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The red leaf blotch of almond, Caused by Polystigma amygdalinum, in Catalonia: Biology and Epidemiology

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Tesis Doctoral

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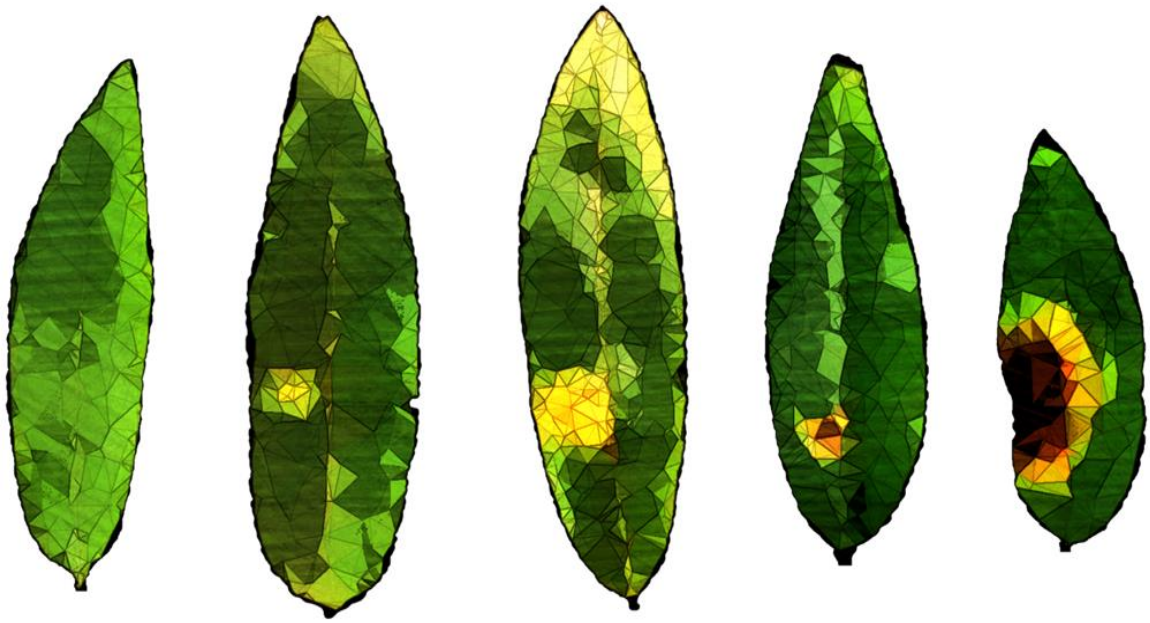

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Knowledge is a weapon, Jon. Arm yourself well before you ride forth to battle.

—G.R.R.M.

Abstract

Major biological aspects of the red leaf blotch (RLB) disease of almond, caused by the ascomycete *Polystigma amygdalinum*, were studied in this PhD Thesis. This disease is one of the most important foliar diseases in most almond-growing regions in the Mediterranean basin and the Middle East, since severe infections may cause a premature defoliation of the tree. Those key aspects were related to i) the epidemiology of *P. amygdalinum* in two almond-growing regions in Spain, ii) the reliable detection and quantification of the pathogen through quantitative real-time PCR (qPCR), in addition to iii) the search for the molecular and biochemical basis for the differential cultivar susceptibility to the RLB.

The primary inoculum of *P. amygdalinum* was available in extended periods in the growing season (January to August). The infectivity period in both Spanish almond-growing areas extended from March to mid-June when temperatures are in the range 10 to 20°C. The incubation period was variable among all years studied (2 to 12 weeks), but in general decreased with time in the season. For the detection and quantification of *P. amygdalinum*, a primer pair was designed based on the ITS region of the fungal rDNA, and this was shown to be highly specific and sensitive, enabling to detect the pathogen accurately in naturally infected leaves showing different stages of RLB development. Detection of a minimum of 12 pg of *P. amygdalinum* DNA, and seven ascospores in artificially-prepared ascospore suspensions were determined to be the limits of detection. The development of a qPCR-based protocol allowed to quantify the amounts of ascospores on plastic tapes which are commonly used in volumetric air samplers as well as the fungal colonization in infected leaves. We explored the plant defence mechanisms related to the cultivar susceptibility by identifying some relevant physical and chemical strategies involved in the almond tolerance to *P. amygdalinum*. Thus, we studied the regulation of seven defence-related genes as well as the lignin deposition in two almond cultivars (tolerant 'Mardía' and susceptible 'Tarraco') with highly differential response to RLB. 'Mardía' displayed an up-regulation of the *CAD* and *DFN1* genes at early stages of RLB symptom expression, with further lignin deposition in the fungal-colonized tissues. In contrast, 'Tarraco' mainly triggered the up-regulation of *HQT* and *LDOX* genes, related to chlorogenic acid and anthocyanin biosynthesis pathways, respectively, while lignin deposition was not clearly noticed.

Our results open a door to future research focused on developing an accurate epidemiological model to predict infection risk events for RLB, by integrating the monitoring of primary

inoculum with the aid of volumetric air samplers and the qPCR-based detection method developed in this thesis. In addition, increasing the knowledge on the genetic and molecular bases of the resistance/tolerance to RLB would help in obtaining new almond cultivars with improved tolerant profiles against the RLB. This way, we aim at facing the control of the red leaf blotch of almond in an integrated and sustainable way.

Resumen

En esta tesis doctoral se han estudiado diversos aspectos biológicos importantes sobre la enfermedad de la mancha ocre del almendro (RLB, por sus siglas en ingles), causada por el hongo ascomiceto *P. amygdalinum*. Esta enfermedad es una de las enfermedades foliares más importantes en la mayoría de las regiones de la cuenca Mediterránea y Oriente medio, donde se cultiva el almendro, ya que las infecciones graves pueden causar la defoliación prematura del árbol. Los aspectos estudiados están relacionados con: i) La epidemiología de *P. amygdalinum* en dos regiones productoras de almendra en España; ii) la detección y cuantificación fiables del patógeno, mediante PCR cuantitativa en tiempo real (qPCR), además de iii) el estudio de las bases bioquímicas y moleculares de la diferente susceptibilidad a la RLB en distintas variedades comerciales de almendros.

El inóculo primario de *P. amygdalinum* se encuentra disponible en periodos prolongados en la etapa de crecimiento del cultivo (de enero a agosto). El periodo de infectividad en las dos zonas estudiadas se extendió desde marzo hasta mediados de junio, cuando las temperaturas se situaron entre los 10 y 20°C. El periodo de incubación fue variable en los años estudiados (de 2 a 12 semanas), pero en general disminuyó conforme transcurría el año. Para la detección y cuantificación de *P. amygdalinum* se diseñaron un par de cebadores específicos en la región ITS del ADN ribosomal del hongo, los cuales demostraron ser altamente sensibles y específicos. Ello permitió detectar el patógeno con bastante exactitud y fiabilidad. Los límites de detección se establecieron en 12 pg de ADN de *P. amygdalinum* y siete ascosporas en suspensiones de esporas preparadas artificialmente. El desarrollo de un protocolo basado en qPCR permitió cuantificar las ascosporas dispuestas en cintas de plástico, utilizadas habitualmente en los captadores de aire volumétricos, así como la colonización del hongo en hojas infectadas. En esta tesis doctoral también se exploraron los mecanismos de defensa del almendro en relación con la susceptibilidad del cultivo a la enfermedad, identificando algunas de las estrategias físicas y químicas implicadas en la tolerancia del almendro a *P. amygdalinum*. De esta manera, se ha estudiado la regulación de siete genes relacionados con la defensa de la planta contra la enfermedad, así como la deposición de lignina en dos variedades de almendro (‘Mardía’, tolerante y ‘Tarraco’, susceptible) con una respuesta claramente diferencial a la RLB. ‘Mardía’ mostró una expresión relativa mayor de los genes *CAD* y *DFN1* en las primeras etapas de la aparición de síntomas de la RLB, y con una mayor disposición de lignina en tejidos colonizados por

el hongo. Por otro lado, 'Tarraco' expresó principalmente los genes *HQT* y *LDOX*, relacionados con la biosíntesis del ácido clorogénico y las antocianinas, respectivamente, mientras que la deposición de lignina no fue apreciada con claridad.

Nuestros resultados abren la puerta para investigaciones futuras centradas en el desarrollo de un modelo epidemiológico preciso para predecir periodos de riesgo de infección de la RLB, integrando el seguimiento del inóculo primario mediante captadores volumétricos de aire y el método de detección del patógeno basado en la qPCR que se ha desarrollado en esta tesis doctoral. Además, la mejora del conocimiento sobre las bases genéticas y moleculares de la resistencia/tolerancia del almendro a la RLB ayudarán a la obtención de nuevas variedades de almendro con mejores perfiles de tolerancia a la RLB. De esta manera, se ponen las bases para hacer frente a la mancha ocre del almendro de una manera integral y sostenible.

Resum

En aquesta tesi doctoral s'han estudiat diversos aspectes biològics importants sobre la malaltia de la taca ocre de l'ametller (RLB, en la seva abreviatura en anglès), causades per l'ascomicet *Polystigma amygdalinum*. Aquesta malaltia és una de les malalties foliars més importants de la majoria de les regions de la conca mediterrània i de l'Orient Mitjà on es cultiva l'ametller, ja que les infeccions greus poden causar la defoliació prematura de l'arbre. Els aspectes estudiats han estat relacionats amb: i) l'epidemiologia de *P. amygdalinum* en dues regions del cultiu de l'ametller a Espanya; ii) la detecció i quantificació fiables del patogen mitjançant PCR quantitativa en temps real (qPCR), a més de iii) l'estudi de les bases molecular i bioquímica de la susceptibilitat diferencial a la RLB de diferents varietats comercials d'ametller.

L'inòcul primari de *P. amygdalinum* es trobava disponible en períodes prolongats de l'època de creixement del cultiu (de gener a agost). El període d'infectivitat a les dues zones estudiades es va estendre de març a mitjan juny, quan les temperatures se situen entre 10 i 20 °C. El període d'incubació va ser variable en els anys estudiats (de 2 a 12 setmanes), però en general va anar disminuint en el transcurs de l'any. Per a la detecció i la quantificació de *P. amygdalinum* es va dissenyar un parell d'encebadors en la regió ITS de l'ADN ribosòmic del fong, i es va demostrar que aquests eren altament específics i sensibles, cosa que permet detectar el patogen amb prou exactitud i fiabilitat. Els límits de detecció es van establir en 12 pg d'ADN de *P. amygdalinum* i set ascòspores en suspensions d'espores preparades artificialment. El desenvolupament d'un protocol basat en qPCR va permetre quantificar les quantitats d'ascospores disposades en cintes de plàstic com les que s'utilitzen habitualment en els mostrejadors d'aire volumètrics, així com també la colonització del fong a fulles infectades. En aquesta tesi també hem explorat els mecanismes de defensa de l'ametller en relació amb la susceptibilitat del cultiu a la malaltia, tot identificant algunes estratègies físiques i químiques implicades en la tolerància de l'ametller a *P. amygdalinum*. D'aquesta forma, s'ha estudiat la regulació de set gens relacionats amb la defensa de la planta enfront de la malaltia, així com la deposició de lignina en dos cultivars d'ametlla ('Mardía', tolerant, i 'Tarraco', susceptible) amb una resposta altament diferencial a la RLB. 'Mardía' va mostrar una activació relativa major dels gens *CAD* i *DFN1* en les primeres etapes de l'expressió de símptomes de la RLB, amb una major deposició de lignina als teixits colonitzats pel fong. Per contra, 'Tarraco' va desencadenar principalment l'activació dels gens *HQT* i *LDOX*,

relacionats amb les vies de la biosíntesi de l'àcid clorogènic i les antocianines, mentre que la deposició de lignina no es va apreciar clarament.

Els nostres resultats obren la porta a investigacions futures centrades en el desenvolupament d'un model epidemiològic precís per a predir esdeveniments de risc d'infecció per a la RLB, tot integrant el seguiment de l'inòcul primari amb l'ajut de captadors volumètrics d'aire i el mètode de detecció del patogen basat en qPCR que s'ha desenvolupat en aquesta tesi. A més, augmentar el coneixement sobre les bases genètiques i moleculars de la resistència/tolerància de l'ametller a la RLB ajudarà de ben segur a l'obtenció de noves varietats d'ametller amb perfils de tolerància a la RLB millorats. D'aquesta manera, es posen les bases pel control de la taca ocre de l'ametller d'una forma integrada i sostenible.

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Chapter 1

Chapter 1: General introduction

1.1 The almond

The almond (*Prunus dulcis* (Mill.) D.A. Webb) is a deciduous tree belonging to the subfamily Prunoideae of the family Rosaceae, one of the largest families of flowering plants with about 3,400 species (Muzzaffar *et al.* 2018). The genus *Prunus* includes important economic tree crops such as apricots (*Prunus armeniaca* L.), cherries (*Prunus avium* L.), European and Japanese plums (*P. domestica* L. and *P. salicina* Lindl., respectively) and peaches (*Prunus persica* (L.) Batsch) (Lee and Wen, 2001). The almond tree grows 4–9 m tall, in early spring produces white or pale pink flowers (3–5 cm diameter) of five petals, and then grows lanceolate leaves, 6–12 cm long and serrated edges. Almond tree is classified together with peach in the subgenus *Amygdalus*, which is differentiated from other subgenera by the corrugated seed shell (Badenes *et al.* 1995). Horticulturally, almonds are classified as a nut in which the edible seed (the kernel) is the commercial product. The fruit is classified as a drupe. It consists of an exocarp (skin), mesocarp (hull), and the shell, which is a hardened endocarp. Almonds are diploid ($2n = 2x = 16$) and this species is one of the most polymorphic fruit tree species (Kester *et al.*, 1991; Socias i Company and Felipe, 1992). This high polymorphism may be due to its predominantly self-incompatibility (Socias i Company, 1998). This self-incompatibility is controlled by a single locus with multiple codominant alleles in the gametophyte (Socias i Company and Felipe 1988; Dicenta and García 1993). However, self-compatible almond cultivars were reported in Puglia region in Italy, which has made self-compatibility one of the main objectives for almond breeding programs in Europe and the USA (Grasselly *et al.* 1981; Vargas *et al.* 1984; Socias i Company and Felipe 1988).

Several *Prunus* species (*P. fenzliana* (Fritsch) Lipsky, *P. bucharica* Korschinsky and *P. kuramica* Korschinsky) have been described as the wild species most closely related to almond (Grasselly 1976b; Browick and Zohary 1996). However, Ladizinsky (1999) accepted only *P. fenzliana* as the only wild ancestor of comercial almond. Additionally, the species *P. webbii* (Spach) Vieh, which is thought to be originated from the Balkan region, has also been described as closely related to cultivated almond (Grasselly 1976a,b; Browick and Zohary 1996).

1.2 Almond crop

1.2.1 Almond crop in the world

The origin of almond is found in central Asia, and this species was subsequently spread toward the Mediterranean Basin, Southwest and Middle Asia, by following ancient trade routes. Almond has been more recently introduced into areas with Mediterranean-type climates, such as California, Chile and Australia (Grasselly, 1976a; López *et al.*, 2006). Almond production was initially concentrated in specific areas, as almond tree is drought-resistant and frost-sensitive. Breeding new, adapted land races has increased the world areas growing almonds (Grasselly and Crossa-Raynaud, 1980).

Almonds are considered one of the most important world tree crops in the nut market, mostly for the following reasons: its good taste, positive health attributes and versatility to be included into new products, mainly in food and cosmetics industries. Almonds are consumed raw, toasted, fried or as an important ingredient in different foodstuffs (Cortés *et al.*, 2018). Currently, two main types of almonds are grown: bitter almonds, mainly managed for oil production, and sweet almonds. Sweet almond is the most consumed type due to its multiple uses, which include both food and cosmetics (Socias i Company and Gradziel 2017).

According to FAO (Food and Agricultural Organization of the United Nations) data, 1,717,328 ha were cultivated with almonds in 2010 worldwide (FAOSTAT, 2019). Only in seven years, the increase in the harvested area was of 208,559 ha approximately, thus highlighting the boom in the almond cultivation around the world. This trend is also reflected in the total production; in 2010, the world production was 1,899,884 tons (t) of nuts with shell, which raised up to 2,239,697 t in 2017 (Figure 1.1). In recent years, expectations of almond crop expansion are optimistic, as an increasing consequence of global nuts consumption, valued at 7 billion USD per year (Henselek, *et al.*, 2018). Moreover, if the consumption trend continues by increasing, production might not be enough to satisfy the demand in a few years (ABA, 2016).

In 2017, Spain was the largest almond-growing area in the world, with 633,562 ha, followed by USA and Tunisia. Nonetheless, USA was the largest producer of almond worldwide with a total production of 1,029,655 t, which represents about 70% of world production. Spain was the second largest producer with 255,503 t, followed by Morocco with 116,923 t.

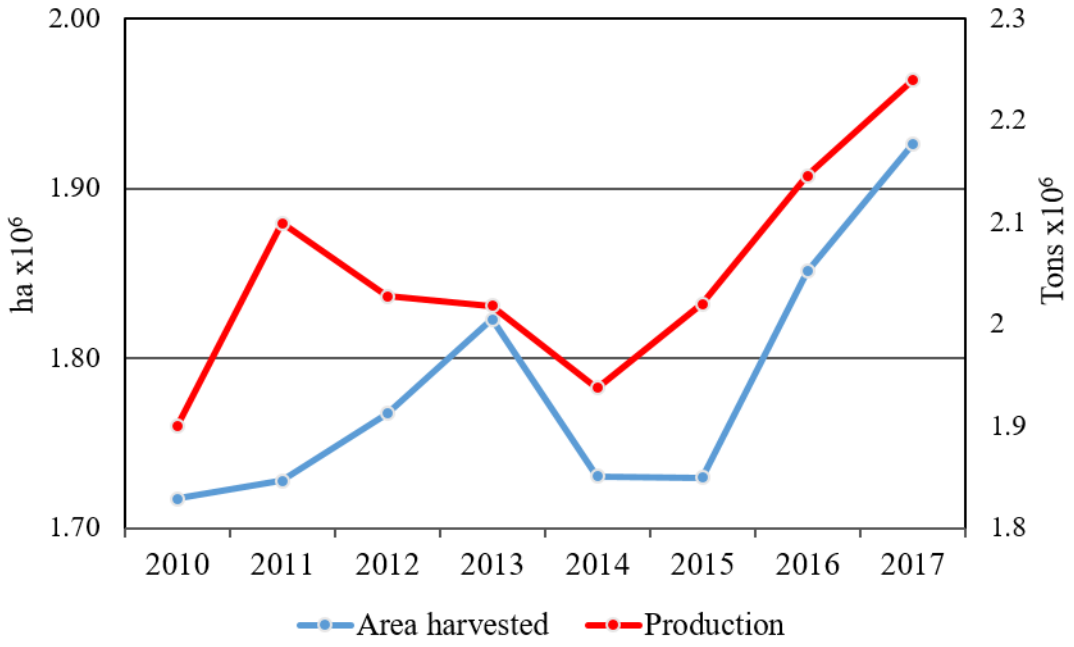

Figure 1.1 Total production and harvested area of almonds (with shell) in the World in 2010 – 2017. (FAOSTAT, 2019).

According to FAO data, the average yield in Spain was 403 kg ha^{-1} in 2017, clearly below the average yield of countries such as Australia (1,983 kg ha^{-1}), Chile (4,071 kg ha^{-1}), Turkey (2,643 kg ha^{-1}) and USA (2,544 kg ha^{-1}), which had best average yields worldwide (FAOSTAT, 2019). These remarkable differences lie mostly in the crop conditions found in these countries (Miarnau *et al.*, 2010). Most almond orchards in USA, Chile and Australia are established in fertile/irrigated soils, whereas in 2010, the 93% of Spanish plantations were found in poor soils under rain-fed conditions, which represent the 7% of the irrigated orchards. In 2016, the percentage of Spanish plantations in poor soils under rain-fed conditions decreased 6 % and the irrigated orchards increase to 13% of the total harvested area (MAPA, 2017), thus indicating that almond orchards in Spain are going through substantial changes.

Figure 1.2 Principal almond-growing regions in Spain, in yellow (MAPA, 2017).

1.2.2 Almond crop in Spain

Almond plays an important social and economic role in Spanish agriculture, as its cropping has been traditionally located in poor, dry areas of Central, Southern and Eastern Spain (Figure 1.2). In recent years, growers are increasingly turning the traditional management of the crop into new plantations with better pruning and fertilizer techniques along with the optimization of irrigation. These innovative cropping practices are applied in new, high-density and productive plantations (Figure 1.3B). The largest almond-growing areas under these intensive cropping conditions are located in Andalusia, Castilla - La Mancha, Comunidad Valenciana, Aragon and Catalonia (Figure 1.2, Table 1.1).

For decades, the most commonly used cultivars in Spain were 'Desmayo Largueta' and 'Marcona'. These cultivars are characterized by their early flowering and self-incompatibility characteristics, together with a greater sensibility to frost and diseases, which have led to reduced almond yields for decades (Miarnau *et al.*, 2010). These characters were also common among other traditional European varieties, which led to start new almond-breeding programs in 1970-1990 in Europe.

Table 1.1 Area and production of almond crop in Spain, 2017 (MAPA (2018).

Region	Harvested area (ha)			Yield (kg ha^{-1})		Production (t)
	Rainfed	Irrigated	Total	Rainfed	Irrigated	
Andalucía	168,905	22,737	191,642	295	2,800	77,525
Aragón	60,295	12,167	72,462	525	2,252	51,479
Baleares	23,822	210	24,032	280	810	5,607
C. Valenciana	78,959	12,332	91,291	362	1,505	42,853
Canarias	149	13	162	201	1,000	242
Cantabria	–	–	–	–	–	–
Castilla y León	1,646	299	1,945	401	1,316	572
C. La Mancha	96,671	17,838	114,509	199	705	20,271
Cataluña	31,060	7,931	38,991	361	962	17,635
Extremadura	4,300	3,638	7,938	598	2,790	3,074
Galicia	–	–	–	–	–	–
La Rioja	8,890	761	9,651	352	1,193	2,631
Madrid	840	–	840	830	–	502
Navarra	1,998	1,663	3,661	204	2,014	3,007
P. de Asturias	–	–	–	–	–	–
País Vasco	75	–	75	850	–	115
R. de Murcia	69,463	6,900	76,363	317	1,510	29,990

These programs focused on two main characters for selection: late flowering and self-compatibility (Socias i Company and Felipe, 2006; Dicenta *et al.*, 2008; Socias i Company *et al.*, 2008), to provide new cultivars with frost resistance and a better production performance, respectively. A single *S* gene controls self-compatibility with multiple codominant alleles (Socias i Company and Felipe 1988; Dicenta and García 1993; López *et al.*, 2006). Crossa-Raynaud and Grasselly (1985) firstly proposed the existence of six different self-incompatibility alleles (S_1, S_2, S_3, S_4, S_7 and S_8) and the self-compatible allele S_f in various European cultivars. Currently, twenty-six *S* alleles of self-incompatibility and eighteen cross-incompatibility groups, formed by cultivars having the same self-incompatibility genotypes (Groups I to XVII and Group O of cultivars with unique genotypes) have been identified (López *et al.*, 2006).

In recent years, it has been possible to obtain a considerable number of new cultivars incorporating those desired characters such as late flowering and self-compatibility. For

Figure 1.3 Almond orchards in Les Borges Blanques, Spain: A) Traditional almond orchard in **r**ain-fed conditions; B) high-density almond orchard under irrigation. Photos by Xavier Miarnau, 2018.

instance, 'Ferraduel', 'Ferragnès' and 'Lauranne' have been obtained by INRA (Institut National de la Recherche Agronomique) in France. In Spain, 'Soleta' and 'Mardía' were obtained by CITA (Centro de Investigación y Tecnología Agroalimentaria, Aragón), whereas 'Penta' and 'Tardona' by CEBAS-CSIC (Centro de Edafología y Biología Aplicada del Segura – Consejo Superior de Investigaciones Científicas, Murcia) and 'Constantí', 'Marinada', 'Tarraco' and 'Vairo' by IRTA (Institut de Recerca i Tecnologia Agroalimentàries, Catalonia) (Batlle *et al.* 2017). With these new cultivars and a better integral crop management, yields can exceed 2,000 kg ha^{-1} of nuts with shell in some cases (Miarnau *et al.*, 2017).

1.3 Almond diseases

Almond is able to face different abiotic stresses, being low fertility soils and water scarcity two of the most frequently found (Oliveira *et al.*, 2018). However, integration of new

cultivars and changes in crop management (intensive cropping), along with global climate change, might lead to the occurrence of new or reemerging biotic stresses, without the required information to control them in an effective way. Almond pathogens involved in biotic stresses are numerous and generally grouped according to the symptoms they cause (root rots, wilts, leaf spots, blights, rusts, smuts) and the plant part being affected (Agrios, 2005). Some of the most important fungal pathogens of almond are summarized below.

1.3.1 Almond root diseases

Poor fertilizer management, in addition to clay soils where waterlogging is common, can favor plant root diseases. Root diseases caused by fungi may represent an important threat for almond growers, especially where diseases are well established in the orchards.

1.3.1.1 Armillaria mellea

Armillaria mellea (Vahl) P. Kumm infects almond roots producing characteristic white-yellowish mycelial mats under the bark, and dark rhizomorphs on the roots. Affected trees usually show symptoms of decline, including crown dieback and early defoliation of small and discolored leaves (Lushaj *et al.*, 2010). New infections are originated by contact of infected roots with the healthy rhizosphere. Furthermore, *A. mellea* can survives on dead roots for several years (Tsopelas, 1999).

1.3.1.2 Phytophthora *species*

Phytophthora affects a large range of plants including almond, that first show symptoms of drought and starvation, causing the plant or tree to grow poorly, produce fewer and smaller fruits, sparse foliage, and twigs dieback. Affected plants then quickly become weakened and susceptible to be attacked by other pathogens that finally kill the whole tree. In older trees, the killing of roots may be slow or rapid, depending on the amount of *Phytophthora* inoculum present in the soil and the prevailing environmental conditions (Agrios, 2005). About 15 *Phytophthora* species are known to occur on almond worldwide (Farr and Rossman, 2019). Most important *Phytophthora* taxa on almond are *P. cactorum* (Lebert and Cohn) J. Schröt. *P. cambivora* (Petri) Buisman, *P. citrophthora* (R.E. Sm. & E.H. Sm.) Leonian, *P. cryptogea* Pethybr. & Laff., and *P. megasperma* Drechsler. Recently, a new species, *P. niederhauserii* Z.G. Abad & J.A. Abad, has been described from fruit tree nurseries and orchards in Spain (Pérez-Sierra *et al.*, 2010; Abad *et al.*, 2014). In general,

Phytophthora species are able to survive in warm or cold winters and dry summers in infected roots or stems, and may also survive in the soil and infect susceptible hosts when both organisms come in contact. More mycelium is produced during wet, cool weather and spread the disease more effectively (Browne and Viveros, 1998).

1.3.1.3 Verticillium dahliae

Verticillium dahliae Kleb. causes defoliation, gradual wilting and death of successive branches, or even an abrupt collapse and death of the entire plant. Trees infected with *Verticillium* are usually stunted and their vascular tissues show a characteristic brownish discoloration (Agrios, 2005). When *Verticillium* wilt first appears in a field, symptoms are mild and local which will grow in strength as more virulent strains of the fungus appear over a few years. The attacks become successively more severe and widespread until the crop has to be discontinued or replaced. *Verticillium dahliae* overwinters in the soil as microsclerotia, which can survive up to 15 years (Wilhelm, 1955; Devay *et al.*, 1974; Krikun *et al.*, 1990).

1.3.2 Almond diseases: aerial part

The fungal diseases of the aerial part of the almond tree are very diverse and may affect more than one structure such as buds, branches, leaves, flowers or fruits.

1.3.2.1 Diaporthe amygdali

Diaporthe amygdali (Delacr.) Udayanga, P.W. Crous & K.D. Hyde causes characteristic brown oval cankers on branches which extend near the buds. When the canker rounds the branch, this dries up and wilts. This pathogen may also produce a toxin –fusicoccin– which stimulates a quick acidification of the plant cell wall, causing an irreversible stomatal closure and a subsequent leaf wilting (Ballio *et al.*, 1964). Infections may occur during rain, high humidity and dew conditions, which causes a water-dripping that drags the spores from one shoot to another and concentrate damage in the lower parts of the tree canopy (Agrios, 2005).

1.3.2.2 Monilinia laxa

Monilinia laxa (Aderh. & Ruhland) Honey can affect the entire aerial parts of almond. First noticeable infections usually occur on flowers, causing their wilting, and subsequent infections expand to fruits, buds, and branches. The fruits turn black and remain mummified on the branches, where *M. laxa* overwinters. In acute lesions, such as cankers on branches, infections can block the normal vascular flow thus inducing the dead of the whole branch.

1.3.2.3 Taphrina deformans

Taphrina deformans (Berk.) Tul. is the causal agent of the leaf curl disease in members of the *Prunus* family. It causes leaf, flower, and fruit deformations (Agrios, 2005). The initial symptoms of *T. deformans* consist of a yellow discolored blotch on leaves, which becomes purplish and hypertrophied with age, usually resulting in an early defoliation of the tree (Pscheidt, 1995). The pathogen overwinters on buds, which promotes new infections in spring through the contact of infected buds with young leaves (Tavares *et al.*, 2004). Longer periods of humidity and cold weather during bud opening produce serious outbreaks (Fitzpatrick, 1934; Mix, 1935), while accumulative defoliations for several seasons can lead to the death of the whole tree (Pscheidt, 1995)

1.3.2.4 Wilsonomyces carpophilus

Wilsonomyces carpophilus (Lév.) Adask., J.M. Ogawa & E.E. Butler, the causal agent of the "shot hole" disease, is responsible for causing small yellow discoloration patches on the almond leaves, which later evolve to necrotic tissues that detach from the leaf, leaving a hole. Shot-hole symptoms can also be seen on fruits as little necrotic blotches, and on branches as small cankers with rubber exudation. *Wilsonomyces carpophilus* overwinters on infected buds or cankers, and the spores may be disseminated by rain and wind.

1.3.2.5 Polystigma amygdalinum

The causal agent of the red leaf blotch (RLB) of almond is the ascomycete *Polystigma amygdalinum* P.F. Cannon. In recent years, it has been increasingly considered as one of the major foliar diseases of this fruit tree species (Tuset and Portilla, 1987; Saad and Masannat, 1997). The RLB reduces plant photosynthetic functioning and may cause the early defoliation of the tree (Cannon, 1996; Shabi 1997), thus committing fruit production in following years (Miarnau *et al.*, 2010; López-López *et al.*, 2016). An expanded description of this disease and its causal agent is given below.

1.4 The red leaf blotch

1.4.1 Geographic distribution

The RLB is widely distributed in the Mediterranean basin and the Middle East (Cannon, 1996); it has been detected in numerous countries: Cyprus, Greece, Iran, Israel, Italy, Lebanon, Libya, Morocco, Portugal, Romania, Spain, and Turkey (Cannon, 1996; Farr and

Rossman, 2019). No occurrences have been reported from other almond-growing areas in the world. First citation of the RLB disease was in the early 1970 in Shiraz, Iran, according to Ashkan and Assadi (1974). In Spain, the RLB occurs in all areas where almonds are grown, from the South to the Ebro valley (Egea *et al.*, 1984; Tuset and Portilla, 1987). However, it has a greater presence in places with a continental weather, away from the coastal line.

1.4.2 The pathogen

The genus *Polystigma* DC (*Phyllachoraceae*) includes fungi which occur on living leaves of *Prunus* species and are characterized by its brightly colored stromata, according to Cannon (1996). This character differentiates *Polystigma* from other genera in the family –*Isothea* Fr. and *Phyllachora* Nitschke ex Fuckel– as stromata in the later genera are usually black. However, Habibi *et al.* (2015) suggested that *P. amygdalinum* should be better accommodated in the *Xylariamycetidae* Subclass of Xylariales, based on phylogenetic analyses. *Polystigma* includes about five species, which can be identified from several morphological features of stromata and reproductive structures. Stroma of *P. amygdalinum* is irregularly shaped, yellow at first, to become orange and finally darken in the central area (Cannon, 1996). *Polystigma amygdalinum* only occurs on living almond leaves, and it is therefore considered a biotrophic pathogen. It is unable to grown on artificial media and needs living plant tissue for the completion of its sexual cycle and spore formation (Perfect

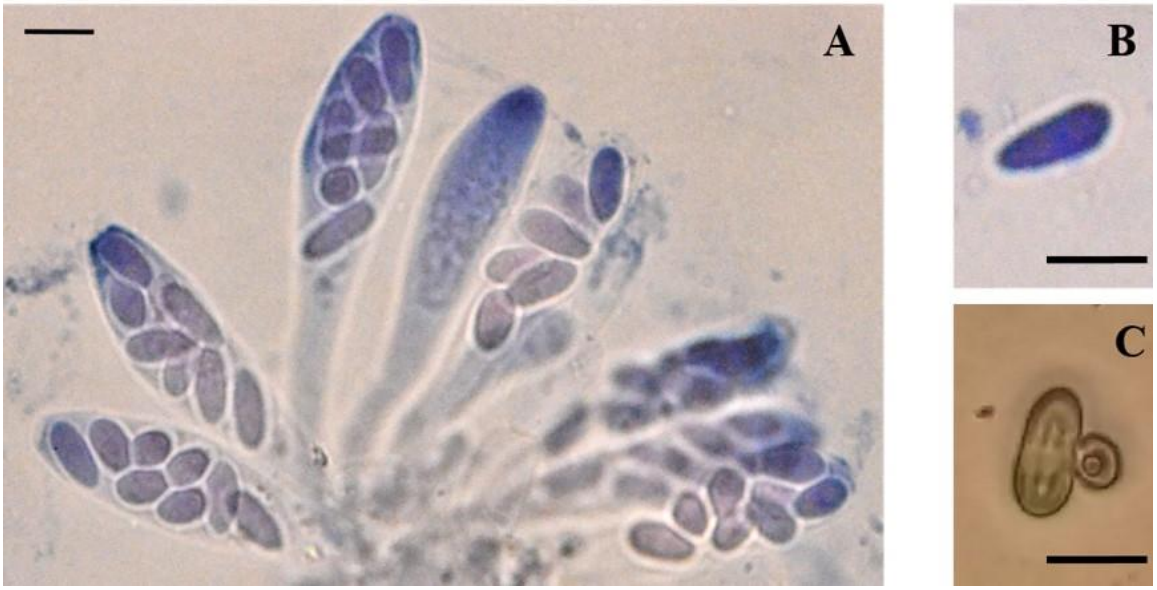

Figure 1.4 *Polystigma amygdalinum*: A) asci and ascospores; B) ascospore; C) germinating ascospore. A and B stained with Trypan Blue, C on PDA. (bar = 10 µm). Photos by Erick Zúñiga.

and Green, 2001). During the first stages of the stromata development, the anamorph stage of *P. amygdalinum* (*Polystigmina* Sacc.) begins with the formation of internal conidiomata, globose pycnidia (80–120 µm), and the production of hyaline curved filiform conidia (22–34 × 0.75–1 µm). *Polystigma amygdalinum* perennates in winter on leaf litter, where the sexual fruiting bodies (perithecia) develop. Asci are clavate, short-stalked, very thin-walled, the apex obtuse to truncate, with an apical ring (74–97 × 18–23 µm), 8-spored (Figure 1.4A). Ascospores are hyaline, aseptate, smooth, without a gelatinous sheath, arranged biseriately, ± cylindrical, one end rounded and the other obtuse to acute, usually slightly constricted in the central region and thin-walled (13–16.5 × 5.5–6.5 µm) (Figure 1.4B).

1.4.3 Life cycle of Polystigma amygdalinum

In spring, *P. amygdalinum* infects young leaves through ascospores released from past-year leaf litter. The first leaf symptoms appear after an incubation period lasting 30–35 days (Banihashemi, 1990; Cannon, 1996; Suzuki *et al.*, 2008), and consisting of irregular yellow blotches (approx. 1-2 cm diam.) which expand and became orange-brown. This stroma became reddish and dark purplish with age, and an occasional leaf curling can be noticeable at this stage (Figure 1.5).

Figure 1.5 Development of red leaf blotch symptoms on almond leaves. From left to right: 1) asymptomatic leaf; 2) initial yellowish discoloration; 3) orange-reddish blotch; 4) initial necrosis of stroma; 5) expanded dark necrosis in a well-developed stroma. Photos by Erick Zúñiga.

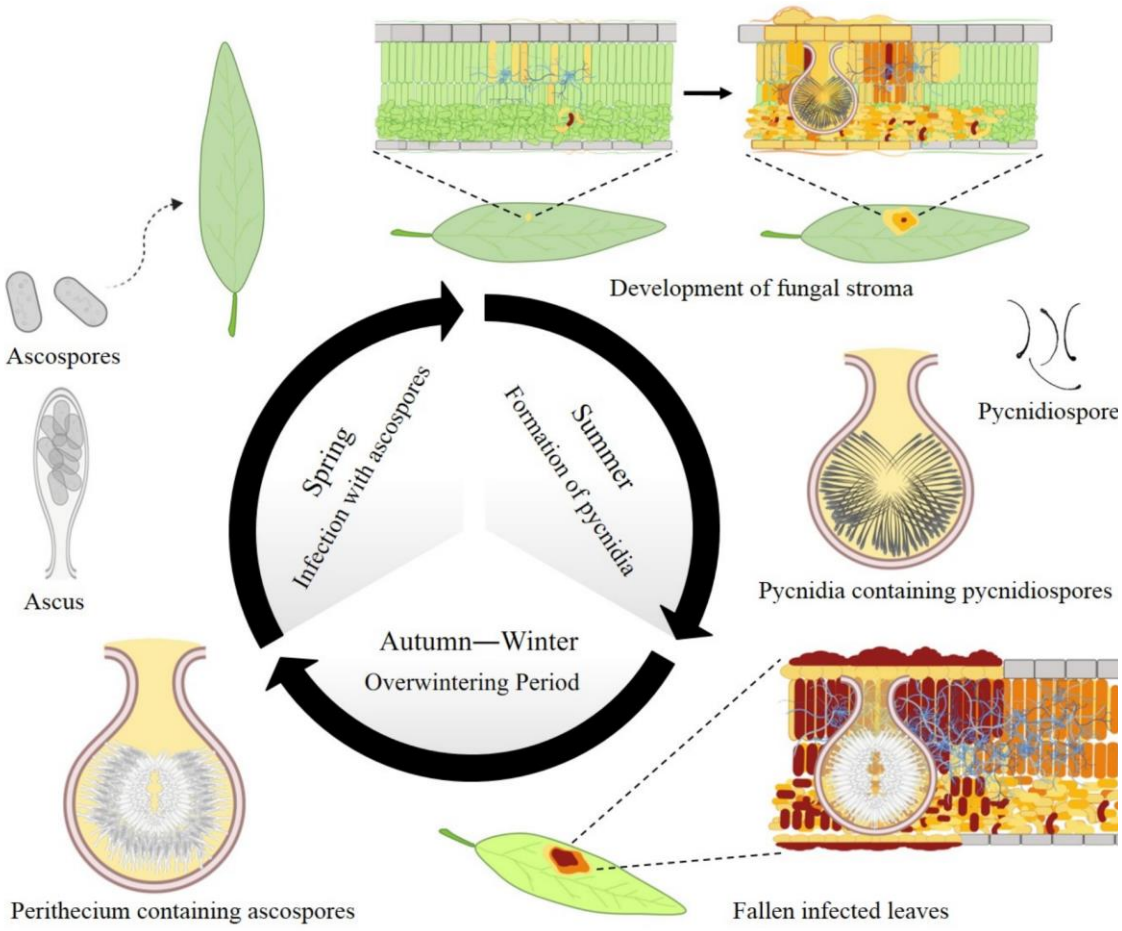

Figure 1.6 Schematic life cycle of *Polystigma amygdalinum* on *Prunus dulcis* leaves assumed by field observations. From: Erick Zúñiga, modified from Suzuki *et al.* (2008).

In summer, the stromatic tissue contains mainly the pycnidial stage. Pycnidiospores are not able to germinate on synthetic culture media and fail to cause secondary infections (Ashkan and Assadi, 1974; Banihashemi, 1990; Saad and Masannat, 1997). The symptomatic leaves fall at the end of summer, earlier than usual and overwinter in the ground in order to produce mature perithecia, ascus and ascospores (Figure 1.6).

The life cycle continues with a new ascospore release in spring and the beginning of new season infections (Banihashemi, 1990; Cannon, 1996; Suzuki *et al.*, 2008). A rapid diagnosis method of RLB foliar symptoms based on analyses of High-Resolution Hyperspectral and Thermal imagery has been developed (López-López *et al.*, 2016).

Banihashemi (1990) reported that, ascospore discharge began at flowering and lasts for six weeks until fruit set, in Iran conditions (late April to early May). In Spain, Lovera *et al.* (2014) observed that the ascospore discharge period was from January to June. There have

been several attempts to grow *P. amygdalinum* it in culture media. The pycnidiospores are neither able to germinate in culture media nor infect *in vivo* leaves, so it is suggested that they are actually spermatogonia, i.e. spores involved in the development of the posterior sexual stage (Banihashemi, 1990; Saad and Masannat, 1997). Ascospores are able to germinate and developed appressorium in culture media (Figure 1.4C). However, they fail to grow other structures (Habibi and Banihashemi, 2015).

1.4.4 Cultivar susceptibility

Egea *et al.* (1984) reported an evaluation of RLB susceptibility among 81 almond cultivars. They classified some traditional Spanish cultivars as high-susceptible to *P. amygdalinum*, such as 'Desmayo Largueta' and 'Marcona'. In Catalonia, Vargas *et al.* (2010) observed remarkably susceptibility differences between cultivars, with 'Guara' classified as high-susceptible whereas 'Vayro' as a high RLB-tolerant. In recent years, additional field evaluations have been integrating newly generated cultivars; visual observations confirmed 'Guara' and 'Tarraco' as high susceptible whereas 'Vayro' and 'Mardía' are highly tolerant (Vargas *et al.* 2011; Marimon *et al.* 2012; Ollero *et al.* 2016a, 2019). In general, late-blooming cultivars are generally more susceptible (Palacio-Bielsa *et al.*, 2017). According to these authors, 'Guara' is highly susceptible; 'Tarraco' 'Francoli', 'Marinada' and 'Masbovera' are less susceptible; whereas 'Constanti', 'Desmayo Largueta', 'Ferraduel', 'Ferragnés', 'Marcona', 'Marinada' and 'Vayro' are variable in their susceptibility. All these susceptibility classifications are based on RLB symptom assessments done in the field. To date, no research has been done on the study of the genetic bases of the RLB susceptibility.

1.4.5 Control management of the red leaf blotch

Little research has been done on the control management of the RLB with fungicides. Banihashemi (1990) evaluated several systemic and non-systemic fungicides (Triforine, Benomyl, Carbendazim, Thiophanate methyl, Bordeaux mixture, Copper hydroxide, Copper oxychloride and Mancozeb), in spray applications from flowering until fruit set. He observed that Triforine at lowest concentrations of 100 mg ml^{-1} was able to reduce infections significantly. Furthermore, applications after flowering were more effective than those made during flowering. The other fungicides were either less effective (Mancozeb), non-effective

(Benomyl), or even increased the level of infection (Carbendazim and Thiophanate methyl). Recently, Almacellas and Marín (2011) suggested application of other fungicides, such as Captan and Thiram, but the authors reported no experimental data. In Spain, only Mancozeb 75% [WG] was allowed by the MAPA (Ministerio de Agricultura, Pesca y Alimentación) in 2018 to control RLB. Mancozeb, a mixture of [[1,2-ethanediylbis [carbamodithioate]] (2-)] manganese with [[1,2-ethanediylbis [carbamodithioate]] (2-)] zinc, belongs to the dithiocarbamates group of fungicides, and specifically to the class of ethylene bisdithiocarbamates (EBDCs). Mancozeb is a multi-site fungicide listed under FRAC (Fungicide Resistance Classification) code list as M3. The direct effect of Mancozeb upon core biochemical processes within the fungus results in inhibition of spore germination (Gullino *et al.*, 2010). However, Lovera *et al.* (2014) pointed that conventional fungicide treatments (before and during flowering) does not control the disease in an effective way. To date, it is still unclear which is the best fungicide for the control of RLB.

Alternatively, a traditional control management of the RLB may be partially reached by 1) burning leaf litter, and 2) applying urea to promote leaf litter decomposition (Lin and Szteinberg, 1992). However, an effective control of RLB can only be reached through an integrated strategy, which should include a detailed knowledge from multiple subjects: i) the life cycle and epidemiology of *P. amygdalinum*, ii) the cultivar susceptibility and genetics of resistance sources, iii) the selection of best fungicides against RLB, and iv) efficient infection risk models to aid in decision support systems.

Chapter 2

Chapter 2: Objectives

This PhD thesis aimed to study some critical aspects of the biology of the red leaf blotch disease of almond, related to the epidemiology, the identification and quantification of its causal agent, as well as the search of plant defense mechanisms involved in cultivar susceptibility. Thus, the following expanded specific objectives were proposed:

- To study the epidemiology of the red leaf blotch of almond, with a special interest in questions related to the primary inoculum development, and the infectivity, incubation and dispersion periods.
- To develop a qPCR-based method for the detection and quantification of *Polystigma amygdalinum* in biological samples.
- To identify relevant molecular and biochemical strategies in cultivars with differential susceptibility to the red leaf blotch.

Chapter 3

Chapter 3: Inoculum and infection dynamics of *Polystigma amygdalinum* in almond orchards in Spain

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3.1 Abstract

Red leaf blotch disease of almond, caused by *Polystigma amygdalinum*, is an important foliar disease in most production regions of the Mediterranean basin and the Middle East, since severe infections may cause a premature defoliation of the tree. Some key aspects on the epidemiology of *P. amygdalinum* were studied in multi-year trials in two almond-growing regions in Spain, which included the seasonal development of perithecia, production and germination of ascospores, along with the disease incubation and plant infectivity periods. Our results showed that primary inoculum was available in extended periods (January to August), with significant differences in ascospore amounts among regions, higher in the southern Andalusia and lower in the northern Catalonia, and years of study. Furthermore, the geographical site, the sampling period and the evaluation year were found significant on the development of *P. amygdalinum* perithecia. Variable ascospore germination rates were observed from April to July, over 15 % but rarely exceeding 30 %. The infectivity period in Catalonia extended from March to mid-June, while in Andalusia from March to May. The incubation period was in a range of 2 to 12 weeks in Catalonia but decreased with time in the season. The environmental conditions of October-January influence the available ascospore amounts in the next season. RLB infection occurs in spring to summer, when temperatures are in the range 10 to 20°C. These results represent the first step in developing a predictive model of the disease that might serve as a tool for the control of the RLB.

3.2 Introduction

Almond (*Prunus dulcis* (Mill.) D.A. Webb) is a traditional and widespread crop in the Mediterranean basin and the Middle East. It is generally considered a marginal crop in this area that is currently grown in dry land under limiting soil and climate conditions, which leads to low yields. Spain is the leading world country in relation to the cultivated area of almonds, with more than 630,000 ha in 2017 (FAOSTAT, 2019; MAPA, 2019). However, the average yield of almonds with shield is slightly over 400 kg ha^{-1}, much lower than yields obtained in the USA, the leading producer country in the world, with an average production of 2,544 kg ha^{-1} (FAOSTAT, 2019). In recent years, the general increase in world nut consumption and price of nuts, and especially of almond, has led to the expansion of a more intensive almond cropping in Spain (Miarnau *et al.*, 2010; 2018). This intensive cropping is characterized by the use of new almond cultivars planted in higher tree density, and supported with proper irrigation, fertilization and pesticide programs to reach higher yields (Vargas *et al.*, 2010; Miarnau *et al.*, 2018). The crop yield in these intensive conditions is high in Spain, about 2,500 kg ha^{-1} in some regions (MAPA, 2019), comparable to that obtained in the USA. However, there is a great concern about the possible increase in the incidence of almond diseases, which have become the main limiting factor of these new plantations (Ollero-Lara *et al.*, 2016a, 2019; Torguet *et al.*, 2016).

Red leaf blotch (RLB) of almond, caused by the ascomycete *Polystigma amygdalinum* P.F. Cannon, is a foliar disease which is widely extended among most almond production regions of Europe and Asia (Cannon, 1996; Farr and Rossman, 2019), and is considered of high economic relevance in several countries (Cannon, 1996; Saad and Masannat, 1997). The disease is endemic in these regions and to date is not known in other almond-growing areas in the world, such as the USA or Australia (Farr and Rossman, 2019). Although RLB is well-known in Spain since old times (González-Fragoso, 1927), its incidence has markedly increased during the last years and is currently considered a re-emerging disease in the new intensive almond plantations (Almacellas, 2014; Ollero-Lara *et al.*, 2016a; Torguet *et al.*, 2016). It has been hypothesized that increased incidence of RLB could be related to the better growing conditions for almond cropping in the new intensive plantations, as well as the use of new late-flowering cultivars (especially 'Guara' in Spain), which are more susceptible to RLB than traditional ones (Ollero-Lara *et al.*, 2016b, 2019). First RLB symptoms appear as pale green to yellowish spots on both leaf sides in spring, turning into yellowish-orange and

later dark brown spots with age. Size of leaf spots increases through the spring and summer seasons and cover almost the whole leaf surface in late summer under favorable weather conditions. These spots are commonly associated with hypertrophy and deformation of leaves caused by the development of the fungal stroma on leaves. Occasionally, severe infections under hot and dry conditions can induce an early leaf fall in summer, thus reducing the photosynthetic activity of trees.

Polystigma amygdalinum is a biotrophic ascomycete, specific to almond, whose asexual state (conidial/spermogonial) belongs to the genus *Polystigmina* (Cannon, 1996). This pathogen was first described in 1845 in Italy as *Septoria rubra* var. *amygdalina,* being later reclassified within the genus *Polystigma* as *P. ochraceum* or *P. rubrum,* mainly due to the color of the fungal stroma in the leaves (Cannon, 1996). The species *P. amygdalinum* differs from other *Polystigma* species, which affect numerous species of the *Prunus* genus, due to its host specificity, stromal coloration and the morphology of fruiting bodies and spores (Cannon, 1996). Because its host specificity, the limited geographical distribution, and the inability to grow in artificial media, research on this fungus and other *Polystigma* species is scarce (Cannon, 1996). A recent phylogenetic study indicated that *P. amygdalinum* might not belong to the Phyllachorales, as had been considered to date, and should be better accommodated in the Xylariomycetidae subclass of Sordariomycetes, close to Xylariales and Trichosphaeriales (Habibi *et al.*, 2015).

The RLB disease is monocyclic and the only inoculum sources are the stromata of affected leaves which fall to the ground in autumn (Banihashemi, 1990; Ollero *et al.*, 2016b; Saad and Masannat, 1997). The sexual stage is developed in winter on the leaf stromata, and later in spring, ascospores are air-spread and can infect new almond leaves (Banihashemi, 1990). In Iran, Ghazanfari and Banihashemi (1976) reported on the influence of autumn-winter weather conditions in perithecia development. Based on these authors, the start of perithecia maturation occurs below 10°C. Banihashemi (1990) showed that ascospore release in Iran is related with rain periods, beginning at flowering and reaching the maximum at petals fall (late April to early May). In Lebanon, ascospore release can occur between February and mid-May (Saad and Masannat, 1997). However, the effect of weather conditions on the infection process is deeply unknown. After infection, leaf spots appear after a latent period of 30-35 days (Banihashemi, 1990; Cannon, 1996; Suzuki *et al.*, 2008). The occurrence of secondary cycles has not been confirmed (Ollero-Lara *et al.*, 2016b; Saad and Masannat,

1997; Shabi, 1997), since conidia do not have infective ability and their only function appears to act as spermatia in the sexual reproduction of the pathogen (Cannon, 1996; Habibi and Banihashemi, 2016).

As a monocyclic disease, the strategies for the management of RLB should be aimed at: 1) reducing the ascospores produced in the affected leaves fallen in autumn, i.e., to reduce inoculum potential, and 2) protecting new almond leaves that develop in spring. Based on several authors (Almacellas, 2014, Arquero *et al.*, 2013, Lin and Szteinberg, 1992), the control measures to achieve the first objective include: *i*) to remove or bury the leaves, *ii*) to favor their decomposition through urea applications and *iii*) to treat fallen leaves with fungicides. However, none of these measures have been evaluated for almond crop in Spain (Ollero-Lara *et al.*, 2016b). The most effective measure to protect the new leaves from RLB infections appears to be the application of fungicides (Almacellas and Marín, 2011; Arquero *et al.*, 2013; Bayt-Tork *et al.*, 2014). For all the above control measures to be effective, it is necessary to know the dynamics of the inoculum potential in fallen leaves, and the conditions influencing ascospore production, dispersal and infection. A decision support system considering the monocyclic pattern of the RLB epidemics might be a helpful tool to optimize fungicide applications.

The objective of this study was to characterize the dynamics of the production, maturation and potential dispersion of *P. amygdalinum* ascospores and correlate the key aspects of the disease with the environmental conditions in two Spanish almond-growing regions, namely Andalusia and Catalonia. These two areas are highly representative of the main almond growing areas in Spain (South and Ebro Valley, respectively).

3.3 Materials and methods

3.3.1 Geographic locations

Three experimental sites were located as follows: one location in Córdoba, Andalusia (S Spain; WGS84 coordinates: UTM 30S X = 341069, Y = 4190753), and two locations in Catalonia (NE Spain), namely Gandesa (UTM 31T X = 284000, Y = 4549200) and Les Borges Blanques (Borges, hereinafter; UTM 31T X = 320870, Y = 4597530). These sites corresponded to experimental almond orchards located at facilities of IFAPA (Andalusia) and IRTA (Catalonia). Orchards consisted in trees of different national and foreign cultivars,

variously arranged and managed under local usual practices. Trees in the orchards were not treated with any chemical product to allow natural infection of leaves by *P. amygdalinum*.

3.3.2 Plant material

For the experiments on primary inoculum monitoring, development of fruiting bodies, and germination of ascospores; fallen leaves of various almond cultivars with distinct RLB symptoms were collected during early autumn (September) in Andalusia and autumn/winter (December to January) in Catalonia. As the experimental orchards consisted of different almond cultivars, collected fallen leaves from the ground could not be classified according to their cultivar origin. In each season, an additional bulk sample of leaves collected in Gandesa was taken to Borges in order to study the eventual environmental influence in samples from different geographic origins. This sample (hereinafter as Borges/Gandesa) was considered as a third categorical location within the Catalonian sites. In all experimental sites, leaves were placed into nylon mesh bags with 90 to 200 leaves. The bags with fewer leaf numbers were processed earlier in the season whereas the bags with higher amounts of leaves were processed later in the season, since natural decomposition of leaves along the time would have left a low sample amount at later stages. The bags were left outdoors in the orchard by nailing them on the ground. A variable number of bags ($N > 13$) were prepared for each experimental site and year.

For the monitoring of natural infections and the estimation of the disease incubation period, 1-year-old plants of the susceptible cultivar ‘Tarraco’ were used. Trap plants were kept in greenhouses while not being exposed to natural RLB infections in the experimental sites, while another group of plants (control plants) never were moved out of the greenhouse. Regardless their geographical location, plants kept in the greenhouses were maintained in 3 liters pots with a peat:perlite mixture (3:1, v:v) (Peat: Floratorf TKS1, Floragard, Germany; Perlite: Europerl, Spain). The substrate was amended with Osmocote Pro 3-4M granular fertilizer (Everris, Heerlen, The Netherlands) at 2.5 g l^{-1}. Plants were irrigated as needed to avoid water stress and never treated with fungicides. Once in the experimental sites, the plants were also irrigated as needed and not treated with fungicides during the exposure period to disease.

3.3.3 Monitoring of primary inoculum

The study of primary inoculum was conducted for three seasons in Andalusia (2014 to 2016) and Catalonia (2015 to 2017). Starting from winter in each year, at the stage of dormant trees, the bags of leaf samples were taken fortnightly to the laboratory till the end of the trial. The sample was oven-dried for 24 h at 35°C. Each sample was weighted (dry weight) and later subdivided into eight equally-weighted subsamples. In Andalusia, all eight subsamples were treated separately for ascospore extraction in distilled water, by crushing leaves in a mortar until getting a homogeneous suspension (about 10 min operation). In Catalonia, an additional ascospore extraction method was tested, by continuous stirring (18 h, room temperature) of a suspension of leaf fragments in water. Thus, in Catalonia, four subsamples were extracted by crushing and the remaining four by stirring. In all cases, variable amounts of distilled water were used according to the sample weight (about 40 ml per g sample). For both extraction methods, final ascospore suspensions were filtered through a 2-folded 60-µm Nylon mesh and ascospores were counted under a microscope (×250) using a hemocytometer (Neubauer chamber). Each subsample was measured four times and eight replicated measurements were done per subsample measurement. Results were expressed as numbers of ascospores per g (dry weight) of leaves after proper calculations. The experimental period covered from January to August for all combinations of year and location.

3.3.4 Development of fruiting bodies

This study was conducted with the leaf samples collected in Catalonia in 2016 and 2017. Prior to the oven-drying of leaf samples, four leaves with well-developed fungal stromata were taken from each sample bag. The outer part of the fungal stroma was cut off with a sterile scalpel and five randomly chosen fruiting bodies from each leaf were individually excised with a hypodermic needle. The fruiting bodies were placed in a water droplet on a microscope slide and bisected to unveil their content, then covered with a cover slip and examined under a light microscope (×250). The fruiting bodies (20 per each location and sampling period) were classified into six different development stages by using modified categories described by Toscano-Underwood *et al.* (2003), as follows: class P (pycnidia, either with conidia or not); class A (perithecium differentiated, asci undifferentiated or beginning differentiation, ascospores undifferentiated); class B (perithecium differentiated,

asci differentiated, ascospores undifferentiated); class C (perithecium differentiated, most asci differentiated, some ascospores (< 8) differentiated); class D (fully matured perithecium, asci differentiated, ascospores (8) fully differentiated); class E (perithecium empty, no asci present, ascospores discharged). The percentages of each developmental status at each monitoring period were calculated.

3.3.5 Germination of ascospores

This study was conducted with the leaf samples collected in Catalonia in 2017. The viability of ascospores was estimated by determining the germination percentages at each sampling period. From the same leaf samples used in the study on fruiting bodies development, a sufficient amount of perithecia containing mature ascospores was obtained. The perithecia were bisected and their content suspended in a 1.5 ml Eppendorf tube containing 1 ml distilled water. A volume (200 µl) of the ascospore suspension was spread over a potato dextrose agar (PDA, Difco™, Becton, Dickinson & Co., Sparks, MD) plate amended with streptomycin sulphate at 100 IU streptomycin ml^{-1} and incubated at 20°C. Fifty randomly-chosen ascospores were counted and classified into germinated and non-germinated categories by using a light microscope (250×), at two intervals, 4 h and 24 h after plating. An ascospore was recorded as germinated when the germ tube was greater than half the width of the ascospore, as similarly described by Habibi and Banihashemi (2015).

3.3.6 Disease incubation and infectivity periods

The disease incubation and infectivity periods were studied in two locations in Andalusia (Córdoba) and Catalonia (Borges), for one (2016) and three (2015 to 2017) years, respectively. Groups of ten 1-year-old 'Tarraco' plants kept in greenhouses at IFAPA and IRTA facilities were exposed to RLB natural infections in two-week periods at the experimental sites, from February to August each year. After the exposure to disease, trap plants were removed from the field, taken back to a greenhouse and kept until the appearance of RLB symptoms. The plants were monitored weekly from February to October. Numbers of apparently healthy and RLB-affected leaves in each plant were recorded, and the proportion of RLB-affected leaves (incidence) was calculated and averaged for each monitoring period. The infectivity period was determined as the period in which RLB-

symptomatic leaves were detected. The incubation period was estimated by determining the time between the initial field exposure and the consistent appearance of disease foliar symptoms.

3.3.7 Weather data

Main meteorological variables, namely temperature (T), relative humidity (RH) accumulated rainfall were recorded daily in the experimental areas throughout the monitored years. Data from three automatic weather stations included in the weather network services of the regional governments were used. The weather station in Gandesa was located less than 100 m away from the experimental area, about 9 km away in the case of Borges, and about 1 km away in the case of Córdoba. All recorded meteorological data were considered as representative of the weather conditions at the experimental sites. Raw meteorological data were summarized with 14 weather variables: maximum, minimum and mean daily T, maximum and mean RH, accumulated rainfall, number of rainy days (days with rainfall ≥ 0.2 mm), accumulated vapor pressure deficit, number of wet days (see below), accumulated low T in wet days (50-T), and the number of days with mean daily T lower than 10ºC, from 10 to 20ºC, and equal or higher than 20ºC. In addition, number of days considered both wet and with mild T (10≤T<20ºC) were also recorded, as those conditions are thought to be potentially optimal for RLB development. Daily vapor pressure deficit (VPD) was calculated from mean daily T and mean RH according to the modified equation of Buck (1981) as described by Rossi *et al.* (2009):

$$VPD\ (hPa) = (1- RH/100) \times 6.11 \times \exp\left[(17.47 \times T_{mean})/239+T_{mean}\right]$$

Days were considered wet when VPD was ≤ 4 hPa or accumulated rainfall ≥ 0.2 mm. The accumulation of low T in wet days was measured as the sum of 50-T only in wet days. The 14 weather variables were calculated for the following time intervals: *i*) from the previous 7- and 14-day periods of each monitoring date, *ii*) from months comprised between June to January, and *iii*) from the subdivisions June-September (stage 1) and October-January (stage 2) in the whole monitoring period indicated in *ii*, and *iv*) from all the infectivity periods of trap plants. This resulted in a total number of 182 weather variables (14 weather variables × 13 time intervals).

3.3.8 Data analysis

Data obtained from the primary inoculum monitoring, and incubation and infectivity periods were analyzed using Statistix v.10 (Analytical Software, Tallahassee, FL). Otherwise stated in text and figures, mean values are shown together with their corresponding standard errors. In the primary inoculum monitoring, factorial ANOVA was performed to test main effects and interactions of location and evaluation year on the ascospores amounts recorded periodically. These comparisons were performed by considering only the crushing method of ascospores extraction, as it was only the common ascospore extraction method used in all studied locations. In an exploratory analysis, and to avoid missing data due to differences in the monitoring start and ending dates among locations, only 13 matching data per year and locations were used, i.e. for the period comprised between mid-February and early-August in all years and locations. In further analyses, made separately on the location basis, all recorded data were used. Data were tested for normality, homogeneity of variances and normally-distributed residual patterns. Logarithmic transformations were carried out when necessary. Treatment means were compared using Fisher's protected least significant difference (LSD) at $\alpha = 0.05$.

A regression model was fitted to describe the relationship between the ascospore counts from the two different ascospores extraction methods used in this study. Spearman's rank correlation coefficients (ρ) were calculated from the following variables: *i*) the ascospore counts obtained by the crushing method in each evaluation period, expressed as a proportion of the total accumulated counts during the selected period comprised between mid-February and early-August (ASC_{rate}), and the weather variables observed within the 7- and 14-day periods previous to each ascospore count, *ii*) the total ascospore counts during the season, total ascospores number in the previous year, weather variables calculated from months comprised between June to January and stages 1 and 2, and *iii*) RLB incidence in trap plants and the weather variables from infectivity periods and ascospores counts at the end of the infectivity period. To avoid misunderstood and random variable associations (Fernández-Escobar *et al.* 2018), only associations with $\rho > 0.500$ and $P < 0.05$ between variables were considered strong and reliable enough. Therefore, only these relationships were considered in this work.

In order to analyze the possible influence of location and sampling period on the development and maturation of *P. amygdalinum* fruiting bodies, an ordinal logistic

regression was performed in R v.3.5.1 (https://www.R-project.org/) with the *clm* (cumulative link models) function included in the package 'ordinal' (Christensen 2018).

3.4 Results

3.4.1 Monitoring of primary inoculum

The time progress of ascospore production on symptomatic almond leaves maintained on the orchard ground varied greatly among locations and years (Figs. 3.1-3.4). In general, the ascospore production period extended from January to August during the three years monitoring periods among the studied areas. A factorial ANOVA test showed that the annual mean amount of RLB potential inoculum, as estimated by the ascospore counts per gram of leaf (hereinafter abbreviated as agl), was significantly influenced by the experimental location ($P = 0.007$), the evaluation year ($P = 0.039$), and their interaction ($P = 0.011$). Because of interactions, further ANOVA tests were performed on data subsets according to their location origin.

During the three years, the highest amount of ascospores was recorded in Córdoba, whereas in Borges and Gandesa there was almost a tenth of ascospores in average as compared to Córdoba. The Borges/Gandesa group did not show a different amount of ascospores than Borges ($P = 0.922$). In Córdoba, higher amounts of ascospores were observed in 2014 and 2015 in comparison to 2016 ($P < 0.001$). For Borges and Gandesa, a higher number of ascospores were observed in 2017 than in 2015 and 2016 ($P < 0.001$). In Córdoba, ascospore counts of *P. amygdalinum* in 2014 were over 8.9×10^6 agl, early at the beginning of the season (January, week 4), and remained in similar values for the rest of the trial (Fig. 3.1A). In 2015, ascospores counts fairly exceeded 2×10^7 agl in a five-month interval (March to July, weeks 10 to 28). In this period, the highest ascospore count was recorded in May, week 18 (Fig. 3.1B). In 2016, ascospores counts remained higher between March and May, and peaked in April (week 15) with $8.3 \times 10^6 \pm 2.6 \times 10^6$ agl. Ascospore counts remained around 1×10^6 agl until the end of the monitoring period (Fig. 3.1C).

In Gandesa, ascospore amounts obtained by leaf crushing in 2015 increased progressively from February (week 6) to the end of April (week 16), up to approximately 5.9 $\times 10^5$ agl. Mean values fluctuated around 4.1×10^5 agl until the end of the season, when the annual maximum amount of ascospores was recorded ($2.5 \times 10^6 \pm 1.6 \times 10^6$ agl) in August. Ascospores counts obtained through the leaf-stirring bath technique were consistently lower than those obtained by crushing (Fig. 3.2A). In 2016, first ascospore detection in Gandesa occurred in mid-March (week 10), although it was recorded from stirred leaves ($1.7 \times 10^4 \pm 6.8 \times 10^3$ agl) and none from crushed leaves. However, the ascospore amounts obtained by leaf crushing in subsequent periods increased progressively up to the annual maximum ($1.5 \times 10^5 \pm 3.4 \times 10^4$ agl), which was recorded by the end of May (week 22). Then, a slow

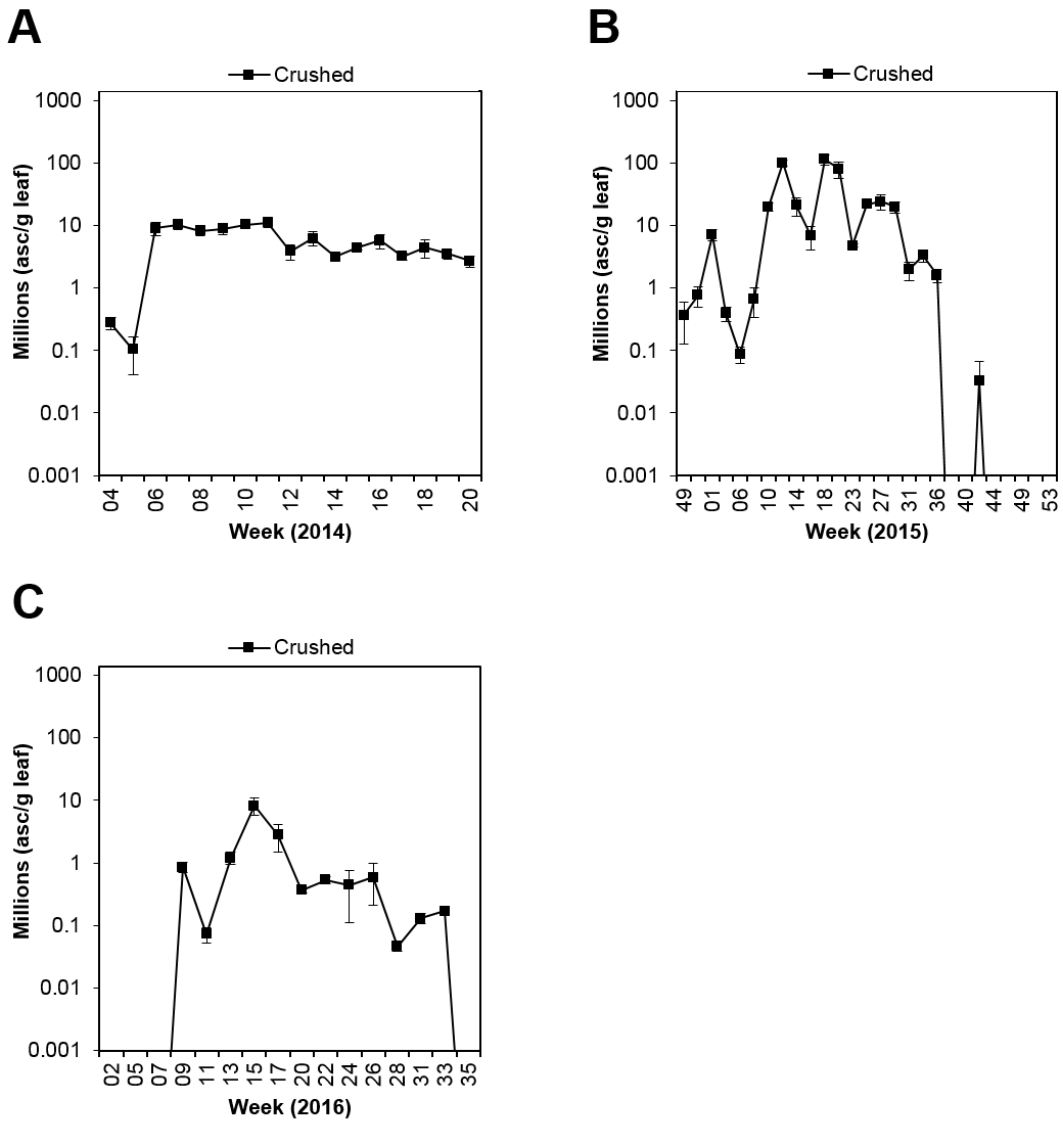

Figure 3.1 Time evolution of *Polystigma amygdalinum* ascospores extracted from infected almond leaves in three consecutive years: A) 2014; B) 2015; and C) 2016, in Córdoba, Spain.

decrease in ascospore counts was recorded until the end of the experimental period (Fig. 3.2B). In 2017, the ascospore counts were in general higher than those of both previous years. After an initial peak observed in February, week 6 ($6.3\times10^5 \pm 2.8\times10^5$ agl), a local minimum was detected in week 10 ($1.4\times10^5 \pm 7.5\times10^4$ agl) and thereafter a progressive increase was recorded to reach a new maximum in April, week 14 ($3.5\times10^6 \pm 6.6\times10^5$ agl). A third peak, i.e. the annual maximum, was detected in mid-June ($7.8\times10^6 \pm 1.4\times10^6$ agl), and counts were subsequently lower (Fig. 3.2C).

In Borges, ascospores obtained by leaf crushing in 2015 were detected from February to mid-July. Two maximum peaks were recorded, the first one at the end of March, week 14

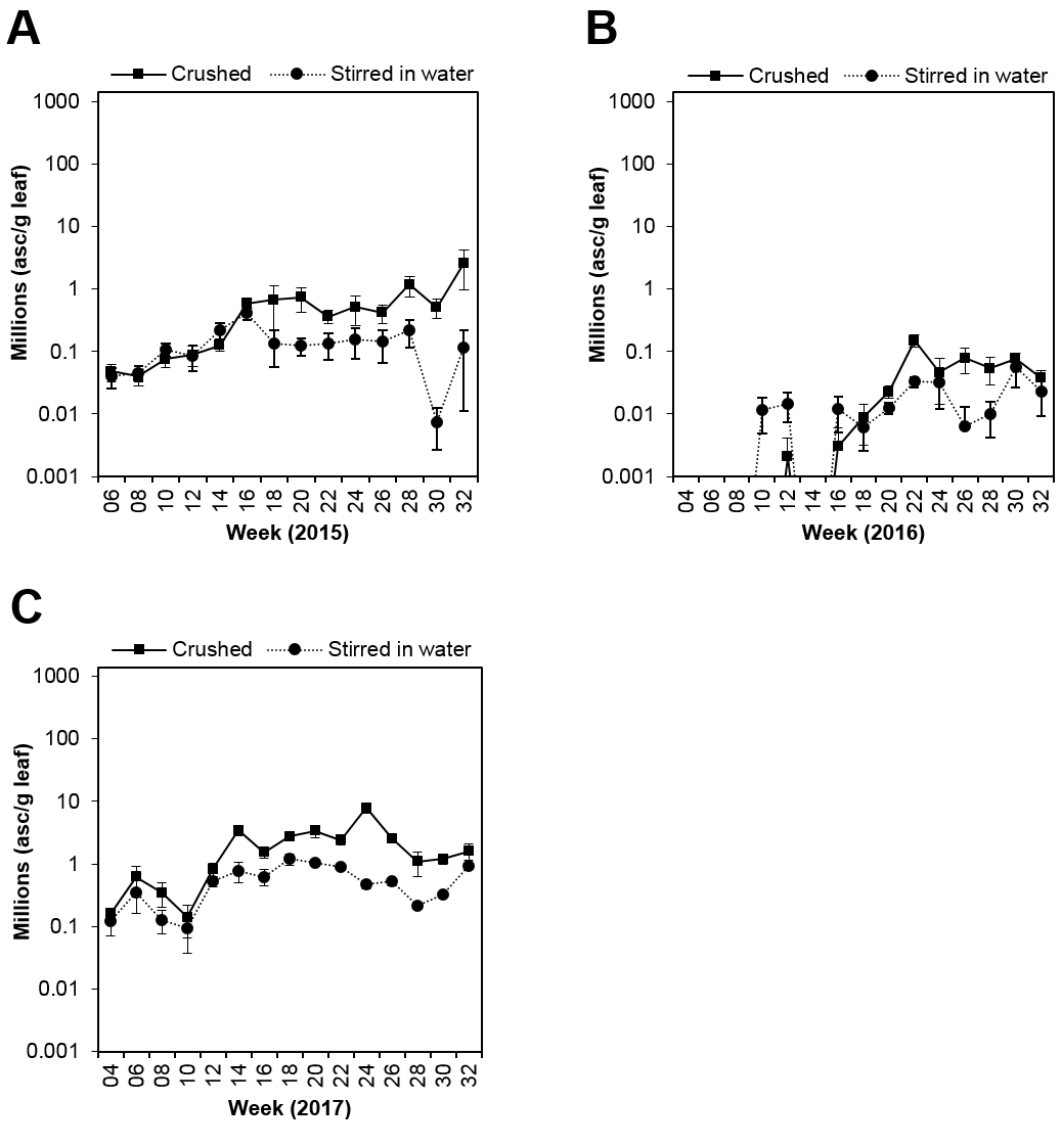

Figure 3.2 Time evolution of *Polystigma amygdalinum* ascospores extracted from infected almond leaves in three consecutive years: A) 2015; B) 2016; and C) 2017, in Gandesa, Spain

($6.1\times10^5 \pm 5.9\times10^5$ agl) and the second one by early May, week 18 ($3.2\times10^5 \pm 1.5\times10^5$ agl), and later a progressively decrease in ascospore counts was observed until the end of the experimental period (Fig. 3.3A). In 2016, ascospores were observed earlier in the season (week 4, late January), but maximum values were recorded consistently from mid-April (week 16) to end of May (week 22), with mean values ranging from 1.2×10^5 to 7.6×10^5 agl. After a local minimum observed in mid-June, remaining counts until the end of the monitoring period were about 2×10^5 agl (Fig. 3.3B).

In 2017, the ascospore counts were in general higher than those of the two previous years, as observed in Gandesa. First, a progressive increase was observed in earlier evaluations (week 4 to 10), when a first peak ($6.4\times10^6 \pm 1.9\times10^6$ agl) was recorded in early March

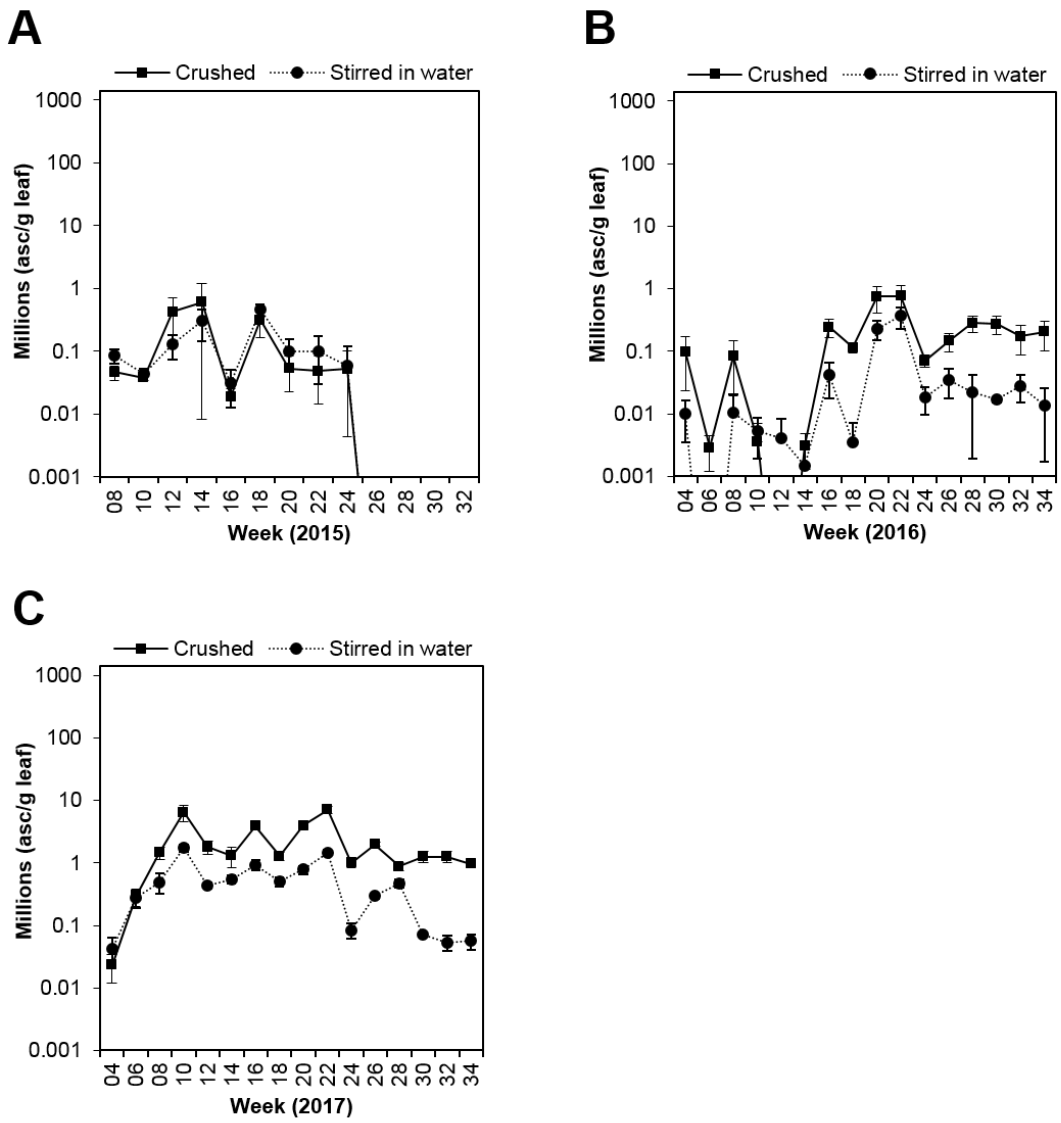

Figure 3.3 Time evolution of *Polystigma amygdalinum* ascospores extracted from infected almond leaves in three consecutive years: A) 2015; B) 2016; and C) 2017, in Les Borges Blanques, Spain

(week 10). After a local peak observed later in week 16 (late April), the maximum ascospore counts ($7.1\times10^6 \pm 8.8\times10^5$ agl) were recorded in the last week of May (Fig. 3.3C). Then, ascospore counts decreased from this date on but remained around 1×10^6 agl until the end of the monitoring period. As observed previously, counts from suspensions obtained through leaf crushing were higher than those obtained by the leaf-stirring bath.

Regarding the ascospore counts in the Borges/Gandesa group, ascospores were detected within all the monitoring period (February to August) in 2015 regardless the extraction method, and high ascospore counts were recorded from end March to end April (mean values around 2×10^5 agl). Three additional peaks were detected in May, June and July, with the

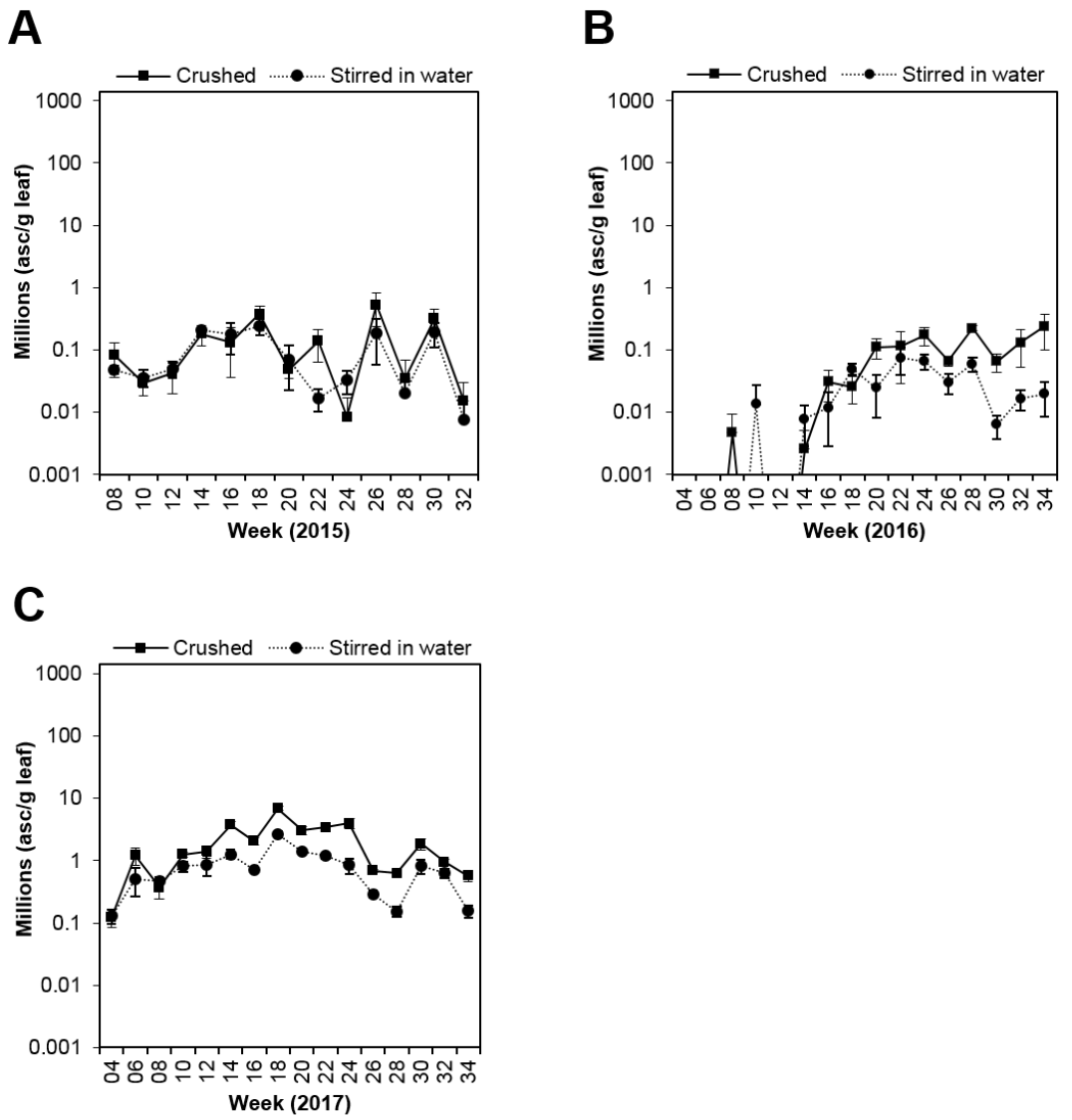

Figure 3.4 Time evolution of *Polystigma amygdalinum* ascospores extracted from infected almond leaves in three consecutive years: A) 2015; B) 2016; and C) 2017, from leaves taken from Gandesa to Les Borges Blanques, Spain.

annual maximum ($5.3\times10^5 \pm 2.9\times10^5$ agl) observed in mid-June, week 26 (Fig. 3.4A). In 2016, consistent detections started in April (week 14) and were progressively increasing in number until mid-June (week 24), with a maximum of $1.7\times10^5 \pm 0.6\times10^5$ agl. Mean values in later periods were around this value until the end of the monitoring period, with lower values for counts on ascospores obtained by leaf-stirring baths (Fig. 3.4B). In 2017, as similarly occurred in Borges and Gandesa, ascospores counts were higher than in previous years. The seasonal highest amount of ascospores ($6.8\times10^6 \pm 4.9\times10^5$ agl) was observed in early May, week 18 (Fig. 3.4C).

A significant linear relationship ($P < 0.001$) between the two ascospore extraction methods was found, as follows: $Ascospores_{(crushing)} = 6.21 \times 10^3 + 23.97 \times Ascospores_{(stirring)}$, with $r^2 = 0.70$. Intercept was not significantly different from zero ($P = 0.457$) whereas slope was significantly different from one ($P < 0.001$). In addition, there were no significant differences between samples from different origin (Borges and Borges/Gandesa) which were obtained with either extraction method ($P = 0.922$).

Associations with $\rho > 0.500$ and $P < 0.05$ between the rate of ascospore amounts per season (ASC_{rate}) and any tested weather variable were not found. On the other hand, the total amount of ascospores per season only correlated significantly with weather-derived variables of stage 2 (October-January), which resulted in ρ values higher than 0.700 ($P < 0.05$), i.e. mean RH ($\rho = 0.767$), accumulated rainfall ($\rho = 0.717$), number of raining days ($\rho = 0.728$) and number of days with mean daily T higher than 20ºC ($\rho = 0.706$). Specifically, in October, the mean of maximum RH ($\rho = 0.783$), the accumulated rainfall ($\rho = 0.733$), the number of days with mean daily T equal or higher than 20ºC ($\rho = 0.706$) and the number of days with mean daily T from 10 to 20ºC ($\rho = -0.792$) were correlated with total amounts of ascospores per season. Nevertheless, the only the latter was negatively correlated as opposed to the rest of positive correlations. In January, accumulated rainfall also showed a positive correlation with the total amount of ascospores per season ($\rho = 0.783$). No more associations were found between the seasonal total ascospore amounts and weather variables for the remaining months of the stage 2.

3.4.2 Development of fruiting bodies

The development and maturation of perithecia along the season was confirmed through the observation that early stages (P, A, B) were prevalent at the beginning of the experimental period whereas later stages (C, D, E) were recorded later in the season (Fig. 3.5). Results from the ordinal logistic regression analysis on the whole dataset showed that all analyzed factors and their interactions, except for the interaction location × week, were significant (*results not shown*). Because of interactions, further ordinal logistic regressions were performed on data subsets according to the location of sampled leaves (Gandesa and Borges). However, datasets from Borges and Borges/Gandesa were combined into a single dataset to evaluate the influence of the geographic origin of samples. Analysis of the ordinal logistic regression for the location Gandesa subset showed the significance in factors sampling period ($P < 0.001$), year ($P < 0.001$), and their interaction ($P < 0.001$). Similarly, analysis of the ordinal logistic regression for the location Borges (Borges and Borges/Gandesa subsets) showed significance in the same factors, i.e. sampling period, evaluation year, and their interaction (all $P < 0.001$). However, the origin of leaf samples was not found significant ($P = 0.262$), nor the interactions: origin × sampling period ($P = 0.618$), origin × evaluation year ($P = 0.262$), and origin × sampling period × evaluation year ($P = 0.618$).

In Gandesa, fully mature asci (D) were observed rarely in 2016, and the percentage of this class never exceeded 20% throughout the assay. In 2017, percentages in class D ascocarps were equal or higher than 40% were detected from mid-May until the end of the experiment (Fig. 3.5A). However, in Borges, mature ascocarps reached 80% at the end of May 2016, and remained in 15-45% until August. In 2017, the percentage of mature fruiting bodies prevailed above 50% from mid-February to the end of the experiment. Moreover, peaks (75%) in class D perithecia were detected in May, weeks 18 and 20 (Fig. 3.5B). In Borges/Gandesa samples, two peaks of fruiting bodies over 50% in class D were recorded in 2016, the first one in June (week 24), and the second one by the end of the experiment. However, in 2017 the pattern of Borges/Gandesa development and maturation of fruiting bodies behave equivalent as the Borges samples, as shown above by the statistical analyses, with percentages above 50% that prevailed in almost every week of evaluation from mid-February on. In Borges/Gandesa, an early peak of class D ascocarps (75%) was detected in late March, week 12 (Fig. 3.5C).

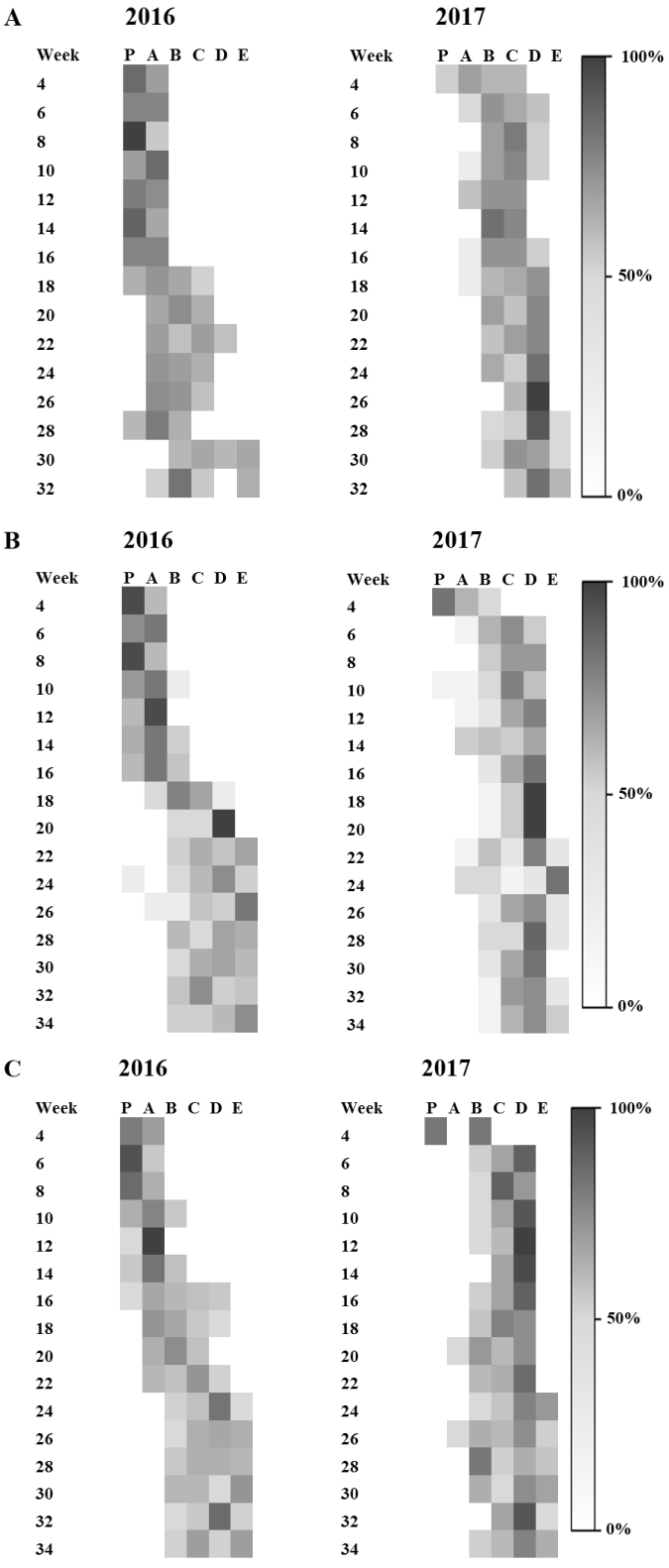

Figure 3.5 Development of *Polystigma amygdalinum* fruiting bodies on almond leaves in two consecutive years and three sample origins, A) Gandesa; B) Borges, C) Borges/Gandesa. Results are shown in a gray scale as percentages of fruiting bodies (N = 20) for each sampling period. Abbreviatures: (P) Pycnidia, no perithecia present; (A) Differentiated perithecium but not mature, asci and ascospores are undifferentiated; (B) Differentiated perithecium but not mature, differentiated asci with undifferentiated ascospores; (C) Differentiated perithecium but not mature, differentiated asci with < 8 ascospores/ascus; (D) Mature perithecium and asci with 8 ascospores/ascus; (E) Empty perithecium, without asci and ascospores.

3.4.3 Ascospore germination

The germination of *P. amygdalinum* ascospores on PDA was observed as earlier as 4 h after plating. However, highest percentages of germinated ascospores were observed at 24 h incubation (Fig. 3.6). In general, germination percentages at 24 h ranged 12 to 44% for all leaf samples origins, with mean values for each leaf origin as follows: Gandesa, 16.6 ± 3.8 %; Borges, 19.2 ± 1.5 %; Borges/Gandesa, 20.9 ± 1.2%. In Gandesa, the highest germination percentage was observed in mid-July (44.0 ± 8.0 %; Fig. 3.6A), whereas in Borges the maximum (28.0 ± 2.0 %) occurred earlier in the season, in mid-April. Ascospores from the Borges group showed consistently 20% and above of germination during the first half of the monitoring period (Fig. 3.6B). Similarly, ascospores from the Borges/Gandesa group (Fig. 3.6C) showed the higher germination percentage in April (30.0 ± 2.0 %), as the samples from Borges did.

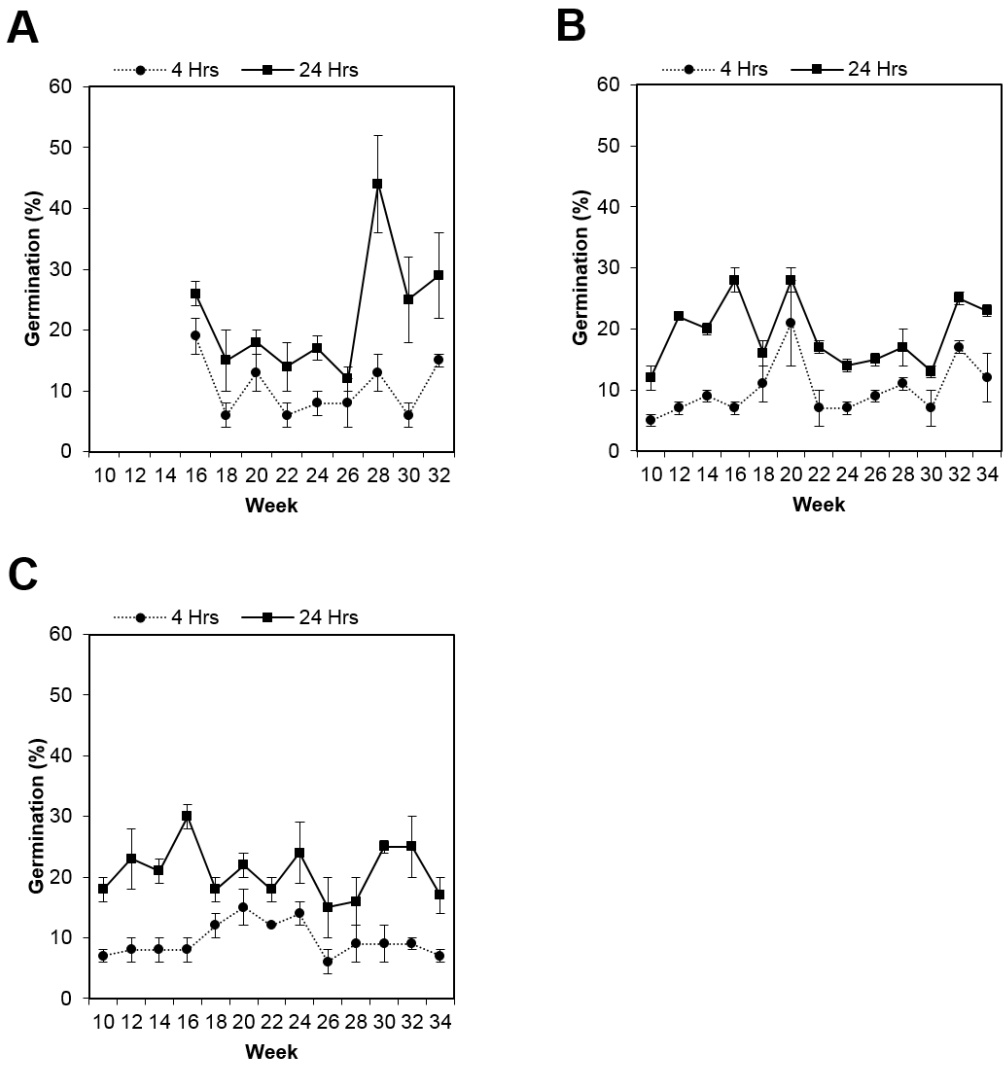

Figure 3.6 Germination percentages of *Polystigma amygdalinum* ascospores (N = 50) from A) Gandesa, B) Les Borges Blanques, and C) leaves taken from Gandesa to Les Borges Blanques.

3.4.4 Disease incubation and infectivity periods

Trap plants exposed in Córdoba and Borges showed that RLB infections occurred from March (week 9) to late July (week 30), although major infection percentages were mainly detected from week 9 to week 18, i.e. from March to early May (Fig. 3.7). Overall incidence in 2016 in Córdoba was higher than in Borges. Infections in Córdoba were well ranging 30 to 70% within the weeks 9 to14 and declined thereafter. Data collected in Borges in three consecutive years (2015 to 2017) indicated that most of the infections occurred between first half of March (week 10) through early May (week 18), with 5 to 35% of the leaves being affected. When considering all three years, infections decreased drastically in June and only sporadic infections were detected later (Fig. 3.7).

No differences in RLB incidence of trap plants (P = 0.064) between Borges (5.54 ± 5.60%) and Córdoba (26.55 ± 6.19%) were detected in 2015. In addition, no differences in RLB incidence of trap plants (P = 0.167) were detected in Borges when comparing all three monitored years. No significant relationship was found between the incidence recorded in each infectivity period and the ascospore amounts at the end of the period based on crushing (P = 0.654) or stirring method (P = 0.479). However, the incidence of RLB in trap plants was positively correlated with the number of days with mean daily T from 10 to 20ºC (10≤T<20 ºC) (ρ = 0.526, P = 0.001) and the number of days both wet and mild T (VPD ≤ 4 hPa or R ≥ 0.2 mm and 10≤T<20 ºC) (ρ = 0.632, P < 0.001).

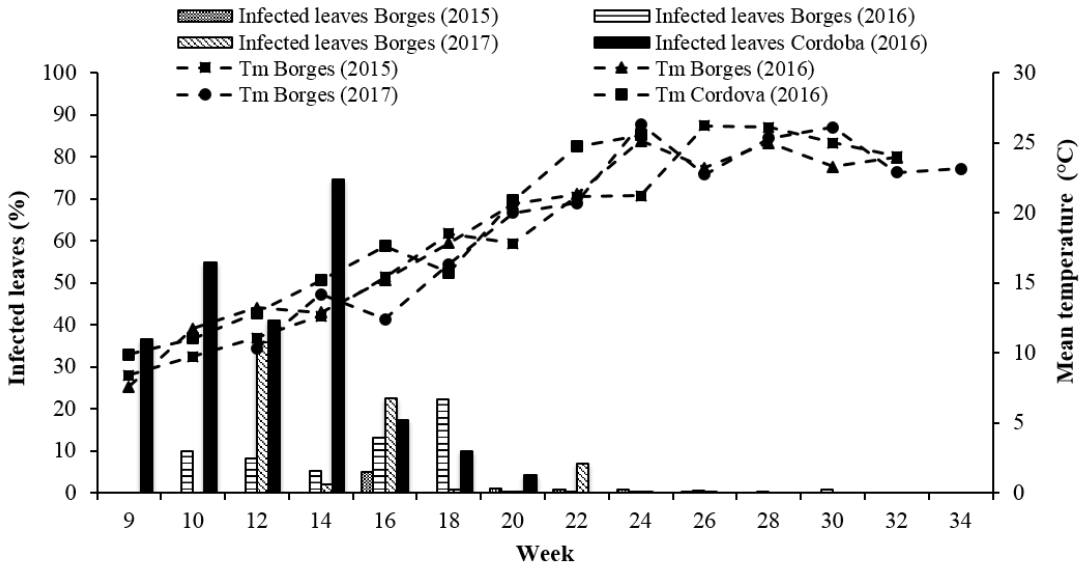

Figure 3.7 Red leaf blotch incidence (%) in 'Tarraco' susceptible almond trees exposed to natural infection periods in Córdoba (2016) and Les Borges Blanques (2015–17). Mean temperatures shown in secondary (right) axes

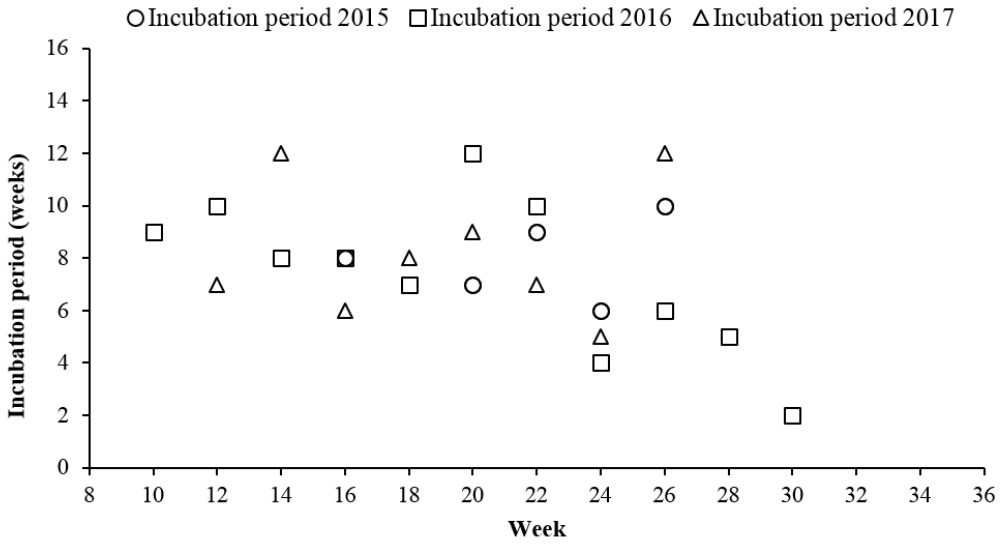

Figure 3.8 Incubation periods of almond red leaf blotch in 'Tarraco' susceptible almond trees exposed in Les Borges Blanques (2015–17).

The incubation periods estimated from the data recorded in 2015 to 2017 in Borges were 6 to 10 weeks in 2015, 2 to 12 weeks in 2016 and 5 to 12 weeks in 2017. The duration of the incubation period tended to decrease from week 20 in 2016, but this trend was not observed in 2015 and 2017 (Fig. 3.8).

3.5 Discussion

Some key aspects of the *P. amygdalinum* epidemiology have been studied from 2013 to 2017 in two almond-growing regions in Spain, namely Andalusia and Catalonia, which included the potential primary inoculum development, and the incubation and infectivity periods. Correlation analyses between biological and meteorological data contributed to a better understanding of the pathogen life cycle on almond.

Previous data about the RLB epidemiology and strategies to control this disease at worldwide level were based on studies conducted in Iran and Lebanon, which reported on the production of ascospores, disease infection periods and the effect of fungicides against *P. amygdalinum* (Ashkan and Assadi, 1974; Banihashemi, 1990; Bayt-Tork *et al.*, 2014; Ghazanfari and Banihashemi, 1976; Saad and Masannat, 1997). In Spain, previous knowledge on the RLB disease include some field observations about symptom incidence and severity, treated in a descriptive way (Almacellas, 2014; Ollero-Lara *et al.*, 2016a,b),

and data on the cultivar susceptibility to RLB (Ollero-Lara *et al.*, 2019). Thus, the current work aimed to increase the knowledge on the dynamics of major aspects of the RLB disease in the Western Mediterranean area, where environmental conditions for almond-growing areas could be different from those of previous studied cases in Iran and Lebanon.

The main period of the potential primary inoculum availability in Córdoba and Borges within the first monitored season (2014 and 2015, respectively) was similar to previous works carried out in Iran and Lebanon, i.e. from April to May (Ashkan and Assadi, 1974; Banihashemi, 1990; Saad and Masannat, 1997). However, dynamics in Gandesa (2015) differed from those results, as the presence of the potential inoculum was detected from February to August, covering almost four additional months. This pattern of an extended period of ascospore availability was repeatedly observed in the following years among the studied almond orchards. This suggests a larger period where primary inoculum of RLB is present in Spain, thus increasing the risk of infections during periods of the year far beyond that previously known in other almond-growing areas.

The amounts of ascospores recorded in this study cannot be directly compared with data on ascospore counts reported earlier by Banihashemi (1990) and Saad and Masannat (1997), since methods in those studies were based on the quantification of airborne ascospores captured on microscope slides coated with petroleum jelly. In our study, the ascospore extraction method from leaves may had influenced the estimated amounts of ascospores, as shown by the regression equation analysis between both extraction methods. Fruiting bodies can be physically broken during the crushing extraction process so that the whole perithecia content is released to the medium, thus resulting in higher ascospore amounts. However, it was observed that both ascospore extraction methods might be easily exchangeable when there is a low amount of ascospores.

Banihashemi (1990) suggested that changing environmental conditions among different growing seasons could influence ascospore release and RLB infections. In this study we observed that total amount of ascospores per season were correlated positively with environmental conditions of previous fall and winter seasons (October to January), especially with variables related to water availability, i.e. rainfall and relative humidity, and, to a lesser extent, to temperature. Apparently, the pathogen benefits from the hydration of fallen leaves and T above 20°C during fall to produce higher inoculum potential during the next season. The month with a higher number of weather variables correlated with ascospore

amounts was October. Similarly, Ghazanfari and Banihashemi (1976) reported a higher number of mature ascocarps being detected in October in Iran. These authors found that *P. amygdalinum* overwinters as ascocarps in leaf litter on the soil and it requires T below 10°C in fall and winter in order to produce mature ascocarps for the next season, which is in contrast with our results, as T above 20°C during fall were related to higher inoculum amounts in the following season. However, it seems that fall and winter environmental conditions play a key role in the dynamics of the disease.

Geographic conditions may also play a major role in primary inoculum development, as confirmed by the sharp differences detected between amounts of potential inoculum among the studied almond-growing areas, i.e. with greater inoculum presence in Andalusia than in Catalonia. Even within the same almond-growing area (Catalonia), differences between localities were observed. These results confirm the hypothesis that RLB of almond needs to be studied in each region where it is reported. Moreover, it is advisable to conduct a multi-year monitoring of the primary inoculum since, as reported here, highly variable records on ascospore amounts can be obtained.

The maturation of *P. amygdalinum* perithecia and ascospores was rather related to local factors occurring during the season than to a geographical origin of samples. However, a disparity between maturity of fruiting bodies and primary inoculum dynamics was detected in some cases. Thus, fruiting bodies reached maturation late in the season in 2016 in all locations while free ascospores were detected from the first weeks of the year until the end of the experiment. We hypothesize that this might have been due to the sample size of the analyzed leaves, which could have been insufficient to adequately represent how fruiting bodies developed in the leaf litter along the season. Gadoury *et al.* (1992) reported a disparity between morphological maturity of ascospores and physiological maturity of asci in *Venturia inaequalis*, the causal agent of apple scab, which could be comparable to our results. The authors found that discharge of ascospores was recorded as early as asci were rated as mature in approximately 10 to 15% of full maturity. However, these criteria must be considered carefully when developing a predictive model on the development of disease epidemics.

In our study, ascospores were able to germinate and develop appressorium but failed to grow further. Our results agree with those obtained by Habibi and Banihashemi (2015), who reported that *P. amygdalinum* was able to germinate and develop appressorium in different

artificial media and temperature conditions. In our study, germination percentages after 24 h incubation were consistently low, i.e. well below 30% in most cases. These low germination percentages could respond to the biotrophic nature of the pathogen (Cannon, 1996; Habibi and Banihashemi, 2015), or it may be due to unknown effects of other environmental factors. Data on ascospore germination could be useful in a first stage for testing fungicides *in vitro*, especially for those involved in the inhibition of ascospore germination. In addition, variables related to ascospore germination could be used in the development of mechanistic predictive models on RLB epidemiology.

The natural RLB infections observed in trap plants occurred between week 9 and 22 (February to May) despite the geographical location of almond orchards. By that time, mean daily T increased roughly from 10 to 20 °C. Infections in Andalusia and Catalonia declined when T raised above 20 °C, thus suggesting that warmer temperatures where inhibiting RLB infections. Several lines of evidence indicate that optimum temperature for ascospore germination among *Phyllachora* species is 10 to 20°C (Banihashemi, 1990; Dittrich *et al.*, 1991; Parbery, 1963) whereas the appressorium formation takes place at a similar temperature range which would promote the infections of susceptible host. However, if T rises above 25°C the appressorium formation would be reduced (Habibi and Banihashemi, 2015).

Based on the associations found in this study, T comprised between 10 and 20ºC promote *P. amygdalinum* infections, mainly when those temperatures are matched with wet conditions. Rainfall and high RH, which enhance wet conditions, could easily promote the required conditions of ascospores spread and subsequent infection. The relevance of both weather variables has been evidenced in a great range of pathosystems (Agrios, 2005). The ability of weather variables built from a combination of T and RH conditions (hydrothermal variables) to explain plant disease development is well-known from a general viewpoint (Lowell *et al.*, 2004) but also in specific pathosystems, such as *Plasmopara viticola*-grape (Rossi *et al.*, 2007) and *Venturia pirina*-pear (Rossi *et al.*, 2009). Nevertheless, correlation analyses must be considered carefully to avoid misleading interpretations related with casual associations or lack of causality between variables (Fernández-Escobar *et al.*, 2018).

Our results showed that the incubation period could be as long as 12 weeks in some occasions, but as season goes this variable trended downward and can be as short as 2 weeks in summer. These results differ from those reported by Ashkan and Assadi (1974), who

estimated a narrower incubation period of 30 to 35 days, whereas Banihashemi (1990) reported a similar period (30 to 40 days).

Although the *P. amygdalinum* is considered as a biotrophic pathogen (Cannon, 1996), other plant pathogens with a similar multistage infection strategy as *P. amygdalinum* are also classified as hemibiotrophic pathogens, such as *Mycosphaerella graminicola* (Fuckel) J. Schröt., *Pyricularia oryzae* Cavara, and *Colletotrichum* (Marshall *et al.*, 2011; Mentlak *et al.*, 2012; O'Connell *et al.*, 2012). The infection strategy of hemibiotrophic pathogens consists of an initial biotrophic phase, with the pathogen spreading inside living host cells without causing noticeable host cell damage, and later switching onto a necrotrophic phase, by killing host cells and completing its life cycle (Luttrell, 1974; Lo Presti *et al.*, 2015). This hemibiotrophic lifestyle can be easily recognized in the life cycle of *P. amygdalinum*: the infection of almond leaves is followed by a variable incubation period (2 to 12 weeks) with no visible RLB symptoms and the subsequent development of the brightly-colored stromata through late spring and summer. This long part of the pathogen cycle occurs on living leaves and corresponds to the biotrophic phase. Afterwards, *P. amygdalinum* switches onto the necrotrophic phase along with the appearance of late symptoms stages: darkening of leaf stroma and pathogen growth causing multiple host cell death (Saad and Masannat, 1997; Zúñiga *et al.*, 2019). Lastly, *P. amygdalinum* continues the necrotic phase on fallen leaves (from October to January), which is previous to the final development and maturation of perithecia and ascospores.

In this work we have studied, during an extended vegetative period of the almond crop, the primary inoculum dynamics, the development of the fruiting bodies, the germination of ascospores as well as the natural infections of RLB. The results reported here can help in building a future predictive model, which would integrate some key biological aspects of *P. amygdalinum* with the environmental conditions met in each almond-growing area. Thus, predicting risk events for RLB infection could help in taking more effective decisions on management programs and control strategies.

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Chapter 4

Chapter 4: A qPCR-based method for detection and quantification of *Polystigma amygdalinum*, the cause of red leaf blotch of almond

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4.1 Abstract

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.

Keywords: *Prunus dulcis*, molecular detection, specific primers.

4.2 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 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.

4.3 Materials and methods

4.3.1 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 4.1). 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.

4.3.2 DNA extraction and sequencing

4.3.2.1 Non-culturable fungi

DNA from *P. amygdalinum* was obtained as follows. The upper part of each fungal stroma was cut off with a sterile scalpel, and 20 fruiting bodies were individually excised with a hypodermic needle and collected into a 1.5 mL capacity 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

Table 4.1 Fungal taxa and DNA samples from culturable and non-culturable almond pathogens obtained in this study.

Fungal taxa	Isolate	Sample	Location[1]	Source	Symptom	GenBank Accession ITS
Alternaria alternata	CJL 877	CR52	GA	leaf	shot-hole	MH205933
A. alternata	CJL 880	CR54	VP	leaf	shot-hole	MH205931
Alternaria sp.	CJL 878	CR55	GA	leaf	shot-hole	MH205934
Cladosporium tenellum	CJL 876	CR53	GA	leaf	shot-hole	MH205932
Coniothyrium sp.	-	CR25	GA	leaf	shot-hole	
Coniothyrium sp.	-	CR26	GA	leaf	shot-hole	
Coniothyrium sp.	-	CR28	GA	leaf	shot-hole	MH205924
Coniothyrium sp.	CJL 866	CR36	GA	leaf	shot-hole	MH205926
Coniothyrium sp.	CJL 864	CR12	BB	twig	scab	MH205920
Diaporthe amygdali	CJL 862	CR14	GA	branch	canker	MH205921
Monilinia laxa	CJL 863	CR11	BB	flower	blight	MH205919
Phoma sp.	CJL 879	CR51	VP	leaf	shot-hole	MH205930
Polystigma amygdalinum	-	TO1	GA	leaf	leaf blotch	MH205935
P. amygdalinum	-	TO5	GA	leaf	leaf blotch	MH205936
P. amygdalinum	-	TO7	GA	leaf	leaf blotch	MH205937
P. amygdalinum	-	TO9	GA	leaf	leaf blotch	MH205938
P. amygdalinum	-	TO10	GA	leaf	leaf blotch	MH205939
P. amygdalinum	-	TO15	GA	leaf	leaf blotch	MH205940
P. amygdalinum	-	TO16	GA	leaf	leaf blotch	MH205941
P. amygdalinum	-	TO18	GA	leaf	leaf blotch	MH205942
Taphrina deformans	-	CR20	BB	leaf	leaf curl	MH205922
T. deformans	-	CR34	CA	leaf	leaf curl	MH205925
Wilsonomyces carpophilus	CJL 869	CR39	BB	leaf	shot-hole	MH205927
W. carpophilus	CJL 872	CR41	BB	leaf	shot-hole	MH205928
W. carpophilus	CJL 875	CR50	BB	leaf	shot-hole	MH205929
W. carpophilus	-	CR22	BB	leaf	shot-hole	MH205923

[1]: All samples collected in Catalonia, Northeast Spain: CA, Cabrils; BB, Les Borges Blanques GA, Gandesa; VP, Vilafranca del Penedès.

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

4.3.2.2 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 capacity 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.

4.3.3 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 4.1).

4.3.4 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 4.1) 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 sensitivity 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).

4.3.5 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 4.1) and from supposedly pathogen-free almond leaves. Each set of qPCR assays included a negative control, without template DNA.

4.3.6 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 addition, 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.

4.3.6.1 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 for DNA 2. Ten-fold dilutions series to $1:10^6$ 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 $1.66 \times 10^5 \pm 0.089 \times 10^5$ (std. error) 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

Table 4.2 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	

with eight pseudoreplicates each). Ten-fold dilution series (until $1:10^5$) 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 4.2). 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.

4.3.7 Validation of the specific qPCR primer pair PamyI2F4/PamyI2R2

4.3.7.1 Case 1: Detection of Polystigma 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.

4.3.7.2 Case 2: Early detection of Polystigma amygdalinum *in asymptomatic and* symptomatic leave

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 4.3, Figure 4.1). An additional group of leaves with characteristic shot-hole lesions caused by *W. carpophilus* 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.

4.3.8 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 $P < 0.05$.

4.4 Results

4.4.1 Design, selection and specificity of Polystigma 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 4.1).

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 4.2). 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 4.1), 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.

4.4.2 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 4.2a). 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 4.2b). 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).

4.4.3 Validation of the specific qPCR primer pair PamyI2F4/PamyI2R2

4.4.3.1 Detection of Polystigma 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 4.2c). 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}.

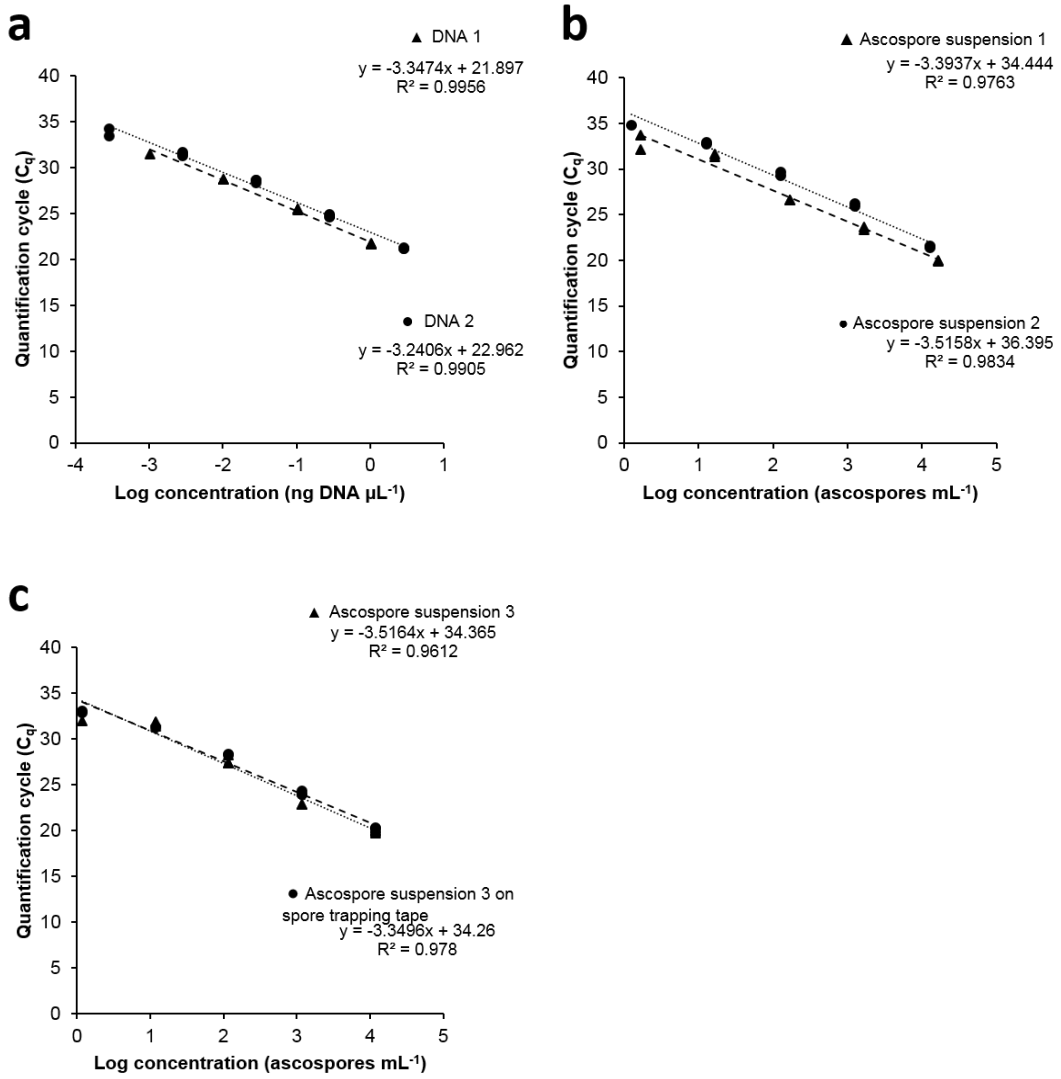

Figure 4.2 Standard regression curves of qPCR tests run with several 10-fold serial dilutions of *Polystigma amygdalinum* genomic DNA obtained from: a) perithecial contents (samples 1 and 2 with initial amounts of, respectively, 1.03 and 2.83 ng DNA µL^{-1}); b) Ascospore suspensions 1 and 2 (respectively $3.33\times10^5 \pm 1.76\times10^5$ and $2.54\times10^5 \pm 1.68\times10^5$ ascospores mL^{-1}); c) Ascospore suspension 3 ($2.35\times10^5 \pm 1.30^5\times10^5$ ascospores mL^{-1}), either processed directly for DNA extraction or after placing ascospores on a plastic spore-trapping tape.

4.4.3.2 Early detection of Polystigma 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 4.3). In general, 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.

Table 4.3 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.

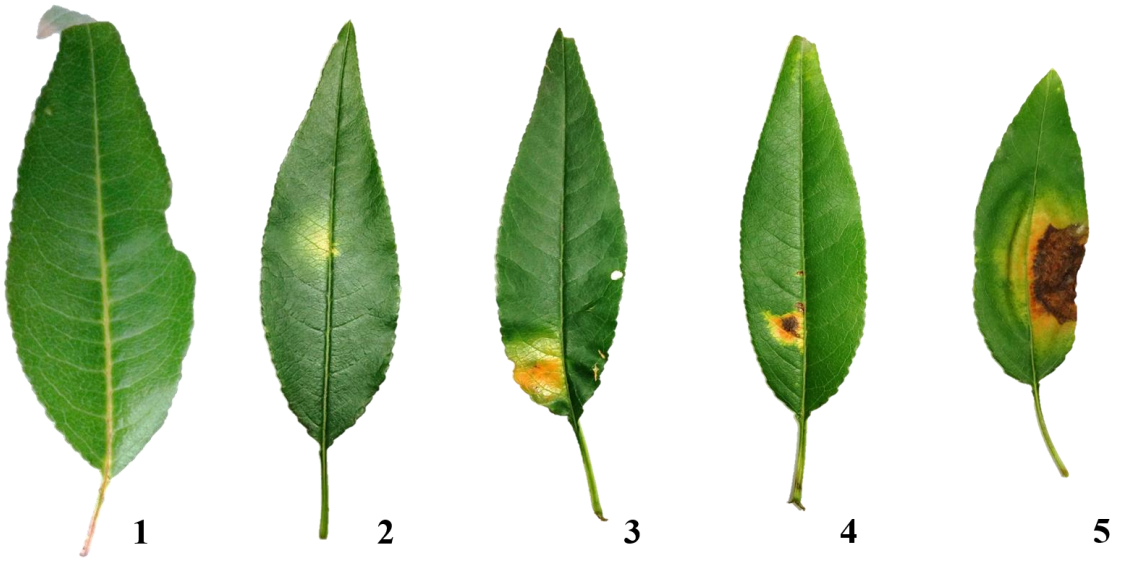

Figure 4.1 Sequential development of the red leaf blotch symptoms on almond leaves. From left to right: 1) asymptomatic leaf; 2) initial yellowish discolouration; 3) orange-reddish blotch; 4) initial darkening of stroma with darkening red blotch; 5) dark, well-developed stroma.

4.5 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 PCR 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 therefore 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.

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Chapter 5

Chapter 5: Lignin biosynthesis as a key mechanism to repress *Polystigma amygdalinum*, the causal agent of the red leaf blotch disease in almond.

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5.1 Abstract

The red leaf blotch (RLB) of almond, caused by the fungus *Polystigma amygdalinum*, is considered as one of the most important leaf diseases of this fruit tree. Differential cultivar susceptibility to the RLB has been described based on field observations, while its molecular and biochemical bases remain unknown to date. We aimed to explore the plant defence mechanisms related to the cultivar susceptibility by identifying some relevant physical and chemical strategies for the pathogen control. Thus, we studied the regulation of seven defence-related genes as well as the lignin deposition in two almond cultivars with highly differential response to RLB: the highly tolerant 'Mardía' and the susceptible 'Tarraco' cultivars. 'Mardía' displayed an up-regulation of the *CAD* and *DFN1* genes at early stages of RLB symptom expression, with further lignin deposition in the fungal-colonized area that was visualized by microscopy. Thus, 'Mardía' uses both physical and chemical responses to effectively repress the pathogen. In contrast, 'Tarraco' triggered the up-regulation of *HQT* and *LDOX* genes, related to chlorogenic acid and anthocyanin biosynthesis pathways, respectively, while lignin deposition was not clearly noticed. This strategy recorded in 'Tarraco' at later stages of RLB symptoms failed to control the fungal infection and colonization. Our results suggested a major role of the phenylpropanoids pathway in the defence response against RLB, by showing that an early production of lignin might be a major mechanism to control the spread of *P. amygdalinum* within the host leaf tissues.

Keywords: Defence genes, Lignin, *Polystigma amygdalinum*, *Prunus dulcis*, Red leaf blotch, Varietal susceptibility.

5.2 Introduction

The red leaf blotch disease (RLB) of almond (*Prunus dulcis*), caused by the ascomycete *Polystigma amygdalinum*, was firstly reported in 1970 in Shiraz, Iran (Ghazanfari and Banihashemi, 1976), and appears to be widely distributed in the Mediterranean region and the Middle East (Cannon, 1996). The RLB is considered one of the most important leaf diseases of almond, since it reduces photosynthetic capacity of the host and may induce an early defoliation of the tree (Kranz, 1962). A subsequent decrease in fruit production has also been reported as caused by the disease (Miarnau and Vargas, 2013; López-López *et al.*, 2016). Leaf infections occur in spring, when ascospores of *P. amygdalinum* are released from previous-year fallen leaves under favorable environmental conditions (Banihashemi, 1990). RLB symptoms are characterized at early stages by diffuse, roundish discoloured blotches (1–2 cm) on the leaves, which expand and turn subsequently into reddish and purplish dark necrosis, as the fungal stromata develop along the season. The typical leaf symptoms appear after a latent period lasting 30–35 days (Banihashemi, 1990; Saad and Masannat, 1997). The symptomatic leaves fall at the end of summer, earlier than usual and overwinter in the ground in order to produce mature perithecia and ascospores in the fungal stromata. The fungus fails to grow in artificial media and sporadic germination of ascospores are observed with no further mycelium development (Habibi and Banihashemi, 2015), which confirms its biotrophic nature.

Little is known about the RLB disease and specifically about the host-pathogen interaction. However, works focused on the disease cultivar sensitivity have reported differential susceptibility of almond cultivars commonly planted in Spain. Thus, most of the settled cultivars in 1980′s were classified as susceptible or most susceptible to *P. amygdalinum* by Egea *et al.* (1984), who checked 81 almond cultivars for RLB susceptibility. In the last decade, different field surveys were carried out to report on the susceptibility of newly generated cultivars to RLB; visual observations have confirmed high susceptibility of ‘Guara’ and ‘Tarraco’ and high tolerance of ‘Vairo’ and ‘Mardía’ (Vargas *et al.*, 2011; Marimon *et al.*, 2012; Ollero *et al.*, 2016). Ollero *et al.* (2016) suggested that differential susceptibility among cultivars could be due to the genetic background of varieties. However, the molecular bases of the biology of cultivar tolerance to RLB are currently unknown.

A plethora of plant signaling pathways are induced by biotic stress composing intricate and cross-talked networks in order to activate the appropriate defence responses (Anderson *et*

al., 2004). The phenylpropanoids pathway, with important secondary-derived compounds related to plant-pathogen interactions, plays a crucial role in plant defence (Dixon *et al.*, 2002). Lignin is one of these compounds whose accumulation is considered an active resistance mechanism of plants against pathogens and pests (Maher *et al.*, 1994; Mauch-Mani and Slusarenko, 1996; Wuyts *et al.*, 2006; Tronchet *et al.*, 2010). Pathogen attack induces deposition of lignin in the walls of host cells and this reinforced structure enhances resistance against pathogen invasion (Agrios, 2005; Bhuiyan *et al.*, 2009). Anthocyanins, also derived from the phenylpropanoids pathway, have been secondarily related to biotic stress (Gould, 2004; Reveles-Torres *et al.*, 2018; Wang *et al.*, 2017). One of the most extensively studied host-defence compounds is the salicylic acid (SA) that is synthetized from multiples pathways including the phenylpropanoids pathway (Dixon *et al.*, 2002). The SA-dependent signaling pathway leads to the expression of certain pathogenesis-related (PR) proteins that are toxic to invading fungal pathogens (Sherif *et al.*, 2012). In addition to PR proteins, small antimicrobial peptides (generally called defensins) work as host defence compounds relevant in the induced defence system (Wilmes *et al.*, 2011). In peach (*Prunus persica*), a defensin 1 (DFN1) has been described as inductor of destabilization and permeabilization of fungal membranes (Nanni *et al.*, 2013). DFN1 production coupled with the synthesis of SA and phenolics is the main response activated in *P. persica* against *Taphrina deformans* (Svetaz *et al.*, 2017). All these pathogen-induced compounds could be generally described as chemical weapons which function is to combat pathogenic agents (Van Loon *et al.*, 2006).

Related to the varietal susceptibility in *Prunus* species, differential levels of phenolic compounds in peach varieties showing differences in susceptibility to the brown rot fungus *Monilinia fructicola* have been reported (Bostock *et al.*, 1999; Lee and Bostock, 2007). To date, no comparable information is available for almond, which reflects the lack of knowledge about the gene regulation induced by biotic stress in this tree species.

According to this preliminary scenario, we aimed to characterize the gene-mediated response as well as the lignin deposition in tolerant and susceptible almond cultivars against the infection of *P. amygdalinum*. The relative expression of key genes from different plant defence pathways and the microscopic visualization of lignin in the fungal infected tissues would indicate us the induced strategies (physical and/or chemical) to repress the disease.

Two Spanish cultivars, 'Mardía' (tolerant) and 'Tarraco' (susceptible) were analyzed along the sequential development of disease symptoms.

5.3 Materials and methods

5.3.1 Plant material

'Mardía' and 'Tarraco' almond trees were sampled in an experimental orchard owned by IRTA and located in Les Borges Blanques, Catalonia, Spain (UTM 31 T, X = 320870, Y = 4597530). Individual samples consisting of three leaves were obtained from RLB symptomatic and asymptomatic leaves with four trees (replicates) sampled for each cultivar. Leaves were collected at two different sampling dates: period A (samples harvested in July 5, 2016 with low RLB incidence), and period B (samples harvested in July 28, 2016 with higher RLB incidence). Collected leaves were wrapped with aluminium foils conveniently labelled, immediately frozen in liquid nitrogen in the field and brought to the laboratory. Frozen samples were finely pulverized in liquid nitrogen and then stored at –80°C until subsequent processing.

5.3.2 Pathogen quantification and visualization in almond leaves

DNA from about 100 mg of the pulverized leaf samples was extracted using the NucleoSpin® Plant II kit (Macherey-Nagel, Düren, Germany) following manufacturer's instructions. Extracted DNA was quantified and quality checked with a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). A specific pair of primers for quantification of *P. amygdalinum* was used: I2F4 [5′- GAAGTCCAATCAAGCCGTAG -3′] and I2R2 [5′-GTTTCACTACGCT CAGAGTC-3′] (forward and reverse, respectively) generating a 99 bp fragment (Zúñiga *et al.*, 2018). Fungal quantification was normalized by the *pre-mRNA splicing factor 7* (*SLU7*) gene of *P. dulcis* used as reference. qPCR was performed on a CFX 384 Real-Time System (Bio-Rad, Hercules, CA, USA). Each reaction contained 5 μL of iTaqTM Universal SYBR®Green Supermix (Bio-Rad, Hercules, CA, USA), 1 μL of the sense and antisense primers and 3 μL of a DNA concentration standardized for all samples. The amplification program in all reactions was performed by pre-incubating the DNA for denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and then 60°C for 30 s. To assess the amplification specificity, melting curve

analysis was always performed at the end of the qPCR reaction, by monitoring the fluorescence from 55 to 95°C, every 0.1°C. Three technical replicates were used in all reactions. In addition, a negative control without DNA template were also included in each reaction run. The fungal amount was estimated indirectly from the relative quantification of the fungus in all samples corresponding to four trees per variety and sampling period.

Mycelial colonization of *P. amygdalinum* in almond infected leaves, was visualized using Trypan-blue staining as follows. Asymptomatic leaf areas as well as areas with typical RLB symptoms, from both cultivars, were cut in sections with a sterile scalpel (˜40 µm thickness) and chlorophyll was removed to enable a clear observation according to the protocol of Liberato *et al.* (2005). For the chlorophyll removal, leaf sections were immersed in 100% glacial acetic acid and absolute ethanol (1:1, v/v), placed in a water bath at 60°C (1 h) and, then dyed with Trypan Blue according to the protocol of Toscano-Underwood *et al.* (2003). Leaves sections, were mounted on microscope slides and observed under a light microscope (Nikon Eclipse E400, Tokyo, Japan) equipped with a digital camera (Nikon).

5.3.3 RNA extraction and cDNA synthesis

Total RNA was extracted from around 100 mg of frozen pulverized leaves using the Maxwell® RSC plant RNA kit (Promega Corporation, Madison, WI, USA) according to manufacturer's instructions. Extracted RNA was quantified and quality checked as described in Section 5.3.2 One microgram RNA was used in a reverse-transcription reaction to obtain cDNA using the cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions.

5.3.4 RT-qPCR and gene expression analyses

The expression of seven selected genes involved in defence response were analyzed by reverse-transcription quantitative PCR (RT-qPCR). The studied defence genes were selected from previous works (Melan *et al.*, 1993; Beckman, 2000; Dixon *et al.*, 2002; Sherif *et al.*, 2012; Shahzad *et al.*, 2013; Martos *et al.*, 2016). These genes encode for defensin 1 (*DFN1*), pathogenesis-related protein 1 (*PR1*) and the lipoxygenase enzyme (*LOX*), that synthesizes products of fatty acid oxidation called oxylipins (Porta and Rocha-Sosa, 2002). Furthermore, genes involved in the phenylpropanoids pathway were also included, such as cinnamate 4-

hydroxylase (*C4H*), hydroxycinnamoyl CoA quinate hydroxycinnamoyl transferase (*HQT*), and cynnamoyl alcohol dehydrogenase (*CAD*). A late structural gene involved in the anthocyanin biosynthesis from the flavonoid pathway, the leucoanthocyanidin dioxygenase (*LDOX*) was additionally selected. The coding sequences for all genes were taken from the Rosaceae database (https:// www.rosaceae.org) and sense and antisense primer pairs (Supplementary Table S5.1) were designed with the aid of the primer-designing tool of GenBank (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The *PR1* primer pair was designed over the almond genome, whereas other reference-guided genomes used were those of *P. persica* (*CAD*, *DFN1*, *HQT*, *LDOX*, *LOX*, and *SLU7*) and *P. armeniaca* (*C4H*). The almond *SLU7* gene was use as reference to normalize the relative expression (RE) of all studied genes, as done in previous works (Chan *et al.*, 2014; Yeap *et al.*, 2014). In addition, *SLU7* showed the most constant expression level as compared to other analysed reference genes in preliminary analyses (*TEF1*, translation elongation factor 1α and *β-TUB*, β-tubulin) (*results not shown*). RE of targeted genes were obtained through the $2^{-\Delta Ct}$ formula as described by Livak and Schmittgen (2001). RE values for each defence-related gene were averaged on the basis of each variety and sampling period.

5.3.5 Validation of designed primers used in RT-qPCR

The suitability of primers designed in this study was tested by sequencing the generated amplicons from *P. dulcis* samples and those from field-collected leaves of *P. persica* and *P. armeniaca.* Leaves of *P. persica* and *P. armeniaca* were collected in 2017 in commercial peach and apricot orchards located in Avinyonet del Penedès, Catalonia (Spain), and processed for RNA extraction and cDNA synthesis as explained above.

Conventional PCR followed by nucleotide sequencing were used to identify and characterize the targeted almond defence-related genes. Reactions were performed in a final volume of 20 µL containing 2 µL H_2O HPLC-grade, 10 µL 2 × PCR Master Mix (Biotools B&M Labs, S.A., Madrid, Spain), 1.5 µL of each primer, and 5 µL of sample cDNA. The PCR were performed in a GeneAmp® 9700 thermal cycler (Applied Biosystems®, Foster City, California, USA), with the following conditions: initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 45 s, primer annealing at 60°C for 60 s, and extension at 72°C for 60 s, and a final extension at 72°C for 5 min. Amplicons were purified using the High Pure PCR Product Purification kit (Roche Diagnostics GmbH,

Manhein, Germany) and sequenced by GATC Biotech (Köln, Germany). Sequences were aligned in pairs of *P. dulcis* and *P. persica* or *P. armeniaca* with the BioEdit Sequence Alignment Editor (Hall, 1999).

The sequenced *P. dulcis* regions were then submitted to the basic local alignment search tool (BLAST) of GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to search for similarities with deposited reference sequences. The percentage of identity with the first significant alignment for each specific region and plant species were recorded.

5.3.6 Histological observation of lignin deposition in almond leaves

The deposition of lignin was studied in RLB-affected leaves of both selected cultivars. Lignin was stained from leaves of both studied cultivars at different stages of the sequential development of RLB symptoms. RLB lesions from leaves with initial yellowish discolorations and further orange-reddish blotches (stages 1, 2 and 3, as described by Zúñiga *et al.*, 2018) were selected. Transverse sections of leaves were prepared and manipulated as explained in Section 5.3.2 for the observation of fungal colonization in leaf tissues. Lignin was dyed with Wiesner staining (Phloroglucinol–HCl) as described by Pradhan Mitra and Loqué (2014) and visualized with an optical microscope. Sixty leaves per cultivar and symptom stage were stained and observed. Asymptomatic almond leaves were also analyzed to determine the basal content of lignin in both cultivars.

5.3.7 Statistical analyses

Data from the relative expression of defence genes were analyzed using lineal modelling in R (R Core Team, 2017) and a multifactorial design approach. The original data were log-transformed prior to the analyses to ensure the normal distribution and homocedasticity of data and the independency of errors. Previously, means of technical replications were calculated for each individual combination of factors to reduce pseudoreplication. A preliminary analysis was done with a saturated model including the fixed effects of cultivar (1 df), sampling period (1 df), symptom expression (1 df), defence genes (6 df) and all their possible interactions. Posterior analyses were done separately for each defence gene but keeping the multifactorial approach. Depending on the ANOVA outputs, the models obtained for each individual gene were later simplified by stepwise deletion of non-

significant factors and interactions using the R anova function and the LRT option. Since all factors and their interactions in the individual gene models had 1 df, the significance was declared at $P < 0.05$ and no further mean comparisons were done.

5.4 Results

5.4.1 Leaf detection of Polystigma amygdalinum

Polystigma amygdalinum was positively detected by qPCR in leaves exhibiting typical symptoms associated to the RLB (Figure 5.1). This result confirmed that the specific primers designed by Zúñiga *et al.* (2018) targeted correctly the fungal DNA in infected almond leaves. The fitted linear model showed statistical significance of the factors cultivar ($P < 0.001$) and symptom expression ($P < 0.001$), along with the interaction cultivar × symptom ($P < 0.001$) on the relative quantification (RQ) of *P. amygdalinum*. However, the period was not found statistically significant including the interactions with the other two factors. The highest fungal amount was quantified in the symptomatic leaves of the susceptible 'Tarraco' as compared to 'Mardía' which RQ values remained close to zero (range: 0.002 to 0.049, despite of the infective stage and period). Mean RQ value in asymptomatic 'Tarraco' leaves were 0.013 and 0.036 at early and late periods, respectively, whereas same figures in symptomatic leaves were dramatically increased, up to 13.229 (early) and 18.902 (late) (Figure 5.1).

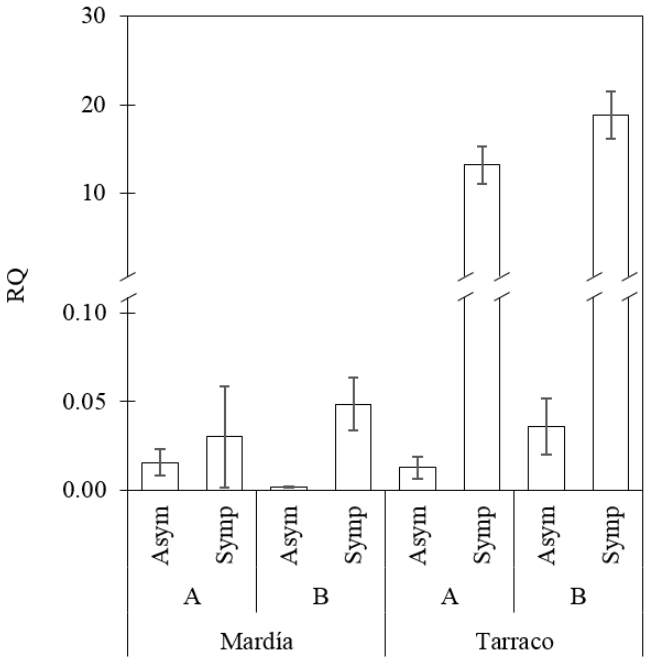

Figure 5.1 Mean values of *Polystigma amygdalinum* relative quantification (RQ) in two almond cultivars, 'Mardía' and 'Tarraco', under early and advanced infection stages. RQ values are shown with a break in Y-axis because of higher observed RQ values in symptomatic 'Tarraco' leaves.
Abbreviations:
RQ, relative quantification;
Asym, asymptomatic leaves;
Symp, symptomatic leaves;
A and B, early and late sampling periods, respectively.
Error bars show the standard error of the mean.

Additionally, the presence of hyphae presumably corresponding to *P. amygdalinum*, in leaves exhibiting RLB typical symptoms, was visualized with the aid of Trypan-blue staining (Figure 5.2). The microscope images confirmed that fungal colonization was limited by the lesion boundaries, without hyphae spreading out of the affected area.

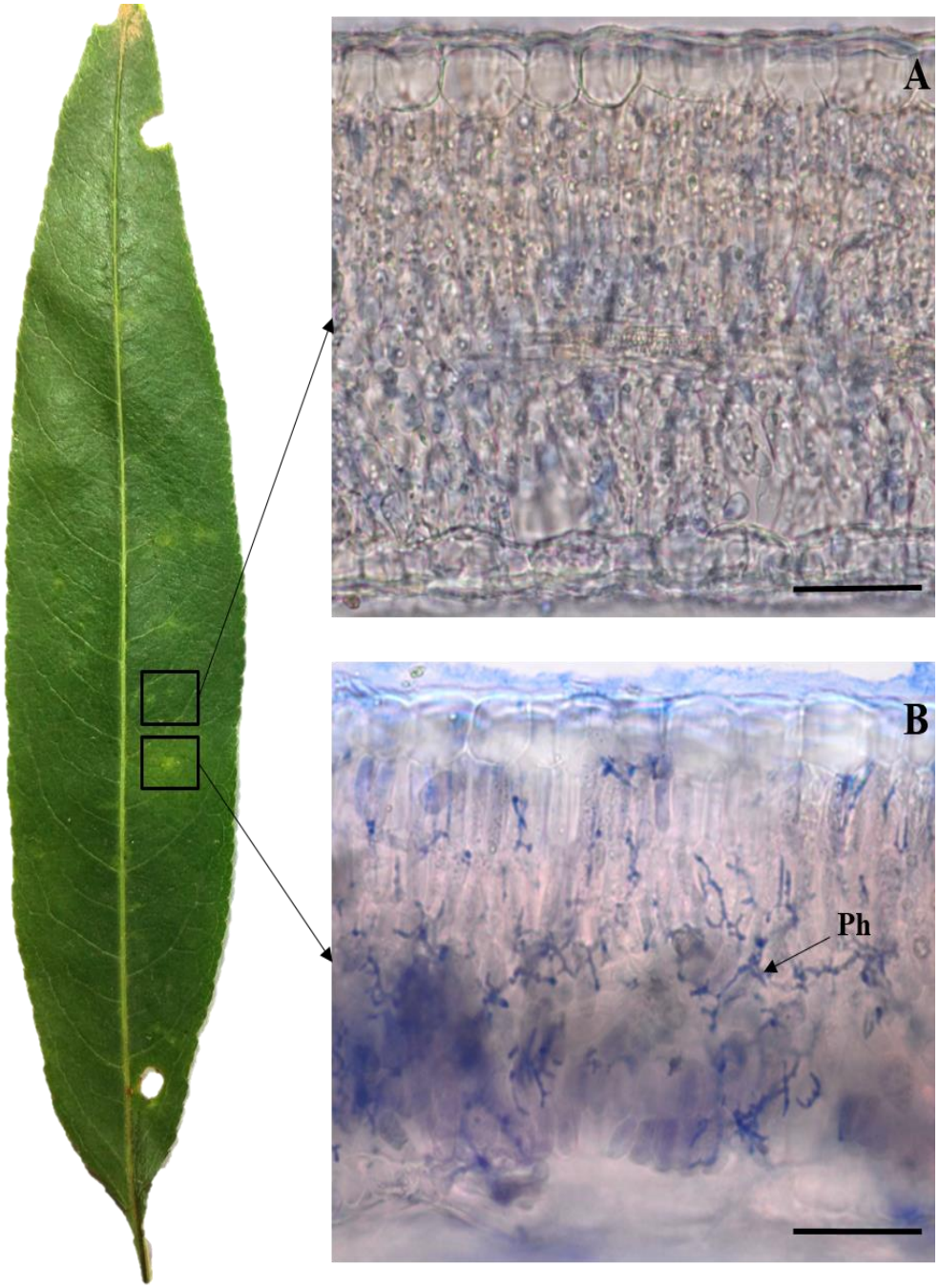

Figure 5.2 Transversal sections of asymptomatic (A) and symptomatic (B) areas after Trypan Blue staining to show the mycelial colonization by *Polystigma amygdalinum* in a representative almond leaf ('Tarraco'). Abbreviations: Ph, *Polystigma amygdalinum* hyphae. Microscope magnification: ×200. Bar = 50 µm.

5.4.2 Suitability of designed primers

The suitability of primers designed in this study to amplify targeted *P. dulcis* genes was checked by different methods. Firstly, band sizes for the seven *P. dulcis* genes were similar to those obtained for the guide genomes of *P. persica* and *P. armeniaca* (*results not shown*). Secondly, the alignments of primers pairs between *P. dulcis* and either *P. persica* or *P. armeniaca* showed a 100% of similarity with the reference-guided genomes (*results not shown*). Finally, BLAST searches at GenBank revealed between 95 and 100% of identity with the same targeted genes belonging to *P. persica* and *P. armeniaca* (Supplementary Table S5.2). These results confirmed that the designed primers were suitable to be used on *P. dulcis*.

5.4.3 Gene expression of defence genes

The mean values of the relative gene expression for each defence gene as a combination of all main considered factors are shown in Figure 5.3. The preliminary ANOVA on the whole dataset of the relative expression of defence genes showed the significance of the factor symptom expression ($P = 0.001$), and the 2-way interaction gene × cultivar ($P = 0.008$). In addition, the factor defence gene and the interaction gene × symptom were near significance ($P = 0.066$ and $P = 0.055$, respectively). Moreover, no 3-way interactions and the 4-way interaction were found significant. A simplified model was consequently fitted by removing all 3-way interactions and higher and reanalyzing the data. The new model showed the significance of factors gene ($P = 0.001$), and symptom expression ($P < 0.001$), and the interactions gene × cultivar ($P < 0.001$), gene × symptom ($P = 0.002$), gene × period ($P = 0.034$), cultivar × period ($P = 0.016$), and symptom × period ($P = 0.007$). These results led us to run separate analyses for each defence gene. The models were again simplified by removing non-significant factors and interactions. Summarized results of the analyses and the significance of factors are shown in Table 5.1

The genes *C4H*, *LOX* and *PR1* did not show significant 2-way interactions of the main factors, and in the latter two genes, a significant cultivar effect was detected in the relative gene expression. *CAD*, *DFN1* and *HQT* genes showed 2-way interactions between the main factors: cultivar × symptom in case of *DFN1* and *HQT*, cultivar × period in case of *CAD*, and symptom × period in case of *CAD* and *DFN1* (Table 6.1, Figure 6.3). Finally, the gene *LDOX* showed a significant 3-way interaction, with no other significance detected among factors.

Table 5.1 F-values (F) and significance (*P*) of the fitted models in the study of expression of defence genes in two almond cultivars ('Mardía' and 'Tarraco') showing differential susceptibility to the red leaf blotch pathogen *Polystigma amygdalinum*.

Gene	Main factors			Interactions				Model		
	CV	*Sym*	*Per*	*CVxSym*	*CVxPer*	*SymxPer*	*CxSxP*	*F*	*Res df*	*P*
C4H	F=1.227 ns	F=26.208 *P*<0.001	F=1.935 ns					10.060	27	<0.001
CAD	F=4.629 *P*=0.041	F=8.876 *P*=0.006	F=0.003 ns	F=0.042 ns	F=5.250 *P*=0.031	F=6.938 *P*=0.014		3.544	25	0.011
DFN1	F=1.661 ns	F=10.443 *P*=0.004	F=0.163 ns	F=4.468 *P*=0.045	F=1.311 ns	F=8.921 *P*=0.006		4.722	24	0.003
HQT	F=6.560 *P*=0.018	F=0.004 ns	F=0.027 ns	F=5.081 *P*=0.034	F=3.825 ns	F=0.069 ns		14.24	22	<0.001
LDOX	F=1.231 ns	F=0.005 ns	F=1.497 ns	F=2.545 ns	F=0.668 ns	F=3.149 ns	F=5.596 *P*=0.028	8.652	21	<0.001
LOX	F=6.429 *P*=0.017	F=0.991 ns	F=3.071 ns					3.501	27	0.028
PR1	F=14.906 *P*<0.001	F=4.276 *P*=0.047	F=17.038 *P*<0.001					12.070	28	<0.001

Abbreviations: CV, cultivar; Sym, symptom expression; Per, sampling period; Res df, Residual degrees of freedom.

The relative expression of genes depending on the infection status showed significant differences in the case of *C4H* and *PR1* genes, with consistent higher expression in symptomatic leaves than in the asymptomatic ones. Regarding the sampling period, this factor was shown to be only significant in the relative expression of *PR1*. Genes *CAD* and *DFN1* showed a similar pattern of expression in 'Mardía', with highest mean values (4.93, 4.23, respectively) observed in symptomatic leaves sampled early in the season, as compared to the remaining values (Figure 5.3B and C).

On the other side, *C4H*, *HQT* and *LDOX* were highly expressed in 'Tarraco' symptomatic leaves sampled as compared to asymptomatic ones (Figure. 5.3A, D and E). 'Tarraco' showed a higher relative expression for *LOX* and *PR1* (mean values over periods and infection status: 2.26 and 2.40, respectively) as compared to 'Mardía' (1.38 and 1.23, respectively). In addition, the sampling period and the infection status were statistically significant in *PR1*, with higher expressions for the late period and, separately, in the symptomatic leaves. Finally, a significant effect of the infection status was detected for *C4H* and *PR1*, thus indicating increased RE at symptomatic leaves compared to the asymptomatic ones (mean values: 2.98 and 0.65, respectively) (Figure 5.3A and G).

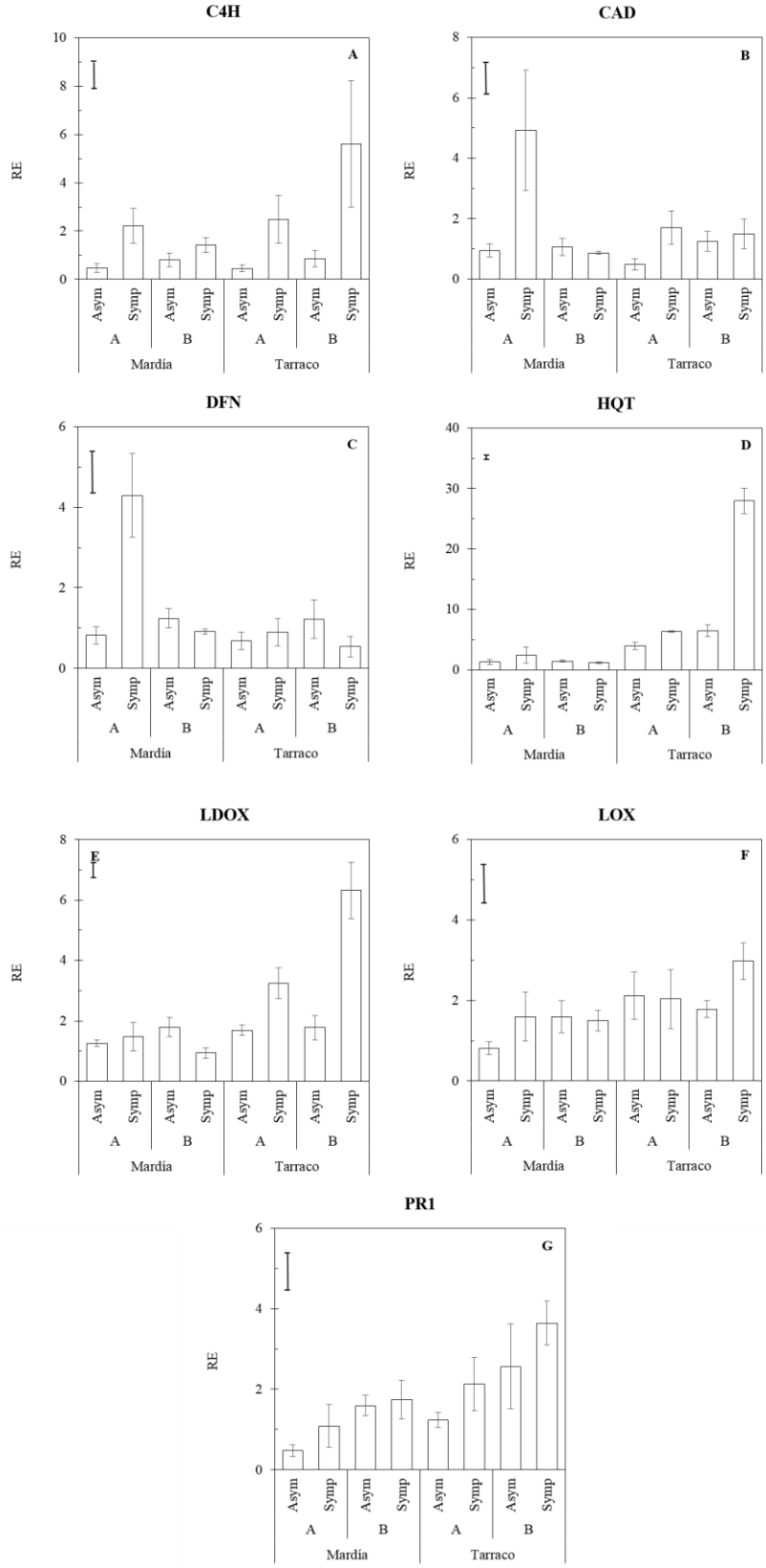

Figure 5.3 Mean values of seven defence genes expression in two almond cultivars, 'Mardía' and 'Tarraco', showing differential susceptibility to *Polystigma amygdalinum*. Abbreviations: RE, relative expression; Asym, asymptomatic leaves; Symp, symptomatic leaves; A and B, early and late sampling periods, respectively. Gene names at the top of each graph. Error bars show the standard error of the mean (N = 4). Least significant difference values shown as bold bars in the top left part of graphics.

5.4.4 Lignin deposition in leaf fungal-infected areas

RLB asymptoms leaves did not show red coloration associated to Wiesner staining of lignin in any cultivar (Figure 5.4A and E). The positive detection of lignin was observed on earlier sampled leaves of 'Mardía' (Figure 5.4B and C).

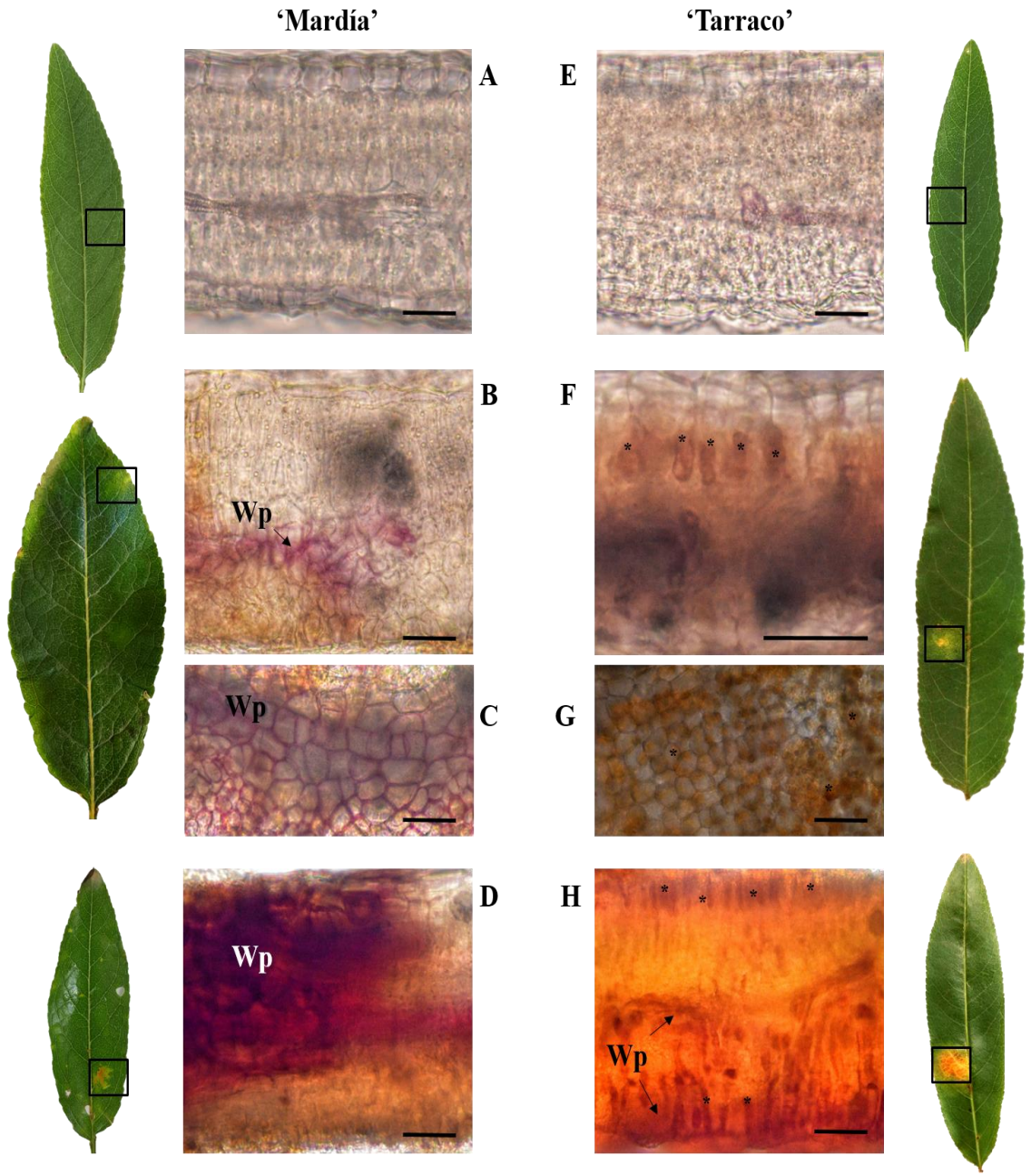

Figure 5.4 Microscope images of asymptomatic, initial and advanced symptoms caused by *Polystigma amygdalinum* infection in 'Mardía' (A to D) and 'Tarraco' (E to H) leaves, stained with Wiesner staining. Representative images from 60 observed leaves per cultivar and stage of symptomatology. A and E, Non-infected; B, C, F, G, initial symptoms; D and H, advanced symptoms. Transversal sections in A, B, D, E, F and H. Upper view in C and G. Legends: Wp, Wiesner-positive; *, Dead cells. Microscope magnification: ×200 (A, B, C, D, E, G, H); ×400 (F). Bar = 50 µm.

Deposition of lignin was clearly localized in cell walls as it can be observed in Figure 5.4C. Similarly, leaves of 'Mardía' with advanced RLB symptoms showed higher lignin accumulation as compared to 'Tarraco' (Figure 5.4D and H, respectively). On the contrary, 'Tarraco' leaves were determined Wiesner-negative in symptomatic leaves collected at early stages of infection, since red coloration was absent in the observed structures (Figure 5.4F and G).

On the advanced symptoms stage (Figure 5.4H), 'Tarraco' presented red lignin coloration more expanded and less accumulated as compared to 'Mardía'. However, symptomatic leaves of 'Tarraco' were characterized by noticeable orange-reddish blotches at both infection stages. Microscopic observation revealed the diffuse presence of this orange color that could not be designated to any plant structure and remained even after chlorophyll removal (Figure 5.4G and H). Additionally, a higher number of swollen and dead cells were found in the RLB symptomatic leaves of 'Tarraco' as compared to 'Mardía'.

5.5 Discussion

Almond susceptibility to RLB has been determined to date based on field observations of symptom incidence and severity (Egea *et al.*, 1984; Saad and Masannat, 1997; Vargas *et al.*, 2011; Marimon *et al.*, 2012; Ollero *et al.*, 2016). So far, the molecular and biochemical bases of this pathogen-plant interaction had not been studied. This is the first approach to understand these susceptibility differences, by analyzing the relative expression of selected putative defence genes of two almond cultivars, 'Mardía' (tolerant to RLB) and 'Tarraco' (susceptible to RLB), under the hypothesis that changes in their transcriptomes would reflect their differential susceptibility response. The seven selected genes studied here comprise five different plant defence strategies: i) phenylpropanoids pathway represented by *C4H*, *HQT* and *CAD* genes, ii) flavonoids pathway by *LDOX* gene, iii) defensin (*DFN1*), iv) pathogen-related protein (*PR1*) and, v) fatty acid oxidation catalyzed by the lipogenase enzyme (*LOX*). Four of the seven selected genes are involved on the phenylpropanoids and flavonoids pathways deriving into the biosynthesis of lignin and anthocyanin, respectively. Thus, the lignin deposition was also visualized, as it is one of the best-known plant mechanism induced after pathogen attack (Maher *et al.*, 1994; Dixon *et al.*, 2002; Tronchet *et al.*, 2010; Xu *et al.*, 2011). The pathogen-induced response is the cell wall lignification of

the infection-surrounding cells that creates a reinforced physical barrier more efficient to confine the pathogen expansion (Agrios, 2005).

Statistical analysis of gene expression by RT-qPCR indicated that 'Mardía' and 'Tarraco' were reliable candidates for a comparative molecular analysis of susceptibility to RLB. Our results revealed a cultivar-dependent expression pattern of the selected genes. Up-regulation of *CAD* in the tolerant cultivar 'Mardía' during the early expression of RLB symptoms was relevant. *CAD* belongs to the phenylpropanoids pathway as a structural biosynthetic enzyme involved in the late step of the monolignol synthesis (Cheng *et al.*, 2017). Pathogen infections strongly activate the phenylpropanoids pathway in plants and it has been widely documented for fungal pathogens (Xu *et al.*, 2011; Dhokane *et al.*, 2016). Compounds derived from the penylpropanoids pathway show a broad-spectrum of antimicrobial activity and some of them have been specifically associated to *Prunus* pathogens (Bostock *et al.*, 1999; Lee and Bostock, 2007; Villarino *et al.*, 2011). Several lines of evidence indicate that the reinforcement of plant cell walls, mainly lignification, is associated to increased tolerance to microbial pathogens (Dixon *et al.*, 2002; Xu *et al.*, 2011; Hu *et al.*, 2018). *CAD* inhibition resulted in reduced lignin content and increased disease susceptibility to *Peronospora parasitica* in *Arabidopsis* (Mauch-Mani and Slusarenko, 1996). In our study, the increased induction of *CAD* would indicate a greater lignin accumulation in the tolerant cultivar. In fact, 'Mardía' displayed lignin deposition around cells in the infected area in leaves with early and more advanced RLB symptoms, as confirmed by the lignin staining. On the contrary, 'Tarraco' leaves did not show lignin accumulation surrounding the cells in the early infection stages, and weak Wiesner-positive staining was observed only in advanced symptoms. This is in accordance with a higher expression of *C4H* and *HQT* in 'Tarraco' at late infection stages; these genes are involved in first steps of the phenyl-propanoids pathway. Accumulation of lignin has been already described in *Prunus* spp. affected by the shot-hole disease (caused by the fungus *Wilsonomyces carpophilus*), specifically in the cells layers nearest the infection (Samuel, 1927). It is suggested that the early intense response in 'Mardía' triggers cell wall lignification that repress the pathogen growth and thus characterize the cultivar with a tolerant profile. Conversely, the accumulation of lignin occurred in advanced infections stages in 'Tarraco', but appeared to be ineffective as the pathogen managed to establish the infection. Furthermore, the increased expression of *DFN1* gene in 'Mardía' could indicate the activation of other defence mechanism such as the production of defensins, peptides with described toxic activity for

pathogens (Stotz *et al.*, 2009). Defensins play a role in plant innate immunity displaying antifungal activity against wide spectrum of pathogens (Nanni *et al.*, 2013). It has been repported that transgenic plants overexpressing defensins have been shown as highly resistant against fungal pathogens (Stotz *et al.*, 2009).

The flavonoids pathway is also activated after pathogen attack with a significant contribution to plant resistance (Treutter, 2005). Flavonoids are classified in six major groups, being anthocyanins one of those groups (Falcone Ferreyra *et al.*, 2012). Similar to lignin, anthocyanins are synthesized from Phenylalanine, and both pathways share the first three steps (Shi *et al.*, 2015). Recently, Usenik *et al.* (2017) showed that *Plum pox virus* (Sharka) promotes the accumulation of anthocyanins among other flavonoid compounds in the necrotic tissues of *Prunus domestica*. A late gen of the anthocyanins pathway is *LDOX*, which several evidences demonstrated its critical role in anthocyanin accumulation in members of the Rosaceae family (Aharoni *et al.*, 2001; Salvatierra *et al.*, 2013). This gene showed a higher expression on symptomatic leaves of the susceptible cultivar when the RLB symptoms were advanced. This resulted in an orange-reddish pigment accumulation in infected 'Tarraco' leaves, as it was described by Tanaka *et al.* (2014) in maize plants challenged by *Ustilago maydis*. Flavonoids were accumulated diffusely in the symptomatic area, not localized to any plant structure, dissimilarly to lignin that is accumulated in cell walls. We hypothesize that an increase in anthocyanin production negatively affected the lignin biosynthetic pathway by reducing the levels of the common precursor p-coumaric acid. This was already evidenced by Van der Rest *et al.* (2006) who studied tomato transgenic plants exhibiting reduced lignin content but higher amounts of chlorogenic acid and rutin. The increased activity of *HQT* observed in our study would deviate the p-coumaric acid to the synthesis of chlorogenic acid instead of lignin that hence reduced its potential protective effects. It was observed that 'Tarraco' activated the production of lignin later than 'Mardía' and also triggered alternative responses (anthocyanins and phenolic acid synthesis) which, all in all, would have reduced lignin accumulation in infected leaves and thus failed to repress the growth and infection of *P. amygdalinum*. This should be further investigated in order to confirm this hypothesis.

The molecular detection of *P. amygdalinum* in leaves was correlated to the cultivar susceptibility observed on the field. Higher amounts of fungal biomass were estimated in 'Tarraco' symptomatic leaves, while pathogen quantification in 'Mardía' was minimal

despite of the infection stage. In both cultivars, *P. amygdalinum* was detected in asymptomatic leaves, although with minimal values. This could be due to: 1) the high sensitivity of the specific primers, as Zúñiga *et al.* (2018) reported a minimum detection of 7 ascospores, and 2) the extreme long latency period (30–35 days) reported for *P. amygdalinum* (Banihashemi, 1990; Saad and Masannat, 1997) which could allow the fungus for a sufficient mycelial accumulation in leaves to be detected by qPCR even in absence of noticeable symptoms.

The already known biology data recorded for *P. amygdalinum* (Banihashemi, 1990; Cannon, 1996; Habibi and Banihashemi, 2015) combined with the images presented in this study would indicate a hemibiotroph lifestyle of the pathogen. Plant pathogens classified as hemibiotrophs show a multistage infection strategy (O'Connell *et al.*, 2012). In this type of pathogen-plant interactions, several genes and cross-talking signals between pathways are involved, such as the SA signaling pathway (effective against biotrophs) and the jasmonic (JA) and ethylene (ET) signaling pathways (effective against necrotrophs) (Kunkel and Brooks, 2002). The genes studied here (*C4H*, *HQT*, *LOX* and *PR1*) are involved in these pathways but it would be risky to jump onto solid conclusions and more genes should be studied. Our preliminary results opens the door to make future investigations focusing on other defence pathways in order to draw a complete schema of the almond strategies to fight against the RLB disease. Moreover, RNAseq could be a comprehensive approach to discover the set of genes participating in the coordinated plant response to the RLB disease.

5.6 Conclusion

The RT-qPCR technique may represent a suitable and economic tool to screen almond cultivars for tolerance to RLB. From our results, it can be concluded that 'Tarraco' and 'Mardía' have different molecular and biochemical strategies to face *P. amygdalinum* that could explain their differential susceptibility to the fungal disease. The tolerant cultivar 'Mardía' displays a lignin accumulation in infected leave tissues and likely the production of antifungal compounds (defensins); a dual strategy that would combine physical and chemical responses to repress the fungal spread during the early expression of RLB symptoms. On the contrary, the susceptible cultivar 'Tarraco' activated later the lignin synthesis likely together with the production of anthocyanin and phenolic acid, showing to

be ineffective in inhibiting fungal growth. The lignin biosynthesis was revealed as a key response to repress the RLB pathogen at early stages of the disease spread.

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5.8 Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interests.

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Appendix A. Supplementary data Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jplph.2019.03.004.

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Table S5.1 of Supplementary Data. Nucleotide sequences and amplified fragments sizes of the primers designed in this study.

Gene	Primer name	Sense	Sequence 5'-> 3'	Fragment size (bp)
Cinnamate 4-hydroxylase (*C4H*)	ParC4H-F	F	AGGTTCGAGAGCGAGGATGA	83
	ParC4H-R	R	TCGAAGCTCTGAGCCAATCG	
Cynnamoyl alcohol dehydrogenase (*CAD*)	PpeCAD-F	F	AGGTGGTTGGTGAGGTGTTG	184
	PpeCAD-R	R	GTGGGTTTGCCATCGGAGTA	
Defensin 1 (*DFN1*)	PpeDFN1-F	F	GAGCGCTCCATGCGTTTATT	98
	PpeDFN1-R	R	CAGGTCCTAGCCTCAGCAAC	
Hydroxycinnamoyl CoA quinate hydroxycinnamoyl transferase (*HQT*)	PpeHQT-F	F	CGGTCTATGAGCCTCAACCC	93
	PpeHQT-R	R	TGGGTAGCTGAGGAAGTCCA	
Leucoanthocyanidin dioxygenase (*LDOX*)	PpeLDOX-F	F	GGTGTCGAAGCTCACACTGA	98
	PpeLDOX-R	R	CAGTGACCCACTTGCCTTCA	
Lipoxygenase (*LOX*)	PpeLOX-F	F	ATCACCGTTGATCCACACCC	146
	PpeLOX-R	R	AGAAACCACTTTGCCCCACA	
Pathogenesis-related protein 1 (*PR1*)	PduPR1-F	F	TAAACGTCGGGCCATTGTCA	82
	PduPR1-R	R	TTGCAGTCGCCAATGTGTTG	
Pre-mRNA splicing factor 7 (SLU7)	PpeSLU7-F	F	TGGAATTGAGGCTGCTGAGG	117
	PpeSLU7-R	R	GTTCCCCAAGTGGCAAGTCT	
β-tubulin (β-TUB)	PpeBTUB-F	F	AGGTTTTCAGGTTTGCCACT	266
	PpeBTUB-R	R	GGGTGCTGAGCTTTAGGGTC	
Translation elongation factor 1α (TEF1)	PpeEF1-F	F	GAACGTGTCTCGGTGGTACA	108
	PpeEF1-R	R	TCCTCTGTGATGGGAGCAGA	

Table S5.2 of Supplementary Data. Homology of amplified gene regions in *Prunus* species according to BLAST searches at GenBank.

Gene	Species	Identities (%)	Nucleotide matches
C4H	*P. dulcis*	96	80/83
	P. armeniaca	98	79/81
CAD	*P. dulcis*	99	183/184
	P. persica	100	184/184
DFN1	*P. dulcis*	98	96/98
	P. persica	98	96/98
HQT	*P. dulcis*	100	41/41
	P. persica	100	41/41
LDOX	*P. dulcis*	99	96/97
	P. persica	100	97/97
LOX	*P. dulcis*	100	144/144
	P. persica	100	144/144
PR1	*P. dulcis*	95	78/82
	P. persica	96	79/82
SLU7	*P. dulcis*	100	117/117
	P. armeniaca	100	117/117
	P. persica	100	117/117

Chapter 6

Chapter 6: General discussion

In this PhD thesis, some key aspects of the red leaf blotch (RLB) disease of almond, caused by the ascomycete *Polystigma amygdalinum*, were studied regarding: i) disease epidemiology (Zúñiga *et al.*, 2019b); ii) detection and quantification of *P. amygdalinum* by qPCR-based methods (Zúñiga *et al.*, 2018), and iii) molecular and biochemical bases of cultivar susceptibility to the RLB (Zúñiga *et al.*, 2019a). The results enabled us to expand the knowledge on major aspects of the RLB in Spain, while keeping the door open to go further on the research on this disease.

Most of the world knowledge about the RLB is based on investigations carried out in Eastern Mediterranean and Middle East countries, namely Iran, Lebanon, and Turkey (Ashkan and Assadi 1974; Banihashemi 1990; Bayt-Tork *et al.* 2014; Cimen and Ertugrul, 2017; Ghazanfari and Banihashemi, 1976; Saad and Masannat 1997). These works focused on the disease incidence in different almond-growing areas, the release of ascospores to the environment, the infection and incubation periods of the disease, and the effectiveness of fungicides against *P. amygdalinum* in filed experiments. On the other hand, previous studies conducted in Spain on the RLB consisted mainly on field observations of symptom incidence and cultivar susceptibility (Almacellas *et al.* 2011; Almacellas 2014; Ollero-Lara *et al.* 2016a,b; Torguet *et al.* 2016; Ollero-Lara *et al.* 2019). As it is suggested that monocyclic diseases should be studied locally at each geographical area where the pathogen is reported (Banihashemi 1990; Saad and Masannat 1997; Vicent *et al.* 2011), because of the high dependency of the disease progress on the local environmental factors, our first research objectives were focused on the RLB epidemiology.

Regarding epidemiological topics of RLB, we studied for the first time in Spain the seasonal development of perithecia, the production and germination of ascospores, along with the disease incubation and plant infectivity periods. We confirmed that the primary inoculum of *P. amygdalinum* is present between January and August, which expands the inoculum availability reported in previous investigations (Ashkan and Assadi 1974; Banihashemi 1990; Saad and Masannat 1997). Significant differences in ascospore amounts among regions were detected, which were higher in the southern Andalusia and lower in the northern Catalonia (over 10 times greater in Andalusia in average). Differences in ascospore

amounts were also detected among the years of study. Furthermore, the geographical site, the sampling period and the evaluation year were also found significant on the development of *P. amygdalinum* perithecia. Statistical analysis showed positive correlations between the amount of ascospores in the season and some weather variables from previous fall and winter seasons, which confirms the major role of environmental conditions and the geographical location on the development of the primary inoculum. Banihashemi (1990) suggested that development of *P. amygdalinum* perithecia occurs between fall and winter, and the environmental conditions of each region might affect the development of primary inoculum. This is in accordance with our observations, as the development and maturity of fruiting bodies of *P. amygdalinum* responded to local factors rather than to the origin of the sample, as well as to differences among years of study. However, no correlations were found between the amounts of ascospores and the seasonal weather conditions, which was not expected and difficult to explain. This reinforces the idea that development of primary inoculum should be further studied as no consistent relationships were found between primary inoculum dynamics and current weather conditions of the season.

Ascospores of *P. amygdalinum* are able to germinate and develop the appressorium *in vitro* but failed to grow further. Our results agree with those previously obtained by Habibi and Banihashemi (2015). In our study, germination percentages after 24 h incubation were consistently low, below 30%. These germination rates could be due to the biotrophic nature of the pathogen (Cannon 1996; Habibi and Banihashemi 2015). Germination of ascospores could be useful in further studies, e.g. as a first stage in testing fungicides *in vitro*, especially for those involved in the inhibition of ascospore germination.

The natural infections of RLB in Spain occur between February to May, regardless of the geographical location of the almond-growing area, which is comparable to previous reports (Banihashemi 1990). Temperatures between 10 to 20 °C, are determinant for RLB infections to occur, and infection frequencies decreased when temperatures raised above 20 °C. This suggests that temperatures higher than 20 °C inhibit RLB infections. Furthermore, infections follow an incubation period, which can be as long as 12 weeks. However, as the season progresses incubation period is reduced, until 2 weeks in some occasions. The incubation periods estimated in this study are longer than those reported in previous investigations, roughly 30 to 40 days (Ashkan and Assadi 1974; Banihashemi 1990).

Although, *P. amygdalinum* is considered as a biotrophic pathogen (Cannon, 1996), plant pathogens with a similar multistage infection strategy are also classified as hemibiotrophic pathogens (Marshall *et al*. 2011; Mentlak *et al*. 2012; O'Connell *et al*. 2012). RLB infection starts with a prolonged incubation period with no visible symptoms (the biotrophic phase), followed by the apparition of symptoms (the necrotrophic phase), i.e. the colored and darkening of leaf stroma and pathogen growth causing multiple host cell death (Saad and Masannat 1997; Zúñiga *et al.* 2019). Subsequently, *P. amygdalinum* continues the necrotic phase on fallen leaves (from October to January), during the development and maturation of perithecia and ascospores. Further investigations needs to be conducted on the subject for elucidate completely the lifestyle of *P. amygdalinum*.

The identification of *P. amygdalinum* relies mostly on recognizing RLB symptoms on affected trees and leaves. A tool for the rapid diagnosis of RLB foliar symptoms was developed by López-López *et al.* (2016), based on the analysis of High-Resolution Hyperspectral and Thermal imagery, but this is an indirect detection method. During the early infections stages, RLB symptoms could be confused with those caused by other foliar pathogens of almond such as *Wilsonomyces carpophilus*, causal agent of shot hole disease of several *Prunus* spp. Consequently, we aimed to develop a reliable and direct method for the detection of *P. amygdalinum*. This was achieved by developing a successful qPCR method using the specific primer pair PamyI2F4/PamyI2R2 (Zúñiga *et al.*, 2018). The primer pair performed successfully in terms of specificity and detection limits. Furthermore, the technique was validated in repeated experiments by quantifying the pathogen in both forms, natural sources (naturally-infected leaves), and 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*, which might be a useful tool for general purposes on detection and quantification of *P. amygdalinum* in biological samples.

In this PhD thesis, we conducted the first molecular approach to understand the differential cultivar susceptibility to RLB, by analyzing the relative expression of selected putative defense genes and lignin deposition in two almond cultivars differing in RLB susceptibility, 'Mardía' (tolerant) and 'Tarraco' (susceptible). Under the hypothesis that changes in their transcriptomes would reflect their differential susceptibility response and would reveal the key defense strategies to face the disease. The studied genes comprised different plant

defense strategies: i) phenylpropanoids pathway, ii) flavonoids pathway, iii) defensin, iv) pathogen-related protein and, v) fatty acid oxidation catalyzed by the lipogenase enzyme.

The relative expression of defense genes in 'Mardía' (tolerant) showed a dual strategy that combined both physical and chemical responses to repress *P. amygdalinum* during the early expression of RLB symptoms. The increased induction of the *CAD* gene (involved in the last step in the synthesis of lignin monomers) was confirmed by the positive Wiesner staining of lignin in cells of leaves with early and more advanced RLB symptoms. This cell wall lignification performs as a physical barrier and confine the pathogen expansion (Agrios, 2005). In addition, the increased expression of the *DFN1* gene promoted the activation of defensins, which play a role in plant innate immunity displaying antifungal activity (Nanni *et al.*, 2013). On the contrary, 'Tarraco' only showed a weak lignin accumulation surrounding the cells in advanced symptoms, thus suggesting that 'Tarraco' activated the production of lignin later than 'Mardía' and triggered alternative responses (anthocyanins and phenolic acid synthesis). The gene *LDOX* (a late gene in the anthocyanins pathway) showed a higher expression on advanced RLB symptoms, which resulted in an orange-reddish pigment accumulation in infected 'Tarraco' leaves, also reported in other members of the Rosaceae family (Aharoni *et al.*, 2001; Salvatierra *et al.*, 2013). We hypothesize that an increase in anthocyanin production negatively affected the lignin biosynthetic pathway, as the former biosynthetic pathway reduced the available levels of the common precursor *p*-coumaric acid. In addition, the increased activity of *HQT* observed in 'Tarraco' would also deviate the *p*-coumaric acid into the synthesis of chlorogenic acid instead of lignin that reduced the potential protective effects of lignin.

Our results point to future research focused on develop an accurate epidemiological model to predict infection risk events for RLB. First step toward this goal would integrate the primary inoculum monitoring with the aid of volumetric air samplers and the qPCR-based detection method developed in this PhD thesis. In addition, search for complete the genetic and molecular bases of the resistance/tolerance to RLB that would help to obtain new almond cultivars with improved tolerant profiles against the RLB. Thus, we aim at facing the control of the red leaf blotch of almond in an integrated and sustainable way.

Chapter 7

Chapter 7: Bibliography of General Introduction and Discussion

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Zúñiga, E., Luque, J., Martos, S., 2019. Lignin biosynthesis as a key mechanism to repress *Polystigma amygdalinum*, the causal agent of the red leaf blotch disease in almond. *Journal of Plant Physiolgy* 236, 96–104.

Chapter 8

Chapter 8: Conclusions

- Results from the study on RLB epidemiology showed larger periods of ascospore presence (January - August), infection periods (Feb - May), incubation periods (2-12 weeks) for *Polystigma amygdalinum* than previously reported.

- Environmental conditions in fall and winter such as accumulate rain, maximum relative humidity, and temperatures above 20°C, promote higher inoculum potential of *Polystigma amygdalinum* during the next season.

- Temperatures between 10 to 20°C in spring and summer are key elements for incidence in new infections. Temperatures higher than 20°C inhibit RLB infections.

- The specific primer pair designed on the ITS region of the *Polystigma amygdalinum* rDNA, enabled the identification and quantification in different types of samples, such as DNA suspensions (12 pg of pathogen DNA), ascospore suspensions (seven ascospores), on plastic trapping tapes and from naturally-infected almond leaves.

- Relative quantification of *Polystigma amygdalinum* in infected leaves of two almond cultivars with different RLB susceptibility showed a relative higher amount of the pathogen in leaves of ‘Tarraco’ (susceptible) than in ‘Mardía’ (tolerant) cultivars.

- The study of molecular and biochemical bases of the pathogen-plant interaction in two almond cultivars with differential RLB susceptibility profiles showed that i) ‘Mardía’ displays a dual strategy: lignin accumulation in infected leave tissues and production of defensins to repress the fungal spread during the early expression of RLB symptoms, and ii) ‘Tarraco’ supposedly activated lignin synthesis at later stages, together with the production of anthocyanins and chlorogenic acid, showing to be ineffective in inhibiting fungal growth

Chapter 9

Chapter 9: Annex

9.1 Scientific publications

- Zúñiga, E.; Luque, J.; Martos, S. (2019). Lignin biosynthesis as a key mechanism to repress *Polystigma amygdalinum*, the causal agent of the red leaf blotch disease in almond. Journal of Plant Physiology. 236:96–104.
- Zúñiga, E.; León, M.; Berbegal, M.; Armengol, J.; Luque, J. (2018). Development of a qPCR-based method for the detection and quantification of *Polystigma amygdalinum*, the causal agent of the red leaf blotch of almond. *Phytopathologia Mediterranea.* 57:257–268.

9.2 Submitted manuscripts to scientific journals

- Zúñiga, E., Romero, J., Ollero-Lara, A., Arquero, O., Miarnau, X., Torguet, L., Trapero, A., Luque, J. 2019. Inoculum and infection dynamics of *Polystigma amygdalinum* in almond orchards in Spain. Submitted to *Plant Disease.*

9.3 Technical publication

- Zúñiga, E., Luque, J., Torguet, L., Miarnau, X. 2017. Biología y epidemiología de la mancha ocre del almendro en Cataluña. *Revista de Fruticultura* 57, 6–15.

9.4 Contribution in congresses

- Zúñiga, E.; Luque, J.; Miarnau, X.; Arquero, O.; Lovera, M.; Ollero, A. Roca, L.F.; Trapero, A. Aspectos epidemiológicos de la mancha ocre del almendro (*Polystigma amygdalinum*) en Andalucía y Cataluña XVIII Congreso de la Sociedad Española de Fitopatología. Palencia, del 20 al 23 de septiembre de 2016. Título de la contribución: Aspectos epidemiológicos de la mancha ocre del almendro (*Polystigma amygdalinum*) en Andalucía y Cataluña.
- Zúñiga, E.; Luque, J.; Miarnau, X.; Arquero, O.; Lovera, M.; Ollero, A.; Roca, L.F.; Trapero, A. Epidemiology of almond red leaf blotch caused by *Polystigma amygdalinum* in Spain. VII International Symposium on almond and pistachios. Organizado por: International Society for Horticultural Science. Adelaide, Australia, del 5 al 9 de noviembre de 2017.

ASPECTOS EPIDEMIOLÓGICOS DE LA MANCHA OCRE DEL ALMENDRO (*Polystigma amygdalinum*) EN ANDALUCÍA Y CATALUÑA

Zúñiga, E.[1]; **Luque, J.**[1]; **Miarnau, X.**[2], **Lovera, M.**[3]; **Arquero, O.**[3]; **Ollero, A.**[4]; **Roca, L.F.**[4]; **Trapero, A.**[4]

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INTRODUCCIÓN

La mancha ocre del almendro, causada por el hongo patógeno biótrofo *Polystigma amygdalinum* P.F. Cannon, es una enfermedad común en zonas de clima continental de España. *Polystigma amygdalinum* causa en las hojas manchas de forma variable, entre 1-2 cm de tamaño, de color amarillo al principio y más tarde pardo-rojizo (Fig.1), que reducen la capacidad fotosintética del árbol y que pueden llegar a producir defoliaciones prematuras.

En este estudio se ha evaluado la producción del inóculo y período de infectividad de *P. amygdalinum*, para conocer mejor la biología del patógeno en dos regiones productoras, Andalucía y Cataluña.

Figura 1 Progresión de las lesiones de *Polystigma amygdalinum* en hoja de almendro.

MATERIAL Y MÉTODOS

Maduración de cuerpos frutíferos de *P. amygdalinum*. De hojas con síntomas de mancha ocre obtenidas en Cataluña (2016) se extrajeron 20 cuerpos fructíferos al azar y se clasificaron de acuerdo con su estadío de desarrollo.

Producción y liberación de ascosporas de *P. amygdalinum*. Esta actividad se llevó a cabo en Andalucía (2014-16) y Cataluña (2015-16). Partiendo de hojas infectadas recolectadas quincenalmente, se realizó una extracción de ascosporas en medio acuoso y éstas se contaron al microscopio (×250).

Estimación de los períodos de infectividad y latencia. En las fincas experimentales de Córdoba (2016) y les Borges Blanques (Cataluña; 2015-16), desde febrero hasta finales de agosto, se expusieron diez plantas de 1 año de edad a infecciones naturales de mancha ocre en períodos de dos semanas. Tras su exposición, las plantas se retiraron del campo y se llevaron a invernadero donde se mantuvieron en condiciones óptimas de riego y temperatura hasta que se observó la aparición de los síntomas de la enfermedad.

RESULTADOS Y DISCUSIÓN

Maduración de ascocarpos

Clase	Estadío de desarrollo		
	Peritecio	*Asco*	*Ascosporas*
P	Ausente	Ausente	Ausentes
P[y]	Ausente	Ausente	Ausentes (conidios)
A	Diferenciado, No maduro	Indiferenciado	Indiferenciadas
B	Diferenciado, No maduro	Diferenciado	Indiferenciadas
C	Diferenciado, No maduro	Diferenciado	Diferenciado; < 8 esporas/asco
D	Maduro	Maduro	Maduro; 8 esporas/asco
E	Vacío	Vacío	Vacío

Gandesa

Estadío de desarrollo						
Sem.	P	P[y]	A	B	C	D	E
4	13	0	7	0	0	0	0
6	10	0	10	0	0	0	0
8	18	0	2	0	0	0	0
10	0	7	13	0	0	0	0
12	0	11	9	0	0	0	0
14	8	6	6	0	0	0	0
16	10	0	10	0	0	0	0
18	5	0	8	6	1	0	0
20	0	0	6	9	5	0	0
22	0	0	7	3	7	3	0
24	0	0	8	7	5	0	0
26	0	0	9	8	3	0	0
28	4	0	11	5	0	0	0
30	0	0	0	4	6	4	6
32	0	0	1	12	2	0	5
34	-	-	-	-	-	-	-

Borges

Estadío de desarrollo						
Sem.	P	P[y]	A	B	C	D	E
4	15	0	5	0	0	0	0
6	9	0	11	0	0	0	0
8	15	0	5	0	0	0	0
10	7	1	11	1	0	0	0
12	2	3	15	0	0	0	0
14	2	4	11	3	0	0	0
16	4	1	11	4	0	0	0
18	0	0	2	10	7	1	0
20	0	0	0	2	2	16	0
22	0	0	0	3	6	4	7
24	0	1	0	2	5	9	3
26	0	0	1	1	4	3	11
28	0	0	0	5	2	7	6
30	0	0	0	2	6	7	5
32	0	0	0	4	9	3	4
34	0	0	0	3	3	5	9

Borges/Gandesa*

Estadío de desarrollo						
Sem.	P	P[y]	A	B	C	D	E
4	12	0	8	0	0	0	0
6	17	0	3	0	0	0	0
8	14	0	6	0	0	0	0
10	5	1	11	3	0	0	0
12	0	1	19	0	0	0	0
14	2	1	13	4	0	0	0
16	1	0	7	5	4	3	0
18	0	0	9	7	3	1	0
20	0	0	6	10	4	0	0
22	0	0	5	4	9	2	0
24	0	0	0	2	4	13	1
26	0	0	0	1	6	7	6
28	0	0	0	3	6	6	5
30	0	0	0	5	5	1	9
32	0	0	0	1	3	14	2
34	0	0	0	2	8	2	8

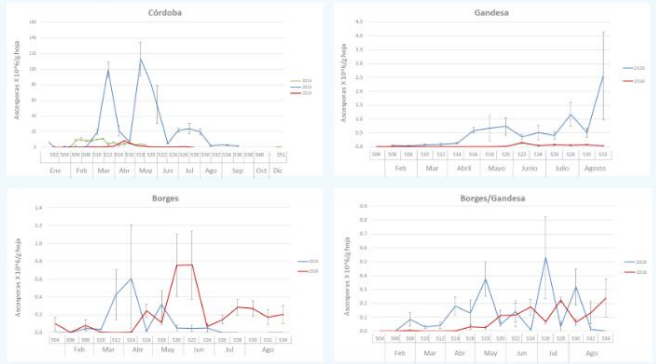
* Borges/Gandesa hace referencia a hojas tomadas en Gandesa y llevadas a Borges en Diciembre de 2015 .

La maduración de los cuerpos fructíferos transcurre de forma ligeramente distinta en las dos localidades.

En Borges, la mayor frecuencia de ascocarpos con ascosporas maduras (Clase D) se registró a finales de Mayo (semana 22), permaneciendo así hasta Agosto (semanas 32-34). En Borges se detectan ascosporas maduras durante más tiempo (10-14 semanas).

En Gandesa, la maduración plena de los cuerpos fructíferos no se observó hasta la semana 30, a finales de Julio.

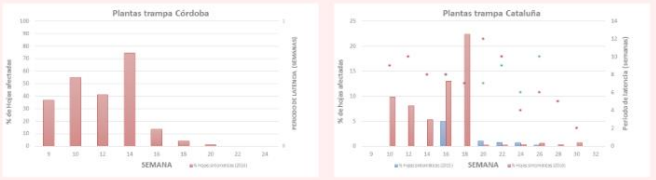
Producción y liberación de ascosporas



Andalucía. En líneas generales, la producción y liberación de ascosporas se inició en Febrero y se extendió hasta finales de Agosto. Sin embargo, los registros absolutos fueron muy distintos según los años: en 2014 los registros no superaron los 12 millones de ascosporas/g hoja (asc/g hoja). En 2015 se registraron tres máximos, entre 80 y 113 millones asc/g hoja. En 2016 los conteos tuvieron una media de 1,3 millones asc/g hoja, con un máximo de 8,3 millones asc/g hoja.

Cataluña. En Gandesa, en el 2015 se registraron conteos de hasta 1,2 millones asc/g hoja. En el 2016 no se superaron las 150.000 asc/g hoja. En Borges, el período de producción y liberación de ascosporas en el 2015 se inició en Marzo y terminó a mediados de Julio, mientras que en el 2016 el período se inició un mes más tarde y se mantuvo con una media de 200.000 asc/g hoja hasta el final del ensayo. En Borges/Gandesa, en el 2015 se dieron tres períodos de producción y liberación de ascosporas entre Abril y Mayo, y principios de Agosto. En el 2016, un único período desde Abril y con una media de unas 74.000 asc/g hoja.

Infectividad y latencia



La infección de las plantas trampa ocurre en distintos períodos según la anualidad y la zona geográfica. El período de latencia es muy variable (4-12 semanas), aunque, se observa un descenso con el aumento de la temperatura semanal media.

Andalucía. Primeros síntomas observados en la semana 9 de 2016 (Marzo), incrementándose hasta la semana 14 (Abril), con cerca del 80 % de hojas afectadas. Descenso brusco en la semana 16 (finales de Abril) y siguientes.

Cataluña. Primeros síntomas observados en la semana 16 de 2015 (Abril); en el 2016, en la semana 10 (Marzo). Las infecciones cayeron bruscamente en la semana 20 (mediados de Mayo) en ambas anualidades.

CONCLUSIONES

Se han observado diferencias notables de índole geográfica y temporal en las zonas estudiadas, en cuanto a la producción y liberación de ascosporas y la infectividad de las mismas. De los datos registrados, parece deducirse una gran presión de la enfermedad en Andalucía en comparación con Cataluña.

Este trabajo ha sido financiado con fondos de un proyecto INIA (RTA2013-00004-C03) y FEDER. Erick Zúñiga recibe financiación de CONACYT (México) para la realización de su tesis doctoral.

FIELD STUDIES ON THE PRIMARY INOCULUM AND EARLY INFECTIONS OF ALMOND RED LEAF BLOTCH (*Polystigma amygdalinum*) IN SPAIN

Zúñiga, E.[1]; Luque, J.[1]; Miarnau, X.[2], Lovera, M.[3]; Arquero, O.[3]; Ollero, A.[4]; Roca, L.F.[4]; Trapero, A.[4]

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INTRODUCTION

The red leaf blotch of almond (RLB), caused by the ascomycete *Polystigma amygdalinum* P.F. Cannon, is a common disease in continental climate areas of Spain and other countries in the Mediterranean basin. Early RLB symptoms are yellow discoloured blotches (1-2 cm) on leaves, which turn red and end in dark necrosis (Fig. 1). The disease usually causes an early defoliation that is conducive to a decrease in fruit production.

This work reports on the results of a coordinated research carried out in southern and northeastern Spain (Andalusia and Catalonia regions, respectively) to monitor the dynamics of the pathogen primary inoculum production and the period of plant infectivity.

Figure 1 Progression of *Polystigma amygdalinum* lesions on almond leaf.

MATERIALS AND METHODS

Development of *P. amygdalinum* fruiting bodies. Trial carried out in Catalonia (2016), in Gandesa and Borges locations. 20 fruiting bodies were randomly selected from leaves with RLB symptoms each two weeks, from January to August. Fruiting bodies were classified according to their stage of development.

Production and release of *P. amygdalinum* ascospores. Trial carried out in Córdoba, Andalusia (2014-16), and same both locations in Catalonia (2015-16) as reported earlier. Ascospores from fortnightly collected infected leaves were extracted in aqueous solutions and counted under a microscope (×250). Data expressed as number of ascospores per g leaf.

Latency and infectivity periods. Trial carried out in Andalusia (2015) and Catalonia (2015-16). Ten 1-year-old plants were exposed to natural infections in two-week periods from February to August. After their exposure to disease, the plants were removed from the field, taken to a greenhouse and kept in optimal growth conditions until the appearance of RLB symptoms.

RESULTS AND DISCUSSION

Development of fruiting bodies

Class	Stage of development		
	Perithecia	*Asci*	*Ascospores*
P	Absent	Absent	Pycnidia, no conidia
P[y]	Absent	Absent	Pycnidia with conidia
A	Differentiated, Not mature	Undifferentiated	Undifferentiated
B	Differentiated, Not mature	Differentiated	Undifferentiated
C	Differentiated, Not mature	Differentiated	Differentiated; < 8 spores/ascus
D	Mature	Mature	Mature; 8 spores/ascus
E	Empty	Empty	Discharged

Gandesa

Week	Development stage P	P[y]	A	B	C	D	E
4	15	0	7	0	0	0	0
6	10	0	10	0	0	0	0
8	18	0	2	0	0	0	0
10	0	7	13	0	0	0	0
12	0	11	9	0	0	0	0
14	8	6	6	0	0	0	0
16	10	0	10	0	0	0	0
18	5	0	8	6	1	0	0
20	0	0	6	9	5	0	0
22	0	0	7	3	7	3	0
24	0	0	8	7	5	0	0
26	0	0	9	8	3	0	0
28	4	0	11	5	0	0	0
30	0	0	0	4	6	4	6
32	0	0	1	12	2	0	5
34	-	-	-	-	-	-	-

Borges

Week	Development stage P	P[y]	A	B	C	D	E
4	15	0	5	0	0	0	0
6	9	0	11	0	0	0	0
8	15	0	5	0	0	0	0
10	7	1	11	1	0	0	0
12	2	3	15	0	0	0	0
14	2	4	11	3	0	0	0
16	4	1	11	4	0	0	0
18	0	0	2	10	7	1	0
20	0	0	0	2	2	16	0
22	0	0	0	3	6	4	7
24	0	1	0	2	5	9	3
26	0	0	1	1	4	3	11
28	0	0	0	0	5	2	7	6
30	0	0	0	0	2	6	7	5
32	0	0	0	0	4	9	3	4
34	0	0	0	0	3	3	5	9

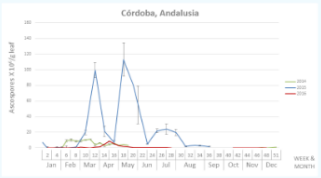
Borges/Gandesa*

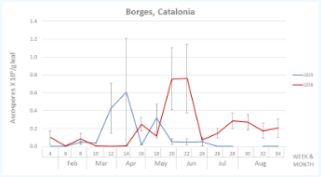
Week	Development stage P	P[y]	A	B	C	D	E
4	12	0	8	0	0	0	0
6	17	0	3	0	0	0	0
8	14	0	6	0	0	0	0
10	5	1	11	3	0	0	0
12	0	1	19	0	0	0	0
14	2	1	13	4	0	0	0
16	1	0	7	5	4	3	0
18	0	0	9	7	3	1	0
20	0	0	6	10	4	0	0
22	0	0	5	4	9	2	0
24	0	0	0	2	4	13	1
26	0	0	0	1	6	7	6
28	0	0	0	3	6	6	5
30	0	0	0	5	5	1	9
32	0	0	0	1	3	14	2
34	0	0	0	2	8	2	8

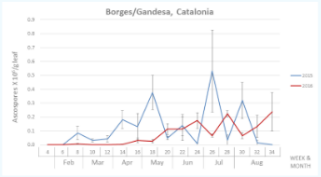
* Borges/Gandesa refers to leaves taken from Gandesa to Borges in December 2015.

The maturation of the fruiting bodies took place slightly different in both sites. In Gandesa, the full maturation of fruiting bodies was not detected before week 30, at the end of July, and with a few Class D fruiting bodies throughout the assay. However, greater frequencies of Class D ascocarps were recorded at the end of May (week 20) in Borges, which remained high until August (weeks 32-34). Additionally, two peaks of Class D fruiting bodies were recorded in Borges/Gandesa, the first one in week 24, and the second one by the end of the assay (week 32).

Production and release of ascospores



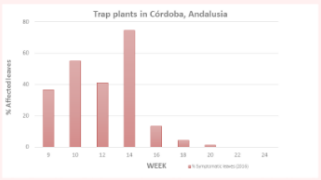


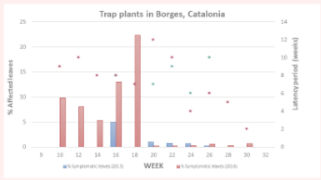


Andalusia. In general terms, the production and release of ascospores began in February and lasted until the end of August. Moreover, absolute recordings were different across the years: in 2014 the countings did not exceed 12 million ascospores/g leaf (asc/g leaf). In 2015 there were three peaks, between 80 and 113 million asc/g leaf. In 2016 the average was low as compared to previous years: 1.3 million asc/g leaf, with a maximum of 8.3 million asc/g leaf.

Catalonia. In Gandesa 2015, up to 1.2 million asc/g leaf were recorded, while in 2016 recordings never exceeded 150,000 asc/g leaf. In Borges 2015, the period of production and release of ascospores started in March and ended in mid-July. However, in 2016 the period started one month later and was maintained with an average of 200,000 asc/g leaf until the end of the assay. In Borges/Gandesa 2015, there were three peaks in ascospore production between April and early August. In 2016, a single peak in April (74,000 asc/g leaf) was recorded.

Latency and infectivity periods





The infection of trap plants occurred in different periods according to the season and the geographical location. The latency period recorded in Catalonia was variable (4-12 weeks), although a clear decrease in latency was observed along the season.

Andalusia. First RLB symptoms were observed in March 2016 (week 9), and increased until April (week 14), with about 80% of affected leaves. A sharp decrease in infected leaves was detected from late April on (week 16).

Catalonia. First RLB symptoms were observed in April 2015 (week 16) but in March 2016 (week 10). Infections decreased dramatically in mid-May (week 20) in both years.

CONCLUSIONS

Significant geographical and seasonal (yearly) differences have been observed in the studied areas, in terms of the production and release of ascospores and their infectivity. Recorded data suggested a great inoculum pressure of the almond red leaf blotch in Andalusia as compared to Catalonia.

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