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Temporal Dispersal Patterns of *Phaeomoniella chlamydospora*, Causal Agent of Petri Disease and Esca, in Vineyards

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ABSTRACT

Although the fungus *Phaeomoniella chlamydospora* is the most commonly detected causal agent of Petri disease and esca, two important fungal grapevine trunk diseases, little is known about the dispersal patterns of *P. chlamydospora* inoculum. In this work, we studied the dispersal of *P. chlamydospora* airborne inoculum from 2016 to 2018 in two viticultural areas of eastern (Ontinyent) and northern (Logroño) Spain. The vineyards were monitored weekly from November to April using microscope slide traps, and *P. chlamydospora* was detected and quantified by a specific real-time quantitative (qPCR) method set up in this work. The method was found to be sensitive, and a good correlation was observed between numbers of *P. chlamydospora* conidia (counted by microscope) and DNA copy numbers (quantified by qPCR). We consistently detected DNA of *P. chlamydospora* at both locations and in all seasons but in different quantities. In most cases, DNA was first detected in the last half of November, and most of the DNA was detected from December to early April. When rain was used as a predictor of *P. chlamydospora* DNA detection in traps, false-negative detections were observed, but these involved only 4% of the total. The dispersal pattern of *P. chlamydospora* DNA over time was best described ($R^2 = 0.765$ and concordance correlation coefficient = 0.870) by a Gompertz equation, with time expressed as hydrothermal time (a physiological time accounting for the effects of temperature and rain). This equation could be used to predict periods with a high risk of dispersal of *P. chlamydospora*.

Keywords: ecology and epidemiology, grapevine trunk diseases, hydro-thermal time, mycology, real-time quantitative PCR, *Vitis vinifera*

Petri disease and esca are grapevine trunk diseases (GTDs) that represent a serious threat to viticulture worldwide (Gramaje et al. 2018). The fungi *Phaeomoniella chlamydospora*, *Phaeoacremonium* spp., and *Cadophora luteo-olivacea* are the main causal agents of Petri disease in young vineyards (Bertsch et al. 2013; Gramaje et al. 2011, 2015, 2018). In mature vineyards, the same fungi together with *Fomitiporia mediterranea* and other basidiomycetes are associated with esca (Bertsch et al. 2013; Cloete et al. 2015; Fischer and González-García 2015).

The etiology of these GTDs is complex because grapevines can be simultaneously infected by different pathogens, and the symptoms caused by these pathogens can overlap (Gramaje et al. 2018). In brief, Petri disease is characterized by the presence of phenolic compounds in the xylem vessels of the trunk (producing dark exudates when the trunk is cut) and dark streaks in longitudinal sections (Gubler et al. 2015). Esca is characterized by the appearance of multiple discolored bands in a 'tiger-stripe' pattern on the foliage. Esca can also have an apoplectic form, characterized by a sudden wilting of shoots, arms, or the entire plant. Internal wood symptoms of esca include black spots in the xylem, brown to black vascular streaking, and a white to yellow soft rot in older vines (Gramaje et al. 2018).

P. chlamydospora is an especially important GTD pathogen because it has been associated with both Petri disease and esca, and because it is the fungus most frequently isolated from affected vines (Bertsch et al. 2013; Gubler et al. 2015). *P. chlamydospora*, which is an anamorphic member of the family Phaeomoniellaceae in the order Phaeomoniellales of the Eurotiomycetes (Pezizomycotina, Ascomycota), has an unknown teleomorph. It produces conidia on conidiophores that arise directly from hyphae but produces conidia also in pycnidia of a *Phoma*-like synanamorph (Chen et al. 2015; Crous and Gams 2000).

P. chlamydospora overwinters as pycnidia in pruning wounds, although mycelium on infected wood can also produce conidia (Baloyi et al. 2016; Edwards and Pascoe 2001; Edwards et al. 2001). From these sources, inoculum of *P. chlamydospora* is aerially dispersed (Eskalen and Gubler 2001; Larignon and Dubos 2000; Quaglia et al. 2009). *P. chlamydospora* conidia may also be dispersed by arthropods (Moyo et al. 2014) and by pruning shears (Agustí-Brisach et al. 2015). The conidia produce germ tubes that enter the plant through pruning wounds (Eskalen et al. 2007; Larignon and Dubos 2000; Serra et al. 2008), although the susceptibility of pruning wounds significantly decreases over time (Elena and Luque 2016; Eskalen et al. 2007; Larignon and Dubos 2000; Serra et al. 2008; van Niekerk et al. 2011). *P. chlamydospora* can also be disseminated with grapevine propagation material

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(Fourie and Halleen 2002; Halleen et al. 2003; Whiteman et al. 2007), and is commonly detected in grafted commercial plants (Bertelli et al. 1998; Giménez-Jaime et al. 2006). In grapevine nurseries, PCR analyses have confirmed the presence of *P. chlamydospora* inoculum in hydration tanks, on grafting tools, and on the substrates used for callusing (Aroca et al. 2010; Edwards et al. 2007; Retief et al. 2006; Ridgway et al. 2002).

It is widely accepted that the infection of pruning wounds by aerial inoculum is the main infection pathway for GTDs (Rolshauen et al. 2010; van Niekerk et al. 2011) but little is known about the dispersal patterns of *P. chlamydospora* conidia. Early studies showed that conidia of *P. chlamydospora* were dispersed throughout the year in France and California (Eskalen and Gubler 2001; Larignon and Dubos 2000) but conidia were trapped only from March to December in vineyards in Italy (Quaglia et al. 2009). In California and Italy, dissemination of conidia occurred mainly during or following rain events (Eskalen and Gubler 2001; Quaglia et al. 2009). These studies, however, provided little information about the effects of environmental conditions on the dispersal dynamics of *P. chlamydospora* conidia. The latter information is essential for identifying periods with a high risk of spore dispersal and for adopting efficient management strategies.

Past studies of the dispersal of *P. chlamydospora* conidia were based on classical microbiological methods such as the microscopic counting of spores from spore traps or the counting of fungal colonies from spore traps on culture media (Eskalen and Gubler 2001; Larignon and Dubos 2000; Quaglia et al. 2009; van Niekerk et al. 2010). These procedures are time consuming and limited in accuracy and sensitivity due to the small size of the spores and their similarity with the conidia of *Phaeoacremonium* spp. and *C. luteo-olivacea* (Crous and Gams 2000; Gramaje et al. 2011, 2015). Real-time quantitative PCR (qPCR) combines specificity with accurate and sensitive measurement of DNA copy number. Several qPCR methods have been developed for *P. chlamydospora* using different chemistries and target regions (Edwards et al. 2007; Martín et al. 2012; Overton et al. 2004; Pouzoulet et al. 2013) but have not been applied to detect and quantify the pathogen's conidia in spore traps.

The aim of this study was to analyze the dynamics of *P. chlamydospora* airborne inoculum in vineyards in relation to weather conditions. For this purpose, we set up a rapid, specific, and highly sensitive qPCR-based method for detection of *P. chlamydospora* DNA. The study had four specific objectives: (i) to develop a simple trapping system compatible with the DNA-based method for detection and quantification of *P. chlamydospora* airborne inoculum, (ii) to study the release dynamics of *P. chlamydospora* in two wine-producing regions of Spain over a 3-year period, (iii) to investigate the relationships between the release dynamics and weather conditions, and (iv) to develop equations for predicting the dispersal patterns of *P. chlamydospora* in vineyards.

MATERIALS AND METHODS

Laboratory samples. Total DNA of a representative *P. chlamydospora* isolate (Pch184) (Tello et al. 2010) obtained from the culture collection of the Instituto Agroforestal Mediterráneo-Universitat Politècnica de València, Spain, was extracted with the EZNA Plant Miniprep Kit (Omega Bio-Tek, Norcross, GA, U.S.A.). Before DNA extraction, the sample was homogenized in 2-ml tubes containing 600 µl of P1 buffer (provided in the kit) and three 3-mm-diameter tungsten carbide beads (Qiagen, Hilden, Germany); the beads facilitated the rupture of mycelia and conidia when the preparation was subjected to vibration in a FastPrep (MP Biomedicals, Santa Ana, CA, U.S.A.) at 50 Hz for 30 s. The concentration (nanograms per microliter) of the genomic DNA (gDNA) obtained was quantified with the Qubit Fluorometric Quantitation kit (Life Technologies, Carlsbad, CA, U.S.A.). Seven 1:10-fold serial dilutions of gDNA were prepared.

For preparation of *P. chlamydospora* conidial suspensions, the fungus was grown on 9-cm Petri dishes in the dark on potato

dextrose agar for 3 weeks at 25°C. Each of three suspensions (designated A, B, and C) was obtained by scraping the mycelia on a Petri dish with 20 ml of sterile water. After the suspensions were passed through cheesecloth and the volume was increased to 200 ml, seven 10-fold dilutions were made from suspensions and a total of 500 µl of each dilution was evenly distributed on a 48-mm-long siliconed (Lanzoni S.r.l., Bologna, Italy) Melinex plastic tape (Burkard Scientific Ltd., Uxbridge, UK) on a glass microscope slide (25 by 76 mm). Concentration of the three conidial suspension dilution series was determined by microscopic counts using a hemocytometer. Sensitivity of this methodology allowed us to calculate concentrations until the third dilution, and measurements for each suspension were repeated three times. A negative control tape was also included in the assay, in which 500 µl of sterile water rather than a conidial suspension was distributed on the tape. The tapes were dried for 24 h before DNA was extracted as described below. Dilution series prepared from suspensions A and B were used to determine the relationship between *P. chlamydospora* conidia counts determined by microscopy and DNA copy number determined by qPCR. Conidial suspension C was used to determine DNA extraction efficiency (i.e., the relationship between *P. chlamydospora* conidia counts determined by microscopy and DNA quantity as determined by qPCR as described below).

Field samples and spore trapping. Vineyards with a history of esca symptoms and positive isolation of *P. chlamydospora*, located in Ontinyent (Alicante region, southeastern Spain) and Logroño (La Rioja region, northern Spain) were selected for the study. Two vineyards were located in Ontinyent; one was planted with cultivar Malvasía, was 30 years old, and was sampled during the 2015–16 growing season; the second was planted with cultivar Monastrell, was 20 years old, and was sampled during the 2016–17 growing season. In Logroño, two vineyards that were less than 500 m apart were sampled; one was planted with cultivar Tempranillo, was 42 years old, and was sampled during the 2015–16 growing season; the second was planted with Tempranillo, was 39 years old, and was sampled during the 2016–17 growing season and also during the 2017–18 growing season. All four vineyards had a traditional low-density, head-trained (bush vines) system, and were managed following the common viticulture practices of each region.

Airborne particles from both locations were collected using glass microscope slide traps. Each trap consisted of a 52-mm-long piece of silicone-coated Melinex tape set to a slide and stuck on the 2-mm side margins. The slide was attached to a structure near the trunk of a grapevine and at a 45° angle relative to the soil surface. Five traps (at least 10 m apart) were deployed in each vineyard and were replaced weekly. Traps were first deployed on 21 November 2015 and 12 November 2015 in Ontinyent, and on 4 November 2015, 2 November 2016, and 1 November 2017 in Logroño; in all cases, trapping ended on 5 May of the following year.

In both locations, standard weather stations (Spectrum Technologies, Inc., Plainfield, IL, U.S.A.) were installed, with sensors at 1 m above the ground. The stations provided an hourly record of air temperature (T, °C), relative humidity (RH, %), rainfall (R, mm), and leaf wetness (W, min).

DNA extraction from laboratory and field samples. Three commercial DNA extraction kits were evaluated for their suitability for the extraction of DNA from microscope slide traps: the EZNA Plant Miniprep kit (Omega Bio-Tek), the Power Plant kit (Qiagen), and the Power Soil kit (Qiagen). In a preliminary study, these kits were compared using nonexposed and field-exposed tapes in spore traps that were artificially inoculated with a *P. chlamydospora* conidial suspension in the laboratory, as described earlier. The tape from each trap was cut into six equal fragments that were placed in a 2-ml tube. Each tube contained the first buffer designated for each kit and about 100 g of 0.5-mm-diameter BashingBeads, which were collected from ZR BashingBead Lysis Tubes (Zymo Research, Irvine, CA, U.S.A.) and which were added to facilitate the rupture of the conidia by vibration in a Fastprep at 50 Hz for 30 s. DNA

extractions were completed following the manufacturer's protocol provided with each kit. Three replicate tapes were extracted for each combination of dilution and kit. DNA integrity was evaluated by electrophoresis in a 1.5% agarose gel with 1× Tris-acetate-EDTA buffer. Gels were stained with 1× GelRed nucleic acid gel stain (Biotium, Hayward, CA, U.S.A.) and visualized under UV light. Concentrations of DNA for all samples were determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Loughbrough, U.K.). Of the three DNA extraction kits, the EZNA Plant Miniprep and Power Plant kits provided more consistent DNA yields than the Power Soil kit (data not shown). Because it was the easier to use than the Power Plant kit, the EZNA kit was used for all experiments.

For laboratory samples (tapes treated with conidial suspensions prepared in the laboratory), tapes were cut and DNA was extracted with the EZNA kit. The DNA extraction product from 500 µl of conidial suspension C placed in the tape was subjected to seven 10-fold dilutions. These DNA samples and those obtained from the dilutions placed directly in the tapes were compared to determine DNA extraction efficiency.

For field samples (tapes that were placed in the vineyards), the 2-mm margins of the long sides of the tape that were fixed to the slide in the trap were removed; this did not change the total capturing surface of the tape. The tapes were subsequently processed for DNA extraction as previously described for laboratory samples. Extracted DNA was kept at −20°C until it was subjected to PCR amplification.

Construction of the standard curve. A standard curve for the quantification of *P. chlamydospora* was constructed using a chemically synthesized single copy of a 360-bp internal fragment of the 18S ribosomal RNA gene that included the annealing sites for Pch1 and Pch2 (Tegli et al. 2000). The 500-ng lyophilized Pch gBlocks (Integrated DNA Technologies Inc. [IDT], Skokie, IL, U.S.A.) was resuspended in 50 µl of TE buffer (Tris and EDTA, pH 8.0; Sigma Aldrich, St. Louis, MO, U.S.A.) following the manufacturer's recommendation to obtain a final concentration of 10 ng/µl.

The total copy number of the Pch gBlocks was determined using the following formula (Lee et al. 2006): number of copies = [6.02 × 10^{23} (copy/mol) × DNA amount (g)]/[DNA length (bp) × 660 (g/mol/bp)]. The 10 ng/µl stock solution of Pch gBlocks was calculated at $2.5 × 10^{10}$ copies. A 10-fold dilution series from $2.5 × 10^{9}$ to 2.5 copies was prepared and used to develop a standard curve with the qPCR conditions described in detail in the next section. Each 25-µl first-round nested-PCR contained 12.5 µl of Premix Ex Taq (2×) (Takara Bio Inc., Shiga, Japan), 0.4 µM each primer, and 2 µl of each standard solution ($5 × 10^{9}$ to 5 copies/reaction). First-round reactions were performed in a Veriti Thermalcycler (Applied Biosystems, Foster City, CA, U.S.A.).

The quantification cycle (Cq) value for each Pch gBlocks standard sample was calculated and analyzed using Rotor-Gene Q Series software (version 2.3.1) to generate a standard curve. The number of copies for each Pch gBlocks standard dilution was plotted against the Cq value, and the resulting regression equations were used to quantify the number of copies of the target gene in the unknown samples. The limit of detection and sensitivity of the qPCR was determined using Pch gBlocks standards and gDNA as templates. The following gDNA concentrations obtained from *P. chlamydospora* isolate (Pch184) were used as templates: $3.7 × 10^{7}$, $3.7 × 10^{6}$, $3.7 × 10^{5}$, $3.7 × 10^{4}$, $3.7 × 10^{3}$, 370, 37, and 3.7 fg/reaction. These gDNA samples were analyzed by qPCR with Pch gBlocks as standards using four replicates in two independent assays following the conditions described below. The nomenclature for interpreting all qPCR results followed the MIQE guidelines as described by Bustin et al. (2009).

qPCR analysis of samples. Because low concentrations of fungal DNA were expected in the samples collected in vineyards, a nested PCR that included a conventional PCR for the first round and a real-time PCR for the second round was used. The number of cycles in which the DNA of the most concentrated dilution was

detected was selected as the number of cycles to be applied in the first amplification reaction of the nested PCR. Optimal primers for the first round were determined by comparing the efficiency of *P. chlamydospore*-specific primers Pch1 and Pch2 combined with universal primers internal transcribed spacer (ITS)4 and ITS1F (Gardes and Bruns 1993; White et al. 1990), respectively. According to the results obtained, the reaction was performed using universal primer ITS1F and Pch2 in the first round and Pch1 and Pch2 in the second round.

The first round was carried out as described earlier. The second round (final volume 25 µl) was carried out on a Rotor-Gene Q 5plex HRM instrument (Qiagen), and the reaction mixture consisted of 12.5 µl of TB Green Premix Ex Taq (2×) (Tli RNaseH Plus; Takara Bio Inc.), 0.4 µM each primer, and 2 µl of the template DNA obtained in the first round. The reaction conditions were initial denaturation at 95°C for 1 min, followed by 20 cycles (for PCR) or 40 cycles (for qPCR) of 95°C for 5 s, 55°C (for PCR) or 62°C (for qPCR) for 30 s, and 72°C for 40 s. Melt peaks were examined to confirm amplification of the correct product. Reactions included the following controls and standards: (i) negative controls with no DNA template in both nested PCR rounds, (ii) the product of the negative control for the first round in the second round, and (iii) Pch gBlocks standard solutions ($5 × 10^{8}$ and $5 × 10^{5}$ copies/reaction). Each laboratory sample was run in four replicates, and fields samples were run in duplicate. Positive products of qPCR obtained from the first field samples analyzed were confirmed by 1.5% agarose gel electrophoresis and were visualized under UV light. Confirmed positive products were sequenced by Macrogen sequencing service (Macrogen Europe, Amsterdam, The Netherlands).

To determine the number of copies amplified by each reaction, the previously developed standard curves were imported using the Rotor-Gene Q software. One of the Pch gBlocks standard solutions included in each qPCR was used to calibrate the imported standard curve. The mean Cq values for each unknown sample were used to calculate the number of copies per reaction.

Linear regression analysis was performed on the number of *P. chlamydospora* conidia counted by microscopy versus the corresponding Cq values and DNA copy number using the function *lm* of the 'stats' package of R v. 3.6.0 (R Core Team 2019).

Dispersal patterns of *P. chlamydospora*. To study the temporal dispersal patterns of *P. chlamydospora*, the proportion of the total seasonal DNA (PSDNA) was calculated for each vineyard and year as the proportion of *P. chlamydospora* DNA found in traps on a particular date (the number of copies per reaction) over the total DNA found over the entire season. PSDNA values were then regressed over time, which was expressed as (i) day of the season (DOS, starting on 1 November, when all vine leaves had fallen), (ii) thermal time (TT), or (iii) hydrothermal time (HTT). TT and HTT are both forms to express the time in physiological units (Lovell et al. 2004), and consisted of sums of daily rates from a function that accounted for the effect of temperature (in the case of TT) or temperature and moisture (in the case of HTT) on the biological process (i.e., the pycnidial development and inoculum dispersal of *P. chlamydospora*). For TT, daily values of relative mycelial growth rate (MGR) were accumulated; MGR was selected because there is no information about the effect of temperature on pycnidial development and inoculum dispersal of *P. chlamydospora*. MGR values range from 0 to 1 and were calculated as a function of temperature, as described below. For HTT, daily values of MGR were also accumulated but MGR = 1 on rainy days (i.e., on days with R > 0 mm). MGR was calculated by regressing data from Tello et al. (2010), who assessed the colony diameter of 57 isolates of *P. chlamydospora* collected in Spain every 2 days during 2 months at temperatures ranging from 5 to 35°C (5°C intervals), then calculated the mean growth rate at each temperature. The effect of temperature on mycelial growth was then described by a β equation of Analytis (1977), in the form $y = a × Teq^{b} (1 − Teq)^{c}$, in which *y* is the growth rate (calculated by dividing the daily average growth at

any temperature by that at the optimal temperature); *a*, *b*, and *c* are the equation parameters; and *Teq* is an equivalent of temperature calculated as $Teq = (T - Tmin)/(Tmax - Tmin)$, in which *T* is the temperature regime and *Tmin* and *Tmax* are minimal and maximal temperatures, respectively, for mycelium growth (5 and 40°C, respectively). The *nls* function of the R 'stats' package was used to estimate the parameters, and the *epi.ccc* function of the R 'epiR' package (Stevenson 2012) was used to calculate concordance correlation coefficient (CCC) (Lin 1989). Parameter estimates were as follows: $a = 30.02 \pm 13.34$, $b = 2.70 \pm 0.34$, and $c = 2.18 \pm 0.28$, with $R^2 = 0.979$ and CCC = 0.991.

Nonlinear logistic and Gompertz equations were fit to the data by using the *nls* function in the following forms (Madden et al. 2007): $y = 1/(1 + a \times e^{-b \times t})$ for nonlinear logistic equations and $y = e^{-a \times e^{-b \times t}}$ for Gompertz equations. In these equations, *y* is the PSDNA, *a* and *b* are the equation parameters, and *t* is the time expressed as either DOS, TT, or HTT. Goodness-of-fit of the different equations was assessed by using the adjusted R^2, the magnitude of the standard error of the equation parameters, the coefficient of residual mass, and the CCC (Lin 1989; Nash and Sutcliffe 1970). The adjusted R^2 was estimated by conducting a linear regression between the observed values (i.e., PSDNA) and the model predicted values; the linear regression was conducted with the *lm* function of the R 'stats' package.

Effect of rain on *P. chlamydospora* dispersal. The Bayes' theorem (Madden et al. 2007) was used to calculate the posterior probability of predicting the presence of *P. chlamydospora* DNA in traps based on the following rainfall cut-off values: ≥0.2, ≥1, ≥2, ≥3, ≥4, and ≥5 mm of rain. DNA presence in a trap and rain during the exposure period of the trap in the vineyard was categorized as 0 (no DNA or R < cut-off value) or 1 (DNA is present or R ≥ cut-off value). Contingency tables (two by two) were prepared in which cells were 0-0 (no DNA and no R), 1-1 (DNA present and R), 0-1 (no DNA and R), and 1-0 (DNA present and no R). The true-positive proportion (TPP), false-negative proportion (FNP), false-positive proportion (FPP), and true-negative proportion (TNP) were then determined for each cut-off value. The prior probabilities of *P. chlamydospora* DNA being present in the trap (i.e., P(O+)) or not (i.e., P(O–)) were computed, and the posterior probability of prediction given to each rainfall cut-off threshold was calculated. To study in more detail the FNP (the cases in which DNA was dispersed without rain), a *t* test was conducted to assess the effect of rain on the quantity of DNA detected (i.e., to evaluate whether the quantity of DNA collected in the periods without rain was different from that collected in the periods with rain). The *t* test was computed by running the *t.test* function of the R 'stats' package; this function performs a Welch two-sample *t* test suitable for nonnormal large populations ($n > 30$) with unequal variances (Ruxton 2006).

RESULTS

Efficiency of the DNA extraction. A significant linear relationship ($P < 0.001$; $R^2 = 0.968$) was found between conidial counts in suspension C and Cq values obtained from two sets of DNA samples (Fig. 1). In one set, DNA was extracted from each of the seven 10-fold dilutions (D1 to D7) of suspension C distributed on tapes. In a second set, the DNA was extracted from the most concentrated dilution (D1) placed on a tape, and the DNA extract (rather than the spore suspension) was subjected to 10-fold dilutions.

qPCR analysis. Melting analysis confirmed the amplification of the correct products, and no amplifications were observed for the negative controls. An $R^2 = 0.99$ and reaction efficiency of 96% were obtained based on the standard curve constructed with 10-fold dilutions of the Pch gBlocks gene fragments ranging from 5×10^9 to 5 copies/reaction (Fig. 2). The qPCR limit of detection was 36 fg of gDNA of *P. chlamydospora* and 50 copies using the Pch gBlocks gene fragments as standards (Tables 1 and 2). A Cq value of 31.85,

which corresponded with the limit of detection, was set up as threshold for the cutoff for false-positive reactions (Table 2).

For conidial suspensions A and B, the number of *P. chlamydospora* conidia as determined by microscopy was significantly related to DNA copy numbers as determined by qPCR (i.e., qPCR provided a good estimate of the number of conidia detected on the tapes;
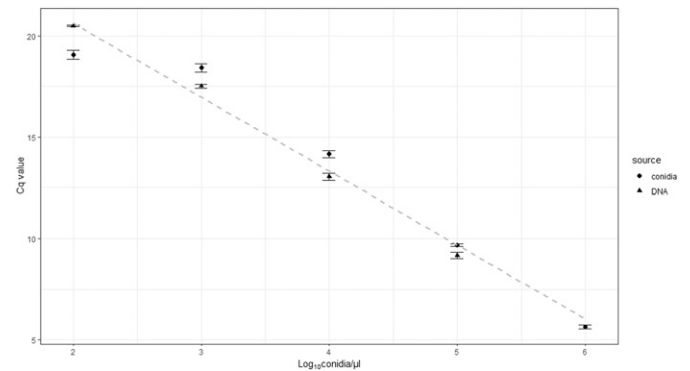
Fig. 1. Relationship between number of *Phaeomoniella chlamydospora* conidia in conidial suspension series C and quantification cycle (Cq) values obtained from two sets of DNA samples. In one set, the spore suspension was diluted (seven 10-fold dilutions, dilution D1 to D7) and placed on tapes before DNA was extracted (dots). In a second set, DNA was extracted from dilution D1 and the extracted DNA was then subjected to 10-fold dilutions (triangles). Values are means ± standard error of four replicates. The gray dashed line represents the linear regression model fit to the data ($y = -3.644x + 27.903$) with $R^2 = 0.968$ and $P < 0.001$.
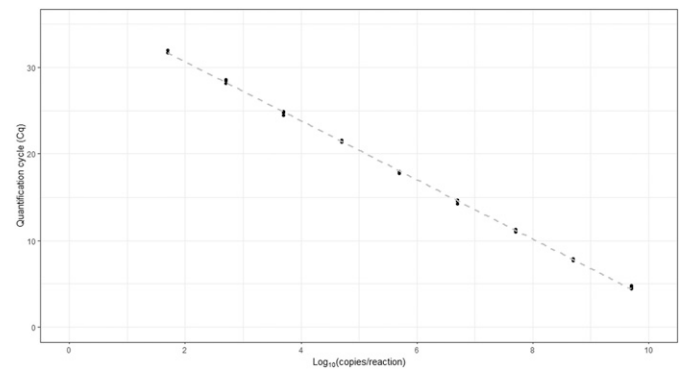
Fig. 2. Standard curve for *Phaeomoniella chlamydospora* inoculum quantification. The curve was constructed using 10-fold dilutions of the Pch gBlocks gene fragments containing from 5×10^9 to 5 copies/reaction. Values are means of four replicates. Reaction efficiency was 96%. The gray dashed line represents the linear regression of the standard curve ($y = -3.409x + 37.479$) with $R^2 = 0.999$ and $P < 0.001$.

TABLE 1. Limit of detection of the quantitative PCR (qPCR) analysis using 10-fold dilutions of the genomic DNA of *Phaeomoniella chlamydospora* (isolate Pch184)

DNA (fg/reaction)	Cq value[a]	Signal ratio[b]
360,000	1.03 ± 0.01	8/8
36,000	3.20 ± 0.01	8/8
3,600	6.31 ± 0.11	8/8
360	9.18 ± 0.09	8/8
36	12.43 ± 0.09	8/8
3.6	Not detected	0/8

[a] Quantification cycle (Cq value) at which fluorescence was detected in the qPCR analysis. The Cq values are the means ± standard error of two independent assays, each with four technical replicates.

[b] Number of positive samples detected out of the total number of reactions performed.

$R^2 = 0.729$ and $P = 0.019$) (Fig. 3). Thus, the quantity of DNA of *P. chlamydospora* found in traps was expressed as the number of conidia per square centimeter of trap.

Dynamics of *P. chlamydospora* DNA dispersal. DNA of *P. chlamydospora* was detected in all of the vineyards and years, although differences were evident in its frequency and quantity. *P. chlamydospora* DNA was detected consistently throughout the season in Ontinyent and Logroño in 2015–16 and 2016–17 but was detected only three times in Logroño in 2017–18 (Figs. 4 and 5).

In Ontinyent in 2015–16, a DNA quantity corresponding to 4.7×10^5 *P. chlamydospora* conidia/cm^2 was detected over the entire sampling period; the DNA was first detected in mid-November, and a low quantity was detected until late February (Fig. 4A). In this season, only 45 mm of rain fell, and *P. chlamydospora* DNA was frequently detected in weeks without rain, mainly during December and January. In Ontinyent in 2016–17, more DNA of *P. chlamydospora* (corresponding to 2.6×10^5 conidia/cm^2) was detected than in the previous season; the DNA was also first detected in mid-November, and peaks occurred in mid-December and late January. In this season, 582 mm of rain fell and was distributed throughout the sampling period; most of the DNA was detected in weeks with rain, except in April (Fig. 4B).

In Logroño in 2015–16, a DNA quantity corresponding to 1.5×10^6 *P. chlamydospora* conidia/cm^2 was detected over the entire

sampling period; most of this DNA was detected from November to the beginning of February. In this season, 300 mm of rain fell and was distributed throughout the season. DNA was not detected in March, although rain was frequent in that month, and DNA was detected only twice in April (Fig. 5A). In Logroño in 2016–17, less *P. chlamydospora* DNA (corresponding to a total of 3.4×10^4 conidia/cm^2) was detected than in the previous season; the DNA was not detected until the beginning of December, and was mainly detected during December and from February to April. In this season, 275 mm of rain fell and was distributed throughout the season; most of the DNA was detected in weeks with rain, except in April (Fig. 5B). In Logroño in 2017–18, a DNA quantity corresponding to 3.18×10^4 *P. chlamydospora* conidia/cm^2 was detected over the entire sampling period. Although 421 mm of rain fell and was distributed throughout the sampling period, the DNA was detected only three times: in mid-November (in a period without rain), at the end of February, and in early April (Fig. 5C).

Dispersal patterns of *P. chlamydospora*. The pattern of DNA dispersal (expressed as PSDNA) over time (expressed as DOS) was similar among locations and years, except for Ontinyent in 2015–16 (Fig. 6A). In the other locations and seasons, the DNA of *P. chlamydospora* was first detected in the second half of November, and most of the DNA was detected from December to early April (Fig. 6A, DOSs 30 to 120). The detection of DNA in Ontinyent began later in 2015–16 than in the other years and locations, and most of the DNA was found from February to April (Fig. 6A, DOSs 120 to 190). The logistic and Gompertz equations relating PSDNA to DOS had $R^2 < 0.5$ and CCC < 0.7 (Fig. 6A; Table 3).

When PSDNA was regressed against TT, the pattern was quite similar among years and locations, with the exception of Ontinyent in 2015–16. Equations relating PSDNA to TT had $R^2 < 0.31$ and CCC < 0.5 (Fig. 6B; Table 3). When HTT was used as the independent variable instead of TT, the pattern of PSDNA was similar for all years and locations, indicating an important role of rainfall in the dispersal of *P. chlamydospore*; both logistic and Gompertz equations had $R^2 > 0.7$ and CCC = 0.87 (Fig. 6C, Table 3; Supplementary Fig. S1).

Effect of rain on *P. chlamydospora* dispersal. Considering the whole dataset (data from 146 weeks from all seasons and vineyards), the DNA of *P. chlamydospora* was detected in 17 of

TABLE 2. Limit of detection of the quantitative PCR (qPCR) analysis using 10-fold dilutions of the Pch gBlocks gene fragments ranging from 5×10^9 to 5 copies/reaction

Copies per reaction	Cq value[a]	Signal ratio[b]
5,000,000,000	4.61 ± 0.07	8/8
500,000,000	7.78 ± 0.06	8/8
50,000,000	11.22 ± 0.07	8/8
5,000,000	14.53 ± 0.09	8/8
500,000	17.92 ± 0.05	8/8
50,000	21.43 ± 0.06	8/8
5,000	24.77 ± 0.10	8/8
500	28.42 ± 0.10	8/8
50	31.85 ± 0.13	8/8
5	Not detected	0/8

[a] Quantification cycle (Cq value) at which fluorescence was detected in the qPCR analysis. The Cq values are the means ± standard error of two independent assays, each with four technical replicates.

[b] Number of positive samples detected out of the total number of reactions performed.
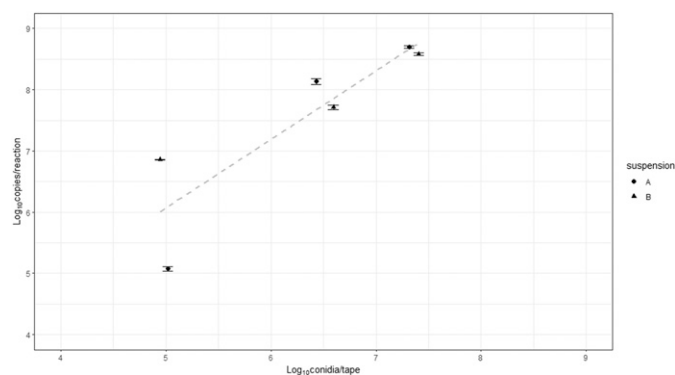

Fig. 3. Relationship between conidia counts obtained using light microscopy from conidial suspension series A and B and DNA copy number of *Phaeomoniella chlamydospora*. Conidia were counted in suspensions using a hemocytometer and a microscope. The suspensions were then added to tapes before DNA was extracted and subjected to quantitative PCR for determination of DNA copy number. Values are means ± standard error of four replicates. The gray dashed line represents the linear regression model fit to the data ($y = 0.808x + 2.679$) with $R^2 = 0.729$ and $P = 0.019$.
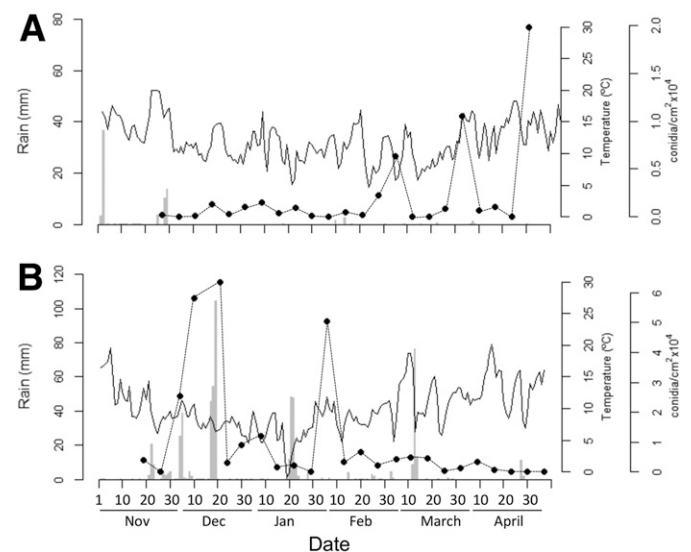

Fig. 4. *Phaeomoniella chlamydospora* inoculum detected on microscope slide traps in two vineyards in Ontinyent, Spain in seasons **A,** 2015–16 and **B,** 2016–17. Black dots indicate the inoculum expressed as the average number of conidia per square centimeter on five traps replaced weekly. The black line and gray bars represent the daily average temperature and the daily accumulated rain, respectively.

23 weeks with no rain (74.0%), and in 68 of 123 weeks with rain (55.3%). In 55 cases, rain was recorded and DNA of *P. chlamydospora* was not (44.7%).

With a cut-off value of R ≥ 0.2 mm, the TPP was 0.80 and the TNP was 0.10, with an overall accuracy of 0.51 (Table 4). When higher rainfall cut-off values were considered as predictors of *P. chlamydospora* dispersal, the overall accuracy decreased, and the posterior probabilities of correct predictions were reduced (Table 4).

The posterior probabilities of correctly predicting *P. chlamydospora* dispersal (P(P+|O+)) and no dispersal (P(P−|O−)) based on R ≥ 0.2 mm were 0.55 and 0.78, respectively. The posterior probability of predicting a dispersal that did not occur (P(P+|O−)) was 0.45 (Table 4), indicating that the use of rain as a predictor of *P. chlamydospora* dispersal generated several false positives. These false positives occurred, for instance, in Rioja in 2015–16, when the repeated late-season rains (in March) did not result in *P. chlamydospora* DNA detection, probably because the inoculum was depleted by previous rains. False negatives also occurred in Rioja in 2017–18, where the quantity of DNA found during the season was very low compared with the other vineyards. Therefore, false positives seem to be related to the scarcity of *P. chlamydospora* inoculum in the vineyard.

The posterior probability of failing to predict the dispersal (P(P−|O+)) was 0.22, indicating that using ≥0.2 mm of rain as a predictor of *P. chlamydospora* dispersal generated some false negatives. Even though false negatives may result in the underestimation of inoculum dispersal in the vineyard and, consequently, an underestimation of a potential infection, the *P. chlamydospora* DNA found in traps during these false negatives accounted for only 4% of the total DNA detected during the study, indicating that, although *P. chlamydospora* can disperse during periods without rain, the inoculum load in these periods may be very low compared with the total inoculum of the season. This was also confirmed by the comparison of the distributions of the conidia trapped in weeks with and without rain (Fig. 7; $P < 0.001$).

DISCUSSION

In the current study, we investigated the temporal dispersal patterns of *P. chlamydospora* in two viticultural areas of eastern (Ontinyent) and northern (Logroño) Spain, during two and three growing seasons, respectively. In these areas and in other areas of Spain, *P. chlamydospora* has been previously isolated from plants in vineyards (Armengol et al. 2001; Gramaje et al. 2009; Tello et al. 2010) and nurseries (Aroca et al. 2010; Gramaje et al. 2009). We consistently detected the DNA of *P. chlamydospora* in spore traps exposed from November to April in both locations and in all seasons, except in Logroño in 2017–18, where the pathogen was detected only three times.

The inoculum of *P. chlamydospora* detected during the season differed substantially among vineyards and years; these differences were probably due to differences in the quantity of primary inoculum which, in turn, can be affected by multiple epidemiological and agronomical factors, including the incidence of GTDs in the vineyard. In this work, we selected commercial vineyards in which vines showed symptoms of esca. However, the incidence of the disease could vary from vineyard to vineyard and from year to year. Differences in the abundance *P. chlamydospora* conidia were especially evident between Logroño in 2015–16 (150×10^4 conidia/cm² of trap) and Logroño in 2016–17 (3.18×10^4 conidia/cm² of trap). Those vineyards were less than 500 m apart, suggesting that the inoculum is mainly dispersed short distances, probably by splashes of raindrops (Aylor 2017).

Despite these differences in the quantities of *P. chlamydospora* DNA found in traps, the dispersal patterns throughout the growing seasons were similar among vineyards and years: the DNA was first detected in the second half of November, and most of the DNA was detected from December to the beginning of April. This pattern of

P. chlamydospora detection generally agrees with previous reports (Eskalen and Gubler 2001; Larignon and Dubos 2000; Quaglia et al. 2009) but differs in some ways. For example, Quaglia et al. (2009) did not trap conidia from January to March, and Larignon and Dubos (2000) did not trap conidia from February to June. Comparison between these and our findings is difficult, because different methodologies were used to detect and quantify the inoculum of *P. chlamydospora*. As in the current research, the two previous studies exposed microscope slides in the vineyards and replaced them weekly. In contrast to the current research, however, the two previous studies then removed the spores with water and plated the suspension on different culture media. Because qPCR is probably more sensitive than plating on culture media, the fact that *P. chlamydospora* was detected over wider periods in the current study than in the two earlier studies is not surprising.

For all years and locations, the dynamics of *P. chlamydospora* dispersal were best explained when time was expressed as HTT. HTT is a physiological time that accounts for the effects of both temperature and rain, and that has been previously used to describe the development of different pathogens, including Botryosphaeriaceae species affecting grapevines (Onesti et al. 2018; Silva et al. 2018). In the equations developed in the current study, moisture was accounted for by rain events. It is plausible that rain can contribute to (i) the development of pycnidia and masses of conidia and (ii) the splash dispersal of conidia from pycnidia. For (i), the rain events were likely associated with periods of high RH that, in other pycnidia-producing fungi, together with moderate temperatures, promote the production of pycnidia and the extrusion of the conidia (Anco et al. 2013; Lalancette et al. 2003; Onesti et al. 2017). In the case of *P. chlamydospora*, no information is available about the
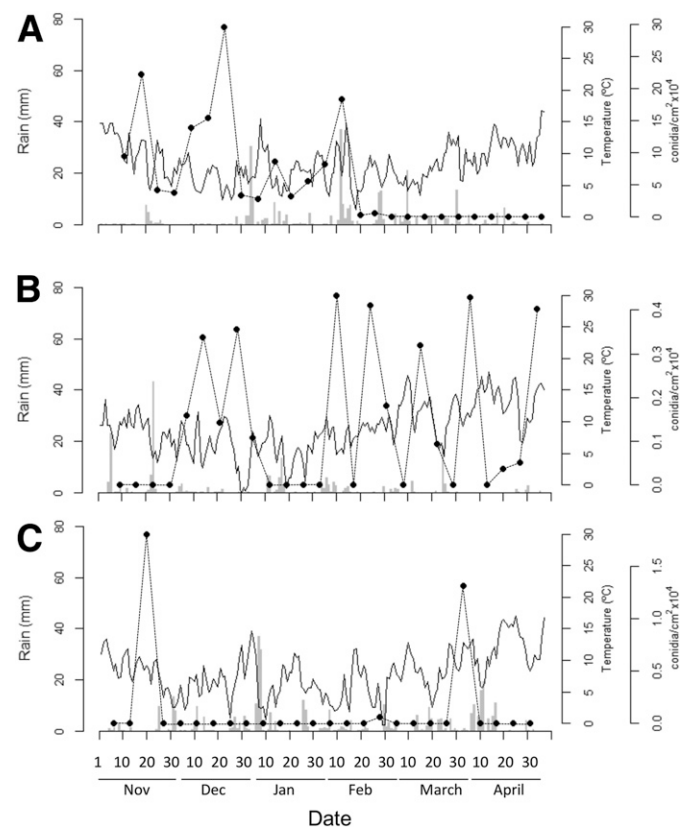
Fig. 5. *Phaeomoniella chlamydospora* inoculum detected in microscope slide traps in two vineyards in Logroño, Spain, in seasons **A,** 2015–16; **B,** 2016–17; and **C,** 2017–18. Black dots indicate the inoculum expressed as the average number of conidia per square centimeter on five traps replaced weekly. The black line and gray bars represent the daily average temperature and the daily accumulated rain, respectively.

effect of weather on the production of pycnidia; in the current study, we inferred the effect of temperature from a previous experiment regarding mycelial growth (Tello et al. 2010). Specific studies are needed to verify whether the temperature relationships for colony growth and the development of pycnidia are similar. Moreover, because environmental conditions may also affect the dispersal of other pathogens associated with Petri disease and esca, studies should also be conducted to determine how the dispersal of these other pathogens is related to environmental conditions.

When rain was evaluated as a predictor of *P. chlamydospora* dispersal, high FNPs and FPPs were observed. FNP was related to cases in which rain was not recorded but *P. chlamydospora* DNA was detected in traps; such dispersal involved only 4% of the total DNA detected throughout the seasons. Aerial dissemination of *P. chlamydospora* in periods with no rain may involve conidia produced by conidiophores extending from hyphae (i.e., not produced in pycnidia) or fragments of cirri that extruded from pycnidia in previous moist periods and that have not been dispersed by rain splashes; as these cirri desiccate and crumble, perhaps their fragments can become airborne. Aerial dissemination of pycnidio-spores in periods with no rain has been previously reported for other pathogens that produce pycnidia (Shulhani and Shtienberg 2018).

An important outcome of this study was the development and testing of a PCR-based method for the detection and quantification of *P. chlamydospora* in spore traps; to our knowledge, no similar methods have been published. Previous studies on conidial dispersal patterns of *P. chlamydospora* relied on the microscopic counting of spores or on the counting of CFU on culture media (Eskalen and Gubler 2001; Larignon and Dubos 2000; Quaglia et al. 2009; van Niekerk et al. 2010). These techniques are time consuming and less specific and sensitive than molecular methods for detecting and quantifying fungal pathogens in the environment (Billones-Baaijens et al. 2018).

In a preliminary experiment in the current study, a previously developed Taqman assay targeting the ITS region (Martín et al. 2012) was tested using gDNA from *P. chlamydospora*, and we found that the sensitivity of detection was low (data not shown). However, we still considered the ITS region to be a preferred target for molecular detection of *P. chlamydospora*. The choice of the locus used for qPCR assays largely depends on the aim of the study. Although multicopy genes allow the detection of lower DNA

TABLE 3. Parameters and goodness-of-fit indexes of the equations used to describe the effect of different physiological units on the proportion of the total seasonal inoculum (PSDNA) of *Phaeomoniella chlamydospora* detected in three vineyards located in Ontinyent and Logroño, Spain, from 2016 to 2018

Units[a]	Equation[b]	Estimated parameters[c]		Goodness of fit[d]		
		a	*B*	R^2	CRM	CCC
DOS	Logistic	6.676 (2.486)	0.025 (0.004)	0.494	−0.006	0.670
	Gompertz	2.537 (0.576)	0.018 (0.003)	0.493	0.006	0.669
TT	Logistic	3.409 (1.200)	0.089 (0.020)	0.289	−0.041	0.482
	Gompertz	1.738 (0.389)	0.068 (0.015)	0.305	0.006	0.499
HTT	Logistic	12.443 (3.915)	0.072 (0.008)	0.771	0.007	0.873
	Gompertz	3.871 (0.765)	0.051 (0.006)	0.765	0.028	0.870

[a] Physiological units. DOS = days of the season starting on 1 November. TT = thermal time; daily values of temperature were accumulated as a function of mycelial growth rate (MGR) as described in Materials and Methods. HTT = hydrothermal time; like TT except that the days with rain take a value of 1, regardless the values of MGR.

[b] Regression equations were $y = 1/(1 + a \times \exp(-b \times t)$ for logistic and $y = \exp[-a \times \exp(-b \times t)]$ for Gompertz, in which *y* is PSDNA, *a* and *b* are the equation parameters, and *t* is the time expressed by the different physiological units.

[c] Standard errors of the estimated parameters are in parentheses.

[d] R^2 = coefficient of determination, CRM = coefficient of residual mass, and CCC = concordance correlation coefficient.
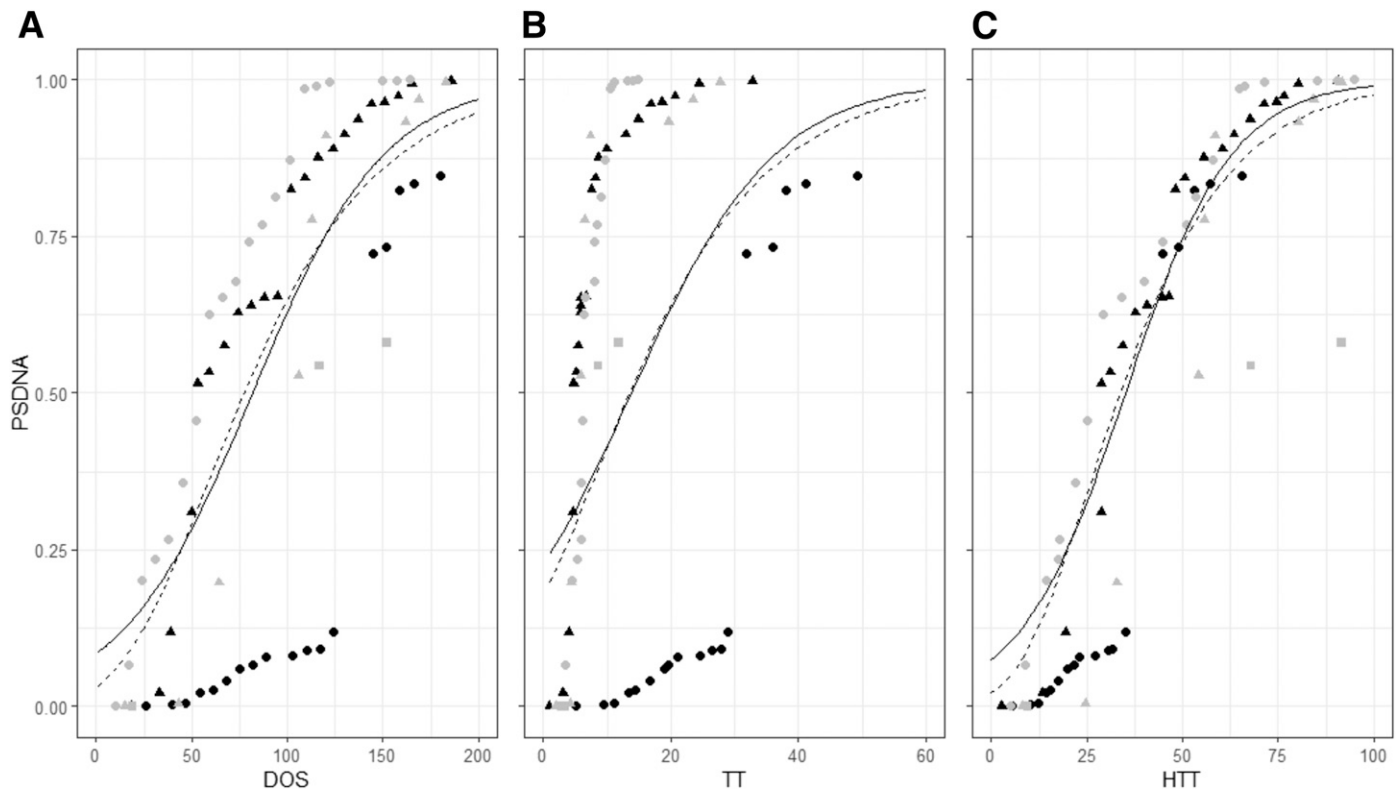

Fig. 6. Proportion of the total seasonal inoculum (PSDNA) of *Phaeomoniella chlamydospora* detected over time on microscope slide traps in vineyards in 2015–16 (dots), 2016–17 (triangles), and 2017–18 (squares). Black and gray symbols indicate vineyards located in Ontinyent and Logroño, respectively. Time is expressed as **A,** day of the year starting on 1 November (DOS); **B,** thermal time (TT); or **C,** hydrothermal time (HTT). Logistic (solid line) and Gompertz (dotted line) equations were fit to the data.

amounts, single-copy genes give more precise measurements of DNA copy number (Longo et al. 2013; Tellenbach et al. 2010). In the qPCR method developed in the current study, we selected the ITS region because we expected the quantity of DNA of *P. chlamydospora* to be low in the spore traps located in the vineyards. For the same reason, we increased the sensitivity of the qPCR by using a nested approach; in this approach, almost the entire locus was initially amplified by conventional PCR, and the resulting product was then quantified with the specific primer combination in a second step. In a previous study, a nested PCR using primers ITS4-ITS6 and Pch1-Pch2 was optimized for detecting *P. chlamydospora* in DNA extracted from soil, water, callusing medium, and grapevine wood (Retief et al. 2006). With the synthetic single copy of the target fragment (gBlocks; IDT) as standards, the qPCR limit of detection obtained in our study was 36 fg of gDNA of *P. chlamydospora* and 50 copies of the target fragment.

In the current study, we used glass microscope slides for the weekly monitoring of the airborne propagules of *P. chlamydospora* in the vineyards and ceramic beads to remove them from the tapes and for tissue lysis according to a protocol described by Billones-Baaijens et al. (2018), with minor modifications. In a preliminary test using nonexposed and field-exposed tapes that were artificially infested with *P. chlamydospora* conidia in the laboratory, the commercial kit selected for DNA extraction was found to be efficient and to provide consistent results. The significant linear relationship between conidial counts in suspension C and Cq values obtained from DNA samples (Fig. 1) confirmed the efficiency of the DNA extraction protocol. The linear relationship between *P. chlamydospora* conidia counts and DNA copy numbers (Fig. 3) enabled us to estimate the number of conidia detected on the tapes.

The equations developed here to describe the dynamics of *P. chlamydospora* dispersal could be used to predict periods of high risk of dispersal of the pathogen; before they are used, however, the equations should be validated with independent data collected in different years, locations, and viticultural systems (Rossi et al. 2010). Identifying the periods of high risk of dispersal may contribute to the practical management of this pathogen. During high risk periods, for instance, pruning should be avoided and pruning wounds should be protected (Berbegal et al. 2020; Gramaje et al. 2018; Mondello et al. 2018). Previous reports have been inconsistent about the best period for pruning in order to reduce the risk of *P. chlamydospora* infection. In South Africa, van Niekerk et al. (2011) indicated that late-winter wounds were more

TABLE 4. Evaluation of rainfall for predicting the detection of *Phaeomoniella chlamydospora* DNA on spore traps placed in three vineyards located in Ontinyent and Logroño, Spain, from 2016 to 2018

Rain[a]	Proportions[b]				Overall accuracy[c]	Posterior probabilities[d]			
	TPP	FNP	FPP	TNP		(P+|O+)	(P–|O–)	(P+|O–)	(P–|O+)
≥0.2	0.80	0.20	0.90	0.10	0.51	0.55	0.78	0.45	0.22
≥1	0.55	0.45	0.69	0.31	0.45	0.53	0.62	0.47	0.38
≥2	0.45	0.55	0.59	0.41	0.43	0.51	0.56	0.49	0.43
≥3	0.40	0.60	0.56	0.44	0.42	0.50	0.54	0.50	0.45
≥4	0.35	0.65	0.56	0.44	0.39	0.47	0.53	0.53	0.47
≥5	0.28	0.72	0.51	0.49	0.37	0.44	0.50	0.56	0.50

[a] Total quantities of rainfall (in millimeters) that were used as cut-off values to define a rain event.

[b] TPP (true-positive proportion, or sensitivity) = periods when rain = 1 and DNA detection = 1 divided by the total number of periods with detection. TNP (true-negative proportion, or specificity) = periods when rain = 0 and DNA detection = 0 divided by the total number of periods with no detection. FPP (false-positive proportion) = periods when rain = 1 and DNA detection = 0 divided by the total number of periods with no detection. FNP (false-negative proportion) = periods when rain = 0 and DNA detection = 1 divided by the total number of periods with detection.

[c] Overall accuracy calculated as the proportion of correct predictions.

[d] P(P+|O+) = posterior probability that *P. chlamydospora* DNA was detected when predicted based on rainfall amount. P(P–|O–) = posterior probability that DNA was not detected when not predicted. P(P+|O–) = posterior probability that DNA was not detected when predicted. P(P–|O+) = posterior probability that DNA was detected when not predicted.
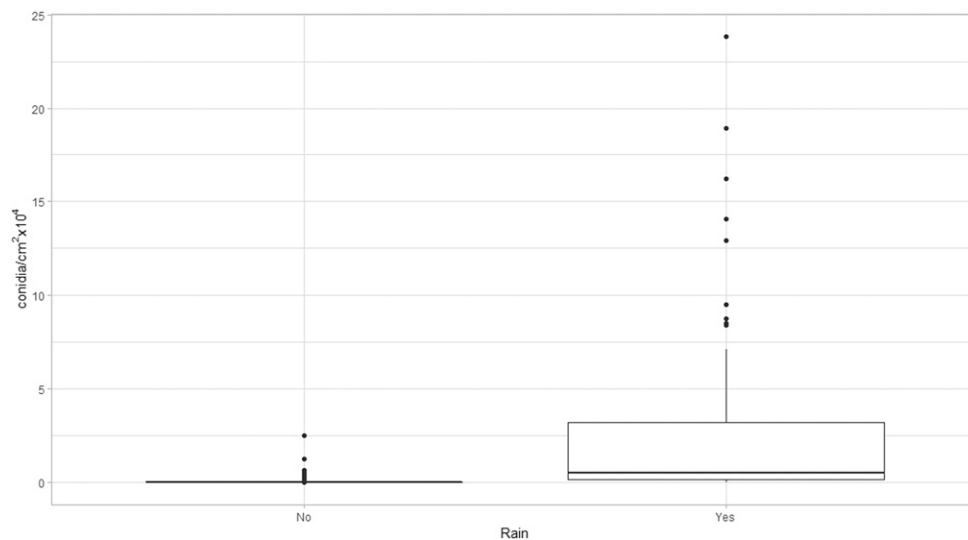

Fig. 7. Boxplots of the distributions of the DNA of *Phaeomoniella chlamydospora* detected on microscope slide traps in weeks without rain ($n = 72$) or with rain ($n = 74$). Quantitative PCR was used to detect and quantify the inoculum, which is expressed as the average number of conidia per square centimeter on five traps replaced weekly. Boxes include the second and third quartiles; the thick black line is the median, whiskers extend to minimum and maximum values, and the dots are the outliers.

susceptible to infection than early-season wounds. In contrast, Larignon and Dubos (2000) in France observed that, with early pruning (December and January), the pathogen was able to infect during a longer period and that infections were more serious than with later pruning. In Italy, Serra et al. (2008) found infections caused by *P. chlamydospora* for up to 4 months after pruning. In California, Eskalen et al. (2007) showed that wounds were susceptible to *P. chlamydospora* throughout the summer and, in Spain, Elena and Luque (2016) did not detect seasonal differences in wound susceptibility to *P. chlamydospora* when fall and winter pruning were compared. Results of our work indicate that the period of highest risk for *P. chlamydospora* may vary from year to year or among locations, depending on weather conditions.

The present research increases our understanding of the epidemiology of the main causal agent of Petri disease and esca, *P. chlamydospora*. Once the equation developed here is validated, it should be incorporated into a decision support system that will help growers adopt effective practices for controlling GTDs (Rossi et al. 2010).

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