Effects of Cultivar Susceptibility, Fruit Maturity, Leaf Age, Fungal Isolate, and Temperature on Infection of Almond by *Colletotrichum* spp.

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Abstract

Almond anthracnose, caused by *Colletotrichum* spp., is a reemerging disease in Spain. To date, little research has been conducted on the factors affecting this disease development. In this study, the effects of cultivar, fruit wounding and maturity, leaf age, fungal isolate, and temperature on almond infection by *Colletotrichum* spp. were evaluated under laboratory-controlled conditions. Inoculations were performed using conidial suspensions of *Colletotrichum acutatum* or *C. godetiae*. Disease severity was higher in wounded than in unwounded fruit. Based on observations of inoculated fruit, Ferraduel and Nonpareil were the most tolerant cultivars, while Tarraco and Penta were the most susceptible cultivars. Four categories of susceptibility (highly susceptible, susceptible, moderately susceptible, and resistant) were distinguished by using the cluster analysis statistical approach. Differences in susceptibility between young and old

leaves were observed, but Nonpareil was consistently the most tolerant cultivar. Significant differences in virulence between *C. acutatum* and *C. godetiae* were observed in inoculated fruit, with *C. acutatum* being the most virulent. Disease development was more severe when inoculations were performed at the fruitlet stage or when the fruit were incubated at approximately 25°C, with respect to other maturity stages and temperatures evaluated. Natural fruit infections were also assessed. Cultivar susceptibility data were compared between laboratory tests and field observations. A significant positive linear correlation was obtained between the susceptibility of the common cultivars evaluated under the two conditions.

Keywords: almond anthracnose, biology, epidemiology, *Prunus dulcis*, tree nuts

Almond (*Prunus dulcis* (Mill.) D. A. Webb) is the most important nut crop in the Mediterranean basin, and this fruit crop is the second in size in Spain after the olive (*Olea europaea* subsp. *europaea* L.). Spain is the world leader with respect to the area of almond planted, with more than 500,000 ha cultivated, of which approximately 140,000 ha are grown in the Andalusia region of southern Spain (Arquero et al. 2013; FAO 2018).

In Spain, almond has been traditionally associated with marginal growing areas characterized by unfavorable climatic, edaphic, and orographic conditions (Arquero et al. 2013). However, during recent years, almond has been introduced as a potential alternative crop in nontraditional almond-growing regions across the country, usually in areas characterized by moderate temperatures in spring and summer and high relative humidity (RH). Thus, a shift from traditional dry-farming cultivation systems to more intensive almond-cropping systems based on high-input strategies has been observed progressively, particularly in southern Spain. This change has been conducted with the objective of enhancing the competitiveness of almond production in the global market (i.e., markedly increasing almond kernel production). Indeed, Spanish almond production currently exceeds 1,000 kg ha⁻¹ in intensive systems, whereas it is

usually less than 150 kg ha⁻¹ in traditional almond-cropping systems (Arquero et al. 2013; Ollero-Lara et al. 2016, 2019).

Consequently, this forced change in almond crop management and the environmental conditions of the new almond-growing regions have favored an increase in the incidence and prevalence of common foliar diseases of this crop such as red leaf blotch (*Polystigma amygdalinum* P. F. Cannon), shot hole (*Wilsonomyces carpophilus* (Lév.) Adask., J. M. Ogawa & E. E. Butler), blossom blight (*Monilia* spp.), and leaf curl (*Taphrina deformans* (Berk.) Tul.), as well as the emergence of new diseases and the reemergence of old ones (Ollero-Lara et al. 2016, 2019). In spring 2014, a serious disease showing typical symptoms of anthracnose was observed in almond orchards in the provinces of Huelva and Seville, Andalusian region. Although there were previous references of almond anthracnose (AA) in the main growing areas of this crop throughout the Iberian Peninsula, it had been considered a secondary disease in Spain and the Mediterranean basin until recently (López-Moral et al. 2017). However, AA has been described as the most important fruit disease of almond in the main growing regions of this crop worldwide, such as Australia, Greece, Israel, and the United States (Adaskaveg and Förster 2000; Adaskaveg et al. 2002b; McKay et al. 2009; Shabi and Katan 1983).

The main characteristic symptom of AA is fruit rot, which consists of depressed, round, and orange or brown lesions that develop on the fruit surface in spring to summer. Abundant production of white mycelia and orange masses of conidia are present on the surface of infected fruit when the disease progresses. Subsequently, fruit mummify and drop prematurely (Förster and Adaskaveg 1999; López-Moral et al. 2017; Peres et al. 2005). Leaf necrosis, defoliation, shoot blight, and branch dieback are observed when serious attacks of AA occur, as a consequence of toxins produced by the pathogen in the infected fruit (Adaskaveg et al. 2012; Förster and Adaskaveg 1999; López-Moral et al. 2017). The disease is caused by *Colletotrichum* spp. belonging to the *Colletotrichum acutatum* J. H. Simmonds species complex, which is composed of 34 phylogenetic species (Damm et al. 2012). Among them, *C. fioriniae* Marcelino & Gouli ex R. G. Shivas & Y. P. Tan (pink colony type) and *C. godetiae* Neerg. (syn. *C. clavatum* Agosteo, Faedda & Cacciola; gray colony type) are the prevalent species causing AA in the main almond-growing regions of the world (Adaskaveg et al. 2002b;

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Guerber et al. 2003; McKay et al. 2009). Recently, López-Moral et al. (2017) reported for the first time *C. acutatum* (pink colony type) associated with AA in southern Spain, where *C. godetiae* was also reported as the most frequent species causing AA. *C. acutatum* has also been previously reported as a causal agent of AA in South Australia (Whitelaw-Weckert et al. 2007).

Disease management is mainly based on fungicide applications from the beginning of the flowering period (pink bud) to the beginning of summer (Adaskaveg and Förster 2000; Adaskaveg et al. 2002b, 2017) and on cultivar resistance (Adaskaveg et al. 2002a; Diéguez-Urbeondo et al. 2011). The latter control measure has been studied in California by evaluating the susceptibility of almond cultivar blossoms, leaves, and fruit to anthracnose under both field and laboratory conditions (Adaskaveg et al. 2002a; Diéguez-Urbeondo et al. 2011). In these previous studies, Nonpareil was significantly more tolerant to AA than other important cultivars used in California such as Carmel, Ne Plus Ultra, and Wood Colony. Field observations of anthracnose development in almond orchards in South Australia also indicated that Nonpareil was the most tolerant cultivar (McKay et al. 2014). In Israel, Striem et al. (1989) evaluated the resistance of four almond cultivars to AA under field conditions by counting the number of infected fruit per tree. These authors indicated that the selection M.D.1 was the cultivar most resistant to AA, followed by the selection 59/4, whereas Poria and Ne Plus Ultra were described as susceptible cultivars.

Little research has been conducted on the environmental factors that affect the development of this disease due to the fact that it has been considered a secondary almond disease in Spain until epidemic outbreaks occurred in 2014 in southern Spain. In addition, cultivar susceptibility to AA has not received much attention until recently, even though a few reports based on field observations in Spain have been published (Dicenta et al. 2010; Egea et al. 1984; López-Moral et al. 2016; Palazón and Palazón 1979; Salazar and Melgarejo 2002). All of these aspects are critical in anthracnose epidemics, and data on how agronomic and environmental factors affect pathogen infection and disease development are essential to optimize disease management strategies. Therefore, the objectives of this study were to (i) determine the influence of cultivar, fruit maturity, leaf age, and temperature after inoculation on infection of almond by *Colletotrichum* spp. and (ii) evaluate differences in pathogenicity between *Colletotrichum* spp. Studies to accomplish both objectives were conducted under laboratory-controlled conditions. In addition, natural fruit infections were also assessed in one experimental field seriously affected by AA, and cultivar susceptibility in the field was compared with results of laboratory tests.

Materials and Methods

Fungal isolates. Six representative fungal isolates of the *C. acutatum* species complex associated with AA in the Andalusia region (southern Spain) were selected for this study. Among them, there were three isolates of *C. acutatum* (Col-536, Col-537, and Col-538) and three isolates of *C. godetiae* (Col-522, Col-548, and Col-555). These two species are representative of the pink and gray colony types, respectively, described in Spain, and all of the isolates used here were previously characterized and tested for pathogenicity by López-Moral et al. (2017). Single-spore cultures from these isolates are maintained in the collection of the Department of Agronomy at the University of Cordoba (Spain). Fungal isolates growing on potato dextrose agar (Difco Laboratories, Detroit, MI, U.S.A.) at 23 ± 2°C under a 12-h diurnal photoperiod of cool fluorescent light (350 µmol m⁻² s⁻¹) for 10 days were used as the inoculum source to prepare conidial suspensions for the inoculations.

Plant material. Detached, healthy, and unwounded almond fruit or leaves of 19 commercial cultivars (Antoñeta, Belona, Constantí, Desmayo largueta, Damar, Ferraduel, Ferragnès, Garrigues, Guara, Lauranne, Marcona, Marinada, Nonpareil, Penta, Soleta, Tarraco, Texas, Tuono, and Vairo) were used in this study. These cultivars were selected because they are among the most representative cultivars currently grown in the new almond plantations of Andalusia and the Mediterranean basin (Arquero et al. 2013). Plant material was

collected from an experimental field (7-year-old trees) located in Córdoba (Alameda del Obispo experimental area), which belongs to the experimental field network established in the Andalusia region by the Andalusian Institute for Research and Formation in Agriculture and Fishery (IFAPA in Spanish).

Cultivar susceptibility. Fruit susceptibility under laboratory-controlled conditions. Prior to conducting fruit inoculations for the different experiments in this study, a preliminary experiment was conducted to evaluate the effect of wounding and the inoculation method on fruit infection. To this end, healthy fruit of susceptible cultivar Guara (maturity stage 4; see below) were washed and surface disinfested following the protocol described by López-Moral et al. (2017). Subsequently, the fruit were placed in humidity chambers (plastic containers, 22 by 16 by 10 cm), not wounded, and inoculated by spraying them until runoff with a conidial suspension (10⁵ conidia ml⁻¹) or were wounded with a sterile needle (0.8 mm in diameter) and then inoculated with a 20-µl drop of a conidial suspension (10⁵ conidia ml⁻¹) per wound. The isolates *C. acutatum* Col-536 and *C. godetiae* Col-522 were used in this experiment. After inoculation, the fruit were incubated at 23 ± 2°C under a 12-h photoperiod at 100% RH for 21 days. Fruit treated with deionized sterile water (DSW) were also included as controls. A completely randomized design with fungal isolates and inoculation methods as independent variables and humidity chambers as replications was used. There were three replicate humidity chambers per isolate and inoculation method combination or control, with 15 fruit per humidity chamber (180 almond fruit in total). The experiment was conducted twice. Although symptoms of AA were produced on inoculated fruit using both inoculation methods, the second method (wounding and drop inoculation) was selected for further analysis because disease symptoms developed earlier and were more localized than those observed in response to the spraying method, making disease assessment easier.

Cultivar susceptibility was evaluated on the fruit (maturity stage 4; see below) of the 19 commercial cultivars listed above. Fruit were washed, disinfested, wounded, and inoculated with a drop of conidial suspension as described above. For inoculations, *C. acutatum* isolate Col-536 was selected because this species was previously described as the most virulent in the infection of detached almond fruit (López-Moral et al. 2017). After inoculation, the fruit were incubated as described above. In addition, fruit treated with DSW were included as a control. The experiment was conducted twice over two consecutive years, and a randomized complete block design with years as blocks, almond cultivars as the independent variable, and humidity chambers as replications was used. For each year, there were three replicate humidity chambers per inoculated cultivar or control, with 15 fruit per humidity chamber (90 almond fruit per cultivar).

Leaf susceptibility under laboratory-controlled conditions. Fully expanded leaves from healthy almond trees of the cultivars Belona, Ferraduel, Guara, Lauranne, Marinada, Nonpareil, Penta, Soleta, Tarraco, and Vairo were harvested at two different stages: young leaves (collected approximately 1 month after bud expansion; thin and light green) and old leaves (collected approximately 3 months after bud expansion; thick and light to dark green). The leaves were washed with 0.02% Tween-20 solution in tap water for 1 min, surface sterilized by immersion in 10% solution of commercial bleach (Cl at 50 g liter⁻¹) for 1 min, and double washed under running tap water. The leaves of each cultivar were placed in humidity chambers and inoculated by spraying until runoff using a conidial suspension (10⁵ conidia ml⁻¹) of *C. acutatum* isolate Col-536. After inoculation, the humidity chambers were incubated as described above. Noninoculated almond leaves sprayed with DSW were included as controls. For each age category (young and old leaves), a completely randomized design with three replicate humidity chambers per inoculated cultivar or control was used, with 12 leaves per humidity chamber (72 leaves per cultivar). The experiment was conducted twice.

Fruit susceptibility under field conditions. The susceptibility of almond fruit to anthracnose under field conditions was evaluated using seven cultivars (Ferraduel, Guara, Lauranne, Marinada, Tarraco,

Tuono, and Vairo) evaluated for fruit infection in the laboratory experiments. Field assessments were conducted during the late spring to summer of two consecutive years (2017 and 2018) in a second experimental orchard (18-year-old trees) also belonging to the IFAPA's experimental field network located in Alameda del Obispo (Córdoba, Spain). This orchard is representative of the edapho-climatic characteristics and the standard crop management of the new almond plantations in Andalusia (Arquero et al. 2013). The experiment consisted of 12 replicate trees per cultivar in a completely randomized block design distributed across six blocks, with two replicate trees per elemental plot (Ollero-Lara et al. 2019).

Interaction between almond cultivar and *Colletotrichum* isolate with respect to fruit infection. According to the results obtained from the fruit susceptibility experiment, fruit (maturity stage 4; see below) of six cultivars (Ferraduel, Guara, Lauranne, Soleta, Tarraco, and Vairo) differing in susceptibility were selected. The fruit were washed, disinfested, and inoculated as described above. In this experiment, inoculations were conducted using a conidial suspension (10^5 conidia ml^{-1}) of each of the six fungal isolates described above. Inoculated fruit were placed in humidity chambers and incubated under the same conditions as described above. Fruit treated with DSW were included as a control. A factorial randomized complete block design with three replicate humidity chambers per cultivar and isolate or control was used. Each humidity chamber contained 15 fruit; therefore, in total, 1,890 fruit were used in this experiment. The experiment was conducted twice.

Effect of fruit maturity on disease development. According to the results obtained from the fruit susceptibility experiment, four cultivars (Ferraduel, Guara, Soleta, and Tarraco), representatives of the four susceptibility categories, were selected to determine the effect of fruit maturity on disease development. Fruit of each cultivar were harvested at different maturity stages during fruit setting according to the experience of the authors: (i) at the fruitlet stage; fruit diameter approximately 1 cm (maturity stage 1); (ii) 15 days after the fruitlet stage (DAFL) (maturity stage 2); (iii) 30 DAFL (maturity stage 3); and (iv) 45 DAFL, at the end of setting, when fruit reached their final size (maturity stage 4). The fruit were washed, disinfested, and inoculated as described in the fruit susceptibility experiment. Inoculations were performed with conidial suspensions (10^5 conidia ml^{-1}) of *C. acutatum* isolate Col-536 and *C. godetiae* isolate Col-522. Inoculated fruit were placed in humidity chambers and incubated under the same conditions as described in the first experiment of this study. Fruit treated with DSW were included as controls. A factorial randomized complete block design with three replicate humidity chambers per cultivar, isolate or control, and maturity stage combination was used. Each humidity chamber contained 15 fruit; therefore, in total, 2,160 fruit were used in this experiment. The experiment was conducted twice.

Effect of temperature on disease development. Almond fruit of cultivars Guara and Lauranne were used in this experiment. The fruit were washed, disinfested, and inoculated as described above. Inoculation was carried out with conidial suspensions (10^5 conidia ml^{-1}) of *C. acutatum* isolate Col-536 and *C. godetiae* isolate Col-522. Inoculated fruit were placed in humidity chambers and incubated at 10, 15, 20, 25, and 30°C under a 12-h photoperiod at 100% RH for 21 days. For each temperature and cultivar, fruit treated with DSW were included as controls. A factorial randomized complete block design with three replicate humidity chambers per cultivar, isolate or control, and temperature combination was used. Each humidity chamber contained 15 fruit; therefore, in total, 1,350 fruit were used in this experiment. The experiment was conducted twice.

Disease assessment. Disease severity (DS) on inoculated wounded fruit was evaluated every 4 days until most of the fruit had 90 to 100% of their surface affected (20 days; five assessments). In each assessment, the percentage of affected fruit surface was determined by comparing both the lesion and fruit mean diameters (average of the major and minor diameters). In all cases, the relative area under the disease progression curve (RAUDPC) was calculated as described by López-Moral et al. (2017). On fruit and leaves inoculated by spraying, DS was evaluated every 3 days until most of the

fruit or leaf surface achieved the maximum value (100%, in approximately 21 or 15 days for fruit or leaves, respectively). Each fruit or leaf was assessed for DS with a 0-to-6 or a 0-to-8 rating scale adapted from the 1-to-6 or 1-to-8 scales previously set up for olive anthracnose (Moral et al. 2008) and olive scab (Viruega et al. 2011), respectively. Rating scales estimated the percentage of tissue surface covered by visible lesions according to the following intervals for each scale value: for fruit, 0 = no visible symptoms; 1 = visible symptoms affecting <25, 2 = 25 to 50, 3 = 50 to 75, and 4 = 75 to 100% of the fruit surface; 5 = fruit completely rotted; and 6 = mummified fruit; and for leaves, 0 = no symptoms and 1 = ≤ 12.5 , 2 = 12.6 to 25, 3 = 26 to 37, 4 = 38 to 50, 5 = 51 to 62, 6 = 63 to 75, 7 = 76 to 87, and 8 = 88 to 100% of the leaf surface affected. A DS index (DSI) was calculated for each replication using the formula $\text{DSI} = [\sum(n_i \times i) / (N \times 6 \text{ or } 8)] \times 100$, where i represents severity (0 to 6 and 0 to 8 for fruit and leaves, respectively), n_i is the number of leaves or fruit with severity i , N is the total number of leaves or fruit, and 6 and 8 are the highest values of the severity rating scale for fruit and leaves, respectively. RAUDPC was calculated for DSI data over time, as described above. Fruit infection under field conditions was assessed by estimating the percentage of affected fruit (disease incidence [DI]) in eight 1-m² areas of the tree canopy. Two 1-m² areas per orientation (north, south, east, and west) were evaluated per tree; approximately 10 to 20% of the tree canopy was checked.

Data analysis. Analysis of variance (ANOVA) and means comparison tests were conducted to determine the effect of wounding on fruit infection, differences in susceptibility of almond cultivars, virulence among isolates, and effect of maturity and temperature on disease development on inoculated fruit. In addition, a nonhierarchical K-means cluster analysis with the initial cluster center method under the assumption of four groups ($k = 4$) was applied to establish the categories of susceptibility of all 19 almond cultivars evaluated in the laboratory on inoculated fruit using the average RAUDPC value. For inoculated leaves, ANOVA and means comparison tests were conducted to determine the differences in susceptibility between almond cultivars and the effect of leaf age on fungal infection and disease development. For the different analyses conducted, RAUDPC was the dependent variable in all cases. Pearson correlation coefficients (r) between young and old leaves, young leaves and fruit, and old leaves and fruit were calculated using the average RAUDPC values of the common cultivars evaluated ($n = 10$) in both experiments in which fruit and leaves were inoculated. Additionally, Pearson's linear correlation was also conducted to compare cultivar susceptibility between artificial and natural fruit infection for the cultivars evaluated ($n = 7$) under both laboratory and field conditions. In the experiment evaluating the effect of fruit maturity stage on disease development, linear regressions were also performed with RAUDPC as the dependent variable and maturity stage as the independent variable. Finally, in the experiment evaluating the effect of temperature on disease development on detached fruit, RAUDPC data from each cultivar were subjected separately to ANOVA to determine the differences among fungal isolates for each temperature. In parallel, because significant differences between cultivars were not observed ($P > 0.05$), data from both cultivars tested (Guara and Lauranne) were combined, and a nonlinear adjustment of the combined data were applied separately for each isolate using the generalized Analytis Beta model to evaluate the variation of the RAUDPC over temperature (López-Moral et al. 2017). Subsequently, for each isolate, RAUDPC values were fitted to a regression curve to estimate the optimum temperature for fruit infection, and ANOVA was also conducted to determine differences between isolates with respect to the adjusted RAUDPC values for the optimum temperature. In all cases, the two runs of the experiment were combined after checking for homogeneity of the experimental error variances by the F test. RAUDPC mean values were compared using Fisher's protected least significant difference test for experiments with independent variables with less than seven levels and Tukey's honestly significant difference tests for experiments with independent variables with seven or more levels (Steel and Torrie 1985). All data were analyzed using Statistix 10 (Analytical Software 2013), with the exception of the K-means

cluster, which was performed using SPSS 16 software (IBM Analytics, Armonk, NY, U.S.A.).

Results

Cultivar susceptibility. *Fruit susceptibility under laboratory-controlled conditions.* Both inoculation methods (unwounded and spraying or wounded and drop deposition) were useful to reproduce characteristic symptoms of AA on fruit. The ANOVA results indicated that there were significant differences in RAUDPC among fungal isolates ($P = 0.0002$) and between inoculation methods ($P = 0.0004$), while the interaction between both variables was not significant ($P = 0.3662$). *C. acutatum* isolate Col-536 was more virulent (RAUDPC = 76.0%) than *C. godetiae* isolate Col-522 (RAUDPC = 44.9%). With respect to the inoculation method, wounded fruit inoculated with a 20- μ l drop of conidial suspension showed significantly higher RAUDPC values (74.4%) than those observed in unwounded fruit inoculated by spraying (46.5%). In addition, the symptoms that developed on wounded fruit were more localized than those observed in unwounded and sprayed fruit, making disease assessment easier.

Regarding the evaluation of cultivar susceptibility, detached fruit of the 19 inoculated cultivars showed a round necrotic lesion with mycelial growth and fruit rot at 3 weeks after inoculation. There were significant differences in RAUDPC among cultivars ($P < 0.0001$). Ferraduel and Nonpareil were the most resistant (RAUDPC = 36.1 and 41.6%, respectively). In contrast, Tarraco was the most susceptible (RAUDPC = 97.3%), followed by Penta (RAUDPC = 90.2%). The remaining cultivars showed moderate levels of susceptibility, with RAUDPC ranging from 81.7 to 60.6% for Diamar and Guara, respectively. Cluster analysis showed four well-distinguished categories of susceptibility among the 19 cultivars, which were classified as highly susceptible (four cultivars; mean RAUDPC = 89.0%), susceptible (nine cultivars; mean RAUDPC = 75.8%), moderately susceptible (four cultivars; mean RAUDPC = 61.0%), and resistant (two cultivars; mean RAUDPC = 38.8%) (Fig. 1; Supplementary Fig. S1).

Leaf susceptibility under laboratory-controlled conditions. Both young and old detached leaves of the 10 cultivars evaluated in this experiment showed irregular necrotic lesions with mycelial growth at 3 weeks after inoculation. Because the interaction between leaf age and cultivar was significant ($P < 0.0001$), ANOVA was conducted separately for each leaf age category. In general, for both young and old leaves, there were significant differences in RAUDPC between cultivars ($P < 0.0001$). Based on observations on young leaves, Nonpareil and Soleta were the most resistant cultivars (RAUDPC = 45.6 and 48.1%, respectively), whereas Ferraduel and Lauranne were the most susceptible (RAUDPC = 90.3 and 84.7%, respectively). The remaining cultivars evaluated showed moderate susceptibility, with RAUDPC ranging from 55.5 to 74.1% for Marinada and Penta, respectively. In contrast, based on observations on old leaves, Soleta was the most susceptible cultivar (RAUDPC = 93.1%), followed by Lauranne, Guara, and Belona. In this case, Nonpareil was also the most resistant (RAUDPC = 50.8%). The remaining cultivars showed moderate susceptibility, with RAUDPC ranging from 55.8 to 79.0% for Tarraco and Vairo, respectively (Fig. 2). Linear correlation analyses showed that there was no significant correlation between young and old leaves ($r = 0.2864$; $P = 0.4225$), young leaves and fruit ($r = -0.0220$; $P = 0.9519$), and old leaves and fruit ($r = -0.4044$; $P = 0.2464$).

Fruit susceptibility under field conditions. No significant differences were observed among the four orientations (north, south, east, and west) of the tree canopy evaluated and in the interaction between orientation and cultivar ($P > 0.05$); therefore, for the comparison between cultivars, the average value of the four orientations was used. Among the seven almond cultivars evaluated, Ferraduel was the most resistant (DI = 4.1%), whereas Tarraco was the most susceptible (DI = 90.0%). A significant positive linear correlation ($r = 0.982$; $P = 0.0001$) was observed between DI (%) and DS (RAUDPC, %) of fruit infection for the seven common almond cultivars evaluated under both field and laboratory conditions (Fig. 3).

Interaction between cultivar and *Colletotrichum* isolates on fruit infection. Factorial ANOVA was conducted separately for each

Fig. 1. Relative area under the disease progression curve (RAUDPC) for detached fruit of 19 almond cultivars. Fruit were inoculated with a conidial suspension of *Colletotrichum acutatum* isolate Col-536. Columns are the mean of two independent experiments, each consisting of three replicated humidity chambers and 15 fruit per humidity chamber. Vertical bars are the standard error of the means. Columns with the same letter do not differ significantly according to Tukey's honestly significant difference test at $P = 0.05$. Column colors represent the different categories of susceptibility determined by cluster analysis: highly susceptible (dark gray), susceptible (light gray), moderately susceptible (white), and resistant (dark-white lines).

fungus species because there were significant differences in RAUDPC for the interaction between fungus species and almond cultivar ($P < 0.0001$). Likewise, for each fungus species, there were significant differences in RAUDPC for the interaction between cultivar

Fig. 2. Relative area under the disease progression curve (RAUDPC) for detached leaves (young and old leaves) of 10 almond cultivars inoculated with a conidial suspension of *Colletotrichum acutatum* isolate Col-536. Columns are the mean of two independent experiments, each consisting of three replicated humidity chambers and 12 leaves per humidity chamber. Vertical bars are the standard error of the means. For each age category, columns with the same letter do not differ significantly according to Tukey's honestly significant difference test at $P = 0.05$.

Fig. 3. Linear correlation between disease incidence (natural fruit infection under field conditions) and severity (RAUDPC; fruit infection under laboratory-controlled conditions) of almond anthracnose in seven cultivars. Symbols represent the average of 12 almond trees, with 400 fruit assessed per tree in the field evaluations, and of six humidity chambers, each containing 15 fruit under artificial inoculation conditions.

and isolate variables ($P = 0.0027$ and $P < 0.0001$ for *C. acutatum* and *C. godetiae*, respectively). Thus, the effect of the isolates on fruit infection was analyzed separately for each fungus species and almond cultivar. In general, even though there were significant differences in RAUDPC between fungus species as well as between isolates within fungus species for each almond cultivar, the susceptibility degree of the different cultivars followed the same pattern in both cases. Tarraco was the most susceptible (RAUDPC average = 96.3 and 84.9% for *C. acutatum* and *C. godetiae*, respectively), followed by Vairo, Soleta, Lauranne, and Guara, whereas Ferraduel was the most resistant (RAUDPC average = 35.5 and 26.4% for *C. acutatum* and *C. godetiae*, respectively) (Table 1).

Effect of fruit maturity on disease development. The effects of fruit maturity on disease development in cultivars Ferraduel, Guara, Soleta, and Tarraco inoculated with *C. acutatum* isolate Col-536 and *C. godetiae* isolate Col-522 are shown in Figure 4. Significant differences in RAUDPC for the variables fungus species, cultivar, and maturity stage, as well as their interactions ($P < 0.0001$ in all cases), were observed. In all cases, *C. acutatum* isolate Col-536 was significantly more virulent than *C. godetiae* isolate Col-522. Thus, factorial ANOVA was conducted separately for each fungus species to evaluate differences between cultivars and maturity stage with respect to disease development. In general, even though there were significant differences in RAUDPC between fungus species for each almond cultivar, the susceptibility degree of the four cultivars agreed with their respective four categories according to cluster analysis (fruit infection experiment). Tarraco was the most susceptible cultivar (RAUDPC average = 73.5 and 63.4% for *C. acutatum* isolate Col-536 and *C. godetiae* isolate Col-522, respectively), followed by Soleta (susceptible) and Guara (moderately susceptible); Ferraduel was the least susceptible (RAUDPC average = 54.0 and 43.7% for *C. acutatum* isolate Col-536 and *C. godetiae* isolate Col-522, respectively). With regard to the effect of fruit maturity, the susceptibility of the fruit decreased significantly with increasing maturity, with the fruitlet stage (maturity stage 1) being the most susceptible. The RAUDPC values for this stage reached almost 100% in all cases (Fig. 4). The regression analysis between DS and fruit maturity showed significant adjustments ($P < 0.05$) for the four cultivars and two isolates evaluated. The R^2 values varied between 0.822 and 0.989. Because all of the fitted regression lines were parallel and differed in their elevation, a linear regression model was fitted for the average of the four cultivars and the two isolates; the following fitting of the global trend was obtained, which was highly significant ($R^2 = 0.934$, $P = 0.0338$): $\text{RAUDPC} = 88.974 - 1.3135 \times \text{DAFL}$.

Effect of temperature on disease development. The effects of temperature on disease development in fruit of cultivars Guara and Lauranne inoculated with *C. acutatum* isolate Col-536 and

Table 1. Disease severity on selected almond cultivars inoculated with three isolates of the two *Colletotrichum* spp. causing almond anthracnose

Isolates ^z	RAUDPC (%) ^y					
	Tarraco	Vairo	Soleta	Lauranne	Guara	Ferraduel
<i>Colletotrichum acutatum</i> (pink)						
Col-536	96.2 ± 2.2 a	87.4 ± 1.8 a	79.7 ± 2.0 a	61.1 ± 1.6 a	58.6 ± 2.2 a	40.8 ± 0.8 a
Col-537	95.6 ± 1.4 a	83.9 ± 2.1 a	73.3 ± 2.8 a	63.4 ± 1.8 a	60.5 ± 2.4 a	35.5 ± 2.2 b
Col-538	97.1 ± 1.0 a	78.7 ± 2.7 a	75.6 ± 2.7 a	61.5 ± 1.7 a	68.2 ± 2.6 a	30.2 ± 1.3 c
Average	96.3 ± 0.8 A	83.3 ± 1.7 B	76.2 ± 1.6 C	62.0 ± 0.9 D	62.4 ± 1.9 D	35.5 ± 1.7 E
<i>C. godetiae</i> (gray)						
Col-522	76.9 ± 1.2 b	68.6 ± 0.9 b	65.5 ± 1.9 a	49.4 ± 1.4 a	38.0 ± 2.0 a	29.6 ± 1.7 a
Col-548	90.1 ± 1.5 a	78.6 ± 1.7 a	62.9 ± 1.1 a	41.3 ± 1.5 b	39.6 ± 1.8 a	25.4 ± 1.7 a
Col-555	87.8 ± 1.3 a	75.2 ± 2.6 ab	66.2 ± 1.8 a	47.6 ± 1.7 a	37.8 ± 1.6 a	24.1 ± 0.8 a
Average	84.9 ± 2.1 A	74.2 ± 1.7 B	64.9 ± 1.0 C	46.1 ± 1.5 D	38.5 ± 1.0 E	26.4 ± 1.1 F

^y Relative area under the disease progress curve (RAUDPC). Data represent the average of two independent sets (experiments) of three replicate humidity chambers with 15 fruit per humidity chamber ± standard error of the means. For each fungus species and almond cultivar, means in a column followed by the same lowercase letter do not differ significantly according to Fisher's protected least significant difference (LSD) test at $P = 0.05$. For each fungus species, the average values of RAUDPC followed by the same uppercase letter do not differ significantly among cultivars according to Fisher's protected LSD test at $P = 0.05$.

^z Fungus species (colony type) and isolate. Average = RAUDPC average of the three isolates of each fungus species inoculated in each almond cultivar ± standard error of the means.

C. godetiae isolate Col-522 are shown in Figure 5. Because the interactions between temperature and cultivar and between temperature and isolate were significant, the effect of temperature on disease development in almond fruit was analyzed separately for each cultivar. Infection and disease progression were observed on inoculated fruit incubated at all temperatures, with 10 and 30°C showing the lowest RAUDPC for both fungal species. Disease development was higher when fruit were incubated at 25°C. Significant differences in virulence were observed between *Colletotrichum* spp. in the two evaluated cultivars ($P < 0.0001$) at all temperatures tested, with the exception of 10°C, where both species showed similar RAUDPC values (isolate Col-536: 11.1 and 9.2% and isolate Col-522: 9.9 and 8.5 for Guara and Lauranne, respectively) (Fig. 5). By applying Beta Analytis, a nonlinear regression model was fitted for the average of the two cultivars and for each isolate separately, obtaining the two following fittings of the global trend, which were highly significant ($R^2 = 0.9977$ and 0.9749 for Col-536 and Col-522, respectively): (i) for Col-536: $\text{RAUDPC} = (4.51 \times 10^{-5}) \times [(T - T_{\min})^{4.05} \times (T_{\max} - T)^{0.94}]$; and (ii) for Col-522: $\text{RAUDPC} = (2.79 \times 10^{-6}) \times [(T - T_{\min})^{4.72} \times (T_{\max} - T)^{1.05}]$; where T = temperature, $T_{\min} = 0.5^\circ\text{C}$, and $T_{\max} = 30.5^\circ\text{C}$ for both fitted models. According to the fitted models, the optimum fruit infection temperature was 24.8 and 25.0°C for *C. acutatum* isolate Col-536 and *C. godetiae* isolate Col-522, respectively. No significant difference in the optimum fruit infection temperature (T_{optimum}) was observed between isolates ($P = 0.3895$), whereas differences in virulence were observed ($P < 0.0001$; mean RAUDPC for $T_{\text{optimum}} = 94.9$ and 59.2% for *C. acutatum* isolate Col-536 and *C. godetiae* isolate Col-522, respectively).

Discussion

To date, little information exists about the agronomic and environmental factors that affect the development of AA in the almond-

growing regions in the Mediterranean basin due to the reemergent character of the disease. Because these aspects are critical in anthracnose epidemics, the effects of cultivar, fruit wounding and maturity, leaf age, and temperature on infection of almond by *Colletotrichum* spp. were determined in this study by artificial inoculations. Moreover, the variability in virulence between *Colletotrichum* spp. isolates in terms of fruit infection was also evaluated. Most of the experiments under laboratory-controlled conditions were conducted by inoculating a representative isolate of *C. acutatum*. Although two species belonging to the *C. acutatum* species complex have been associated with AA in southern Spain (*C. acutatum* and *C. godetiae*), *C. acutatum* was selected here due to its higher virulence in terms of affecting almond fruit, as previously described by López-Moral et al. (2017). The incidence of anthracnose was also assessed during two consecutive epidemic years in an experimental orchard of almond cultivars.

The effect of wounding on fruit infection was first evaluated to determine the best inoculation method to reproduce symptoms and to facilitate disease assessment. Disease development was significantly more severe on wounded fruit that were drop inoculated than on non-wounded fruit inoculated by spraying. Our results are similar to those obtained by Adaskaveg and Hartin (1997), who also tested these two inoculation methods under laboratory and field conditions in California. Our results demonstrated that wounding is not essential for almond infection by *Colletotrichum* spp. In previous studies evaluating the pathogenicity of *C. acutatum* against mango, avocado, almond, and apple fruit, wounds were only necessary on mango fruit to obtain infection (Freeman and Shabi 1996). In general, although wounding is not essential to reproduce anthracnose symptoms in most of the susceptible fruit crops, the inoculation method selected in the present study is the most common protocol used in laboratory

Fig. 4. Relative area under the disease progression curve (RAUDPC) for detached fruit of cultivars Ferraduel, Guara, Soleta, and Tarraco inoculated with **A**, *Colletotrichum acutatum* isolate Col-536 and **B**, *C. godetiae* isolate Col-522. For each isolate, cultivar, and maturity stage, columns are the mean of two independent experiments, each consisting of three replicated humidity chambers and 15 fruit per humidity chamber. Vertical bars are the standard error of the means. For each isolate and cultivar, columns with different letters differ significantly according to Fisher's least significant difference test at $P = 0.05$.

Fig. 5. Relative area under the disease progression curve (RAUDPC) for detached fruit of cultivars **A**, Guara and **B**, Lauranne inoculated with *Colletotrichum acutatum* isolate Col-536 and *C. godetiae* isolate Col-522 and incubated at 10, 15, 20, 25, and 30°C . For each cultivar, temperature, and isolate, columns are the mean of two independent experiments, each consisting of three replicated humidity chambers and 15 fruit per humidity chamber. Vertical bars are the standard error of the means. For each cultivar and temperature, columns with the same letter do not differ significantly according to Fisher's least significant difference test at $P = 0.05$.

experiments to evaluate *Colletotrichum* infections on a broad range of hosts (Peres et al. 2005).

In the laboratory, Ferraduel and Nonpareil were the cultivars most tolerant to *C. acutatum*, while Tarraco and Penta were the most susceptible. Our results pertaining to the resistance of Nonpareil to AA on inoculated fruit agrees with that obtained by Adaskaveg et al. (2002a), who evaluated the susceptibility of the main commercial almond cultivars to AA in California. These authors observed differences in the susceptibility of almond cultivar blossoms, leaves, and fruit under both field and laboratory conditions, with Nonpareil being the most tolerant cultivar (Adaskaveg et al. 2002a). In addition, McKay et al. (2014) monitored anthracnose development in the cultivars Nonpareil and Price for three growing seasons in an almond orchard in South Australia. These authors also demonstrated that Nonpareil was the cultivar most tolerant to the disease (McKay et al. 2014). Concerning cultivars showing intermediate susceptibility, our results agree with those obtained by Palazón and Palazón (1979), who indicated that Desmayo largueta and Marcona are moderately susceptible to AA. Nevertheless, these same authors indicated that Garrigues was the cultivar most susceptible to the disease, whereas our results suggested that some of the new cultivars such as Tarraco, Penta, and Vairo are more susceptible to AA than Garrigues. Because much of the information available on the susceptibility of almond varieties to anthracnose is outdated (Palazón and Palazón 1979; Striem et al. 1989) and new varieties are continually being introduced from breeding programs (CITA 2018; Dicenta et al. 2010; Egea et al. 2003; Romero et al. 2011a,b; Vargas et al. 2014), evaluation of the susceptibility of these new varieties, at least under artificial inoculation conditions as in this study, is essential to prevent anthracnose epidemics in the near future.

A critical aspect of the results of the artificial inoculations of cultivars is their correspondence with the severity of the disease in the field. However, our field observations were in concordance with those obtained from inoculated fruit for the seven cultivars evaluated. Among these cultivars, Ferraduel was the most resistant and Tarraco was the most susceptible. This good correlation between field and laboratory observations for the sample of seven cultivars belonging to the four categories of susceptibility confirms the validity of the results obtained in the laboratory. Although further field observations are needed to deliver more conclusive results, data from the laboratory experiments represent a first acceptable approach regarding the anthracnose susceptibility of the current almond cultivars used in the Mediterranean basin.

It is interesting to note that Guara and Tuono showed identical values of RAUDPC for inoculated fruit. Our results are in concordance with those obtained by Ollero-Lara et al. (2019), who observed the same level of susceptibility of these two cultivars against the four most common almond fungal diseases (red leaf blotch, shot hole, blossom blight, and leaf curl) in southern Spain. This similarity between the studies reinforces the results obtained recently by Dicenta et al. (2015), who characterized these two cultivars with identical genetic profiles.

When fruit were inoculated with both *C. acutatum* and *C. godetiae* isolates, significant differences in virulence between both species were observed, with *C. acutatum* being the most virulent. Nevertheless, all six cultivars evaluated in this experiment showed the same susceptibility degree to both fungal species, and this result coincides with the susceptibility pattern observed in the first experiment on inoculated fruit conducted in the present work. Differences in susceptibility to *C. acutatum* and *C. godetiae* were previously described by López-Moral et al. (2017) for inoculated almond fruit as well as apple and olive fruit, also demonstrating differences in virulence between hosts and between isolates of *Colletotrichum* spp. from almond and olive. Likewise, special attention must be given to this finding in future research because anthracnose is associated with a broad range of species of *Colletotrichum* affecting many crops, and differences in virulence between fungal species and between isolates within species from different hosts and geographic origins have been described (Damm et al. 2012; Moral et al. 2007, 2014; Talhinhás et al. 2018).

In the laboratory experiments on leaves, Nonpareil was also the cultivar most tolerant to *C. acutatum*. Previous studies conducted by Diéguez-Urbeondo et al. (2011), who inoculated blossoms and leaves of four almond cultivars grown in California, also indicated that Nonpareil was the cultivar most resistant to AA. However, no correlations for susceptibility were observed in this study between young and old leaves or between leaves of the two ages and fruit. Thus, different patterns of susceptibility among the cultivars were observed in experiments conducted on inoculated leaves and fruit; the most relevant difference was noticed in Soleta, which was the second most resistant cultivar after Nonpareil based on young leaves and the most susceptible based on old leaves.

The effect of maturity on disease development showed that the fruitlet stage was the most susceptible for all cultivars tested. Our results corroborated the difference in susceptibility between cultivars with respect to inoculated fruit as well as the difference in virulence between *C. acutatum* and *C. godetiae* based on fruit infection. Although the increase in resistance to AA with increasing fruit maturity has previously been reported (Palazón and Palazón 1979; Striem et al. 1989), the extreme susceptibility of the youngest fruit for all evaluated cultivars shows the high vulnerability of the first stages of fruit development and the need to protect these initial stages through fungicide treatments.

The effect of temperature on disease development was evaluated using the fruit of cultivars Guara and Lauranne by inoculating them with the two species *C. acutatum* and *C. godetiae*. These two cultivars were selected because they are representative of the most important commercial cultivars used in the new almond plantations in Spain (Arquero et al. 2013) and because their susceptibility to *Colletotrichum* spp. was almost the same in previous trials (López-Moral et al. 2017). Differences in susceptibility between the two cultivars were not observed at any of the temperatures tested, whereas differences in virulence between fungal species were observed at all temperatures tested, with the exception of 10°C. In all cases, *C. acutatum* was more virulent than *C. godetiae*, as described above in the other experiments of this study. All of these results agree with those presented by López-Moral et al. (2017). The optimum fruit infection temperature was 24.8 and 25.0°C for *C. acutatum* isolate Col-536 and *C. godetiae* isolate Col-522, respectively. Our results are similar to those obtained by López-Moral et al. (2017), who evaluated the effect of temperature on mycelial growth development of these same *Colletotrichum* isolates in vitro. These authors indicated that the optimum growth temperatures of *C. acutatum* isolate Col-536 and *C. godetiae* isolate Col-522 were 23.7 and 22.4°C, respectively. In addition, Diéguez-Urbeondo et al. (2011) evaluated the effect of temperature on *C. acutatum* infection of almond blossoms and leaves at 10, 15, and 20°C and observed that disease progressed much more rapidly at 15 or 20°C compared with 10°C. These authors also indicated that visible symptoms were observed earlier when inoculated plant material was incubated at 15 or 20°C (10 days after inoculation) than at 10°C (20 days after inoculation). Similar results were obtained in our study on almond fruit: a 9-day delay in the onset of the first symptoms was observed at 10 and 30°C compared with 15, 20, and 25°C. In addition, a previous study conducted in Israel by Striem et al. (1989) also showed different growth rates on inoculated almond fruit incubated at 15, 22, and 27°C. The results demonstrated that the optimum growth temperature for fungal infection and development on the fruit surface ranged from 22 to 27°C, depending on the cultivar (Striem et al. 1989). These latter results suggest that further research is needed to elucidate the effect of temperature on *Colletotrichum* fruit infection depending on almond cultivar and *Colletotrichum* spp., as well as fungal isolates within species from different geographic origins.

To our knowledge, this is the first study to evaluate the effect of several environmental, agronomic, and biotic factors and their interactions on almond infection by *Colletotrichum* spp. All of this information will be helpful in improving our knowledge about how these factors affect pathogen infection and disease development to develop optimum control management strategies against the disease. Cultivar resistance offers an economically and ecofriendly sound alternative

to chemical control with minimal environmental impact in crop protection. Likewise, this present study is also relevant because it results in a first approach to evaluate the susceptibility of almond cultivars against anthracnose in Spain. The variation in susceptibility to the disease found among cultivars should be considered by farm advisors when new almond plantations are established in the future. In particular, this is essential in growing areas where almond has been introduced as an alternative crop in intensive farming systems, mainly in regions with moderate temperatures and high RH (e.g., the Guadalquivir Valley in the Andalusia region, southern Spain). According to our results, many of the new varieties are susceptible to AA, and only Ferraduel and Nonpareil can be planted in growing areas favorable for AA epidemics in Spain and the Mediterranean basin. However, cultivar behavior also depends on the environmental conditions (i.e., humidity, rainfall, frost incidence, and so on), crop system (irrigated or nonirrigated land), and field conditions (i.e., planting density, mechanization, and so on), which could markedly affect productivity. Thus, in parallel with these aspects, further research monitoring AA development under different field conditions and in different geographic areas during consecutive growing seasons is needed to improve knowledge of cultivar resistance against the disease and elucidate environmental factors and cultural practices that favor disease development under field conditions.

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