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Running page heading: Molecular detection of *Podosphaera pannosa*

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

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1 ABSTRACT

2 A molecular qPCR-based method was developed to detect and quantify *Podosphaera pannosa*,
3 the main causal agent of peach powdery mildew. A primer pair was designed to target part of
4 the ITS region of the fungal ribosomal DNA, which proved to be highly specific and sensitive. A
5 minimum of 2.81 pg μL^{-1} of *P. pannosa* DNA and 6 conidia mL^{-1} in artificially-prepared conidia
6 suspensions were found to be the limit of detection. Moreover, a quantification of conidia
7 placed on plastic tapes commonly used in volumetric air samplers was performed. Regression
8 equations on conidia quantification obtained either from aqueous conidia suspensions or
9 conidia placed on plastic tapes were similar. The protocol was further validated in field
10 conditions by estimating the number of *P. pannosa* conidia obtained with an air sampler, by
11 both microscopical and molecular quantification. Both techniques coincided in detecting the
12 peaks of conidia production during a 4-month sampling period, and a significant correlation (r
13 = 0.819) was observed between both quantification methods. Additionally, the molecular
14 method was applied to detect the latent fungal inoculum in different plant parts of peach
15 trees. The pathogen was detected mainly on the bark of affected twigs, and to a lesser extent,
16 in foliar buds. The method developed here can be applied in the study of *P. pannosa*
17 epidemiology and can help in improving the management of this pathogen through its early
18 detection and quantification.

19

20 KEYWORDS

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

22 INTRODUCTION

23 The ascomycete *Podosphaera pannosa* (Wallr.) de Bary is one of the causal agents of
24 powdery mildew, which occurs mainly on the *Prunus* and *Rosa* genera of Rosaceae (Farr and
25 Rossman 2019; Takamatsu *et al.* 2010). Other powdery mildew species can be found on
26 different peach fruit morphologies such as nectarines and flat fruits albeit rarely, such as *P.*
27 *clandestina*, *P. leucotricha*, and *P. tridactyla* (Farr and Rossman 2019). However, *P. pannosa* is
28 widely recognized as the main causal agent of the peach powdery mildew (PPM). In this
29 species, the pathogen infects green parts of the tree, e.g. fruits, leaves, buds, and twigs (Grove
30 1995; Ogawa and English 1991), where a distinguishable white-greyish mycelium develops on
31 the surface of the affected part. Severe infections of PPM on fruits makes them unacceptable
32 to industry, thus causing important economic losses. *Podosphaera pannosa* has been reported
33 from over 40 peach-growing countries in the world (Amano 1986; Farr and Rossman 2019).
34 The fungus overwinters in peach as dormant mycelium in latent buds (Ogawa and English
35 1991; Toma *et al.* 1998; Weinhold 1961; Yarwood 1957), and the ascocarps (chasmothecia) are
36 usually found in the mycelium infecting twigs and leaves (Butt 1978). Primary PPM infections
37 occur in spring, when primary inoculum is available and favourable weather conditions are
38 met. However, precise experimental data on the environmental conditions needed for primary
39 PPM infections are scarce (Toma *et al.* 1998; Weinhold 1961). Air-dispersed conidia released
40 from primary-established colonies are responsible for secondary infections that extend over
41 the vegetative growing season of peach tree (Grove 1995; Jarvis *et al.* 2002). In general, PPM
42 spreads rapidly in seasons when a relatively cold and humid spring is followed by a dry
43 summer (Toma *et al.* 1998). Previous studies reported on the optimal temperature and relative
44 humidity (about 21 °C and 70-95 % RH, respectively) for pathogen development (Grove 1995;
45 Toma *et al.* 1998). Regarding the infection of *P. pannosa* on *Rosa*, Longrée (1939) described
46 similar optimal conditions for *P. pannosa* infection (21 °C and between 75-99 % RH). The
47 control of PPM can be achieved efficiently through the foliar application of fungicides (Grove

48 1995; Hollomon and Wheeler 2002; Ogawa and English 1991), which usually starts at petals fall
49 or the beginning of fruit set and continues periodically (Grove 1995; Reuveni 2001). These
50 fungicide applications are done on a calendar basis (Ogawa and English 1991) since
51 epidemiological models on PPM infection risk are scarce. Recently, a decision support system
52 to initiate fungicide applications programs has been proposed (Marimon *et al.* 2020).

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

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

70

71 **MATERIALS AND METHODS**

72 **Experimental orchards**

73 Three experimental peach and nectarine orchards owned by IRTA and located in
74 Catalonia, Spain, were used in this study (Alcarràs, 41°36'33"N, 0°26'45"E; Cabrils, 41°31'7"N,
75 2°22'34"E; and Mollerussa, 41°37'8"N, 0°52'2"E). The orchard located in Alcarràs was an
76 'Autumn free' nectarine orchard, whereas orchards in Cabrils and Mollerussa were planted
77 with 'Early Gold' peach and 'Texas' almond interspecific progenies that are known to be
78 susceptible to PPM (Donoso *et al.* 2016). These orchards were managed using cultural
79 practices, such as pruning, soil management and nutrient supply, according to the guidelines of
80 Spanish Integrated Production Management practices (MAPA 2002). No fungicide treatments
81 were applied during the experimental period (spring to summer) to allow natural infections of
82 *P. pannosa*, which were known to occur in the orchards.

83 **Plant material**

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

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

104 **Fungal material**

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

110 **DNA extraction**

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

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

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

128 **Primer design**

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

145 **qPCR conditions**

146 Optimal qPCR conditions were set up as follows: for a final volume of 25 μ L each
147 reaction, products and concentrations were 10 μ L SYBR Premix Ex Taq™ TliRNase H Plus
148 (Takara), 0.4 μ M of each specific forward and reverse primers, 5 μ L of template DNA and HPLC-
149 grade deionized water to reach final volume. qPCR was carried out on a Rotor-Gene Q 5plex
150 thermal cycler (Qiagen, Hilden, Germany) with the following temperature and timing profile:
151 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.
152 After the final amplification cycle, the temperature was held at 72 °C for 90s. The melting
153 curve analysis was performed raising the temperature from 72 °C to 95 °C, increasing 1 °C
154 every 5 s with continuous measurement of fluorescence at 510 nm wavelength. All reactions
155 were run in triplicate and using genomic DNA extracted from *P. pannosa* conidia suspensions
156 as positive controls, and negative controls with no DNA template.

157 **Analytical specificity and sensitivity tests**

158 The primer pair specificity was checked *in silico* and *in vitro*. *In silico*, specificity for the
159 primer pair PpanITS1-F/PpanITS1-R was evaluated with the Primer-BLAST tool
160 (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). *In vitro*, specificity was tested by
161 analysing qPCR amplifications of 28 DNA samples obtained from six *Podosphaera* species other
162 than *P. pannosa* and occurring on several Rosaceae and non-Rosaceae species, which included
163 20 samples from the earlier described herbarium material, and fresh samples of *P. leucotricha*
164 (n = 5, from apple), and *P. tridactyla* (n = 3, from plum), both collected at IRTA Cabrils facilities.
165 Identity of those fungi different from *P. pannosa* was confirmed by sequencing their rDNA ITS
166 region using the forward primers ITS1F (Gardes and Bruns, 1993) and the reverse primer ITS4
167 (White et al., 1990) in single PCR, and using methods described elsewhere (Luque et al. 2005).
168 All qPCR reactions involved in the specificity test were carried out in triplicate and included
169 negative and positive controls of *P. pannosa*.

170 The primer pair sensitivity was evaluated according to the protocols described by
171 Armbruster and Pry (2008). Two independent DNA samples (DNA 1 and DNA 2) and three
172 independent conidia suspensions (CS 1, CS 2 and CS 3) were prepared and used in the
173 experiments. The DNA samples were obtained from conidia suspensions and later serially-
174 diluted, whereas the CS samples were serially-diluted before DNA extraction. In both cases,
175 DNA was extracted from the resulting conidia suspensions using the method described above.
176 The measured DNA concentrations for DNA 1 and DNA 2 samples were (mean \pm std. error) 25.4
177 \pm 3.8 ng μL^{-1} and 33.9 \pm 4.6 ng DNA μL^{-1} , respectively. Ten-fold dilutions series down to 1:10⁵
178 were prepared and subsequently used in the qPCR assays. For each CS sample, amounts of
179 conidia were determined from four measurements with five pseudoreplicates using a
180 haemocytometer. Initial conidia concentrations for CS1 to CS3 samples were 5.87 \pm 0.212 \times 10⁵
181 conidia mL⁻¹, 3.13 \pm 0.136 \times 10⁵ conidia mL⁻¹, and 8.06 \pm 0.274 \times 10⁵ conidia mL⁻¹, respectively.
182 For each suspension, ten-fold dilution series down to 1:10⁵ were prepared. The DNA from each
183 dilution point was extracted as described earlier. All DNA samples were amplified with the
184 primer pair designed in this study and using the qPCR conditions described above, and by
185 additionally including 0.4 μL of ROX Reference Dye in each reaction. All qPCR reactions were
186 performed using a StepOne™ Real-Time PCR System thermal cycler (Life Technologies,
187 Carlsbad, CA, USA). Three technical replicates were run for each biological sample, and three
188 replicates of deionized water template were included in each reaction plate as negative
189 controls. After each qPCR, a melting curve was performed to verify the targeted amplification
190 product. A homogeneous melting peak at 88°C indicated that the amplified targeted ITS1
191 region was specific for *P. pannosa*. For each DNA and CS samples, a standard curve was
192 calculated by plotting the quantification cycle values (C_q) against the logarithm of the DNA or
193 conidia concentration at each dilution point. The amplification efficiency (AE), intercept, slope,
194 and determination coefficient (r^2) were calculated for each standard curve obtained in this
195 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 1:10⁵ 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

221 proposed by the Spanish Aerobiological Network (REA) (Galán *et al.* 2007): each daily fragment
222 was stained with acid lactofuchsin and mounted on a glass slide. Microscope samples were
223 examined using a microscope (model Eclipse E400, Nikon Corporation, Toquio, Japan) at 250x
224 and only conidia that were morphologically compatible with *P. pannosa* were considered, *i.e.*
225 conidia containing fibrosin refractive bodies (Braun *et al.* 2002), and measuring 12-15 x 20-27
226 μm (Horst and Cloyd 2007). Final number of conidia per day was estimated from the examined
227 surface (about 45% of the total strip surface) and expressed as conidia m^{-3} . For qPCR
228 quantification, daily samples were cut into six equally-sized pieces and put into a 1.5 mL
229 Eppendorf tube. DNA was extracted and amplified according to the protocols described in this
230 study. Additionally, a positive control from CS 4 (dilution 1:10²) was included in the qPCR plate.
231 The quantification of conidia for each daily sample was calculated using the standard curve
232 obtained from the CS 4 suspension placed on a plastic tape. Samples matching the following
233 criteria were excluded from the analyses: *i*) C_q values higher than the LOB threshold
234 determined in this study, *i.e.* 35 cycles, *ii*) C_q values showing a standard deviation higher than
235 0.5 between technical replicates, and *iii*) replicates with a highly-different melting temperature
236 from that observed in the specific primer evaluation tests. Quantification of trapped conidia
237 using qPCR was expressed in conidia m^{-3} after proper conversion factors were applied on the
238 values obtained from the standard curve analysis (expressed in conidia mL^{-1}). Conversion
239 factors considered were: *i*) the volumetric ratio of sampler (10 L air min^{-1}), *ii*) the final volume
240 of DNA extracted from daily samples (100 μL), and *iii*) the standard curve data obtained from
241 the CS 4 conidial suspension as explained above.

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

243 Three biological replicates of different peach plant parts (leaves, leaf buds, floral buds
244 and twig barks) were detached from each collected branch. Samples were accurately examined
245 using a stereomicroscope (10x) 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, $12.43 \pm$
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 \pm
265 standard error of the mean are reported when appropriate.

266

267 **RESULTS**

268 **Primer design**

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

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

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

284 *P. pannosa* was detected and quantified in two independent DNA samples (DNA 1 and
285 DNA 2) obtained from *P. pannosa* conidia. A clear linear relationship was obtained between
286 the C_q values and the logarithm of DNA concentrations (Fig. 1a). Parameters for the standard
287 curves for DNA 1 and DNA 2 are described in Table 2. Both equations (Fig. 1a) had significant
288 slopes ($P < 0.001$) that showed to be equal ($P = 0.56$). Three independent conidia suspensions
289 were also quantified using qPCR (Fig. 1b). The standard regression curve parameters for
290 conidia suspensions CS 1, CS 2 and CS 3 are described in Table 2. Slopes for the equations of
291 the three conidia suspensions did not show significant differences among them ($P = 0.72$).
292 After these experiments, an arbitrary LOD was established at 2.81 ± 0.49 pg DNA μL^{-1} and 6 ± 2
293 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

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

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

363 The detection and quantification of airborne *P. pannosa* conidia using a volumetric air
364 sampler coupled with the qPCR method was successfully performed. When compared with the
365 microscopical observation of trapped conidia on plastic tapes, the molecular technique
366 allowed: *i*) to determine the period when *P. pannosa* conidia are present in the air, similar to
367 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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413

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419

420 Compliance with ethical standards

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

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

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

425

426 Authors' contributions

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

431

432 **Availability of data and material**

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

435

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547 detection and quantification of *Polystigma amygdalinum*, the cause of red leaf blotch
548 of almond. *Phytopathologia Mediterranea*, 57, 257-268.

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

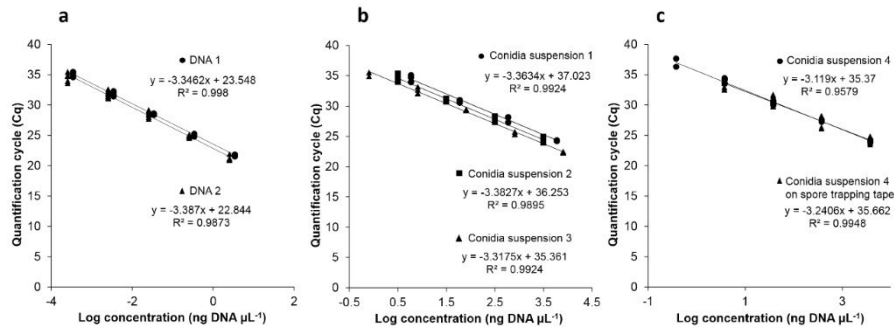
568

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

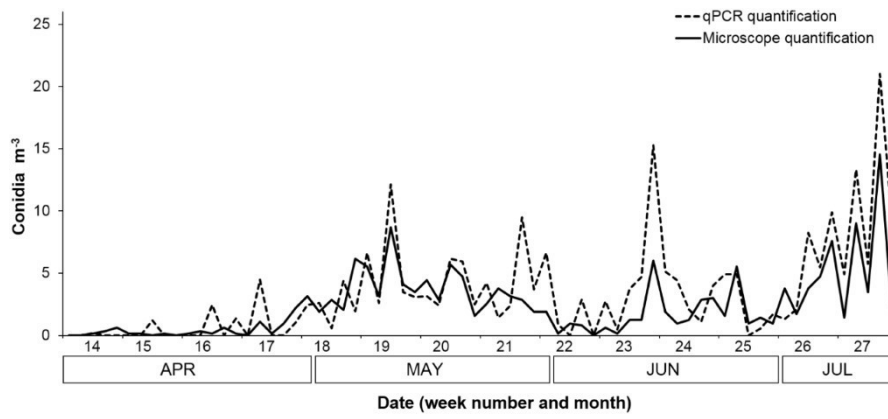
573 **Fig. 3** Correlation between the estimated amounts of conidia (conidia m^{-3}) obtained
574 through qPCR quantification (x) and microscopy examination (y)

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



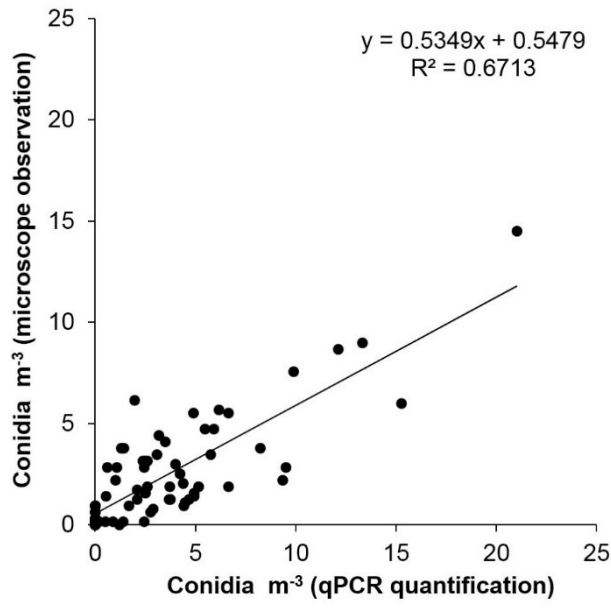
576

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



578

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



580