RESEARCH PAPERS

A qPCR-based method for detection and quantification of *Polystigma amygdalinum*, the cause of red leaf blotch of almond

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Summary. Red leaf blotch of almond, caused by the fungus *Polystigma amygdalinum*, results in early defoliation of trees and decreases fruit production in many Mediterranean and Middle Eastern countries. A latent period of 30–40 d has been reported for this pathogen before disease symptoms are expressed, which makes targeted fungicide control difficult. A quantitative real-time PCR detection method was developed to detect and quantify the fungus in biological samples, to assist early detection. A primer pair was designed based on the ITS region of the fungal rDNA, and this was highly specific and sensitive, enabling detection of a minimum of 12 pg of *P. amygdalinum* DNA and seven ascospores in artificially-prepared ascospore suspensions. A protocol was also developed to quantify ascospores on plastic tapes which are commonly used in volumetric air samplers. The detection limit for these samples was the same as for ascospore quantification in aqueous suspensions. The pathogen was also quantified in naturally infected leaves showing different stages of disease development, including early stages of leaf infection with doubtful visual identifications. Future practical applications of the method developed here are discussed in view of improving the management of red leaf blotch of almond.

Key words: *Prunus dulcis*, molecular detection, specific primers.

Introduction

The Ascomycete *Polystigma amygdalinum* P.F. Cannon (Phyllachorales), the causal agent of red leaf blotch (RLB) of almond (*Prunus dulcis* (Mill.) D.A. Webb), is widely distributed in the Mediterranean basin and the Middle East. The pathogen is known to occur in Cyprus, Greece, Iran, Israel, Italy, Lebanon, Libya, Morocco, Portugal, Romania, Spain, and Turkey (Cannon, 1996; Farr and Rossman, 2018). RLB of almond is one of the most serious foliar diseases of this fruit species, causing early defoliation (Cannon, 1996; Shabi 1997) and subsequent decreases in fruit production (Miarnau *et al.*, 2010; López-López *et al.*, 2016).

Ascospores of *P. amygdalinum* are released from host leaves that have fallen in the previous year, under favourable conditions in spring, thus promoting first infections to occur. In Iran, Banihashemi (1990) reported that ascospores are discharged from host flowering (early March) to fruit set (mid April). Saad and Masannat (1997) reported that ascospore discharge in the Halat region, Lebanon, occurred before flowering (early February) until fruit set (mid May), but maximum ascospore release coincided with that described by Banihashemi (1990). A latent period, i.e. the time from initial leaf infection to appearance of disease symptoms, has been reported for *P. amygdalinum*. Ashkan and Assadi (1974) estimated a latent period of 30–35 d, whereas Banihashemi (1990) reported a latent period of 30–40 d under natural conditions, and 30–35 d in greenhouse conditions.

RLB symptoms are characterized at early stages by diffuse, roundish chlorotic blotches on both sides of the

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affected host leaves. At these very early stages, when blotches are a few mm in diameter, symptoms could be confused with those caused by other foliar pathogens of almond (Zúñiga, *personal observation*), such as *Wilsonomyces carpophilus* (Lév.) Adask., J.M. Ogawa & E.E. Butler, which causes shot hole disease of several *Prunus* spp. (Farr and Rossman, 2018). As infection progresses, the leaf spots grow and turn yellow-orange and later into red and purple dark lesions. Fungal stromata develop in the affected leaves, where pycnidia develop in summer (Ghazanfari and Banihashemi, 1976). Conidia formed in pycnidia are probably non-infectious (Banihashemi, 1990), as has been reported for the close relative species *P. fulvum* Pers. ex DC. (Suzuki *et al.*, 2008). At late stages of disease development, leaves curl and become necrotic. The diseased leaves fall at the end of summer, earlier than for healthy leaves (Cannon, 1996), and these overwinter on the soil surface to produce mature perithecia and ascospores in the *P. amygdalinum* stromata. The mature ascospores are released in spring of the following year, establishing a new cycle of the pathogen (Suzuki *et al.*, 2008). *Polystigma amygdalinum* is a biotrophic fungus that does not grow in culture media, although germination of ascospores on media has been observed (Banihashemi, 1990; Cannon, 1996; Habibi and Banihashemi, 2015).

Identification of *P. amygdalinum* relies mostly on recognizing RLB symptoms on plants, and by studying morphological characteristics of the pathogen through microscope observations. This is challenging, tedious and can lead to misinterpretations, especially at early stages of disease development. A technique has been developed for rapid diagnosis of foliar symptoms of RLB, based on analyses of High-Resolution Hyperspectral and Thermal imagery (López-López *et al.*, 2016). This was able to separate between asymptomatic trees and those affected either by early or advanced stages of RLB development. However, other approaches such as molecular tools for the rapid diagnosis of *P. amygdalinum* have not been fully developed. Habibi *et al.* (2015) obtained several DNA sequences from this species, which were used in a phylogenetic study to show that *P. amygdalinum* should be better accommodated in the Xylariomycetideae subclass (Xylariales) of the Sordariomycetes.

Molecular detection tools allow rapid and sensitive pathogen diagnoses, either *in vitro* or from plant tissue or environmental samples (Popov *et al.*, 2011; Shishido *et al.*, 2012; Scarlett *et al.*, 2013). Detection methods based on quantitative real-time PCR (qPCR)

confer some advantages compared with other techniques, including reduced analysis time, increased sensitivity and specificity, and prevention of laboratory contamination due to post-PCR product manipulations (Rubio *et al.*, 2017).

Early detection of the RLB pathogen would improve strategies for control of the disease, by designing improved fungicide programmes which could use plant protection products at the time of infection occurrence. However, the 30–40 d latent period of the disease, together with the unspecific symptoms seen at early stages of disease development, make it difficult to determine the exact times of infection.

The primary aim of the research described here was to design a specific primer pair for detection and quantification of *P. amygdalinum* using qPCR. In addition, two practical evaluations were conducted to validate the method: firstly, to develop, in laboratory conditions, a detection and quantification protocol for ascospores trapped on plastic tapes commonly used in volumetric air samplers, and secondly, to detect the pathogen in naturally infected host leaves at different stages of disease development.

Materials and methods

Fungal material

Fallen leaves of unknown almond varieties with distinct *P. amygdalinum* stromata were collected in December 2014 in Gandesa, Catalonia, Spain. Other fungal pathogens known to occur on almond, such as *Alternaria alternata* (Fr.) Keissl., *Monilinia laxa* (Aderh. & Ruhland) Honey, *Diaporthe amygdali* (Delacr.) Udayanga, Crous & K.D. Hyde, and *W. carpophilus*, as well as undetermined species of *Alternaria*, *Cladosporium*, and *Coniothyrium* were isolated on culture medium from diseased almond leaves, fruits and twigs collected in 2016 from additional locations in Catalonia (Table 1). The fungi were isolated on potato dextrose agar (PDA, Difco™, Becton, Dickinson and Co.) amended with ampicillin at 100 mg L^{-1}, and incubated at 25 ± 2°C in darkness. Pure cultures were obtained by subculturing of hyphal tips.

DNA extraction and sequencing

Non-culturable fungi

DNA from *P. amygdalinum* was obtained as follows. The upper part of each fungal stroma was cut off with

Table 1. Fungal taxa and DNA samples from culturable and non-culturable almond pathogens obtained in this study.

Fungal taxa	Isolate	Sample	Location[a]	Source	Symptom	GenBank Accession ITS
Alternaria alternata	CJL 877	CR52	Gandesa	leaf	shot-hole	MH205933
A. alternata	CJL 880	CR54	Vilafranca del Penedès	leaf	shot-hole	MH205931
Alternaria sp.	CJL 878	CR55	Gandesa	leaf	shot-hole	MH205934
Cladosporium tenellum	CJL 876	CR53	Gandesa	leaf	shot-hole	MH205932
Coniothyrium sp.	-	CR25	Gandesa	leaf	shot-hole	
Coniothyrium sp.	-	CR26	Gandesa	leaf	shot-hole	
Coniothyrium sp.	-	CR28	Gandesa	leaf	shot-hole	MH205924
Coniothyrium sp.	CJL 866	CR36	Gandesa	leaf	shot-hole	MH205926
Coniothyrium sp.	CJL 864	CR12	Les Borges Blanques	twig	scab	MH205920
Diaporthe amygdali	CJL 862	CR14	Gandesa	branch	canker	MH205921
Monilinia laxa	CJL 863	CR11	Les Borges Blanques	flower	blight	MH205919
Phoma sp.	CJL 879	CR51	Vilafranca del Penedès	leaf	shot-hole	MH205930
Polystigma amygdalinum	-	TO1	Gandesa	leaf	leaf blotch	MH205935
P. amygdalinum	-	TO5	Gandesa	leaf	leaf blotch	MH205936
P. amygdalinum	-	TO7	Gandesa	leaf	leaf blotch	MH205937
P. amygdalinum	-	TO9	Gandesa	leaf	leaf blotch	MH205938
P. amygdalinum	-	TO10	Gandesa	leaf	leaf blotch	MH205939
P. amygdalinum	-	TO15	Gandesa	leaf	leaf blotch	MH205940
P. amygdalinum	-	TO16	Gandesa	leaf	leaf blotch	MH205941
P. amygdalinum	-	TO18	Gandesa	leaf	leaf blotch	MH205942
Taphrina deformans	-	CR20	Les Borges Blanques	leaf	leaf curl	MH205922
T. deformans	-	CR34	Cabrils	leaf	leaf curl	MH205925
Wilsonomyces carpophilus	CJL 869	CR39	Les Borges Blanques	leaf	shot-hole	MH205927
W. carpophilus	CJL 872	CR41	Les Borges Blanques	leaf	shot-hole	MH205928
W. carpophilus	CJL 875	CR50	Les Borges Blanques	leaf	shot-hole	MH205929
W. carpophilus	-	CR22	Les Borges Blanques	leaf	shot-hole	MH205923

[a] All samples collected in Catalonia, Northeast Spain.

a sterile scalpel, and 20 fruiting bodies were individually excised with a hypodermic needle and collected into a 1.5 mL Eppendorf tube. DNA from each sample was individually extracted using the E.Z.N.A Plant Miniprep Kit (Omega Bio-Tek), with some modifications in the protocol as follows: in a preliminary step, 700 µL of buffer P1 (E.Z.N.A Plant Miniprep Kit) and

approx. 0.15 g of glass beads (diam. 500–750 µm; (Acros Organics) were added to the fungal material and vortexed together for 15 min at 50 Hz in a Vortex-Gene 2 (MoBio Laboratories) for cell lysis. The subsequent extraction steps were conducted according to the manufacturer instructions.

Fungal material of *Taphrina deformans* (Berk.)

Tul. was obtained by repeatedly washing the surface of fresh symptomatic almond leaves with a 5% Chelex-100 solution, with the aid of a micropipette, to release the fungal spores into the solution. Sample volumes of 200 μL each were prepared for the subsequent DNA extraction. DNA was extracted according to Leus *et al.* (2006) with some modifications. Suspensions of fungal material were autoclaved (120°C for 1 h). After autoclaving, the solutions were centrifuged for 5 min (10,000× *g*), and 140 μL of the supernatant was collected from each sample.

DNA concentrations were measured using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). Extracted DNA was stored in -20°C until further use.

Culturable fungi

DNA was extracted from fungal isolates following the protocols of Rogers and Blendich (1985) and Henrion *et al.* (1992), with some modifications. A sample (20–30 mg fresh weight) of an active growing colony (age 7–14 d, depending on the fungal species) was obtained and put into a 1.5 mL Eppendorf tube. A volume of 600 μL of 2% CTAB (100 mM Tris–HCl pH 8, 1400 mM NaCl, 20 mM EDTA pH 8) was added to each sample, and the sample was crushed with a micropestle and incubated for 40 min at 65°C, and then centrifuged (10,000× *g* for 5 min) at room temperature. The upper phase of each sample was transferred to a new Eppendorf tube and mixed with one volume of chloroform, centrifuged (10,000× *g* for 5 min) and the upper phase was transferred into a new tube. The DNA was precipitated by adding 1.5 volumes of cold (-20°C) isopropanol, mixing and keeping the samples in a freezer at -20°C for 30 min. Samples were centrifuged at 10,000× *g* for 30 min at 4°C. The supernatant of each sample was discarded, the pellet was washed with 200 μL of ice-cold ethanol (70% v/v) and centrifuged again (5,000× *g* for 5 min at 4°C). After centrifugation, the supernatant was carefully discarded, the pellet was dried and resuspended in 100 μL of HPLC grade H_2O. DNA concentrations were measured as described above, and extracted DNA was stored at -20°C until further use.

Sequencing of fungal DNA

The internal transcribed spacer (ITS) region of ribosomal DNA was amplified by PCR, using the primers ITS1F (Gardes and Bruns, 1993) and ITS4 (White *et al.*, 1990). Reactions were performed in a final volume of 25 μL containing 12.5 μL of 2× PCR Master Mix (Takara, Takara Bio Europe SAS), 0.2 μM of each primer, 4 μL of template DNA (20–200 ng DNA), and 6.5 of HPLC-grade H_2O to reach the final volume. PCRs were performed in a GeneAmp® 9700 thermal cycler (Applied Biosystems), with the following conditions: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 20 s, primer annealing at 55°C for 30 s, and extension at 72 °C for 40 s, and a final extension at 72°C for 7 min. PCR products (3 μL) were separated by gel electrophoresis in 2% agarose gel in 0.5× TBE buffer (54 g Tris-base, 27.5 g boric acid, 0.5 M EDTA, pH 8). They were then stained with SYBR Safe DNA gel stain (Life Technologies) and visualized using a UV transilluminator. PCR products were purified using the QIAquick® PCR purification kit (Qiagen GmbH) prior to sequencing (GATC Biotech). Both strands were sequenced using the primer pair (above). Sequences obtained in this study were deposited in GenBank and matched with reference sequences using BLAST to confirm tentative fungal identifications (Table 1).

Design of qPCR primers

The ITS region was considered suitable for the design of qPCR primers for two reasons: (1) the high copy numbers of the ITS region allow for the easy detection of low quantities of the pathogen, and (2) the large number of reference sequences available in GenBank. ITS sequences of *P. amygdalinum* obtained in this study (Table 1) and 14 additional ITS sequences of *Polystigma* deposited at GenBank (eight of *P. amygdalinum*: KC756360 to KC756366 and JQ995323; three of *P. pusillum* Syd. & P. Syd.: KX451899, KX451907 and KX451922; one of *P. rubrum* (Pers.) DC: KY594023; and two of *Polystigma* sp.: KC966927 and KX451916) were aligned using the Clustal W algorithm (Thompson *et al.*, 1994) within the MEGA7 software package (Kumar *et al.*, 2016). Sequence regions suitable for a *P. amygdalinum*-specific analysis were identified in polymorphic zones of the alignment. The regions identified were also analysed using IDT´s PrimerQuest Tool (https://eu.idtdna.com/PrimerQuest/Home/Index), with the default design parameters for qPCR Intercalating Dyes for the designed primer pairs. Four primer pairs were designed and, after optimization of qPCR conditions, their specificity and sensitiv-

ity were tested. The four primer pairs were obtained from Macrogen.

To optimize the qPCR reaction conditions, different annealing temperatures (60 and 64ºC), primer concentrations (100, 200 and 400 μM of each primer) and Takara qPCR SYBR Premixes (SYBR Premix Ex Taq and SYBR Premix Ex Taq II) were tested with examination of amplification plots, dissociation curves and primer-dimer formation (*data not shown*). The optimized reaction contained (final volume of 25 μL) 12.5 μL SYBR Premix Ex Taq™ (Takara Bio Inc.), 0.4 μM of each specific forward and reverse primers, 5 μL of template DNA and ultrapure sterile water (Chromasolv Plus, Sigma-Aldrich). The optimized PCR cycling conditions were 95°C for 30 s, 40 cycles each at 95°C for 5 s, and 60°C for 30 s, in which the fluorescence signal was measured. After the final amplification cycle, the melting curve profiles were obtained by raising the temperature from 72 to 95°C, increasing 1°C every 5 s with continuous measurement of fluorescence at wavelength 510 nm. All reactions were performed in triplicate and were carried out on a Rotor-Gene Q 5plex thermal cycler (Qiagen).

Specificity tests

Specificity of the primer pairs was checked *in silico* analysis with the Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Each primer pair was tested for suitability for the qPCR amplification of portion of *P. amygdalinum* ITS and for the presence of primer dimers by analysing dissociation curves and visualizing qPCR products on 3% agarose gels (MetaPhor® Agarose) under UV light. The DNA samples used in qPCR tests included the DNA extracts from *P. amygdalinum* samples, as well as from other pathogens of almond (Table 1) and from supposedly pathogen-free almond leaves. Each set of qPCR assays included a negative control, without template DNA.

Analytical sensitivity

The parameters limit of blank (LOB), limit of detection (LOD) and limit of quantification (LOQ) were calculated for each qPCR assay involving the assessment of the analytical sensitivity of the method. These parameters were estimated according to the guideline EP17 of the Clinical and Laboratory Standards Institute described by Armbruster and Pry (2008). In ad-

dition, for each calculated regression equation of the fitted qPCR standard curves the values of intercept, slope, determination coefficient (r^2) and amplification efficiency (AE) are reported. The quantification cycle (C_q) values corresponding to detection limits are reported when appropriate.

DNA preparation for qPCR assays

Two independently replicated genomic DNA samples of *P. amygdalinum* obtained from perithecium contents, designated DNA 1 and DNA 2, were used. The DNA from each sample was extracted using the E.Z.N.A Plant Miniprep Kit as described above. The measured DNA concentrations were 1.03 ng genomic DNA μL⁻¹ for DNA 1 and 2.83 ng μL⁻¹ for DNA 2. Ten-fold dilutions series to 1:10⁶ were prepared and subsequently used in the qPCR assays, as described below. Additionally, perithecia of *P. amygdalinum* were obtained from stromata as described above, and were placed in water droplets on a microscope slide and bisected to release ascospores. Two independently replicated ascospore suspensions, designated 1 and 2, were prepared from the collected ascospores, and these were quantified using a light microscope (×250 magnification) and a Neubauer chamber. Initial concentrations were $1.66 \times 10^5 \pm 0.089 \times 10^5$ (SE) ascospores mL⁻¹ for Suspension 1, and $1.27 \times 10^5 \pm 0.084 \times 10^5$ ascospores mL⁻¹ for Suspension 2 (N = four measurements with eight pseudoreplicates each). Ten-fold dilution series (until 1:10⁵) of each ascospore suspension were prepared. The DNA from each sample was extracted using the E.Z.N.A Plant Miniprep Kit as described above. Samples were stored at -20°C until further use in the qPCR assays (below).

Quantitative real-time PCR assays

Standard curves for the qPCR assays for the two DNA sources, perithecial content and ascospore suspensions, were carried out with a StepOne™ Real-Time PCR System thermal cycler and using the StepOne software v. 2.3 (Life Technologies). DNA samples were amplified by qPCR using the optimized conditions described above, with primers PamyI2F4 and PamyI2R2 (Table 2). The standard curve was obtained by plotting the C_q values versus the logarithm of the quantity of the serially diluted DNA. Three technical replicates were used for each biological sample, and HPLC water template was used as the negative control in every experiment.

Table 2. Main characteristics of the primer pairs designed for the detection and quantification of *Polystigma amygdalinum*.

Target region (ITS)	Primer name	Sense	Sequence (5'-3')	Product size (bp)
ITS1	PamyI1F1	Forward	CGTGAAAGCACGTTTCATCC	114
	PamyI1R1	Reverse	CACTTCAGATGCATGGTTCAAG	
ITS1	PamyI1F2	Forward	CCGTGGACGTGAAAGCA	108
	PamyI1R2	Reverse	TGGTTCAAGACGGGTTTGG	
ITS2	PamyI2F3	Forward	CCGTAGGCTTGCCGTTG	75
	PamyI2R3	Reverse	GCTCAGAGTCGCCACGA	
ITS2	PamyI2F4	Forward	GAAGTCCAATCAAGCCGTAG	99
	PamyI2R2	Reverse	GTTTCACTACGCTCAGAGTC	

Validation of the specific qPCR primer pair PamyI2F4/PamyI2R2

Case 1: Detection of P. amygdalinum *on spore-trapping tapes*

Starting from a single ascospore suspension (Ascospore suspension 3) containing $1.17 \times 10^5 \pm 0.07 \times 10^5$ ascospores mL^{-1}, two 10-fold dilution series were prepared until $1:10^5$ of initial concentration. DNA was extracted from each ascospore suspension of the first dilution series. For the second dilution series, 500 µL of each ascospore suspension were placed onto a 48-mm-long Melinex® 200 gauge (TEKRA) clear plastic tape, previously coated with a thin uniform film of silicone solution (Lanzoni). The tapes were dried at room temperature for 24 h in a laminar flow cabinet, and DNA from each ascospore suspension on tape was individually extracted with the E.Z.N.A Plant Miniprep Kit. The qPCR was subsequently performed in a StepOne™ Real-Time PCR System thermal cycler using the primer pair PamyI2F4/PamyI2R2 and with the optimized conditions described above. Three technical replicates were used for each dilution series point.

Case 2: Early detection of P. amygdalinum *in asymptomatic and symptomatic leaves*

Apparently healthy and RLB-symptomatic leaves were obtained from unknown almond varieties in an experimental orchard owned by IRTA. The orchard was located in Les Borges Blanques, Catalonia, Spain (UTM 31T X = 320870, Y = 4597530). The orchard was not treated with fungicides to allow natural pathogen infections. Samples were classified according to the stage of disease development in the leaves (Table 3, Figure 1). An additional group of leaves with characteristic shot-hole lesions caused by *W. carphilus* was added to the samples, since very early shot-hole and RLB stages can sometimes be undistinguishable. In all leaf samples, 1 cm^2 of plant tissue was cut, washed three times with sterile distilled water, and placed into a 1.5 mL Eppendorf. DNA was extracted using the E.Z.N.A Plant Miniprep Kit (Omega Bio-Tek), by adding 0.15 g of glass beads (500–750 µm) and two 3-mm Tungsten balls (Qiagen). The qPCR was then performed with the optimized conditions described above. Three technical replicates were used for each biological sample.

Statistical analyses

Output data corresponding to the fitted qPCR standard curves equations, including intercept, slope, r^2 and AE, were obtained from the software of the thermal cyclers used in this study. The comparisons of regression equation slopes were performed using linear modelling in R (R Core Team, 2017), and specifically with the analysis of covariance using the aov function. Statistical significance was declared at $P < 0.05$.

Table 3. Detection of *Polystigma amygdalinum* in asymptomatic and symptomatic almond leaves through quantitative real-time PCR.

Red leaf blotch development stage	Number of samples	Number of positive samples	C_q[a]
Asymptomatic leaves	9	3	30.274 ± 0.893[b]
Initial or unclear symptoms	3	3	21.523 ± 1.656
Yellowish discoloured blotches	3	3	18.323 ± 0.202
Orange discoloured blotches	3	3	18.869 ± 0.654
Reddish blotches	3	3	17.320 ± 0.188
Dark necroses (mature stroma)	3	3	15.318 ± 0.545

[a] Values corresponding to positive samples.
[b] Mean ± standard error values.
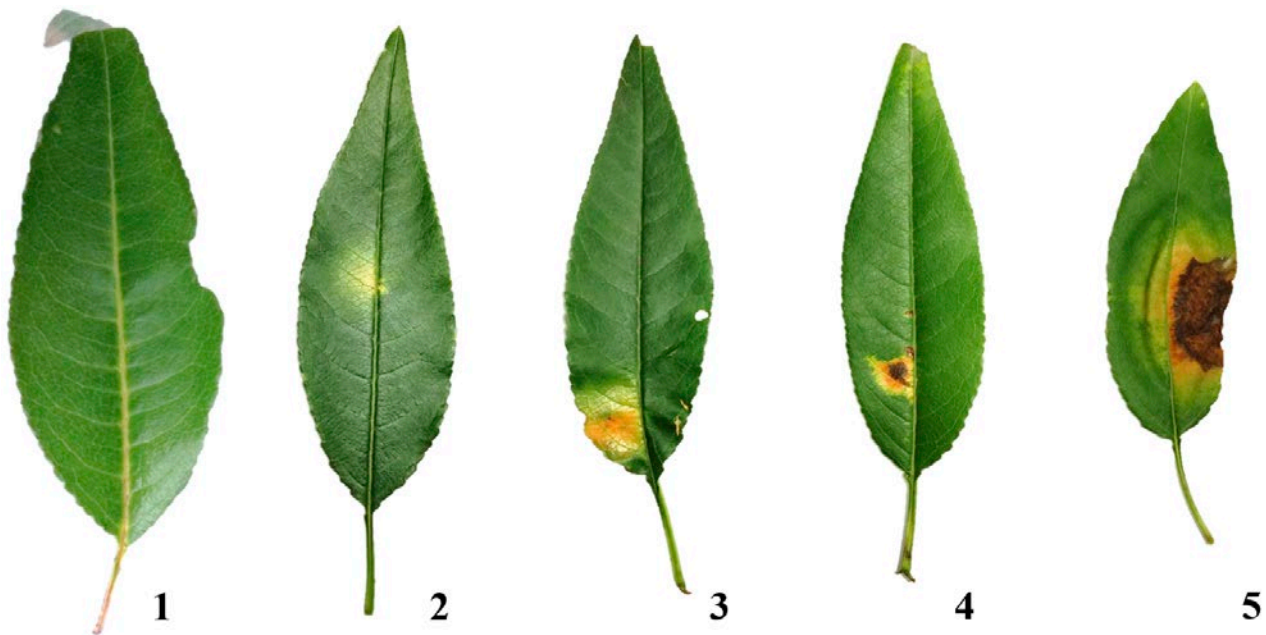
Figure 1. Sequential development of the red leaf blotch symptoms on almond leaves. From left to right: 1) asymptomatic leaf; 2) initial yellowish discolouration; 3) orange-reddish blotch; 4) initial darkening of stroma with darkening red blotch; 5) dark, well-developed stroma.

Results

Design, selection and specificity of *P. amygdalinum* specific primers

The BLAST analysis of eight *P. amygdalinum* ITS sequences obtained in this study (TO1, TO5, TO7, TO9, TO10, TO15, TO16 and TO18) showed 99 to 100% similarity with accessions of *P. amygdalinum* deposited in GenBank (KC756360 to KC756366 and JQ995323). The similarity with ITS sequences of other *Polystigma* species (KC966927, KX451899, KX451907, KX451916, KX451922 and KY594023) was between 75 to 91%. The new sequences obtained in this study were deposited at GenBank (Table 1).

For the design of the *P. amygdalinum* specific primers, 22 ITS sequences of *Polystigma* spp. were aligned. The ITS region showed three polymorphic sites among the *P. amygdalinum* sequences, but they showed high polymorphism with respect of sequences of other *Polystigma* species. Four primer pairs were designed, two in each ITS1 and ITS2 regions (Table 2). The analysis with the Primer-BLAST tool showed identity only with *P. amygdalinum* ITS sequences. Primer specificity was tested with genomic DNA extractions from eight *P. amygadalinum* samples and 19 additional DNA extracts representing eight fungal taxa associated to *P. dulcis* (Table 1), as well as from DNA extracted from supposedly pathogen-free almond leaves. All primer pairs amplified only the target DNA of *P. amygdalinum*, and the final selection of the best primer pair was based on the lowest estimated C_q value and the melting curve results. The finally selected primer pair (PamyI2F4/PamyI2R2) is located in the ITS2 region and amplified a fragment of 99 bp. Amplifications and amplicon sizes were checked on 3% Metaphor agarose gel (*data not presented*). Sequences of the qPCR products were identical to those of regions delimited by the designed primer pairs. All extracted DNAs were amplified with the primer pair ITS1F/ITS4 to discard inhibition issues in the PCR.

Efficiency assays and quantification of fungal DNA and ascospore suspensions

In all qPCR tests performed in this study, estimated LOB values were always less than the corresponding LOD values in each qPCR test, so they are not reported here, according to Armbruster and Pry (2008). The average C_q value for the LOB in all the experiments was established at 35.07 cycles.

DNA from *P. amygdalinum* was detected and quantified successfully in two independent 10-fold dilution series of extracted DNA from perithecium contents: until dilution $1:10^3$ for the first sample (DNA 1) and to $1:10^4$ for the second sample (DNA 2). The efficiency and accuracy to detect *P. amygdalinum* were markedly reduced below these dilutions. There was a clear linear relationship between the logarithm of the DNA concentrations and the C_q values (Figure 2a). The standard regression curve of the first sample (DNA 1, with intercept = 21.897, slope = -3.347, r^2 = 0.996, and AE = 98.95 %) showed a LOD down to 9.36 pg DNA μL^{-1}, with a mean C_q value at LOD ($C_{q\ LOD}$) = 28.68, and a LOQ = 21.32 pg DNA μL^{-1}. The

standard regression curve of the second DNA sample (DNA 2, with intercept = 22.962, slope = -3.240, r^2 = 0.990, AE = 103.51 %) showed a LOD = 2.37 pg DNA μL^{-1}, with $C_{q\ LOD}$ = 31.47 and LOQ = 7.38 pg DNA μL^{-1}. Both equations had equal, statistically similar slopes (P = 0.50). The LOD was therefore determined to be 12 pg DNA mL^{-1}.

Quantification of ascospores in aqueous suspensions using qPCR showed reliable detection, to seven ascospores mL^{-1}. In lower concentration dilutions, detection efficiency and accuracy decreased (Figure 2b). The standard regression curve of the first sample (Ascospore suspension 1, with intercept = 34.444, slope = -3.394, r^2 = 0.976, AE = 97.09 %) showed the following characteristic values: LOD = 7.38 ascospores mL^{-1}, $C_{q\ LOD}$ = 31.49, LOQ = 18.62 ascospores mL^{-1}. The standard regression curve of the second ascospore suspension sample (Ascospore suspension 2, with intercept = 36.395, slope = -3.516, r^2 = 0.983, AE = 92.50 %) showed the following values: LOD = 10.34 ascospores mL^{-1}, $C_{q\ LOD}$ = 32.82, LOQ = 75.22 ascospores mL^{-1}. Both equations had equal, statistically similar slopes (P = 0.63).

### Validation of the specific qPCR primer pair PamyI2F4/PamyI2R2

Detection of P. amygdalinum *in spore-trapping tapes*

Ascospores of *P. amygdalinum* were detected and quantified both from ascospore suspensions and ascospores placed on plastic tapes. No reliable amplifications were detected below $1:10^4$ dilution. The regression equations for the two standard curves showed no statistical differences (P = 0.55) in their slopes (Figure 2c). First standard curve (Ascospore suspension 3 not placed on plastic tape, with intercept = 34.364, slope = 3.516, r^2 = 0.961, AE = 92.48 %) showed the following values: LOD = 6.06 ascospores mL^{-1}, $C_{q\ LOD}$ = 31.60, and LOQ = 57.78 ascospores mL^{-1}. Similarly, the second standard curve (Ascospore suspension 3 placed on plastic tape, with intercept = 34.26, slope = -3.349, r^2 = 0.978, AE = 98.86 %) showed the following values LOD = 7.72 ascospores mL^{-1}, $C_{q\ LOD}$ = 31.28, and LOQ = 16.52 ascospores mL^{-1}.

Early detection of P. amygdalinum *in asymptomatic and symptomatic leaves*

Polystigma amygdalinum was detected in 100 % of samples with clear RLB symptoms, regardless of the developmental stage of the disease (Table 3). In gener-
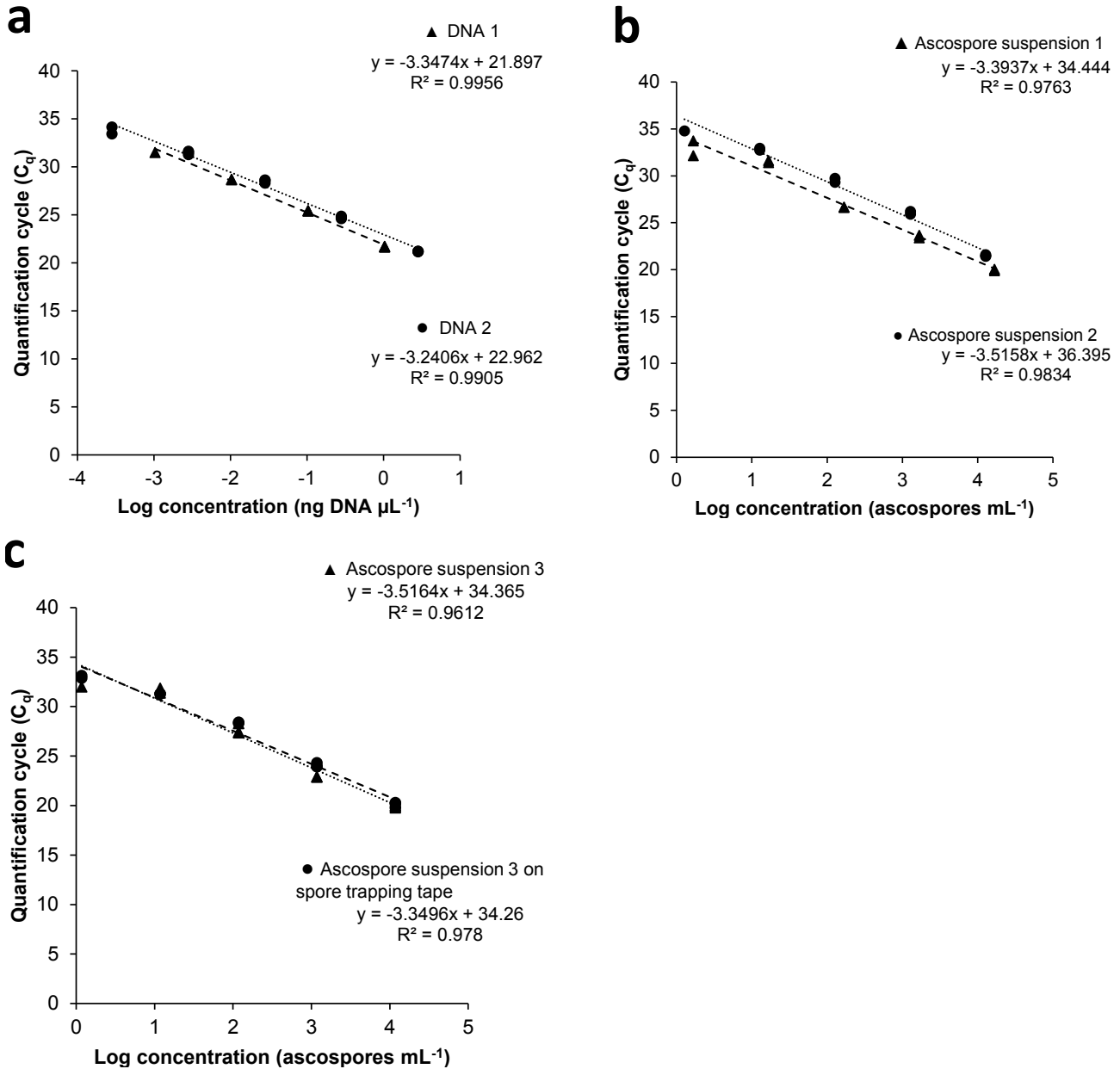
Figure 2. Standard regression curves of qPCR tests run with several 10-fold serial dilutions of *Polystigma amygdalinum* genomic DNA obtained from: a) perithecial contents (samples 1 and 2 with initial amounts of, respectively, 1.03 and 2.83 ng DNA/μL); b) Ascospore suspensions 1 and 2 (respectively $3.33 \times 10^5 \pm 1.76 \times 10^5$ and $2.54 \times 10^5 \pm 1.68 \times 10^5$ ascospores mL^{-1}); c) Ascospore suspension 3 ($2.35 \times 10^5 \pm 1.30^5 \times 10^5$ ascospores mL^{-1}), either processed directly for DNA extraction or after placing ascospores on a plastic spore-trapping tape.

al, the results showed that the older the symptom the lower was the C_q at which *P. amygdalinum* was detected. C_q values of these samples ranged from approx. 18, in yellowish-orange discoloured blotches, down to 15.32 in fully mature stromata. However, in the presence of initial or unclear symptoms, the C_q was slightly greater (21.52), but, again, the pathogen was detected in 100% of cases. *Polystigma amygdalinum* was detected

also in approx. one third of the asymptomatic leaves, with mean C_q = 30.27 in those positive samples. Additionally, in shot-hole affected leaves, *P. amygdalinum* was detected towards the end of the reactions in all six samples analysed, with mean C_q = 32.21.

Discussion

The qPCR method developed in this study, which used the specific primer pair PamyI2F4/PamyI2R2, successfully detected and quantified *Polystigma amygdalinum*, the fungus which causes red leaf blotch of almond. The method was validated in repeated experiments by quantifying the pathogen both from natural sources (naturally infected leaves), and from artificially prepared samples (ascospores placed on plastic spore-trapping tapes). To the best of our knowledge, this is the first time that a qPCR-based method has been reported for detection and quantification of *P. amygdalinum*.

Regarding the specificity of the primer pair PamyI2F4/PamyI2R2, amplification was detected from all DNA samples of *P. amygdalinum* tested in this study, while no other fungal species (eight taxa) potentially associated with diseased almond leaves, twigs and flowers were detected in any case. The sensitivity of the primer pair PamyI2F4/PamyI2R2, gave LOD values of approx. 12 pg DNA of *P. amygdalinum* and seven ascospores per reaction. The LOD values estimated both for ascospore suspensions and ascospores placed on spore-trapping tapes were comparable (6-7 ascospores), thus indicating that the plastic tapes did not interfere with the DNA extraction protocol in those samples.

The fungal DNA was isolated from samples used in the validation tests using a commercially available extraction kit. This was an efficient and simple method to obtain the required fungal DNA, which in turn enabled a standardized routine in sample analyses. However, if this qPCR method was further used to quantify *P. amygdalinum* in infected leaves, then a normalization of the fungal DNA with the plant DNA abundance should be implemented. When the fungal DNA is quantified on the basis of leaf surface area or leaf fresh weight, as in this preliminary validation assay, the validity of the qPCR results could be affected by host tissue deterioration and variable DNA extraction yields among samples (Valsesia *et al*., 2005; Moretti *et al*., 2015), thus leading to overestimation of the fungal biomass.

Polystigma amygdalinum was detected in all of leaf samples with shot-hole symptoms, although the estimated C_q values were greater than those for samples positively infected with the RLB pathogen, and those from asymptomatic almond leaves. Furthermore, these C_q values were comparable to the LOD values reported for the detection of the pathogen, therefore suggesting that initial symptomless infections by *P. amygdalinum* were already present in leaves by the time they were collected. Alternatively, the *P. amygdalinum* ascospores may not have washed from the leaves during the laboratory processing, and these could have resulted in positive detection of the pathogen. The specificity of the primer set for the detection of *P. amygdalinum* was demonstrated *in silico* and *in vitro*, as no amplifications were observed for the DNA extracted from the shot-hole pathogen and other species commonly found on almond leaves. Moreover, at the end of each reaction in all experiments, a melting curve analysis was performed to ensure accurate quantification of the target product and discarding of non-specific fluorescents signal due to unspecific amplifications. These results indicate that either latent infections or single ascospores may be present in the leaves at the time of collection. This also confirms that RLB and shot-hole diseases can coexist and persistently affect almond leaves in the same orchard.

The qPCR method developed in this study can help in early and rapid detection of *P. amygdalinum* in the field, either from collected latent-infected leaves or from spore trapping samplers that can be monitored periodically. The latent period that has been reported for the RLB of almond is 30 to 40 d (Ashkan and Assadi, 1974; Banihashemi, 1990). However, there are no available data from Spain to confirm that the disease latent period in this country is the same as reported. The molecular tool developed in this study, combined with controlled artificial inoculations, could be used to establish the period of latent infections of *P. amygdalinum* under different crop conditions. Current methods to identify either initial infection stages in the field or *P. amygdalinum* ascospores through microscopic observation are usually time-consuming and demand highly specialized personnel. With the new qPCR technique, these issues can be efficiently resolved. Thus, fungicide applications could commence at the correct times, optimizing the use of plant protection products while enhancing their effectiveness against the disease. This qPCR method could there-

fore serve as a valuable decision support tool for effective disease management in almond orchards.

The qPCR-based method could also be used in almond breeding programmes. As stated by Moretti *et al.* (2015), accurate quantification of a pathogen in diseased plant tissues can be an indicator of the degree of host susceptibility. By combining pathogen quantification in host tissues with the assessment of RLB severity for whole plants, accurate evaluation of varieties for susceptibility or tolerance to disease could be achieved. In tolerant genotypes, symptom attenuation is not correlated with reductions in pathogen growth, as is the case for plant resistance (Moretti *et al.*, 2015).

Future research is needed to implement PCR detection of *P. amygdalinum* under field conditions, while maintaining efficacy of the detection method. This molecular tool will also help to increase knowledge of the biology and management of the red leaf blotch of almond.

Acknowledgements

Literature cited

Armbruster D.A. and T. Pry, 2008. Limit of blank, limit of detection and limit of quantitation. *The Clinical Biochemist Reviews* 29 (suppl. 1), S49–S52.

Ashkan M. and P. Assadi, 1974. Red blotch of almond (*Polystigma ochraceum*) in Iran. *Iranian Journal of Plant Pathology* 10, 49–63.

Banihashemi Z., 1990. Biology and control of *Polystigma ochraceum*, the cause of almond red leaf blotch. *Plant Pathology* 39, 309–315.

Cannon P.F., 1996. Systematics and diversity of the Phyllachoraceae associated with Rosaceae, with a monograph of *Polystigma. Mycological Research* 100, 1409–1427.

Farr D.F. and A.Y. Rossman, 2018. Fungal Databases, U.S. National Fungus Collections, ARS, USDA. Retrieved January 16, 2018, from https://nt.ars-grin.gov/fungaldatabases/

Gardes M. and T.D. Bruns, 1993. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. *Molecular Ecology* 2, 113–118.

Ghazanfari J. and Z. Banihashemi, 1976. Factors influencing as-

cocarp formation in *Polystigma ochraceum. Transactions of the British Mycological Society* 66, 401–406.

Habibi A. and Z. Banihashemi, 2015. Ascospore germination and appressorium formation *in vitro* of *Polystigma amygdalinum* and its survival period. *Iranian Journal of Plant Pathology* 51, 461–469.

Habibi A., Z. Banihashemi and R. Mostowfizadeh-Ghalamfarsa, 2015. Phylogenetic analysis of *Polystigma* and its relationship to *Phyllachorales. Phytopathologia Mediterranea* 54, 45–54.

Henrion B., F. Le Tacon and F. Martin, 1992. Rapid identification of genetic variation of ectomycorrhizal fungi by amplification of ribosomal RNA genes. *New Phytologist* 122, 289–298.

Kumar S., G. Stecher and K. Tamura, 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for bigger datasets. *Molecular Biology and Evolution* 33, 1870–1874.

Leus L., A. Dewitte, J. Van Huylenbroeck, N. Vanhoutte, E. Van Bockstaele and M. Hofte, 2006. *Podosphaera pannosa* (syn. *Sphaerotheca pannosa*) on *Rosa* and *Prunus* spp.: Characterization of pathotypes by differential plant reactions and ITS sequences. *Journal of Phytopathology* 154, 23–28.

López-López M., R. Calderón, V. González-Dugo, P.J. Zarco-Tejada and E. Fereres, 2016. Early detection and quantification of almond red leaf blotch using high-resolution hyperspectral and thermal imagery. *Remote Sensing* 8(4), 276, 23 pp. doi:10.3390/rs8040276

Miarnau X., F.J. Vargas, R. Montserrat and S. Alegre, 2010. Aspectos importantes en las nuevas plantaciones de almendro en regadío. *Revista de Fruticultura* (Extraordinario 2010), 94–103.

Moretti C., M. Quaglia, M. Cerri, D.E. Nicosia and R. Buonaurio, 2015. A real-time PCR assay for detection and quantification of *Botrytis cinerea* in *Pelargonium × hortorum* plants, and its use for evaluation of plant resistance. *European Journal of Plant Pathology* 143, 159–171.

Popov A.P., A.A. Belov, N.E. Ivanushkina, I.L. Tsvetkov and A.S. Konichev, 2011. Molecular genetic determinants of intraspecific polymorphism of the phytopathogenic fungus *Cryphonectria parasitica. Russian Journal of Genetics* 47, 262–271.

R Core Team, 2017. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/

Rogers S.O. and A.J. Blendich, 1985. Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. *Plant Molecular Biology* 5, 69–76.

Rubio S., A. Barnes, K. Webb and J. Hodgetts, 2017. A real-time PCR assay for improved rapid, specific detection of *Cryphonectria parasitica. Annals of Applied Biology* 171, 52–61.

Saad A.T. and K. Masannat, 1997. Economic importance and cycle of *Polystigma ochraceum*, causing red leaf blotch disease of almond, in Lebanon. *Bulletin OEPP/EPPO Bulletin* 27, 481–485.

Scarlett K., L. Tesoriero, R. Daniel and D. Guest, 2013. Detection and quantification of *Fusarium oxysporum* f. sp. *cucumerinum* in environmental samples using a specific quantitative PCR assay. *European Journal of Plant Pathology* 137, 315–324.

Shabi E., 1997. Disease management of the almond pathogens *Glomerella cingulata, Polystigma ochraceum* and *Tranzschelia*

pruni-spinosae. *Bulletin OEPP/EPPO Bulletin* 27, 479–480.

Shishido M., I. Kubota and H. Nakamura, 2012. Development of real-time PCR assay using TaqMan probe for detection and quantification of *Rosellinia necatrix* in plant and soil. *Journal of General Plant Pathology* 78, 115–120.

Suzuki Y., S. Hatakeyama, Y. Harada and K. Tanaka, 2008. *Polystigma fulvum*, a red leaf blotch pathogen on leaves of *Prunus* spp., has the *Polystigmina pallescens* anamorph/andromorph. *Mycoscience* 49, 395–398.

Thompson J.D., D.G. Higgins and T.J. Gibson, 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22, 4673–4680.

Valsesia G., D. Gobbin, A. Patocchi, A. Vecchione, I. Pertot and C. Gessler, 2005. Development of a high-throughput method for quantification of *Plasmopara viticola* DNA in grapevine leaves by means of quantitative real-time polymerase chain reaction. *Phytopathology* 95, 672–678.

White T.J., T. Bruns, S. Lee and J. Taylor, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: a Guide to Methods and Applications* 38, 315–322.