A qPCR-based method for the detection and quantification of the peach powdery mildew (*Podosphaera pannosa*) in epidemiological studies.

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ABSTRACT

A molecular qPCR-based method was developed to detect and quantify *Podosphaera pannosa*, the main causal agent of peach powdery mildew. A primer pair was designed to target part of the ITS region of the fungal ribosomal DNA, which proved to be highly specific and sensitive. A minimum of 2.81 pg μL^{-1} of *P. pannosa* DNA and 6 conidia mL^{-1} in artificially-prepared conidia suspensions were found to be the limit of detection. Moreover, a quantification of conidia placed on plastic tapes commonly used in volumetric air samplers was performed. Regression equations on conidia quantification obtained either from aqueous conidia suspensions or conidia placed on plastic tapes were similar. The protocol was further validated in field conditions by estimating the number of *P. pannosa* conidia obtained with an air sampler, by both microscopical and molecular quantification. Both techniques coincided in detecting the peaks of conidia production during a 4-month sampling period, and a significant correlation (r = 0.819) was observed between both quantification methods. Additionally, the molecular method was applied to detect the latent fungal inoculum in different plant parts of peach trees. The pathogen was detected mainly on the bark of affected twigs, and to a lesser extent, in foliar buds. The method developed here can be applied in the study of *P. pannosa* epidemiology and can help in improving the management of this pathogen through its early detection and quantification.

KEYWORDS

aerobiology, epidemiology, molecular technique, powdery mildew, *Prunus persica*

INTRODUCTION

The ascomycete *Podosphaera pannosa* (Wallr.) de Bary is one of the causal agents of powdery mildew, which occurs mainly on the *Prunus* and *Rosa* genera of Rosaceae (Farr and Rossman 2019; Takamatsu et al. 2010). Other powdery mildew species can be found on different peach fruit morphologies such as nectarines and flat fruits albeit rarely, such as *P. clandestina*, *P. leucotricha*, and *P. tridactyla* (Farr and Rossman 2019). However, *P. pannosa* is widely recognized as the main causal agent of the peach powdery mildew (PPM). In this species, the pathogen infects green parts of the tree, e.g. fruits, leaves, buds, and twigs (Grove 1995; Ogawa and English 1991), where a distinguishable white-greyish mycelium develops on the surface of the affected part. Severe infections of PPM on fruits makes them unacceptable to industry, thus causing important economic losses. *Podosphaera pannosa* has been reported from over 40 peach-growing countries in the world (Amano 1986; Farr and Rossman 2019). The fungus overwinters in peach as dormant mycelium in latent buds (Ogawa and English 1991; Toma et al. 1998; Weinhold 1961; Yarwood 1957), and the ascocarps (chasmothecia) are usually found in the mycelium infecting twigs and leaves (Butt 1978). Primary PPM infections occur in spring, when primary inoculum is available and favourable weather conditions are met. However, precise experimental data on the environmental conditions needed for primary PPM infections are scarce (Toma et al. 1998; Weinhold 1961). Air-dispersed conidia released from primary-established colonies are responsible for secondary infections that extend over the vegetative growing season of peach tree (Grove 1995; Jarvis et al. 2002). In general, PPM spreads rapidly in seasons when a relatively cold and humid spring is followed by a dry summer (Toma et al. 1998). Previous studies reported on the optimal temperature and relative humidity (about 21 °C and 70-95 % RH, respectively) for pathogen development (Grove 1995; Toma et al. 1998). Regarding the infection of *P. pannosa* on *Rosa*, Longrée (1939) described similar optimal conditions for *P. pannosa* infection (21 °C and between 75-99 % RH). The control of PPM can be achieved efficiently through the foliar application of fungicides (Grove

1995; Hollomon and Wheeler 2002; Ogawa and English 1991), which usually starts at petals fall or the beginning of fruit set and continues periodically (Grove 1995; Reuveni 2001). These fungicide applications are done on a calendar basis (Ogawa and English 1991) since epidemiological models on PPM infection risk are scarce. Recently, a decision support system to initiate fungicide applications programs has been proposed (Marimon et al. 2020).

Rapid and reliable detection and quantification of *P. pannosa* in biological samples might contribute to a better understanding of its life cycle and therefore to improve its management. The detection of airborne inoculum of powdery mildews has been made traditionally through air-sampling devices combined with microscopical observations (Cao et al. 2015; Grove 1991). However, this method is time-consuming and non-specific for the identification and quantification of airborne plant pathogens (Dung et al. 2018; Falacy et al. 2007). Otherwise, coupling spore traps with DNA-based assays is faster, more specific and sensitive, and a reliable alternative to the conventional detection of airborne plant pathogens through microscopical observations (Kunjeti et al. 2016), including powdery mildews (Falacy et al. 2007; Thiessen et al. 2016).

The main objective of the current study was to develop a real-time qPCR assay for detection and quantification of *P. pannosa* in biological samples, including the design of a species-specific primer pair. In addition, two further practical applications were conducted in peach orchards to detect and quantify (*i*) the airborne inoculum of *P. pannosa* in spore traps, and (*ii*) the primary inoculum of *P. pannosa* in host plant material. The protocol reported here could be used in future applied studies, e.g. those including the need for a rapid and accurate detection and quantification of *P. pannosa*.

MATERIALS AND METHODS

Experimental orchards

Three experimental peach and nectarine orchards owned by IRTA and located in Catalonia, Spain, were used in this study (Alcarràs, 41°36'33''N, 0°26'45''E; Cabrils, 41°31'7"N, 2°22'34"E; and Mollerussa, 41°37'8''N, 0°52'2''E). The orchard located in Alcarràs was an 'Autumn free' nectarine orchard, whereas orchards in Cabrils and Mollerussa were planted with 'Early Gold' peach and 'Texas' almond interspecific progenies that are known to be susceptible to PPM (Donoso et al. 2016). These orchards were managed using cultural practices, such as pruning, soil management and nutrient supply, according to the guidelines of Spanish Integrated Production Management practices (MAPA 2002). No fungicide treatments were applied during the experimental period (spring to summer) to allow natural infections of *P. pannosa*, which were known to occur in the orchards.

Plant material

Specificity and sensitivity tests. In order to obtain conidia suspensions of *P. pannosa*, symptomatic peach fruits and leaves were collected in summer 2017 in the Mollerussa orchard. Samples were stored in a portable cooler and taken to the laboratory for further processing. All field samples were processed in the laboratory within 48 h after collection. For the specificity experiment, fresh leaves of apple and plum trees infected with powdery mildew were obtained and treated similarly as the peach samples to get conidia suspensions. Additional herbarium material used in this experiment, consisting of six powdery mildew species occurring on various hosts, was kindly provided by Dr Josep Girbal (Universitat Autònoma de Barcelona, Bellaterra, Spain) as follows: three samples of *Podosphaera aphanis*, collected on *Alchemilla alpina*, *Alchemilla vulgaris*, and *Potentilla reptans*, respectively; one sample of *P. clandestina* from *Crataegus monogyna*; one sample of *P. fusca* from *Cucurbita pepo*, and two from *Cucumis sativus*; six samples of *P. leucotricha* from *Malus domestica*; two samples of *P. macularis* from *Humulus lupulus*, and five of *P. tridactyla* from *Prunus cerasifera*.

Latent mycelium detection. Five trees per each experimental orchard located in Alcarràs and Mollerussa, and three trees from the orchard located in Cabrils were used. At the end of summer 2016, eight sight-heighted branches preferably showing PPM symptoms were selected and marked in each tree. The apical part of each branch (about 40 cm) was covered with a plastic mesh to retain leaves from falling, and the mesh was tied to prevent its accidental opening. In January 2017, all selected branches were collected and kept at 4 ºC until further processing.

Fungal material

Powdery mildew conidia were collected from the symptomatic plant parts by repeatedly washing away the plant infected surface with 1.5 mL of sterile 5% Chelex-100 (Bio-Rad, Hercules, CA, USA) aqueous suspension. Each sample volume was collected separately in 1.9 mL Eppendorf tubes and conidia concentration was measured using a Neubauer haemocytometer. Samples were stored at 4°C for further DNA extraction.

DNA extraction

Conidia suspensions. DNA was extracted from conidia suspensions using the short protocol from the E.Z.N.A. Plant DNA Kit (Omega Bio-tek, Norcross, GA, USA), with modifications described by Zúñiga et al. (2018) as follows: 0.15 g of 500-750 µm glass beads (Acros Organics, Geel, Belgium) were added to 700 µL of the extraction buffer in each sample, and the samples were vortexed for 15 min at 50 Hz. DNA quality and concentration were checked and measured with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). DNA samples were stored at -20 °C until further use.

Spore trap samples. DNA was extracted from the air-exposed plastic tapes used in the spore-trapping device (see below) by following the short protocol of the E.Z.N.A. Plant DNA Kit (Omega Bio-tek). Extraction, and DNA quantity and quality checking were conducted as described above and DNA was stored at -20 °C until further use.

Plant tissues. Before DNA extraction, all fresh peach samples (i.e. leaf and flower buds, leaves, and bark from twigs) were oven-dried at 35 °C until constant weight. Herbarium samples were processed for DNA extraction with no previous oven-drying. Fungal DNA was extracted from those plant tissues using the E.Z.N.A. Plant DNA Kit (Omega Bio-tek), following the dried plant samples protocol and the sample homogenization step with glass beads. DNA checking was also conducted as earlier described and DNA was stored at -20 °C until further use.

Primer design

Primers were designed to target the Internal Transcribed Spacer (ITS) in the ribosomal DNA region. Two representative ITS sequences of *P. pannosa* samples, namely 'Ppan53' and 'Ppan92', were obtained in this study (Table 1). These sequences were selected from a previous screening analysis involving 31 *P. pannosa* samples obtained from *P. persica* and *Rosa* (Luque, *unpublished*). Sequences were included in a matrix together with 29 additional sequences retrieved from GenBank (Table 1), as follows: 4 from *P. pannosa*; 10 from phylogenetically closer species such as *P. aphanis* (n = 3), *P. clandestina* (n = 4), and *P. spiraeae* (n = 3); and 15 sequences from other *Podosphaera* species, namely *P. fusca* (n = 5), *P. tridactyla* (n = 8) and *P. leucotricha* (n = 2). The identical sequences were grouped by Sequencher software 5.0 (Gene Codes Corp., Ann Arbor, Michigan), using the Assemble algorithm with the 100% Minimum Match parameter. Sequences were aligned using ClustalW (Thompson et al., 1994) with default settings and posterior manual adjustments were made when necessary. Regions with polymorphisms and suitable for specific primer design were identified, and later analysed with the PrimerQuest tool (IDT, URL: https://eu.idtdna.com/PrimerQuest/Home/Index) using the default parameters. The primer pair PpanITS1-F/PpanITS1-R was obtained.

qPCR conditions

Optimal qPCR conditions were set up as follows: for a final volume of 25 µL each reaction, products and concentrations were 10 µL SYBR Premix Ex Taq™ TliRNase H Plus (Takara), 0.4 µM of each specific forward and reverse primers, 5 µL of template DNA and HPLC-grade deionized water to reach final volume. qPCR was carried out on a Rotor-Gene Q 5plex thermal cycler (Qiagen, Hilden, Germany) with the following temperature and timing profile: an initial denaturation at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s. After the final amplification cycle, the temperature was held at 72 ºC for 90s. The melting curve analysis was performed raising the temperature from 72 ºC to 95 °C, increasing 1 °C every 5 s with continuous measurement of fluorescence at 510 nm wavelength. All reactions were run in triplicate and using genomic DNA extracted from *P. pannosa* conidia suspensions as positive controls, and negative controls with no DNA template.

Analytical specificity and sensitivity tests

The primer pair specificity was checked *in silico* and *in vitro*. *In silico*, specificity for the primer pair PpanITS1-F/PpanITS1-R was evaluated with the Primer-BLAST tool (htpps://www.ncbi.nlm.nih.gov/tools/primer-blast/). *In vitro*, specificity was tested by analysing qPCR amplifications of 28 DNA samples obtained from six *Podosphaera* species other than *P. pannosa* and occurring on several Rosaceae and non-Rosaceae species, which included 20 samples from the earlier described herbarium material, and fresh samples of *P. leucotricha* (n = 5, from apple), and *P. tridactyla* (n = 3, from plum), both collected at IRTA Cabrils facilities. Identity of those fungi different from *P. pannosa* was confirmed by sequencing their rDNA ITS region using the forward primers ITS1F (Gardes and Bruns, 1993) and the reverse primer ITS4 (White et al., 1990) in single PCR, and using methods described elsewhere (Luque et al. 2005). All qPCR reactions involved in the specificity test were carried out in triplicate and included negative and positive controls of *P. pannosa*.

The primer pair sensitivity was evaluated according to the protocols described by Armbruster and Pry (2008). Two independent DNA samples (DNA 1 and DNA 2) and three independent conidia suspensions (CS 1, CS 2 and CS 3) were prepared and used in the experiments. The DNA samples were obtained from conidia suspensions and later serially-diluted, whereas the CS samples were serially-diluted before DNA extraction. In both cases, DNA was extracted from the resulting conidia suspensions using the method described above. The measured DNA concentrations for DNA 1 and DNA 2 samples were (mean ± std. error) 25.4 ± 3.8 ng μL^{-1} and 33.9 ± 4.6 ng DNA μL^{-1}, respectively. Ten-fold dilutions series down to $1:10^5$ were prepared and subsequently used in the qPCR assays. For each CS sample, amounts of conidia were determined from four measurements with five pseudoreplicates using a haemocytometer. Initial conidia concentrations for CS1 to CS3 samples were $5.87 \pm 0.212 \times 10^5$ conidia mL^{-1}, $3.13 \pm 0.136 \times 10^5$ conidia mL^{-1}, and $8.06 \pm 0.274 \times 10^5$ conidia mL^{-1}, respectively. For each suspension, ten-fold dilution series down to $1:10^5$ were prepared. The DNA from each dilution point was extracted as described earlier. All DNA samples were amplified with the primer pair designed in this study and using the qPCR conditions described above, and by additionally including 0.4 µL of ROX Reference Dye in each reaction. All qPCR reactions were performed using a StepOne™ Real-Time PCR System thermal cycler (Life Technologies, Carlsbad, CA, USA). Three technical replicates were run for each biological sample, and three replicates of deionized water template were included in each reaction plate as negative controls. After each qPCR, a melting curve was performed to verify the targeted amplification product. A homogeneous melting peak at 88°C indicated that the amplified targeted ITS1 region was specific for *P. pannosa*. For each DNA and CS samples, a standard curve was calculated by plotting the quantification cycle values (C_q) against the logarithm of the DNA or conidia concentration at each dilution point. The amplification efficiency (AE), intercept, slope, and determination coefficient (r^2) were calculated for each standard curve obtained in this study. Then, the limit of blank (LOB), limit of detection (LOD), and limit of quantification (LOQ)

196 were calculated according to the EP17 guideline of the Clinical and Laboratory Standards
197 institute (Armbruster and Pry 2008).

198 **Validation of the specific qPCR primer pair PpanITS1-F/PpanITS1-R**

199 *Case 1: Detection of* P. pannosa *airborne inoculum in spore traps*

200 Starting from a conidia suspension (CS 4) containing $3.73 \pm 0.22 \times 10^5$ conidia mL^{-1}, two
201 independent 10-fold dilution series were prepared until $1:10^5$ of the initial concentration, with
202 three replicates per dilution. For the first dilution series, DNA for each dilution and replicates
203 was extracted as described earlier. Regarding the second dilution series, 500 µL from each
204 dilution and replicate was placed on a Melinex (TEKRA, New Berlin, WI, USA) polyester plastic
205 strip (19 x 48 mm) previously treated with silicone solution (Lanzoni, Bologna, Italy) on one
206 side. Plastic strips were dried overnight in a laminar airflow cabinet at room temperature.
207 Finally, DNA was extracted and amplified according to the protocol described in this study.
208 Three technical replicates were run per sample. Standard curves for each of two replicates
209 were obtained and used in further quantification of *P. pannosa* conidia trapped on plastic
210 tapes.

211 In a subsequent experiment, daily airborne conidia of *P. pannosa* were tracked in the
212 peach orchard located in Mollerussa using a Hirst-type, 7-day recording volumetric spore
213 sampler VPPS 2000 (Lanzoni). The spore sampler was placed from April to July 2018 in the
214 vicinity of trees that had shown PPM infections in previous years. Sampler orifice was located
215 0.5 m above ground level and the volumetric ratio adjusted at 10 L air min^{-1}. Plastic tapes
216 treated with the silicon solution were replaced weekly and taken to the laboratory for
217 subsequent processing. Exposed tapes were cut into seven 48-mm pieces, each one
218 corresponding to 1-day period. Each daily fragment was further cut longitudinally into two
219 equal-sized segments: one half-part was used for microscopic observation whereas the other
220 half was used for the qPCR analysis. For microscopic observation, samples were processed as

proposed by the Spanish Aerobiological Network (REA) (Galán et al. 2007): each daily fragment was stained with acid lactofuchsin and mounted on a glass slide. Microscope samples were examined using a microscope (model Eclipse E400, Nikon Corporation, Toquio, Japan) at 250x and only conidia that were morphologically compatible with *P. pannosa* were considered, i.e. conidia containing fibrosin refractive bodies (Braun et al. 2002), and measuring 12-15 x 20-27 µm (Horst and Cloyd 2007). Final number of conidia per day was estimated from the examined surface (about 45% of the total strip surface) and expressed as conidia m^{-3}. For qPCR quantification, daily samples were cut into six equally-sized pieces and put into a 1.5 mL Eppendorf tube. DNA was extracted and amplified according to the protocols described in this study. Additionally, a positive control from CS 4 (dilution $1:10^2$) was included in the qPCR plate. The quantification of conidia for each daily sample was calculated using the standard curve obtained from the CS 4 suspension placed on a plastic tape. Samples matching the following criteria were excluded from the analyses: *i*) C_q values higher than the LOB threshold determined in this study, *i.e.* 35 cycles, *ii*) C_q values showing a standard deviation higher than 0.5 between technical replicates, and *iii*) replicates with a highly-different melting temperature from that observed in the specific primer evaluation tests. Quantification of trapped conidia using qPCR was expressed in conidia m^{-3} after proper conversion factors were applied on the values obtained from the standard curve analysis (expressed in conidia mL^{-1}). Conversion factors considered were: *i*) the volumetric ratio of sampler (10 L air min^{-1}), *ii*) the final volume of DNA extracted from daily samples (100 µL), and *iii*) the standard curve data obtained from the CS 4 conidial suspension as explained above.

Case 2: Detection of the primary inoculum of P. pannosa *in host plant material*

Three biological replicates of different peach plant parts (leaves, leaf buds, floral buds and twig barks) were detached from each collected branch. Samples were accurately examined using a stereomicroscope (10×) to detect symptoms and signs compatible with *P. pannosa*

246 infections. When those compatible structures were detected, an optical microscope was used
247 to ascertain the presence of mycelium and chasmothecia, and a sample (about 12 mg) was
248 taken for DNA extraction and further qPCR amplification. Sample weights according to sample
249 origins were as follows: 11.97 ± 0.19 mg for leaves, 12.07 ± 0.23 mg for foliar buds, 12.43 ±
250 0.22 mg for floral buds, and 11.52 ± 0.16 mg for twig barks. Samples were separately put into
251 1.5 mL Eppendorf tubes and DNA extraction and qPCR quantification were done according to
252 the methods described in this study. Three technical replicates per biological sample were run
253 and two types of negative controls were used: DNA from *in vitro*, no symptomatic *P. persica*
254 leaves and deionized water template. The quantification of DNA for each sample was
255 calculated using the DNA 1 solution.

256 **Statistical analyses**

257 Output data corresponding to the fitted qPCR standard curves equations, including
258 intercept, slope, r^2 and AE, were obtained from the software of the thermal cyclers used in this
259 study. Further statistical analyses were performed using the *stats* package included in R (R
260 Core Team 2019). The analysis of covariance was used to compare the regression equation
261 slopes of the standard curves when appropriate. Lineal modelling including correlation and
262 regression analyses was used to study the relationship between the amounts of trapped
263 conidia in aerobiological samples estimated through either the microscopical or qPCR
264 approaches. Statistical significance in all analyses was declared at $\alpha < 0.05$. Values of mean ±
265 standard error of the mean are reported when appropriate.

266

267 **RESULTS**

268 **Primer design**

The design of *P. pannosa* specific primers was performed through the alignment of the ITS region of 31 unique sequences of powdery mildew fungi (Table 1). Several nucleotide polymorphisms were detected among species at two polymorphic regions that allowed to design forward and reverse primers at those sites. The forward and reverse primers were named PpanITS1-F and PpanITS1-R, respectively, and amplified a region of 155 bp at the ITS 1 region. The amplified product showed a melting temperature at 88 °C. Sequences for the PpanITS1-F and PpanITS1-R primers were 5'-CCACCCGTGTGAACTGAATT-3' and 5'-CCGTTGTTGAAAGTTTTACTTATTAAGTT-3', respectively.

Specificity and sensitivity of the primer pair PpanITS1-F/PpanITS1-R

Specificity tests were performed using the primer pair PpanITS1-F/PpanITS1-R for the amplification of several *Podosphaera* species. Only DNA samples from *P. pannosa* were amplified with the specific primers, showing a single peak around 88°C in the melting curve analysis, whereas no amplification was observed for other non-*P. pannosa* samples. In order to discard false positives and to confirm *P. pannosa* identification, amplified products were checked in 2 % agarose gels and further sequenced (*data not shown*).

P. pannosa was detected and quantified in two independent DNA samples (DNA 1 and DNA 2) obtained from *P. pannosa* conidia. A clear linear relationship was obtained between the C_q values and the logarithm of DNA concentrations (Fig. 1a). Parameters for the standard curves for DNA 1 and DNA 2 are described in Table 2. Both equations (Fig. 1a) had significant slopes ($P < 0.001$) that showed to be equal ($P = 0.56$). Three independent conidia suspensions were also quantified using qPCR (Fig. 1b). The standard regression curve parameters for conidia suspensions CS 1, CS 2 and CS 3 are described in Table 2. Slopes for the equations of the three conidia suspensions did not show significant differences among them ($P = 0.72$). After these experiments, an arbitrary LOD was established at 2.81 ± 0.49 pg DNA μL^{-1} and 6 ± 2 conidia mL^{-1}. Estimated LOB values, as described by Armbruster and Pry (2008), are not

294 reported for all the above qPCR assays since they were lower than LOD values in all cases.
295 Mean C_q corresponding to LOB was established at 35 cycles for all the reactions performed in
296 this study.

297 **Validation of the specific primer pair PpanITS1-F/PpanITS1-R**

298 *Case 1: Detection of* P. pannosa *airborne inoculum in spore traps*

299 Ten-fold dilution series from suspension CS 4, with and without placing on spore-
300 trapping tapes, were successfully detected until 10^{-3} dilution. The standard regression curve
301 parameters for both types of samples are described in Table 2. Slopes for both standard curves
302 did not show significant differences ($P = 0.29$) (Fig. 1c). Regarding the detection of *P. pannosa*
303 in periodical air samplings, the fungus was successfully detected and quantified in the spore-
304 trapping tape samples collected from April to July 2018 (Fig. 2). Propagules of *P. pannosa* were
305 firstly detected at the beginning of the third sampling week, corresponding to mid-April.
306 Thereafter, abundance of airborne conidia was fluctuating along the season, with spontaneous
307 peaks, and achieved the seasonal maximum (17.0 conidia m^{-3} from microscope observations
308 and 21.1 conidia m^{-3} from qPCR analysis) by mid-July. Both estimation methods, either by
309 microscope observation or qPCR analyses, followed a similar time pattern in conidia detection.
310 Furthermore, a linear regression equation ($P < 0.001$, $r^2 = 0.6713$) was adjusted between the
311 above variables (Fig. 3), with the following parameters: $y = 0.548 + 0.535x$, where y = conidia
312 quantified through microscopical observation, and x = conidia quantified through qPCR. From
313 the regression equation, lower levels of conidia were observed (about 53 %) through
314 microscope as compared to qPCR quantification. We hypothesize that those low recordings
315 from visual identifications, as compared to molecular quantifications, could be explained by: *i*)
316 large amounts of particles (dust, pollens, other fungal spores…) in the trapping tape which
317 could have interfered with the microscopical identification of *P. pannosa* conidia in the

318 samples, and *ii*) an eventual degradation of *P. pannosa* conidia, thus making difficult the
319 morphological identification of the species.

320 *Case 2: Detection of the primary inoculum of* P. pannosa *in host plant material*

321 Detection and quantification tests done with samples of leaves, twigs, and foliar and
322 floral buds were performed using the detection threshold C_q LOD = 30.79, as determined in the
323 analytical sensitivity test. Trees in orchards located in Alcarràs and Cabrils did not show any
324 visual symptom of PPM infection in 2017. Furthermore, none of samples collected in those
325 orchards showed positive qPCR detections of *P. pannosa* (*data not shown*). Regarding the
326 samples collected in Mollerussa, results indicated that the pathogen was not detected from
327 dried leaf and floral bud tissues (Table 3). In contrast, leaf buds showed to be infected with the
328 pathogen in average in 42.5 % cases (range: 25 to 75 %), although this finding could not be
329 confirmed through visual examination as no distinguishable fungal structures could be
330 detected under the stereomicroscope. Mean DNA concentration of PPM in sampled leaf bud
331 tissues ranged from 0.02 to 3.90 ng g^{-1} dried tissue. All twig samples from the orchard located
332 in Mollerussa showed clear PPM symptoms on their surface. Examined samples showed one to
333 seven visible lesions with symptoms, 0.6 to 216 mm in length, and with the presence of
334 chasmothecia in 60% of samples (84 out of 140 total examined lesions). Mean DNA
335 concentration of PPM in sampled twig tissues ranged from 37.74 to 96.27 ng g^{-1} dried tissue,
336 about 50 times greater than in foliar bud tissues.

337

338 **DISCUSSION**

339 A qPCR-based protocol was developed for the specific detection and quantification of *P.*
340 *pannosa* in biological samples. A specific primer pair, named PpanITS1-F/PpanITS1-R, was
341 designed and successfully validated using both artificially-prepared (e.g. conidia suspensions)
342 and environmental samples (e.g. spore-trapping tapes from a volumetric air sampler, and

different plant tissues). To the best of our knowledge, this is the first time that a molecular qPCR-based tool for the detection and quantification of *P. pannosa* is developed. The primer pair targeting the ITS region designed in this study proved to be highly specific, as indicated by the positive detection of *P. pannosa* DNA and the negative amplification of DNA from other *Podosphaera* species, either from Rosaceae hosts (*P. aphanis*, *P. clandestina*, *P. leucotricha*, and *P. tridactyla*) or non-Rosaceae hosts (*P. fusca*, and *P. macularis*). The ITS region has been proved to be appropriate for studying genetic variation at species level in powdery mildew fungi belonging to the genus *Podosphaera* (Ito and Takamatsu 2010). Thus, few nucleotide differences in the ITS sequences could be associated to *Prunus* specialization within the *Podosphaera tridactyla* complex (Cunnington et al. 2005). Moreover, Leus et al. (2006) showed that one single nucleotide difference in the ITS sequences of *P. pannosa* isolates distinguished different host-specific groups on *Rosa* and *Prunus* species.

Regarding the detection thresholds obtained in this study, they were set at 2.81 ± 0.49 pg DNA $\mu L^{-1}$and 6 ± 2 conidia mL^{-1}. Previous studies on the detection threshold for other powdery mildew species have been reported elsewhere. Thus, Falacy et al. (2007) reported 10 conidia as the detection threshold for the grapevine powdery mildew, *Erysiphe necator*, in a single PCR reaction mixture. In addition, Sholberg et al. (2005) reported that 20 to 30 conidia of *P. leucotricha*, the apple powdery mildew, could be detected using a DNA macroarray. The results obtained in this study are therefore comparable to those of previous studies, albeit using different analysis techniques.

The detection and quantification of airborne *P. pannosa* conidia using a volumetric air sampler coupled with the qPCR method was successfully performed. When compared with the microscopical observation of trapped conidia on plastic tapes, the molecular technique allowed: *i*) to determine the period when *P. pannosa* conidia are present in the air, similar to how it is done with microscope examination, and *ii*) to obtain a reliable quantification of

368 airborne conidia, as shown by the high correlation found between the quantifications
369 conducted through microscope and molecular approaches. Furthermore, molecular detection
370 using specific primers allowed us to overcome some important limitations which are not
371 uncommon in the microscope examination of aerobiological samples: *i*) the required time of
372 handling and posterior microscope observation of samples (Dung et al. 2018), *ii*) the
373 morphological similarity of conidia from different powdery mildew species (Braun 1987), which
374 makes difficult species differentiation and therefore demands trained skills to analysts, and *iii)*
375 the inaccurate identification due to co-location of overlapping structures that can disfigure
376 spore morphology (Mahaffee and Stoll 2016). Thus, the present study reports on a rapid and
377 reliable detection and quantification method for PPM airborne propagules.

378 The detection and quantification of pathogen overwintering structures in different plant
379 tissues was also studied. Chasmothecia of *Podosphaera* species perennate in winter as fruiting
380 bodies immersed in the mycelium attached to the host (Jarvis et al. 2002). In *P. clandestina*, on
381 sweet cherry, chasmothecia survive on senescent leaves, on fallen leaves on the orchard floor
382 and in tree bark crevices (Grove, 1991). In the case of *P. pannosa*, Ogawa and English (1991)
383 reported the formation of chasmothecia on twigs and stems, most frequently around the
384 thorns on rose. In the case of peach infections, several authors suggested that the fungus
385 overwinters as mycelium deep within the buds, from where infected shoots arise after the
386 spring budburst (Yarwood 1957; Weinhold 1961). However, to date, no molecular detection of
387 PPM in overwintering structures had been described. In our study, the use of the specific
388 primer pair PpanITS1-F/PpanITS1-R confirmed that the pathogen is mostly present on the
389 surface of twigs, where mycelium and chasmothecia were also clearly detected by visual
390 examinations. Besides twigs, *P. pannosa* was detected in lower concentrations in foliar bud
391 tissues, where the pathogen mycelium was supposedly detected using a stereomicroscope
392 (Weinhold 1961). Conversely to what we expected, no positive detection of *P. pannosa* from
393 autumn leaves was confirmed. In that scenario, first spring infections could be developed

394 either from airborne ascospores released from chasmothecia present on twigs and shoots, or
395 from latent mycelium inside bud tissues.

396 In recent years, the study of epidemiology of air-borne pathogens has been done
397 through their detection and quantification by molecular-based techniques, which have been
398 proved more effective than conventional ones to answer complex questions regarding the
399 biology of tree fruit pathogens (Michailides et al. 2005). Considering that molecular detection
400 is a rapid, sensible and specific, our methodology can detect and quantify *P. pannosa*
401 propagules well before the visual occurrence of disease symptoms in the orchard. In
402 conclusion, the method developed here can be applied in the study of the PPM epidemiology,
403 and therefore it can help in improving the management of this disease through the early
404 detection and quantification of the pathogen.

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Compliance with ethical standards

Conflicts of interest/Competing interests: The authors declare that they have no conflict of interest.

Research involving Human Participants and/or Animals: Not applicable.

Informed consent: All authors read and approved the final manuscript.

Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Neus Marimon, Maela León, Mónica Berbegal, and Jordi Luque. The first draft of the manuscript was written by Neus Marimon and Jordi Luque. All authors revised all previous versions of the manuscript.

Availability of data and material

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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549 **TABLES**

550 **Table 1** GenBank accession numbers of sequences used to design a specific primer pair for the
551 detection and quantification of *Podosphaera pannosa*

Fungal taxa	Sample designation	Host	Country	GenBank ITS[a]
Podosphaera aphanis	S_Italy3	*Fragaria* sp.	Italy	GU942447
Podosphaera aphanis	R_Eng_Kent2	*Rubus* sp.	UK	GU942461
Podosphaera aphanis	R_Sco1b	*Rubus* sp.	UK	GU942462
Podosphaera clandestina	MUMH 1868	*Crataegus* sp.	Argentina	AB525932
Podosphaera clandestina	30111	*Phlox drummondii*	Italy	HQ844621
Podosphaera clandestina	P-G	*Prunus avium*	Belgium	DQ139434
Podosphaera clandestina	BC-1	*Prunus serotina*	Mexico	KJ158161
Podosphaera fusca	Unknown	*Cucurbita pepo*	USA	AF011321
Podosphaera fusca	SqPl-1	*Eupatorium fortunei*	China	JX546297
Podosphaera fusca	MAY1	*Euryops pectinatus*	Spain	EU424056
Podosphaera fusca	UC1512289	*Taraxacum officinale*	USA	AF011320
Podosphaera fusca	PF001	*Trichosanthes kirilowii*	South Korea	HQ683746
Podosphaera leucotricha	MUMH 468	*Malus domestica*	Japan	AB027231
Podosphaera leucotricha	N4-08	*Prunus persica*	Serbia	HM579839
Podosphaera pannosa	Ppan53	*Prunus persica*	Spain	*MN796128*
Podosphaera pannosa	R-A	*Rosa* sp.	Belgium	DQ139410
Podosphaera pannosa	R-D	*Rosa* sp.	Belgium	DQ139430
Podosphaera pannosa	Ppan92	*Rosa* sp.	Spain	*MN796129*
Podosphaera pannosa	UCB	*Rosa* sp.	USA	AF011322
Podosphaera pannosa	UC1512288	*Rosa* sp.	USA	AF011323
Podosphaera spiraeae	TPU-1825	*Spiraea cantoniensis*	Japan	AB026143
Podosphaera spiraeae	HMQAU 13013	*Spiraea japonica*	China	KF500426
Podosphaera spiraeae	TPU-1877	*Spiraea thunbergii*	Japan	AB026153
Podosphaera tridactyla	MUMH 247	*Photinia beauverdiana*	Japan	AB026147
Podosphaera tridactyla	VPRI 19864	*Prunus armeniaca*	Australia	AY833657
Podosphaera tridactyla	UC1512290	*Prunus armeniaca*	USA	AF011318
Podosphaera tridactyla	VPRI 19238	*Prunus cerasifera*	Australia	AY833656
Podosphaera tridactyla	VPRI 22157	*Prunus laurocerasus*	Switzerland	AY833654
Podosphaera tridactyla	P-S	*Prunus lusitanica*	Belgium	DQ139435
Podosphaera tridactyla	VPRI 22158	*Prunus lusitanica*	Switzerland	AY833655
Podosphaera tridactyla	KUS-F26292	*Prunus salicina*	South Korea	JQ517296

552 **[a]:** Accession numbers obtained in this study are shown in italics.

553 **Table 2** Parameters for the standard curves obtained in this study (see text for details). LOD
554 and LOQ parameters are expressed as pg DNA μL^{-1} for DNA 1 and DNA 2 samples, and as
555 conidia mL^{-1} for conidia suspensions (CS)

Standard curve name	Intercept	Slope	r^2	Efficiency (%)	LOD	C_q LOD	LOQ	C_q LOQ
DNA 1	23.548	-3.346	0.998	98.99	2.31	31.78	6.86	30.79
DNA 2	22.844	-3.387	0.987	97.35	3.29	30.38	8.94	29.78
CS 1	37.023	-3.363	0.992	98.30	5.30	35.89	9.57	34.51
CS 2	36.253	-3.383	0.990	97.52	2.90	34.68	7.58	33.28
CS 3	35.361	-3.318	0.992	100.00	10.50	31.98	16.65	31.31
CS 4	35.683	-3.248	0.995	103.17	6.90	32.94	17.21	31.66
CS 4 tape	35.370	-3.119	0.958	109.22	7.20	32.69	40.57	30.35

556

557

558 **Table 3** Detection and quantification of *Podosphaera pannosa* in different plant tissues (N =8

559 per tree) collected in a peach orchard located in Mollerussa, Spain

Plant part	Tree	No. Positive detections[a]	C_q[b]	Fungal DNA biomass ($ng \cdot g^{-1}$ dry tissue)
Leaf bud	1	2	29.06 ± 0.13	0.02
	2	3	22.19 ± 0.50	2.11
	3	4	21.30 ± 2.80	3.90
	4	6	23.08 ± 3.24	1.15
	5	2	25.94 ± 2.39	0.16
Floral bud	1	0	> C_q LOD	n.d.[c]
	2	0	> C_q LOD	n.d.
	3	0	> C_q LOD	n.d.
	4	0	> C_q LOD	n.d.
	5	0	> C_q LOD	n.d.
Twig	1	8	17.18 ± 1.75	69.36
	2	8	18.07 ± 1.26	37.74
	3	8	17.62 ± 2.62	51.37
	4	8	16.71 ± 2.63	96.27
	5	8	18.05 ± 1.56	38.15
Leaf	1	0	> C_q LOD	n.d.
	2	0	> C_q LOD	n.d.
	3	0	> C_q LOD	n.d.
	4	0	> C_q LOD	n.d.
	5	0	> C_q LOD	n.d.

560 **[a]:** Number of samples with positive detection of *P. pannosa*. **[b]:** Mean ± std. err. values. **[c]:** n.d.,

561 not determined.

562 **FIGURE CAPTIONS**

563

564 **Fig. 1** Standard regression curves obtained from qPCR assays involving 10-fold serial
565 dilutions from a) DNA extracted from conidia suspensions, DNA 1 and DNA 2; b) conidia
566 suspensions CS 1, CS 2 and CS 3; c) conidia suspension CS 4 either placed or not on a spore-
567 trapping tape

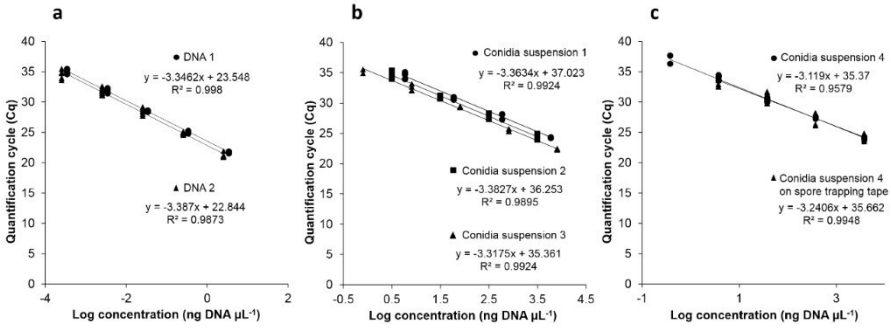
568

569 **Fig. 2** Daily values of airborne conidia trapped using a volumetric spore sampler (conidia
570 m^{-3}), estimated either from microscopic examination (solid line) or qPCR quantification
571 (dashed line). Time expressed as week number of the year and month

572

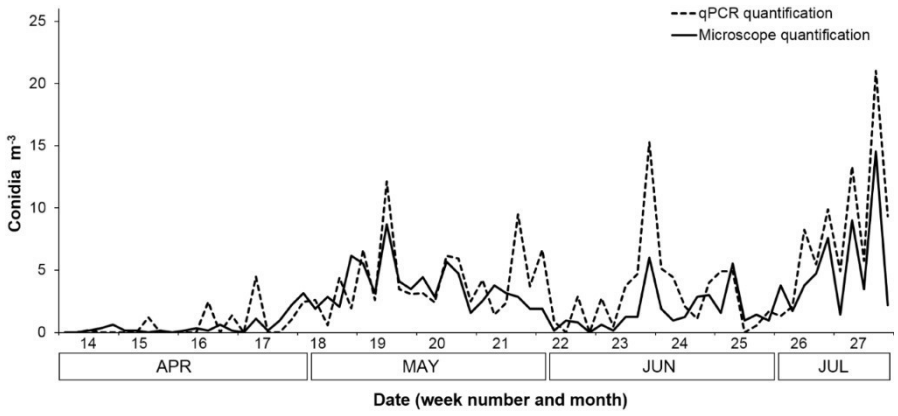
573 **Fig. 3** Correlation between the estimated amounts of conidia (conidia m^{-3}) obtained
574 through qPCR quantification (*x*) and microscopy examination (*y*)

575 **Fig. 1 - Low resolution for reviewing purposes**



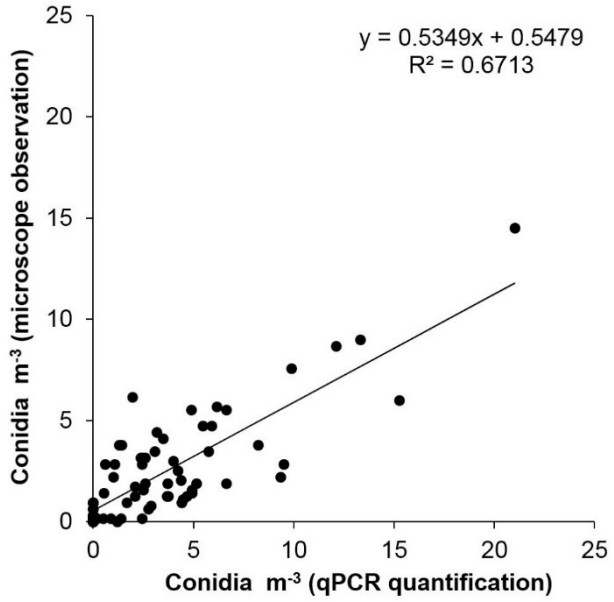
576

577 **Fig. 2 - Low resolution for reviewing purposes**



578

579 **Fig. 3 - Low resolution for reviewing purposes**



580