Identification of inoculum sources of *Fusicladium eriobotryae* in loquat orchards in Spain

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Abstract *Fusicladium eriobotryae* is the causal agent of loquat scab, the main disease damaging fruit, leaves and young twigs of this crop. A two growing seasons study (2015-2016 and 2016-2017) was carried out in two loquat orchards (cv "Algerie") to determine the inoculum sources of *F. eriobotryae* by direct observation of conidia, pathogen isolation on culture media and detection using a new real time PCR protocol developed in this study. One-year-old twigs, fruit peduncles and fruit mummies were randomly sampled three times per growing season on each orchard, and inflorescences only at flowering. Conidia of *F. eriobotryae* were not found and the isolation of the pathogen was neither possible from any sample in both seasons. Specific primers FUG2F and FUG2R, were designed to detect and quantify DNA of *F. eriobotryae* on plant material, with a limit of detection (LOD) established at 48.6 fg/µl. The DNA of the pathogen was not detected by real time PCR in fruit mummies neither inflorescences, but it was detected in fruit peduncles and twigs in the season 2016-2017 with concentrations ranging from 50 to 2742 fg/µl, confirming that this two loquat organs might act as potential inoculum sources for *F. eriobotryae*. The detection of *F. eriobotryae* only in this season agrees with the predictions of an epidemiological model for this pathogen. Our results indicate that in years with a high disease pressure, fruit twigs and peduncles might act as a source of inoculum of new infections the following year.

Key words Loquat scab, epidemiology, pathogen detection, qPCR.

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Compliance with ethical standards

Human and Animal Rights. The authors declare that ethical standards have been followed and that no human participants or animals were involved in this research.

Introduction

Loquat (*Eriobotrya japonica* Lindl.) is a fruit tree grown in regions with subtropical climate, including China, Japan and the Mediterranean basin, being Spain, Israel, Italy and Turkey the major producing countries in this area (Caballero and Fernández 2002; Lin 2007; Janick 2011). Loquat scab is the main disease affecting loquat in Spain and the whole Mediterranean region (Sánchez-Torres et al. 2009; Gladieux et al. 2010; González-Domínguez et al. 2017). This disease damages fruits and leaves, and young twigs can also be affected when the disease is severe (Sánchez-Torres et al. 2009; Gladieux et al. 2010). The symptoms first appear as circular chlorotic spots, which become olive-coloured and velvety as they increase in size (Sánchez-Torres et al. 2007b; 2009). Scabby fruits are unsuitable for sale, resulting in significant economic losses (Soler et al. 2007). *Fusicladium eriobotryae* (Cavara) Sacc. is the causal agent of loquat scab. This pathogen has been reported in the Mediterranean region and also in USA and Chile (Raabe and Gardner 1972; Sánchez-Torres et al. 2009; Acuña 2010). *Fusicladium* spp. are the anamorphic stages of the ascomycete genus *Venturia* De Not., but the sexual stage of *F. eriobotryae* has never been found in nature (Gladieux et al. 2010). Within the genus *Venturia*, many species have been also described as causal agents of scab in other fruit trees such as *Venturia inaequalis* (Cooke) G. Winter and *Venturia pyrina* Aderh., causal agents of apple and pear scab, respectively, *Venturia carpophila* E.E. Fisher on peach (*Prunus domestica* L.) or *Fusicladium oleagineum* (Castagne) Ritschel & U. Braun on olive (*Olea europea* L.) (González-Domínguez et al. 2017).

Several studies have elucidated some of the most relevant aspects relative to the biology and epidemiology of *F. eriobotryae* (Prota 1960; Ptskialadze 1968; Salerno et al. 1971; Raabe and Gardner 1972; Sánchez-Torres et al. 2009; González-Domínguez et al. 2013; 2014a; 2014b). These studies confirmed *F. eriobotryae* as a pathogen only dispersed by rain that requires mild temperatures and long wet periods to infect loquat trees. Once infection has occurred, the fungus grows under the cuticle and conidiophores erupt through it producing new conidia (Sanchez-Torres et al. 2009; González-Domínguez et al. 2014b).

Based on the above information, González-Domínguez et al. (2014a) described the life cycle of this pathogen. The conidia produced by the oversummering lesions (the pathogen does not overwinter, because loquat blooms in autumn, develops fruit in winter, and ripens in early spring) serve as the only form of primary inoculum, which are dispersed by rain splash, infecting twigs, young leaves and/or loquat fruits. Secondary infections are caused by conidia produced on lesions during the entire tree-growing season as

long as favourable environmental conditions occur. However, these authors pointed out the lack of knowledge regarding the potential inoculum sources of *F. eriobotryae* in loquat orchards.

For some other *Venturia* spp., the overwintering (or oversummering) process has been seldom studied (González-Domínguez et al. 2017). Considering the *Venturia* spp. in which only the asexual stage is known, *F. effusum* G. Winter overwinters on pecan trees mainly on the surface of twigs and nuts as stromata, which sporulate profusely in early spring (Demaree 1924). In the case of *F. oleagineum* (Castagne) Ritschel & U. Braun, the most important inoculum sources are the infected leaves remaining in the olive canopy (Graniti 1993; Viruega et al. 2013), because the fungus does not produce conidia on fallen, scabbed leaves (Viruega et al. 2013). Regarding *F. eriobotryae*, González-Domínguez et al. (2014a) hypothesized that the fungus probably oversummers as mycelium and/or conidia in lesions on branches and leaves and in mummified fruits. Rodriguez (1983) and Gisbert et al. (2006) previously stated that flowers are susceptible to *F. eriobotryae*; however, symptoms on inflorescences have not been properly described and the pathogen has never been isolated from them. Thus, specific experiments and methodologies should be developed to better understand the importance of these tree organs as inoculum sources, the ability of the fungus to sporulate on them, and their epidemiological role.

Isolation of *F. eriobotryae* by conventional methods from infected tissue on culture media is difficult due to its slow growth, making the process time-consuming (up to 6 weeks) and prone to contaminations by other fungal species (Sánchez-Torres et al. 2009). New methodologies based on molecular tools provide several advantages over traditional detection methods because of its specificity, sensitivity and fastness. Polymerase chain reaction (PCR)-based protocols have become a valuable tool for the detection and diagnose of plant-pathogenic fungi. Sánchez-Torres et al. (2009) designed specific primers at the region of glyceraldehyde 3-phosphate dehydrogenase gene (G3PD), which were able to clearly separate strains of *F. eriobotryae* from other related species, namely *F. carpophilum*, *V. inaequalis*, and *V. pyrina*. Moreover, a nested-polymerase chain reaction protocol (nested-PCR) has been recently developed by González-Domínguez et al. (2015) for *F. eriobotryae*-specific identification from infected loquat tissues.

An additional step in molecular DNA based methods is the development of quantitative real time PCR (qPCR) protocols for quantifying phytopathogenic fungi. Two qPCR protocols were developed and validated to quantify *V. inaequalis* in apple infected leaf tissue by Däniels et al. (2012) and Gusberti et al. (2012). However, a qPCR-based protocol has not been set up yet to detect *F. eriobotryae* in loquat, which

could be useful to develop epidemiological studies for this species. Thus, the general aim of this work was to determine the inoculum sources of *F. eriobotryae* in loquat orchards. The specific objectives were: (i) to develop a qPCR protocol to detect and quantify *F. eriobotryae* on plant material and (ii) to analyse samples of different tree organs and growing seasons by means of; direct observation of conidia, pathogen isolation, and detection using qPCR. The results will contribute to elucidate the epidemiological role of the primary inoculum sources of *F. eriobotryae*.

Material and methods

Design of specific primers to detect *F. eriobotryae* using qPCR

Specific primers were designed to detect DNA of *F. eriobotryae* using qPCR, based on a partial sequence of the G3PD gene. G3PD nucleotide sequences of different *F. eriobotryae* isolates and other *Venturia* species were downloaded from NCBI (Table 1) and aligned with the software MEGA v.7 (Kumar et al. 2016) with default options. The alignment of sequences obtained was used to identify conserved and variable regions. Species-specific primers were designed on the basis of *F. eriobotryae* regions of divergence. Primer conditions were set as follows: G/C content between 40 and 60%, maximum (self) complementarity of 3, maximum 3' (self) complementarity of 0 (no primer dimer formation), melting temperature of 60°C (±1°C), primer size of 20 bp (±2) and PCR product size between 75 and 125 bp. The characteristics of each primer were evaluated with the DNAMAN software (Lynnon BioSoft, Montreal, Canada) and were synthesized by Isogen Life Science (Utrecht, The Netherlands).

Primers specificity

Specificity of the primers was tested with a subset of isolates of *F. eriobotryae*, *V. inaequalis*, *V. pyrina* and *F. oleagineum*, because of its high genetic similarity (Gladieux et al. 2010), and with other fungal species commonly found in the process of isolation from loquat samples (Table 1). In the case of *F. eriobotryae*, isolates were representative of different locations. Reaction conditions such as annealing temperature, $MgCl_2$ or primer concentrations were adjusted experimentally to optimize the amplification. Optimized qPCR reactions were prepared containing 2 µL of DNA extracted from each sample, 1× of SYBR Green Master Mix: SYBR_Premix Ex Taq_II (Tli RNase H Plus) (Takara Bio Inc., Shiga, Japan) and 0.4 µM of each primer. The reaction mix was adjusted to a final volume of 20 µL with sterile distilled water. qPCR analysis were performed using the Rotor-Gene 6000 real-time rotary analyser (Qiagen, Hilden, Germany) and the program consisted of a first denaturation at 95°C for 10 s, followed by 50 cycles of

denaturing at 95°C for 5 s and annealing at 60°C for 45 s. The generated data were analysed using Rotor-Gene 6000 Series software v. 1.7 (Qiagen).

Real time PCR sensitivity

Sensitivity of qPCR assay was assessed by determining the minimum amount of *F. eriobotryae* that could be detected. A standard curve was constructed with *F. eriobotryae* isolate FeV40 (Table 1). For DNA extraction, fungal mycelia of FeV40, grown on potato dextrose agar (PDA, Biokar-Diagnostics, Zac de Ther, France) for 6 weeks at 25°C in darkness, was scraped from the surface of the plate with a sterile scalpel. Total DNA was extracted using the DNeasy Power Plant Pro Kit (Qiagen) following the manufacturer's instructions and mycelia was previously homogenized with 4 steel beads of 2.38 mm and 2 of 3 mm diameter (Qiagen) using a FastPrep-24TM5G (MP Biomedicals, California, USA) at 5 m/s for 20 s twice. Total DNA extracted from the isolate was quantified with Invitrogen Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and sevenfold serially diluted, yielding concentrations from 48.60 ng/µL to 4.86 fg/µL. qPCR analyses were performed with the different dilutions as explained above and the standard curve was generated following the MIQE guidelines (Bustin et al. 2009), by plotting quantification cycle (C_q) values obtained for each specific DNA concentration, versus the logarithm of the initial concentration of isolate DNA. The mean DNA concentration and the standard deviation were determined from four DNA repeats per DNA concentration. The amplification efficiency (E) and the coefficient of determination (R^2) of the standard curve were obtained using the Rotor-Gene 6000 Series software v. 1.7 (Qiagen) and the limit of detection (LOD) was calculated from the last dilution in which DNA of *F. eriobotryae* was successfully amplified, accompanied by a melting curve peak temperature specific to *F. eriobotryae*.

Loquat trees sampling

A two growing seasons study (2015-2016 and 2016-2017) was carried out in two loquat orchards located at Callosa d'En Sarrià, Alicante province, south-eastern Spain. Both plots were planted with the cultivar Algerie; orchard A, covered with a polyethylene mesh, was 20 years old, occupied 0.49 ha, and was drip irrigated (two drippers per tree) and; orchard B, uncovered, was 25 years old, and occupied 0.32 ha. Prior to the experiments and during both growing seasons, both orchards did not receive fungicide applications to manage scab, but otherwise received standard cultural practices. Different loquat organs were randomly sampled three times per growing season on each orchard at: flowering, fruit development and fruit maturity

stages (Table 2). Per each sampling date and orchard: 10-one year old twigs, 10 fruit peduncles and 10 fruit mummies were selected. Ten inflorescences were also collected at the first sampling date, performed during the flowering period. Each sample was divided into two subsamples; one was used for direct conidia observation and isolation of *F. eriobotryae* and the other was stored at −20°C for molecular detection.

Conidia observation and fungal isolation

2 g of each subsample were washed in 20 mL of sterile water and briefly vortexed for 5 s. The liquid suspension obtained was centrifuged for 15 min at 5000 rpm. The supernatant was discarded and the resulting pellets were collected in 2 mL tubes to centrifuge again for 5 min at 10000 rpm. Each pellet was used to direct conidia observation under microscope and it was subsequently stored at −20°C for molecular procedures. The washed subsamples were placed in a humid chamber for 3 days to enhance *F. eriobotryae* growth and sporulation. Then, small tissue fragments were surface sterilized with ethanol 70% and plated on potato dextrose agar (PDA, Biokar-Diagnostics) supplemented with 0.5 g/L of streptomycin sulphate (Sigma-Aldrich, St. Louis, Missouri, USA) for the pathogen isolation. The plates were incubated at 20ºC in darkness for 15 days and daily assessed for the presence of *F. eriobotryae* based on culture morphology (Schubert et al. 2003; Sánchez-Torres et al. 2007a).

DNA extraction

All the samples stored at −20°C (plant material and pellets obtained from the washings) were used for the molecular detection. Forty mg per sample were used to perform the total DNA extraction using the DNeasy Power Plant Pro Kit (Qiagen). Prior to perform the DNA extraction following the manufacturer's instructions, plant material samples were homogenized as previously described and pellet samples were grinded using a 0.5 mL volume of ceramic beads of 0.5 mm diameter (Zymo Research, Irvine, California, USA). Extracted DNA was kept at −20°C until its use in qPCR analysis.

Detection of *F. eriobotryae* in plant material and pellet samples by qPCR assay

qPCR analyses were conducted with the designed specific primers to detect and quantify *F. eriobotryae* using the Rotor-Gene 6000 real-time rotary analyser (Qiagen). Reactions were prepared following the optimized conditions established when designing the primers, containing 2 µL of DNA extracted from each sample (plant material and pellets obtained from the washings). Each set of reactions included two replicates of each sample, a negative control (water) and a positive control with DNA extracted from a pure

culture of *F. eriobotryae* FeV40. To detect the presence of PCR inhibitors in the DNA extractions, an extra set of reactions was included, with two more replicates per each sample, adding 2 µL FeV40 DNA (internal positive controls). All qPCR reactions were performed as previously established. The DNA concentration of the pathogen in each sample was quantified using the standard curve, based on C_q values obtained in the qPCR reactions. The mean DNA concentration and the standard deviation were determined from the two DNA replicates per sample. Melting curves were examined to ensure that unespecific products were amplified.

When the internal positive controls (reactions with DNA of the sample and DNA of FeV40) were not amplified, the DNA of the sample was diluted 1/10 to avoid the inhibitions observed (Schrader et al. 2012). The new dilutions were used for a new qPCR reaction adding the extra set of reactions with the DNA of FeV40 (internal positive controls) at the same conditions as indicated above. When the internal positive controls did not show any amplification, the same DNA dilution was used to perform a nested-real time PCR. The first reaction of the nested-real time PCR was a conventional PCR reaction and was carried out in a thermocycler Veriti™ 96 well Thermal Cycler (Applied Biosystems, Foster City, California, USA) with the primers G3PD-For 5′-TCGGTCGTATTGGACGTATC-3′ and G3PD-Rev 5'-GACCTTGGCCATGTATGCTA-3' (Sanchez-Torres et al. 2009). Reactions contained 1× PCR Buffer, 2.5 mM $MgCl_2$, 0.8 mM of dNTP, 0.4 mM of each primer, 1 U Hot Began Taq DNA polymerase (Canvax Biotech, Córdoba, Spain) and 1 µL DNA sample. The reaction mix was adjusted to a final volume of 20 µL with sterile distilled water. A negative control (water) and a positive control (DNA of FeV40) were also added. The programme used consisted of an initial step of 1 min at 95°C, followed by 20 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 30 s and elongation at 72°C for 40 s. A final extension was performed at 72°C for 10 min. The PCR product obtained was used as template DNA for the second round, which was a qPCR reaction carried out with the specific primers designed in this study at the same conditions described before. A negative control (water), a positive control (DNA of FeV40) and internal positive controls (DNA of the sample and DNA of FeV40) were also added.

Disease progress of loquat scab predicted by an epidemiological model

To predict the disease progress during the two growing seasons in which samples were collected, a mechanistic weather based model, specific for loquat scab, was run as described by González-Domínguez et al. (2014a). The model splits the disease cycle of *F. eriobotryae* into different state variables; changes

209 from one state to the following one is based on the environmental conditions and run by means of
210 mathematical equations. Briefly, the model assumes that conidia of *F. eriobotryae* are always present, and
211 predicts that any measurable rain (i.e., R > 0.2 mm in 1 h) triggers an infection process. The model predicts
212 the development of each infection in time following an infection rate (dependent on hourly values of
213 temperature and leaf wetness) (González-Domínguez et al. 2014a). The accumulated value of these
214 infection rates was used to estimate the progress of the disease during both growing seasons (González-
215 Domínguez et al. 2018).

216 The model was run from 1st October 2015 to 30th March 2016, and from 1st October 2016 to 28th May 2017.
217 A software developed by the Regional Agrometeorological Service (http://riegos.ivia.es/) was used to
218 operate the model and hourly weather data was obtained from a weather station located in Callosa d'En
219 Sarrià, Alicante, southeastern Spain.

220 **Results**

221 Primers design, specificity and sensibility

222 Complete homogeneity of the studied G3PD region was observed within the isolates of *F. eriobotryae*. On
223 the basis of *F. eriobotryae* regions of divergence compared with other related species, two primers were
224 designed named FUG2F 5'-GAATGAGAAGACCTACGACC-3' and FUG2R 5'-
225 AAGTCAGTAGTCTGTATGA-3'. The qPCR assay performed under optimized conditions with a melting
226 temperature of 60°C, showed standard fluorescence amplification representing exponential growth and
227 amplified a specific product of 110 bp for all *F. eriobotryae* isolates tested (Table 1). Amplicons were not
228 obtained from samples of *V. inaequalis*, *V. pyrina* and *F. oleagineum*, neither from samples of other fungal
229 species commonly found in the process of isolation (Table 1). The standard curve, constructed with serial
230 dilutions of the DNA of the isolate FeV40, revealed that the primers designed in the present study were
231 quite accurate over a linear range and high correlations between C_q and DNA quantities were obtained with
232 R^2 value of 0.99832 and reaction efficiency of 1.00 (Fig. S1). The standard curve showed that the minimum
233 concentration of *F. eriobotryae* DNA detectable was at C_q value of the dilution D6 thus, the limit of
234 detection (LOD) was established at 48.6 fg/µL.

235 Conidia observation and fungal isolation

236 Conidia of *F. eriobotryae* were not observed in the pellets obtained from any of the samples. However, a
237 high presence of conidia of other fungal saprophytes belonging to different genera, such as *Alternaria*,
238 *Aspergillus*, *Cladosporium* and *Penicillium* were observed, especially abundant in fruit mummies and
239 twigs. Similar results were obtained from isolations on culture media, where it was not possible to obtain
240 colonies of *F. eriobotryae*, but saprophytes were also prevalent.

241 Molecular detection of *F. eriobotryae*

242 Analysis for qPCR inhibitors in the extracted DNA from the different plant material and pellet samples
243 indicated the presence of inhibitions in orchards A and B in both growing seasons. Those samples were
244 diluted 1/10 and analysed again with qPCR. The dilution performed successfully solved the inhibitions in
245 almost the all samples, except for the fruit mummies. Thus, a nested-real time PCR was performed with the
246 DNA diluted from all the fruit mummy samples (plant material and pellets obtained from the washings),
247 including the ones that did not show inhibition. After nested-PCR analysis, all internal controls were
248 positive, solving the inhibitions previously observed.

249 Real time PCR results confirmed the presence of *F. eriobotryae* in plant material and pellet samples during
250 the second growing season (2016-2017). DNA of *F. eriobotryae* was detected in twigs during the second
251 sampling (in one sample of plant material and one pellet sample, both collected in orchard B), and during
252 the third sampling (in five samples of plant material and three pellet samples, both also collected in orchard
253 B). During the third sampling, the DNA of the pathogen was detected in fruit peduncles (in one sample of
254 plant material, collected in orchard A and in two pellet samples from orchard B). The concentration of *F.*
255 *eriobotryae* DNA in twig samples ranged from 50 to 2742 fg/µL, except for one plant material sample and
256 one pellet sample, where the observed concentrations were close to the LOD (Table 3). The DNA of the
257 pathogen detected in fruit peduncles samples was not possible to quantify due to the low reproducibility
258 obtained in the results (Table 3).

259 Disease progress of loquat scab predicted by the epidemiological model

260 During the first (2015-2016) and the second (2016-2017) season, 217 mm and 961 mm of rain were
261 recorded, respectively (Fig. 1a; 2a). The accumulated infection rate predicted by the model was higher for
262 the second growing season compared with the first one (Fig. 1b; 2b). During the first growing season, the
263 accumulated infection rate increased slightly only in two periods: during the last two weeks of October and
264 at the end of March (Fig. 1b). However, during the second growing season two noticeable increases in the

accumulated infection rate were observed in mid-October, from November 20th to December 20th, and during the third week of January (Fig. 2b). The model predicted a final value of the accumulated infection rate 3 times higher for the second season, compared with the first one. A daily increase of the accumulated infection rate higher than 0.2 (considered as high risk) was observed 5 times the first growing season and 11 times the second one (Fig. 1b; 2b). *Fusicladium eriobotryae* DNA was detected in the second and third sampling of the second growing season (Fig. 2b).

Discussion

The identification of inoculum sources of fungal pathogens is an important step to elucidate the life cycle of these organisms. This study contributes to determine which organs of loquat trees might act as potential inoculum reservoirs of inoculum for *F. eriobotryae* in loquat orchards in Spain.

A specific pair of primers (FUG2F and FUG2R) were designed to detect and quantify *F. eriobotryae* by qPCR analysis. The two primers developed here showed a high specificity to detect *F. eriobotryae*, amplifying a large number of isolates but not from other related fungal species. A specific primer Fusic1 F, used in combination with the universal primer EF1-986R, was previously designed for a nested-polymerase chain reaction in the elongation factor EF1-α gene (González-Domínguez et al. 2015). However, a qPCR-based protocol had not been set up yet to detect *F. eriobotryae* in loquat. Sánchez-Torres et al. (2009) designed primers in the region of G3PD gene, sowing high variability between *F. eriobotryae* and close related species such as *V. inaequalis*. These results showed the G3PD as the most suitable region for the design of highly specific primers thus, the primers used in the present study were designed in that region.

The standard curves developed showed that this set of new primers were able to detect DNA of *F. eriobotryae* when concentrations were equal or greater than 48.6 fg/µL. Däniels et al. (2012) and Gusberti et al. (2012) developed and validated two qPCR protocols to quantify *V. inaequalis* in apple infected leaf tissue, with a limit of detection of 100 fg and 50 fg per reaction, respectively. Based on the present results, the specific primers designed to detect *F. eriobotryae* were sensitive enough to be used in the qPCR analysis performed in this study.

It was not possible to observe conidia of *F. eriobotryae* neither from liquids obtained washing different loquat organs, or attempting fungal isolation from these organs. However, mycelial saprophytes belonging to the genera *Alternaria*, *Aspergillus*, *Cladosporium* and *Penicillium* were found; these

294 saprophytes were more abundant in twigs and fruit mummies than in fruit peduncles and inflorescences.
295 *Fusicladium eriobotryae* is a slow-growing pathogen, with a maximum *in vitro* growth rate of 0.49 mm/day
296 at 20ºC (González-Domínguez et al. 2013), making very difficult its isolation by conventional techniques
297 due to the contamination by other fast-growing saprophytes (Sánchez-Torres et al. 2009). For this reason,
298 a real time PCR assay was designed to detect and quantify the DNA of *F. eriobotryae* in each type of loquat
299 organ as a more reliable alternative to microscopic examination.

300 González-Domínguez et al. (2014a) assumed that *F. eriobotryae* oversummers in mummified fruits that
301 remain in the tree after harvest. However, in the present study, the DNA of this fungus was not detected in
302 any sample of fruit mummies collected during the two growing seasons 2015-2016 and 2016-2017. Loquat
303 mummies are not comparable to those caused by pathogens such as *Monilinia* spp. in stone fruit (Villarino
304 et al. 2010), or *Botrytis cinerea* on grapevine (Ciliberti et al. 2015), in which the fungus causes a quick
305 generalized colonization and rot of the fruits, thus becoming important sources of inoculum. In loquat, *F.*
306 *eriobotryae* grows only subepidermically, causing superficial lesions that progress slowly (González-
307 Domínguez et al. 2013). In this case, the mummies are fruits that remain attached to the trees, but suffer a
308 progressive decomposition process mainly caused by the action of saprophytes such as *Alternaria*,
309 *Cladosporium*, *Rhizopus*, etc. Tissues in these fruits are highly degraded and likely not colonized by the
310 pathogen, difficulting both the isolation and the detection by DNA. The efficiency of PCR reactions could
311 have also been influenced by chemical compounds of different nature present in this type of plant tissue,
312 co-extracted with the DNA of the sample (Schena et al. 2013). To avoid the risk of false negatives, internal
313 controls were added in the qPCR analysis to detect PCR inhibitors. Inhibition problems were observed
314 when analysing fruit mummy and some twig samples. The dilutions and the nested qPCR analysis
315 performed with the DNA of the false negatives solved the inhibitions observed in twig and fruit mummy
316 samples, respectively, avoiding the previous difficulties in the detection of *F. eriobotryae* DNA in these
317 type of organs. Some other studies have also experienced inhibitions when detecting DNA of pathogenic
318 fungi in problematic samples such as soil or necrotic tissues (Cullen et al. 2001; Bilodeau et al. 2012; Pilotti
319 et al. 2012; Ghasemkhani et al. 2016). Further research would be necessary to set up a protocol able to
320 detect *F. eriobotryae* in tissues where the presence of inhibitors is more likely without using DNA dilutions
321 or nested qPCR approaches.

322 *Fusicladium eriobotryae* DNA was neither found in inflorescences samples collected in both
323 growing seasons. Moreover, before the inflorescences were sampled, the epidemiological model predicted

2 and 3 days with high risk of *F. eriobotryae* infection (i.e., daily increase in the accumulated infection rate>0.2) in the first and the second season, respectively, indicating that environmental conditions were suitable for infections. The ability of this pathogen to infect loquat inflorescences has been remained unclear; despite Rodriguez (1983) and Gisbert et al. (2006) stated that inflorescences are susceptible to this pathogen, inflorescence symptoms have not been properly described and *F. eriobotryae* has never been isolated from them. The results obtained in the present study suggest that loquat inflorescences should not be considered inoculum sources of this pathogen.

Fusicladium eriobotryae DNA was detected and quantified using qPCR analysis in plant material and pellet samples of twigs collected during the second and the third sampling dates of the second growing season. Both samplings were preceded by a period of high disease pressure, consequence of the several rain events recorded in this period (González-Domínguez et al. 2014a). Despite the fact that qPCR assays are unable to discriminate between DNA from viable and non-viable cells, the results obtained in the present study suggested that loquat twigs could be a primary inoculum source of *F. eriobotryae*. This organ has been considered the main source of inoculum in case of *V. carpophila* (Scherm et al. 2008; Lalancette et al. 2012). The pathogen DNA was also detected in some samples of fruit peduncles during the third sampling date of the second growing season. This result suggests that fruit peduncles might also act as a source of inoculum of new infections the following year.

Currently, cultural practices recommend to reduce the inoculum level focusing on the removal of fruit mummies, as suggested for other pathogens which cause fruit diseases (van Leeuwen et al. 2002). This management practice has resulted in an effective reduction of primary inoculum of the pathogen early in the season; however, based on the results of this work, the treatment of twigs and fruit peduncles at the end of the season could be also considered in the years with a high pressure of the disease.

Reference list

Acuña, R. P. (2010). Compendio de bacterias y hongos de frutales y vides en Chile. Servicio Agrícola y Ganadero, Santiago de Chile, Chile.

Bilodeau, G. J., Koike, S. T., Uribe, P., & Martin, F. N. (2012). Development of an assay for rapid detection and quantification of *Verticillium dahliae* in soil. Phytopathology, 102, 331–343.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Hipley, G. L., Vandesompele, J., & Wittwer, C. T. (2009). The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clinical Chemistry, 55, 611–622.

Caballero, P., & Fernández, M. A. (2002). Loquat, production and market. Options Méditerranéennes Serie A, 58, 11–20.

Ciliberti, N., Fermaud, M., Languasco, L., & Rossi, V. (2015). Influence of fungal strain, temperature, and wetness duration of infection of grapevine inflorescences and young berry clusteres by *Botrytis cinerea*. Phytopathology, 105, 325–333.

Cullen, D. W., Lees, A. K., Toth, I. K., & Duncan, J. M. (2001). Conventional PCR and real-time quantitative PCR detection of *Helminthosporium solani* in soil and on potato tubers. European Journal Plant Pathology, 107, 387–398.

Daniëls, B., De Landtsheer, A., Dreesen, R., Davey, M. W., & Keulemans, J. (2012). Real-time PCR as a promising tool to monitor growth of *Venturia* spp. in scab-susceptible and -resistant apple leaves. European Journal of Plant Pathology, 134, 821–833.

Demaree, J. (1924). Pecan scab with special reference to sources of the early spring infections. Journal of Agriculture Research, 28, 321–330.

Ghasemkhani, M., Holefors, A., Marttila, S., Dalman, K., Zborowska, A., Rur, M., Rees-George, J., Nybom, H., Everett, K. R., Scheper, R. W. A., & Garkava-Gustavsson, L. (2016). Real-time PCR for detection and quantification, and histological characterization of *Neonectria ditissima* in apple trees. Trees, 30, 1111–1125.

Gisbert, A. D., Besoain, X., Llácer, G., & Badenes, M. L. (2006). Protección de cultivo II, Enfermedades. In M. Agustí, C. Reig, & P. Undurraga (Eds.), *El Cultivo del Níspero Japonés* (pp. 227–246). Valencia, Spain: Gráficas Alcoy.

Gladieux, P., Caffier, V., Devaux, M., & Le Cam, B. (2010). Host specific differentiation among populations of *Venturia inaequalis* causing scab on apple, pyracantha and loquat. Fungal Genetics and Biology, 47, 511–521.

Graniti, A. (1993). Olive scab: a review. EPPO Bulletin, 23, 377–384.

González-Domínguez, E., Rossi, V., Armengol, J., & García-Jiménez, J. (2013). Effect of environmental factors on mycelial growth and conidial germination of *Fusicladium eriobotryae*, and the infection of loquat leaves. Plant Disease, 97, 1331–1338.

González-Domínguez, E., Armengol, J., & Rossi, V. (2014a). Development and validation of a weather-based model for predicting infection of loquat fruit by *Fusicladium eriobotryae*. PLoS One, 9, e107547.

González-Domínguez, E., Rossi, V., Michereff, S. J., García-Jiménez, J., & Armengol, J. (2014b). Dispersal of conidia of *Fusicladium eriobotryae* and spatial patterns of scab in loquat orchards in Spain. European Journal of Plant Pathology, 139, 849–861.

González-Domínguez, E., León, M., Armengol, J., & Berbegal, M. (2015). A nested polymerase chain reaction protocol for in planta detection of *Fusicladium eriobotryae*, causal agent of loquat scab. Journal of Phytopathology, 163, 415–418.

González-Domínguez, E., Armengol, J., & Rossi, V. (2017). Biology and epidemiology of *Venturia* species affecting fruit Crops: a review. Frontiers in Plant Science, 8, 1496.

Gusberti, M., Patocchi, A., Gessler, C., & Broggini, G. A. L. (2012). Quantification of *Venturia inaequalis* growth in *Malus* × *domestica* with quantitative real-time polymerase chain reaction. Plant Disease, 96, 1791–1797.

Janick, J. (2011). Predictions for loquat improvement in the next decade. Acta Horticulturae, 887, 25–30.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution, 33, 1870–1874.

Lalancette, N., McFarland, K., & Burnett, L. (2012). Modelling sporulation of *Fusicladium carpophilum* on nectarine twig lesions: relative humidity and temperature effects. Phytopathology, 102, 421–428.

Lin, S. Q. (2007). World loquat production and research with special reference to China. Acta Horticulturae, 750, 37–44.

403 Martínez-Calvo, B. J., Badenes, M. L., Llacer, G., Bleiholder, H., Hack, H., & Meier, U. (1999).
404 Phenological growth stages of loquat tree (*Eriobotrya japonica* (Thunb) Lindl.). Annals of Applied
405 Biology, 134, 353–357.

406 Pilotti, M., Lumia, V., Di Lernia, G., & Brunetti, A. (2012). Development of Real-Time PCR for in wood-
407 detection of *Ceratocystis platani*, the agent of canker stain of *Platanus* spp. European Journal of
408 Plant Pathology, 134, 61–79.

409 Prota, U. (1960). Ricerche sulla «ticchiolatura del Nespolo del Giappone e sul suo agente (*Fusicladium*
410 *eriobotryae* Cav.). I. Observazioni sull'epidemiologia della malattia e sui caratteri morfo-biologici
411 del parassita in Sardegna. Studi di Sassari, 8, 175–196.

412 Ptskialadze, L. (1968). The causal agent of loquat scab and its biological characteristics. Review of Applied
413 Mycology, 47, 268.

414 Raabe, R., & Gardne, M. W. (1972). Scab of pyracantha, loquat, Toyon and Kageneckia. Phytopathology,
415 62, 914–916.

416 Rodríguez, A., (1983). El cultivo del níspero en el valle del Algar-Guadalest. Sociedad Cooperativa de
417 Crédito de Callosa d'En Sarrià, Alicante, Spain.

418 Salerno, M., Somma, V., & Rosciglione, B. (1971). Ricerche sull'epidemiologia della ticchiolatura del
419 nespolo del giappone. Technology Agriculture, 23, 947–956.

420 Sánchez-Torres, P., Hinarejos, R., & Tuset, J. J. (2007a). *Fusicladium eriobotryae*: hongo causante del
421 moteado del níspero en el Mediterráneo español. Boletín de Sanidad Vegetal. Plagas, 33, 89–98.

422 Sánchez-Torres, P., Hinarejos, R., & Tuset, J. J. (2007b). Identification and characterization of *Fusicladium*
423 *eriobotryae*: fungal pathogen causing mediterranean loquat scab. Acta Horticulturae, 750, 343–347.

424 Sánchez-Torres, P., Hinarejos, R., & Tuset, J. J. (2009). Characterization and pathogenicity of *Fusicladium*
425 *eriobotryae*, the fungal pathogen responsible for loquat scab. Plant Disease, 93, 1151–1157.

426 Schena, L., Li Destri Nicosia, M. G., Sanzani, S. M., Faedda, R., Ippolito, A., & Cacciola, S. O. (2013).
427 Development of quantitative PCR detection methods for phytopathogenic fungi and oomycetes.
428 Journal of Plant Pathology, 95, 7–24.

429 Scherm, H., Savelle, A. T., Boozer, R. T., & Foshee, W. G. (2008). Seasonal dynamics of conidial
430 production potential of *Fusicladium carpophilum* on twig lesions in south eastern peach orchards.
431 Plant Disease, 92, 47–50.

432 Schrader, C., Schielke, A., Ellerbroek, L., & Johne, R. (2012). PCR inhibitors – occurrence, properties and
433 removal. Journal of Applied Microbiology, 113, 1014–1026.

434 Schubert, K. S., Ritschel, A. R., & Braun, U. B. (2003). A monograph of *Fusicladium* s. lat.
435 (Hyphomycetes). Schlechtendalia, 9, 1–132.

436 Soler, E., Martínez-Calvo, J., Llácer, G., & Badenes, M. L. (2007). Loquat in Spain: Production and
437 Marketing. Acta Horticulturae, 750, 45–47.

438 van Leeuwen, G. C. M., Holb, I. J., & Jeger, M. J. (2002). Factors affecting mummification and sporulation
439 of pome fruit infected by *Monilinia fructigena* in Dutch orchards. Plant Pathology, 51, 787–793.

440 Villarino, M., Melgarejo, P., Usall, J., Segarra, J. & De Cal, A. (2010). Primary inoculum sources of
441 *Monilinia* spp. in Spanish peach orchards and their relative importance in brown rot. Plant disease,
442 94, 1048–1054.

443 Viruega, J. R., Moral, J., Roca, L. F., Navarro, N., & Trapero, A. (2013). *Spilocaea oleagina* in olive groves
444 of southern Spain: survival, inoculum production, and dispersal. Plant Disease, 97, 1549–1556.

Figure captions

Fig. 1 Daily weather data (rain, temperature, and relative humidity) (a) and predicted seasonal dynamics of the accumulative infection rate of *Fusicladium eriobotryae* (b), during the first growing season 2015-2016. Rhombus indicates the sampling dates

Fig. 2 Daily weather data (rain, temperature, and relative humidity) (a) and predicted seasonal dynamics of the accumulative infection rate of *Fusicladium eriobotryae* (b), during the second growing season 2016-2017. Rhombus indicates the sampling dates. Filled symbols indicate the sampling dates where DNA of *F. eriobotryae* was detected

Fig. S1 Standard regression curve plots of the qPCR analysis. A dilution series of *Fusicladium eriobotryae* FeV40 DNA spanning six orders of magnitude (1/10, 1/100, 1/1,000, 1/10,000, 1/100,000 and 1/1,000,000) amplified with the primers FUG2F and FUG2R was used to generate the standard curve

Table 1 *Fusicladium eriobotryae* isolates and other fungi used in this study to determine the specificity of the species-specific primers

Fungal species	Source[a]	Host	Location	Collection code	GenBank[b]	Year
Fusicladium carpophilum	CBS	*Prunus* sp.	Switzerland	CBS 497.62	EU744580.1	1961
F. eriobotryae	IVIA	*Eriobotrya japonica*	Valencia, Spain	CA1	EU744567.1	2008
	IVIA	*E. japonica*	Valencia, Spain	CAC31	EU744568.1	2008
	IVIA	*E. japonica*	Valencia, Spain	CAC32		2008
	IVIA	*E. japonica*	Valencia, Spain	CAC31	EU744569.1	2008
	IVIA	*E. japonica*	Valencia, Spain	CAC32	EU744570.1	2008
	IVIA	*E. japonica*	Valencia, Spain	CAC61		2008
	IVIA	*E. japonica*	Valencia, Spain	CAC62	EU744571.1	2008
	IVIA	*E. japonica*	Valencia, Spain	E5	EU744574.1	2008
	IVIA	*E. japonica*	Valencia, Spain	E6	EU744575.1	2008
	IVIA	*E. japonica*	Valencia, Spain	FE-40	EU744572.1	2008
	IVIA	*E. japonica*	Valencia, Spain	FE-41	EU744573.1	2008
	IAM	*E. japonica*	Valencia, Spain	FE-52[c]	-	2011
	IAM	*E. japonica*	Granada, Spain	FE-65[c]	-	2011
	IAM	*E. japonica*	Castellón, Spain	FE-107[c]	-	2011
	IAM	*E. japonica*	Valencia, Spain	FE-112[c]	-	2011
	IAM	*E. japonica*	Alicante, Spain	FE-118[c]	-	2011
	IAM	*E. japonica*	Castellón, Spain	FE-124[c]	-	2011
	IAM	*E. japonica*	Castellón, Spain	FE-132[c]	-	2011
	IAM	*E. japonica*	Alicante, Spain	FE-137[c]	-	2011
	IAM	*E. japonica*	Alicante, Spain	FE-142[c]	-	2011
	IAM	*E. japonica*	Alicante, Spain	FE-257[c]	-	2011
	IAM	*E. japonica*	Alicante, Spain	FE-270[c]	-	2011
	IAM	*E. japonica*	Valencia, Spain	FEV-40[c]	-	2011
	IVIA	*E. japonica*	Valencia, Spain	ST1	EU744566.1	2008
Venturia inaequalis	CBS	*Malus sylvestris*	The Netherlands	CBS 330.65[c]	-	1965
	CBS	*M. sylvestris*	The Netherlands	CBS 476.61	EU744577.1	-
	CBS	*M. sylvestris*	The Netherlands	CBS 593.70	EU744578.1	-
	CBS	*M. sylvestris*	The Netherlands	CBS 595.70[c]	EU744576.1	1970
	CBS	*M. sylvestris*	The Netherlands	CBS 815.69[c]	-	1965
	CBS	*M. sylvestris*	The Netherlands	CBS 813.69[c]	EU744579.1	1969
V. pyrina	CBS	*Pyrus communis*	The Netherlands	CBS 331.65[c]	EU744582.1	1965
Aspergillus sp.	IAM	*E. japonica*	Valencia, Spain	ASP [c]	-	2008
Cladosporium sp.	IAM	*E. japonica*	Valencia, Spain	CLA [c]	-	2008
Alternaria sp.	IAM	*E. japonica*	Valencia, Spain	ALT-1 [c]	-	2008
Phomopsis sp.	IAM	*E. japonica*	Valencia, Spain	PHO-1 [c]	-	2008
Mycosphaerella sp.	IAM	*E. japonica*	Valencia, Spain	MHY [c]	-	2008
Phyllosticta sp.	IAM	*E. japonica*	Valencia, Spain	PHYL [c]	-	2008
Stemphylium sp.	IAM	*E. japonica*	Valencia, Spain	STEM [c]	-	2008
Pestalotiopsis sp.	IAM	*E. japonica*	Valencia, Spain	PEST [c]	-	2008
Penicillium sp.	IAM	*E. japonica*	Valencia, Spain	PENI [c]	-	2008

[a]Isolates were obtained from: CBS, Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands; IAM, Culture Collection of the Instituto Agroforestal Mediterráneo, UPV, Valencia, Spain; IVIA, Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain

[b]GenBank accession number of ITS sequences used for primer design

[c] Isolates used to determine specificity of the qPCR protocol

- No information available

Table 2 Sampling dates and phenological states of the two orchards in both growing seasons

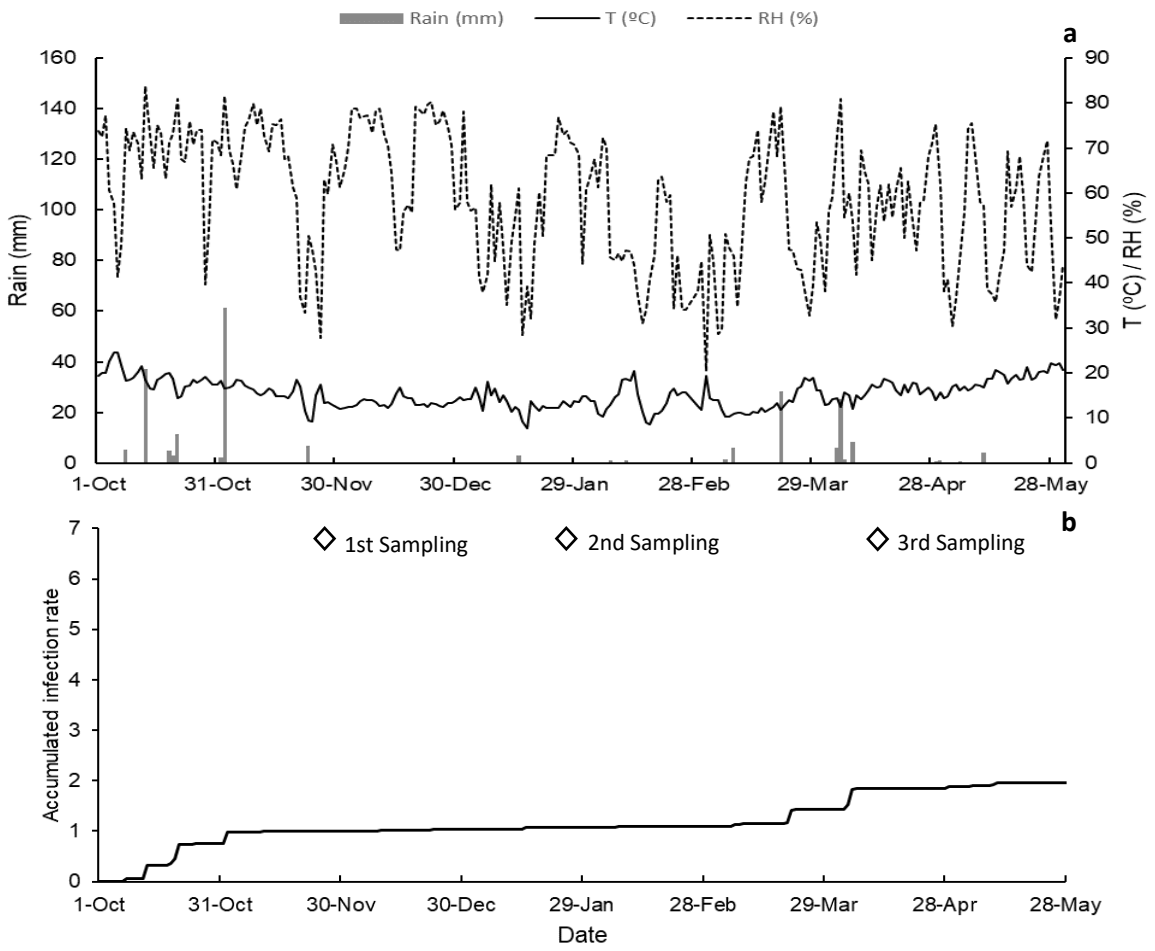
		Phenological state	
Growing season	Date	Orchard A	Orchard B
2015/2016	27/11	605-607[a]	601-605
	27/01	701-709	701
	11/04	805-807	801-805
2016/2017	18/11	605-607	601-605
	7/02	701-709	701
	11/04	805-807	801-805

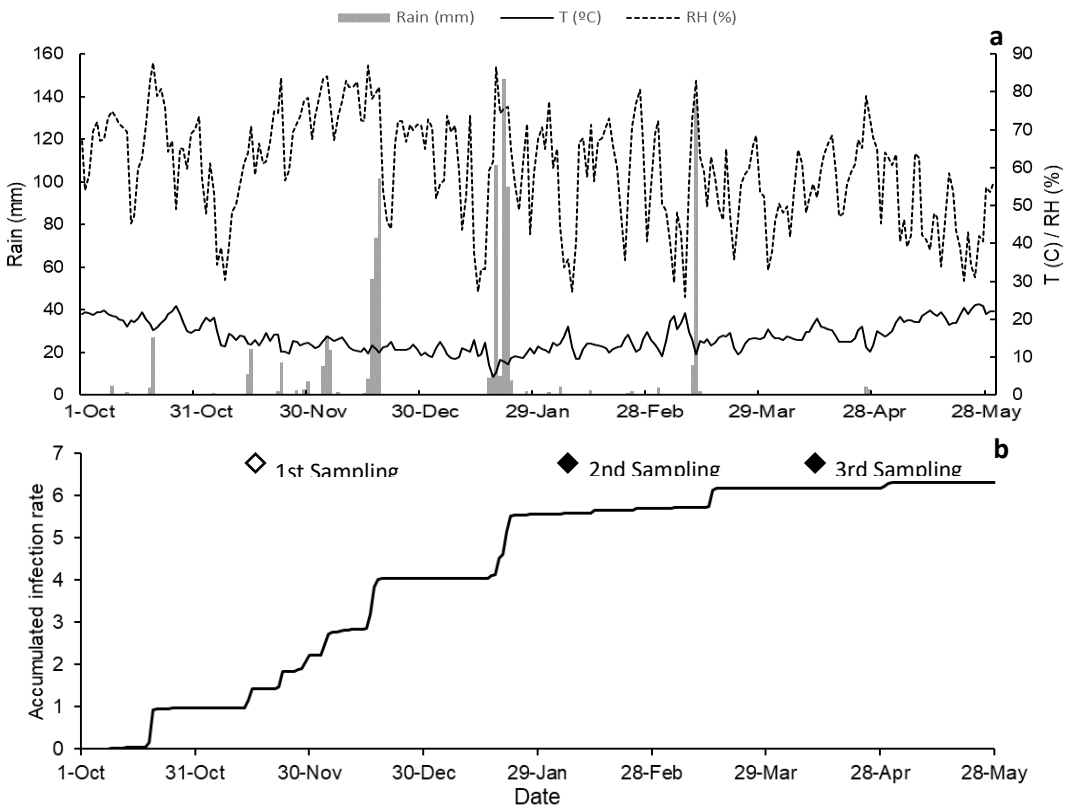
[a]Phenological state according to Martínez-Calvo et al. (1999). State 601 corresponds to beginning of flowering: 10% of flowers open, state 701 corresponds to the fruit with a 10% of the final size and state 801 corresponds to the beginning of fruit colouring (colour break)

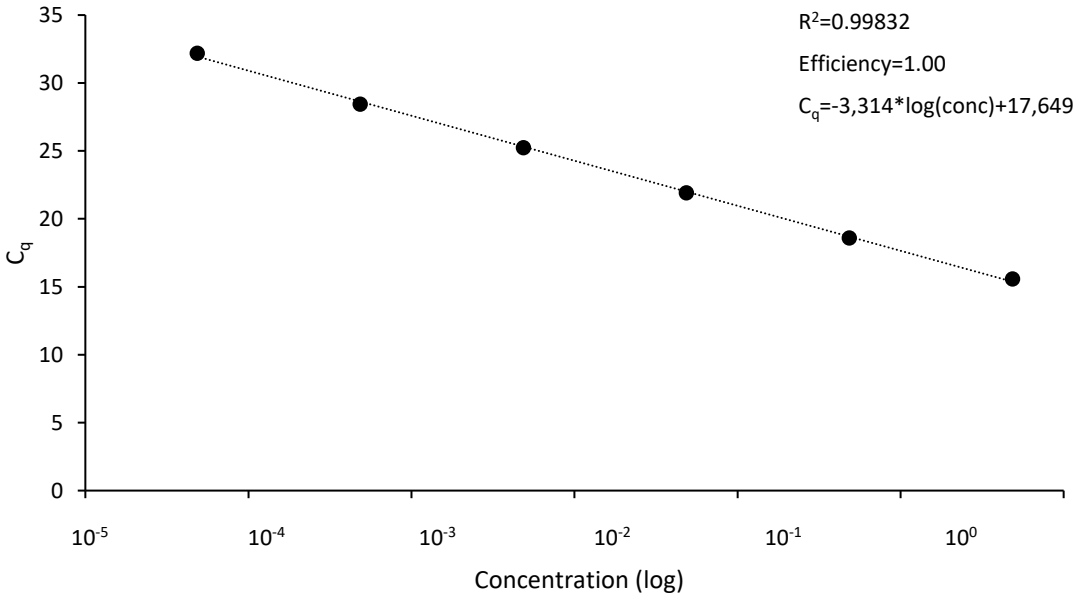
Table 3 Detection of *Fusicladium eriobotryae* in twigs and fruit peduncles samples collected from orchard A and B during the second growing season 2016/2017

	Twigs				Fruit peduncles			
	Plant material		Pellet		Plant material		Pellet	
Sampling date	Number of detections	Concentration of DNA (fg/µL)	Number of detections	Concentration of DNA (fg/µL)	Number of detections	Concentration of DNA (fg/µL)	Number of detections	Concentration of DNA (fg/µL)
07-feb	1 (orchard B)	2742±667	1 (orchard B)	15±3,5	1 (orchard A)	-[a]	2 (orchard B)	-
11-abr	5 (orchard B)	48±8	1 (orchard B)	32,5±9,5				
		96,5±24,5						
		12,5±3,5						
		226±58						
		50±25						

[a] DNA of *Fusicladium eriobotryae* detected but not possible to quantify


Rain (mm)
T (ºC)
RH (%)
a
Rain (mm)
T (ºC) / RH (%)
1-Oct
31-Oct
30-Nov
30-Dec
29-Jan
28-Feb
29-Mar
28-Apr
28-May
b
1st Sampling
2nd Sampling
3rd Sampling
Accumulated infection rate
Date


Rain (mm)
T (ºC)
RH (%)
a
Rain (mm)
T (C) / RH (%)
1-Oct
31-Oct
30-Nov
30-Dec
29-Jan
28-Feb
29-Mar
28-Apr
28-May
b
1st Sampling
2nd Sampling
3rd Sampling
Accumulated infection rate
Date


R²=0.99832
Efficiency=1.00
Cq=-3,314*log(conc)+17,649
Cq
Concentration (log)