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

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Abstract

Red leaf blotch (RLB) 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). Significant differences in ascospore amounts among regions, higher in the southern Andalusia and lower in the northern Catalonia, and years of study were detected. The factors geographical location, sampling period and 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 RLB infectivity period in Catalonia extended from March to mid-June, while in Andalusia from March to May. The incubation period was mainly in a range of 5 to 10 weeks in Catalonia. The environmental conditions of October-January influence the available ascospore amounts in the next season. RLB infection occurs in spring to summer, when mean temperatures are in the range 10 to 20°C. These results represent the first step in developing a prediction model of the disease that might serve as a tool for the control of RLB.

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 leads the world in area under almond cultivation, with more than 630,000 ha of almonds grown in 2017 (FAOSTAT 2019; MAPA 2019). However, the Spanish annual yield of almonds with shell is slightly over 400 kg ha^{-1}, much lower than those obtained in the USA, the leading producer country in the world with an average production over 2,500 kg ha^{-1} (FAOSTAT 2019). In recent years, an intensive almond cropping in Spain has been driven by a general increase in world nut consumption (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 (Miarnau et al. 2018; Vargas et al. 2010), as comparable to that obtained in the USA (MAPA 2019). However, there is a great concern about an eventual 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), where it 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 increased worryingly 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, which 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 *P. amygdalinum* 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

95 Xylariomycetidae subclass of Sordariomycetes, close to Xylariales and Trichosphaeriales
96 (Habibi et al. 2015).

97 The RLB disease is monocyclic and the only inoculum sources are the stromata of
98 affected leaves which fall to the ground in autumn (Banihashemi 1990; Ollero et al. 2016b;
99 Saad and Masannat 1997). The sexual stage is developed in winter on the leaf stromata, and
100 later in spring, ascospores are air-spread and can infect new almond leaves (Banihashemi
101 1990). In Iran, Ghazanfari and Banihashemi (1976) reported on the influence of autumn-
102 winter weather conditions in perithecia development. Based on these authors, the start of
103 perithecia maturation occurs below 10°C. Banihashemi (1990) showed that ascospore
104 release in Iran is related with rain periods, beginning at flowering and reaching the
105 maximum at petals fall (late April to early May). In Lebanon, ascospore release can occur
106 between February and mid-May (Saad and Masannat 1997). However, the effect of weather
107 conditions on the infection process is deeply unknown. After infection, leaf spots appear
108 after an incubation period of 30-35 days (Banihashemi 1990; Cannon 1996; Suzuki et al.
109 2008). The occurrence of secondary cycles has not been confirmed (Ollero-Lara et al.
110 2016b; Saad and Masannat 1997; Shabi 1997), since conidia do not have infective ability
111 and their only function appears to act as spermatia in the sexual reproduction of the
112 pathogen (Cannon 1996; Habibi and Banihashemi 2016).

113 As a monocyclic disease, control management of RLB should be aimed at: (*i*)
114 reducing primary inoculum potential, i.e. ascospores produced in the affected leaves fallen
115 in previous autumn, and (*ii*) protecting new almond leaves growing in the season. Based on
116 several authors (Almacellas, 2014, Arquero et al. 2013, Lin and Szteinberg, 1992), the
117 control measures to achieve the first objective include: (*i*) to remove or bury the leaves, (*ii*)
118 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). Regarding the second objective, application of fungicides appears to be the most effective measure to protect the new leaves from RLB infections (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).

Materials and Methods

Geographical 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.

141 Trees in the orchards were not treated with any chemical product to allow natural infection
142 of leaves by *P. amygdalinum*.
143 **Plant material.** For the experiments on primary inoculum monitoring, development of
144 fruiting bodies, and germination of ascospores; fallen leaves of various almond cultivars
145 with distinct RLB symptoms were collected during early autumn (September) in Andalusia
146 and autumn/winter (December to January) in Catalonia before each specific experiment. As
147 the experimental orchards consisted of different almond cultivars, collected fallen leaves
148 from the ground could not be classified according to their cultivar origin. In each season, an
149 additional bulk sample of leaves collected in Gandesa was taken to Borges in order to study
150 the eventual environmental influence in samples from different geographical origins. This
151 sample (hereinafter as Borges/Gandesa) was considered as a third location within the
152 Catalonian sites. In all experimental sites, leaves were placed into nylon mesh bags with 90
153 to 200 leaves per bag. The bags with fewer leaf numbers were processed earlier in the
154 season whereas the bags with higher amounts of leaves were processed later in the season,
155 since natural decomposition of leaves along the time would have left a low sample amount
156 at later stages. The bags were left outdoors in the orchard by nailing them on the ground.
157 Various numbers of bags ($N > 13$) were prepared for each experimental site and year.
158 For the monitoring of natural infections and the estimation of the disease incubation
159 period, 1-year-old plants of the susceptible cultivar 'Tarraco' grafted onto 'GF-677'
160 rootstock were used. Trap plants were kept in greenhouses while not being exposed to
161 natural RLB infections in the experimental sites, while another group of plants (control
162 plants) never were moved out of the greenhouse. Regardless their geographical location,
163 plants kept in the greenhouses were maintained in 3 liters pots with a peat:perlite mixture
164 (3:1, v:v) (Peat: Floratorf TKS1, Floragard, Germany; Perlite: Europerl, Spain). The

165 substrate was amended with Osmocote Pro 3-4M granular fertilizer (Everris, Heerlen, The
166 Netherlands) at 2.5 g l^{-1}. Plants were irrigated as needed to avoid water stress and never
167 treated with fungicides both in the greenhouse and in the experimental sites.
168 **Monitoring of primary inoculum.** The study of primary inoculum was conducted for
169 three seasons in Andalusia (2014 to 2016) and Catalonia (2015 to 2017). Starting from
170 winter in each year, at the stage of dormant trees, the bags of leaf samples were taken
171 fortnightly to the laboratory till the end of the trial. The sample was oven-dried for 24 h at
172 35°C. Each sample was weighted (dry weight) and later subdivided into eight equally-
173 weighted subsamples. In Andalusia, all eight subsamples were treated separately for
174 ascospore extraction in distilled water, by crushing leaves in a mortar until getting a
175 homogeneous suspension (about 10 min operation). In Catalonia, an additional ascospore
176 extraction method was tested, by continuous stirring (18 h, room temperature) of a
177 suspension of leaf fragments in water. Thus, in Catalonia, four subsamples were extracted
178 by crushing and the remaining four by stirring. In all cases, various amounts of distilled
179 water were used according to the sample weight (about 40 ml per g sample). For both
180 extraction methods, final ascospore suspensions were filtered through a 2-folded 60-µm
181 Nylon mesh and subsamples of ascospore suspensions were examined under a microscope
182 (×250) using a hemocytometer (Neubauer chamber). Ascospores of *P. amygdalinum* were
183 unambiguously identified through their morphology features and counted. Each subsample
184 was measured four times and eight replicated measurements were done per subsample
185 measurement. Results were expressed as numbers of ascospores per g (dry weight) of
186 leaves after proper calculations. The experimental period covered from January to August
187 for all combinations of year and location.

188 **Development of fruiting bodies.** This study was conducted with the leaf samples collected
189 in Catalonia in 2016 and 2017. Prior to the oven-drying of leaf samples, four leaves with
190 well-developed fungal stromata were taken from each sample bag. The outer part of the
191 fungal stroma was cut off with a sterile scalpel and five randomly chosen fruiting bodies
192 from each leaf were individually excised with a hypodermic needle. The fruiting bodies
193 were placed in a water droplet on a microscope slide and bisected to unveil their content,
194 then covered with a cover slip and examined under a light microscope (×250). The fruiting
195 bodies (20 per each location and sampling period) were classified into six different
196 developmental stages by using modified categories described by Toscano-Underwood et al.
197 (2003), as follows: class P (pycnidia, either with conidia or not); class A (perithecium
198 differentiated, asci undifferentiated or beginning differentiation, ascospores
199 undifferentiated); class B (perithecium differentiated, asci differentiated, ascospores
200 undifferentiated); class C (perithecium differentiated, most asci differentiated, some
201 ascospores (< 8) differentiated); class D (fully matured perithecium, asci differentiated,
202 ascospores (8) fully differentiated); class E (perithecium empty, no asci present, ascospores
203 discharged). The percentages of each developmental status at each monitoring period were
204 calculated.
205 **Germination of ascospores.** This study was conducted with the leaf samples collected in
206 Catalonia in 2017. The viability of ascospores was estimated by determining the
207 germination percentages at each sampling period. From the same leaf samples used in the
208 study on fruiting bodies development, a sufficient amount of perithecia containing mature
209 ascospores was obtained. The perithecia were bisected and their content suspended in a 1.5
210 ml Eppendorf tube containing 1 ml distilled water. A volume (200 µl) of the ascospore
211 suspension was spread over a potato dextrose agar (PDA, Difco™, Becton, Dickinson &

212 Co., Sparks, MD) plate amended with streptomycin sulphate at 100 IU streptomycin ml^{-1}
213 and incubated at 20°C. Fifty randomly-chosen ascospores per location and sampling date
214 were counted (five replicates, 10 ascospores each) and classified into germinated and non-
215 germinated categories by using a light microscope (250×), at two intervals, 4 h and 24 h
216 after plating. An ascospore was recorded as germinated when the germ tube was greater
217 than half the width of the ascospore, as similarly described by Habibi and Banihashemi
218 (2015).
219 **Disease infectivity and incubaton periods.** The disease infectivity and incubation periods
220 were studied in two locations in Andalusia (Córdoba) and Catalonia (Borges), for one
221 (2016) and three (2015 to 2017) years, respectively. For each location and year, a group of
222 130 one-year-old 'Tarraco' plants was kept in a greenhouse at IFAPA and RTA facilities,
223 away from an eventual exposure to natural RLB infections. From February to August each
224 year, 10 different plants from the group were brought every two weeks to the experimental
225 orchards and left to be RLB-infected under natural conditions. This resulted in a total of 13
226 recordings per each year and location. After the 2-week exposure to the disease, trap plants
227 were removed from the field and taken back to the greenhouse, where they were monitored
228 weekly from February to October to check for the expression and evolution of RLB
229 symptoms. Numbers of apparently healthy and RLB-affected leaves in each plant were
230 recorded, and the proportion of RLB-affected leaves (incidence) was calculated and
231 averaged for each monitoring period. The infectivity period was determined as the period in
232 which RLB-symptomatic leaves were detected along the experiment. The incubation period
233 was estimated by determining the time (in weeks) between the initial exposure in the
234 orchards and the consistent appearance of disease foliar symptoms minus one week, in
235 order to correct the 2-week exposure interval with its intermediate point.

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).

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 using analytical tools of the statistical package. 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).

Results

Monitoring of primary inoculum. An exploratory 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. In general, the ascospore production period extended from January to August among the studied areas and seasons (Fig. 1). Highest amounts of ascospores were recorded in Córdoba, whereas those values of Borges and Gandesa were about a tenth of

307 the amounts recorded in Córdoba. The Borges/Gandesa group did not differ from Borges
308 and Gandesa ($P = 0.922$) in terms of mean annual ascospore amounts. In Córdoba, higher
309 amounts of ascospores were observed in 2014 and 2015 in comparison to 2016, whereas in
310 Borges and Gandesa a higher amount of ascospores was observed in 2017 than in previous
311 years (all $P < 0.001$).

312 In Córdoba, mean ascospore amounts of *P. amygdalinum* obtained by leaf crushing
313 were in the range from 1×10^6 to 6×10^6 agl (in 2014 and 2016), whereas about a 10-fold
314 higher annual mean, i.e. 2×10^7 agl, was estimated in 2015 (Fig. 1A). In 2014, ascospores in
315 Córdoba were recorded early at the beginning of the season in January and remained quite
316 stable at about 1×10^7 agl thereafter. In 2015, higher amounts of ascospores were mainly
317 detected in a six-month interval, i.e. March to August, and ranged between 1×10^6 and
318 1×10^8 agl within this period. In 2016, ascospores were detected between March and June,
319 and peaked up to 8×10^6 agl in April (Fig. 1A). In the Catalan locations, Borges and
320 Gandesa, the presence of ascospores was detected from January to August, with some
321 exceptions: no recordings after mid-June in 2015 in Borges, and none or occasional low
322 recordings at the beginning of the season in 2016 in Gandesa and Borges/Gandesa (Fig.
323 1B,C,D). Mean annual ascospore amounts obtained from crushed leaves were mostly in the
324 range from 1×10^5 to 1×10^6 in 2015 and 2017, and between 1×10^4 and 1×10^5 in 2016, with
325 several occasional peaks along the seasons. Occurrence of those peak values in ascospore
326 amounts was variable among locations and years, and a pattern of peak occurrence was not
327 clearly observed (Fig. 1). Dynamics of the RLB inoculum potentials along the season were
328 similar in the cases of Gandesa and Borges/Gandesa in 2015 and 2016, and slightly
329 different from Borges within the same years. However, dynamics of ascospore amounts in
330 2017 were similar for the three Catalan leaf sources (Fig. 1B,C,D).

331 Ascospores amounts obtained through the stirring-bath technique were consistently
332 lower than those obtained by crushing (Fig. 1B,C,D). A significant linear relationship
333 ($P < 0.001$, $R^2 = 0.6721$) between the log-transformed data of the two ascospore extraction
334 methods was found as follows: $\log(\text{Ascospores}_{\text{stirring}}) = 1.1619 + 0.7128 \times \log(\text{Ascospores}_{\text{crushing}})$. The equivalent power function was therefore: $\text{Ascospores}_{\text{stirring}} = 14.5180 \times (\text{Ascospores}_{\text{crushing}})^{0.7128}$. Moreover, there were no significant differences
337 between samples from different origin (Borges and Borges/Gandesa) which were obtained
338 with either extraction method ($P = 0.621$ and $P = 0.497$ for crushing and stirring,
339 respectively).
340 Associations with $\rho > 0.500$ and $P < 0.05$ between the rate of ascospore amounts per
341 season (ASC_{rate}) and any tested weather variable were not found. On the other hand, the
342 total amount of ascospores per season was only significantly correlated with weather-
343 derived variables of stage 2 (October-January), which resulted in significant ($P < 0.05$) ρ
344 values higher than 0.700 in the following cases: mean RH ($\rho = 0.767$), accumulated rainfall
345 ($\rho = 0.717$), number of raining days ($\rho = 0.728$) and number of days with mean daily T
346 higher than 20ºC ($\rho = 0.706$). Moreover, in October, the mean of maximum RH ($\rho = 0.783$),
347 the accumulated rainfall ($\rho = 0.733$), and the number of days with mean daily T equal or
348 higher than 20ºC ($\rho = 0.706$) were positively correlated with the total amount of ascospores
349 per season. On the other side, only the number of days with daily mean T from 10 to 20ºC
350 in October was negatively correlated ($\rho = -0.792$) with the total amount of ascospores. In
351 January, accumulated rainfall also showed a positive correlation with the total amount of
352 ascospores per season ($\rho = 0.783$). No more associations were found between the seasonal
353 total ascospore amounts and weather variables for the remaining months of the stage 2.

354 **Development of fruiting bodies.** The development and maturation of perithecia along the
355 season was confirmed through the observation that immature stages (A, B) were prevalent
356 at the beginning of the experimental period whereas mature stages (C, D, E) were mainly
357 recorded later in the season (Fig. 2). Results from the ordinal logistic regression analysis on
358 the whole dataset showed that all analyzed factors and their interactions, except for the
359 interaction location × week, were significant (*results not shown*). Because of interactions,
360 further ordinal logistic regressions were performed on data subsets according to the location
361 of sampled leaves. In addition, datasets from Borges and Borges/Gandesa were combined
362 into a single dataset to evaluate the influence of the geographical origin of samples.
363 Separate analyses of each location subsets showed the significance of factors sampling
364 period, year, and their interaction (all $P < 0.001$). However, the origin of leaf samples in the
365 Borges and Borges/Gandesa subset was not found significant ($P = 0.262$), nor the
366 interactions: origin × sampling period ($P = 0.618$), origin × evaluation year ($P = 0.262$), and
367 origin × sampling period × evaluation year ($P = 0.618$).
368 In Gandesa, fully mature perithecia (D) were observed rarely in 2016, and the
369 percentages of this class never exceeded 20% throughout the assay. However, mature
370 perithecia with percentages equal or higher than 40% were detected in 2017 from early-
371 May (week 18) until the end of the experiment (Fig. 2A). In Borges, proportion of mature
372 ascocarps were more frequently detected than in Gandesa. Thus, class D ascocarps reached
373 80% at mid-May 2016 (week 20), and remained in the range 15 to 45% until August. In
374 2017, the percentage of mature perithecia observed in Borges prevailed above 50% from
375 late-February (week 8) to the end of the experiment (Fig. 2B). Regarding the
376 Borges/Gandesa samples, maturation of ascocarps behaved similarly as those from Borges,
377 as shown earlier by the non-significance of the geographical origin factor and its

interactions in the statistical analyses. Thus, percentages above 50% in class D perithecia in 2017 prevailed in almost every week from mid-February on (Fig. 2C).

Ascospore germination. Ascospores of *P. amygdalinum* germinated on PDA as earlier as 4 h after plating, but highest percentages of ascospore germination were observed at 24 h incubation (Fig. 3). 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 (44.0 ± 8.0 %) was observed in mid-July (week 28), whereas in Borges the maximum (28.0 ± 2.0 %) occurred in mid-April (week 16). Ascospores from the Borges group showed consistently 20% and above of germination during the first half of the monitoring period (Fig. 3). Similarly, ascospores from the Borges/Gandesa group showed the higher germination percentage (30.0 ± 2.0 %) in April, just as the samples from Borges did.

Disease infectivity and incubaton periods. Trap plants exposed in Córdoba and Borges showed that RLB infections occurred from March (week 9) to late July (week 30), although higher infection percentages were mainly detected from week 9 to week 18, i.e. from March to early May (Fig. 4). Moreover, the incidence of RLB in trap plants was positively correlated with the number of days with mean daily T from 10 to 20ºC ($10 \leq T < 20$ ºC) ($\rho = 0.526$, $P = 0.001$) and the number of days both wet and mild T (VPD ≤ 4 hPa or R ≥ 0.2 mm, and $10 \leq T < 20$ ºC) ($\rho = 0.632$, $P < 0.001$).

Overall incidence in Córdoba was higher than in Borges in 2016, as infections in Córdoba were well ranging 30 to 70% within the weeks 9 to14, whereas equivalent values in Borges were ranging between 5 and 20% (Fig. 4). However, no differences in mean RLB incidence of trap plants (P = 0.064) between Borges (5.54 ± 5.60%) and Córdoba (26.55 ±

6.19%) were detected. Data collected in Borges in three consecutive years (2015 to 2017) indicated that infections decreased drastically in June and only sporadic infections were detected later, coinciding with daily mean T over 20 °C in this period (Fig. 4). In addition, no differences in RLB incidence of trap plants (P = 0.167) were detected in Borges when comparing all three monitored years.

The incubation periods estimated from the data recorded in 2015 to 2017 in Borges were mostly between 6 and 10 weeks, but extreme values (from 2 to 12) were occasionally recorded (Fig. 5). The duration of the incubation period tended to decrease from week 20 in 2016 (Fig. 5), but this trend was not observed in 2015 and 2017, since correlations were not significant (*data not shown*).

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, the disease infection period and the control of RLB using fungicides (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

(Almacellas 2014; Ollero-Lara et al. 2016a; Ollero-Lara et al. 2016b), and cultivar susceptibility (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 our country, where environmental conditions for almond-growing areas could be different from those of previously studied cases in Iran and Lebanon.

In previous research conducted in Iran and Lebanon, a main period from April to May was reported for the potential primary inoculum availability (Ashkan and Assadi 1974; Banihashemi 1990; Saad and Masannat 1997), which coincides with that observed in Córdoba and Borges in 2014 and 2015, respectively. However, an extended period of ascospore availability, i.e. from February to August, was repeatedly observed in later seasons in our study. This suggests a larger period where primary inoculum of RLB can be present in Spain, thus increasing the potential risk of infections during favorable periods. The amounts of ascospores recorded in this study cannot be compared with data on ascospore counts reported by Banihashemi (1990) and Saad and Masannat (1997), since methods in those latter studies were based on the quantification of captured airborne ascospores. In our study, the ascospore extraction methods from leaves may have overestimated the amounts of available ascospores, especially when extracting ascospores by crushing. Fruiting bodies can be physically broken when leaves were crushed so that the whole perithecia content is released to the medium and higher ascospore amounts can be therefore recorded.

Banihashemi (1990) suggested that changing environmental conditions 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

449 water availability and, to a lesser extent, to temperature. Thus, *P. amygdalinum* benefits
450 from the hydration of fallen leaves and T above 20°C during fall, mainly in October, to
451 produce higher inoculum potentials during the next season. However, Ghazanfari and
452 Banihashemi (1976) reported that *P. amygdalinum* requires T below 10°C in fall and winter
453 to favor ascocarp development in the next season, which is in contrast with our results.
454 Further research is therefore needed to clarify the influence of fall and winter
455 environmental conditions on the seasonal dynamics of the disease. However, correlations
456 must be treated with caution to avoid spurious associations (Fernández-Escobar et al.
457 2018). Geographical conditions may also play a major role in the primary inoculum
458 development, as confirmed by the sharp differences in annual potential inoculum amounts
459 observed in Andalusia and Catalonia. These results confirm the idea that RLB of almond
460 needs to be studied in each region where it is reported. Moreover, it is advisable to conduct
461 a multi-year monitoring of the primary inoculum since, as reported here, highly variable
462 ascospore amounts can be recorded among seasons.

463 The maturation of *P. amygdalinum* perithecia and ascospores was rather related to
464 seasonal weather conditions than to the geographical origin of samples. However, a
465 disparity between maturity of fruiting bodies and primary inoculum dynamics was detected
466 in some cases. Thus, perithecia reached maturation late in the season in 2016 in all
467 locations while free ascospores were detected from the first weeks of the year until the end
468 of the experiment. We hypothesize that this might have been due to the sample size of the
469 analyzed leaves, which could have been insufficient to adequately represent how fruiting
470 bodies developed in the leaf litter along the season. Gadoury et al. (1992) reported a
471 disparity between morphological maturity of ascospores and physiological maturity of asci
472 in the apple scab fungus, *Venturia inaequalis*, which could be comparable to our results.

473 The authors found that discharge of ascospores was recorded as early as asci were rated as
474 mature in approximately 10 to 15% of full maturity.
475 In our study, ascospores were able to germinate but failed to grow further, in
476 agreement with data reported by Habibi and Banihashemi (2015). However, germination
477 percentages after 24 h incubation were consistently low, well below 30% in most cases.
478 These low germination percentages could be related to the biotrophic nature of the
479 pathogen (Cannon 1996; Habibi and Banihashemi 2015) or even to unknown
480 environmental factors. Data on ascospore germination could be useful in a first stage for
481 testing fungicides *in vitro* against *P. amygdalinum*, as well as in the development of
482 mechanistic predictive models on RLB epidemiology.
483 The natural RLB infections observed in trap plants occurred between week 9 and 22
484 (February to May), despite the geographical location of almond orchards. Moreover, we
485 found T between 10 ºC and 20 ºC promoted infection by *P. amygdalinum*, particularly
486 when associated with wetness conditions. Rainfall and high RH could provide adequate
487 moisture for ascospore dispersal and subsequent infection. The importance of T and
488 moisture (hydrothermal variables) is well-characterized in many pathosystems (Agrios,
489 2005), and are generally known to explain plant disease development (Lowell et al. 2004),
490 such as in the *Plasmopara viticola*-grape (Rossi et al. 2007) and the *Venturia pirina*-pear
491 (Rossi et al. 2009) pathosystems. Moreover, infections in Andalusia and Catalonia declined
492 when T raised above 20 °C, thus suggesting that warmer temperatures where inhibiting
493 RLB infections. It is known that optimum temperature for ascospore germination and
494 appressorium formation among *Phyllachora* species is 10 ºC to 20 °C (Banihashemi 1990;
495 Dittrich et al. 1991; Parbery 1963), and that T above 25 °C inhibits appressorium formation
496 (Habibi and Banihashemi 2015). These data would be compatible with fewer infections of

497 *P. amygdalinum* being detected in summer. Our results showed that the incubation period
498 mostly ranged between 5 and 10 weeks, but can be as long as 12 weeks in spring and as
499 short as 2 weeks in summer. These results differ clearly from those reported by Ashkan and
500 Assadi (1974), who estimated a narrower incubation period of 30 to 35 days, and those by
501 Banihashemi (1990), who reported a similar period (30 to 40 days).

502 Although *P. amygdalinum* has been considered as a biotrophic pathogen (Cannon,
503 1996), other plant pathogens with a similar multistage development as *P. amygdalinum* are
504 classified as hemibiotrophic pathogens, such as *Mycosphaerella graminicola* (Fuckel) J.
505 Schröt., *Pyricularia oryzae* Cavara, and *Colletotrichum* spp. (Marshall et al. 2011; Mentlak
506 et al. 2012; O'Connell et al. 2012). This hemibiotrophic lifestyle can be easily recognized
507 in *P. amygdalinum*: (*i*) a long initial biotrophic phase, with the pathogen spreading inside
508 living host cells without causing noticeable host cell damage in spring and summer, and (*ii*)
509 a short necrotrophic phase in which pathogen growth causes multiple host cell death and
510 the darkening of leaf stroma (Saad and Masannat 1997; Zúñiga et al. 2019). Lastly, *P.*
511 *amygdalinum* continues the necrotic phase on fallen leaves (from October to January),
512 which is previous to the final development and maturation of perithecia and ascospores.

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

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Figure captions

Fig. 1. Dynamics of *Polystigma amygdalinum* ascospores extracted from infected almond leaves during a 3-year monitoring period conducted in two almond-growing regions in Spain. Specific locations and monitoring periods: A) Córdoba (2014-16), B) Gandesa (2015-17), Les Borges Blanques (2015-17), and D) leaves taken from Gandesa to Les Borges Blanques (2015-17)

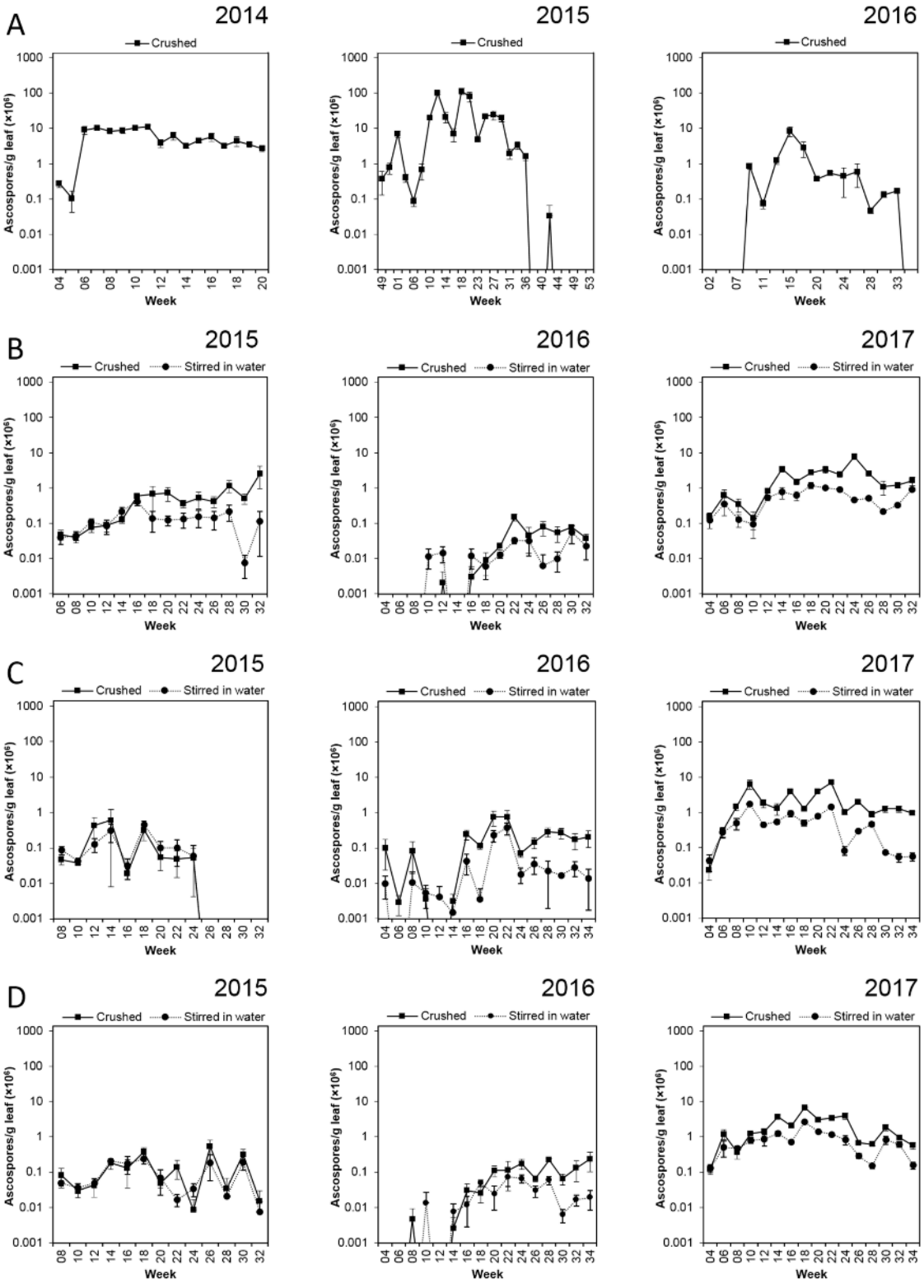
Fig. 2. Development of *Polystigma amygdalinum* fruiting bodies on almond leaves in two consecutive years and for three sample origins (Gandesa, Les Borges Blanques and Borges/Gandesa). Results are shown in a gray scale as percentages of fruiting bodies (N = 20) for each sampling period. Abbreviations: (P) Pycnidia, no perithecia present; (A) Differentiated immature perithecium, with undifferentiated asci and ascospores; (B) Immature perithecium with differentiated asci and undifferentiated ascospores; (C) Immature perithecium and differentiated asci with < 8 ascospores/ascus; (D) Mature perithecium and asci with 8 ascospores/ascus; (E) Empty perithecium, without asci and ascospores

Fig. 3. Germination percentages of *Polystigma amygdalinum* ascospores (N = 50) recorded from three leaf sample origins (Gandesa, Les Borges Blanques and Borges/Gandesa).

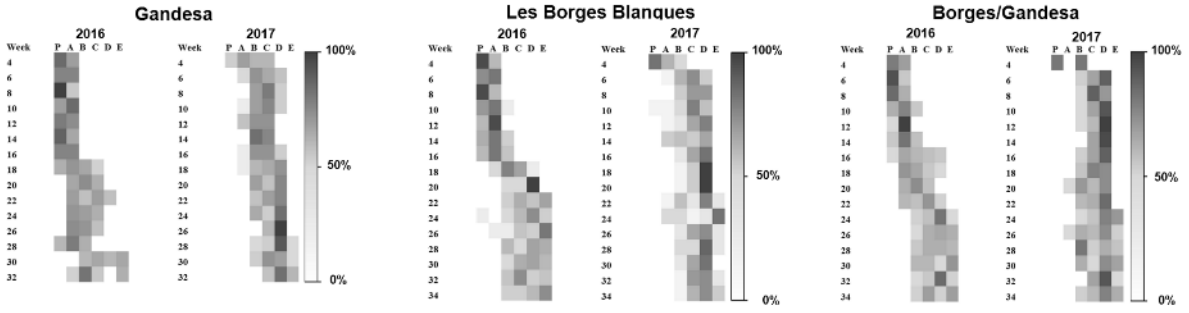
Fig. 4. Red leaf blotch incidence (%) in 'Tarraco' susceptible almond trees exposed to natural infection periods in Córdoba (2016) and Les Borges Blanques (2015–17). Mean temperatures shown in the secondary (right) axe

Fig. 5. Incubation periods of almond red leaf blotch in 'Tarraco' susceptible almond trees exposed to natural infections in Les Borges Blanques (2015–17)

Fig. 1.


A
2014
2015
2016
Crushed
B
2015
2016
2017
Crushed
Stirred in water
C
2015
2016
2017
Crushed
Stirred in water
D
2015
2016
2017
Crushed
Stirred in water
Ascospores/g leaf (×10⁶)
Week

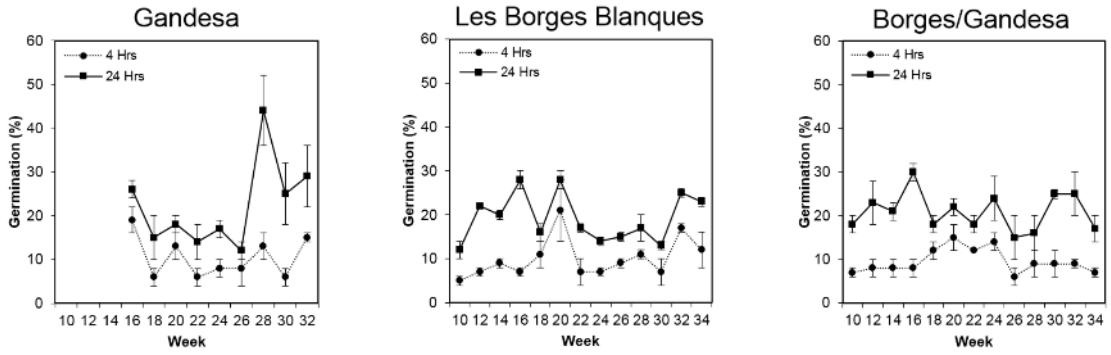
664 **Fig. 2.**



665

666

667 **Fig. 3.**



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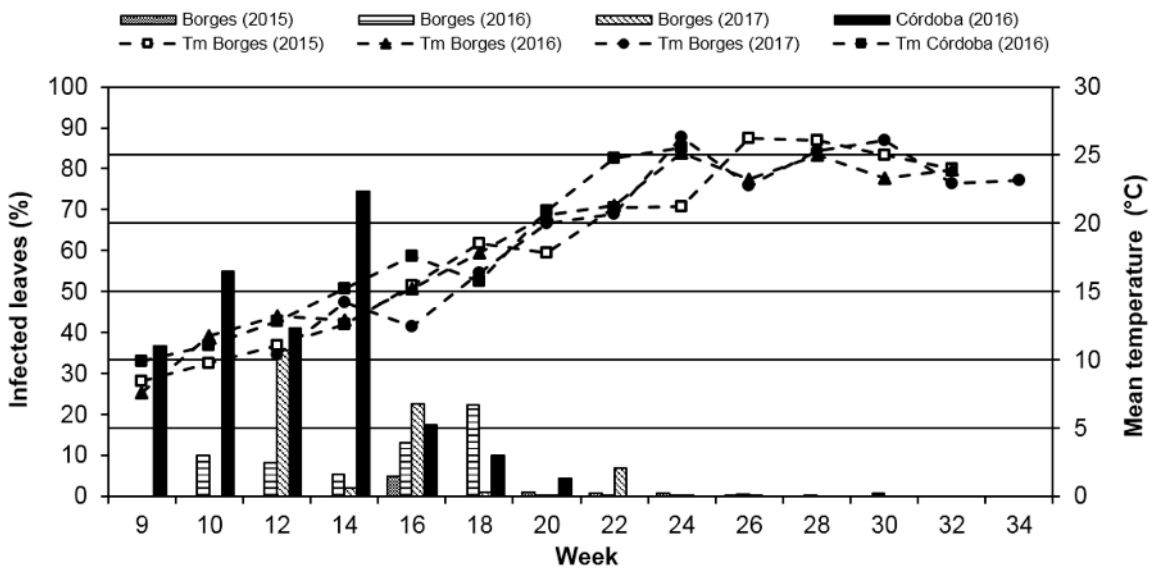
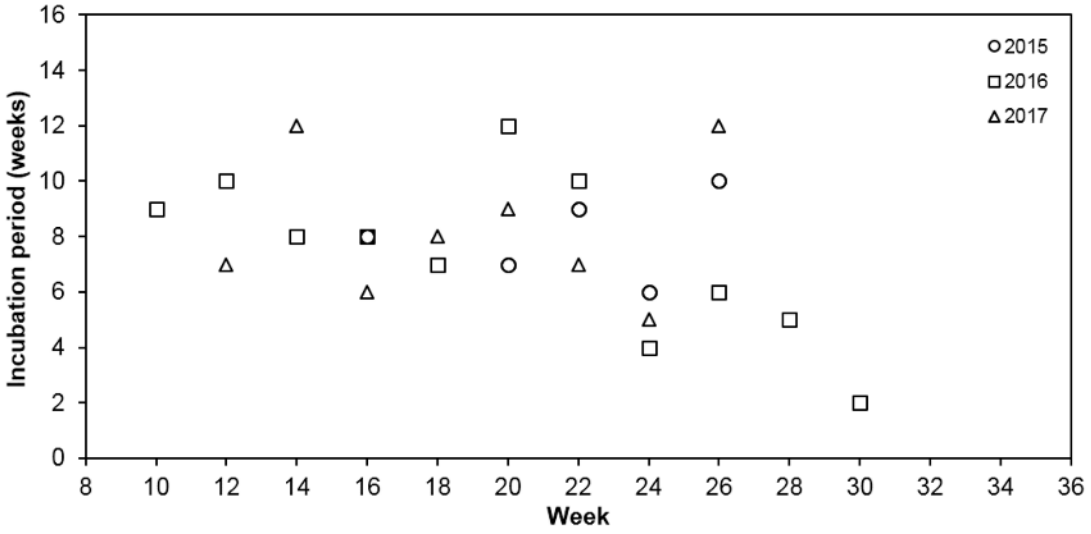
670 **Fig. 4.**


Borges (2015)
Borges (2016)
Borges (2017)
Córdoba (2016)
Tm Borges (2015)
Tm Borges (2016)
Tm Borges (2017)
Tm Córdoba (2016)
Infected leaves (%)
Mean temperature (°C)
Week
0
10
20
30
40
50
60
70
80
90
100
0
5
10
15
20
25
30
9
10
12
14
16
18
20
22
24
26
28
30
32
34

671

672 **Fig. 5.**


Incubation period (weeks)
Week
2015
2016
2017
0
2
4
6
8
10
12
14
16
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36

673