early detection of *R. mali* on fruits at harvest or during the first phases of shelf life. Moreover, coupling with spore trap might be of special interest to understand the life cycle of this pathogen and to give first insights on its epidemiology, and to build up an effective disease management strategy.

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412 **References**

413

414 1. Adams, P. S. 2006. Data analysis and reporting. In Real-time PCR, ed. M. Tevfik Dorak. New
415 York: Taylor & Francis, p. 65–88.
416 2. Bakhshi, M., and Arzanlou, M. 2017. Multigene phylogeny reveals a new species and novel
417 records and hosts in the genus *Ramularia* from Iran. Mycol. Prog. 16:703–712.
418 3. Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., et al. 2009.
419 The MIQE guidelines: Minimum information for publication of quantitative real-time PCR
420 experiments. Clin. Chem. 55:611–622.
421 4. Bustin, S. A., and Nolan, T. 2004. Pitfalls of quantitative real- time reverse-transcription
422 polymerase chain reaction. J. Biomol. Tech. 15:155–166.
423 5. Daniëls, B., de landtsheer, A., Dreesen, R., Davey, M. W., and Keulemans, J. 2012. Real-time
424 PCR as a promising tool to monitor growth of *Venturia* spp. in scab-susceptible and -resistant
425 apple leaves. Eur. J. Plant Pathol. 134:821–833.
426 6. Fischer, J. M. M., Savi, D. C., Aluizio, R., May De Mio, L. L., and Glienke, C. 2017.
427 Characterization of *Monilinia* species associated with brown rot in stone fruit in Brazil. Plant
428 Pathol. 66:423–436.
429 7. Food and Agriculture Organization of the United Nations (FAO). 2022. FAOSTAT, FAO's
430 food and agriculture database. Available at: http://www.fao.org/faostat/en/#data/QCL
431 [Accessed June 22, 2022].
432 8. Gianetti, G., Grosso, S., Ortalda, E., and Di Mauro, G. 2012. Segnalata in Piemonte *Ramularia*
433 *eucalypti* su mele frigoconservate. Inf. Agrar. 9:72–73.
434 9. Gianetti, G., Ortalda, E., and Grosso, S. 2016. Nuove acquisizioni su bioepidemiologia e
435 distribuzione di *Ramularia mali* - Quaderni dell'agricoltura on line. Available at:
436 https://quaderniagricoltura.regione.piemonte.it/articoli/analisi-e-ricerche/9-articolo-
437 secondario-4.html [Accessed June 22, 2022].

438 10. Giordani, L., Grosso, S., Vittone, G., and Nari, L. 2011. Rinvenuta in Piemonte su pere
439 frigoconservate *Ramularia eucalypti*. Inf. Agrar. 25:66–67.
440 11. Hariharan, G., and Prasannath, K. 2021. Recent Advances in Molecular Diagnostics of Fungal
441 Plant Pathogens: A Mini Review. Front. Cell. Infect. Microbiol. 10:600234.
442 12. Huang, W. W., Ge, X. Y., Huang, Y., Chai, X. T., Zhang, L., Zhang, Y. X., et al. 2021. High-
443 yield strain of fusidic acid obtained by atmospheric and room temperature plasma mutagenesis
444 and the transcriptional changes involved in improving its production in fungus *Fusidium*
445 *coccineum*. J. Appl. Microbiol. 130:405–415.
446 13. Kumari, S., Tayal, P., Sharma, E., and Kapoor, R. 2014. Analyses of genetic and pathogenic
447 variability among *Botrytis cinerea* isolates. Microbiol. Res. 169:862–872.
448 14. Lindner, L. 2013. Phytopathologische Diagnostik, Jahresbericht 2013. Available at:
449 http://www.laimburg.it/en/projects-publications/diagnostics.asp [Accessed June 22, 2022].
450 15. Liu, J., Huang, S., Sun, M., Liu, S., Liu, Y., Wang, W., Zhang, X., Wang, H., and Hua, W.
451 2012. An improved allele-specific PCR primer design method for SNP marker analysis and
452 its application. Plant Methods 8:34.
453 16. McCartney, H. A., Foster, S. J., Fraaije, B. A., and Ward, E. 2003. Molecular diagnostics for
454 fungal plant pathogens. Pest Manag. Sci. 59:129–142.
455 17. McGrann, G. R. D., Andongabo, A., Sjökvist, E., Trivedi, U., Dussart, F., Kaczmarek, M., et
456 al. 2016. The genome of the emerging barley pathogen *Ramularia collo-cygni*. BMC
457 Genomics 17:584.
458 18. Mcmullen, M., and Petter, F. 2014. Report of the EPPO Workshop on setting Ct cut-off values
459 for real-time PCR. EPPO Bull. 44:165.
460 19. EPPO. 2019. PM 7/98 (4) Specific requirements for laboratories preparing accreditation for a
461 plant pest diagnostic activity. EPPO Bull. 49:530–563.
462 20. Pfaffl, M. 2004. Quantification strategies in real-time PCR. In A-Z of quantitative PCR, ed.
463 Stephen A. Bustin. La Jolla, CA, USA, p. 87–112.

21. Prencipe, S., Siciliano, I., Gatti, C., Garibaldi, A., Gullino, M. L., Botta, R., et al. 2018. Several species of *Penicillium* isolated from chestnut flour processing are pathogenic on fresh chestnuts and produce mycotoxins. Food Microbiol. 76:396–404.

22. Prencipe, S., Sillo, F., Garibaldi, A., Gullino, M. L., and Spadaro, D. 2020. Development of a sensitive TaqMan qPCR assay for detection and quantification of *Venturia inaequalis* in apple leaves and fruit and in air samples. Plant Dis. 104:2851–2859.

23. Rahman, M. D. M., Lim, S. J., and Park, Y. C. 2022. Development of Single Nucleotide Polymorphism (SNP)-Based Triplex PCR Marker for Serotype-specific *Escherichia coli* Detection. Pathogens 11:115.

24. Sanzani, S.M., Li Destri Nicosia, M.G., Faedda, R., Cacciola, S.O. and Schena L. 2014. Use of quantitative PCR detection methods to study biocontrol agents and phytopathogenic fungi and oomycetes in environmental samples J. Phytopathol. 162:1-13.

25. Schena, L., Nigro, F., Ippolito, A., and Gallitelli, D. 2004. Real-time quantitative PCR: A new technology to detect and study phytopathogenic and antagonistic fungi. Eur. J. Plant Pathol. 110:893–908.

26. Schena, L., Li Destri Nicosia, M.G., Sanzani, S.M., Faedda, R., Ippolito, A., and Cacciola, S.O. 2013. Development of quantitative PCR detection methods for phytopathogenic fungi and oomycetes. J. Plant Pathol. 95:7-24.

27. Schena, L., Abdelfattah, A., Mosca, S., Li Destri Nicosia, M.G., Agosteo, G.E., and Cacciola, S.O. 2017. Quantitative detection of *Colletotrichum godetiae* and *C. acutatum sensu strictu* in the phyllosphere and carposphere of olive during four phenological phases. Eur. J. Plant Pathol. 149:337-347.

28. Spadaro D., Agustí N., Franco Ortega S., Hurtado Ruiz M.A. (2020) Diagnostics and identification of diseases, insects and mites. In: Integrated pest and disease management in greenhouse crops (Gullino M.L., Albajes R., Nicot P., eds.), Springer, Dordrecht, The Netherlands, 231-258.

490 29. Spadaro, D., Torres, R., Errampalli, D., Everett, K., Ramos, L., Mari, M. 2019. Postharvest
491 Diseases of Pome Fruit. In: Postharvest pathology of fresh horticultural produce (Palou L.,
492 Smilanick J.L., eds.), CRC Press, Boca Raton, FL, USA, 55-109.
493 30. Stam, R., Münsterkötter, M., Pophaly, S. D., Fokkens, L., Sghyer, H., Güldener, U., et al.
494 2018. A new reference genome shows the one-speed genome structure of the barley pathogen
495 *Ramularia collo-cygni*. Genome Biol. Evol. 10:3243–3249.
496 31. Taylor, J. M. G., Paterson, L. J., and Havis, N. D. 2010. A quantitative real-time PCR assay
497 for the detection of *Ramularia collo-cygni* from barley (*Hordeum vulgare*). Lett. Appl.
498 Microbiol. 50:493–499.
499 32. Turzhanova, A., Khapilina, O. N., Tumenbayeva, A., Shevtsov, V., Raiser, O., and Kalendar,
500 R. 2020. Genetic diversity of *Alternaria* species associated with black point in wheat grains.
501 Peer J. 8:e9097.
502 33. Videira, S. I. R., Groenewald, J. Z., Braun, U., Shin, H. D., and Crous, P. W. 2016. All that
503 glitters is not *Ramularia*. Stud. Mycol. 89:49–163.
504 34. Videira, S. I. R., Groenewald, J. Z., Verkley, G. J. M., Braun, U., and Crous, P. W. 2015a.
505 The rise of *Ramularia* from the *Mycosphaerella* labyrinth. Fungal Biol. 119:823–843.
506 35. Videira, S. I. R., Groenewald, J. Z., Kolecka, A., van Haren, L., Boekhout, T., and Crous, P.
507 W. 2015b. Elucidating the *Ramularia eucalypti* species complex. Persoonia 34:50–64.
508 36. Wang, J., Jacobs, J. L., Byrne, J. M., and Chilvers, M. I. 2015. Improved diagnoses and
509 quantification of *Fusarium virguliforme*, causal agent of soybean sudden death syndrome.
510 Phytopathology. 105:378–387.
511 37. Wieczorek, T. M., Jørgensen, L. N., Hansen, A. L., Munk, L., and Justesen, A. F. 2014. Early
512 detection of sugar beet pathogen *Ramularia beticola* in leaf and air samples using qPCR. Eur.
513 J. Plant Pathol. 138:775–785.

514 38. Xu, T., Yao, Z., Liu, J., Zhang, H., Din, G. M. U., Zhao, S., et al. 2020. Development of
515 droplet digital PCR for the detection of *Tilletia laevis*, which causes common bunt of wheat,
516 based on the SCAR marker derived from ISSR and real-time PCR. Sci. Rep. 10:16106.
517 39. Zijlstra, C., Lund, I., Justesen, A. F., Nicolaisen, M., Jensen, P. K., Bianciotto, V., et al. 2011.
518 Combining novel monitoring tools and precision application technologies for integrated high-
519 tech crop protection in the future (a discussion document). Pest Manag. Sci. 67:616–625.

Tables

Table 1. Species, host, source of isolation and origin of the fungal species used in this study to develop the qPCR assay.

Species	Strain ID	Host	Source	Origin
Ramularia mali	MG 2C; A1; 1/1; 1/2; 2/7; 2/9; 3/1; 3/6; 3/8; 3/10; 4/1; 4/4; 4/5; 4/6; 5/2; 5/4; 5/6; 8/3;	*Malus domestica* 'Golden'	Fruit	Italy
R. mali	8/7; 8/9; 8/11; 9/1; 9/2; 9/7; 9/12; 10/1; 10/2; 11/1; 11/4; 11/5; 11/10; 12/1; 12/3; 12/8	*Malus domestica* 'Ambrosia'	Fruit	Italy
R. mali	CBS 129581	*Malus domestica* 'Ambrosia'	Fruit	Italy
Ramularia grevilleana	CBS 719.84	*Fragaria ananassa*	-	New Zealand
Ramularia vizellae	CBS 115981	*Malus* dead leaf litter	Leaf	The Netherlands
Ramularia malicola	CBS 119227	*Malus* sp.	-	USA, Missouri
Ramularia eucalypti	CBS 120726	*Corymbia grandifolia*	Leaf	Italy
Ramularia glennii	CBS 120727	*Corymbia grandifolia*	Leaf	Italy
Venturia inaequalis	CBS 815.69	*Malus sylvestris*	Fruit	The Netherlands
Venturia asperata	IRHS 2345	*Malus domestica*	Fruit	France
Monilia fructicola	MON1	*Malus domestica*	Leaf	Italy
Monilia laxa	BM8	*Prunus avium*	Fruit	Italy
Colletotrichum fioriniae	CVG174	*Salvia leucantha*	Leaf	Italy
Epicoccum nigrum	CDS51	*Fragaria ananassa*	Leaf	Italy
Stemphylium sp.	SDIVB	*Pyrus communis*	Fruit	Italy
Cladosporium cladosporioides	CLAD1	*Malus domestica*	Leaf	Italy
Alternaria aff. arborescens	GR8	*Prunus avium*	Fruit	Italy
Alternaria aff. arborescens	MALT1	*Malus domestica*	Leaf	Italy
Alternaria alternata	CDS032	*Solanum lycopersicum*	Leaf	Italy
Botrytis cinerea	CDS002	*Fragaria ananassa*	Stem	Italy
Fusarium sp.	FUS31	*Malus domestica*	Leaf	Italy
Penicillium expansum	PEN6	*Malus domestica*	Fruit	Italy
Penicillium expansum	X5	*Castanea sativa*	Fruit	Albania
Golubevia pallescens	CVG625	*Malus domestica*	Fruit	Italy
G. pallescens	CVG414	*Malus domestica*	Fruit	Italy
G. pallescens	CVG704	*Malus domestica*	Fruit	Italy
Tilletiopsis washingtoniensis	CVG604	*Malus domestica*	Fruit	Italy

528 **Table 2.** Primer pairs designed on *rpb2* and *cmdA* genes used in this study for the detection of *R.*
529 *mali.* In bold: conserved polymorphisms in all *R. mali* strains.
530

Primers name	Amplicon size (bp)	Gene	Sequence (5'-3')	End-point PCR annealing temperature
396F/736R	378	*rpb2*	GACTTTATGCGACA**A**CGTGG GCTCTATCCTCTTCGCTTATACC**G**	65
503F/736R	339	*rpb2*	GTACGACCCTGTT**T**TGGAGC GCTCTATCCTCTTCGCTTATACC**G**	60
396F/503R	59	*rpb2*	GACTTTATGCGACA**A**CGTGG GCTCCAA**A**ACAGGGTCGTAC	60
135F/396R	297	*rpb2*	GGGTGATCAAAAGAAAGCGTC**G** AGATCCATACCACG**T**TGTCG	60
135F/503R	327	*rpb2*	GGGTGATCAAAAGAAAGCGTC**G** GCTCCAA**A**ACAGGGTCGTAC	60
503FN/772R	342	*rpb2*	CTGGAAGAGTACGACCCTGTT**T** GACCAAGCCCATCCAGCC**A**	65
503FN/736R	378	*rpb2*	CTGGAAGAGTACGACCCTGTT**T** GCTCTATCCTCTTCGCTTATACC**G**	65
cmdFC/cmdRC	228	*cmdA*	CTCGGCCAGAACCCCTC**G** CTCTCCAATACTCGTCATGAC**G**	65
cmdFL/cmdRL	237	*cmdA*	TCGCTCGGCCAGAACCCCTC**G** CAACTTCTCTCCAATACTCGTCATGAC**G**	65

531
532

533 **Table 3**. Results obtained (Cq values ± standard deviation) and the estimated number of *R. mali*
534 cells/µL ± standard deviation for the asymptomatic *Malus domestica* 'Jeromine', 'Gala', 'Golden
535 Delicious', 'Opal' and 'Ambrosia' samples obtained using the qPCR assay.
536

Sample ID	Cultivar	Year of sampling	Cq mean ± SD[a]		Mean number of *R. mali* cells/µL ± SD[a]	
JERA	Jeromine	2020	31.43	0.68	30.99	± 11.94
JERB	Jeromine	2020	30.09	0.15	62.13	± 6.23
JERC	Jeromine	2020	32.41	0.48	13.30	± 4.23
JERD	Jeromine	2020	Negative	-	-	-
JERE	Jeromine	2020	Negative	-	-	-
JERF	Jeromine	2020	Negative	-	-	-
JERG	Jeromine	2020	Negative	-	-	-
GALA	Gala	2020	32.79	0.15	9.76	± 0.98
GALB	Gala	2020	31.45	0.69	30.60	± 11.91
GALC	Gala	2020	30.93	0.30	33.10	± 6.46
GALD	Gala	2020	34.25	0.64	3.78	± 1.58
GALE	Gala	2020	Negative	-	-	-
GALF	Gala	2020	Negative	-	-	-
GALG	Gala	2020	Negative	-	-	-
GOA	Golden delicious	2020	Negative	-	-	-
GOB	Golden delicious	2020	35.18	0.44	1.89	± 0.65
GOC	Golden delicious	2020	36.24	0.66	2.09	± 1.13
OP1	Opal	2020	34.34	1.12	3.28	± 2.70
OP2	Opal	2020	35.12	1.57	2.09	± 2.26
OP3	Opal	2020	35.01	1.17	1.24	± 3.08
A1	Ambrosia	2020	Negative			-
A2	Ambrosia	2020	35.02	1.23	5.39	± 2.93
A3	Ambrosia	2020	36.47	1.80	0.99	± 1.02
A4	Ambrosia	2020	37.34	0.54	11.39	± 6.54
A5	Ambrosia	2020	Negative	-	-	-
A6	Ambrosia	2020	35.41	1.01	1.73	± 1.19
A7	Ambrosia	2020	Negative	-	-	-
A8	Ambrosia	2020	Negative	-	-	-
A9	Ambrosia	2020	33.26	0.53	7.43	± 2.53
A10	Ambrosia	2020	34.28	0.67	4.58	±1.60
A11	Ambrosia	2020	35.42	0.77	1.77	± 0.89
A12	Ambrosia	2020	35.76	0.97	1.03	± 0.88
AMBA	Ambrosia	2018	34.60	0.60	2.97	± 1.17
AMBB	Ambrosia	2018	33.13	0.48	7.86	± 2.51
AMBC	Ambrosia	2018	30.01	0.42	63.88	± 17.97

Sample ID	Cultivar	Year of sampling	Cq mean ± SD[a]		Mean number of *R. mali* cells/µL ± SD[a]	
AM1	Ambrosia	2018	34.21	0.89	5.51	± 0.77
AM13	Ambrosia	2018	35.58	0.14	1.48	± 0.14
AM14	Ambrosia	2018	35.97	0.79	1.07	± 0.72
AM15	Ambrosia	2018	34.72	0.41	2.68	± 0.73
ST1	Story Inored	2020	Negative	-	-	-
ST2	Story Inored	2020	Negative	-	-	-
ST3	Story Inored	2020	35.23	0.27	1.89	± 0.34
ST5	Story Inored	2020	34.89	0.71	2.52	± 1.01

[a] The values are expressed as the mean ± SD (n=9)

539 **Figure captions**
540
541 **Figure 1.** Best scoring Maximum Likelihood tree based on the concatenated RNA polymerase II
542 second largest subunit and calmodulin sequence datasets. The numbers at the major nodes indicate
543 the bootstrap value from 1000 bootstrapped datasets. Branches with lower bootstrap values than 70%
544 are not shown. Phylogeny was rooted by *R. nyssicola* (CBS 127665) as reported in Videira et al.
545 (2015b). Evolutionary analyses were conducted using MEGA, version 6.
546
547 **Figure 2**. Standard curve using genomic DNA of *R. mali* CBS 129581 strain showing the correlation
548 between the *R. mali* DNA amount (from 10 ng to 100 fg) and the Cq values from three qPCR runs
549 performed in triplicates. Error bars represent the standard deviation of Cq values between runs.
550
551 **Figure 3**. Standard curve using genomic DNA of *R. mali* CBS 129581 strain diluted in 10 ng of *Malus*
552 *domestica* DNA 'Ambrosia' and 'Golden Delicious' respectively. Figure showed the correlation
553 between the *R. mali* DNA amount and the Cq values obtained from three qPCR runs performed in
554 triplicates. Error bars represent the standard deviation of Cq values between runs.
555
556 **Figure 4.** Quantification of *R. mali* in *Malus domestica* 'Ambrosia' leaf disks artificially inoculated
557 at different concentrations, from 5×10^3 conidia/mL to 5 conidia/mL , with qPCR assay. Runs were
558 performed in triplicates. Error bars represent the standard deviation of Cq values between runs.
559
560 **Figure 5.** Detection and quantification of *R. mali* in symptomatic 'Ambrosia' fruits with the qPCR
561 assay. Runs were performed in triplicates. Error bars represent the standard deviation of Cq values
562 between runs.
563

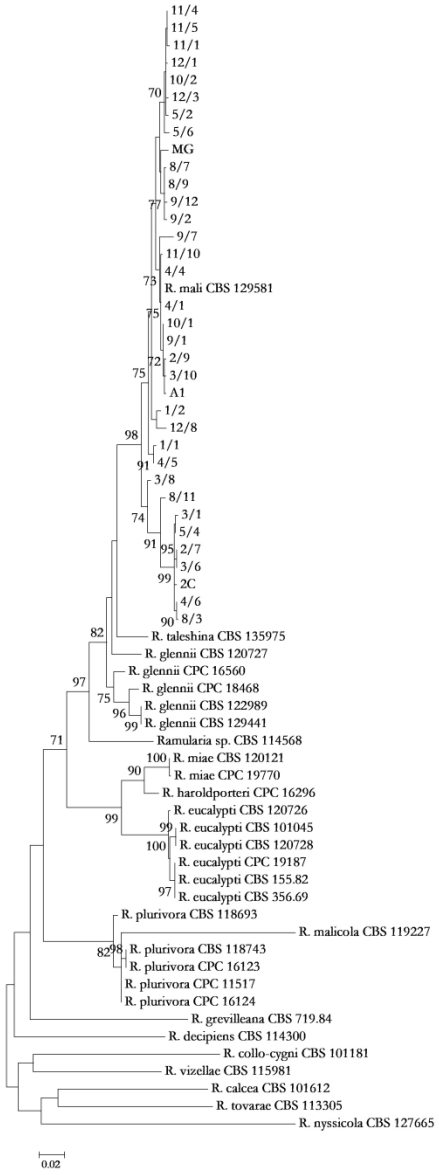

Figure 1. Best scoring Maximum Likelihood tree based on the concatenated RNA polymerase II second largest subunit and calmodulin sequence datasets. The numbers at the major nodes indicate the bootstrap value from 1000 bootstrapped datasets. Branches with lower bootstrap values than 70% are not shown. Phylogeny was rooted by *R. nyssicola* (CBS 127665) as reported in Videira et al. (2015b). Evolutionary analyses were conducted using MEGA, version 6.

190x338mm (300 x 300 DPI)

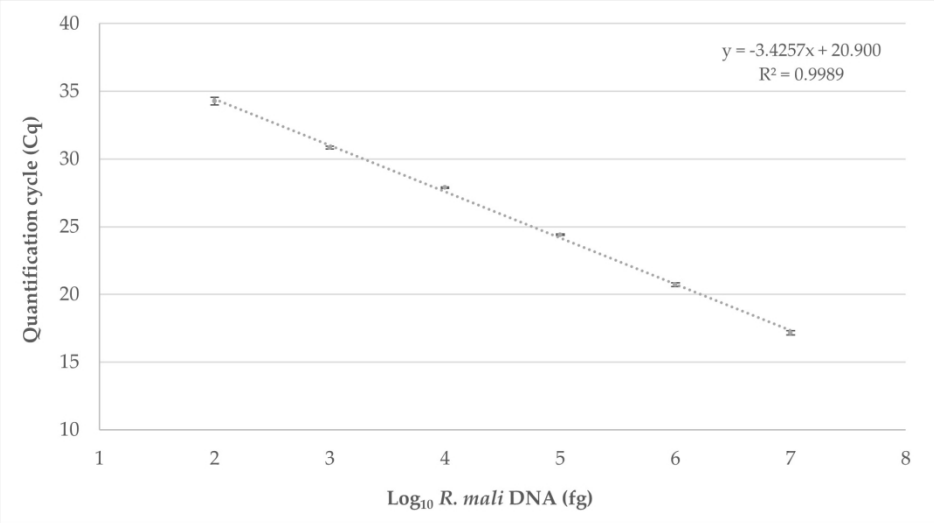

Figure 2. Standard curve using genomic DNA of R. mali CBS 129581 strain showing the correlation between the *R. mali* DNA amount (from 10 ng to 100 fg) and the Cq values from three qPCR runs performed in triplicates. Error bars represent the standard deviation of Cq values between runs.

178x100mm (300 x 300 DPI)

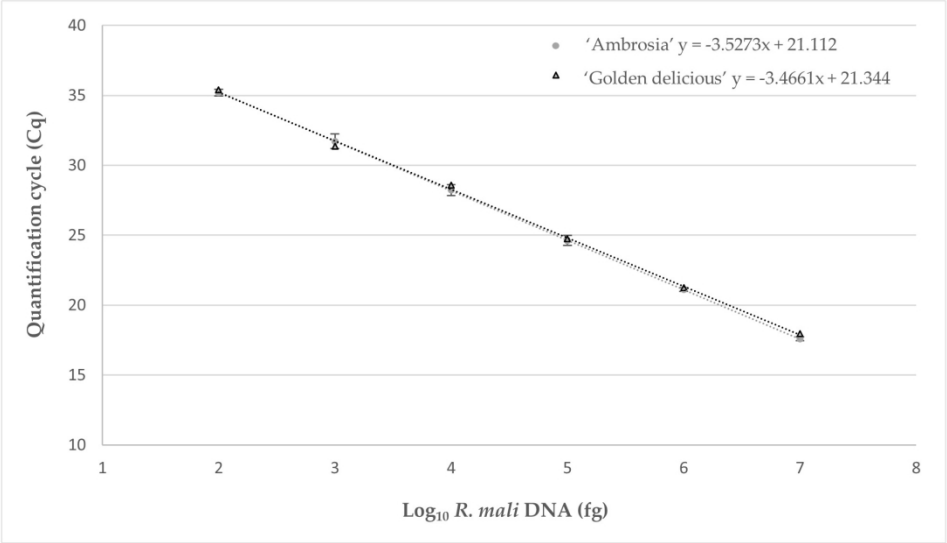

Figure 3. Standard curve using genomic DNA of *R. mali* CBS 129581 strain diluted in 10 ng of *Malus domestica* DNA 'Ambrosia' and 'Golden Delicious' respectively. Figure showed the correlation between the *R. mali* DNA amount and the Cq values obtained from three qPCR runs performed in triplicates. Error bars represent the standard deviation of Cq values between runs.

178x100mm (300 x 300 DPI)

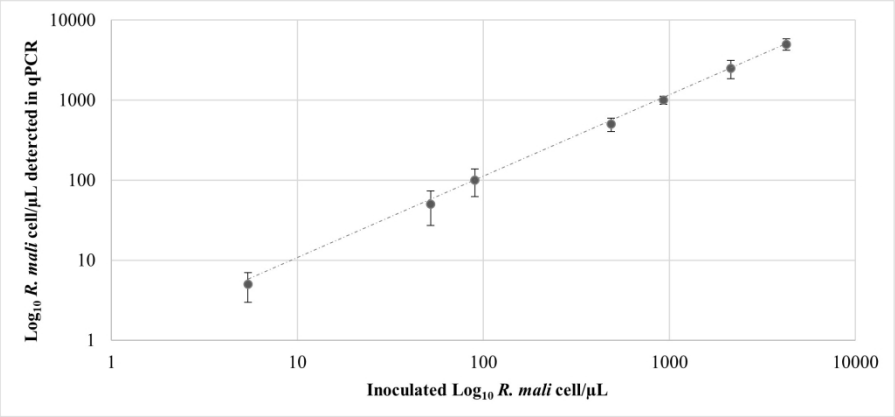

Figure 4. Quantification of *R. mali* in *Malus domestica* 'Ambrosia' leaf disks artificially inoculated at different concentrations, from 5×103 conidia/mL to 5 conidia/mL , with qPCR assay. Runs were performed in triplicates. Error bars represent the standard deviation of Cq values between runs.

208x100mm (300 x 300 DPI)

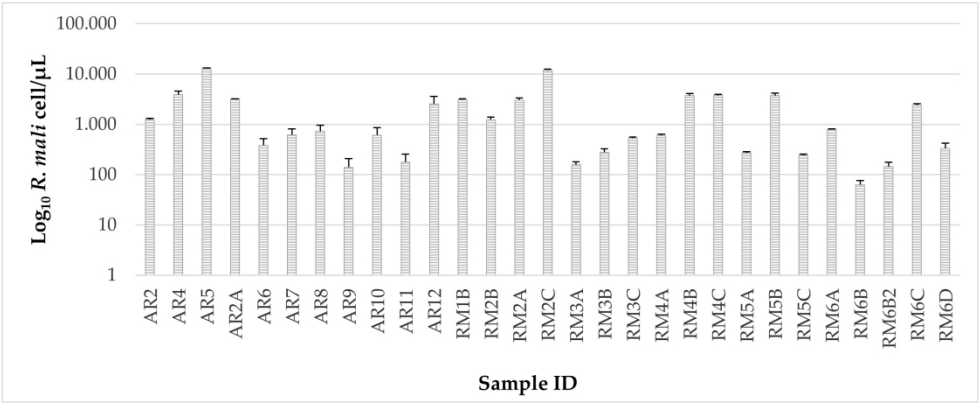

Figure 5. Detection and quantification of *R. mali* in symptomatic 'Ambrosia' fruits with the qPCR assay. Runs were performed in triplicates. Error bars represent the standard deviation of Cq values between runs.

208x100mm (300 x 300 DPI)

Supplementary material

Supplementary Table 1. Sample ID and accession numbers of the RNA polymerase II second largest subunit and calmodulin genes for all the *Ramularia* strains used for the phylogenetic analyses; *R. mali* sequences obtained in this study are in bold.

Species	Sample ID	*rpb2* accession numbers	*cmdA* accession numbers
R. calcea	CBS 101612	KJ504656	KJ504512
R. collo-cygni	CBS 101181	KJ504657	KJ504657
R. decipiens	CBS 114300	KJ504658	KJ504514
R. eucalypti	CBS 155,82	KJ504660	KJ504516
R. eucalypti	CBS 356,69	KJ504661	KJ504517
R. eucalypti	CBS 101045	KJ504662	KJ504518
R. eucalypti	CBS 120726 ^{T}, CPC 13043	KJ504663	KJ504519
R. eucalypti	CBS 120728, CPC 13304	KJ504664	KJ504520
R. eucalypti	CPC 19187	KJ504668	KJ504523
R. glenii	CBS 120727, CPC 13046	KJ504638	KJ504498
R. glenii	CBS 122989, CPC 15195	KJ504639	KJ504639
R. glenii	CBS 129441 ^{T}	KJ504640	KJ504500
R. glenii	CPC 16560	KJ504643	KJ504501
R. glenii	CPC 18468	KJ504646	KJ504503
R. gravilleana	CBS 719.84	KP894662	KP894881
R. haroldporteri	CBS 137272 ^{T}, CPC 16296	KJ504637	KJ504497
R. mali	CBS 129581 ^{T}	KJ504649	KJ504506
R. mali	MG	**OM417524**	**OM966598**
R. mali	2C	**OM417525**	**OM966599**
R. mali	A1	**OM417526**	**OM966600**
R. mali	1/1	**OM417527**	**OM966601**
R. mali	1/2	**OM417528**	**OM966602**
R. mali	2/7	**OM417529**	**OM966603**
R. mali	2/9	**OM417530**	**OM966604**
R. mali	3/1	**OM417531**	**OM966605**
R. mali	3/6	**OM417532**	**OM966606**
R. mali	3/8	**OM417533**	**OM966607**
R. mali	3/10	**OM417534**	**OM966608**
R. mali	4/1	**OM417535**	**OM966609**
R. mali	4/4	**OM417536**	**OM966610**
R. mali	4/5	**OM417537**	**OM966611**
R. mali	4/6	**OM417538**	**OM966612**
R. mali	5/2	**OM417539**	**OM966613**
R. mali	5/4	**OM417540**	**OM966614**
R. mali	5/6	**OM417541**	**OM966615**
R. mali	8/3	**OM417542**	**OM966616**
R. mali	8/7	**OM417543**	**OM966617**
R. mali	8/9	**OM417544**	**OM966618**

Species	**Sample ID**	***rpb2* accession numbers**	***cmdA* accession numbers**
R. mali	8/11	**OM417545**	**OM966619**
R. mali	9/1	**OM417546**	**OM966620**
R. mali	9/2	**OM417547**	**OM966621**
R. mali	9/7	**OM417548**	**OM966622**
R. mali	9/12	**OM417549**	**OM966623**
R. mali	10/1	**OM417550**	**OM966624**
R. mali	10/2	**OM417551**	**OM966625**
R. mali	11/1	**OM417552**	**OM966626**
R. mali	11/4	**OM417553**	**OM966627**
R. mali	11/5	**OM417554**	**OM966628**
R. mali	11/10	**OM417555**	**OM966629**
R. mali	12/1	**OM417556**	**OM966630**
R. mali	12/3	**OM417557**	**OM966631**
R. mali	12/8	**OM417558**	**OM966632**
R. malicola	CBS 119227	KX288635	KX289099
R. miae	CBS 120121 ^{T}, CPC 12736	KJ504672	KJ504525
R. miae	CPC 19770	KJ504676	KJ504528
R. nyssicola	CBS 127665 ^{T}	KJ504636	KJ504496
R. plurivora	CBS 118693, CPC 12206	KJ504650	KJ504507
R. plurivora	CBS 118743 ^{T}, CPC 12207	KJ504651	KJ504508
R. plurivora	CPC 11517	KJ504652	KJ504509
R. plurivora	CPC 16123	KJ504653	KJ504510
R. plurivora	CPC 16124	KJ504654	KJ504511
Ramularia sp.	CBS 114568	KJ504659	KJ504515
R. taleshina	CBS 135975 ^{T}	KY967397	-
R. tovarae	CBS 113305	KJ504678	KJ504529
R. vizellae	CBS 115981	KP894722	KP894928

- Sequence not available

Supplementary Table 2. Results obtained (Cq values ± standard deviation) with the DNA (≃1-10 ng) of *R. mali* strains used to evaluate the specificity of the qPCR assay.

Sample ID	Species	Year of isolation	City	Ct Mean ±SD[a]
MG	*R. mali*	2019	Provence (France)	17.36 ± 0.30
2C	*R. mali*	2011	Centallo (CN)	19.34 ± 0.29
A1	*R. mali*	2011	Centallo (CN)	18.65 ± 0.41
1/1	*R. mali*	2019	Villafalletto (CN)	17.61 ± 0.03
1/2	*R. mali*	2019	Villafalletto (CN)	17.43 ± 0.10
2/7	*R. mali*	2019	Villafalletto (CN)	19.36 ± 0.12
2/9	*R. mali*	2019	Villafalletto (CN)	18.13 ± 0.13
3/1	*R. mali*	2019	Villafalletto (CN)	18.88 ± 0.05
3/6	*R. mali*	2019	Villafalletto (CN)	19.22 ± 0.84
3/8	*R. mali*	2019	Centallo (CN)	19.36 ± 0.62
3/10	*R. mali*	2019	Centallo (CN)	19.98 ± 0.96
4/1	*R. mali*	2019	Centallo (CN)	19.19 ± 0.50
4/4	*R. mali*	2019	Centallo (CN)	18.98 ± 0.85
4/5	*R. mali*	2019	Centallo (CN)	17.95 ± 0.18
4/6	*R. mali*	2019	Centallo (CN)	18.49 ± 0.82
5/2	*R. mali*	2019	Scarnafigi (CN)	18.23 ± 0.45
5/4	*R. mali*	2019	Scarnafigi (CN)	19.45 ± 0.72
5/6	*R. mali*	2019	Scarnafigi (CN)	19.87 ± 0.01
8/3	*R. mali*	2020	Saluzzo (CN)	16.69 ± 0.18
8/7	*R. mali*	2020	Saluzzo (CN)	19.09 ± 0.64
8/9	*R. mali*	2020	Saluzzo (CN)	17.81 ± 0.37
8/11	*R. mali*	2020	Saluzzo (CN)	17.59 ± 0.32
9/1	*R. mali*	2020	Caraglio (CN)	18.70 ± 0.48
9/2	*R. mali*	2020	Caraglio (CN)	17.80 ± 0.75
9/7	*R. mali*	2020	Caraglio (CN)	18.54 ± 0.56
9/12	*R. mali*	2020	Caraglio (CN)	19.26 ± 0.72
10/1	*R. mali*	2020	Villafalletto (CN)	19.02 ± 0.44
10/2	*R. mali*	2020	Villafalletto (CN)	18.16 ± 0.91
11/1	*R. mali*	2020	Saluzzo (CN)	19.01 ± 0.52
11/4	*R. mali*	2020	Saluzzo (CN)	19.36 ± 0.45
11/5	*R. mali*	2020	Saluzzo (CN)	19.39 ± 0.70
11/10	*R. mali*	2020	Saluzzo (CN)	19.03 ± 0.30
12/2	*R. mali*	2020	San Benigno (CN)	15.36 ± 0.29
12/3	*R. mali*	2020	San Benigno (CN)	18.26 ± 0.54
12/8	*R. mali*	2021	San Benigno (CN)	16.61 ± 0.39

[a] The values are expressed as the mean ± SD (n=9)

Supplementary Table 3. Results obtained (Cq values ± standard deviation) and the estimated number of *R. mali* cells/µL ± standard deviation for the symptomatic *Malus* × *domestica* 'Ambrosia' samples obtained using the qPCR assay.

Sample ID	Cultivar	Year of sampling	Cq mean ± SD[a]	Mean number of *R. mali* cells/µL ± SD[a]
AR2	Ambrosia	2018/2019	25.56 ± 0.07	1.25×10^3 ± 60.25
AR4	Ambrosia	2018/2019	23.87 ± 0.26	3.89×10^3 ± 674.13
AR5	Ambrosia	2018/2019	22.05 ± 0.01	1.31×10^4 ± 120.25
AR2A	Ambrosia	2018/2019	24.16 ± 0.01	3.20×10^3 ± 23.10
AR6	Ambrosia	2018/2019	27.36 ± 0.53	3.82×10^2 ± 133.48
AR7	Ambrosia	2018/2019	26.63 ± 0.46	6.21×10^2 ±190.77
AR8	Ambrosia	2018/2019	26.40 ± 0.48	7.27×10^2 ± 232.65
AR9	Ambrosia	2018/2019	28.90 ± 0.74	1.40×10^2 ± 66.58
AR10	Ambrosia	2018/2019	26.68 ± 0.63	6.12×10^2 ± 250.39
AR11	Ambrosia	2018/2019	28.52 ± 0.68	1.79×10^2 ± 79.11
AR12	Ambrosia	2018/2019	24.55 ± 0.63	2.56×10^3 ± 104.76
RM1B	Ambrosia	2019/2020	24.19 ± 0.05	3.12×10^3 ± 105.09
RM2B	Ambrosia	2019/2020	25.57 ± 0.19	1.24×10^3 ± 154.42
RM2A	Ambrosia	2019/2020	24.24 ± 0.17	3.03×10^3 ± 338.67
RM2C	Ambrosia	2019/2020	22.19 ± 0.05	1.20×10^4 ± 415.39
RM3A	Ambrosia	2019/2020	28.63 ± 0.21	1.59×10^2 ± 22.37
RM3B	Ambrosia	2019/2020	27.79 ± 0.25	2.79×10^2 ± 46.18
RM3C	Ambrosia	2019/2020	26.83 ± 0.09	5.31×10^2 ± 31.29
RM4A	Ambrosia	2019/2020	26.65 ± 0.09	5.98×10^2 ± 37.48
RM4B	Ambrosia	2019/2020	23.93 ± 0.14	3.74×10^3 ± 352.34
RM4C	Ambrosia	2019/2020	23.92 ± 0.09	3.75×10^3 ± 224.16
RM5A	Ambrosia	2019/2020	27.83 ± 0.09	2.71×10^2 ± 15.99
RM5B	Ambrosia	2019/2020	23.93 ± 0.17	3.73×10^3 ± 421.69
RM5C	Ambrosia	2019/2020	28.00 ± 0.09	2.42×10^2 ± 14.74
RM6A	Ambrosia	2019/2020	26.23 ± 0.04	7.95×10^2 ± 22.95
RM6B	Ambrosia	2019/2020	30.02 ± 0.34	6.28×10^1 ± 14.12
RM6B2	Ambrosia	2019/2020	28.78 ± 0.33	1.44×10^2 ± 32.04
RM6C	Ambrosia	2019/2020	24.58 ± 0.10	2.41×10^3 ± 163.39
RM6D	Ambrosia	2019/2020	27.54 ± 0.41	3.35×10^2 ± 91.85
Blank	Water	-	00.00 ± 0.00	-

[a] The values are expressed as the mean ± SD (n=9)

Appendix I - Working protocol for diagnosis of *Ramularia mali* with specific qPCR assay

1. Nucleic acid extraction

DNA is extracted (a) from 100 mg of fresh-weight mycelium taken with a scalpel blade from a culture of the fungus, grown on MEA at 25±1 °C in the dark for 15 days, or (b) from 100 mg of fresh-weight fruit peel, cut with a scalpel. Fresh-weight mycelium is then extracted according to the manufacturer's instructions, with Omega E.Z.N.A. Fungal DNA Mini Kit (VWR, USA), with the final elution step with 50 µL of Elution Buffer. Fresh-weight fruit peel is freeze-dried and ground with liquid nitrogen, then DNA is extracted with E.Z.N.A. Plant DNA kit (VWR) following the manufacturer's instructions, with the final elution step with 50 µL of Elution Buffer.

DNA is stored at 2-8 °C for immediate use or at approximately - 20 °C for later use.

2. Quantitative Polymerase Chain Reaction (qPCR)

2.1 General information

- This qPCR assay is used for the detection of *Ramularia mali* in symptomatic or asymptomatic apple fruits or using a pure culture of the fungus.
- The primers are designed in the calmodulin gene (sequences of the calmodulin gene are available in Genbank, accession numbers OM966598 to OM966632, and KJ504506).
- Amplicon size: 237 bp.
- Primer names and sequences: cmdFL 5'-TCGCTCGGCCAGAACCCCTCG-3'
  cmdRL 5'-CAACTTCTCTCCAATACTCGTCATGACG-3'
- Amplification is considered positive until 37 amplification cycles. After 37 cycles, amplification of the species *R. glennii*, *R. malicola* and *R. eucalypti* occasionally occur.
- The assay is able to quantify from 10 ng to 100 fg of target DNA, with a LOD quantification cycle (Cq) of 34.40 ± 0.32.

2.2 Master Mix

Reactions are carried out in a final volume of 10 µL, with 1 µL of DNA (~10 ng), 0.25 µL of each primer (working solution 1 µM) and 5 µL of SsoAdvanced Universal SYBR Green Supermix (working solution 2x; Bio-Rad, USA).

Water is prepared by autoclaving deionized or distilled water, or using 0.20 µm filter, or using a sterile nuclease-free water.

2.3 PCR conditions

95 °C for 10 min, followed by 40 cycles of annealing temperature (63 °C) for 1 min and 95 °C for 15 s. After each run, one cycle of melting curve step was conducted by ramping the temperature from 60 °C to 90 °C.

2.4 Procedural information

In each qPCR experiment, a negative and a positive control and 10-fold dilutions of standard DNA, at least in triplicate should be included.

To monitor contamination during nucleic acid extraction, each extraction should include a known uninfected sample, if this is not possible, perform the extraction only with buffers reagents.

To monitor contamination during the preparation of the reaction mix, each run of amplification should include a negative control using nuclease-free water to prepare the reaction mix.

To monitor the efficiency of amplification each run of amplification should include a positive control, DNA of target extracted from pure culture or extracted from a symptomatic sample, and 2 or 3 10-fold dilutions of standard DNA (target DNA from a reference strain) with known amplification cycles.

3. Further information

To quantify the target DNA in different samples, a standard curve is used as internal control and carried out in triplicate. The Cq values obtained by qPCR are interpolated with the generated standard curve. As the genome size of *R. mali* is not available, the approximate number of cells/µl is calculated by dividing the DNA quantity by the mean weight of the genome of other sequenced *Ramularia* species (0.0000351 ng; McGrann et al., 2016; Stam et al., 2018; Huang et al., 2021).

For early detection of *R. mali* on asymptomatic apples, sampling could be carried out at harvest and after one or two months of storage. For general diagnostic purpose, the qPCR assay was validated on samples cold-stored for 4 months with no influence on results obtained with the qPCR assay.