Influence of temperature and moisture duration on pathogenic life-history traits of predominant haplotypes of *Fusarium circinatum* on *Pinus* spp. in Spain

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Keywords: pitch canker disease, Monterey pine, virulence, infection period, sporulation, germination, cardinal temperatures, melanization

Funding: Projects RTA 2013-00048-C03-01 and RTA2017-00063-C04-01, Ministerio de Economia y Competitividad (Subprograma de Proyectos de I+D

orientado a los Recursos y Tecnologias Agrarias en coordinación con las Comunidades Autonomas).

ABSTRACT

Pathogen life-history traits influence epidemic development and pathogen adaptive ability to interact with their hosts in different environments. Reduced traits variation may compromise pathogen evolutionary potential which is particularly important for introduced pathogens. *Fusarium circinatum* (cause of Pine Pitch Canker) is an invasive fungal pathogen in Europe, with current distribution restricted to forest stands of *Pinus radiata* and *P. pinaster* in northern Spain and Portugal. This study aimed to quantify pathogenic traits of Spanish isolates of *F. circinatum*, with two of the strains representing the two dominant haplotypes in the Spanish population. Disease severity was measured on *P. radiata*, analyzing the influence of temperature and moisture duration on infection as well as the influence of temperature on spore germination, sporulation and mycelial growth. Results indicated that the isolate representing the most common haplotype caused more severe disease on *Pinus radiata* at 25 and 30ºC compared to the second most common haplotype, but less severe disease at 15ºC. Spore germination was higher for the most common haplotype, which produced more spores at 20 and 25ºC. The isolate showed hyphal melanization at 5ºC, which has been associated with survival and may be important since no resting structures have been described for *F. circinatum*. Our study determined that longer moisture periods during infection result in more severe

disease from 7 to 24 h, regardless of the isolate virulence. This is the first study on virulence of the most abundant haplotypes of *F. circinatum* in Spain as affected by temperatures and moisture.

Variation in pathogen life-history traits influence spatial and temporal dynamics of epidemics (Barrett et al. 2008). In particular, variation in pathogenicity (defined as the ability of a pathogen to cause disease in a particular host (Sacristán and García-Arenal 2008)) and virulence (defined as the degree of damage to the host (Sacristán and García-Arenal 2008)) are essential traits in disease epidemiology (Laine and Barrès 2013). The relationship between processes in disease epidemiology and pathogen evolution is not well understood (Grenfell et al. 2004; Barrett et al. 2008), but the interaction determines various outcomes, including success of pathogen adaptation to new environments and ability to overcome host resistance (Laine 2007; Barrett et al. 2009; Desprez-Loustau et al. 2016). Therefore, variation in pathogen life history traits, and especially those related to virulence (Lannou 2012), will determine the evolutionary potential of pathogens (Linde 2010).

In the case of introduced or exotic pathogens, where a new population is initiated by a small number of individuals, the reduced genetic variation in the population may constrain adaptation to the new environment. Multiple pathogen

introductions of divergent populations may counteract this effect, and therefore, increase evolutionary potential (Desprez-Loustau et al. 2016). Once an invasive pathogen becomes established in a new environment or interacts with a new host, it may cause a high disease incidence and/or severe disease since host-pathogen co-evolution has not occurred (Ennos 2015).

Fusarium circinatum Nirenberg and O'Donnell is an invasive fungal pathogen in Europe and is the cause of Pine Pitch Canker (PPC) disease. PPC is established in northern Spain and Portugal (EPPO 2011), affecting forest stands of *Pinus radiata* D. Don and *P. pinaster* Ait., but it has potential to spread and establish in other European countries (EFSA 2010; Möykkynen et al. 2015). In fact, there have been sporadic reports of PPC affected trees in France and Italy (EPPO 2011), although the disease was eradicated after exceptional measures were taken (Vettraino et al. 2018). In Spain, the disease was officially reported for the first time in 2005 (Landeras et al. 2005), and has been restricted to the Atlantic area where *P. radiata*, one of the most susceptible species to PPC (Iturritxa et al. 2012, 2013), is grown. The pathogen is responsible for significant damage which affects tree growth and wood quality (Wingfield et al. 2008). It represents a major threat to countries where pines susceptible to *F. circinatum* are grown or planted, and it is considered one of the most devastating pine pathogens worldwide (Wingfield et al., 2008).

A global population genetic analysis of *F. circinatum* revealed that the Spanish population has two predominant haplotypes, each occurring in one of the two clusters in which the pathogen population is structured (Berbegal et al. 2013).

Population differentiation into two clusters is compatible with two independent pathogen introductions of each predominant haplotype in Spain, that subsequently underwent clonal divergence (Berbegal et al. 2013). The results were further supported by evidence of linkage disequilibrium. Indeed, earlier diversity studies and the lack of observed sexual structures of *F. circinatum* under natural conditions support the existence of clonal propagation for most populations worldwide (Iturritxa et al. 2011; Wingfield et al. 2008). Each of the clusters exclusively grouped isolates of only one of the two mating types, either MAT-1 or MAT-2 (Berbegal et al. 2013). Among the total of 15 haplotypes identified in Spain, 27% were grouped in one of the clusters, including one of the most commonly found in Spain (MLG32), a haplotype also detected in France. The remaining 73% of the haplotypes grouped in the second cluster which includes the second most common haplotype observed in Spain (MLG59), a haplotype also detected in Portugal (Berbegal et al. 2013). All clusters identified by the multivariate analysis to assess global population structure included haplotypes from populations in the USA, indicating North America as a potential source of introductions (Berbegal et al. 2013).

Life-history traits of plant pathogens are grouped in two categories, those related to the epidemic phase and involve interaction with the host; and those involved in the survival phase that usually occurs outside the host (Le May et al. 2020). Pathogenic traits in a plant-pathogen system during the epidemic phase refer to stages in the pathogen life cycle (Pariaud et al. 2009). Measurement allows comparison of virulence among different strains and is usually estimated through

laboratory assays on individual plants under controlled conditions during a single pathogen cycle. The pathogenic traits that are measured include (Pariaud et al. 2009; Lannou 2012): infection efficiency, latent period, spore production rate, infectious period, and lesion size, all traits that are related to virulence. Disease severity (area of plant tissue that is symptomatic) may be measured and used as a proxy for pathogen virulence (Pariaud et al. 2009). In this case, disease severity includes infection efficiency (probability that a spore deposited on a receptive host surface produces a lesion) and lesion size (surface area that produces spores) (Lannou 2012).

Expression of pathogenic traits is influenced by environmental conditions (Milus et al. 2006; Lannou 2012). The environment affects not only pathogen biology, but also its interaction with the host, as it influences resistance and tolerance mechanisms (Barrett et al. 2009; Lively 2006). The effect of environment on pathogen infection and dispersion has been studied (Barrett et al. 2009), especially the effects of temperature and moisture (Barrett et al. 2009; Lannou 2012). For the specific case of *F. circinatum*, there is scarce information on the precise effects of environmental factors on the pathogen biology. Temperature affects spore germination and mycelial growth (Mullett et al. 2017; Inman et al. 2008). Conidia germinate over a wide range of temperatures with high relative humidity, with an optimum between 20 and 25ºC and mycelia grow at temperatures above 5ºC (Mullett et al. 2017) or 10ºC (Inman et al. 2008), the optimum being approximately 25ºC. Conidia of *F. circinatum* enter plants through wounds (Wingfield et al. 2008). The

134 effect of increased relative humidity on infection frequency from inoculated wounds
135 was studied, but no effect was found (Sakamoto and Gordon 2006).

136 The goal of this work was to quantify and compare pathogenic life-history
137 traits of several Spanish isolates of *F. circinatum*, with two of the isolates
138 representing the two most common haplotypes in the Spanish population. We
139 investigated the effect of temperature and moisture duration on disease
140 development to determine the influence on the expression of pathogenic traits. The
141 study was performed on *P. radiata* since it is the most susceptible host to *F.*
142 *circinatum* (Iturritxa et al. 2012; Martín-García et al. 2019), and one of the most
143 important pine species in northern Spain. We also measured spore germination and
144 sporulation as influenced by temperature, and we defined the cardinal temperatures
145 (maximum, minimum and optimum) for mycelial growth *in vitro*.

146

147 ## MATERIALS AND METHODS

148 **Fungal isolates and inoculum production.** Three isolates of *F. circinatum* were
149 selected for study (Table 1). One isolate was collected in Kortezubi (Bizkaia, País
150 Vasco) in northern Spain in 2004 (Iturritxa et al. 2011), and the other two isolates
151 were collected in Castropol (Asturias) and Galicia, respectively in 2005, during
152 surveys of areas in northern Spain affected by PPC (Pérez-Sierra et al. 2007). Two
153 of the isolates were chosen as representing the first and second most abundant
154 haplotypes in Spain, MLG32 (isolate ID 7) and MLG59 (isolate ID 26), respectively

(Berbegal et al. 2013), and each occur in one of the two genetic clusters (clusters 1 and 2, respectively) of the pathogen population in Spain. Additionally, MLG32 has been detected in all Spanish regions where PPC populations have been studied. The third isolate (MLG62, isolate ID 9), is the second most abundant haplotype in cluster 2 and was collected from *P. nigra* Arnold, a pine species significantly less susceptible to *F. circinatum* compared to *P. radiata* (Iturritxa et al. 2013) and representing a pine host with a more restricted distribution in northern Spain compared to *P. radiata*.

The isolates of *F. circinatum* were stored on colonized filter paper (Whatman no 1) at -20ºC at the Instituto Nacional Investigación y Tecnología Agraria y Alimentaria (INIA, Madrid, Spain) laboratory, and were cultured on potato dextrose agar (PDA, Oxoid, Unipath Ltd., Bedford, UK) in Petri-plates for 10 days at 25 ºC in the dark. The cultures were subcultured to fresh PDA and incubated for 7 to 10 days at 20ºC in the dark for production of mycelium, and used as the experiments required.

Spore suspensions of each isolate of *F. circinatum* were prepared by adding 20 mL of sterile distilled water per plate, scraping the culture surface, and filtering the suspension through two layers of sterile cheese cloth. Spore concentration was estimated with a hemocytometer and adjusted to the desired concentration for the experiment. Ten-fold dilutions of a 10^3 spores per mL suspension were prepared, and 100 µl of the suspension were plated on PDA for further confirmation of spore

viability and actual concentration used in the disease severity and *in vitro* germination trials (with three replicate plates for each experiment).

Effect of temperature and moisture duration on severity of PPC. To measure the effect of temperature and moisture duration on disease development, seedlings of *P. radiata* were purchased from Eskalmendi Nursery (Alava, Spain) and inoculated with either isolates 7 or 26. Pine seedlings were provided in 35-pot trays (7 × 5 pots of 5 × 5 × 12 cm size each) grown in a 1:1 mixture of blond and black peat with 3.5 g per liter of mineral fertilizer (14:8:13 NPK, ®Osmocote, Scotts Miracle-Gro Company, Marysville, OH). For each tray, 14 plants corresponding to two rows of a tray (14 × 2 isolates) were inoculated with one isolate each, and the remaining 7 plants were used as a water control. Approximately 5 cm was cut from the shoot tips for inoculation of the youngest growth by using a hand-held sprayer pumped twice to dispense a total volume of 2.4 mL on each plant at a concentration of 10^5 spores/mL. During inoculation, plants corresponding to each treatment were shielded using a vertical panel. The experiment design was a split-split-plot. For each growth chamber temperature (10, 15, 20, 25 or 30 ºC), all five trays (with plants previously inoculated with each of the two isolates) corresponding to each moisture duration (0, 7, 16, 24 and 48 hours), were incubated simultaneously in the growth chamber in the dark. After inoculation, moisture (high humidity) was achieved by covering the plants with a plastic bag, the inside of which was previously wetted. When a moisture treatment period ended, the plastic bags were removed and the tray transferred to a greenhouse and maintained at 25/20ºC day/night with a 12 h

198 photoperiod until the end of the experiment. The order of tested temperature, tray
199 location in the incubator, and isolate within each tray was randomly assigned. The
200 experiment was performed twice, the first experiment was conducted in April 2017
201 using 12-month-old plants, and the second experiment in September 2017 with 16-
202 month-old plants. Height of the inoculated seedlings was 30 to 50 cm, depending on
203 age. Lesion length (LL) was measured periodically every 3 to 5 days and at the end
204 of the experiment (ca. 35 days after inoculation). None of the water control plants in
205 the tray were infected, showing no contamination during inoculation or incubation.

206 Data on disease severity under different temperatures and moisture durations
207 were analyzed as a split-split-plot design. The temperature factor (whole plot
208 treatment with 5 levels) was split by the moisture duration factor (split plots with 5
209 levels), which was further split by inoculation with the two isolates of *F. circinatum*.
210 Thus, for the two factors, moisture duration and isolate, the experimental unit was
211 the tray, while for the temperature factor, the experimental unit was all five trays.
212 Lesion length (LL) was natural log transformed (1+LL) to improve normality of
213 studentized residuals (back-transformed values are presented in the results as LL =
214 $e^{(value)}$ -1). For each isolate and tray, the standardized area under the disease
215 progress curve (sAUDPC) was calculated by dividing the AUDPC by the duration of
216 the trial and compared similarly. Analysis of variance was performed using proc
217 mixed (SAS Ver. 9.4, Cary, NC). Pairwise differences of least squares means
218 between levels of each factor were calculated using t-tests and checked for
219 significance at P = 0.05.

Incubation period (days after inoculation to first symptoms of shoot necrosis) was recorded for the second trial. Number of days were adjusted to a Poisson distribution with a log-link function, with predictor variables of temperature, moisture duration and isolate, and all second-order interactions. Analysis was performed using proc genmod in SAS.

Effect of temperature and time on spore germination and sporulation. Spore germination was measured by preparing spore suspensions of isolates 7, 9 and 26 adjusted to 10^5 spores/mL. For each isolate and temperature-incubation time combination, four 40 uL droplets were placed on both sides of two microscope slides in Petri dishes, and incubated in a moist chamber at 5, 10, 20, 25 and 30ºC in the dark for 6, 24 or 48 hours. At each time point, coverslips were placed over the droplets to allow microscopic examination. The experiment was performed twice. Five images were captured at random points across each droplet using a digital color camera (Olympus Color-View I, Olympus Life Science Europe GmbH, Hamburg, Germany) mounted on the microscope (100x) (Olympus CX41)). Using the images, the number of germinated and non-germinated spores were counted. A spore was considered germinated when the length of the germ tube was at least as long as the conidium. A total of 20 spores were counted per image. Over all treatments, a total of 64,474 spores were counted, of which 16,980 had germinated (26.3% of the total).

Data were analyzed by logistic regression in SAS, using a second-degree response surface for the logit of the proportion of germinated spores with respect to the time and temperature variables. Isolates and trials were used as block factors.

The interaction between isolates and time or temperature were not included in the model as they were not significant. Parameters were estimated by the maximum likelihood method. Second-order terms were highly significant, so they were included in the model. A receiver operator curve (ROC) was used to evaluate concordance of prediction, and a deviance and a Hosmer-Lemeshow test were calculated to assess goodness of fit for the model.

The percentage of spore germination at 48 h was compared in a two-way analysis of variance, with temperature and isolate as factors. Percentage of spore germination (p) was transformed to the arc sine (square root of p/100) to improve normality of studentized residuals (back-transformed values are presented in the results as $\text{sine}^2(p*100)$. Means were compared using a multiple range test (Least Significant Difference, LSD) with significance at P = 0.05.

Sporulation was measured by counting the number of spores produced for isolates 7 and 26 at both 20 and 25ºC. Fourteen 5 mm-agar plug with mycelium were taken from a Petri-plate of each isolate of *F. circinatum* grown in the dark for 18 days, and were agitated briskly using a magnetic stirrer in a flask containing 150 mL sterile distilled water for 30 sec. The number of spores were counted using a hemocytometer based on two counts per flask, with two replicate flasks per treatment. Results were compared in a two-way analysis of variance, with temperature and isolate as factors. Means were compared using a multiple range test (LSD) with significance at P = 0.05.

263 We combined data on spore germination and sporulation for each isolate to
264 describe a “spore viability trait”, which was calculated at a specific temperature as
265 the product of the proportion of spores germinated (mean of germinated spores at
266 6, 24 and 48 h) and the number of spores produced.

267 **Effect of temperature on mycelial growth.** Mycelial growth rate (MGR) of isolates
268 7, 9 and 26 (Table 1) was determined by transferring an agar plug (5 mm diameter)
269 with actively growing mycelium to the center of a fresh Petri plate with PDA. The
270 radial perpendicular dimensions of the colony were measured every 2 days (after
271 growth at the colony edge was >1 mm) until the colony had grown to the edge of the
272 plate. For each isolate, there were 8 replicate plates at each temperature, 5, 10, 20,
273 25, 30 and 40ºC. Cultures were incubated in the dark. The experiment had a split-
274 plot design and was performed three times. Thus, the split-plot design was
275 comprised of temperature (five levels) as the whole plots in a randomized block
276 design with three blocks (the three trials). Isolate of *F. circinatum* (with three levels)
277 was the split-plot factor in a randomized design, with eight replicates (plates). The
278 growth rate was estimated over the period that growth was linear and calculated as
279 the difference between the first and last measurements of the mean of the 4 radii for
280 that period of linear growth divided by the number of days between the two
281 recordings. Either no growth or very limited mycelial growth was recorded at 40ºC,
282 so it was not included in the analysis.

283 Analysis of variance was performed using a mixed model (proc mixed) in SAS.
284 Isolate was considered a fixed factor to explore its interaction with temperature. The

best adjusted model (based on AIC values) considered different variance for each temperature. A generalized linear model was also used to fit the MGR data, but residual analysis unambiguously supported the mixed model. Estimated LS means for each isolate and temperature were compared using pairwise t-tests and checked for significance at P = 0.05.

RESULTS

Effect of temperature and moisture duration on *Pinus radiata* infection. Both isolates caused disease at all combinations of temperature and moisture duration tested, including 0 h of moisture. However, disease (measured either as sAUDPC or final lesion length) that developed in the inoculated *P. radiata* seedlings due to the effect of temperature was dependent on the inoculated isolate of *F. circinatum* (interaction term F = 7.53, p = 0.0004; Table 2). In contrast, moisture duration was not dependent on the isolate (interaction term F = 0.66, p = 0.6254) and had a significant effect on final lesion length (F = 2.93, p = 0.0467), but not on sAUDPC (F = 2.62, p = 0.0655). Final lesion length was greater when inoculated with isolate 7 at temperatures of 25 and 30ºC (2.68 and 2.81 cm, respectively) compared to isolate 26. But mean lesion length caused by isolate 7 (0.98 cm) were shorter than those due to isolate 26 (2.61 cm) at 15ºC (Fig. 1). At 10 and 20ºC, there were no significant differences in lesion size between the isolates. A similar pattern was observed for sAUDPC. The sAUDPC for isolate 7 was greater at 25 and 30 ºC (1.84 and 1.70,

306 respectively) and lower at 15ºC than the sAUDPC for isolate 26 (1.28). Effects of
307 temperature on each isolate revealed differences in sAUDPC for isolate 7 between
308 temperatures of 15 and 25 ºC (0.74 and 1.84, respectively). Lesions were longest
309 with a 7 h moisture period (2.5 cm, Fig. 2). It was the only moisture period that
310 resulted in significantly longer lesions compared to those at the 0 h (1.2 cm) or 48-h
311 (1.5 cm) moisture period.

312 Incubation period was found to be similar for both isolate 7 and 26, being
313 25.05 days (standard error [se] ± 0.322) and 25.03 days (se ± 0.401), respectively.
314 Incubation period depended on temperature (Wald chi-square = 61.539, $p < 0.001$)
315 and on its interaction with moisture duration (Wald chi-square = 30.202, $p = 0.017$).
316 No other term of the regression model was significant. Incubation period increased
317 as temperature decreased, and was similar at 20, 25 and 30ºC (Table 3).

318 **Effect of temperature and time on spore germination and sporulation.** Spore
319 germination was observed at 5ºC, but germination depended on the isolate of *F.*
320 *circinatum* tested. At 5ºC, isolate 7 had not germinated at 6 or 24 h, but had at 48 h
321 (it had the highest germination rate overall at 48 h). Germination of isolates 9 and 26
322 began at 6 h, but was very low (<0.3%). At 10 ºC, spores of isolate 7 germinated at
323 24 and 48 h with the highest germination rate overall (Table 4). Mean spore
324 germination at 48 h was affected by temperature ($F = 72.78$, $p <0.000$), isolate ($F =$
325 8.39, $p = 0.0004$) and the interaction of temperature and isolate ($F = 2.54$, $p =$
326 0.0144). Germination of isolate 7 was significantly higher compared to the other

isolates at 10 and 20ºC. Isolates were not significantly different at any other temperature (Fig 3).

The regression model that best described spore germination was a second-order response surface for the logit of the proportion of germinated spores (p) with respect to time (t) and temperature (T):

$$\text{logit (p)} = \mu + \alpha_i + \beta_j + \lambda_1 t + \lambda_2 T + \lambda_3 tT + \lambda_4 t^2 + \lambda_5 T^2$$

where μ is the overall mean, α is the main effect of experiment i (*i=1,2*), and β is the main effect of isolate (*j =1,2,3* for the isolates 7, 9 and 26 respectively). The percentage of concordance for the model was 84.4%. The area under the ROC was 0.848. Both the concordance and ROC are indicative of a reasonable fit. However, the deviance test and the Hosmer-Lemeshow test were significant, showing that the model did not fit the data. Estimates of the model parameters and their 95 % confidence intervals are presented (Table 5). As indicated by the model, the odds for proportion of germinated spores of isolate 7 and isolate 9 are, respectively 3.2 and 1.9 times higher than that of isolate 26. Odds for germination are 1.8 times for each 1ºC increase, and 1.3 times for each increase of 1 h.

For all isolates maximum spore germination as a proportion was observed at 41.1 h at a temperature of 27.9 ºC. For isolate 7 the proportion was 0.7712 [95% CIs = 0.7624, 0.7797], for isolate 9 0.6595 [95% CIs = 0.6498, 0.6690], and for isolate 26 was 0.5094 [95% CIs = 0.4989, 0.5199]. The overall mean proportion of germinated spores was 0.6543 (95% CIs = 0.6468, 0.6617).

Isolate 7 produced more spores compared to isolate 26 at both 20 and 25ºC (isolate and temperature main effects: F = 13.37, p = 0.0033, and F = 13.05, p = 0.0036, respectively). Mean percentage germination at both 20 and 25ºC was higher for isolate 7 (isolate and temperature main effects: F = 14.8, p = 0.0002, and F = 0.96, p = 0.329, respectively) (Fig. 4). Spore production and germination variables were combined as a measure of spore viability: spore viability was higher for isolate 7 compared to isolate 26 (0.9 and 0.5 viable spores/mL at 25 ºC, and 0.69 and 0.19 viable spores /mL at 20 ºC, respectively).

Effect of temperature on mycelial growth. MGR of all isolates increased with temperature from 5 to 25ºC and decreased at 30ºC. The slope depended on isolate of *F. circinatum* and thus, resulted in a significant interaction between isolates and temperature (F = 21.98, p <.0001). Maximum MGR was at 25ºC for all isolates, although mean values did not differ significantly at 20ºC or 30ºC depending on the isolate (Table 6). MGR at 30ºC slowed for all isolates except isolate 9, which had a mean MGR not significantly different from that at 20 and 25 ºC.

Minimum MGR was at 5ºC, but depended on isolate. After 185 days of incubation at 5ºC, isolate 7 ceased growth, isolate 9 was still growing on 7 of 8 plates, and isolate 26 was still growing on 3 of 8 plates. Under the microscope the mycelium presented hyphal thickening and melanization (Fig. 5). Some of the apparently inactive mycelium was transferred to fresh plates of PDA and incubated at 25ºC and 5ºC. Growth recovered, showing the mycelium was alive, with an average rate for the 3 isolates of 3 cm over 11 days at 25ºC, and 2.95 cm over 218 days at 5 ºC,

370 respectively. At a temperature of 40ºC, no mycelial growth was observed for any of
371 the isolates up to ca. 10 days. However, after 10 days the agar dried out.

372

373 DISCUSSION

374 In this study, isolate 7 of *F. circinatum* caused more severe PPC on *P. radiata*
375 compared to isolate 26 at 25 and 30ºC, but less severe disease at 15ºC. Spore
376 germination was also higher for isolate 7, as was maximum germination (77.1%).
377 Moreover, isolate 7 produced more spores compared to isolate 26 at 20 and 25ºC.
378 Thus, we conclude that isolate 7 is more virulent than isolate 26, at least at 25 to
379 30ºC. Virulence of isolates 7 and 9 to *P. radiata* have been tested previously (Iturritxa
380 et al. 2012, 2013), and in that study lesion lengths were similar for both isolates
381 when inoculated at 18 ± 5ºC. Isolate 9 has been tested on other pine spp. (Perez
382 Sierra et al. 2007; Mullet et al. 2017). However, this is the first virulence study
383 comparing isolates 7 and 26 of *F. circinatum* at 10 to 30ºC with different moisture
384 durations. The two isolates represent the first and second most abundant haplotypes
385 in Spain. The isolate 7 haplotype accounts for 56% of the Spanish population of *F.*
386 *circinatum*, and 94% of one of the two population genetic clusters identified
387 (Berbegal et al. 2013). The isolate 26 haplotype represents 21% of the population,
388 and 54% within the genetic cluster with which it is associated (Berbegal et al. 2013).

389 High relative humidity is considered an important factor for successful
390 infection of pine by spores of *F. circinatum* (Sakamoto and Gordon 2006; EFSA et

al. 2020), although specific conditions are unknown. In a survey of 50 pine stands in Cantabria, trees with PPC were found more frequently in proximity to the coast (Blank et al. 2019). Distance to a coastline was also found to be an important predictor of habitat suitability for *F. circinatum* in Spain (Serra-Varela et al. 2017), presumably due to the higher relative humidity close to the coast. Laboratory and controlled-environment experiments provide information on disease epidemiology and pathogen biology, and can be useful to help understand pathogen evolution and to improve disease prediction systems. Sakamoto and Gordon (2016) inoculated *P. radiata* with *F. circinatum* in the field and although infection frequencies tended to be higher at 100% RH compared to at ambient humidity, the differences were not significant. However, there was a significant effect of trial on infection frequency, indicating the variability inherent to artificial inoculations. Our study determined that regardless of the isolate virulence, moisture periods from 7 to 24 h after inoculation resulted in the longest lesions of PPC, while LL was less at 0 and 48 h. A low disease severity at 48 h is unexpected given that spore germination was highest for all isolates at approximately this time. By 48 h post inoculation, plant defenses have likely been elicited (Hernandez-Escribano et al. 2020; Carrasco et al. 2017) including induction of pathogenesis-related proteins and other proteins in *P. radiata* (Carrasco et al. 2017). We observed disease at 0 h of moisture (which resulted in the shortest lesions), when spores have barely germinated. This may be due to inoculation by spray requiring a drying period of approx. 1 h at room temperature prior to transfer to the greenhouse. A moisture period of 1 h is likely sufficient for infection and

413 subsequent disease development. Maximum severity was achieved with 7 h of
414 moisture.

415 Isolate interacted with temperature but not with moisture duration. Pathogenic
416 traits of *F. circinatum* were assessed only on the highly susceptible species *P.*
417 *radiata*, so potential host genotype interactions were not considered (Lannou 2012),
418 but in general terms, we can infer a better adaptation to warmer temperatures for
419 isolate 7 compared to isolate 26, based on significantly more severe disease at 25
420 and 30ºC. Incubation period was influenced by temperature during infection, but as
421 in previous studies (Miller et al. 1998) we did not find differences between isolates.
422 This corroborates previous results that suggest latent period (number of days to first
423 sporulation) is a better estimate of virulence (Pariaud et al. 2009; Lannou 2012).
424 Latency measures the potential number of disease cycles (Miller et al. 1998) which
425 is related to pathogen virulence. We could not determine the latent period in our
426 study as sporulation is not easy to observe on stem lesions of PPC on *P. radiata* and
427 had not occurred by the time the experiments were terminated.

428 Results indicated that optimum temperature for MGR of *F. circinatum* is
429 approximately 25ºC, depending on the isolate. This is in agreement with findings of
430 Inman et al. (2008) who tested mycelial growth at 10 to 25ºC of 14 isolates from
431 North America, Spain and Japan and found all isolates grew most rapidly at 25°C.
432 Mullet et al. (2017) reported that the MGR at 25 ºC can depend on the isolate. Unlike
433 some other studies (Quesada et al. 2019), we found differences between 25 and
434 30ºC for two of the three isolates studied. In our study, isolate 9 had the highest

435 MGR at all temperatures except 5ºC. This, coupled with the higher germination rate
436 at 30ºC compared to isolates 7 and 26, suggests this isolate has preference for
437 warmer temperatures when tested *in vitro*. The results are in broad agreement with
438 a previous study evaluating phenotypic characteristics of *F. circinatum* in Spain
439 (Berbegal et al. 2013; Mullet et al. 2017). Mullet et al. (2017) concluded that MLG62
440 (isolate 9) and MLG59 had slower growth rates at 10 and 25ºC but faster at 35ºC
441 when compared with MLG32 (isolate 7). Isolate 26 grew more *in vitro* than isolate 7
442 at 30º C, which was not congruent with the results of the PPC lesion severity
443 experiment. Unlike MGR, disease severity is influenced not only by the isolate but
444 also by its interaction with the plant and the environmental conditions. Some isolates
445 of *F. circinatum* may be adapted to warmer conditions. Changes in climate provide
446 trait selection for pathogen evolution (Ghelardini et al. 2016), and thus affect
447 pathogen distribution (Garbelotto and Pautasso 2012; Vaumourin and Laine 2018).
448 For example, the population of *Puccinia striiformis* f.sp. *tritici* has adapted to warmer
449 temperatures in the south-central USA. Recently collected isolates are more virulent
450 (a shorter latent period and higher germination at 18ºC compared to 12ºC) (Milus et
451 al. 2006).

452 A temperature of 5ºC was not lethal to *F. circinatum*, but the fungus ceased
453 growth. Growth did not occur at 40ºC. Other studies showed that mycelial growth
454 ceases at 40ºC, although it was not lethal (Mullet et al. 2017). At 5ºC we observed
455 hyphal melanization with isolate 7. Melanin multifunctionality is well documented in
456 fungi, including their role in pathogenicity as well as protection against extreme

environmental conditions (Gessler et al. 2014) . Survival structures have yet to be described for *F. circinatum*, so the observed melanisation of hyphae warrants further study.

PPC is established in northern Spanish on *P. radiata* and *P. pinaster* (Iturritxa et al. 2013). *P. pinaster* is a native species to the Mediterranean region and is economically and ecologically important. *P. radiata* is an exotic species, planted extensively due to its rapid growth. We contend that if temperatures increase in regions where *F. circinatum* occurs, those isolates (e.g. isolate 7) better adapted to warm temperatures will dominate the population. Also warmer conditions may lead to increased host plant vulnerability (Ramsfield et al. 2016). Under heat and drought stress, trees may be less able to respond with an effective defense (Ennos 2015). *Pinus pinaster* is less susceptible to *F. circinatum* than the introduced *P. radiata*, but will likely experience heat and drought stress that may reduce its defense to better adapted isolates of *F. circinatum*.

The isolates we used were sampled in 2004 and 2005, early in the epidemic in Spain (although a technical report suggest that the disease may have been present earlier). Isolate 7 represented a unique haplotype (MLG32) in north-east Spain (Berbegal et al. 2013), while the remaining haplotypes, including representative isolates 9 and 26, were present in north-west Spain. The idea that the two most abundant haplotypes (MLG32 and MLG59) in Spain represent two independent introductions that underwent clonal divergence is supported by the genetic structure and the lack of sexual reproduction (Berbegal et al. 2013). Since

the pathogen was introduced, we expect that population virulence has evolved (Pariaud et al. 2009; Laine and Barrès 2013; Ennos 2015). The virulence-transmission trade-off hypothesis (Sacristán and García-Arenal 2008) predicts that if virulence, which is assumed to be correlated to within-host multiplication, results in severe host damage and mortality, it will transmit less. According to the theory, after the pathogen is established in a new host, reduced virulence will evolve (Desprez-Loustau et al. 2016). However, a trend to a reduced virulence is not always observed (Desprez-Loustau et al. 2016; Sacristán and García-Arenal 2008). Monocultures of plantations with low genetic diversity and/or high tree density may allow rapid reproduction and transmission, even for virulent strains (Desprez-Loustau et al. 2016: Ennos 2015). *P. radiata* plantations are plentiful in north-east Spain, where a unique haplotype (isolate 7) that is highly virulent predominates . Thus, rapid transmission may have occurred since pathogen introduction, and lower virulence might not be evolving in *F. circinatum* in Spain.

The differential genetic resistance that pine species have in Spain can also drive pathogen evolution (Iturritxa et al. 2012). As noted, *P. radiata* is more susceptible than *P. pinaster* (Iturritxa et al. 2013), which provides selection for the pathogen to adapt (Sacristán and García-Arenal 2008). Pathogen adaptation to different populations within a host species (local adaptation) may also occur (Sacristán and García-Arenal 2008). Isolate 7 of *F. circinatum* has been screened against several provenances of *P. pinaster* (Elvira-Recuenco et al. 2014) and northern Spanish provenances were the most resistant. So, differential resistance

between species and among provenances of *P. pinaster* may result in adaptation of *F. circinatum*. The changes can be tracked by studying virulence-related traits for both historic and current isolates. Only then can evolutionary changes in the pathogen population truly be ascertained.

ACKNOWLEDGEMENTS

We thank M.T. Morales Clemente for her valuable technical assistance and Marta Ballesteros for her assistance in measurements. This research was supported by projects RTA 2013-00048-C03-01 and RTA2017-00063-C04-01, Ministerio de Economia y Competitividad (Subprograma de Proyectos de I+D orientado a los Recursos y Tecnologias Agrarias en coordinación con las Comunidades Autonomas).

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630
631

632 TABLE 1. Spanish isolates of *Fusarium circinatum* used to study the effects of
633 temperature and moisture duration on pathogenic life-history traits of the fungus
634

ID	Collection year	Host species	Geographic origin	Haplotype [c]	Mating type[c]	Cluster[c]	% total[d]	% cluster[e]
7 [a]	2004	*P. radiata*	Kortezubi (Bizkaia)	MLG32	MAT-2	1	56	94
26 [b]	2005	*P. radiata*	Castropol (Asturias)	MLG59	MAT-1	2	21	54
9 [b]	2005	*P. nigra*	Galicia	MLG62	MAT-1	2	6	15

635

636 [a] Colección Española de Cultivos Tipo (CECT), reference number 20759, Valencia,
637 Spain.
638 [b] Isolates from culture collections maintained at the Instituto Agroforestal
639 Mediterráneo, Universitat Politècnica de Valencia, Spain. Isolates 26 and 9 have ID
640 214 and ID 253, respectively (Perez-Sierra et al. 2007).
641 [c] Haplotype, mating type, and cluster according to Berbegal et al. (2013)
642 [d] Percentage of isolates of that haplotype relative to the total population studied
643 (N=131) according to Berbegal et al. (2013)
644 [e] Percentage of isolates of that haplotype relative to the population within each
645 cluster (N=79 for cluster 1 and N=52 for cluster 2) according to Berbegal et al. (2013).
646

TABLE 2. Type 3 fixed effects included in the mixed model analysis of a split-split-plot design[a] to study effect of temperature and moisture duration on severity of pine pitch canker (measured as lesion length at the end of the trial and as sAUDPC[b]) caused by two isolates (7 and 26) of *Fusarium circinatum* on seedlings of *Pinus radiata*

Fixed effect	**Df num[b]**	**Df denom[b]**	**Final lesion length**		**sAUDPC[b]**	
			F-value	**Pr > F**	**F-value**	**Pr > F**
Temperature (T)	4	4	0.12	0.9686	0.48	0.7508
Moisture Duration (M)	4	20	2.93	**0.0467[c]**	2.62	0.0655
T*M	16	20	0.22	0.9984	0.27	0.9949
Isolate (Isol)	1	25	3.64	0.0678	5.13	**0.0324**
T*Isol	4	25	7.53	**0.0004**	5.62	**0.0023**
M*Isol	4	25	0.66	0.6254	1.03	0.4088
T*M*Isol	16	25	1.09	0.4157	1.26	0.2957

[a] Split-split-plot design with temperature and moisture period for the whole plot and subplot treatments, respectively.

[b] Df num and Df denom are the degrees of freedom for numerator and denominator, respectively; sAUDPC is the standardized Area Under Disease Progress Curve

[c] p-values in bold are significant (P≤0.05)

659 TABLE 3. Effect of temperature on the incubation period (IP) for development of PPC
660 on *Pinus radiata* plants inoculated with two isolates (7 and 26) of *Fusarium*
661 *circinatum*

Temp (ºC)	IP mean[y] (days)	Std error
10	28.40 a[z]	0.727
15	26.89 a	0.630
20	23.52 b	0.444
25	23.60 b	0.419
30	23.22 b	0.615

662

663 [y] IP mean value for the two isolates tested. Data by isolate were pooled because no
664 significant differences between isolates were detected.
665 [z] Means followed by the same letter do not differ significantly ($P \leq 0.05$) according to
666 the Wald chi-square test for the pairwise mean difference.
667

668 TABLE 4. Effect of temperature and period of incubation on the percentage of spores
669 germinated for three isolates of *Fusarium circinatum*

670

Temperature (ºC)	**Isolate ID**	**6h**	**24h**	**48h**
5	7	0 ± 0 [a]	0 ± 0	2.0 ± 2.57
	9	0.2 ± 0.45	0.1 ± 0.14	1.1 ± 1.59
	26	0.3 ± 0.74	0.7 ± 1.32	0.3 ± 0.76
10	7	0.1 ± 0.14	13.5 ± 15.74	24.5 ± 24.93
	9	0.1 ± 0.22	3.9 ± 6.5	9.0 ± 23.13
	26	0.5 ± 0.68	0.8 ± 0.88	2.6 ± 2.74
20	7	3.5 ± 3.12	60.7 ± 24.0	79.5 ± 17.04
	9	0.2 ± 0.45	41.5 ± 37.08	50.2 ± 27.84
	26	11.3 ± 29.13	14.8 ± 12.55	40.9 ± 27.67
25	7	15.8 ± 6.97	67.1 ± 29.38	71.1 ± 22.90
	9	0.1 ± 0.25	52.9 ± 28.05	60.7 ± 19.49
	26	9.9 ± 18.80	20.4 ± 19.12	61.6 ± 16.72
30	7	29.0 ± 13.69	64.5 ± 21.88	55.8 ± 22.32
	9	16.6 ± 15.20	51.5 ± 20.65	75.8 ± 18.56
	26	4.0 ± 8.24	46.0 ± 26.43	53.7 ± 24.98

671

672 [a] mean values of eight measures ± standard error

673 TABLE 5. Statistical analysis of parameters estimated by logistic regression[a] of the
674 proportion of germinated spores of *Fusarium circinatum* over different periods of time
675 and at different temperatures (both continuous regressors), and with trial and isolate
676 as main factors

Effect	**Estimate[c]**	**Wald Chi-sq[d]**	**95 % confidence limits**		**Exp(est)[e]**
Trial [b]	0.1422	42.3862	0.0994	0.1850	1.153
Isolate 7 [b]	0.1772	1789.587	1.1228	1.2319	3.245
Isolate 9 [b]	0.6233	578.181	0.5725	0.6741	1.865
Time (t) (hours)	0.2618	1873.28	0.2501	0.2738	1.299
Temperature (T)	0.5956	1768.30	0.5680	0.6236	1.814
t*T	-0.0026	290.738	-0.00293	-0.00233	0.997
t^2	-0.0023	1389.46	-0.00241	-0.00217	0.998
T^2	-0.0087	1222.43	-0.00921	-0.00824	0.991

677 [a] The logistic regression model was estimated using the maximum likelihood
678 method, based on 16980 germinated spores out of 64478.
679 [b] Second trial and isolate 26 were used as references in the parameterization of the
680 categorical variables.
681 [c] Estimate refers to the regression coefficient in the logistic model.

682 [d] The Wald chi-square is the statistic used to check for significance of the estimate.

683 All terms in the model were significant with Pr>ChiSq <0.0001.

684 [e] Exp (est) is $e^{estimate}$, which represents the odds ratio of that parameter.

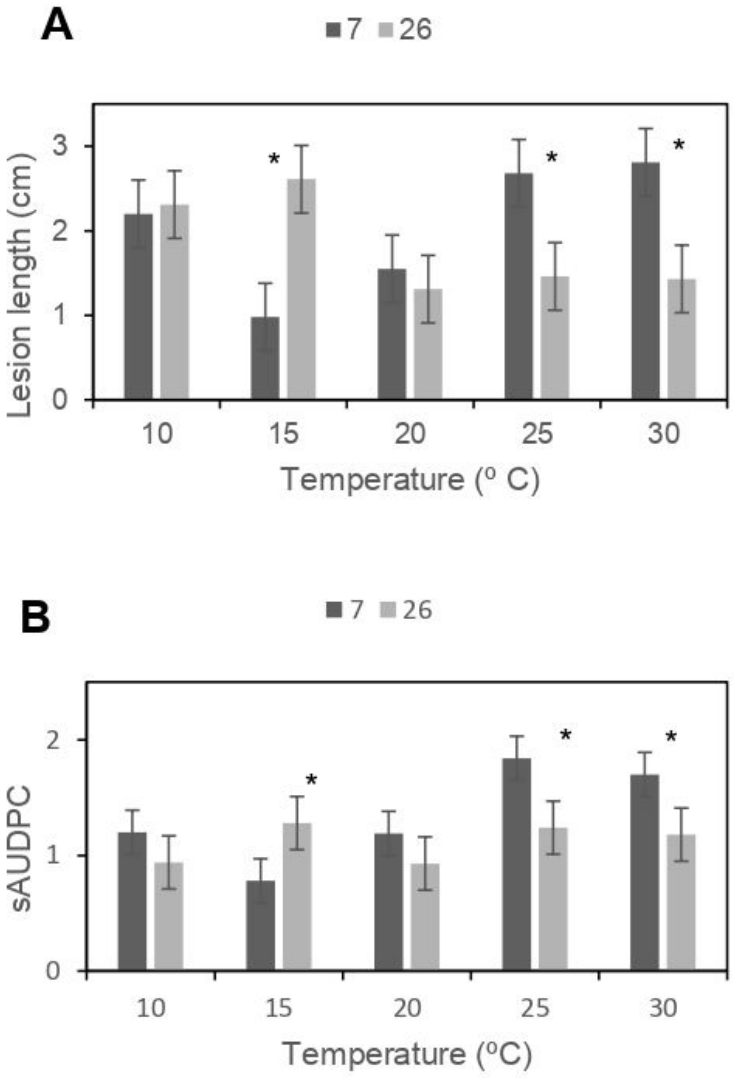
685 TABLE 6. Mean mycelial growth rate (mm/day) for three isolates of *Fusarium*
686 *circinatum* measured on potato dextrose agar at different five different temperatures
687

Temp (ºC)	**Isolate 7**	**Isolate 9**	**Isolate 26**	**Mean (std error)**
5	0.035[y] D[z] a[z]	0.026 C b	0.021 D c	0.027 (0.0186) D
10	0.094 C b	0.113 B a	0.089 C b	0.099 (0.0189) C
20	0.269 A b	0.332 A a	0.305 AB ab	0.302 (0.0208) AB
25	0.303 A b	0.363 A a	0.347 A ab	0.338 (0.0211) A
30	0.175 B c	0.322 A a	0.259 B b	0.252 (0.0193) B
Mean (std error)	0.175 (0.0098) c	0.231 (0.0098) a	0.204 (0.0098) b	

688 [y] Mean values of three trials with 8 replicates for each isolate and temperature.
689 Standard errors are 0.0186, 0.0195, 0.0248, 0.0252 and 0.0206 for individual values
690 at temperatures of 5, 10, 20, 25 and 30ºC, respectively.
691 [z] Means followed by the same letter do not differ significantly ($P \leq 0.05$) according to
692 an LSD test. Uppercase letters are for comparisons within a column and lowercase
693 letters for comparisons within a row.
694

695 **Fig. 1. A.** Pine pitch canker lesion length at the end of the trial, and **B.** Standardized
696 Area Under Disease Progress Curve (sAUDPC) for different temperatures during
697 infection with two isolates (7 and 26) of *Fusarium circinatum* for seedlings of *Pinus*
698 *radiata*. * indicates a significant ($P \leq 0.05$) difference between isolates at that
699 temperature using a pairwise t-test of the estimated least square-mean difference.
700 Error bars represent standard errors.

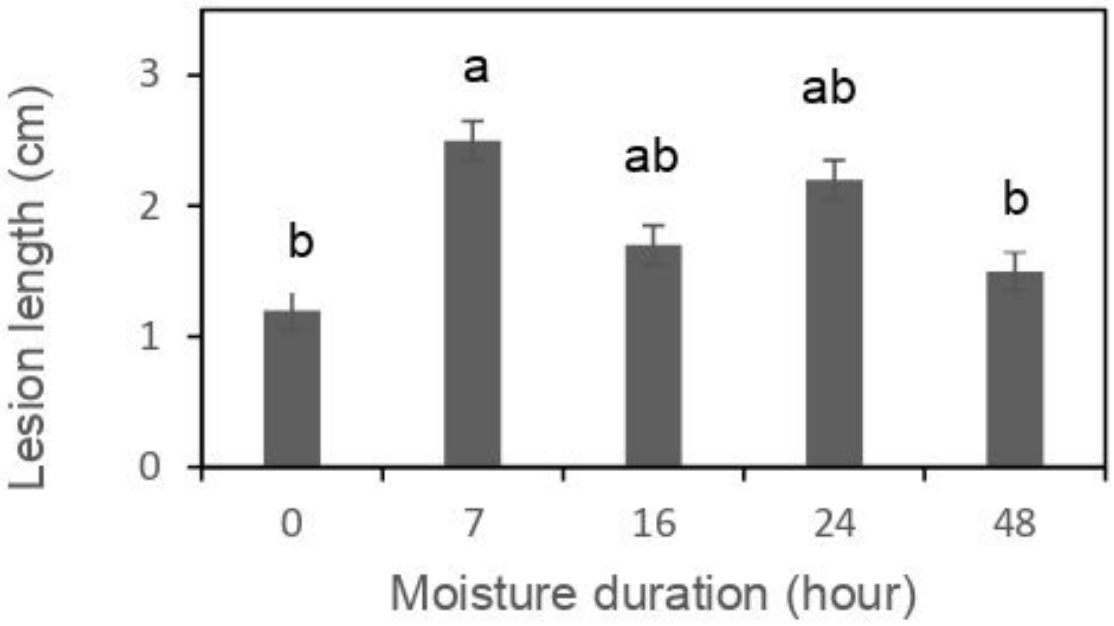
701



702

703 **Fig. 2.** The effect of moisture duration during infection on lesion length of pine pitch
704 canker at the end of the trial. Seedlings of *Pinus radiata* were inoculated with
705 *Fusarium circinatum*. Means with the same letter do not differ significantly ($P \leq 0.05$)
706 according an LSD test. Error bars represent standard errors.

707

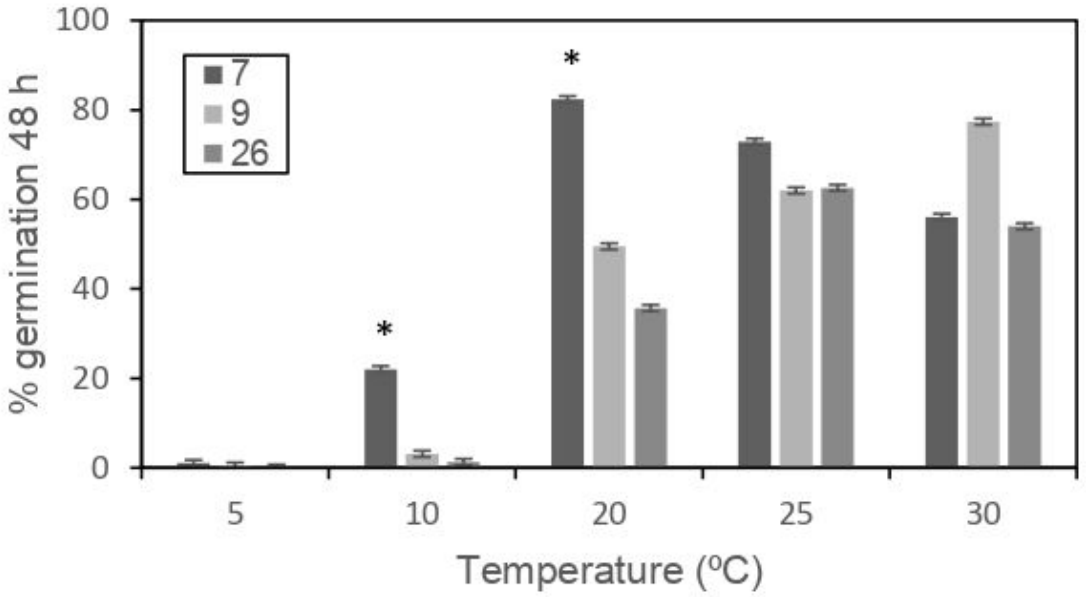


708

709

710 **Fig. 3.** Effect of temperature on the percentage of spore germination of three isolates
711 (7, 9 and 26) of *Fusarium circinatum* measured at 48 hours. * indicates a significant
712 difference between isolate spore germination at that temperature according to an
713 LSD test ($P \leq 0.05$). Least square-mean values are back-transformed from values of
714 percentage germination (p) transformed to the arc sine (square root of p/100) for
715 ANOVA test. The interaction isolate*temperature was significant (p-value = 0.014).
716 Error bars represent standard errors.

717

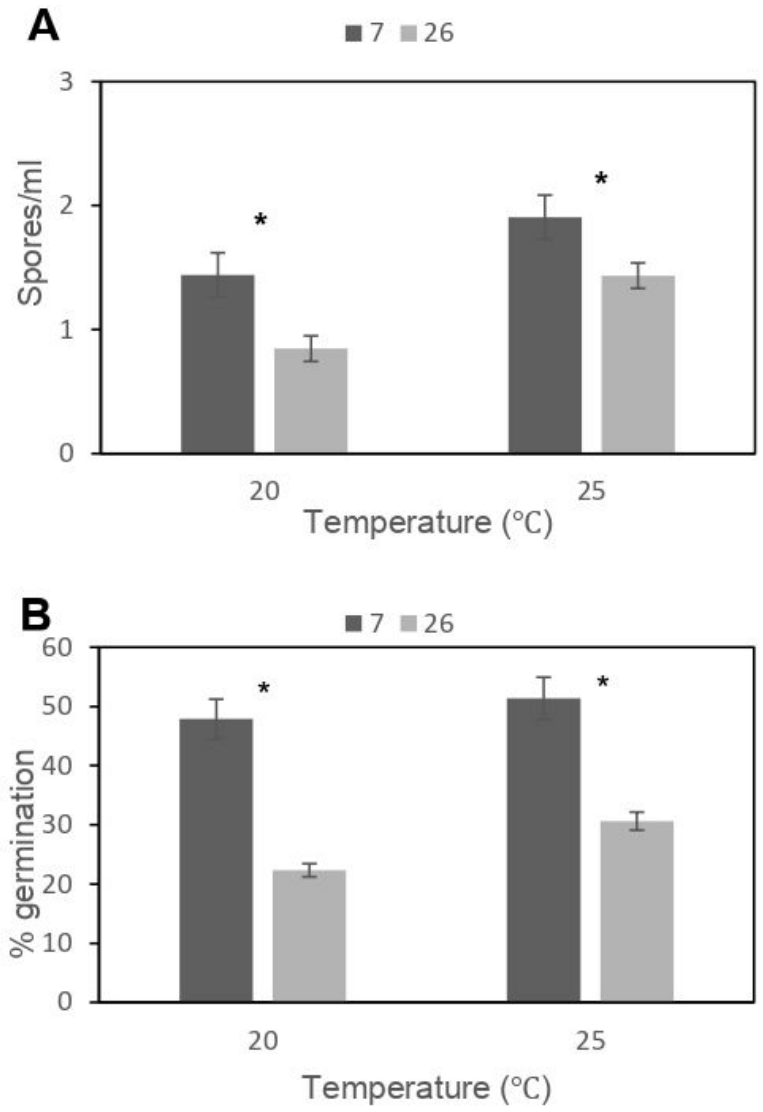


718

719

720 **Fig. 4.** Differences in **A.** sporulation, and **B.** spore germination for two isolates (7
721 and 26) of *Fusarium circinatum* at two temperatures, 20 and 25ºC. * indicates
722 significant differences between isolates based on a two-way analysis of variance and
723 an LSD test $P \leq 0.05$). Error bars represent standard errors.

724



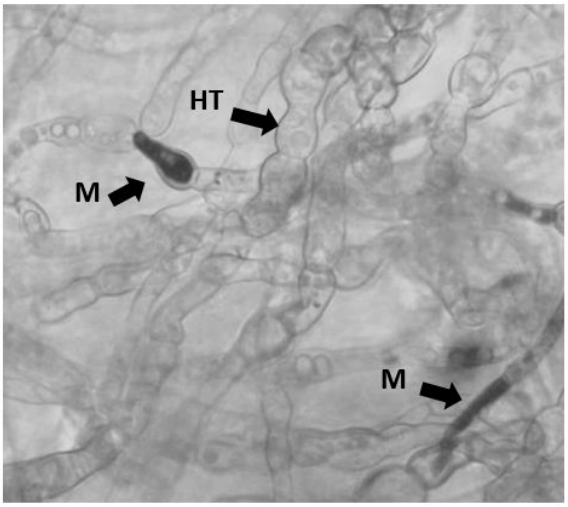
725

726

727 **Fig. 5.** Melanization (M) and hyphal thickening (HT) observed in *Fusarium circinatum*

728 (isolate 7) after growing on potato dextrose agar for 185 days at 5ºC (400x)

729



730