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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 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 microscopic and molecular quantification. Both techniques detected simultaneously the peaks of conidia production during a 4-month sampling period, and a significant correlation (r = 0.772) was observed between both quantification methods. Additionally, the molecular method was applied to detect 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 that occurs on the *Prunus* and *Rosa* genera of Rosaceae (Farr and Rossman 2019; Takamatsu et al. 2010). Other powdery mildew species are rarely found on peach, 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). *Podosphaera* species infect 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 *P. pannosa* on fruit make them unacceptable to industry, thus causing significant economic losses. This species 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 under favourable weather conditions. 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 the optimal temperature and relative humidity (RH) for pathogen development to be at approximately 21 °C and 70-95 % RH, respectively (Grove 1995; Toma et al. 1998). Regarding the infection of *P. pannosa* on *Rosa*, Longrée (1939) described similar optimal conditions for infection (21 °C and between 75-99 % RH). The control of PPM can be achieved efficiently through periodical applications of foliar fungicides (Grove 1995; Hollomon and Wheeler 2002; Ogawa and English 1991), which usually starts at petals fall or the beginning of fruit set (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 (one sample each) 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 phylogenetically close to *P. pannosa* and 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 (1.3 to 1.9 m above ground level) 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 February 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 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 20 µL each reaction, products and concentrations were 10 µL SYBR Premix Ex Taq™ TliRNase H Plus (Takara), 0.4 µL of each specific forward and reverse primers (at 10 µM), 5 µL of template DNA and HPLC-grade deionized water to reach the 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 °C for 90s. The melting curve analysis was performed raising the temperature from 72 °C 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 the fungi that were different from *P. pannosa* was confirmed by sequencing their rDNA ITS region using the forward primer ITS1F (Gardes and Bruns, 1993) and the reverse primer ITS4 (White et al., 1990) using the methods described by Luque et al. (2005). All qPCR reactions involved in the specificity test were carried out in triplicate and included negative and positive (Ppan53) 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 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 ± 0.136 × 10^5 conidia mL^{-1}, and 8.06 ± 0.274 × 10^5 conidia mL^{-1}, respectively. For each suspension, ten-fold dilution series down to 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) were calculated according to the EP17 guideline of the Clinical and Laboratory Standards institute (Armbruster and Pry 2008).

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

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

Starting from a conidia suspension (CS 4) containing 7.47 ± 0.45 × 10^4 conidia mL^{-1}, two independent 10-fold dilution series were prepared until 10^{-5} of the initial concentration, with three replicates per dilution. For the first dilution series, DNA for each dilution and replicates was extracted as described earlier. Regarding the second dilution series, 500 µL from each dilution and replicate was placed on a Melinex (TEKRA, New Berlin, WI, USA) polyester plastic strip (19 x 48 mm) previously treated with silicone solution (Lanzoni, Bologna, Italy) on one side. Plastic strips were dried overnight in a laminar airflow cabinet at room temperature.

208 Finally, DNA was extracted and amplified according to the protocol described in this study.
209 Three technical replicates were run per sample. Standard curves for each of two replicates
210 were obtained and used in further quantification of *P. pannosa* conidia trapped on plastic
211 tapes.

212 In a subsequent experiment, daily airborne conidia of *P. pannosa* were tracked in the
213 peach orchard located in Mollerussa using a Hirst-type, 7-day recording volumetric spore
214 sampler VPPS 2000 (Lanzoni). The spore sampler was placed from 6 April to 10 July 2018 in the
215 vicinity of trees that had shown PPM infections in previous years. Sampler orifice was located
216 0.5 m above ground level and the volumetric ratio adjusted at 10 L air min^{-1}. Plastic tapes
217 treated with the silicon solution were replaced weekly and taken to the laboratory for
218 subsequent processing. Exposed tapes were cut into seven 48-mm pieces, each one
219 corresponding to 1-day period. Each daily fragment was further cut longitudinally into two
220 equal-sized segments: one half-part was used for microscopic observation whereas the other
221 half was used for the qPCR analysis. For microscopic observation, samples were processed as
222 proposed by the Spanish Aerobiological Network (REA) (Galán et al. 2007): each daily fragment
223 was stained with acid lactofuchsin and mounted on a glass slide. Microscope samples were
224 examined using a microscope (model Eclipse E400, Nikon Corporation, Toquio, Japan) at 250x
225 and only conidia that were morphologically compatible with *P. pannosa* were considered, i.e.
226 conidia containing fibrosin refractive bodies (Braun et al. 2002), and measuring 12-15 x 20-27
227 µm (Horst and Cloyd 2007). Final number of conidia per day was estimated from the examined
228 surface (about 45% of the total strip surface) and expressed as conidia m^{-3}. For qPCR
229 quantification, daily samples were cut into six equally-sized pieces and put into a 1.5 mL
230 Eppendorf tube. DNA was extracted and amplified according to the protocols described in this
231 study. Additionally, a positive control from CS 4 (dilution 10^{-2}) was included in the qPCR plate.
232 The quantification of conidia for each daily sample was calculated using the standard curve
233 obtained from the CS 4 suspension placed on a plastic tape. Samples matching at least one of

234 the following criteria were excluded from further conidia quantification: *i*) Only one technical
235 replicate with acceptable values of C_q (C_q < 35 cycles) and melting temperature (T_m = 88 °C), *ii*)
236 replicates with a mean T_m highly different from 88 °C, and *iii*) replicates with acceptable C_q and
237 T_m values but showing a standard deviation (SD) higher than 0.5 between technical replicates.
238 Cases *i*) and *ii*) resulted in a negative quantification (zero) whereas case *iii*) resulted in an
239 undetermined value (missing). Quantification of trapped conidia using qPCR was expressed in
240 conidia m^{-3} after proper conversion factors were applied on the values obtained from the
241 standard curve analysis. Conversion factors considered were: *i*) the volumetric ratio of sampler
242 (10 L air min^{-1}), *ii*) the final volume of DNA extracted from daily samples (100 µL), and *iii*) the
243 equation of the standard curve obtained from the CS 4 conidial suspension placed on a plastic
244 tape.
245 *Case 2: Detection of the primary inoculum of* P. pannosa *in host plant material*
246 Three biological replicates of different peach plant parts (leaves, leaf buds, floral buds
247 and twig barks) were detached from each collected branch. Samples were carefully examined
248 using a stereomicroscope (10×) to detect symptoms and signs compatible with *P. pannosa*
249 infections. When those compatible structures were detected, an optical microscope was used
250 to ascertain the presence of mycelium and chasmothecia, and a sample (about 12 mg) was
251 taken for DNA extraction and further qPCR amplification. Sample weights according to sample
252 origins were as follows: 11.97 ± 0.19 mg for leaves, 12.07 ± 0.23 mg for foliar buds, 12.43 ±
253 0.22 mg for floral buds, and 11.52 ± 0.16 mg for twig barks. Samples were separately put into
254 1.5 mL Eppendorf tubes and DNA extraction and qPCR quantification were done according to
255 the methods described in this study. Three technical replicates per biological sample were run
256 and two types of negative controls were used: DNA from *in vitro*, no symptomatic *P. persica*
257 leaves and deionized water template. The quantification of DNA for each sample was
258 calculated using the DNA 1 solution.
259 **Statistical analyses**

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

RESULTS

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 the design of 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 from a known *P. pannosa* positive control (Ppan53) were amplified with the specific primers, showing a single peak around 88°C in the melting curve analysis. No amplification was observed for other non-*P. pannosa* samples.

286 *P. pannosa* was detected and quantified in two independent DNA samples (DNA 1 and
287 DNA 2) obtained from *P. pannosa* conidia. A clear linear relationship was obtained between
288 the C_q values and the logarithm of DNA concentrations (Fig. 1a). Parameters for the standard
289 curves for DNA 1 and DNA 2 are described in Table 2. Both equations (Fig. 1a) had significant
290 slopes ($P < 0.001$) of similar gradient ($P = 0.56$). Three independent conidia suspensions were
291 also quantified using qPCR (Fig. 1b). The standard regression curve parameters for conidia
292 suspensions CS 1, CS 2 and CS 3 are described in Table 2. Slopes for the equations of the three
293 conidia suspensions did not show significant differences among them ($P = 0.72$). After these
294 experiments, an arbitrary LOD was established at 2.81 ± 0.49 pg DNA μL^{-1} and 6 ± 2 conidia mL^{-1}
295 . Estimated LOB values, as described by Armbruster and Pry (2008), are not reported for all
296 the above qPCR assays since they were lower than LOD values in all cases. Mean C_q
297 corresponding to LOB was established at 35 cycles for all the reactions performed in this study.
298 **Validation of the specific primer pair PpanITS1-F/PpanITS1-R**
299 *Case 1: Detection of* P. pannosa *airborne inoculum in spore traps*
300 Ten-fold dilution series from suspension CS 4, with and without placing on spore-
301 trapping tapes, were successfully detected until 10^{-3} dilution. The standard regression curve
302 parameters for both types of samples are described in Table 2. Slopes for both standard curves
303 did not show significant differences ($P = 0.29$) (Fig. 1c). Regarding the detection of *P. pannosa*
304 in periodical air samplings, 12 daily samples were discarded (10 samples with technical
305 replicates showing SD > 0.5, and two samples with lesser than two acceptable technical
306 replicate each), and 32 daily samples were negative, from a total of N = 96. The fungus was
307 successfully detected and quantified from April to July 2018 (Fig. 2). Propagules of *P. pannosa*
308 were firstly detected at the beginning of the third sampling week, corresponding to mid-April.
309 Thereafter, abundance of airborne conidia was fluctuating throughout the season, with
310 spontaneous peaks, and achieved the seasonal maximum (14.5 conidia m^{-3} from microscope
311 observations and 21.0 conidia m^{-3} from qPCR analysis) by mid-July. Both estimation methods,

312 either by microscope observation or qPCR analyses, followed a similar time pattern in conidia
313 detection (Fig. 2). Furthermore, a linear regression equation ($P < 0.001$, $r^2 = 0.5957$) was
314 adjusted between the microscopic and qPCR variables (Fig. 3), with the following parameters: y
315 = 0.766 + 0.508x, where y = conidia quantified through microscopical observation, and x =
316 conidia quantified through qPCR. From the regression equation, lower levels of conidia were
317 observed (about 50 %) through microscope as compared to qPCR quantification.
318 *Case 2: Detection of the primary inoculum of* P. pannosa *in host plant material*
319 Detection and quantification tests done with samples of leaves, twigs, and foliar and
320 floral buds were performed using the detection threshold C_q LOD = 30.79, as determined in the
321 analytical sensitivity test. Trees in orchards located in Alcarràs and Cabrils did not show any
322 visual symptom of PPM infection in 2017. Furthermore, none of samples collected in those
323 orchards showed positive qPCR detections of *P. pannosa* (*data not shown*). Regarding the
324 samples collected in Mollerussa, the pathogen was not detected from dried leaf and floral bud
325 tissues (Table 3). In contrast, leaf buds showed to be infected with the pathogen on average in
326 42.5 % cases (range: 25 to 75 %), although this finding could not be confirmed through visual
327 examination as no distinguishable fungal structures could be detected under the
328 stereomicroscope. Mean DNA concentration of PPM in sampled leaf bud tissues ranged from
329 0.02 to 3.90 ng g^{-1} dried tissue. All twig samples from the orchard located in Mollerussa
330 showed clear PPM symptoms. Examined samples showed one to seven visible lesions with
331 symptoms, 0.6 to 216 mm in length, and with the presence of chasmothecia in 60% of samples
332 (84 out of 140 total examined lesions). Mean DNA concentration of PPM in sampled twig
333 tissues ranged from 37.74 to 96.27 ng g^{-1} dried tissue, about 50 times greater than in foliar bud
334 tissues.
335
336 **DISCUSSION**

A qPCR-based protocol was developed for the specific detection and quantification of *P. pannosa* in biological samples. A specific primer pair, named PpanITS1-F/PpanITS1-R, was designed and successfully validated using both artificially-prepared (e.g. conidia suspensions) 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* was 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 shown 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 with *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. Falacy et al. (2007) reported 10 conidia as the detection threshold for the grapevine powdery mildew, *Erysiphe necator*, in a single PCR reaction mixture. 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 based on 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

363 microscopic observation of trapped conidia on plastic tapes, the molecular technique was able
364 to determine the period when *P. pannosa* conidia are present in the air, as similarly done with
365 microscope examination. In addition, the qPCR method was successfully used to obtain a
366 reliable quantification of airborne conidia, as shown by the high correlation between the
367 quantifications conducted through microscope and molecular approaches. Furthermore,
368 molecular detection using specific primers allowed us to overcome some important limitations
369 which are not uncommon in the microscope examination of aerobiological samples: *i*) the
370 required time of handling and posterior microscope observation of samples (Dung et al. 2018),
371 *ii*) the morphological similarity of conidia from different powdery mildew species (Braun 1987),
372 which makes difficult species differentiation and therefore demands trained skills to analysts,
373 and *iii)* the inaccurate identification due to co-location of overlapping structures that can
374 disfigure spore morphology (Mahaffee and Stoll 2016). Thus, the present study reports on a
375 rapid and reliable detection and quantification method for PPM airborne propagules. We
376 additionally hypothesize that low quantifications based on visual identifications, as compared
377 to molecular quantifications, may be due to *i*) the occassional large amounts of particles (dust,
378 pollens, other fungal spores…) present in the trapping tape which could have interfered with
379 the microscopic identification of *P. pannosa* conidia, and *ii*) an eventual degradation of *P.*
380 *pannosa* conidia, thus making difficult the morphological identification of the species.
381 The detection and quantification of pathogen overwintering structures in different plant
382 tissues was also studied. Chasmothecia of *Podosphaera* species perennate in winter as fruiting
383 bodies immersed in the mycelium attached to the host (Jarvis et al. 2002). In *P. clandestina*, on
384 sweet cherry, chasmothecia survive on senescent leaves, on fallen leaves on the orchard floor
385 and in tree bark crevices (Grove, 1991). In the case of *P. pannosa*, Ogawa and English (1991)
386 reported the formation of chasmothecia on twigs and stems, most frequently around the
387 thorns on rose. In the case of peach infections, several authors suggested that the fungus
388 overwinters as mycelium deep within the buds, from where infected shoots arise after the

389 spring budburst (Yarwood 1957; Weinhold 1961). However, to date, no molecular detection of
390 PPM in overwintering structures had been described. In our study, the use of the specific
391 primer pair PpanITS1-F/PpanITS1-R confirmed that the pathogen is mostly present on the
392 surface of twigs, where mycelium and chasmothecia were also clearly detected by visual
393 examinations. Besides twigs, *P. pannosa* was detected in lower concentrations in foliar bud
394 tissues, where the pathogen mycelium was previously detected using a stereomicroscope
395 (Weinhold 1961). Conversely to what we expected, no positive detection of *P. pannosa* from
396 autumn leaves was confirmed. In that scenario, first spring infections could be developed
397 either from airborne ascospores released from chasmothecia present on twigs and shoots, or
398 from latent mycelium inside bud tissues.
399 In recent years, the study of epidemiology of air-borne pathogens has increasingly been
400 based on the pathogen detection and quantification by molecular-based techniques, which
401 helped to answer complex questions regarding the biology of tree fruit pathogens (Michailides
402 et al. 2005). The methodology developed in our study can be applied in the study of the PPM
403 epidemiology, and therefore it can help in improving the management of this disease through
404 the early detection and quantification of the pathogen.
405
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Compliance with ethical standards

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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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546 **TABLES**

547 **Table 1** GenBank accession numbers of sequences used to design a specific primer pair for the

548 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

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

550 **Table 2** Parameters for the standard curves obtained in this study

Standard curve name	Intercept	Slope	r^2	Efficiency (%)	LOD[a]	C_q LOD[b]	LOQ[c]	C_q LOQ[d]
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

551 LOD and LOQ parameters expressed as pg DNA μL^{-1} for DNA 1 and DNA 2 samples, and as

552 conidia mL^{-1} for conidia suspensions (CS). [a]: LOD, Limit of detection. [b]: Cq LOD, Quantification

553 cycle at LOD. [c]: LOQ, Limit of quantification. [d]: Cq LOQ, Quantification cycle at LOQ.

554

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

556 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[c]	n.d.[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.
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.

557 **[a]:** Number of samples with positive detection of *P. pannosa*. **[b]:** C_q, Quantification cycle,

558 expressed as mean ± std. err. [c]: C_q LOD, C_q of sample greater than C_q determined for the limit

559 of detection (LOD). [d]: n.d., not determined.

560 **FIGURE CAPTIONS**

561

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

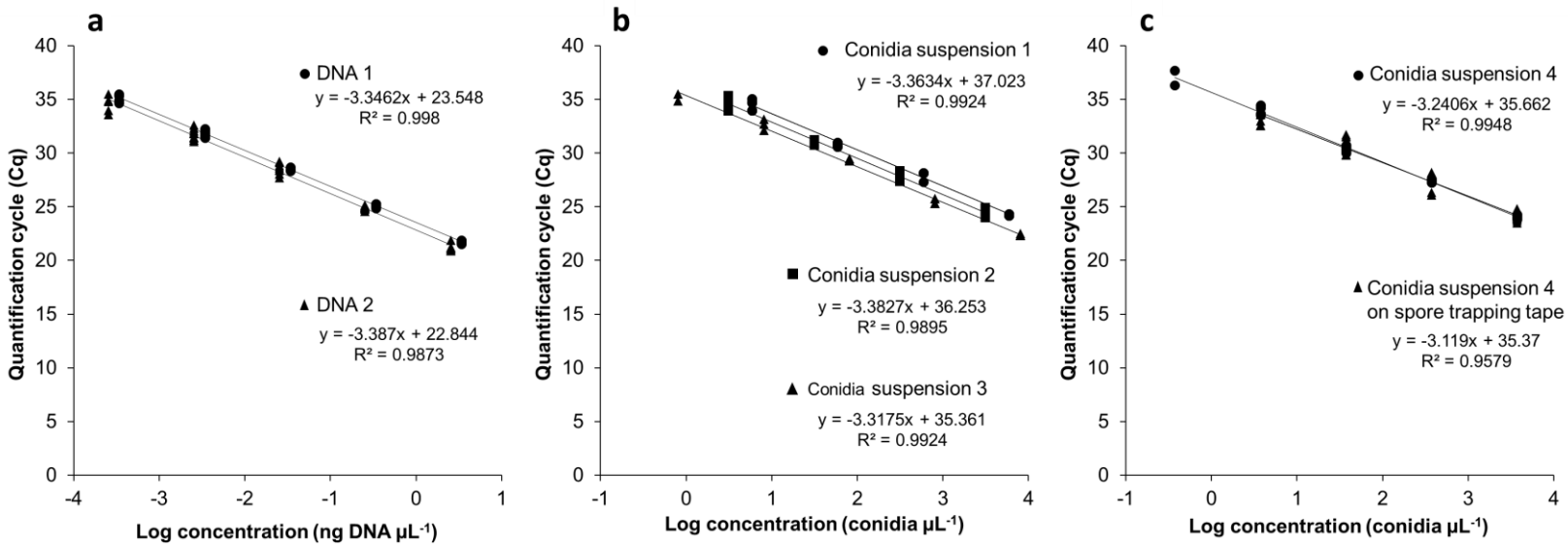
566

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

570

571 **Fig. 3** Correlation between the estimated amounts of conidia (conidia m^{-3}) obtained
572 through qPCR quantification (*x*) and microscopy examination (*y*) of airborne conidia trapped in
573 a peach orchard (Mollerussa, Catalonia, Spain) in the period April to July 2018 (N = 96 days)

574

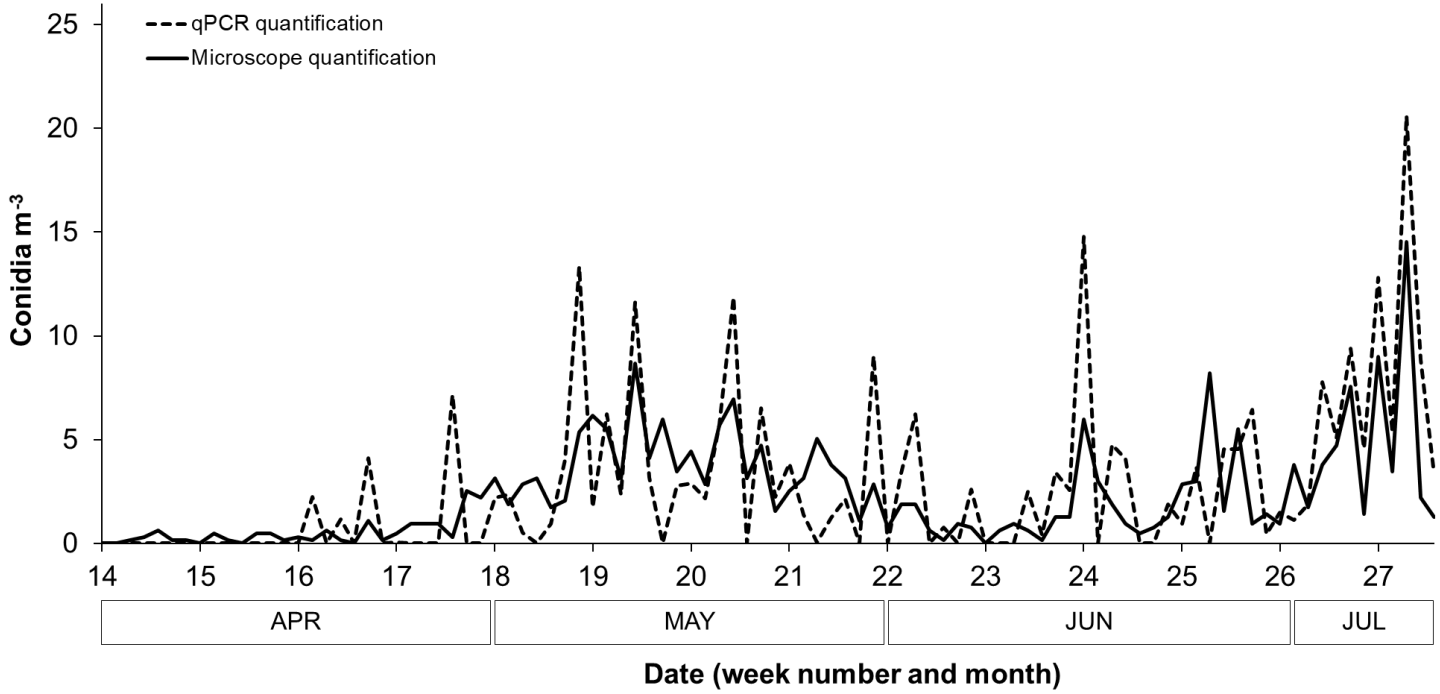

a
DNA 1
y = -3.3462x + 23.548
R² = 0.998
DNA 2
y = -3.387x + 22.844
R² = 0.9873
Quantification cycle (Cq)
Log concentration (ng DNA µL-1)
b
Conidia suspension 1
y = -3.3634x + 37.023
R² = 0.9924
Conidia suspension 2
y = -3.3827x + 36.253
R² = 0.9895
Conidia suspension 3
y = -3.3175x + 35.361
R² = 0.9924
Quantification cycle (Cq)
Log concentration (conidia µL-1)
c
Conidia suspension 4
y = -3.2406x + 35.662
R² = 0.9948
Conidia suspension 4 on spore trapping tape
y = -3.119x + 35.37
R² = 0.9579
Quantification cycle (Cq)
Log concentration (conidia µL-1)

575

576

577

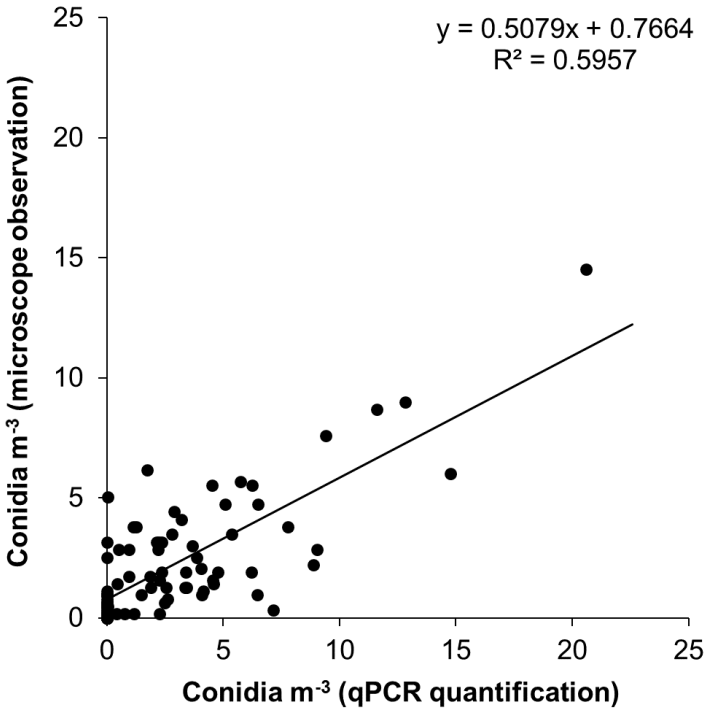
578


qPCR quantification
Microscope quantification
Conidia m-3
25
20
15
10
5
0
14
15
16
17
18
19
20
21
22
23
24
25
26
27
APR
MAY
JUN
JUL
Date (week number and month)

579

580

581


y = 0.5079x + 0.7664
R² = 0.5957
Conidia m-3 (microscope observation)
Conidia m-3 (qPCR quantification)
0
5
10
15
20
25

582

583