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Study of diseases caused by *Diaporthe amygdali* and oomycetes on almond crops

Doctoral Thesis by

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Summary

In recent years, almond (*Prunus dulcis*) cultivation in Spain is going through a very favorable situation. The recent modernization and intensification of almond cultivation has made it possible to increase yields in new plantations significantly.

Changes in the management of new almond plantations, with the use of new varieties and the intensification of cultivation techniques, could play an important role in the incidence of diseases associated with the crop, whether known or emerging, which compromise the productivity. Due to this, it is necessary to deepen the knowledge of these diseases in order to optimize management and control measures.

This PhD Thesis focuses on two important phytopathological problems which affect the cultivation of almond trees in Spain. The first is *Diaporthe amygdali*, causal agent of twig canker and shoot blight disease and the second is related to oomycetes associated with root and crown rot of almond trees. In general, these diseases have been little studied in Spain.

First of all, we studied the role of weather conditions on the dispersal of *D. amygdali* inoculum in almond crops in Mediterranean conditions. For that purpose, a qPCR assay for the detection and quantification of aerial inoculum of *D. amygdali*, including the design of a pair of specific primers for this species, was developed. This methodology was used to study the dynamics of *D. amygdali* inoculum in spore traps placed at two almond orchards from different locations in two growing seasons (2019-2020 and 2020-2021). A two-part Hurdle model was built with the recorded climatic data; the first part related the presence or absence of *D. amygdali* DNA (Bernoulli or qualitative) and the other considered the average concentration of DNA of the fungus (Gamma or quantitative). After the analysis, it was observed that some weather variables affected positively and others negatively on both parts of the model. The temperature effect was related to daily thermal amplitude; wider thermal ranges reduced DNA concentration, while narrower thermal ranges increased DNA detection. The days with average relative humidity higher than 80% had a negative effect on the concentration of *D. amygdali* DNA. Rainfall had a positive influence on both parts of the model, confirming the contribution of precipitation in the dispersal of inoculum. Finally, wind speed variable positively influenced both parts of the model, in both growing seasons.

In order to have a holistic approach to the epidemiology of *D. amygdali* infecting almond trees, the previous study was complemented with the evaluation of the susceptibility to this fungus of almond tree varieties widely used in the

Mediterranean basin. In laboratory tests, twigs from 25 almond cultivars were inoculated with four isolates of *D. amygdali*, while field inoculations involved the inoculation of growing shoots of almond cultivars grafted onto 'GF-677' rootstock over four years. In both type of experiments, inoculum consisted of agar plugs with mycelium, which were inserted underneath the bark and the lesion lengths caused by the fungus were measured. All evaluated cultivars showed necrotic lesions in response to *D. amygdali*, confirming their susceptibility. Cluster analysis categorized cultivars as susceptible or very susceptible. Agronomic traits, such as blooming and ripening times, were linked to cultivar susceptibility. Late blooming, very late blooming, and early to medium ripening cultivars exhibited high susceptibility.

Regarding phytopathogenic oomycetes, a study was carried out to determine the prevalence of these pathogens in almond producing areas in Spain. Surveys were carried out between 2018 and 2020 in 6 provinces of Spain to collect and identify oomycetes associated with root and crown rot symptoms on almond trees. A total of 104 oomycete isolates were obtained from diseased trees, and sequencing of the internal transcribed spacer (ITS) region identified species from different genera including *Globisporangium*, *Phytophthora* (*Ph*.), *Phytopythium* (*Pp*.), and *Pythium*. *Phytopythium vexans* and *Ph. niederhauserii* were the most common species. Pathogenicity tests on one-year-old almond seedlings of 'Garnem' rootstock revealed severe symptoms, including defoliation, wilting, and dieback, with some plants dying when inoculated with *Pp. vexans* and *Ph. niederhauserii*. Some isolates of *Ph. niederhauserii* significantly reduced the dry weight of the roots compared with the control, but this effect was not observed in seedlings inoculated with *Pp. vexans*. These results provide new information about the oomycete species present in almond crops in Spain and highlight the importance of carrying out frequent phytosanitary surveys for a better knowledge of potential risks posed by these soil-borne pathogens.

This preliminary study, in which the pathogenicity of oomycetes was determined on a specific rootstock commonly used in the production of the almond crop, was expanded in the last experimental section of this thesis, which aimed to evaluate the pathogenicity of 12 soil-borne oomycete species (including *Globisporangium*, *Phytophthora*, and *Phytopythium*) on three commonly used *Prunus* hybrid rootstocks ('Garnem', 'GF-677', and 'Rootpac-40') in the Mediterranean Basin. Pathogenicity tests with 15 oomycete isolates were conducted on 1-year-old rootstock seedlings. Ninety days after inoculation, disease symptoms were evaluated on a severity scale, and the Area Under the Disease Progression Curve and the survival probability of the inoculated seedlings were calculated. All the isolates were pathogenic to the rootstock seedlings and were re-isolated from root lesions. For each rootstock large

differences in virulence were detected among the different oomycete species and isolates of *Ph. niederhauserii*. *Phytophthora multivora* and *Pp. helicoides* were generally the most virulent. These findings improve the knowledge about the pathogenicity of frequently isolated oomycete species in *Prunus* spp. orchards and highlight potential risks for these crops.

In this PhD thesis, a comprehensive understanding of some of the main phytopathological challenges affecting almond cultivation in Spain has been achieved. From comprehending the meteorological conditions influencing the dispersal of *D. amygdali* inoculum to assessing the susceptibility of diverse almond varieties, and ultimately, investigating thoroughly the prevalence and pathogenicity of oomycetes in almond roots on different rootstocks. These findings significantly contribute to the knowledge necessary for optimizing management and control measures of these diseases. By delving into these aspects, the way is paved toward more effective and sustainable strategies in almond crop management, emphasizing the importance of considering both climatic factors and agronomic characteristics in decision-making for almond tree health in Spain.

Resumen

En los últimos años, el cultivo del almendro (*Prunus dulcis*) en España, está atravesando una situación muy favorable. La reciente modernización e intensificación del cultivo ha permitido aumentar significativamente los rendimientos de las nuevas plantaciones.

Los cambios en el manejo de las plantaciones de almendro, con el uso de nuevas variedades y la intensificación de las técnicas de cultivo, podrían jugar un papel importante en la incidencia de enfermedades asociadas al cultivo, ya sean conocidas o emergentes, que comprometan la productividad. Debido a esto, es necesario profundizar en el conocimiento de estas enfermedades para optimizar las medidas de manejo y control.

Esta Tesis Doctoral se centra en dos problemas fitopatológicos importantes que afectan al cultivo del almendro en España. El primero es el hongo *Diaporthe amygdali*, agente causal del chancro de las ramas y muerte de los brotes, y el segundo está relacionado con oomicetos asociados con la pudrición de la raíz y cuello de los almendros. En general, estas enfermedades han sido poco estudiadas en España.

En primer lugar, estudiamos el papel de las condiciones climáticas en la dispersión del inóculo de *D. amygdali* en cultivos de almendro en condiciones mediterráneas. Para ello, se desarrolló un ensayo qPCR para la detección y cuantificación de inóculo aéreo de *D. amygdali*, incluyendo el diseño de un par de cebadores específicos para esta especie. Esta metodología se utilizó para estudiar la dinámica del inóculo de *D. amygdali* en trampas de esporas colocadas en dos huertos de almendros de diferentes ubicaciones, en dos temporadas de crecimiento (2019-2020 y 2020-2021). Con los datos climáticos registrados se construyó un modelo Hurdle de dos partes; la primera parte relacionó la presencia o ausencia de ADN de *D. amygdali* (Bernoulli o cualitativa) y la otra consideró la concentración promedio de ADN del hongo (Gamma o cuantitativa). Después del análisis, se observó que algunas variables climáticas incidieron positivamente y otras negativamente, en ambas partes del modelo. El efecto de la temperatura estuvo relacionado con la amplitud térmica diaria; los rangos térmicos más amplios redujeron la concentración de ADN, mientras que los rangos térmicos más estrechos aumentaron la detección de ADN. Los días con un promedio de humedad relativa superior al 80% tuvieron un efecto negativo en la concentración de ADN de *D. amygdali*. Las precipitaciones tuvieron una influencia positiva en ambas partes del modelo, confirmando la contribución de la lluvia en la dispersión del inóculo. Finalmente, la variable

velocidad del viento influyó positivamente en ambas partes del modelo, en ambas temporadas de crecimiento.

Con el fin de tener un enfoque holístico de la epidemiología de *D. amygdali* infectando almendros, el estudio anterior se complementó con la evaluación de la susceptibilidad a este hongo de variedades de almendro ampliamente utilizadas en la cuenca mediterránea. En ensayos de laboratorio, se inocularon ramitas de 25 cultivares de almendro con cuatro aislados de *D. amygdali*, mientras que las inoculaciones de campo implicaron la inoculación de brotes en crecimiento de cultivares de almendro injertados en el portainjerto 'GF-677', durante cuatro años. En ambos tipos de experimentos, el inóculo consistió en discos de agar con micelio, que se insertaron debajo de la corteza y se midió la longitud de las lesiones causadas por el hongo. Todos los cultivares evaluados mostraron lesiones necróticas en respuesta a *D. amygdali*, confirmando su susceptibilidad. El análisis de conglomerados clasificó los cultivares como susceptibles o muy susceptibles. Las características agronómicas, como el tiempo de floración y maduración, se vincularon con la susceptibilidad de los cultivares. Los cultivares de floración tardía, muy tardía y de maduración temprana a media, mostraron una alta susceptibilidad.

Respecto a los oomicetos fitopatógenos, se realizó un estudio para determinar la prevalencia de estos patógenos en zonas productoras de almendro en España. Se realizaron prospecciones entre 2018 y 2020 en seis provincias españolas, para recolectar e identificar oomicetos asociados con síntomas de pudrición de raíz y cuello en almendros. Se obtuvieron un total de 104 aislados de oomicetos procedentes de árboles enfermos, y la secuenciación de la región espaciadora transcrita interna (ITS) identificó especies de diferentes géneros, incluidos *Globisporangium*, *Phytophthora* (*Ph*.), *Phytopythium* (*Pp*.) y *Pythium*. *Phytopythium vexans* y *Ph. niederhauserii* fueron las especies más frecuentes. Las pruebas de patogenicidad en plantas de almendro de un año de edad injertadas en el portainjerto 'Garnem' mostraron síntomas graves, como defoliación, marchitez y muerte regresiva, y algunas plantas murieron cuando se inocularon con *Pp. vexans* y *Ph. niederhauserii*. Algunos aislados de *Ph. niederhauserii* redujeron significativamente el peso seco de las raíces en comparación con el control, pero este efecto no se observó en plantas inoculadas con *Pp. vexans*. Estos resultados aportan nueva información sobre las especies de oomicetos presentes en los cultivos de almendro en España y destacan la importancia de realizar estudios fitosanitarios frecuentes para un mejor conocimiento de los riesgos potenciales que suponen estos patógenos del suelo.

Este estudio previo, en el que se determinó la patogenicidad de oomicetos sobre un portainjerto específico comúnmente utilizado en la

producción del cultivo del almendro, se amplió en la última sección experimental de esta tesis, que tuvo como objetivo evaluar la patogenicidad de 12 especies de oomicetos presentes en el suelo. (incluidos *Globisporangium*, *Phytophthora* y *Phytopythium*) en tres portainjertos híbridos de *Prunus* utilizados frecuentemente ('Garnem', 'GF-677' y 'Rootpac-40') en la cuenca mediterránea. Se realizaron pruebas de patogenicidad con 15 aislados de oomicetos en plantas de portainjertos de 1 año de edad. Noventa días después de la inoculación se evaluaron los síntomas de la enfermedad con una escala de severidad y se calculó el Área Bajo la Curva de Progresión de la Enfermedad y la probabilidad de supervivencia de las plantas inoculadas. Todos los aislados fueron patógenos para las plántulas de los portainjertos y se volvieron a aislar de las lesiones de las raíces. Para cada portainjerto se detectaron grandes diferencias en virulencia entre las diferentes especies de oomicetos y aislados de *Ph. niederhauserii*. *Phytophthora multivora* y *Pp. helicoides* fueron generalmente los más virulentos. Estos hallazgos mejoran el conocimiento sobre la patogenicidad de especies de oomicetos aisladas frecuentemente en plantaciones de *Prunus* spp. y revelan los riesgos potenciales para este cultivo.

En esta tesis doctoral se ha logrado un mejor conocimiento de algunos de los principales desafíos fitopatológicos que afectan al cultivo del almendro en España. Desde comprender las condiciones meteorológicas que influyen en la dispersión del inóculo de *D. amygdali* hasta evaluar la susceptibilidad de diversas variedades de almendros y, en última instancia, investigar a fondo la prevalencia y patogenicidad de los oomicetos presentes en raíces de almendros en diferentes portainjertos. Estos hallazgos contribuyen significativamente al conocimiento necesario para optimizar las medidas de gestión y control de estas enfermedades. Profundizando en estos aspectos se abren puertas a estrategias más efectivas y sostenibles en la gestión del cultivo del almendro, destacando la importancia de considerar tanto los factores climáticos como las características agronómicas en la toma de decisiones para la sanidad del almendro en España.

Resum

En els últims anys, el cultiu d'ametller (*Prunus dulcis*) a Espanya està travessant una situació molt favorable. La recent modernització i intensificació del conreu ha permès augmentar notablement els rendiments de les noves plantacions.

Els canvis en la gestió de les plantacions d'ametller, amb l'ús de noves varietats i la intensificació de les tècniques de cultiu, podrien tenir un paper important en la incidència de malalties associades al cultiu, ja siguin conegudes o emergents, que comprometin la productivitat. Per això, cal aprofundir en el coneixement d'aquestes malalties per tal d'optimitzar les mesures de gestió i control.

Aquesta tesi doctoral se centra en dos problemes fitopatològics importants que afecten el cultiu de l'ametller a Espanya. El primer és el fong *Diaporthe amygdali*, agent causal de la malaltia del cancre i mort de les branques, i el segon està relacionat amb els oomicets associats a la podridura de l'arrel i del coll dels ametllers. En general, aquestes malalties han estat poc estudiades a Espanya.

En primer lloc, hem estudiat el paper de les condicions meteorològiques en la dispersió de l'inòcul de *D. amygdali* en cultius d'ametllers en condicions mediterrànies. Amb aquesta finalitat, es va desenvolupar un assaig qPCR per a la detecció i quantificació d'inòcul aeri de *D. amygdali*, inclòs el disseny d'un parell d'encebadors específics per a aquesta espècie. Aquesta metodologia es va utilitzar per estudiar la dinàmica de l'inòcul de *D. amygdali* en trampes d'espores col·locades en dos horts d'ametllers de diferents indrets en dues temporades de creixement (2019-2020 i 2020-2021). Es va construir un model Hurdle de dues parts amb les dades climàtiques registrades; la primera part relacionava la presència o absència d'ADN de *D. amygdali* (Bernoulli o qualitatiu) i l'altra considerava la concentració mitjana d'ADN del fong (Gamma o quantitativa). Després de l'anàlisi, es va observar que algunes variables meteorològiques afectaven positivament i altres negativament en ambdues parts del model. L'efecte de la temperatura estava relacionat amb l'amplitud tèrmica diària; rangs tèrmics més amplis van reduir la concentració d'ADN, mentre que rangs tèrmics més estrets van augmentar la detecció d'ADN. Els dies amb una mitjana d'humitat relativa superior al 80% van tenir un efecte negatiu en la concentració d'ADN de *D. amygdali*. Les precipitacions van tenir una influència positiva en ambdues parts del model, confirmant la contribució de la pluja en la dispersió de l'inòcul. Finalment, la variable de velocitat del vent va influir

positivament en ambdues parts del model, en ambdues temporades de creixement.

Per tal de tenir un enfocament holístic de l'epidemiologia de *D. amygdali* infectant ametllers, l'estudi anterior es va complementar amb l'avaluació de la susceptibilitat a aquest fong de varietats d'ametller molt utilitzades a la conca mediterrània. En proves de laboratori, es van inocular branquetes de 25 cultivars d'ametller amb quatre aïllats de *D. amygdali*, mentre que les inoculacions de camp van implicar la inoculació de brots en creixement de cultivars d'ametller empeltats al portaempelt 'GF-677' durant quatre anys. En ambdós tipus d'experiments, l'inòcul consistia en discs d'agar amb miceli, que s'introduïen sota l'escorça i es mesuraven les longituds de les lesions provocades pel fong. Tots els cultivars avaluats van mostrar lesions necròtiques en resposta a *D. amygdali*, confirmant la seva susceptibilitat. L'anàlisi de conglomerats va categoritzar els cultivars com a susceptibles o molt susceptibles. Els trets agronòmics, com ara els temps de floració i maduració, estaven relacionats amb la susceptibilitat del conreu. Els cultivars de floració tardana, molt tardana i de maduració precoç a mitjana van mostrar una alta susceptibilitat.

Pel que fa als oomicets fitopatògens, es va realitzar un estudi per determinar la prevalença d'aquests patògens a les zones productores d'ametllers d'Espanya. Entre el 2018 i el 2020 s'han realitzat prospeccions a 6 províncies d'Espanya per recollir i identificar oomicets associats a símptomes de podridura de l'arrel i del coll d'ametllers. Es van obtenir un total de 104 aïllats d'oomicets d'arbres malalts i la seqüenciació de la regió de l'espaiador transcrit intern (ITS) va identificar espècies de diferents gèneres com *Globisporangium*, *Phytophthora* (*Ph*.), *Phytopythium* (Pp*.)* i *Pythium*. *Phytopythium vexans* i *Ph. niederhauserii* eren les espècies més comunes. Les proves de patogenicitat en plàntules d'ametller d'un any empeltades en el portaempelts 'Garnem' van revelar símptomes greus, com ara defoliació, marciment i mort, amb algunes plantes que van morir quan es van inocular amb *Pp. vexans* i *Ph*. *niederhauserii*. Alguns aïllats de *Ph. niederhauserii* van reduir significativament el pes sec de les arrels en comparació amb el control, però aquest efecte no es va observar en les plàntules inoculades amb *Pp. vexans*. Aquests resultats aporten nova informació sobre les espècies d'oomicets presents als cultius d'ametllers a Espanya i posen de manifest la importància de dur a terme prospeccions fitosanitàries freqüents per a un millor coneixement dels possibles riscos que presenten aquests patògens del sòl.

Aquest estudi previ, en què es va determinar la patogenicitat dels oomicets en un portaempelt específic utilitzat habitualment en la producció del cultiu d'ametllers, es va ampliar en l'últim apartat experimental d'aquesta tesi, que tenia com a objectiu avaluar la patogenicitat de 12 espècies d'oomicets del

sòl (incloent-hi *Globisporangium*, *Phytophthora* i *Phytopythium*) en tres portaempelts híbrids de *Prunus* d'ús habitual ('Garnem', 'GF-677' i 'Rootpac-40') a la conca mediterrània. Es van realitzar proves de patogenicitat amb 15 aïllats d'oomicets en plàntules de portaempelt d'un any. Noranta dies després de la inoculació, es van avaluar els símptomes de la malaltia en una escala de gravetat i es va calcular l'àrea sota la corba de progressió de la malaltia i la probabilitat de supervivència de les plàntules inoculades. Tots els aïllats van ser patògens per a les plàntules de portaempelt i es van tornar a aïllar de les lesions de les arrels. Per a cada portaempelt es van detectar grans diferències de virulència entre les diferents espècies d'oomicets i aïllats de *Ph. niederhauserii*. *Phytophthora multivora* i *Pp*. *helicoides* eren en general els més virulents. Aquestes troballes milloren el coneixement sobre la patogenicitat d'espècies d'oomicets aïllades amb freqüència a plantacions de *Prunus* spp. i destaquen els riscos potencials per a aquests cultius.

En aquesta tesi doctoral s'ha aconseguit una millor comprensió d'alguns del principals reptes fitopatològics que afecten el cultiu d'ametller a Espanya. Des de la comprensió de les condicions meteorològiques que influeixen en la dispersió de l'inòcul de *D. amygdali* fins a l'avaluació de la susceptibilitat de diversos cultivars d'ametller i, finalment, investigar a fons la prevalença i la patogenicitat dels oomicets a les arrels d'ametller en diferents portaempelts. Aquestes troballes contribueixen significativament al coneixement necessari per optimitzar les mesures de gestió i control d'aquestes malalties. Aprofundint en aquests aspectes, s'obren les portes a estratègies més efectives i sostenibles en la gestió dels cultius d'ametller, destacant la importància de tenir en compte tant els factors climàtics com les característiques agronòmiques en la presa de decisions per a la sanitat de l'ametller a Espanya.

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Chapter 1

GENERAL INTRODUCTION

Data of *Prunus* species cultivation in the world.

In the last 10 years, the cultivation of edible species of the genus *Prunus*, such as almond (*Prunus dulcis* (Miller) D. A. Webb), apricots (*Prunus armeniaca* L.), cherries (*Prunus cerasus* L.), nectarines (*Prunus persica* var. *nucipersica*), peaches (*Prunus persica* L.), plums (*Prunus domestica* L.) and sloes (*Prunus spinosa* L.), has experienced a sustained growth worldwide. World productive data of the *Prunus* crops, such as the harvested area, production, and yield per ha, are shown in Figure 1.1. The global harvested area has increased by 7.3%, being this fact probably responsible for 16.3% of the increase in total world production in the same period. Regarding the total yield of *Prunus* crops, this has decreased considerably in the last four years, by almost 13% since 2018 (FAOSTAT, 2023).

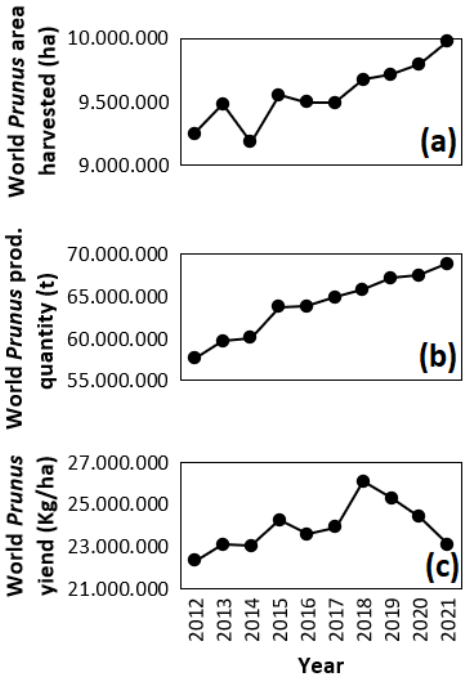


Figure 1.1 Worldwide statistical data of *Prunus* spp. (almonds, apricots, cherries, nectarines, peaches, plums, and sloes) cultivation from 2012 to 2021. (a) *Prunus* area harvested; (b) *Prunus* production quantity and (c) *Prunus* yield (FAOSTAT, 2023).

Different reasons can explain the decrease in yield experienced by some *Prunus* crops: changes in the pattern of per capita consumption in a population, which decreases the market price of the product, decreasing the profitability of its production if it is maintained in the maximum productive potential (Crisoto et al., 2006), in addition to the losses caused by diseases in crops, affecting yield and consequently total production (Savary and Global Plant Health Assessment Project (GPHA), 2023).

Data of almond cultivation in Spain and worldwide.

Productive data on almond cultivation in Spain and worldwide are shown in Figure 1.2. Almond production worldwide has experienced a 22% increase in harvested area, with a total yield per hectare 20% higher than that obtained 10 years ago, which has meant an increase in the total world production of shelled almonds above 26% (FAOSTAT, 2023).

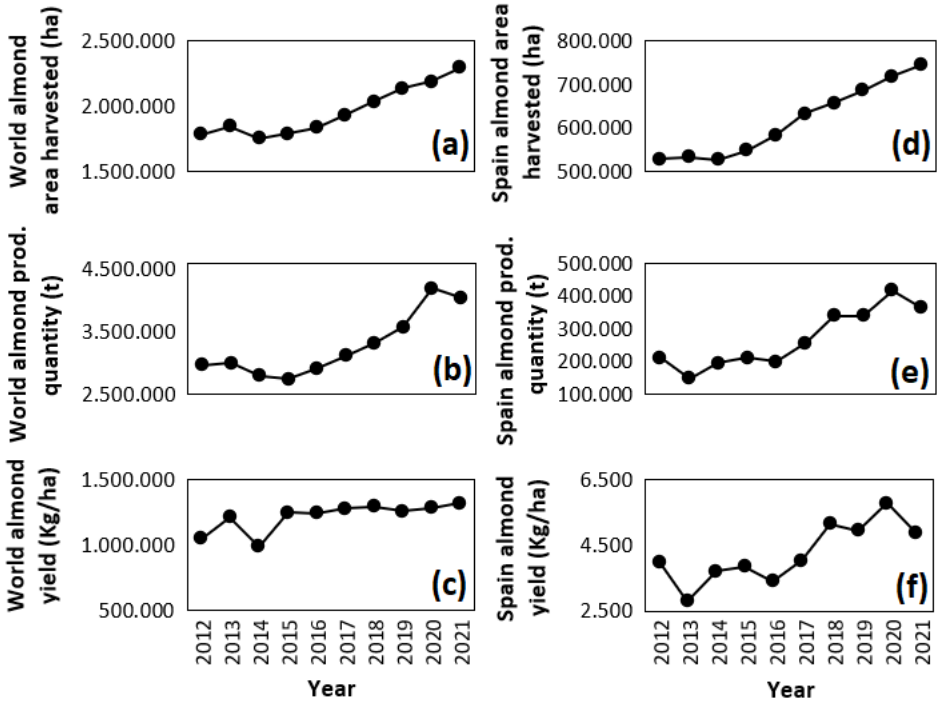


Figure 1.2 Statistical data of almond cultivation in the world (a, b and c) and in Spain (d, e and f) from 2012 to 2021. Worldwide area harvested (a and d), quantity of production (b and e) and yield (c and f) (FAOSTAT, 2023).

In the case of almond production in Spain (Figure 1.2), although, in the last 10 years, the harvested area and the total production have increased by 29% and 42%, respectively, yields have not grown in the same way, presenting greater fluctuations in value. In fact, in the last 10 years, almond yields in Spain increased only 18.5%, being this increase lower than the world yield increase (FAOSTAT, 2023).

The harvested area and the yield of the main almond-producing countries in the world are presented in Figure 1.3. This Figure shows that Spain has the largest area harvested for almonds worldwide, 720,000 ha, 30% more than the United States (USA), which has 506,000 ha. Nevertheless, the yield of the latter is seven times greater than that of Spain, reaching 46 t/ha (FAOSTAT, 2023).

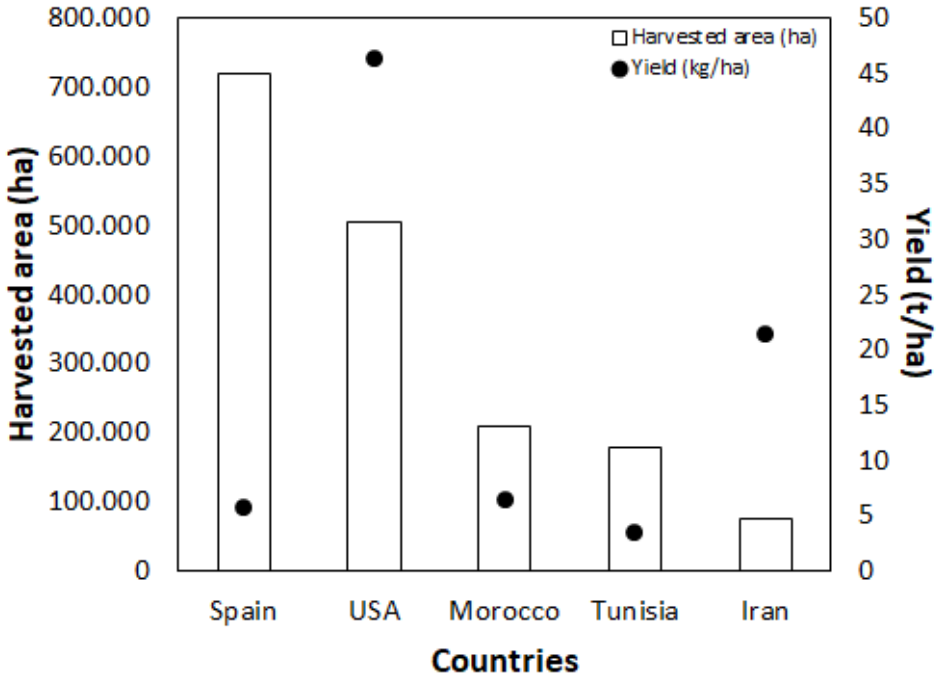


Figure 1.3 Statistical data of almond cultivation in the main producing countries of the world. Solid bars represent the harvested area, and the black dots represent the yield obtained during the year 2020 (FAOSTAT, 2023).

According to López and Peña (2019), the difference in yields between the USA and Spain is due to the fact that both countries present contrasting production models. In general, the cultivation of almonds in Spain occurs on marginal, rainfed or deficit-irrigated soils, using old indeterminate cultivars, which

present commercial problems and lack of uniformity in the harvest (Felipe, 2000). In contrast, the United States produces almond trees intensively, with localized irrigation calculated to maximize harvest, with high-yield cultivars and a uniform harvest. Moreover, Salazar and Melgarejo (2002) indicate that the low yield of Spanish almond orchards is also caused to a greater extent because many plantations have been established in inland areas of the country, where the presence of frost causes intermittent productions, in which only one year can be productive out of every 3 or 4. These authors add that there are also almond growing areas in Spain, where winter cold hours are lacking, which can also cause significant production losses. The main almond growing areas in Spain are shown in Figure 1.4. The region with the greatest coverage of almond plantations in Spain corresponds to Andalucía, followed by Castilla La Mancha, Murcia, Aragon and the Comunidad Valenciana (MAPA, 2022). The plantations are located mainly on the Mediterranean coast because they present a lower risk of spring frosts (López and Peña, 2019).

Figure 1.4 Geographical distribution of almond growing areas in Spain (adapted from Gradziel et al., 2017).

Taxonomy, botany, origin and distribution of almond crops.

The almond [*P. dulcis* (Mill.) D. A. Webb] belongs to the genus *Prunus*, which includes all stone fruit trees. This genus belongs to the Rosales order, in the Rosaceae family. Formerly this species was named as *Amygdalus communis* L., *Prunus amygdalus* (Batsch) and *Prunus communis* (Archangeli) (Agustí, 2010; Muncharaz, 2017; Socias i Company et al., 2017; Salazar and Melgarejo, 2002).

Almond is a very heterogeneous species, both in its morphological and physiological characteristics, as well as in the genetic structure of its different cultivars (Socias i Company et al., 2017).

Regarding its botanical morphological characteristics, this species has a taproot initially, with abundant ramifications later that can explore the soil between 5 and 8 times the volume of its crown, giving the tree the ability to adapt to poor and dry soils, making it efficient in the absorption of water and nutrients. The roots have a very weak epidermis initially, but it regenerates quickly. When the plant grows, the roots become strongly lignified, giving it a firm and rigid anchorage (Salazar and Melgarejo, 2002; Muncharaz, 2017).

The trunk can vary in size depending on the cultivar. When the plant is young, the lignified areas of the bark are smooth and light brown in color. Over time, the wood cracks and acquires a dark brown color, which is very characteristic of this species (Salazar and Melgarejo, 2002). The trunk has an upright growth habit, with a tendency to droop. The morphology of the canopy, like most of the aerial characteristics of the crop, will depend on the grafted cultivar, the vigor induced by the rootstock, the height of the graft and the type of pruning applied (Socias i Company et al., 2017), in addition to the productive techniques in which it is cultivated, including irrigation, fertilization and pruning (Salazar and Melgarejo, 2002).

The leaves of the almond tree are generally smaller and elongated than those of the peach tree, as well as being darker and flatter, although there are differences between cultivars (Socias i Company et al., 2017).

The flowers of the almond tree are hermaphrodite, pentamerous and of variable colors between white and pink. It is important to highlight that the almond tree is one of the earliest species to open its flower buds within the genus *Prunus* and these sprout before the wood buds (Salazar and Melgarejo, 2002; Agustí, 2010). One of the main problems with almond cultivation is self-incompatibility, that is, this plant is incapable of self-pollination, so a minimum of two cultivars with coincident blooms must be cultivated to ensure cross-pollination. In addition, it is advisable to add hives to favor entomophilous pollination. Currently there are self-compatible cultivars, now used in all almond

genetic improvement programs by various research groups to induce autogamy (López and Peña, 2019). The leaves and flowers grow in two types of branches, those of wood when there are only vegetative buds that give rise to only leaves, and mixed branches when the development of the vegetative bud provides branches with leaves and flowers (Muncharaz, 2017).

As for the almond fruit, the edible part is the seed, which is found inside the lignified endocarp commonly called the shell (Agustí, 2010). The characteristics of the seed change according to the cultivar used, but commercially the shape, thickness, fat content, fibrosity, sugar composition, aromaticity and industrial characteristics are important as a response to toasting or frying (Salazar and Melgarejo, 2002).

Regarding its origin and distribution, this species is native to the mountainous areas of western and central Asia, what we currently know as Azerbaijan, Uzbekistan and Afghanistan. Subsequently, the almond tree was spread throughout the Mediterranean basin by the Phoenicians, Greeks, Romans and Arabs, approximately 4.000 years ago. Currently the almond crops are present throughout the world including the regions of the Mediterranean countries, the Central Valley of California, eastern and central Asia, the slopes of the Himalayas and some areas of the southern hemisphere including Chile, Argentina, South Africa and Australia. The great ability to adapt to mild winters and hot, dry summers have allowed this crop to prosper in areas other than its geographical origin, with Mediterranean climate conditions being optimal for its production (Muncharaz, 2017; Socias i Company et al., 2017).

Production conditions and paradigm shift in Spanish almond production.

The almond tree, being a fruit tree of temperate zones, demands certain edaphoclimatic factors necessary for its optimal development and agronomic production, including the formation of vegetative and floral buds, flowering, and the ripening of the fruits. These factors are mainly light intensity, photoperiod, temperature, cold hours, type of soil and moisture content (Alonso, 2017).

The almond tree is sensitive to lack of light since it directly affects the development of the plant. Those leaves that receive less light intensity will perform deficient photosynthesis, withering over time. Light deficiencies translate directly into a decrease in yield, so crop pruning is essential to control any losses that may be caused. Due to the above, the almond tree behaves badly in dense plantations and with poor pruning management (Muncharaz, 2017). Regarding the photoperiod, it plays a key factor in the fall of the leaves in autumn and

therefore in the beginning of the stage of resistance to low winter temperatures (Alonso, 2017).

Regarding temperature, its optimal production range is between 25-30˚C, limiting its growth and development below 15˚C and above 35˚C (Agustí, 2010). Due to the early flowering and the rapid development of its fruits, it is very sensitive to the low temperatures produced in winter and during spring, so it is recommended to avoid locating plantations at the bottom of valleys, which is where cold air layers are deposited (Salazar and Melgarejo, 2002; Muncharaz, 2017). Nevertheless, the almond tree requires a certain number of cold hours when it is in its dormant stage, that is, in winter. These values change depending on the cultivar used, some being few demanding such as 'Verdereta' or 'Pons' (100-150 cold hours), to some very demanding of this factor such as 'Ferragnès' (350-400 cold hours) (Alonso, 2017; Muncharaz, 2017).

Regarding the edaphic requirements of the almond tree, the main advantage of this crop is its tolerance to active limestone and drought (due to its root system that goes deep into the soil), although its productivity improves significantly with irrigation (Alonso, 2017; Muncharaz, 2017). However, it does not tolerate saline or clayey soils, because the latter have poor drainage, favoring the development of root diseases (Salazar and Melgarejo, 2002), so it requires soils with a light or sandy-loam texture (Agusti, 2010).

In the last decade, the cultivation of the almond tree in the Mediterranean basin is going through a favorable transition, due to the application of new technologies which improve the traditional cultivation practices. This is modifying the old almond productive paradigm, which associated this crop with marginal areas, with unfavorable climatic, edaphic and/or orographic conditions, use of free rootstocks, established in rainfed agriculture, without seeking profitability beyond own consumption (Bielsa and Rubio-Cabetas, 2018).

The new semi-intensive and intensive almond production model focuses on maximizing yields. For this purpose, various improvements have been made. First, an active varietal renewal process, thanks to various Spanish and international almond breeding programs. The new almond cultivars obtained in the Spanish breeding programs aim to improve the quality of the fruit (size, shape, weight, protein, oil, and fatty acid content), in addition to trying to solve the problems of early flowering, self-incompatibility and susceptibility to airborne and soil pathogens (Batlle et al., 2017). All this, together with the development of new rootstocks conferring less vigor to the cultivars, allows to increase the almond planting density with respect to the traditional model, giving rise to almond trees more adapted to the soil and productive systems of the main production areas. On the other hand, the evolution in mechanized pruning and harvesting systems has allowed the intensification of almond cultivation, also

advancing their entry into production with respect to conventional pruning systems (Iglesias et al., 2021).

Rootstocks and cultivars.

Most root-grown *Prunus* crops, which were formerly used to grow stone and almond fruit trees, are very susceptible to oomycete infections (Browne and Mircetich, 1995), especially on fine-textured, high-density soils bulk density or poor drainage (Reighard and Loreti, 2008). Currently these infections continue to be a factor that limits production in almond crops, which makes the choice of an adequate rootstock a key factor (Tsipouridis et al., 2005).

Due to the above, during the 1970s, those almond trees that grew on their own roots or those that were grafted onto frank almond rootstocks were replaced by interspecific hybrid rootstocks of species of the genus *Prunus* (Bielsa and Rubio-Cabetas, 2018), which considerably improved the qualities of adaptation or tolerance to heavy soils, waterlogging, alkalinity, drought, vigor and to a lesser extent the tolerance to soilborne pathogens (Reighard and Loreti, 2008; Gainza et al., 2015; Bielsa and Rubio-Cabetas, 2018), as is the case of the rootstocks 'Nemaguard' and 'Nemared', which are widely used for their resistance to nematodes of the genus *Meloidogyne* (Nyczepir, 1991). Although this renewal for rootstocks with better productive characteristics was carried out in the main producing areas of almond trees and other *Prunus* in the world, the change in yield improvement was more noticeable in the Mediterranean basin, based on the change in the productive system, with more ambitious productive objectives than the precarious traditional production systems (Rubio-Cabetas et al., 2017).

The most frequently used rootstocks in Spain during the years 2014-2015 are shown in Figure 1.5. In the Mediterranean basin and more specifically in Spain, the almond x peach hybrid 'GF-677' has been the most widely used rootstock in recent times (Arquero et al., 2002), although the rootstock 'Garnem' (also a hybrid of almond tree x peach tree) is also being widely used for traditional replanting orchards, characterized by low densities, where the high vigor conferred by the rootstock to the variety is desirable (Rubio-Cabetas et al., 2017). However, for semi-intensive or intensive growing conditions, these rootstocks generate lower yields than those with dwarfing characteristics (Iglesias et al., 2021). In the case of high-density crops, complex hybrids such as those of the ‘Rootpac’ series are being chosen, inducing a high yield per tree, similar to that of more vigorous rootstocks such as 'Garnem' and 'GF-677' (Jiménez et al., 2011).

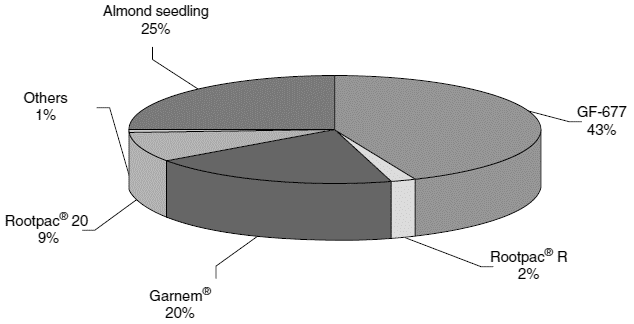


Figure 1.5 Almond rootstocks used in Spain during the years 2014-2015 (Rubio-Cabetas et al., 2017).

The main almond cultivars planted in Spain come mostly from Spanish almond breeding programs, such as the Centro de Edafología y Biología Aplicada–Consejo Superior de Investigaciones Científicas (CEBAS–CSIC), Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA) and Institut de Recerca i Tecnologia Agroalimentàries (IRTA), and from French breeding programs such as the Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE). The cultivars developed by these breeding programs have focused on seeking desirable characteristics for current almond production such as self-fertility and mostly late flowering, covering a wide fruit maturation calendar (Iglesias et al., 2021). These authors indicate that the cultivars most frequently planted in the Iberian Peninsula in recent years are 'Soleta', 'Avijor', 'Guara', 'Vairo', 'Belona', 'Marinada' and more recently 'Penta', added to the American cultivars 'Nonpareil' and 'Monterey'. In addition, in areas of traditional almond production that present little or no risk of frost, 'Marcona' continues to be cultivated, which is a benchmark for quality, or 'Desmayo Llargueta' (Iglesias, 2020).

Diseases caused by fungi and oomycetes in almond trees.

The traditional almond production system in Spain did not pay special attention to the phytosanitary problems of the crop. However, at present, possibly the most important of the threats to which almond cultivation is subjected, due to the high damage it is causing in production, is the presence of diseases affecting the canopy and the root system. In fact, in many of the new areas of expansion of the almond tree, its viability is being compromised by the presence of diseases, some unknown or rare, that were already known from traditional areas (Almacellas and Marín, 2011; Lovera et al. al. 2016; Torguet et al. 2016; Torguet et al., 2019).

Almond crop can be affected by several fungal diseases, such as red leaf blotch (*Polystigma amygdalinum* P.F. Cannon), shot hole (*Wilsonomyces carpophilus* (Lév.) Adask., J.M. Ogawa & E.E. Butler), brown rot and blossom blight (*Monilinia* spp.), and leaf curl (*Taphrina deformans* (Berk.) Tul.) (Miarnau et al., 2021; Ollero-Lara et al., 2019; Teviotdale et al., 2002), as well as by the reemergence of old ones such as anthracnose (*Colletotrichum* spp.) (López-Moral et al., 2019), and the new branch canker and dieback diseases caused by trunk pathogens (Gramaje et al., 2012; Holland et al., 2021; Olmo et al., 2016).

Moreover, branch canker and shoot blight caused by *Diaporthe amygdali* (Delacr.) Udayanga, Crous & K.D. Hyde and the proliferation of infections caused by oomycetes due to soil waterlogging problems, are widespread in Mediterranean countries and seriously compromise crop productivity (Adaskaveg 2002; Diogo et al., 2010; León et al., 2020). In general, these are diseases that have not been studied in detail until now in Spain; perhaps in line with the low economic value that the crop had had when cultivated in a traditional way. Changes in the management of new plantations, with the use of new varieties and the intensification of cultivation techniques, could play an important role in the incidence of these diseases. Therefore, it is necessary to expand the knowledge of their biology and epidemiology, and the development of effective control measures.

Branch cankers and shoot blight caused by *Diaporthe amygdali.*

Diaporthe amygdali is the causal agent of branch cankers and shoot blight disease in almond and peach trees (León et al., 2020). The disease caused by this fungus is characterized by rapid drying of twigs from late winter to early spring. As the disease progresses, brown lesions are formed that initially form in the buds of non-lignified shoots, necrosing the tissue, transforming into a sinking canker. When temperatures rise in the summer, the asexual structures of this fungus, called pycnidia, develop under the epidermis (Tuset and Portilla, 1989; Adaskaveg, 2002). The symptoms caused by the disease on almond plants are shown in Figure 1.6.

Regarding the life cycle of this pathogen, the infection is more favored in spring, with a greater development of cankers. The fungus penetrates the cortical tissues through wounds or through the petiole insertion zone, colonizing the node or internode tissues (Tuset, 2000). *Diaporthe amygdali*, through a toxin called fusicoccin, manages to interfere in the stoma closure process in the leaves, causing the branches to wilt in spring (Adaskaveg, 2002). The pycnidia formed in the areas with cankers, develop masses of spores in structures called cirri, which will be dispersed to nearby branches by insects and more frequently by rain (Tuset, 2000).

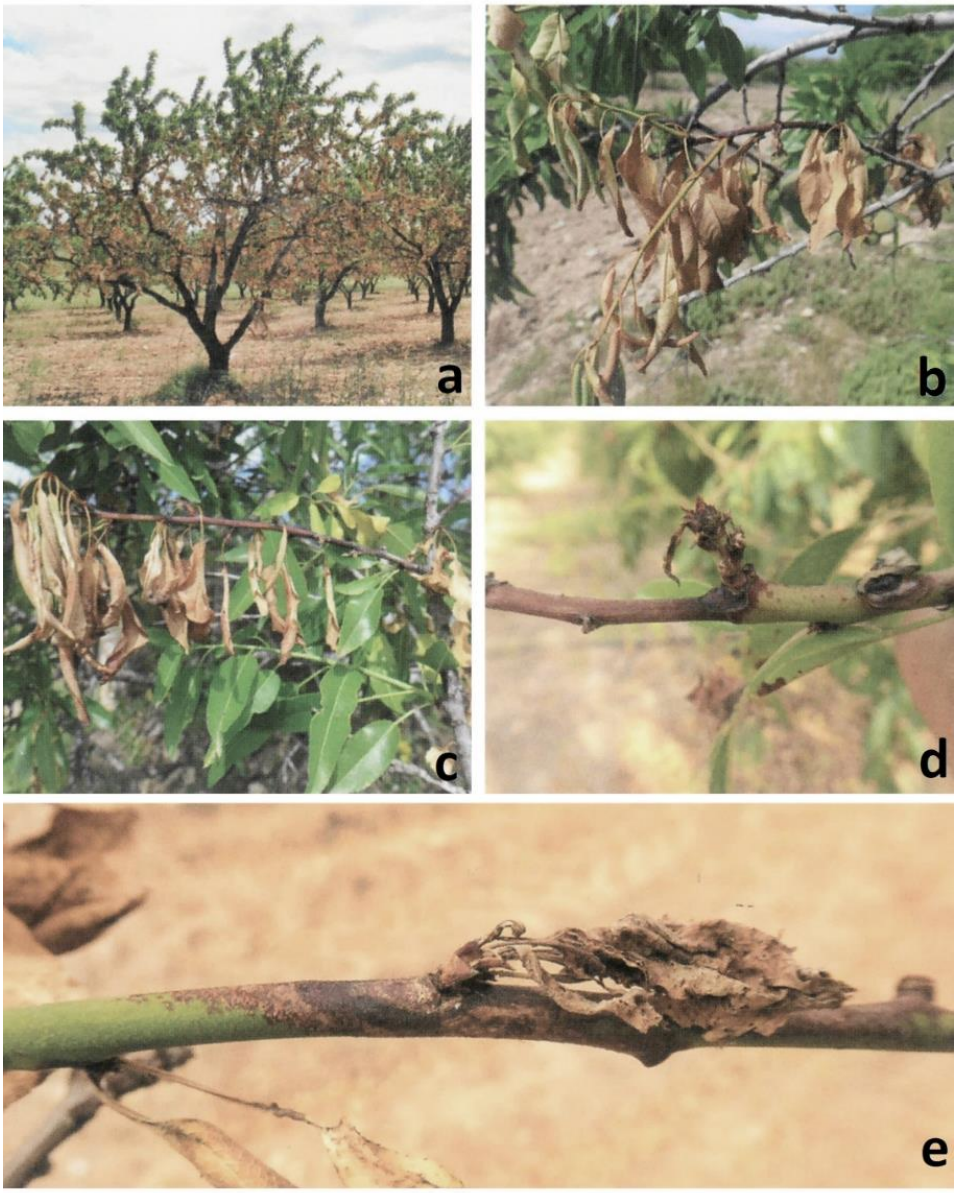


Figure 1.6 Symptoms in almond trees caused by the fungus *Diaporthe amygdali*. (a) Generalized wilting and progressive death; (b and c) desiccation of branches; (d) twig canker and (e) twig canker with abundant production of pycnidia (Barrios, 2021).

The species *D. amygdali* was first described as *Fusicoccum amygdali* Delacr., associated with cankers on almonds in France (Delacroix, 1905). Later, Tuset and Portilla (1989) re-examined the type specimen of *F. amygdali* and, based on morphology and symptomatology, reclassified this fungus in the genus *Phomopsis* as *P. amygdali* (Delacr.) J.J Tuset and M.T. Portilla. In addition, they also considered *P. amygdalina* Canonaco as a synonym of *P. amygdali*. Subsequently, Udayanga et al., (2012) re-evaluated phylogenetic species recognition in the genus *Diaporthe* using a multi-locus phylogeny based on the Internal Transcribed Spacer (ITS) region of rDNA and partial sequences of Translation Elongation Factor 1-α (tef-1α), β-tubulin (tub) and calmodulin (cal) genes. In this study, *P. amygdali* was transferred to the genus *Diaporthe* as *D. amygdali* based on multilocus DNA sequence data.

Root and crown rot caused by oomycetes.

Root and crown rot in almond trees is a disease that in general is confined to the root area of the plant and the base of the trunk. The symptoms begin with the production of necrosis and reddish-brown darkening in the affected tissues. When the infection is advanced, a brown canker forms at the base of the trunk, with abundant gum production, which results in the yellowing of the leaves and entire branches in the treetop, which subsequently dry, due to the collapse of the vascular bundles of the plant. The drying of the branches is accelerated and aggravated when the crop is found in fine-textured soils and waterlogged by persistent rains, especially in spring or early summer with the first rises in temperature (Browne and Doster, 2002; Salazar and Melgarejo, 2002; Muncharaz, 2017). The symptoms produced by this disease on almond trees are shown in Figure 1.7.

This disease is caused by different species of oomycetes, which, until a few years ago, were all included in the genus *Phytophthora (Ph)*. But, recently additional genera of oomycetes have been involved in this type of infection in almond trees and other cultivated *Prunus*. Browne et al., (2015) indicated that there are more than 10 species of *Phytophthora* causing root rot and cankers on the crown and trunk of almond trees. Some of the species identified in the main almond growing areas of the world are: *Ph. cactorum, Ph. cambivora, Ph. chlamydospora, Ph. cinnamomi, Ph. citricola, Ph. citrophthora, Ph. cryptogea, Ph. dreschsleri, Ph. megasperma, Ph. nicotianae, Ph. niederhauserii, Ph. plurivora* and *Ph. syringae* (Wicks and Lee, 1986; Wicks, 1989; Browne and Viveros, 1997; Browne and Doster, 2002; Sahragard and Banihashemi, 2006; Pérez-Sierra et al., 2010; Kurbetli and Değirmenci, 2011; Azizi et al., 2013; Abad et al., 2014; Browne et al., 2015; Çiftçi et al., 2016; Kurbetli and Yilmaz, 2016; Türkölmez et al., 2016, Browne et al., 2020).

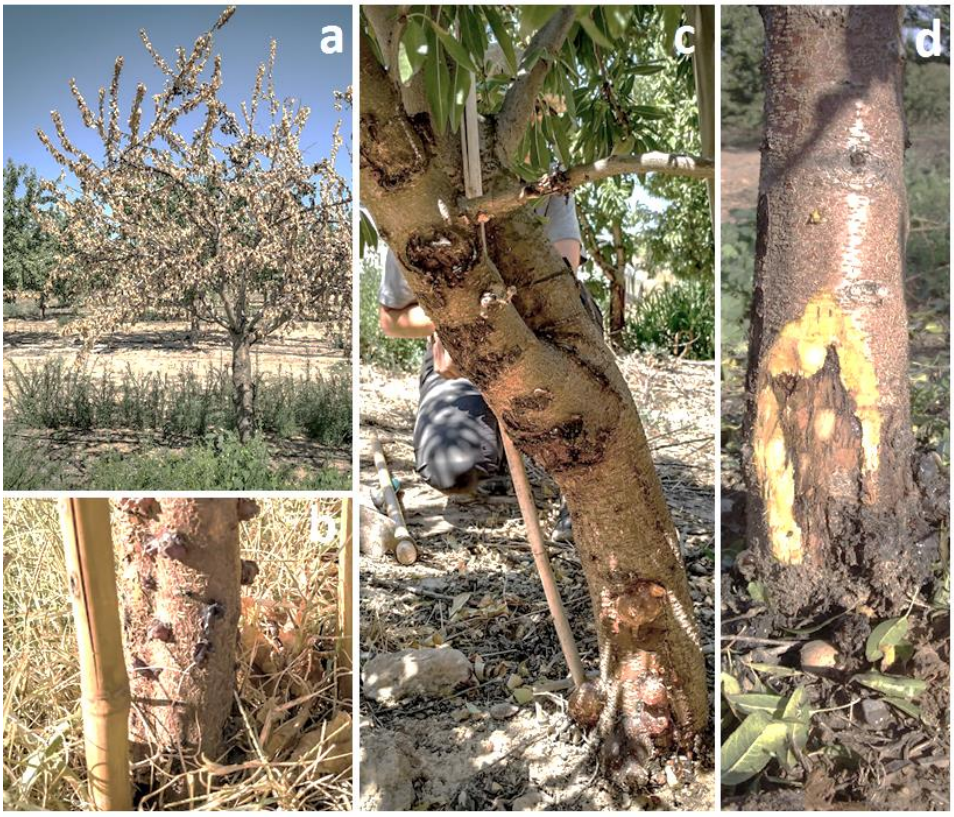


Figure 1.7 Symptoms caused by phytopathogenic oomycetes on almond trees: (a) Decline and regressive death; (b and c) Gum exudations; and (d) cankers at the base of the trunk (Clavero et al., 2023).

During the last decade, there have been continuous revisions of the genus *Pythium*, reclassifying some of its species in the new genus *Phytopythium* (*Pp*.), which comprises the species of clade K in Levesque and De Cock (2004) and is morphologically and phylogenetically between *Pythium* and *Phytophthora* (Bala, 2010). Similarly, Uzuhashi et al. (2010) showed that the genus *Pythium* Pringsh. is a nonmonophyletic group divided into five clades, each one characterized by sporangial morphology. As a result, the genus *Pythium* (*sensu lato*) was divided into five genera, four of which are new. The genus *Globisporangum* (*G.*) corresponds to clade 4. Later, some species of these new genera were described as causing infections with symptoms similar to those caused by *Phytophthora* spp. in almond, as *Pp. helicoides* (Azizi et al., 2013), *Pp. litorale* (Javadi and Sharifnabi, 2016), *G. ultimum* var. *ultimum* and *G. irregulare* (Browne et al., 2019).

The infection cycle of these phytopathogenic oomycetes in almond orchards starts with the introduction of infected material from the nurseries or infested substrate. Once the oomycetes have infected the crop through their highly mobile flagellated spores, called zoospores, they can easily spread out by cultural practices, plant debris, or through irrigation water (Pérez-Sierra et al., 2012). In prolonged periods in which the crop soil remains waterlogged the production and release of zoospores increases, having a direct relationship with the severity of the disease (Browne and Doster, 2002).

References

Abad, Z.G., Abad, J.A., Cacciola, S.O., Pane, A., Faedda, R., Moralejo, E., Perez-Sierra, A., Abad-Campos, P., Alvarez-Bernaola, L.A., Bakonyi, J., Józsa, A., Herrero, M., Burgess, T., Cunnington, J.H., Smith, I.W., Balci, Y., Blomquist, C., Henricot, B., Denton, G., Spies, C., Mcleod, A., Belbahri, L., Cooke, D., Kageyama, K., Uematsu, S., Kurbetli, I. and Değirmenci, K. 2014. *Phytophthora niederhauserii* sp. nov., a polyphagous species associated with ornamentals, fruit trees and native plants in 13 countries. Mycologia 106: 431-447.

Adaskaveg, J. 2002. Phomopsis canker and fruit rot. In: Compendium of nut crop diseases in temperate zones. Teviotdale, B., Michailides, T., Pscheidt, J., Eds.; APS Press: St. Paul, MN, USA, 2002; pp. 27-28.

Agustí, M. 2010. Fruticultura. Editorial Mundi Prensa. Madrid. 507pp.

Almacellas, J. and Marín, J. 2011. Control de plagas y enfermedades en el cultivo del almendro. Vida rural 332: 68-74.

Alonso, J. 2017. Environmental requirements. In: Almonds: Botany, Production and Uses; R. Socias i Company and T. M. Gradziel, Eds.; CAB International, Boston, MA; pp. 254-278.

Arquero, O., Navarro, A., Navarro, C., Fernández, J., Gallego, J. and Oliva, A. 2002. El cultivo del almendro en Andalucía. Fruticultura Profesional 126: 5–14.

Azizi, Z., Amini, J., Shaykh-Eslami, M. and Abasi, S. 2013. Pathogenicity of some isolates of *Pythium* and *Phytophthora* on detached shoots and seedlings of almond. Iran. J. Plant. Pathol. 49: 33-39.

Bala, K. 2010. *Phytopythium* Abad, de Cock, Bala, Robideau, Lodhi & Levesque, gen. nov. and *Phytopythium sindhum* Lodhi, Shahzad & Levesque, sp. nov. Persoonia 24: 136-137.

Barrios, G. 2021. Guía de campo, plagas y enfermedades del almendro. Phytoma. Valencia, España. 65pp.

Batlle, I., Dicenta, F., Socias i Company, R., Gradziel, T., Wirthensohn, M., Duval, H. and Vargas, F. 2017. Classical genetics and breeding. In: Almonds, botany, production and uses; R. Socias i Company and T. M. Gradziel, Eds.; CAB International, Boston, MA; pp. 111–148.

Bielsa, B. and Rubio-Cabetas, M. 2018. La elección del patrón en la almendricultura moderna. Rev. Frut. 65: 92-113.

Browne, G. and Doster, M. 2002. *Phytophthora* Diseases. In: Compendium of nut crop diseases in temperate zones. Teviotdale, B., Michailides, T. and Pscheidt, J., Eds.; APS Press: St. Paul, MN, USA, 2002; pp. 3-6.

Browne, G. and Mircetich, S. 1995. *Phytophthora* root and crown rots. In: Compendium of stone fruit diseases; Ogawa, J., Zehr, E., Bird, G., Ritchie, D., Uriu, K. and Uyemoto, J., Eds.; APS Press: St. Paul, MN, USA, 1995, pp. 38-40.

Browne, G. and Viveros, M. 1997. Diverse symptoms and tree losses caused by *Phytophthora* spp. in California almonds. Acta Hortic. 470: 570-575.

Browne, G., Ott, N. and Fichtner, E. 2019. First report of *Phytopythium helicoides* causing root rot on peach rootstock in California. Plant. Dis. 103: 2968.

Browne, G., Ott, N., Forbes, H., Yaghmour, M. and Milliron, L. 2020. First report of *Phytophthora chlamydospora* causing crown and root rot on almond in California. Plant. Dis. 104:2033-2033.

Browne, G., Schmidt, L. and Brar, G. 2015. First report of *Phytophthora niederhauserii* causing crown rot of almond (*Prunus dulcis*) in California. Plant. Dis. 99:1863.

Çiftçi, O., Türkölmez, Ş., Derviş, S. and Serçe, Ç. 2016. First report of canker and root rot of almond caused by *Phytophthora plurivora* in Turkey. Plant. Dis. 100:1507.

Clavero, I., Castillo, P., Moreno, M., Gramaje, D., Abad-Campos, M., Beluzán, F., Armengol, J. y Palomares, J. 2023. Enfermedades de patógenos de suelo en plantaciones de *Prunus*. Datos actuales y avances en la investigación sobre su control. Phytoma 346:44-54.

Crisosto, C., Crisosto, G., Echeverria, G. and Puy, J. 2006. Segregation of peach and nectarine (*Prunus persica* (L.) Batsch) cultivars according to their organoleptic characteristics. Postharvest Biol. Technol. 39:10-18.

Delacroix, G. 1905. Sur une maladie des amandiers en Provence. Bull. Trimest. Soc. Mycol. Fr. 21:180-185.

Diogo, E., Santos, J. and Phillips, A. 2010. Phylogeny, morphology and pathogenicity of *Diaporthe* and *Phomopsis* species on almond in Portugal. Fungal Divers. 44:107-115.

FAOSTAT, Food and Agriculture Organization of the United Nations. 2023. Available online: < https://www.fao.org/faostat/en/#data/QCL > (accessed on 15 January 2023).

Felipe, A. 2000. El Almendro I: El Material Vegetal, 1st ed.; Mira Editores: Zaragoza, Spain, 461 pp.

Gainza, F., Opazo, I., Guajardo, V., Meza, P., Ortiz, M., Pinochet, J. and Muñoz, C. 2015. Rootstock breeding in *Prunus* species: Ongoing efforts and new challenges. Chil. J. Agric. Res. 75:6-16.

Gradziel, T., Curtis, R. and Socias i Company, R. 2017. Production and growing regions. In: Almonds: Botany, Production and Uses; R. Socias i Company and T. M. Gradziel, Eds.; CAB International, Boston, MA; pp. 70-86.

Gramaje, D., Agustí-Brisach, C., Pérez-Sierra, A., Moralejo, E., Olmo, D., Mostert, L., Damm, U. and Armengol, J. 2012. Fungal trunk pathogens associated with wood decay of almond trees on Mallorca (Spain). Persoonia 28:1-13.

Holland, L., Trouillas, F., Nouri, M., Lawrence, D., Crespo, M., Doll, D., Duncan R., Holtz, B., Culumber, C., Yaghmour, M., Niederholzer, F., Lightle, D., Jarvis-Shean, K., Gordon, P. and Fichtner, E. 2021. Fungal pathogens associated with canker diseases of almond in California. Plant Dis. 105:346-360.

Iglesias, I. 2020. El almendro en España, agricultura eficiente, almendros, cultivos. Agromillora. Available online: < https://www.agromillora.com/olint/el-almendro-en-espana/ > (accessed on 17 February 2023).

Iglesias, I., Foles, P. and Oliveira, C. 2021. El cultivo del almendro en España y Portugal: Situación, innovación tecnológica, costes, rentabilidad y perspectivas. Rev. Frutic. 81: 6-49.

Javadi, N. and Sharifnabi, B. 2016. *Phytopythium litorale*, the causal agent of almond root and crown rot in Iran. In: Proceedings of 22nd Iranian Plant Protection Congress, Karaj, Iran, 27–30 August, College of Agriculture and Natural Resources: Karaj, Iran, pp. 27-30.

Jiménez, S., Pinochet, J., Romero, J., Gogorcena, Y., Moreno, M. and Espada, J. 2011. Performance of peach and plum based rootstocks of different vigour on a late peach cultivar in replant and calcareous conditions. Sci. Hortic. 129:58-63.

Kurbetli, I. and Değirmenci, K. 2011. First report of *Phytophthora* taxon *niederhauserii* causing decline of almond in Turkey. New Dis. Rep. 23:14.

Kurbetli, I. and Yılmaz, A. 2016. Almond decline caused by *Phytophthora megasperma* in southeastern Anatolian region of Turkey. J. Turk. Phytopathol. 45:13-20.

León, M., Berbegal, M., Rodríguez-Reina, J. M., Elena, G., Abad-Campos, P., Ramón-Albalat, A., Olmo, D., Vicent, A., Luque, J., Miarnau, X., Agustí-Brisach, C., Trapero, A., Capote, N., Arroyo, F., Avilés, M., Gramaje, D., Andrés-Sodupe, M. and Armengol, J. 2020. Identification and characterization of *Diaporthe* spp. associated with twig cankers and shoot blight of almonds in Spain. Agron. 10:1062.

Levesque, C. A., & De Cock, A. W. (2004). Molecular phylogeny and taxonomy of the genus *Pythium*. Mycol. Res. 108:1363-1383.

López, P. and Peña, M. 2019. El almendro. Guía práctica de cultivo. Ediciones Mundi-Prensa. 297pp.

López-Moral, A., Agustí-Brisach, C., Lovera, M., Luque, F., Roca, L., Arquero, O. and Trapero, A. 2019. Effects of cultivar susceptibility, fruit maturity, leaf age, fungal isolate, and temperature on infection of almond by *Colletotrichum* spp. Plant Dis. 103:2425-2432.

López-Moral, A., Agustí-Brisach, C., Raya, M., Lovera, M., Roca, L., Luque, F., Arquero, O., Trapero, A. 2016 La antracnosis del almendro, susceptibilidad varietal en Andalucía. Vid. Rur. 423:56-62.

Lovera, M., Serrano, N. and Arquero, O. 2016 El cultivo del almendro en Andalucía. Horticultura núm. especial 2016:30-37.

MAPA Ministerio de Agricultura, Pesca y Alimentación. 2022. Encuesta sobre superficies y rendimientos de cultivo. España. Available online: < https://www.mapa.gob.es/es/estadistica/temas/estadisticas-agrarias/agricultura/esyrce/ > (accessed on 3 February 2022).

Miarnau, X., Zazurca, L., Torguet, L., Zúñiga, E., Batlle, I., Alegre, S. and Luque, J. 2021. Cultivar susceptibility and environmental parameters affecting symptom expression of red leaf blotch of almond in Spain. Plant Dis. 105:940-947.

Muncharaz-Pou, M. 2017. El almendro. Manual técnico. Ediciones Mundi-Prensa. 438pp.

Nyczepir, A. 1991. Nematode management strategies in stone fruits in the United States. J. Nematol. 23:334.

Ollero-Lara, A., Agustí-Brisach, C., Lovera, M., Roca, L., Arquero, O. and Trapero, A. 2019. Field susceptibility of almond cultivars to the four most common aerial fungal diseases in southern Spain. Crop Prot. 121:18-27.

Olmo, D., Armengol, J., León, M. and Gramaje, D. 2016. Characterization and pathogenicity of Botryosphaeriaceae species isolated from almond trees on the island of Mallorca (Spain). Plant Dis. 100:2483-2491.

Pérez-Sierra, A., Mora-Sala, B., León-Santana, M., García-Jiménez, J. and Abad-Campos, P. 2012. Enfermedades causadas por *Phytophthora* en viveros de plantas ornamentales. Bol. Sanid. Veg. Plagas. 38:143-156.

Pérez-Sierra, A., León, M., Álvarez, L., Alaniz, S., Berbegal, M., García-Jiménez, J. and Abad-Campos, P. 2010. Outbreak of a new *Phytophthora* sp. associated with severe decline of almond trees in eastern Spain. Plant. Dis. 94:534-541.

Reighard, G.L. and Loreti, F. 2008. Rootstock development. In: Peach, botany, production and uses. Layne, D. and Bassi, D., Eds. CAB International, Wallingford, Oxfordshire, UK, 2008; pp. 193-220.

Rubio-Cabetas, M., Felipe, A. and Reighard. G. L. 2017. Rootstock development. In: Almonds, botany, production and uses; Socias i Company, R. and Gradziel, T., Eds.; CAB International, Boston, MA, 2017; pp. 209-227.

Sahragard, N. and Banihashemi, Z. 2006. Evaluation of resistance of some almond genotypes and cultivars to *Phytophthora cactorum*. Iran. J. Plant. Pathol. 42:97-99.

Salazar, D. and Melgarejo, P. 2002. Cultivos leñosos: frutales de zonas áridas. El cultivo del almendro. Ed. Mundi-Prensa, Madrid. 307pp.

Savary, S. and Global Plant Health Assessment Project (GPHA). 2023. A global assessment of the state of plant health. Plant Dis. 107:3649-3665.

Socias i Company, R., Ansón, J. and Espiau, M. 2017. Taxonomy, botany and physiology. In: Almonds, botany, production and uses; Socias i Company, R. and Gradziel, T., Eds.; CAB International, Boston, MA, 2017; pp. 1-42.

Teviotdale, B., Michailides, T. and Pscheidt, J. 2002. Compendium of nut crop diseases in temperate zones. American Phytopathological Society. St. Paul, MN.

Torguet, L., Batlle, I., Alegre, S. and Miarnau, X. 2016 Nuevas plagas y enfermedades emergentes, una amenaza para el cultivo del almendro en España. Rev. Frut. 49:152-165.

Torguet, L., Maldonado, M. and Miarnau, X. 2019. Importancia y control de las enfermedades en el cultivo del almendro. Agri. Rev. Agropec. Gan. 1026:72-77.

Tsipouridis, C., Thomidis, T., Elena, K. and Isaakidis, A. 2005. Effect of peach cultivars, rootstocks and *Phytophthora* on iron chlorosis. World J. Agric. Sci. 1:137-142.

Türkölmez, Ş., Derviş, S., Çiftçi, O. and Ulubaş Serçe, Ç. 2016. First report of *Phytophthora chlamydospora* causing root and crown rot on almond (*Prunus dulcis*) trees in Turkey. Plant. Dis. 100:1796.

Tuset, J. 2000. Chancros en ramas causados por *Phomopsis* spp. In: Enfermedades de los frutales de pepita y hueso. Monografía de la Sociedad Española de Fitopatología nº3; Montesinos, E., Melgarejo, P., Cambra, M. and Pinochet, J., Eds.; Ediciones Mundi-Prensa, Madrid, Spain, 2000; pp 72-73.

Tuset, J. and Portilla, M. 1989. Taxonomic status of *Fusicoccum amygdali* and *Phomopsis amygdalina*. Can. J. Bot. 67:1275-1280.

Udayanga, D., Liu, X., Crous, P., McKenzie, E., Chukeatirote, E. and Hyde, K. 2012. A multi-locus phylogenetic evaluation of *Diaporthe* (*Phomopsis*). Fungal Divers. 56:157-171.

Uzuhashi, S., Kakishima, M. and Tojo, M. 2010. Phylogeny of the genus *Pythium* and description of new genera. Mycoscience 51:337-365.

Wicks, T. 1989. Susceptibility of almond and cherry rootstocks and scions to *Phytophthora* species. Aust. J. Exp. Agric. 29:103-109.

Wicks, T. and Lee, T. 1986. *Phytophthora* crown rot of almond trees. Aust. J. Agric. Res. 37:277-287.

Chapter 2

Chapter 2

OBJECTIVES

The current favorable situation of almond crops in Spain has generated changes in its production systems. The higher planting density and the incorporation of drip irrigation, characteristic of intensive crops, have generated the proliferation of air-borne diseases such as cankers caused by *Diaporthe amygdali* and the emergence of diseases caused by soil-borne oomycetes, which were unfrequent in rainfed crops.

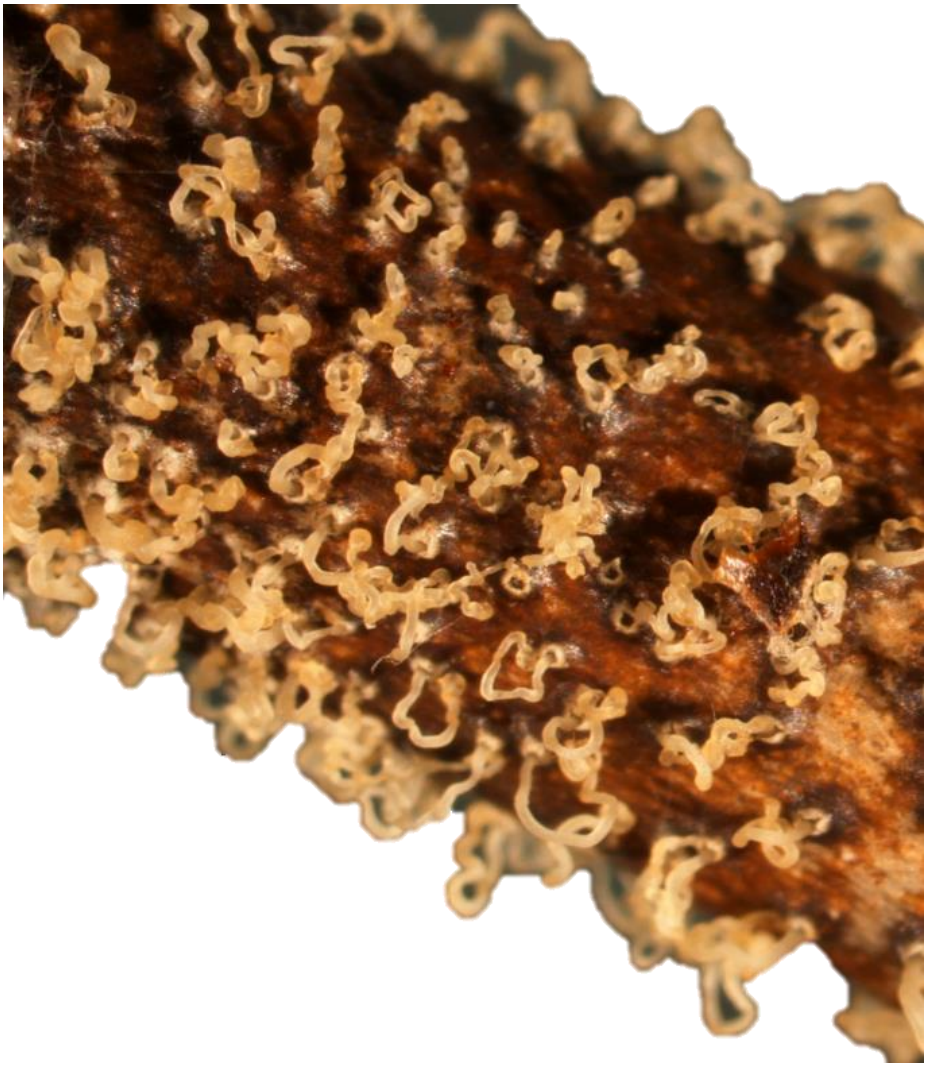
In this context, the main objectives of this Doctoral Thesis are to:

- Elucidate the role of weather conditions on the dispersal of *D. amygdali* inoculum on almond crops in Mediterranean conditions (**Chapter 3**).

- Evaluate the susceptibility of 25 European and American almond cultivars to the infection caused by *Diaporthe amygdali*, under field and laboratory conditions (**Chapter 4**).

- Isolate and identify the main oomycete species associated with root and crown rot symptoms of almond trees in the main almond-producing areas in continental Spain. Moreover, the pathogenicity of the most frequent species to a rootstock widely used in the cultivation of *Prunus* spp. will be evaluated (**Chapter 5**).

- Evaluate the pathogenicity and virulence of 12 species of oomycetes isolated from almond orchards on three *Prunus* hybrid rootstocks widely used for the cultivation of stone fruit and almond trees in the Mediterranean Basin (**Chapter 6**).



Chapter 3

Chapter 3

Effect of weather variables on the inoculum dispersal of *Diaporthe amygdali*, causal agent of twig canker and shoot blight of almonds.

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Abstract. *Diaporthe amygdali* is the causal agent of twig canker and shoot blight disease on almond. The main objective of this study was to elucidate the role of weather conditions on the dispersal of *D. amygdali* inoculum on almond crops in Mediterranean conditions. For that purpose, a quantitative PCR (qPCR) assay for the detection and quantification of *D. amygdali*, including the design of a pair of specific primers for this species, was developed. This methodology was used to study the dynamics of *D. amygdali* inoculum in spore traps placed at two almond orchards from different locations in two growing seasons (2019 to 2020 and 2020 to 2021). Weather variables were also recorded. Two-part Hurdle models, which include a qualitative part (Bernoulli), with a binary response, and a quantitative part (Gamma), were used to study the relationships of DNA concentration of *D. amygdali* on the traps with weather variables. The temperature effect was related to the daily thermal amplitude; narrower thermal ranges increased DNA detection, while wider thermal ranges reduced DNA concentration. Days with average relative humidity higher than 80% had a negative effect on the concentration of *D. amygdali* DNA. Rainfall had a positive influence on both parts of the model, confirming the contribution of precipitation in the dispersal of inoculum. Finally, wind speed positively influenced both parts of the models in both growing seasons. The relationships between weather

variables and inoculum dispersal of *D. amygdali* will assist to develop a decision support system to optimize the management of twig canker and shoot blight disease on almond.

INTRODUCTION

Diaporthe amygdali (Delacr.) Udayanga, Crous & K.D. Hyde is the causal agent of twig canker and shoot blight disease on almond and peach (Adaskaveg 2002; León et al. 2020). This disease is characterized by a rapid desiccation of the shoots, flowers and leaves after infections produced between the end of winter and the beginning of spring (Tuset and Portilla 1989; Adaskaveg 2002; Diogo et al. 2010; Varjas et al. 2017b). Almond buds that were infected by *D. amygdali*, develop reddish-brown necrotic lesions that sink, forming a canker, in which sometimes small gummy exudates can be observed (Zehr 1995). When these lesions grow, cankers ring the twigs and the distal shoots eventually die, triggering a generalized wilting of the canopy. Then, the pathogen remains under the bark of the cankers during the dormancy period (Adaskaveg 2002). Some authors have mentioned that *D. amygdali* can cause leaf necrosis and fruit rot, but these infections are considered of relatively minor importance (Adaskaveg et al. 1999, 2008). Because the shoots infected by *D. amygdali* correspond to the growth of the year, their death results in substantial yield losses, which in peach crops can reach up to 30% of the total yield (Lalancette and Polk 2000; Lalancette and Robison 2002).

Although the pathogenicity of *D. amygdali* has been widely described in species of the genus *Prunus* such as almond, peach and nectarine, worldwide (Adaskaveg et al. 1999, 2008; Álvarez et al. 2014; Beluzán et al. 2021, 2022; Besoain et al. 2000; Cabrita et al. 2004; Dai et al. 2012; Farr et al. 1999; León et al. 2020; Michailides and Thomidis 2006; Uddin et al. 1997; Uddin and Stevenson 1998; Varjas et al. 2017a), infections caused by this fungus have also been reported in other crops from different botanical families, such as grapevine in South Africa (Mostert et al. 2001), the ornamental plant *Pieris japonica* in the United States (US) (Bienapfl and Balci 2013), walnut and Asian pear in China (Bai et al. 2015; Meng et al. 2018) and blueberry in Portugal (Hilário et al. 2021), showing the polyphagous character of this fungus.

In general, the studies about the biology of *D. amygdali* and epidemiology of twig canker and shoot blight disease are scarce. Lalancette and Robison (2001) and Lalancette et al. (2003), unraveled some essential aspects about the development of the disease on peach crops in New Jersey, US. These authors indicated that *D. amygdali* is a fungus that uses wounds as an access route to the plant. Infections can occur through the wounds generated by leaf fall

in autumn and the wounds produced in spring by the sprouting, and the fall of flowers and fruits, or directly through young shoots (Adaskaveg et al. 2008). The incubation period from infection until the start of canker formation lasts approximately one month. In autumns and winters with relatively warm temperatures, weather conditions will allow a rapid development of twig cankers and death of spring shoots. On the contrary, cold winters delay canker growth and shoot death is often observed in summer. Pycnidia of *D. amygdali*, in which conidia are produced, develop in the cankers (Adaskaveg et al. 2008). In general, the number of pycnidia produced by *D. amygdali* is proportional to the size of the canker. Lalancette and Robison (2001) and Lalancette et al. (2003), found that both the average size of the canker and the number of pycnidia have a sinusoidal behavior during the year, with minimum amplitudes in spring (due to low temperatures in winter) and maximum amplitudes in autumn (due to high temperatures in summer). Nevertheless, the studies by Lalancette and Robison (2001) on peach showed that pycnidia of *D. amygdali* can produce large amounts of conidia throughout the year, even under suboptimal environmental conditions, assuring a relatively high amount of available conidia during the periods of greatest susceptibility of the host. On almond, α-conidia are more prevalent, but occasionally β-conidia can be formed (Tuset and Portilla 1989).

Several studies have shown that the production of *D. amygdali* conidia through exudates called cirri depends mostly on weather variables such as temperature and relative humidity (Lalancette et al. 2003). These structures can grow slowly at 5ºC and rapidly above 10ºC (Zehr 1995). Lalancette et al. (2003) showed that cirrus clouds can grow in a wider range of temperatures, ranging from 0 to 37ºC, with an optimum between 19 and 20ºC. These authors pointed out that the maximum production of conidia occurred between 22 and 23ºC and that it requires a minimum of 16 hours with relative humidity above 95%. This may be a key factor in the epidemiology of twig canker and shoot blight disease since low temperatures and high relative humidity (which allow infection by *D. amygdali*) can delay the rate of natural wound healing after leaf abscission (Lalancette et al. 2003). When rain events occur, conidia are aerially dispersed by water splashes (Fontaine et al. 2022), infecting other neighboring plant organs and trees, being able to germinate on humid surfaces with temperatures between 5 and 36ºC (Zehr 1995).

Most information about sporulation and conidia dispersal of *D. amygdali,* and the epidemiology of twig canker and shoot blight disease has been obtained on peach orchards under continental climate conditions or from laboratory experiments using peach isolates. Thus, the main objective of this study was to elucidate the role of weather conditions on the dispersal of *D. amygdali* inoculum on almond crops in Mediterranean conditions, where it is one of the main

diseases affecting this crop (León et al. 2020). To accomplish with this objective, the following experiments were carried out: i) to develop a quantitative PCR (qPCR) assay for the detection and quantification of aerial inoculum of *D. amygdali*, including the design of a pair of specific primers for this species; ii) to study the dynamics of *D. amygdali* inoculum in spore traps from different locations and growing seasons using qPCR; and iii) to study the relationships of DNA concentration of *D. amygdali* on spore traps with weather variables.

MATERIAL AND METHODS

Experimental almond orchards and spore trapping. A 2-season field study (2019 to 2020 and 2020 to 2021) was undertaken in two almond orchards. Orchard A was located in Palma (Balearic Islands) and orchard B in Moncada (Valencia province), Spain. Twig canker and shoot blight disease is endemic in the study regions and the orchards had a record of low to moderate disease severity. Fungicides were not sprayed during the duration of the experiments. Orchard A was planted with the cultivar 'Ferragnès' and it was 9 years old. Orchard B was planted with cultivar 'Marcona' and was 20 years old. Both orchards were grafted onto 'GF-677' and had a low planting density (typical of non-intensive crops), and they were managed following the common practices of each region.

Although the disease was naturally present in the orchards, to assure a proper inoculum amount and reduce experimental variability the system adapted by Onesti et al. (2018) was used. Each spore trap (Fig. 3.1) consisted of a 1.5 L capacity plastic bottle with a funnel (10 cm in diameter) inserted in its cap. Inside the funnel, in the first third of the upper part, 200 g of pieces of naturally infected almond shoots with cankers of *D. amygdali* were placed as a source of inoculum, contained between two plastic nets, allowing the movement of air and rain through the infected wood. On the sides of the funnel, two clips were installed that held the glass microscope slides (75 × 25 mm) at an angle of 45º with respect to the vertical axis of the funnel, at a distance of 10 cm from the inoculum source. A 50 × 12 mm piece of silicone-coated (Lanzoni s.r.l., Bologna, Italy) Melinex tape (Tesa, Hamburg, Germany) was placed on the inner side of each microscope slide. The sampler was designed to trap spores washed off by rainfall (these spores would flow into the bottle) and to trap spores dispersed by air currents or by splashed droplets (these spores would stick to the adhesive tape on the microscope slides) from the inoculum source.

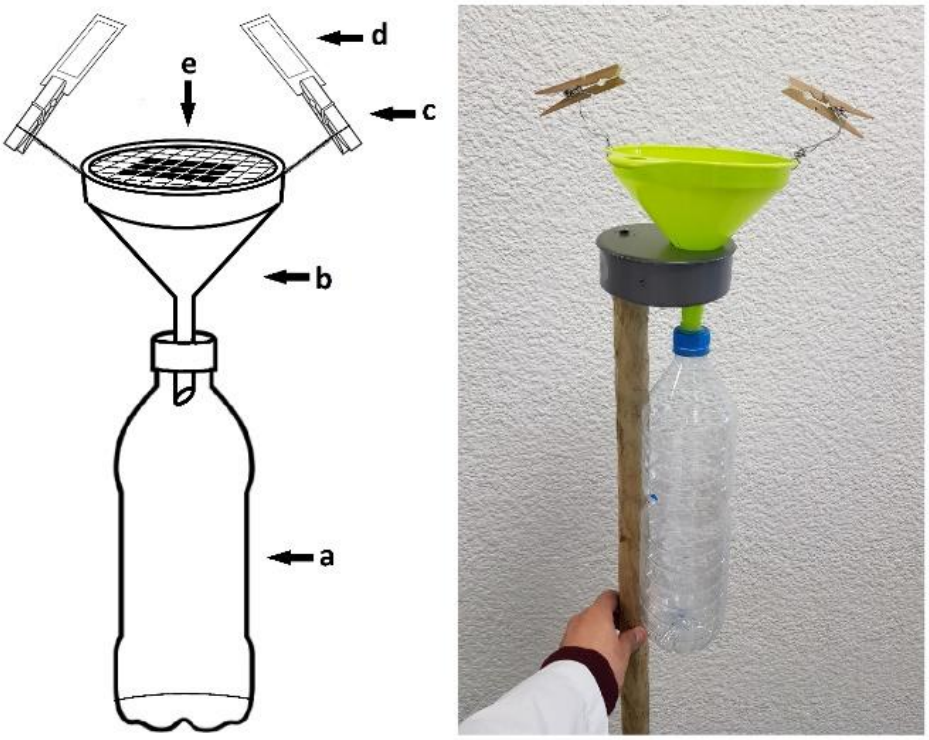

Figure 3.1 Schematic representation (left) and real (right) of the spore trap that was used to study the dynamics of *Diaporthe amygdali* conidia released from pieces of naturally infected almond shoots with cankers. The spore trap is composed of: (a) removable plastic bottle, (b) plastic funnel, (c) wooden clip anchored to the sides of the funnel, (d) glass microscope slides with silicone-coated tape and (e) inoculum source.

The wood pieces of almond shoots naturally infected with *D. amygdali* were collected each season in autumn, prior to setting up each trial, from almond orchards with a history of high disease severity (León et al. 2020). The presence of *D. amygdali* on the shoot pieces was confirmed by isolation in culture medium, followed by DNA extraction from the fungal colonies and amplification by PCR, and subsequent identification by sequencing. For isolation, wood fragments with cankers were cut from the affected branches, washed under running tap water, surface disinfected for 1 min in a 1.5% sodium hypochlorite solution and rinsed twice in sterile distilled water. Small pieces of affected tissues taken from the margin of the lesions were plated on potato dextrose agar (PDA; Biokar-Diagnostics, Zac de Ther, France) supplemented with 0.5 g/L of streptomycin sulphate (Sigma-Aldrich, St. Louis, MO, USA) (PDAS). Plates were incubated at

25 °C in the dark for 7 to 10 d, and all colonies were transferred to PDA. Mycelium was scraped from 10-day-old fungal cultures grown on PDA medium. Total fungal DNA was extracted using the E.Z.N.A. Plant DNA Kit (Omega Bio-tek, Norcross, GA, USA), following the manufacturer's short protocol instructions.

The ITS region and fragments of β-tubulin (tub) and translation elongation factor 1-alpha (tef-1α) genes were amplified and sequenced. Amplification by polymerase chain reaction (PCR) was performed in a total volume of 25 µL using HotBegan™ Taq DNA Polymerase (Canvax Biotech SL, Córdoba, Spain), according to the manufacturer's instructions on a Peltier Thermal Cycler-200 (MJ Research). One reaction was composed of 2.5 µL of 10× PCR Buffer B, 2.5 µL of MgCl2 (25 mM), 2.5 µL of dNTPs (8 mM), 1 µL of each primer (10 µM), 0.2 µL of HotBegan Taq DNA Polymerase (5 U/µL), 1 µL of purified template DNA and 14.3 µL of nuclease-free water. The thermal cycle consisted of an initial step of 3 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing for 30 s and elongation at 72 °C for 45 s. A final extension was performed at 72 °C for 5 min. The primers pairs and the annealing temperatures (Ta) for each locus were as follows: ITS1-F and ITS4 for ITS (Ta = 55 °C) (Gardes and Bruns 1993; White et al. 1990), EF1-688F and EF1-1251R for tef-1α (Ta = 55 °C) (Alves et al. 2008) and for tub BtCadF and BtCadR or T1 and BT2b (Ta = 55 °C for both pairs) (Glass and Donaldson 1995; O'Donnell and Cigelnik 1996; Travadon et al. 2015). PCR products were analyzed by 1% agarose gel electrophoresis, purified and sequenced by Macrogen Inc. (Madrid, Spain) using both PCR primers. Each consensus sequence was assembled using Sequencher software 5.0 (Gene Codes Corp., Ann Arbor, Michigan).

Six and four spore traps were randomly deployed in the inter-row space of the almond orchards A and B, respectively. Each spore trap was placed on a wooden stake 180 cm long, at a height of 150 cm above the ground. The spore traps in both orchards were sampled weekly during the months of November through May, during the 2019 to 2020 and 2020 to 2021 growing seasons, except in 2020 in Valencia, which it was sampled only until March. The bottles of the spore traps were replaced after every rain event (i.e., ≥0.1 mm of rain).

In orchard A, the spore traps were installed for 174 days (11/28/2019 to 05/20/2020) in the first evaluation season and 182 days (11/16/2020 to 05/17/2021) in the second season. In orchard B, the spore traps were installed for 117 days (11/22/2019 to 03/18/2020) in the first season and 169 days (11/16/2020 to 05/04/2021) in the second season. Air temperature (ºC), relative humidity (%), total rainfall (mm) and wind speed (km/h) were recorded every 1 hour by a weather station located in orchard A, and another station located 3.5

km from orchard B (Regional Agrometeorological Service, http:/riegos.ivia.es/). Both weather stations were 1.5 m above the ground.

Design of specific primers to detect D. amygdali using qPCR and assessment of their specificity and sensitivity. Specific primers were designed to detect DNA of *D. amygdali* using qPCR, based on tef-1α gene. The tef-1α sequences of Spanish isolates of *D. amygdali* and other *Diaporthe* species frequently isolated from almond trees in Spain were chosen from León et al. (2020). Sequences were aligned together with tef-1α sequences of other *Diaporthe* species closed related to *D. amygdali* retrieved from GenBank (Table 3.1) using ClustalW (Thompson et al. 1994) with default settings. Posterior manual adjustments were made when necessary. Regions with polymorphisms and suitable for specific primer design were identified, and later analyzed with the OligoAnalyzer tool (IDT, URL: https://eu.idtdna.com/calc/analyzer) using the default parameters. The designed primer pair qDamy_EF-F and qDamy_EF-R was obtained and were tested for their theoretical specificity to *D. amygdali* using NCBI Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Ye et al. 2012) prior to *in vitro* testing. The primers were synthesized by Metabion International AG (Germany).

Table 3.1. GenBank accession numbers of sequences used to design a specific primer pair for the detection and quantification of *Diaporthe amygdali.*

Fungal species	Strain	Host	Country	GenBank Acc. tef-1α
D. amygdali	DAL-4	*Prunus dulcis*	Spain	MT006772
	CBS 126679	*Prunus dulcis*	Portugal	KC343748
	CBS 111811	*Prunus dulcis*	South Africa	KC343745
D. eres	DAL-102	*Prunus dulcis*	Spain	MT007104
	CBS 138594	*Ulmus laevis*	Germany	KJ210550
	CBS 109767	*Acer campestre*	Austria	KC343801
D. foeniculina	DAL-10	*Prunus dulcis*	Spain	MT006963
	DAL-165	*Prunus dulcis*	Spain	MT006984
	CBS 111553	*Foeniculum vulgare*	Spain	KC343827
	CBS 187.27	*Camellia sinensis*	Italy	KC343833
D. phaseolorum	DAL-222	*Prunus dulcis*	Spain	MT007103
	CBS 113425	*Olearia* cf. *rani*	New Zealand	KC343900
	CBS 116019	*Caperonia palustris*	USA	KC343901

To optimize the qPCR reaction conditions, different primer concentrations (200 and 400 nM of each primer), PCR protocols (2-steps or 3-steps) and annealing temperatures (60 and 64ºC for 2-steps or 55ºC for 3-steps) were tested with examination of amplification plots, dissociation curves and visualizing

qPCR products on 3% MetaPhor® Agarose gels under UV light (data not shown). After the final amplification cycle, the melting curve profiles were obtained by raising the temperature from 72 to 95°C, increasing 1°C every 5 s with continuous measurement of fluorescence at wavelength 510 nm. All reactions were performed in triplicate and were carried out on a Rotor-Gene Q 5plex thermal cycler (Qiagen).

The *in vitro* testing specificity of the designed primers was done using DNA extractions of selected isolates of all *Diaporthe* species recovered from almond in Spain (León et al. 2020). Additionally, other fungal isolates frequently isolated from almond trees in Spain (Gramaje et al. 2012; Ollero-Lara et al. 2019; Olmo et al. 2015) were included in the analyses [*Alternaria* sp. (n=1), *Cladosporium* sp. (n=1), *Colletotrichum gloeosporioides* (n=2), *C*. *godetiae* (n=1), *Collophorina hispanica* (n=2), *Cytospora* sp. (n=4), *Diplodia seriata* (n=3), *Eutypa lata* (n=1), *Monilinia fructicola* (n=1), *M. laxa* (n=4), *Neofusicoccum luteum* (n=1), *N. mediterraneum* (n=1), *N. parvum* (n=2), *Phaeoacremonium iranianum* (n=2), *Pleurostomophora richardsiae* (n=1), *Polystigma amygdalinum* (n=3), *Stemphylium vesicarium* (n=1) and *Valsa* sp. (n=1)]. Total DNA from these fungi was extracted as described before.

Sensitivity of qPCR assay was assessed by determining the minimum amount of DNA of *D. amygdali* that could be detected. A standard curve was constructed with the genomic DNA of *D. amygdali* isolate DAL-4 extracted as described before. Total DNA was quantified with Invitrogen Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and 10-fold serially diluted, yielding concentrations from 14.20 ng/µL to 1.42 fg/µL. qPCR analyses were performed with the different dilutions as explained above and the standard curve was generated following the MIQE guidelines (Bustin et al. 2009), by plotting quantification cycle (Cq) values obtained for each specific DNA concentration, versus the logarithm of the initial concentration of genomic DNA. The standard deviation was determined from four DNA repeats per DNA concentration. The amplification efficiency (E) and the coefficient of correlation (R^2) of the standard curve were obtained using the Rotor-Gene 6000 Series software v. 1.7 (Qiagen). The limit of detection (LOD) was estimated as the lowest concentration of DNA of *D. amygdali* that yielded 100% positive results on all replicates included in the qPCR reactions in our conditions, accompanied by a melting curve peak temperature specific to *D. amygdali* amplicon.

Quantitative PCR assays of D. amygdali on spore traps. Each siliconized tape, collected from the spore traps, was cut into six equal fragments that were placed in a 2 ml tube. Each tube contained the first buffer of E.Z.N.A Plant DNA Kit (Omega Bio-tek, Norcross, GA, USA) and about 100 mg of 0.5-mm-diameter

Bashing Beads, which were collected from ZR Bashing Bead Lysis Tubes (Zymo Research, Irvine, CA, U.S.A.) and which were added to facilitate the rupture of the conidia by vibration in a FastPrep-24TM5G (MP Biomedicals, Santa Ana, California, USA) at 5 m/s for 15 s twice. DNA extractions were completed following the manufacturer's protocol provided with the kit. The efficiency of DNA extraction from the tapes was previously tested in the laboratory. To do this, tapes equivalent to those of the spore traps were prepared, impregnated with serial dilutions, using 100 µL each, from an initial solution with a concentration of 1E06 conidia per mL and processed as previously mentioned.

The water samples collected from the removable plastic bottles were manually shaken for 30 s (to homogenize the sample) and 100 ml of the total was collected, as a representative sample of the content of the bottle. Each liquid sample (100 ml) was centrifuged for 15 min at 232 relative centrifugal force (rcf); supernatant was carefully discarded leaving a small amount of liquid to resuspend the pellet and transfer it to an Eppendorf tube. The resuspended sample pellet was centrifuged again for 5 min at 930 rcf, the supernatant was discarded and the pellet was stored at -20°C. Total DNA was extracted as mentioned before for the silicone tapes.

The DNA extracted from each tape and water sample was quantified using the standard curve, based on Cq values obtained in the qPCR reactions, in Rotor-Gene Q 5plex thermal cycler, according to the qPCR conditions for the specific primers designed, described previously. The mean DNA concentration and the standard deviation were determined from the two DNA replicates per sample. Melting curves were examined to ensure that non-specific products were not amplified.

Relationships of DNA concentration of D. amygdali on spore traps with weather variables. The relationships among DNA concentrations of *D. amygdali* and weather variables were studied using Hurdle two-part models (Martin et al. 2005), in which the response variable is decomposed into two independent sub-processes, one describing the observation process and another one considering the conditional-to-presence continuous process.

In particular, if Y_t represents the occurrence of an observation in the field at time *t*, and Z_t represents the conditional-to-observed DNA concentration at time *t*, the two-part model that relates both processes with the possible covariates of interest can be represented as:

$$Y_i \sim Ber(\pi_i)$$
$$logit(\pi_i) = \beta_0 + \beta_1 X_i^{(1)} + \cdots + \beta_p X_i^{(p)}$$
$$Z_i \sim Ga(a_i, b_i)$$
$$E(Z_i) = \mu_i = \frac{a_i}{b_i}$$
$$log(\mu_i) = \gamma_0 + \gamma_1 X_i^{(1)} + \cdots + \gamma_p X_i^{(p)}$$

where the probability of occurrence π_t is linked through the usual logit link to a set of possible weather variables (covariates) of interest expressed via a linear predictor; the mean DNA concentration $\mu_t = (a_i/b_i)$ is linked through its logarithm to another set of possible weather variables of interest; *Ga* represents a Gamma distribution, chosen with the idea of dealing with the positive values of the DNA concentration (although other positive continuous distributions could have been used); and, finally, note that the parameters of both linear predictors have been chosen to be different allowing different relationships between the responses and the weather variables of interest.

Inference in the model was made within the Bayesian paradigm. In particular, models were fitted using the integrated nested Laplace approximation (INLA, Rue et al., 2009), which drastically reduces the computational costs of the traditional simulation-based Markov Chain Monte Carlo methods. INLA was implemented through the R-INLA package (Martins et al., 2013) for R (R Core Team, 2023).

Due to the random effect that is prevalent to the distinct experiments performed, two models have been fitted: one per growing seasons of study (i.e. 2019 to 2020 and 2020 to 2021). The random effect of the study site (i.e. Orchards A and B) over the intercept was also included. In this work, we used the deviance information criterion (DIC), which is a generalization of the Akaike information criterion (AIC) developed for Bayesian model comparison (Spiegelhalter et al. 2002), and the Watanabe-Akaike information criterion (WAIC) (Watanabe 2010). DIC and WAIC are the sum of two components, one of them quantifying model fit and the other evaluating model complexity. The models with the lowest values of DIC and WAIC were selected.

RESULTS

Design of qPCR primers for D. amygdali and assessment of their specificity and sensitivity. The design of *D. amygdali* specific primers was performed through the alignment of the tef-1α region of selected *Diaporthe* species (Table 3.1). Two polymorphic regions were selected for the design of forward and reverse primers: qDamy_EF-F (5’ TTC TAT GAT ACG CGC TCA CC 3’) and

qDamy_EF-R (5' CGT TAG CGA ATG TCC AAC ATA AT 3'). The amplified product was 98 bp long.

The optimized reaction (final volume of 20 µL) contained 10 µL TB Green Premix Ex Taq™ (Tli RNaseH Plus) (Takara Bio Inc., Kusatsu, Japan), 0.4 µM of each specific forward and reverse primers, 2 µL of template DNA and ultrapure sterile water (Chromasolv Plus; Sigma-Aldrich, Steinheim, Germany). The optimized PCR cycling conditions were 95°C for 30 s, 40 cycles each at 95°C for 5 s, 55°C for 30 s and 72°C for 30 s. The fluorescence signal was measured in the amplification step of each cycle. The amplified product showed a melting temperature at 84.7°C.

The analysis with the Primer-BLAST tool showed identity only with *D. amygdali* tef-1α sequences. Primer specificity was confirmed with genomic DNA extractions from *Diaporthe* and non-*Diaporthe* species. Only DNA from *D. amygdali* isolates was amplified with the specific primers, showing a single peak around 84.7 °C in the melting curve analysis. No amplification was observed for other non-*D. amygdali* samples. Amplifications and amplicon sizes were checked on 3% Metaphor agarose gel (data not presented).

Linearity was observed across the whole range used with a correlation coefficient (R^2) of 0.99198. The slope of the standard curve was -3.54071, which corresponds to an amplification efficiency of 91.615% (Fig. 3.2). The limit of detection, defined as the lowest DNA concentration that could be detected using SYBR Green qPCR method, was 14.2 fg μL^{-1}, which corresponds to a Cq of 32.01.

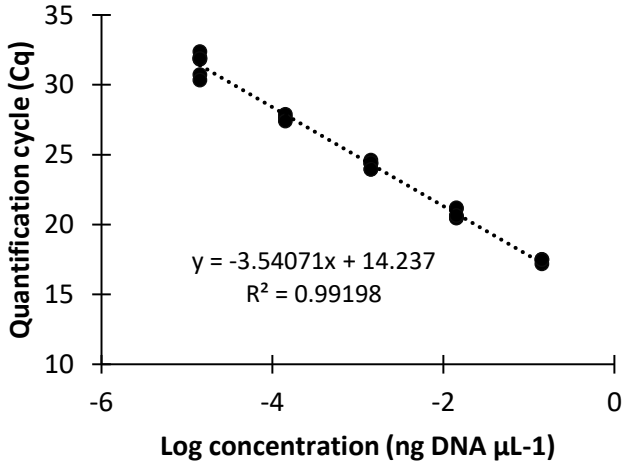

Figure 3.2 Standard regression curves obtained from qPCR assays involving 10-fold serial dilutions from a DNA extracted from pure culture.

Quantitative PCR assays of D. amygdali on spore traps. The efficiency of DNA extraction from the tapes previously tested in the laboratory, showed a linear response, with a value of E = 87% and R^2 = 0.99651. The mean concentration values of *D. amygdali* DNA, detected in the spore traps in orchards A and B throughout the 2019 to 2020 and 2020 to 2021 growing seasons, are shown in Fig. 3.3.

Regarding the concentration of DNA detected in silicone-coated tapes, during the first growing season (2019 to 2020), the highest average values of DNA were found in the week 14 (with a value of 1.1E-04 ng/µL) and 2 (2.0E-04 ng/µL) in orchards A and B, respectively. Regarding the second growing season (2020 to 2021), the highest average values of DNA were found in the week 25 (with a value of 1.7E-03 ng/µL) and 1 (8.3E-02 ng/µL) in orchards A and B, respectively.

Regarding the concentration of DNA detected in rain water (bottles), during the first growing season (2019 to 2020), the highest average values of DNA were found in the week 14 (with a value of 9.9E-02 ng/µL) and 2 (2.0E-02 ng/µL) in orchards A and B, respectively. Regarding the second growing season (2020 to 2021), the highest average values of DNA were found in the week 2 (with a value of 2 ng/µL) and 12 (3.7E-02 ng/µL) in orchards A and B, respectively.

Weather variables in the experimental almond orchards. The values of the weather variables registered in both growing seasons are graphed in Fig. 3.4. During the 2019 to 2020 and 2020 to 2021 growing seasons, Orchards A and B experienced somewhat different weather conditions, displaying some variability in temperatures, relative humidity, wind speed, and rainfall across both growing seasons.

During the 2019 to 2020 growing season, in orchard A, temperature ranged between a mean-minimum of 6.6°C and a mean-maximum of 22°C; relative humidity varied between a mean-minimum value of 45% and a mean-maximum value of 95%; wind speed reached a mean-maximum of 12 km/h; and rainfall had maximum value of 58 mm. In orchard B, temperature ranged between a mean-minimum of 6°C and a mean-maximum of 20°C; relative humidity varied between a mean-minimum value of 48% and a mean-maximum value of 92%; wind speed reached a mean-maximum of 19 km/h; and rainfall had maximum value of 63 mm.

Regarding the 2020 to 2021 growing season, in orchard A, temperature ranged between a mean-minimum of 4°C and a mean-maximum of 21°C; relative humidity varied between a mean-minimum value of 43% and a mean-maximum value of 92%; wind speed reached a mean-maximum of 12 km/h; and rainfall had

maximum value of 23 mm. In orchard B, temperature ranged between a mean-minimum of 4°C and a mean-maximum of 20°C; relative humidity varied between a mean-minimum value of 48% and a mean-maximum value of 93%; wind speed reached a mean-maximum of 16 km/h; and rainfall had maximum value of 25 mm.

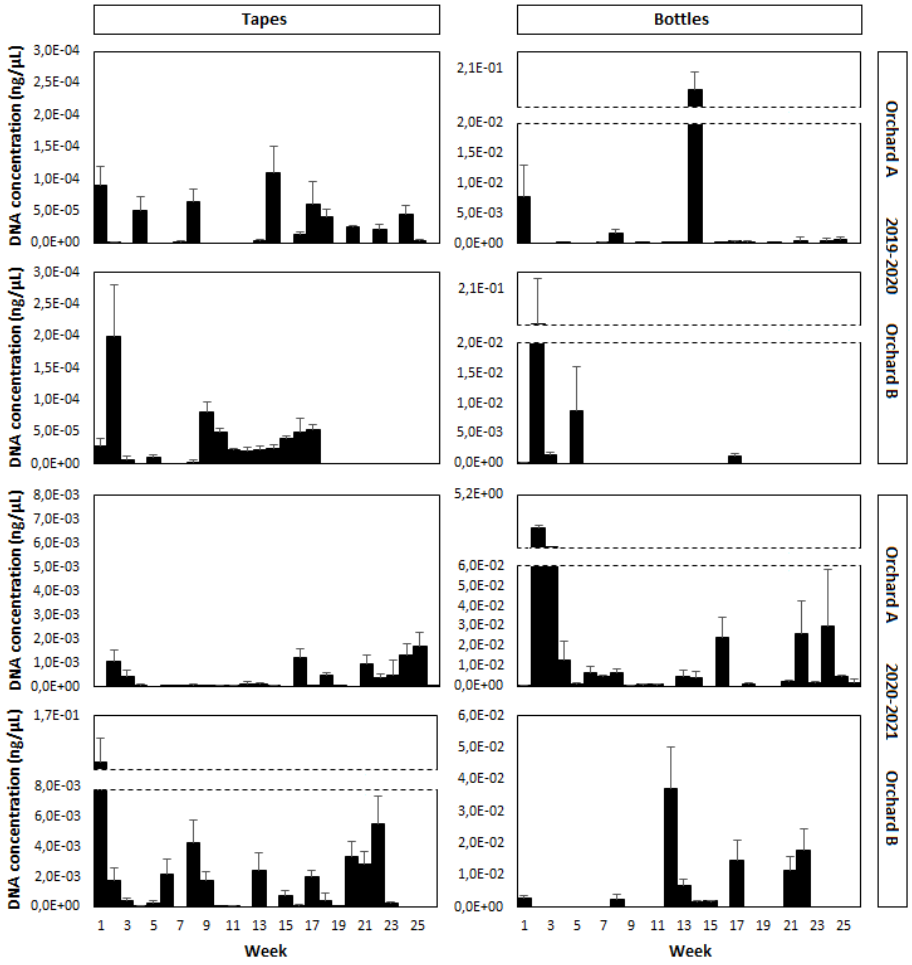

Figure 3.3 Black solid bars show the DNA concentration (ng/µL) of *Diaporthe amygdali* obtained by qPCR with specific primers from spore traps composed of removable plastic bottles and glass microscope slides with silicone-coated tape. The traps were located in two orchards: A (Palma, Balearic Islands) and B (Moncada, Valencia), during the 2019 to 2020 and 2020 to 2021 growing seasons. The weekly values of DNA correspond to the mean of four and six spore traps in orchard A and B, respectively. The vertical bars indicate the standard error of the means.

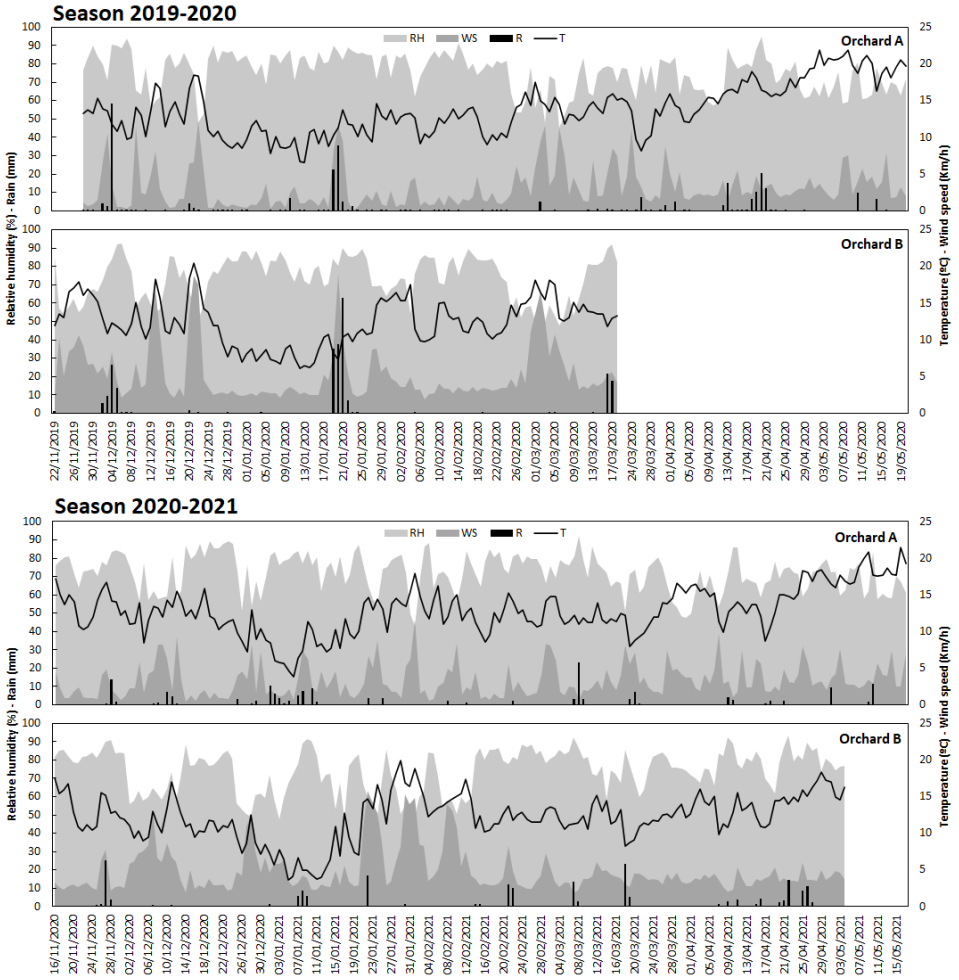

Figure 3.4 Weather variables registered in the experimental almond orchards: Orchard A (Palma, Balearic Islands) and Orchard B (Moncada, Valencia). The light-grey shade represents the relative humidity (%) and the dark-grey shade represents the wind speed (km/h). Vertical bars represent rainfall (mm) and continuous black line represents temperature (ºC). All values plotted represent mean values calculated based on 24 daily values.

Relationships of DNA concentration of D. amygdali on spore traps with weather variables. Only an average of n = 13 samples were collected from the removable plastic bottles in each orchard and season. This sample size was too small for inferential statistics, so these data were analyzed descriptively only. In

the siliconized tapes collected from the spore traps, the DNA concentrations of *D. amygdali* were prone to zero value observations with non-favorable conditions and so they are measured in the [0,∞) interval, resulting in semicontinuous datasets. One way to tackle this kind of data is via two-part models described before (Martin et al. 2005). The selected models minimized the WAIC, which equals -465.06 in the model for the growing season 2019 to 2020 and -446.853 in the model for the 2020 to 2021 growing season (Annex I).

For each period of study, the following models were selected:

2019 to 2020 growing season:

$$Y_i \sim Ber(\pi_i), i = 1,2,\dots n$$
$$logit(\pi_i) = -33.213 - 1.422 \cdot x_{i1} + 2.051 \cdot x_{i2} + 2.754 \cdot x_{i3} + 3.671 \cdot x_{i5} + 2.208 \cdot x_{i7}$$
$$Z_i \sim Ga(a_i, b_i)$$
$$log(\mu_i) = \alpha_{site} - 8.531 - 0.301 \cdot x_{i2} + 0.396 \cdot x_{i7}$$
$$\alpha_{OrchardA} = 9.981 \cdot 10^{-5}, \alpha_{OrchardB} = -9.981 \cdot 10^{-5}$$

2020 to 2021 growing season:

$$Y_i \sim Ber(\pi_i), i = 1,2,\dots n$$
$$logit(\pi_i) = 0.697 \cdot x_{i1} - 0.741 \cdot x_{i4} + 1.238 \cdot x_{i8}$$
$$Z_i \sim Ga(a_i, b_i)$$
$$log(\mu_i) = \alpha_{site} - 0.434 \cdot x_{i2} - 0.317 \cdot x_{i4} - 0.195 \cdot x_{i6} + 0.525 \cdot x_{i7} + 0.045 \cdot x_{i8}$$
$$\alpha_{OrchardA} = -8.595 \cdot 10^{-4}, \alpha_{OrchardB} = 8.595 \cdot 10^{-4}$$

Where x corresponds to the observations of each relevant covariable:

- x_1: Number of days with maximum temperature between 19ºC and 23ºC.
- x_2: Temperature range (calculated as average maximum temperature minus average minimum temperature).
- x_3: Number of days with minimum temperature above 10ºC and maximum temperature below 25ºC.
- x_4: Number of days with mean relative humidity higher than 80%.
- x_5: Number of days with rainfall higher than 0.2 mm.
- x_6: Maximum wind speed.
- x_7: Number of days with mean wind speed higher that 3 km/h.
- x_8: Total rainfall

DISCUSSION

A qPCR-based protocol was developed for the specific detection and quantification of *D. amygdali* inoculum from biological samples. A specific primer pair, named qDamy_EF-F/qDamy_EF-R, was designed and successfully validated. This is the first time that a molecular qPCR-based tool for the detection and quantification of *D. amygdali* has been developed. The primer pair targeting the tef-1α designed in this study proved to be highly specific, as indicated by the positive detection of *D. amygdali* DNA and no amplification of DNA from other *Diaporthe* species and other fungal species frequently isolated from almond trees in Spain. This methodology, together with a recent one developed for *Polystigma amygdalinum* causal agent of red leaf blotch (Zúñiga et al. 2018), can be applied in the study of the epidemiology of the diseases caused these pathogens on almond crops, improving their management through its early detection and quantification.

In this work, we used a two-part model approach to study the relationships between DNA concentration of *D. amygdali* inoculum on spore traps and weather variables that affect the probability of detecting and quantifying it. Data were obtained through field experiments carried out in two distant Spanish locations during two consecutive almond growing seasons.

Datasets from inoculum and plant disease dynamics studies are often characterized by large proportion of zero. This results in problems of overdispersion, which in most cases cannot be overcome using different probability distributions, including Poisson and negative binomial, or even applying zero-inflated models (Martin et al. 2005). Regression quantiles models can accommodate a relative wide range of data distributions and deal with datasets having a large proportion of zero values (Bassimba et al. 2014; Vaz et al. 2008). Nevertheless, these models only perform properly in the upper quantiles where most of the non-zero data are situated, so in practice zero values are not considered in model fitting. In contrast, the two-part Hurdle models used in this study include a qualitative part (Bernoulli), with a binary response, that is, it detects the presence or absence of DNA, and a quantitative part (Gamma) that estimates the average DNA concentration of *D. amygdali*, for each growing season. Thus, all the data and their associated information are considered in model fitting. Hurdle models are widely used in ecology (Martin et al. 2005), but little explored in plant disease epidemiology.

After the analysis of our data in each growing season, it was observed that some weather variables affected positively and others negatively, on both parts of the model. According to our results, temperature was considered into three different covariates: x_1= Number of days with maximum temperature

between 19ºC and 23ºC; x_2= Temperature range; and x_3= Number of days with minimum temperature above 10ºC and maximum temperature below 25ºC. In the 2019 to 2020 season, x_1 had a negative effect whereas x_2 and x_3 had a positive effect on the detection of *D. amygdali* DNA in the Bernoulli part. In the 2020 to 2021 season only x_1 was included in the Bernoulli part of the model, with a positive effect. In addition, x_2 had a negative effect on the quantification of the average DNA concentration, in the Gamma part of the model in both growing seasons. The effects of temperature seem to originate from the greater or lesser daily thermal amplitude. When we consider the covariate x_1, where the number of days have maximum temperatures between 19 and 23°C, the average temperatures and those of the rest of the day must be below this range, which negatively influenced the probability of detecting *D. amygdali* DNA in 2019 to 2020 and positively in 2020 to 2021. This effect was previously studied by Udin and Reynolds (1995), who found lower disease development in peach trees, measured as the Area Under the Disease Progress Curve (AUDPC), when temperatures are below 20°C, indicating that these conditions are also less favorable for pathogen development. Lalancette and Robison (2001) indicated that, in periods of low winter temperatures, the sporulation capacity of pycnidia and cirrus formation (measured as the number of spores per canker) is significantly lower than in summer, where it reaches its maximum production. It is important to note that Lalancette and Robison (2001) reported a significant difference between the number of pycnidia produced in the first and the second year of their study, in which approximately half of the pycnidia were produced. This agrees with the wide variability of DNA detected in our spore traps between the two seasons evaluated.

When considering a greater thermal amplitude during the day, expressed in the covariate x_2, a negative effect is observed mainly in the Gamma or quantitative part of the model, in both growing seasons evaluated, decreasing the average DNA concentration of *D. amygdali* on those days in which the fungus is detected. This effect may be due to extreme temperatures such as daily minimum and maximum temperatures. This again agrees with Udin and Reynolds (1995), who indicated that temperatures of 10 and 30°C, which are far from the optimum for growth and sporulation of *D. amygdali*, are less favorable for canker development in peach trees than 20°C. Therefore, canker development, pycnidia formation and sporulation are closely related processes governed by a similar temperature range (Lalancette and Robison 2001). Lalancette et al. (2003) evaluated the sporulation of *D. amygdali* under different temperatures. Both the formation of cirrus and the production of conidia were observed from 0 to 37°C, with an optimum at 22°C, but decreased considerably as temperatures approached the extremes. This effect was previously reported

from *in vitro* studies of *D. eres*, in which a reduction in the development rate of the fungus was observed when the temperature increased from 25 to 30°C or decreased from 25 to 15°C (Thomidis and Michailides 2009). Nevertheless, it is important to note the ability of *Diaporthe* spp. to survive under extreme temperatures (–20 and +35°C), allowing its subsequent vegetative growth and dispersal under very diverse climatic conditions worldwide (Abramczyk et al. 2020).

On the contrary, when considering a lower thermal amplitude, expressed in the covariate x_3, defined as the number of days with minimum temperature above 10ºC and maximum temperature below 25ºC, it increased the probability of detecting *D. amygdali* DNA during the 2019 to 2020 growing season. This effect was reported by Zehr (1995), who indicated that the cirrus clouds produced by the pycnidia of *D. amygdali* grow rapidly with temperatures above 10°C. Furthermore, Lalancette et al. (2003) found that this fungus increases its sporulation rate between 10 and 25°C, thus also increasing the probability of detecting *D. amygdali* DNA, according to the qualitative or Bernoulli part of our model.

Regarding relative humidity, expressed by the covariate x_4 of the model, defined as the number of days with mean relative humidity higher than 80%, it had a negative effect on the concentration of *D. amygdali* DNA in both parts of the model during the 2020 to 2021 growing season. This result may seem contradictory to what is reported in the literature about the positive effect of relative humidity on the production of fungal spores (Agrios 2005). Nevertheless, it is important to note that both relative humidity and temperature should not be considered separately in the models, but rather together. Studies by Agostini et al. (2003), in which lemon and grapefruit seedlings were inoculated with *D. citri* conidia, the infection by this fungus was very low with 4 h of leaf humidity, moderate with 8 to 16 h, and reached maximum levels at 24 h or more of leaf humidity, at 22°C average temperature. In the study by Lalancette et al. (2003) it was observed that most of the sporulation of *D. amygdali* occurred between 19 and 23°C, but only when peach twigs inoculated with this fungus were exposed between 16 and 48 h at relative humidity above 95%. A study conducted by Anco et al. (2013) on the biology of *D. neoviticola* (synonym *Phomopsis viticola*) in grapevines, examined the relationships among fungal sporulation, temperature, and duration of humidity. The results of this study indicated that fungal sporulation increased significantly between 18 and 25°C after at least 47 hours of continuous humidity. In contrast, it was observed that sporulation decreased considerably when the duration of humidity is less than 12 hours. These findings suggest that for sporulation it is essential that humidity conditions must be maintained for a prolonged period. This is why days with 80% average relative

humidity (as in our study) may be insufficient for the production and dispersal of *D. amygdali* conidia, negatively affecting both parts of the model.

Regarding rainfall, which is expressed by the covariates x_5 and x_8 (defined as: number of days with rain greater than 0.2 mm and total precipitation, respectively), it was observed that the first had a positive influence on the Bernoulli part of the model during the 2019 to 2020 growing season, and the second also a positive influence in both parts of the model during the 2020 to 2021 growing season. This effect was previously reported by Linders et al. (1996), who demonstrated the contribution of rain splashes for the spread of the disease caused by *D. adunca* in *Plantago lanceolata*. These authors indicated that when a raindrop hits a surface with mature pycnidia (formed cirri), thousands of droplets are produced that carry conidia. These droplets can initiate new infections by being thrown onto uninfected spikes of the same plant or plants adjacent to the inoculum source. Studies by Agostini et al. (2003) indicated that rain was a key factor for the release of conidia of *D. citri* favoring their emergence from pycnidia on dead citrus twigs. However, if environmental conditions are favorable, that is, if the duration of moisture in the leaves is maintained for a long time (24 hours or more) and temperatures are relatively high (24 to 28°C), weekly rainfall is not necessary for infection (Agostini et al. 2003). Recent field investigations, carried out by Battilani et al. (2018) in hazelnuts corroborate this relationship, because an increase in the amount of rainfall during the crop growing season was associated with a higher incidence of hazelnuts affected by *Diaporthe* spp. Furthermore, a positive and significant correlation was established between rainfall and the incidence of infections caused by *Diaporthe* spp., which further confirms the contribution of precipitation in the dispersal of conidia.

Finally, regarding wind speed, considered into two different covariates (x_6= Maximum wind speed and x_7= Number of days with mean wind speed higher that 3 km/h), the covariate x_6 negatively influenced the quantitative part of the model, only in the 2020 to 2021 growing season. This may be because high wind speeds can reduce relative humidity, directly affecting the sporulation of pycnidia, which requires prolonged periods high relative humidity (Anco et al. 2013; Lalancette et al. 2003). In addition, Troutt and Levetin (2001) indicated that spores that grow in fruiting bodies (such as *D. amygdali* conidia) are considered moist air spores, being released in response to high relative humidity and rain, without depending on wind speed, in contrast with dry air spores, such as *Alternaria* spp., *Cladosporium* spp. or *Epicoccum* spp., that are disseminated under conditions of low humidity and high wind speeds (Li and Kendrick 1995; Troutt and Levetin 2001). The x_7 covariate seems to be more relevant, positively influencing the qualitative or Bernoulli part of the 2019 to 2020 growing season

and the quantitative or Gamma part in both growing seasons. The effect of wind speed on disease development has been also associated to the physical damage caused high wind speeds to the plant organs, which favors *D. amygdali* infections through wounds (Adaskaveg et al. 2008; Lalancette and Robison 2001; Lalancette et al. 2003) caused by collisions and friction between branches (Agrios 2005). This effect was demonstrated by Kim et al. (2015) in citrus trees, indicating that high wind speed was sufficient to cause mechanical damage to the trees, favoring infection by *D. citri*.

This study contributes to the understanding of the relationships among different weather variables and the inoculum dispersal of *D. amygdali*, which is essential for twig canker and shoot blight management on almond in Mediterranean conditions. These findings may be useful to develop an epidemiological model that could be incorporated into a decision support system aiming to optimize disease control.

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References

Abramczyk, B., Król, E., Zalewska, E. and Zimowska, B. 2020. The influence of temperature and fungal community on growth and sporulation of *Diaporthe* from fruit plants. Acta Sci. Pol. Hortorum Cultus, 19(5).

Adaskaveg, J. 2002. Phomopsis Canker and Fruit Rot. Pages 27–28 in: Compendium of nut crop diseases in temperate zones. Teviotdale, B., Michailides, T., Pscheidt, J., eds. APS Press: St. Paul, MN, USA.

Adaskaveg, J., Förster, H., and Connell, J. 1999. First report of fruit rot and associated branch dieback of almond in California caused by a *Phomopsis* species tentatively identified as *P. amygdali*. Plant Dis. 83:1073-1073.

Adaskaveg, J., Schnabel, G., and Förster, H. 2008. Diseases of peach caused by fungi and fungal-like organisms: biology, epidemiology and management. Pages 352-406 in: The Peach: botany, production and uses Layne DR, Bassi D., eds. Wallingford, CAB International.

Agostini, J., Bushong, P., Bhatia, A. and Timmer, L. 2003. Influence of environmental factors on severity of citrus scab and melanose. Plant Dis. 87:1102-1106.

Agrios, G. 2005. Plant Pathology, 5th ed. Elsevier Academic Press, Burlington, MA. 922 pp.

Álvarez M., Perdomo, E., Martínez, E., Mondino, P., and Alaniz, S. 2014. *Phomopsis amygdali* principal agente causal de la viruela de la púa en durazneros y nectarinos en Uruguay. In: Abstracts, 13th National Congress of Hortifruticulture, 91p.

Alves, A., Crous, P., Correia, A., and Phillips, A. 2008. Morphological and molecular data reveal cryptic speciation in *Lasiodiplodia theobromae*. Fungal Divers. 28:1-13.

Anco, D., Madden, L. and Ellis, M. 2013. Effects of temperature and wetness duration on the sporulation rate of *Phomopsis viticola* on infected grape canes. Plant Dis. 97:579-589.

Bai, Q., Zhai, L., Chen, X., Hong, N., Xu, W., and Wang, G. 2015. Biological and molecular characterization of five *Phomopsis* species associated with pear shoot canker in China. Plant Dis. 99:1704-1712.

Battilani, P., Chiusa, G., Arciuolo, R., Somenzi, M., Fontana, M., Castello, G. and Spigolon, N. 2018. *Diaporthe* as the main cause of hazelnut defects in the Caucasus region. Phytopathol. Mediterr. 57:320-333.

Bassimba, D. D. M., Mira, J. L., and Vicent, A. 2014. Inoculum sources, infection periods, and effects of environmental factors on Alternaria brown spot of mandarin in Mediterranean climate conditions. Plant Dis. 98:409-417.

Beluzán, F., Miarnau, X., Torguet, L., Zazurca, L., Abad-Campos, P., Luque, J., and Armengol, J. 2022. Susceptibility of almond (*Prunus dulcis*) cultivars to twig canker and shoot blight caused by *Diaporthe amygdali*. Plant Dis. 106:1890-1897.

Beluzán, F., Olmo, D., León, M., Abad-Campos, P., and Armengol, J. 2021. First Report of *Diaporthe amygdali* Associated with Twig Canker and Shoot Blight of Nectarine in Spain. Plant Dis. 105:3300.

Besoain, X., Briceno, E., and Piontelli, E. 2000. *Fusicoccum* sp. as the cause of canker in almond trees and susceptibility of three cultivars. Fitopatología 35:176-182.

Bienapfl, J., and Balci, Y. 2013. Phomopsis blight: a new disease of *Pieris japonica* caused by *Phomopsis amygdali* in the United States. Plant Dis. 97:1403-1407.

Bustin, S., Benes, V., Garson, J., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M., Hipley, G., Vandesompele, J., and Wittwer, C. 2009. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55:611–622.

Cabrita, L., Neves, A., and Leitão, J. 2004. Evaluation of resistance to *Phomopsis amygdali* in almond. In Proceedings of the XIth Eucarpia symposium on fruit breeding and genetics, Vols 1 and 2 (Vol. 663, pp. 235-238). International Society for Horticultural Science.

Dai, F., Zeng, R., and Lu, J. 2012. First report of twig canker on peach caused by *Phomopsis amygdali* in China. Plant Dis. 96:288-288.

Diogo, E. L., Santos, J. M., & Phillips, A. J. 2010. Phylogeny, morphology and pathogenicity of *Diaporthe* and *Phomopsis* species on almond in Portugal. Fungal Divers. 44:107-115.

Farr, D., Castlebury, L., and Pardo-Schultheiss, R. 1999. *Phomopsis amygdali* causes peach shoot blight of cultivated peach trees in the southeastern United States. Mycologia 91:1008-1015.

Fontaine, S., Caddoux, L., Remuson, F., and Barrès, B. 2022. Thiophanate-methyl and carbendazim resistance in *Fusicoccum amygdali*, the causal agent of constriction canker of peach and almond. Plant Pathol. 71:785-792.

Gardes, M., and Bruns, T. 1993. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Mol. Ecol. 2:113-118.

Glass, N., and Donaldson, G. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. AEM 61:1323-1330.

Gramaje, D., Agustí-Brisach, C., Pérez-Sierra, A., Moralejo, E., Olmo, D., Mostert, Damm, U., and Armengol, J. 2012. Fungal trunk pathogens associated with wood decay of almond trees on Mallorca (Spain). Pers.: Mol. Phylogeny Evol. Fungi 28:1-13.

Hilário, S., Santos, L., and Alves, A. 2021. Diversity and Pathogenicity of *Diaporthe* Species Revealed from a Survey of Blueberry Orchards in Portugal. Agriculture 11:1271.

Kim, K., Kim, G., Son, K. and Koh, Y. 2015. Outbreaks of Yuzu Dieback in Goheung Area: possible causes deduced from weather extremes. Plant Pathol. J. 31:290-298.

Lalancette, N., and Polk, D. 2000. Estimating yield and economic loss from constriction canker of peach. Plant Dis. 84:941-946.

Lalancette, N., and Robison, D. 2001. Seasonal availability of inoculum for constriction canker of peach in New Jersey. Phytopathology 91:1109-1115.

Lalancette, N., and Robison, D. 2002. Effect of fungicides, application timing, and canker removal on incidence and severity of constriction canker of peach. Plant Dis. 86:721-728.

Lalancette, N., Foster, K., and Robison, D. 2003. Quantitative models for describing temperature and moisture effects on sporulation of *Phomopsis amygdali* on peach. Phytopathology 93:1165-1172.

León, M., Berbegal, M., Rodríguez-Reina, J., Elena, G., Abad-Campos, P., Ramón-Albalat, A., Olmo, D., Vicent, A., Luque, J., Miarnau, X., Agustí-Brisach, C., Trapero, A., Capote, N., Arroyo, F., Avilés, M., Gramaje, D., Andrés-Sodupe, M., and Armengol, J. 2020. Identification and characterization of *Diaporthe* spp. associated with twig cankers and shoot blight of almonds in Spain. Agronomy 10:1062.

Li, D. and Kendrick, B. 1995. A year-round study on functional relationships of airborne fungi with meteorological factors. Int. J. Biometeorol. 39:74-80.

Linders, E., Van Damme, J. and Zadoks, J. 1996. Transmission and overseasoning of *Diaporthe adunca* on *Plantago lanceolata*. Plant Pathol. 45:59-69.

Martin, T., Wintle, B., Rhodes, J., Kuhnert, P., Field, S., Low-Choy, S., Tyre, A. and Possingham, H. P. 2005. Zero tolerance ecology: improving ecological inference by modelling the source of zero observations. Ecol. Lett. 8:1235-1246.

Martins, T. G., Simpson, D., Lindgren, F. and Rue, H. 2013. Bayesian computing with INLA: new features. Comput. Stat. Data Anal. 68-83:434-450.

Meng, L., Yu, C., Wang, C., and Li, G. 2018. First report of *Diaporthe amygdali* causing walnut twig canker in Shandong province of China. Plant Dis. 102:1859-1859.

Michailides, T., and Thomidis, T. 2006. First report of *Phomopsis amygdali* causing fruit rot on peaches in Greece. Plant Dis. 90:1551-1551.

Mostert, L., Crous, P., Kang, J., and Phillips, A. 2001. Species of *Phomopsis* and a *Libertella* sp. occurring on grapevines with specific reference to South Africa: morphological, cultural, molecular and pathological characterization. Mycologia 93:146-167.

O'Donnell, K., and Cigelnik, E. 1996. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. Mol. Phylogenet. Evol. 7:103-116.

Ollero-Lara, A., Agustí-Brisach, C., Lovera, M., Roca, L., Arquero, O., and Trapero, A. 2019. Field susceptibility of almond cultivars to the four most common aerial fungal diseases in southern Spain. Crop Prot. 121:18-27.

Olmo, D., Armengol, J., León, M., and Gramaje, D. 2015. Pathogenicity testing of lesser-known fungal trunk pathogens associated with wood decay of almond trees. Eur. J. Plant Pathol. 143:607-611.

Onesti, G., González-Domínguez, E., Manstretta, V., and Rossi, V. 2018. Release of *Guignardia bidwellii* ascospores and conidia from overwintered grape berry mummies in the vineyard. Aust. J. Grape Wine Res. 24:136-144.

R Core Team. 2023. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available online <https://www.R-project.org/> (accessed on 10 May 2021)

Rue, H., Martino, S., and Chopin, N. 2009. Approximate Bayesian inference for latent Gaussian models by using integrated nested Laplace approximations. J. Roy. Stat. Soc. B Met. 71:319-392.

Spiegelhalter, D. J., Best, N. G., Carlin, B. P., and Van Der Linde, A. 2002. Bayesian measures of model complexity and fit. J. Roy. Stat. Soc. B Met. 64:583-639.

Thomidis, T. and Michailides, T. 2009. Studies on *Diaporthe eres* as a new pathogen of peach trees in Greece. Plant Dis. 93:1293-1297.

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

Travadon, R., Lawrence, D., Rooney-Latham, S., Gubler, W., Wilcox, W., Rolshausen, P., and Baumgartner, K. 2015. *Cadophora* species associated with wood-decay of grapevine in North America. Fungal Biol. 119:53-66.

Troutt, C. and Levetin, E. 2001. Correlation of spring spore concentrations and meteorological conditions in Tulsa, Oklahoma. Int. J. Biometeorol. 45:64-74.

Tuset, J., and Portilla, M. 1989. Taxonomic status of *Fusicoccum amygdali* and *Phomopsis amygdalina*. Canad. J. Bot. 67:1275-1280.

Uddin, W. and Reynolds, K. L. 1995. Phomopsis twig blight of peach: Pathogenicity and influence of temperature on disease development. (Abstr.) Phytopathology 85:1127.

Uddin, W., and Stevenson, K. 1998. Seasonal development of Phomopsis shoot blight of peach and effects of selective pruning and shoot debris management on disease incidence. Plant Dis. 82:565-568.

Uddin, W., Stevenson, K., and Pardo-Schultheiss, R. 1997. Pathogenicity of a species of *Phomopsis* causing a shoot blight on peach in Georgia and evaluation of possible infection courts. Plant Dis. 81:983-989.

Varjas, V., Izsépi, F., Nagy, G., Pájtli, É., and Vajna, L. 2017a. Phomopsis blight on almond in Hungary (pathogen: *Phomopsis amygdali*, teleomorph: *Diaporthe amygdali*). Növényvédelem 53:532-538 (In Hungarian).

Varjas, V., Vajna, L., Izsépi, F., Nagy, G., and Pájtli, É. 2017b. First report of *Phomopsis amygdali* causing twig canker on almond in Hungary. Plant Dis. 101:1674.

Vaz, S., Martin, C. S., Eastwood, P. D., Ernande, B., Carpentier, A., Meaden, G. J., and Coppin, F. 2008. Modelling species distributions using regression quantiles. J. Appl. Ecol. 45:204-217.

Watanabe, S. 2010. Asymptotic equivalence of Bayes cross validation and widely applicable information criterion in singular learning theory. J. Mach. Learn. Res. 11:3571-3594.

White, T., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungi ribosomal RNA genes for phylogenetics. Pages 315-322 in: PCR Protocols. A Guide to Methods and Applications Innis, M., Gelfand, D., Sninsky, J., White, T., eds. Academic Press: Millbrae, CA, USA.

Ye, J., Coulouris G., Zaretskaya I., Cutcutache I., Rozen S., and Madden T. 2012. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinform. 13:1-11.

Zehr, E. 1995. Constriction canker. Pages 31-32 in: Compendium of stone fruit diseases Ogawa, J., Bird, G., Ritchie, D., Uriu, K., and Uyemoto, J., eds. The American Phytopathological Society, St. Paul, MN, USA.

Zúñiga, E., León, M., Berbegal, M., Armengol, J. and Luque, J. 2018. A qPCR-based method for detection and quantification of *Polystigma amygdalinum*, the cause of red leaf blotch of almond. Phytopathol. Mediterr. 57:257-268.

ANNEX I

The tables below show the Hurdle models with the lowest WAIC or DIC values for the 2019 to 2020 and 2020 to 2021 growing seasons. Since Hurdle models are structured in two parts (Bernoulli regression and Gamma regression), they are shown separately. The WAIC or DIC of a Hurdle model equals the sum of the WAIC or DIC from each of the two parts.

The models in the tables below include an intercept and the following covariates:

- TMax: Average maximum temperature.
- D.TMax19-23: Number of days with maximum temperature between 19ºC and 23ºC.
- T.range: Temperature range (calculated as average maximum temperature minus average minimum temperature).
- D.T25T10: Number of days with minimum temperature higher than 10ºC and maximum temperature lower than 25ºC.
- D.RH>80: Number of days with mean relative humidity higher than 80%.
- rainfall: Total volume of precipitation.
- D.PP>0.2: Number of days with rainfall higher than 0.2 mm
- wMax: Average maximum wind speed.
- DW>3: Number of days with mean wind speed higher that 3 km/h.
- site: random factor of the study site.

Table A. Hurdle models for the DNA concentration of *Diaporthe amygdali* based on environmental variables (fixed effects) and the random effect site for the growing seasons 2019 to 2020, ordered according to their WAIC value.

Bernoulli regression	**DIC**	**WAIC**
intercept + D.TMax19-23 + T.range + D.T25T10 + D.PP>0.2 + DW>3	22.71762	24.53697
intercept + D.TMax19-23 + T.range + D.T25T10 + D.PP>0.2 + DW>3 + site	22.70721	24.53941
intercept + D.TMax19-23 + T.range + D.PP>0.2 + DW>3	24.25936	26.59853
intercept + D.TMax19-23 + T.range + D.T25T10 + D.PP>0.2 + wMax + DW>3	23.30486	26.61148
intercept + D.TMax19-23 + T.range + D.T25T10 + D.PP>0.2 + wMax + DW>3 + site	23.29311	26.61417
intercept + D.TMax19-23 + T.range + D.PP>0.2 + DW>3 site	24.25813	26.61545
Gamma regression		
intercept + T.range + DW>3 + site	-470.8396	-471.3913
intercept + T.range + DW>3	-470.8129	-471.3610
intercept + T.range + rainfall + DW>3 + site	-469.1093	-469.9431
intercept + T.range + D.RH>80 + DW>3 + site	-469.5481	-469.9190
intercept + T.range + rainfall + DW>3	-469.0807	-469.9140
intercept + T.range + D.RH>80 + DW>3	-469.5170	-469.8852

Table B. Hurdle models for the DNA concentration of *Diaporthe amygdali* based on environmental variables (fixed effects) and the random effect site for the growing seasons 2020 to 2021, ordered according to their WAIC value.

Bernoulli regression	**DIC**	**WAIC**
D.TMax19-23 + D.RH>80 + rainfall	26.60221	27.37664
D.TMax19-23 + D.RH>80 + rainfall + site	26.60524	27.37932
D.RH>80 + D.PP>0.2 + DW>3	27.66027	27.88215
D.RH>80 + D.PP>0.2 + DW>3 + site	27.66495	27.88781
Tmax + D.TMax19-23 + rainfall + wMax + DW>3	26.21678	27.96816
Tmax + D.TMax19-23 + rainfall + wMax + DW>3 + site	26.22538	27.97448
Gamma regression	**DIC**	**WAIC**
T.range + D.RH>80 + wMax + DW>3 + rainfall + site	-493.7708	-492.4949
T.range + D.RH>80 + wMax + DW>3 + rainfall	-492.7076	-492.3975
T.range + D.RH>80 + wMax + DW>3 + site	-492.9345	-490.9345
T.range + D.RH>80 + wMax + DW>3	-492.7076	-490.8013
intercept + T.range + D.RH>80 + rainfall + wMax + DW>3 + site	-491.9065	-490.629
intercept + T.range + D.RH>80 + rainfall + wMax + DW>3	-491.7421	-490.5142

Table C. Hurdle models for the DNA concentration of *Diaporthe amygdali* based on environmental variables (fixed effects) and the random effect site for the growing seasons 2019 to 2020, ordered according to their DIC value.

Bernoulli regression	**DIC**	**WAIC**
D.TMax19-23 + T.range + D.RH>80 + rainfall + D.PP>0.2 + DW>3 + site	-1011.4269	534.4286
TMax + rainfall + D.PP>0.2 + DW>3 + site	-1005.9235	284.4728
intercept + T.range + D.T25T10 + rainfall + D.PP>0.2 + wMax + site	-967.7315	194.8492
intercept + T.range + D.RH>80 + rainfall + D.PP>0.2 + wMax + DW>3 + site	-945.2307	134.2574
intercept + TMax + rainfall + D.PP>0.2 + DW>3 + site	-921.7569	128.6532
intercept + T.range + rainfall + D.PP>0.2 + wMax + DW>3 + site	-918.2065	143.6426
Gamma regression	**DIC**	**WAIC**
intercept + T.range + DW>3 + site	-470.8396	-471.3913
intercept + T.range + DW>3	-470.8129	-471.3610
intercept + T.range + D.RH>80 + DW>3 + site	-469.5481	-469.9190
intercept + T.range + D.RH>80 + DW>3	-469.5170	-469.8852
intercept + T.range + rainfall + DW>3 + site	-469.1093	-469.9431
intercept + T.range + rainfall + DW>3	-469.0807	-469.9140

Table D. Hurdle models for the DNA concentration of *Diaporthe amygdali* based on environmental variables (fixed effects) and the random effect site for the growing seasons 2020 to 2021, ordered according to their DIC value.

Bernoulli regression	**DIC**	**WAIC**
Tmax + D.RH>80 + rainfall + wMax + DW>3 + site	-497.7624	45.57082
rainfall + D.PP>0.2 + site	-450.6316	45.68554
intercept + rainfall + wMax + DW>3 + site	-421.3088	46.08236
D.TMax19-23 + D.T25T10 + D.RH>80 + rainfall + wMax + site	-421.1320	38.45607
D.RH>80 + rainfall + D.PP>0.2 + wMax + DW>3 + site	-398.8066	55.61422
D.TMax19-23 + rainfall + D.PP>0.2 + wMax + site	-384.3576	86.57172
Gamma regression		
T.range + D.RH>80 + rainfall + wMax + DW>3 + site	-493.7708	-492.4949
T.range + D.RH>80 + rainfall + wMax + DW>3	-493.6103	-492.3975
T.range + D.RH>80 + wMax + DW>3 + site	-492.9345	-490.9345
T.range + D.RH>80 + wMax + DW>3	-492.7076	-490.8013
T.range + D.T25T10 + D.RH>80 + rainfall + wMax + DW>3 + site	-492.3715	-490.4523
T.range + D.T25T10 + D.RH>80 + rainfall + wMax + DW>3	-492.3214	-490.4124

Chapter 4

Chapter 4

Susceptibility of almond (*Prunus dulcis*) cultivars to twig canker and shoot blight caused by *Diaporthe amygdali*

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Abstract. Twenty-five almond cultivars were assessed for susceptibility to *Diaporthe amygdali*, causal agent of twig canker and shoot blight disease. In laboratory experiments, growing twigs were inoculated with four *D. amygdali* isolates. Moreover, growing shoots of almond cultivars grafted onto INRA 'GF-677' rootstock were used in four-year field inoculations with one *D. amygdali* isolate. In both type of experiments, inoculum consisted of agar plugs with mycelium, which were inserted underneath the bark and the lesion lengths caused by the fungus were measured. Necrotic lesions were observed in the inoculated almond cultivars both in laboratory and field tests, confirming the susceptibility of all the evaluated cultivars to all the inoculated isolates of *D. amygdali*. Cultivars were grouped as susceptible or very susceptible according to a cluster analysis. The relationship between some agronomic traits and cultivar susceptibility was also investigated. Blooming and ripening times were found relevant variables to explain cultivars performance related to *D. amygdali* susceptibility. Late and very late blooming, and early and medium ripening cultivars were highly susceptible to *D. amygdali*. Our results may provide valuable information that could assist in ongoing breeding programs of this crop and additionally in the selection of cultivars for new almond plantations.

INTRODUCTION

During the last 15 years, almond (*Prunus dulcis* (Mill.) D.A. Webb) crop has been experiencing a very favorable period worldwide (Gradziel et al., 2017).

Consumption of almonds has several positive connotations with respect to health, as they are rich in nutrients like vitamin E, proteins, mono-unsaturated fatty acids, poly-unsaturated fatty acids, magnesium, potassium, and dietary fibers, which have been linked to lower cardiometabolic disease risk (Kalita et al., 2018). This fact, together with the opening of new markets in Asia, has resulted in an increase in both almond demand and prices (INC 2020). Moreover, almond growing in the Mediterranean area is currently evolving from a marginal rainfed crop to a very productive and profitable one, with new cultivars and production systems, thus increasing its planted area (Maldonado et al., 2019).

Spain stands out with the largest almond area in the world, with 718,540 ha (MAPA 2020), but yields per ha are below those obtained by other countries with less planted area such as the USA and Australia (FAOSTAT 2021). This represents a new challenge for Spanish almond growers, who aim at improving their orchard yields by opting for cultivars with favorable agronomic characteristics for intensive production (i.e., increased planting density, mechanized harvesting, and the use of drip irrigation). In addition, in recent years the crop is experiencing an active process of varietal renewal (Batlle et al., 2017). The new almond cultivars obtained in Spanish breeding programs aim to improve fruit quality (size, shape, weight, protein, oil content and stability and fatty acids), while selecting for late flowering, self-fertility bearing precocity, and tolerance to pathogens (Batlle et al., 2017). Nevertheless, potential yield of almond in Spain can be reduced by the reemergence of pests and diseases that were not usual in traditional almond growing or just showed a low impact on production, and by the low number of fungicides currently authorized for the control of almond pests and diseases (Torguet et al., 2019).

Almond crop can be affected by several fungal diseases, such as red leaf blotch (*Polystigma amygdalinum* P.F. Cannon), shot hole (*Wilsonomyces carpophilus* (Lév.) Adask., J.M. Ogawa & E.E. Butler), brown rot and blossom blight (*Monilinia* spp.), and leaf curl (*Taphrina deformans* (Berk.) Tul.) (Miarnau et al., 2021; Ollero-Lara et al., 2019; Teviotdale et al., 2002), as well as by the reemergence of old ones such as anthracnose (*Colletotrichum acutatum* J.H. Simmonds) (López-Moral et al., 2019), and the new branch canker and dieback diseases caused by trunk pathogens (Gramaje et al., 2012; Holland et al., 2021; Olmo et al., 2016). Among them, twig canker and shoot blight caused by *Diaporthe amygdali* (Delacr.) Udayanga, Crous & K.D. Hyde is widespread in the Mediterranean countries, and seriously compromise crop productivity (Adaskaveg 2002; Diogo et al., 2010; León et al., 2020). A recent study conducted in Spain, in which 225 *Diaporthe* isolates from almond orchards were characterized by a multilocus DNA sequence analysis, confirmed *D. amygdali* as a key pathogen of almond in Spain (Hilário et al., 2021; León et al., 2020).

Symptoms of twig canker and shoot blight disease caused by *Diaporthe* spp. are characterized by the quick desiccation of buds, flowers and leaves after infections produced in late winter or early spring. The new shoots developing from infected buds usually wilt and die (Adaskaveg 2002; Varjas et al., 2017b). Brown lesions (1 to 5 cm diameter), initially formed around buds on green shoots, further develop into annual sunken cankers, sometimes with a gummy exudate, as well as withering of twigs (Adaskaveg 2002). As a result, leaves wilt and, when the disease is severe, defoliation may occur. In summer, pycnidia develop just under the dry canker bark (Adaskaveg 2002).

Studies on the susceptibility of almond cultivars to fungal diseases are increasing in literature, mainly within the last decade. In Spain, Egea et al., (1984) carried out an evaluation of the susceptibility to red leaf blotch with 81 almond cultivars. In California, Gradziel and Wang (1994) evaluated the fruit susceptibility of different almond cultivars to *Aspergillus flavus* Link, and Diéguez-Uribeondo et al., (2011) determined the susceptibility of four almond cultivars to *C. acutatum*. In Australia, Horsfield and Wicks (2014) studied the susceptibility of 34 almond cultivars to the rust pathogen *Tranzschelia discolor* (Fuckel) Tranzschel & M.A. Litv. in field conditions following natural and artificial infections. In Spain, López-Moral et al., (2019) evaluated the susceptibility of 19 almond cultivars to *C. acutatum* and *C. godetiae* Neerg., and additional studies have evaluated the susceptibility of early and late flowering almond cultivars to foliar diseases caused by *Monilinia laxa* (Aderh. & Ruhland) Honey, *P. amygdalinum, T. deformans* and *W. carpophilus* (Miarnau et al., 2021; Ollero-Lara et al., 2019).

Regarding *D. amygdali*, its pathogenicity to almond trees has been widely documented (Adaskaveg et al., 1999; Diogo et al., 2010; León et al., 2020; Teviotdale et al., 2002; Varjas et al., 2017b), and the susceptibility of almond cultivars to this pathogen has also been investigated. In Chile, *D. amygdali* was inoculated in three almond cultivars ('Carmel', 'Nonpareil' and 'Price'), being 'Nonpareil' and 'Price' more susceptible than 'Carmel' (Besoain et al., 2000). In Portugal, a local almond cultivar ('Barrinho Grado') showed a higher tolerance to *D. amygdali* than 'Ferragnès' (Cabrita et al., 2004). In Spain, Vargas and Miarnau (2011) evaluated more than 70 almond cultivars and 36 selections in field conditions with natural infections, and showed a broad gradient of susceptibility to Diaporthe dieback among cultivars. In Hungary, pathogenicity tests were carried out in 162 almond genotypes with *D. amygdali* (Varjas et al., 2017a). Thirty-one of them were found to be highly tolerant according to 4-year observations. Specifically, 'Budatétényi-70' and 'Tétényi keményhéjú' cultivars showed a significantly higher tolerance to this pathogen compared with other

Hungarian cultivars, and the results also showed a wide range of variability among the genotypes and cultivars studied.

The main objective of this research was to obtain new information about the susceptibility of a collection of 25 almond cultivars to *D. amygdali*, with experiments conducted both *in vitro* and *in vivo* conditions. We focused our attention on evaluating the susceptibility to *D. amygdali* of the most recently-obtained Spanish cultivars in the last two decades, in order to provide breeders and farmers with tools to obtain and grow more tolerant cultivars in the future. Additionally, some of the most planted cultivars in Europe, including France and Italy, and the USA were included in our trials for comparison purposes.

MATERIALS AND METHODS

Almond cultivars. In this study, twenty-five almond cultivars were assessed for susceptibility to *D. amygdali*. Fifteen cultivars were obtained from three different Spanish breeding programs: seven from Institut de Recerca i Tecnologia Agroalimentàries (IRTA) ('Constantí', 'Francolí', 'Glorieta', 'Marinada', 'Masbovera', 'Tarraco', and 'Vairo') (Vargas and Romero 1994; Vargas et al., 2008); four from Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA) ('Belona', 'Guara', 'Mardía', and 'Soleta') (Dicenta et al., 2015; Felipe and Socias i Company 1987; Socias i Company and Felipe 2006; Socias i Company et al., 2008) and four from Centro de Edafología y Biología Aplicada–Consejo Superior de Investigaciones Científicas (CEBAS-CSIC) ('Antoñeta', 'Marta', 'Penta', and 'Tardona') (Dicenta et al., 2008; Dicenta et al., 2018; Egea et al., 2000). Three cultivars were obtained from Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE), France ('Ferraduel', 'Ferragnès', and 'Lauranne') (Grasselly 1991; Grasselly and Duval 1997). Two traditional cultivars widely planted in Spain, 'Desmayo Largueta' and 'Marcona' (Felipe 2000), one Italian cultivar commonly planted in some Mediterranean countries, 'Tuono' (Dicenta et al., 2015; Felipe 2000), and four American cultivars ('Fritz', 'Independence', 'Monterey' and 'Nonpareil') (Batlle et al., 2017) were also included in this study. A single clone per cultivar was used in both laboratory and field evaluations.

Fungal isolates. Four fungal isolates of *D. amygdali* (DAL-4, DAL-34, DAL-138 and DAL-174) were used in the laboratory evaluation, and one isolate of *D. amygdali* (DAL-138) was used in the field inoculations. All isolates were obtained from diseased almond shoots showing twig cankers and shoot blight in different almond growing areas of Spain, and characterized as described in previous studies (Hilário et al., 2021; León et al., 2020). The isolates were stored in 15%

glycerol solution at -80 ºC in 1.5 mL cryovials in the fungal collection of the Instituto Agroforestal Mediterráneo–Universitat Politècnica de València (IAM-UPV) (Spain). The fungal inocula used in the laboratory and field inoculations were obtained by previously growing the isolates on potato dextrose agar (PDA; Biokar-Diagnostics, Zac de Ther, France) for 10 d at 26 ºC in the dark.

Laboratory evaluation. In 2020, growing twigs (30 cm long) of the 25 almond cultivars used in this study were obtained from IRTA facilities located in Les Borges Blanques, Lleida, northeastern Spain (UTM coordinates: WGS84 Datum, 31 T x=320870, y=4597530), and they were inoculated with isolates DAL-4, DAL-34, DAL-138 and DAL-174. The twigs were surface sterilized by immersion in 70% ethanol for 30 s, 1.5% sodium hypochlorite solution for 1 min, and again in ethanol 70% for 30 s. Then, they were air-dried in a laminar flow cabinet. Wounds were made in the center of each twig with a 5-mm cork borer. Mycelium agar plugs (5-mm-diameter), which were obtained from active 15-day-old colonies of the *D. amygdali* isolates growing on PDA, were inserted under the bark and the wounds were sealed with Parafilm. Inoculated twigs were kept in an upright position with their lower ends immersed in 1 L jars with 500 mL of sterile water in a growth chamber at 23 ºC with 12 h light per day. The twigs were covered with a plastic bag during the first 7 days to keep a moist environment. Five twigs per isolate were used and a control was prepared using uncolonized PDA plugs. Jars were arranged in a completely randomized design and the water was changed every 3 days. Lesion lengths were measured 15 days after inoculation. The experiment was repeated once.

Immediately after lesion measurements, two representative shoots per inoculated isolate and repetition were surface sterilized as described above. Small internal fragments were cut from the margin of the healthy and necrotic tissue and placed onto PDA supplemented with 0.5 g/L of streptomycin sulphate (PDAS). Plates were incubated at 25 °C in the dark for 7 to 10 days, and all fungal growth resembling *D. amygdali* were transferred to PDA for morphological identification to satisfy Koch's postulates.

Field evaluation. The 21 European cultivars used in this study were grafted onto INRA 'GF-677' rootstock and planted in December 2009 as bare root trees (1 m in height) at the IRTA facilities previously indicated. The experimental plot consisted of 16 trees per each cultivar. The trees were planted at 4 m × 2 m (distances between and within rows, respectively) and pruned as a central axis. The orchard was drip-irrigated, and pruning, soil management, and fertilization were based on the Spanish Integrated Production Management practices (BOE 2002). No fungicide treatments were applied during the experimental period.

Every year in July 2012-2015, six growing shoots were randomly chosen per cultivar. All shoots were located outside the tree in a north-east orientation and were about 30-35 cm long. An incision (1.5 to 2 cm long) was made in the basal part of each shoot with a scalpel and the bark partially removed. A colonized agar plug (~5-mm-diameter), obtained from the margin of a 15-day-old colony of DAL-138, was placed on the wound with the mycelium facing the inner wood tissues, and the wound was sealed with Parafilm. Non-inoculated controls were prepared using uncolonized PDA plugs. About 3-4 weeks after inoculation, the lesion length caused by the fungus, upwards and downwards from the inoculation point, was measured. The pathogen was reisolated from three of the inoculated shoots per cultivar, as it has been described above for the laboratory trial. The experiment was repeated four times within the years 2012 to 2015.

Data analyses. Lesion length means were calculated for each isolate and cultivar. These values were additionally grouped and analyzed according to four common agronomic traits: blooming time, ripening time, tree vigor and branching density (Table 4.1). Blooming and ripening times were classified into four levels (early, medium, late, and very late), whereas branching density and vigor were similarly classified into four levels (low, medium, high, and very high).

Analysis of variance (ANOVA) assumptions were checked prior to the analysis and data were transformed (squared) to meet analysis requirements. One-way ANOVA was performed to detect any statistically significant effect ($P \leq 0.05$) of the cultivar variable on the lesion length caused by the fungus. The Least Significant Difference (LSD) test was further used to compare the mean lesion length of each cultivar. All calculations were performed using Statgraphics Centurion XVI (Statgraphics Technologies, Inc., The Plains, VA, USA).

In addition, a cluster analysis was conducted in R (R Core Team 2021) to characterize the response of the almond cultivars to the inoculation with *D. amygdali* isolates; this was based on a combined analysis of all mean lesion lengths obtained in the field and laboratory experiments. The optimal number of clusters was estimated using the function *NbClust* of the *NbClust* package (Charrad et al., 2014). The cluster analysis was performed using the function *pam* in the *cluster* package, which specifically uses the Partitioning Among Medoids (PAM) algorithm (Kaufman and Rousseeuw 2009). The results were visualized using the *fviz_cluster* function of the *factoextra* package (Kassambara and Mundt 2020), which combines the clustering results with a Principal Component Analysis of the original data matrix. The cluster means obtained in this analysis were compared with the Student's *t*-test.

Table 4.1 Blooming time, ripening time, tree vigor and branching density of all tested cultivars [a].

Cultivar	Blooming time[b]	Ripening time[b]	Vigor[c]	Branching density[c]
'Antoñeta'	Medium	Early	Very high	High
'Belona'	Medium	Medium	Very high	Low
'Constantí'	Late	Medium	High	Medium
'Desmayo Largueta'	Early	Very late	Medium	Very high
'Ferraduel'	Medium	Medium	Medium	High
'Ferragnès'	Medium	Medium	High	Medium
'Francolí'	Medium	Medium	High	Medium
'Fritz'	Early	Late	High	Medium
'Glorieta'	Medium	Medium	High	Medium
'Guara'	Medium	Early	Medium	Low
'Independence'	Early	Early	High	Medium
'Lauranne'	Late	Early	Medium	Medium
'Marcona'	Early	Medium	High	High
'Mardía'	Very late	Medium	High	Low
'Marinada'	Late	Late	Low	Low
'Marta'	Medium	Medium	Very high	Medium
'Masbovera'	Medium	Medium	High	Medium
'Monterey'	Early	Late	High	Medium
'Nonpareil'	Early	Medium	High	Medium
'Penta'	Very late	Early	Medium	High
'Soleta'	Medium	Very late	Medium	Very high
'Tardona'	Very late	Early	Medium	Very high
'Tarraco'	Late	Very late	High	Low
'Tuono'	Medium	Early	Medium	Low
'Vairo'	Medium	Medium	Medium	Medium

[a] Agronomic traits adapted from Felipe (2000), Arquero et al. (2013), and Miarnau et al. (2016).

[b] Blooming and ripening time: early, medium, late, and very late.

[c] Vigor and branching density: low, medium, high, and very high.

RESULTS

Laboratory evaluation. Inoculation of twigs of 25 almond cultivars with four *D. amygdali* isolates resulted in necrotic lesions and canker development in all inoculated twigs of all cultivar and isolate combinations. Lesions were variable in length depending on the cultivar studied and the isolate used (Figure 4.1). The uninoculated controls did not show any measurable lesion and the fungus was not reisolated in any case. Therefore, lesion length data for non-inoculated controls are not included in Figure 4.1.

The significance of the interaction between cultivar and isolate factors ($P \leq 0.01$) was confirmed through a two-way ANOVA on the whole dataset (results not shown). Therefore, one-way ANOVA analyses were conducted separately for each isolate. ANOVA results indicated that significant differences ($P \leq 0.05$) in mean lesion lengths among cultivars were detected for each isolate. Mean lesion lengths ranged from 7 cm in 'Ferragnès' inoculated with isolate DAL-138 to 24 cm in 'Penta' inoculated with isolate DAL-34. Some cultivars, such as 'Soleta' and 'Penta', usually showed longer mean lesions with the four isolates of *D. amygdali*. In contrast, 'Desmayo Largueta' usually showed shorter lesions. Regarding the mean lesion length caused by each isolate, the minimum mean lesion value recorded for DAL-4 was 11.6 cm in 'Ferragnès' and the maximum 23.6 cm in 'Constantí'. In the case of DAL-34, minimum and maximum mean lesion values were 9.7 cm and 24.0 cm, obtained in 'Marta' and 'Penta', respectively. In the case of DAL-138, minimum and maximum mean lesion values were 7.0 cm and 18.4 cm, obtained in 'Ferragnès' and 'Glorieta', respectively. Regarding DAL-174, minimum and maximum mean lesion values were 11.2 cm and 23.6 cm, obtained in 'Fritz' and 'Tardona', respectively.

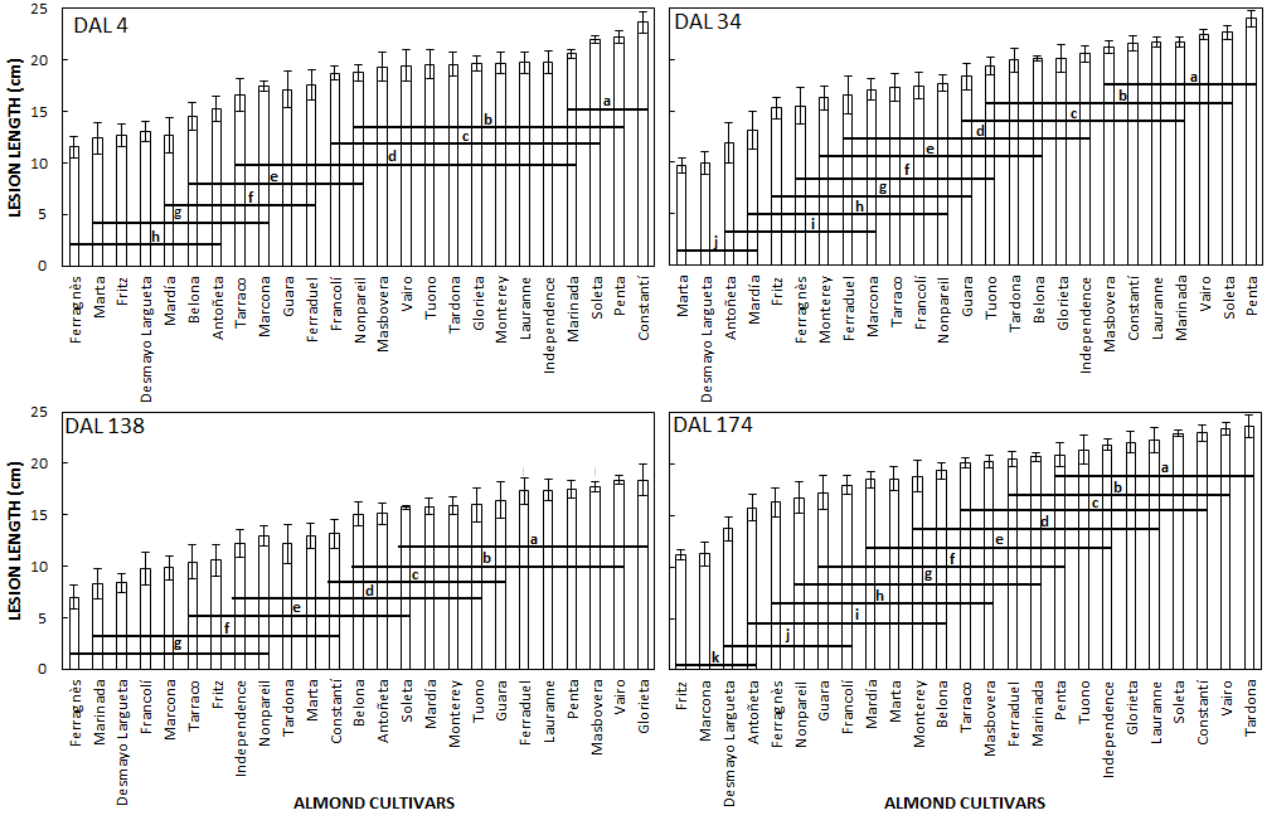

Figure 4.1 Mean lesion length caused by four isolates of *D. amygdali* (DAL-4, DAL-34, DAL-138 and DAL-174) on 25 almond cultivars 15 days after inoculation in laboratory conditions. The vertical bars represent the standard error of the mean. The letters in the horizontal bars indicate significant differences (LSD; $P \leq 0.05$) among the cultivar means.

Mean lesion lengths caused by four *D. amygdali* isolates in 25 almond cultivars grouped according to the four agronomic traits are shown in Figure 4.2 Regarding the effect of blooming time, in all *D. amygdali* isolates the lowest lesion lengths were obtained in the early-blooming cultivars whereas late-blooming cultivars showed longer lesions, although with no consistent differences between means across cultivars. Regarding the ripening time, the longest lesions were observed in early-ripening cultivars with a trend to decrease in late-ripening cultivars, with or without statistically significant differences depending on the isolate. In the case of vigor, the longest mean lesions were observed in the low vigor cultivars for isolates DAL-4, DAL-34 and DAL-174, with a general trend to decrease within the cultivars with higher vigor classes. In contrast, cultivars inoculated with isolate DAL-138 behaved the opposite to the other *D. amygdali* isolates, as low-vigor cultivars inoculated with DAL-138 showed shorter mean lesion values than the other groups. Finally, when the cultivars were grouped by branching density, no statistically significant differences among groups were found, except for isolate DAL-174, in which the cultivars with high branching density showed the shorter mean lesion value, statistically significant when compared to the rest of the groups.

Field evaluation. Mean lesion lengths caused by *D. amygdali* DAL-138 on 21 almond cultivars in field trials are shown in Figure 4.3 In general, a range of variation was found, being 'Tardona' the cultivar with the longest mean lesion length (5.41 cm), and 'Tarraco' the one with the smallest lesion length (4.03 cm). The remaining cultivars showed intermediate mean lesion lengths in a progressive trend (Figure 4.3).

According to the agronomic traits of blooming and ripening times, early-blooming cultivars showed the shortest lesions whereas early-ripening cultivars showed the longest lesions (Figure 4.4), as similarly observed in the laboratory trial. Regarding the vigor, the shortest mean lesion lengths were obtained in high vigor cultivars, but differences with the means of low and very high vigor cultivars were not statistically significant. Finally, no significant differences were detected among groups when cultivars were grouped according to the branching density.

Susceptibility groupings. Cluster analysis (Figure 4.5) separated the 21 evaluated cultivars into two well-defined different groups, which were statistically different according to Student's *t*-test comparisons between the mean lesion lengths of each group. These two groups were classified as very susceptible (longer lesions), which included 'Belona', 'Constantí', 'Ferraduel', 'Glorieta', 'Guara', 'Lauranne', 'Marinada', 'Masbovera', 'Penta', 'Soleta', 'Tardona', 'Tuono',

'Vairo', 'Francolí', and 'Tarraco'; and susceptible (shorter lesions), including 'Antoñeta', 'Desmayo Largueta', 'Ferragnès', 'Marcona', 'Mardía', and 'Marta'.

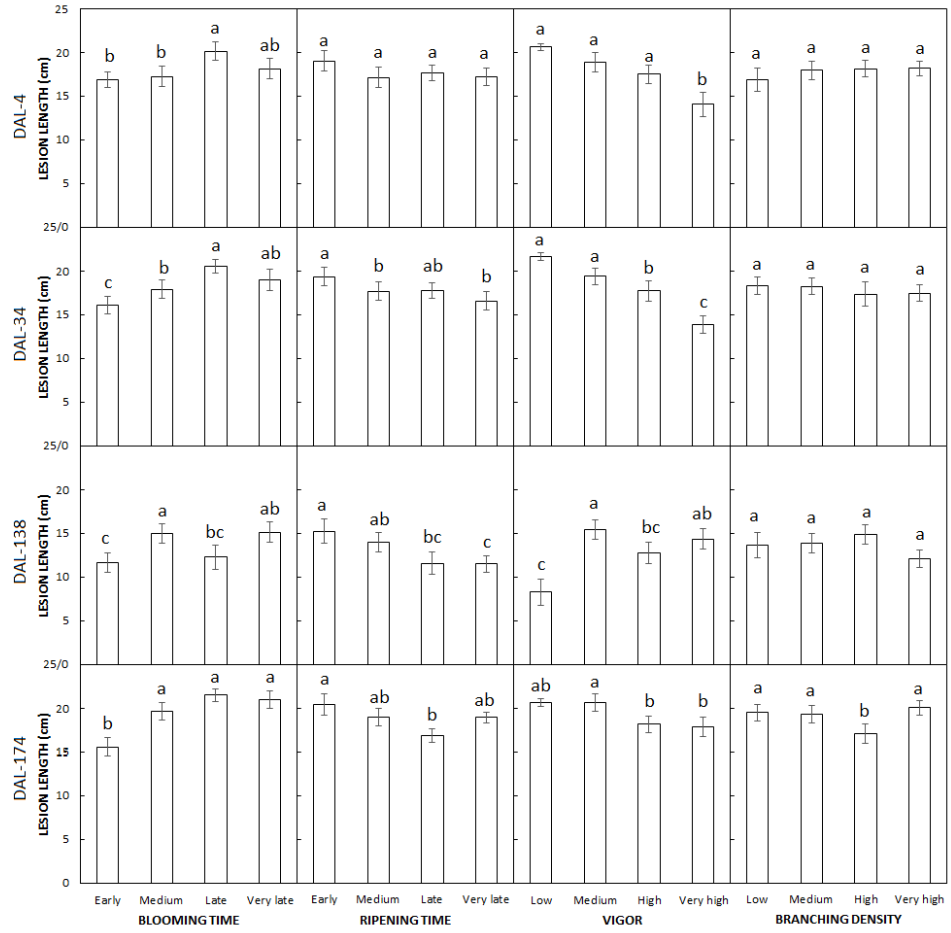

Figure 4.2 Mean lesion length caused by four isolates of *D. amygdali* (DAL-4, DAL-34, DAL-138 and DAL-174) on 25 almond cultivars 15 days after inoculation in laboratory conditions. Cultivars were grouped by blooming time, ripening time, vigor and branching density. The vertical bars represent the standard error of the mean. The letters indicate significant differences (LSD; $P \leq 0.05$) between the level means of each grouping factor.

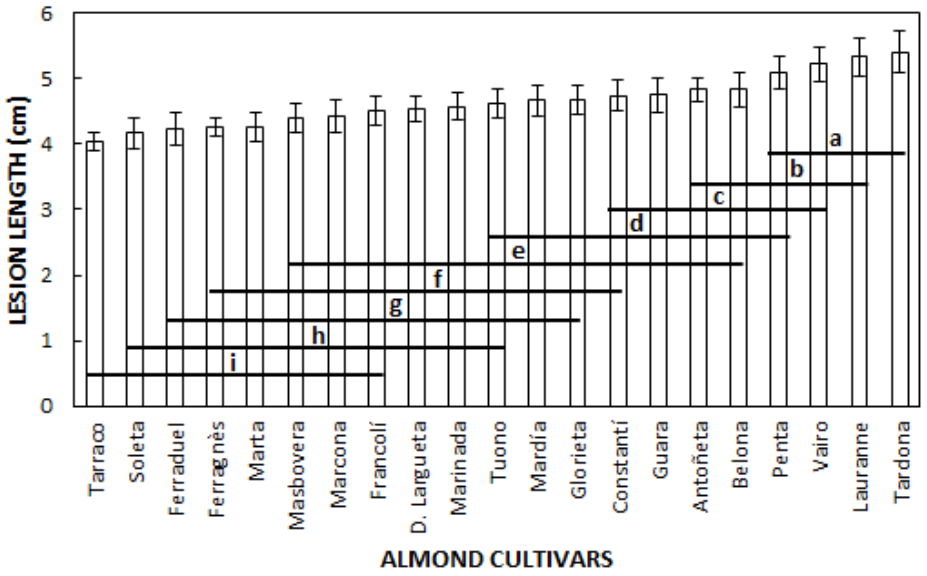

Figure 4.3 Mean lesion length caused by *D. amygdali* DAL-138 on 21 almond cultivars 3-4 weeks after inoculation in field conditions. The vertical bars represent the standard error of the mean. The horizontal bars with different letters indicate significant differences (LSD; $P<0,05$) among the cultivar means.

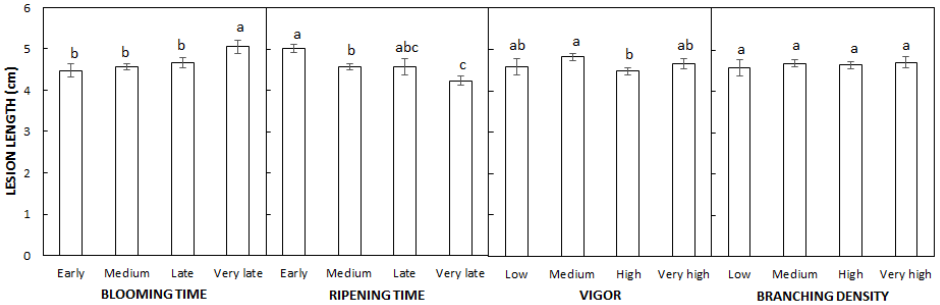

Figure 4.4 Mean lesion length caused by *D. amygdali* DAL-138 in 21 almond cultivars 3-4 weeks after inoculation in field conditions. Cultivars were grouped by blooming time, ripening time, vigor and branching density. The vertical bars represent the standard error of the mean. The letters indicate significant differences (LSD; $P\leq0.05$) between the level means of each grouping factor.

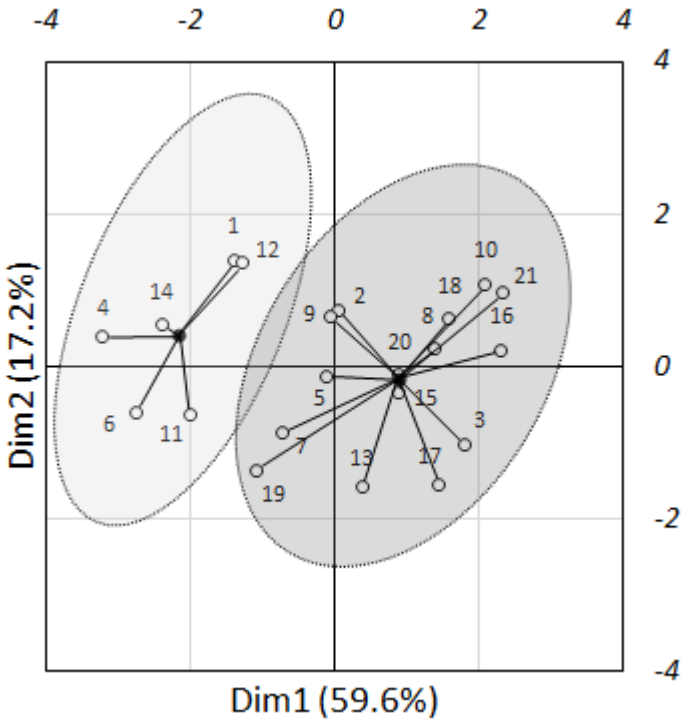

Figure 4.5 Cluster analysis of the mean lesion length caused by four *Diaporthe amygdali* isolates (DAL-4, DAL-34, DAL-138 and DAL-174) and one isolate (DAL-138) in laboratory and field experiments, respectively, on 21 almond cultivars: (1) 'Antoñeta', (2) 'Belona', (3) 'Constantí', (4) 'Desmayo Largueta', (5) 'Ferraduel', (6) 'Ferragnès', (7) 'Francolí', (8) 'Glorieta', (9) 'Guara', (10) 'Lauranne', (11) 'Marcona', (12) 'Mardía', (13) 'Marinada', (14) 'Marta', (15) 'Masbovera', (16) 'Penta', (17) 'Soleta', (18) 'Tardona', (19) 'Tarraco', (20) 'Tuono', and (21) 'Vairo'. Two categories of susceptibility were defined as follows: susceptible (light gray) and very susceptible (gray). Ellipses include the 95% confidence interval for the centroids (black solid dots).

DISCUSSION

Necrotic lesions and cankers observed in the inoculated almond cultivars both in laboratory and field tests coincided with those described as characteristic for twig canker and shoot blight disease caused by *D. amygdali* (Adaskaveg 2002; Diogo et al., 2010; León et al., 2020). Our results evidenced the

susceptibility of all the cultivars evaluated to all the inoculated isolates of *D. amygdali*. Lesion length measurements showed a wide range of variation among cultivars in all experiments. Moreover, in the laboratory evaluation there were differences in pathogenicity among *D. amygdali* isolates as previously reported by Diogo et al. (2010) and León et al. (2020), when these authors inoculated this pathogen on the cultivars 'Ferragnès' and 'Vairo', respectively.

Almond cultivars were grouped as susceptible or very susceptible according to a cluster analysis. It is interesting to remark that cultivars classified as very susceptible showed approximately a 30% increase in mean lesions length compared to those susceptible. We intentionally avoided the use of the concepts like tolerant or very tolerant when classifying cultivars for their susceptibility to *D. amygdali*, because we think that colonization of almond twig tissues by *D. amygdali* was biologically relevant among all cultivars. Nevertheless, the cultivar susceptibility/tolerance concept can be easily managed by farmers and agronomists if cultivars are placed into distinct ordinal classes (Pataky et al., 2011), and this was the goal of the cluster analysis used in this study.

Previous works had already studied the susceptibility of almond cultivars to *D. amygdali* (Besoain et al., 2000; Cabrita et al., 2004; Diogo et al., 2010; Vargas and Miarnau 2011; Varjas et al., 2017a), with some of them also included in our study. Besoain et al. (2000) evaluated the cultivar 'Nonpareil', which showed significant lesions when inoculated with *D. amygdali* on both non-lignified and semi-lignified almond tissues, thus being considered as susceptible. These results agree with those obtained in our study, which confirm an intermediate susceptibility of 'Nonpareil' for all *D. amygdali* isolates. Later, Cabrita et al. (2004), evaluated the susceptibility of the Portuguese 'Barrinho Grado' and the French 'Ferragnès' cultivars to *D. amygdali*, showing that 'Ferragnès' was more susceptible than the Portuguese cultivar because it showed longer lesions in artificially inoculated twigs, in inoculations with either mycelium plugs or conidial suspensions. Diogo et al. (2010) confirmed the susceptibility of 'Ferragnès' to *D. amygdali*, when they compared the lesions caused by this fungus with those caused by *D. foeniculina* (syn. *D. neotheicola* A.J.L. Phillips & J.M. Santos), being the mean length of lesions of the first species significantly longer. Similar results were obtained in our studies, in which the cultivar 'Ferragnès' showed considerable lesions in both laboratory and field tests. In Spain, Vargas and Miarnau (2011) established five categories of susceptibility among 70 almond cultivars after conducting a study on naturally-infected trees. The cultivars ranged from very susceptible for the Spanish cultivars 'Desmayo Largueta' and 'Marcona', and the French ones 'Ferragnès' and 'Lauranne', to very tolerant for the cultivars 'Masbovera' and 'Tarraco'. This is in contrast with our results, in

which these last two cultivars were considered very susceptible. The other cultivars included in the evaluation of Vargas and Miarnau (2011) had intermediate susceptibility ranges; for instance, 'Antoñeta' and 'Marta' resulted susceptible, in agreement with our results. Data regarding the high susceptibility of 'Lauranne' to *D. amygdali* reported by Vargas and Miarnau (2011) are also consistent with our results. It is also important to note that cultivars 'Ferraduel', 'Glorieta', 'Marinada', 'Masbovera', 'Nonpareil', 'Tarraco', and 'Vairo' evaluated in this study did not exactly match the susceptibility range assigned by Vargas and Miarnau (2011) (i.e., medium to very tolerant).

Some disagreements in cultivar susceptibility among different evaluation studies can be due to the type of inoculation (artificial vs. natural). In artificial inoculations some natural barriers from the cultivar are eliminated, with the wounds facilitating the introduction of the pathogen. In contrast, each cultivar can behave differently in response to the pathogen penetration under natural conditions. For instance, Mathew et al. (2018) compared different inoculation methods to study the aggressiveness of *D. helianthi* Munt.-Cvetk., Mihaljč. & M. Petrov isolates causing Phomopsis stem canker of sunflower. These authors found a significant interaction between inoculation methods and isolates, confirming that the inoculation method influenced the disease caused by *D. helianthi*, and pointed out that although inoculation by mycelial plugs has many advantages, such as the efficiency to detect significant differences in the severity of the disease, and the efficient use of space and the time required to inoculate the plants, it does not replicate the natural infection process by *Diaporthe* spp. Ghimire et al. (2019), stated that inoculation methods have a significant impact on the development of symptoms caused by some *Diaporthe* species on soybean, indicating that wound-based inoculation methods resulted in the greatest disease severity ratings.

Regarding the relationship between agronomic traits and cultivar susceptibility, blooming and ripening times were found relevant variables to explain cultivars performance related to *Diaporthe* dieback susceptibility. Late and very late blooming, and early and medium ripening cultivars, such as 'Constantí', 'Lauranne', 'Penta', and 'Tardona' were highly susceptible to *D. amygdali*. These later cultivars are releases from different breeding programs which share late blooming and early ripening time as two major desired goals (Batlle et al., 2017), but these selected characters seem to be related to a higher susceptibility to *D. amygdali*. Moreover, these four cultivars have been obtained from crosses of 'Tuono' (Pérez de los Cobos et al., 2021), an Italian cultivar classified as susceptible in our study and also in previous ones (Martins et al., 2005; Vargas and Miarnau, 2011).

It is generally agreed that vigor of an organism and its susceptibility to disease are antithetic variables, meaning that one increases as the other diminishes, and also that cultural practices aiming at improving the vigor of the plant often help increase its tolerance to pathogens (Agrios 2005; Raines 1922). This is in agreement with our results because, in general, we observed longest lesions in low vigor cultivars although, in the particular case of the laboratory experiment, this was depending on the inoculated isolate. To the best of our knowledge, very few studies have addressed the influence of agronomic traits on the disease tolerance of fruit tree cultivars to dieback diseases. Willingham et al. (2004) reported a contradictory observation: avocado (*Persea americana* Mill.) fruits from non-vigorous tress affected by root rot pathogens were less susceptible to anthracnose caused by *C. gloeosporioides* (Penz.) Penz. & Sacc. than the fruits from healthy vigorous trees. This was related to a 40% increase in the concentration of calcium (Ca) in the flesh of fruits from non-vigorous trees, but their size makes them unmarketable. In our case, the relationship of blooming and ripening times, and vigor with an eventual increased susceptibility of almond cultivars to *D. amygdali* remains to be further investigated.

Information about the susceptibility of almond cultivars to different fungal pathogens could assist in ongoing breeding programs of this crop, in order to achieve simultaneous tolerance to several economically important fungal pathogens. But certainly, it is in short term when the information generated in this study can be very valuable by selecting less susceptible almond cultivars to *Diaporthe* spp. for the new almond orchard plantations and, specifically, in the Iberian Peninsula.

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References

Adaskaveg, J., Förster, H. and Connell, J. 1999. First report of fruit rot and associated branch dieback of almond in California caused by a *Phomopsis* species tentatively identified as *P. amygdali*. Plant Dis. 83:1073-1073.

Adaskaveg, J. 2002. Phomopsis canker and fruit rot. In: Compendium of nut crop diseases in temperate zones. Teviotdale, B., Michailides, T., Pscheidt, J., Eds.; APS Press: St. Paul, MN, USA, 2002; pp. 27–28.

Agrios, G. 2005. Plant Pathology 5th Ed. Elsewier Academic Press, Burlington, MA 01803, USA: 922 pp.

Arquero, O., Belmonte, A., Casado, B., Cruz, M., Espadafor, M., Fernández, J., Gallego, J., García, A., Lorite, I., Lovera, M., Parra, M., Ramírez, A., Roca, L., Romacho, F., Romero, J., Salguero, A., Santos, C., Serrano, N., Trapero, A., Urquiza, F. and Viñas, M. 2013. Manual del almendro. Consejería de Agricultura, Pesca y Desarrollo Rural. Junta de Andalucía. Sevilla; 78 pp.

Batlle, I., Dicenta, F., Socias i Company, R., Gradziel, T., Wirthensohn, M., Duval, H. and Vargas, F. 2017. Classical genetics and breeding. In: Almonds, botany, production and uses; R. Socias i Company and T. M. Gradziel, Eds.; CAB International, Boston, MA; pp. 111–148.

Besoain, X., Briceno, E. and Piontelli, E. 2000. *Fusicoccum* sp. as the cause of canker in almond trees and susceptibility of three cultivars. Fitopatología 35:176-182.

BOE, 2002. Real decreto 1201/2002, de 20 de noviembre, por el que se regula la producción integrada de productos agrícolas. Boletin Oficial del Estado, Madrid.. Available online <https://www.boe.es/eli/es/rd/2002/11/20/1201> (accessed on 15 June 2021).

Cabrita, L., Neves, A. and Leitão, J. 2004. Evaluation of resistance to *Phomopsis amygdali* in almond. In Proceedings of the XIth Eucarpia symposium on fruit breeding and genetics, Vols 1 and 2 (Vol. 663, pp. 235-238). International Society for Horticultural Science.

Charrad, M., Ghazzali, N., Boiteau, V. and Niknafs, A. 2014. NbClust: An R package for determining the relevant number of clusters in a data set. J. Stat. Softw. 61:1-36.

Dicenta, F., Ortega, E., Martínez-Gómez, P., Sánchez-Pérez, R., Martínez-García, P., Cremades, T., Gambín, M. and Egea, J. 2008. Almond breeding programme in CEBAS–CSIC, in Murcia (Spain). XIV GREMPA Meeting, Athens, Greece, 30 March-4 April, 2008. Book of abstracts: 20.

Dicenta, F., Sánchez-Pérez, R., Rubio, M., Egea, J., Batlle, I., Miarnau, X., Palasciano, M., Lipari, E., Confolent, C., Martínez, P. and Duval, H. 2015. The origin of the self-compatible almond 'Guara'. Sci. Hortic. 197:1-4.

Dicenta, F., Cremades, T., Martínez-García, P., Martínez-Gómez, P., Ortega, E., Rubio, M., Sánchez-Pérez, R., López-Alcolea, J. and Egea, J. 2018. 'Penta' and 'Makako': two extra-late flowering self-compatible almond cultivars from CEBAS-CSIC. HortScience 53:1700-1702.

Diéguez-Uribeondo, J., Förster, H. and Adaskaveg, J. 2011. Effect of wetness duration and temperature on the development of anthracnose on selected almond tissues and comparison of cultivar susceptibility. Phytopathology 101:1013-1020.

Diogo, E., Santos, J. and Phillips, A. 2010. Phylogeny, morphology and pathogenicity of *Diaporthe* and *Phomopsis* species on almond in Portugal. Fungal Divers. 44:107-115.

Egea, L., García, J., Egea, J. and Berenguer, T. 1984. Premières observations sur une collection de 81 variétés d'amandiers située dans le sud-est espagnol. Options Mediterraneennes 84:13-25.

Egea, J., Dicenta, F., Berenguer, T. and García, J. 2000. 'Antoñeta' and 'Marta' almonds. HortScience 35:1358-1359.

Felipe, A. and Socias i Company, R. 1987. 'Aylés', 'Guara' and 'Moncayo' almonds. HortScience 22, 961-962.

Felipe, A. 2000. El almendro: El material vegetal. Mira Editores, S.A. Zaragoza, Spain.

FAOSTAT, Food and Agriculture Organization of the United Nations. 2021. Available online: <http://www.fao.org/faostat/en/#home> (accessed on 15 June 2021).

Ghimire, K., Petrović, K., Kontz, B., Bradley, C., Chilvers, M., Mueller, D., Smith, D., Wise, K. and Mathew, F. 2019. Inoculation method impacts symptom development associated with *Diaporthe aspalathi, D. caulivora*, and *D. longicolla* on soybean (*Glycine max*). Plant Dis. 103:677-684.

Gradziel, T. and Wang, D. 1994. Susceptibility of California almond cultivars to aflatoxigenic *Aspergillus flavus*. HortScience 29:33-35.

Gradziel, T; Curtis, R. and Socias i Company, R. 2017. Production and growing regions. In: Almonds, botany, production and uses. R. Socias i Company and T. M. Gradziel, eds. CAB International, Boston, MA. pp 70–86.

Gramaje D., Agustí-Brisach C., Pérez-Sierra A., Moralejo E., Olmo D., Mostert L., Damm U. and Armengol J. 2012. Fungal trunk pathogens associated with wood decay of almond trees on Mallorca (Spain). Persoonia 28:1-13.

Grasselly, C. 1991. Avijor 'Lauranne'. L'Arboriculture Fruitière 436:75.

Grasselly, C. and Duval, H. 1997. L'Amandier. CTFIL, Paris, France.

Hilário, S., Santos, L. and Alves, A. 2021. *Diaporthe amygdali*, a species complex or a complex species?. Fungal Biol. 125:505-518.

Holland, L., Trouillas, F., Nouri, M., Lawrence, D., Crespo, M., Doll, D., Duncan R., Holtz B., Culumber C., Yaghmour M., Niederholzer F., Lightle D., Jarvis-Shean K., Gordon P. and Fichtner, E. 2021. Fungal pathogens associated with canker diseases of almond in California. Plant Dis. 105:346-360.

Horsfield, A. and Wicks, T. 2014. Susceptibility of almond cultivars to *Tranzschelia discolor*. Australas. Plant Pathol. 43:79-87.

International Nut and Dried Fruit Council (INC). 2020. Nuts & dried fruits: statistical Yearbook 2019/2020. International Nut and Dried Fruit Council. Available on line <https://www.nutfruit.org/files/tech/1587539172_INC_Statistical_Yearbook_2019-2020.pdf>. (accessed on 01 January 2021)

Kalita, S., Khandelwal, S., Madan, J., Pandya, H., Sesikeran, B. and Krishnaswamy, K. 2018. Almonds and cardiovascular health: A review. Nutrients 10:468.

Kassambara, A. and Mundt, F. 2020. factoextra: Extract and visualize the results of multivariate data analyses. R package version 1.0.7. Available online: <https://CRAN.R-project.org/package=factoextra> (accessed on 10 april 2021).

Kaufman, L. and Rousseeuw, P. 2009. Finding groups in data: An introduction to cluster analysis. Wiley Series in probability and statistics, Vol. 344. John Wiley & Sons, Hoboken, New Jersey, USA. 342 pp.

León, M., Berbegal, M., Rodríguez-Reina, J. M., Elena, G., Abad-Campos, P., Ramón-Albalat, A., Olmo, D., Vicent, A., Luque, J., Miarnau, X., Agustí-Brisach, C., Trapero, A., Capote, N., Arroyo, F., Avilés, M., Gramaje, D., Andrés-Sodupe, M. and Armengol, J. 2020. Identification and characterization of *Diaporthe* spp. associated with twig cankers and shoot blight of almonds in Spain. Agron. 10:1062.

López-Moral, A., Agustí-Brisach, C., Lovera, M., Luque, F., Roca, L., Arquero, O. and Trapero, A. 2019. Effects of cultivar susceptibility, fruit maturity, leaf age, fungal isolate, and temperature on infection of almond by *Colletotrichum* spp. Plant Dis. 103:2425-2432.

Maldonado, M., Torguet, L., Girabet, R., Zazurca, L., Martinez, G. and Miarnau, X. 2019. Nuevos modelos productivos para la intensificación del almendro. Vida Rural 472:44-49.

MAPA Anuario de Estadística Agraria. 2020. Available online <https://www.mapa.gob.es/es/estadistica/temas/estadisticas-grarias/agricultura/superficies-producciones-anuales-cultivos/> (accessed on 15 June 2021)

Martins, M., Sarmento, D., Batlle, I., Vargas, F. and Oliveira, M. 2005. Development of SCAR/CAPS markers linked to tolerance/sensitivity to *Fusicoccum* in almond. Options Méditerranéennes CIHEAM/IAMZ 63:187-191.

Mathew, F., Jordahl, J., Gulya, T. and Markell, S. 2018. Comparison of greenhouse-based inoculation methods to study aggressiveness of *Diaporthe helianthi* isolates causing Phomopsis stem canker of sunflower (*Helianthus annuus*). Plant Health Prog. 19:92-96.

Miarnau, X., Torguet, L., Batlle, I., Romero, A., Rovira, M. and Alegre, S. 2016. Comportamiento agronómico y productivo de las nuevas variedades de almendro. Revista de Fruticultura 49:42-59.

Miarnau, X., Zazurca, L., Torguet, L., Zúñiga, E., Batlle, I., Alegre, S. and Luque, J. 2021. Cultivar susceptibility and environmental parameters affecting symptom expression of red leaf blotch of almond in Spain. Plant Dis. 105:940-947.

Ollero-Lara, A., Agustí-Brisach, C., Lovera, M., Roca, L., Arquero, O. and Trapero, A. 2019. Field susceptibility of almond cultivars to the four most common aerial fungal diseases in southern Spain. Crop Prot. 121:18-27.

Olmo, D., Armengol, J., León, M. and Gramaje, D. 2016. Characterization and pathogenicity of Botryosphaeriaceae species isolated from almond trees on the island of Mallorca (Spain). Plant Dis. 100:2483-2491.

Pataky, J., Williams, M., Headrick, J., Nankam, C., Du Toit, L. and Michener, P. 2011. Observations from a quarter century of evaluating reactions of sweet corn hybrids in disease nurseries. Plant Dis. 95:1402-1506.

Pérez de los Cobos, F., Martínez-García, Pedro J.; Romero, A., Miarnau, X., Eduardo I., Howad, W., Mnejja, M., Dicenta, F., Socias i Company, R., Gradziel, T., Whirthensohn, M., Duval, H., Holland, D., Arús, P., Vargas, F. and Batlle, I. 2021. Pedigree analysis of 220 almond genotypes reveals two world mainstream breeding lines based on only three different cultivars. Hortic. Res. 8:1-11.

R Core Team. 2021. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available online <https://www.R-project.org/> (accessed on 15 June 2021)

Raines M. 1922. Vegetative vigor of the host as a factor influencing susceptibility and resistance to certain rust diseases of the higher plants. Am. J. Bot. 9:183-203.

Socias i Company, R. and Felipe, A. 2006. 'Belona' and 'Soleta', two new autogamous almonds. Nucis 13:9-12.

Socias i Company, R., Kodad, O., Alonso, J. and Felipe, A. 2008. 'Mardía' almond. HortScience 43:2240-2242.

Teviotdale, B., Michailides, T. and Pscheidt, J. 2002. Compendium of nut crop diseases in temperate zones. American Phytopathological Society. St. Paul, MN.

Torguet, L., Maldonado, M. and Miarnau, X. 2019. Importancia y control de las enfermedades en el cultivo del almendro. Agricultura: Revista Agropecuaria y Ganadera 1026:72-77.

Vargas, F. and Miarnau, X. 2011. Field susceptibility to fusicoccum canker of almond cultivars. Acta Hortic. 912:751-755.

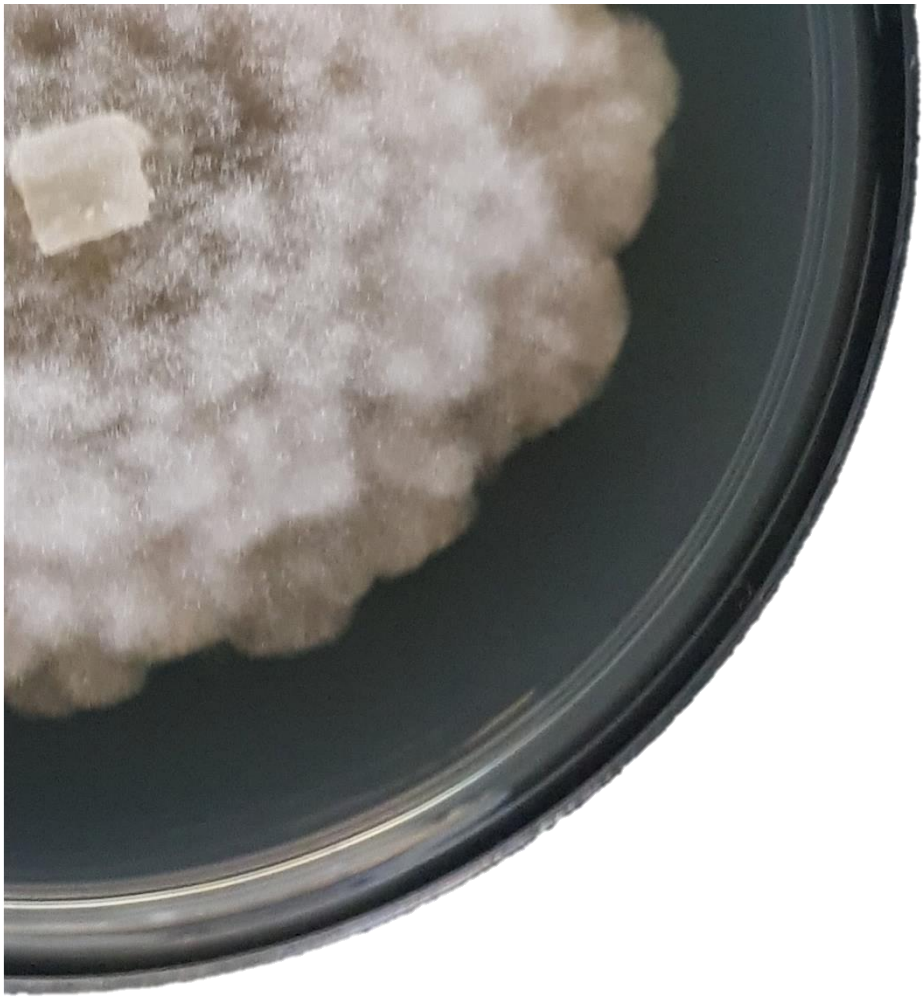
Vargas F. and Romero, M. 1994. 'Masbovera', 'Glorieta', and 'Francolí', three new almond varieties from IRTA. Acta Hortic. 373:75-82.

Vargas, F., Romero, M., Clavé, J., Vergés, J., Santos, J. and Batlle, I. 2008. 'Vayro', 'Marinada', 'Constantí' and 'Tarraco' almonds. HortScience 43:535-537.

Varjas, V., Izsépi, F., Nagy, G., Pájtli, É. and Vajna, L. 2017a. Phomopsis blight on almond in Hungary (pathogen: *Phomopsis amygdali*, teleomorph: *Diaporthe amygdali*). Növényvédelem 53:532-538 (In Hungarian).

Varjas, V., Vajna, L., Izsépi, F., Nagy, G. and Pájtli, É. 2017b. First report of *Phomopsis amygdali* causing twig canker on almond in Hungary. Plant Dis. 101:1674.

Willingham, S., Coates, L., Cooke, A. and Dean, J. 2004. Tree vigour influences disease susceptibility of 'Hass' avocado fruits. Australas. Plant Pathol. 33:17-21.



Chapter 5

Chapter 5

Survey of oomycetes associated with root and crown rot of almond in Spain and pathogenicity of *Phytophthora niederhauserii* and *Phytopythium vexans* to 'Garnem' rootstock

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Abstract. From 2018 to 2020, surveys of oomycetes associated with root and crown rot of almond (*Prunus dulcis*) were conducted on diseased young almond trees in commercial orchards and nurseries in six provinces of Spain. A total of 104 oomycete isolates were obtained from plant and soil samples, which were identified by sequencing the internal transcribed spacer (ITS) region of the ribosomal DNA. Diverse species belonging to the genera *Globisporangium*, *Phytophthora*, *Phytopythium* and *Pythium* were found, *Phytopythium vexans* and *Phytophthora niederhauserii* being the most frequent. The pathogenicity of these two species to one-year-old almond seedlings of 'Garnem' (*P. dulcis* x *P. persica*) rootstock was studied. All seedlings inoculated with *Pp. vexans* and *Ph. niederhauserii* isolates showed severe symptoms at the late stage of the pathogenicity test (defoliation, wilting and dieback) and several plants died. Some isolates of *Ph. niederhauserii* significantly reduced the dry weight of the roots compared with the control, but this effect was not observed in seedlings inoculated with *Pp. vexans*. These results provide new information about the oomycete species present in almond crops in Spain and highlight the importance of carrying out frequent phytosanitary surveys for a better knowledge of potential risks posed by these soil-borne pathogens.

INTRODUCTION

In the Mediterranean area, almond crops (*Prunus dulcis* (Miller) D.A. Webb. syn. *Prunus amygdalus* Batsch) are cultivated mainly in marginal soils and rainfed conditions, quite the opposite to what happens in the USA and Australia, the two major almond producers (Gradziel et al., 2017), where almond crop is grown under irrigation conditions. Specifically, in Spain, this crop has been traditionally planted throughout the Mediterranean coastline, where lighter and poor soils are found and it has been cultivated mainly in dry conditions (Felipe, 2000). It was not until about the 2000s that almond crops began to be planted in irrigated lands in new Spanish cropping areas, with heavier and clayey soils, until then used to grow other fruit trees or extensive crops (Miarnau et al., 2016).

In this new Spanish almond crop paradigm, some already known diseases and new ones have emerged, mainly soil-borne diseases (Torguet et al., 2019). The incorporation of irrigation systems together with the exploration of diverse soil types (previously destined for other uses) has increased the root system problems related to waterlogging cases associated with root asphyxia and the proliferation of certain root and crown diseases. In addition, the 'GF-677' and 'Garnem' rootstocks, both hybrids of *P. dulcis* x *P. persica*, used massively up to now in almond crops mainly in irrigated orchards but also in dry crop conditions, are susceptible to soil-borne diseases (Rubio-Cabetas et al., 2017). New hybrid rootstocks obtained from crosses of almond or peach with plum species have appeared in recent years to avoid these problems (Iglesias and Carbó, 2006; Pinochet, 2010; Rubio-Cabetas et al., 2017). These new materials have certain tolerance or resistance to soil-borne diseases, but it is important to note that the area planted with them is still reduced.

Almond trees can be affected by several soil-borne pathogens, such as *Armillaria mellea* (Vahl: Fr.) P. Kumm*., Rosellinia necatrix* Prill. and some oomycetes (Teviotdale et al., 2002). Diseases caused by oomycetes present in the soil can affect the crown and the roots of almond trees in both the nursery and established orchards. Multiple routes can spread out these diseases from the nurseries with infected plant material and infested substrate. Once established in the orchard, they can spread rapidly through cultural practices, plant debris, or through irrigation water (Pérez-Sierra et al., 2012). Plant pathogenic oomycetes have evolved large arsenals of secreted proteins, termed effectors, that act as weapons in order to successfully infect plants, which respond by multiple defense actions, including the strengthening of physical barriers, production of antimicrobial molecules and programmed cell death. Oomycetes can counteract the plant's immunity mechanisms, interfering or

suppressing numerous biochemical signaling components, disabling the host's defense actions, thus managing to invade its tissues (Wang et al., 2019).

Pinto de Torres and Mircetich (1976) reported, in Chile, the presence of cankers caused by *Globisporangium ultimum* at the base of the trunk in 2-year-old almond trees. In Iran, Azizi et al. (2013) studied the pathogenicity towards almonds of *Pythium* (*Py*.) (*sensu lato*) and *Phytophthora* (*Ph*.) isolates obtained from soil and roots of young almond trees. These authors identified *Ph. cactorum*, *Py. aphanidermatum* and *Phytopythium* (*Pp*.) *helicoides* as pathogenic species for this host, causing death in almond seedlings. Later, in this same country, Javadi and Sharifnabi (2016) reported *Pp. litorale* as the pathogen causing root and crown rot of almond trees. This information was confirmed by Browne et al. (2019), who indicated that diverse *Pythium* and *Phytopythium* species have been associated with the suppression of growth in the replanting of *Prunus* orchards grafted onto 'Nemaguard' rootstock, but their pathogenicity to almond or peach rootstocks was only established for *G. ultimum* var. *ultimum* and *G. irregulare*.

Many authors have reported diverse species of the genus *Phytophthora* causing root infection on almond rootstocks. Browne et al. (2015) indicated that more than 10 species of *Phytophthora* attack almond trees, causing root rot and cankers on the root crown, trunk, or scaffolds. Earlier works by Wicks and Lee (1986) and Wicks (1989) in Australia reported the isolation of *Ph. cambivora*, *Ph. citrophthora*, *Ph. cryptogea* and *Ph. megasperma* from either crown cankers or the soil around the crown of declining almond trees. These authors were only able to test the pathogenicity of *Ph. cambivora* in almond seedlings grown in artificially infested soil. Later, Browne and Viveros (1997) reported *Ph. megasperma*, *Ph. cactorum* and *Ph. citricola* associated with crown rot on dying almond trees in California, which were often of nonbearing age. Subsequent studies in Iran confirmed the pathogenicity of *Ph. cactorum* in a local almond rootstock (Sahragard and banihashemi, 2006). In Spain, a new *Phytophthora* sp. associated with 2-year-old almond plants was detected in a Valencian nursery in 2007. Symptoms associated with this pathogen were chlorosis, wilting, cankers and profuse stem gumming (Pérez-Sierra et al., 2010). From the infected plant material, it was possible to isolate *Phytophthora* taxon *niederhauserii*, which was confirmed as the causal agent of the disease by means of a pathogenicity test. Later, Kurbetli and Değirmenci (2011) detected this pathogen for the first time, in almond orchards in Turkey. The description of this new species was completed by Abad et al. (2014), who established *Ph. niederhauserii* as its definitive name. This oomycete is capable of infecting at least 33 species from 25 different families in both agricultural ecosystems and ornamental plant nurseries (Abad et

al., 2014). Later, in 2015, *Ph. niederhauserii* was first detected, causing almond crown rot in California (Browne et al., 2015). Finally, more recent publications have reported symptoms of cankers and root and crown rot in almond trees, associated with *Ph. megasperma* (Kurbetli and Yilmaz, 2016) and *Ph. plurivora* (Çiftçi et al., 2016) in Turkey; and wilting and decreased vigor caused by *Ph. chlamydospora* in Turkey (Türkölmez et al., 2016) and California (Browne et al., 2020).

There is a lack of updated information about the diversity of oomycetes infecting almond trees in Spain. Thus, the objectives of this study are: (i) to survey, isolate and identify oomycetes from almond trees showing root and crown rot symptoms in the main producing areas of Spain; and (ii) to study the pathogenicity to 'Garnem' rootstock of the most frequent species found.

MATERIAL AND METHODS

Field and Nursery Survey and Sampling. From 2018 to 2020, diseased young almond trees, of 1–3 years old, showing the symptoms root necrosis and root rot, cankers at the crown area, gum exudates at the base of the trunk and general decline (leaf yellowing, defoliation and wilting) (Figure 5.1), were surveyed in commercial orchards and nursery fields in Spain. The sampling sites were selected in order to represent the main almond-growing regions and different environmental conditions. A total of 149 samples was collected in six provinces, Córdoba (4 samples), Huelva (n = 5), Lleida (n = 39), Sevilla (n = 9), Toledo (n = 10) and Valencia (n = 82) (Figure 5.2). Each sample was composed of the base of the trunk of the symptomatic tree, the entire root system and from 2 to 4 kg of adjacent soil.

Isolation of Oomycetes from Plant and Soil Samples. Samples were brought to the laboratory for analysis. The crown area and the roots were carefully washed under running tap water to rinse the oil away. Segments from the crown and primary and secondary roots showing symptoms of browning and necrosis were cut. These plant tissue fragments were disinfected with 70% ethanol for 30 s, then rinsed with sterile distilled water and allowed to dry on absorbent paper. Pieces from 1 to 2 mm long were cut in a laminar flow chamber and seeded in Petri dishes with Corn Meal Agar with Pimaricin + Ampicillin + Rifampicin + Pentachloronitrobenzene + Benomyl (CMA-PARPB) medium and CMA-PARPBH (CMA-PARPB corrected with 0,069 g/L of Hymexazol) and then incubated at 25 °C for 72 h (Jeffers and Martin, 1986). Pure cultures were obtained by transferring the mycelial growth of the margins of colonies to potato dextrose

agar medium (PDA; Biokar-Diagnostics, Beauvais, France) for their subsequent identification (Erwin and Ribeiro, 1996).

Figure 5.1 Symptoms observed in field and nursery samples of almond trees: (a) leaf yellowing; (b) canker and crown rot; (c) general decline; (d) gum exudates at the base of the trunk; (e) root and crown rot.

For the isolation of oomycetes from the soil, two trapping modalities were applied. For the first, the soil was placed in 10 × 30 × 10 cm plastic buckets, then moistened and saturated with distilled water. *Hedera helix* L., *Robinia pseudoacacia* L. and *Pittosporum tobira* (Thunb.) W.T. Aiton young leaves were added to the surface of the water and incubated in a growth chamber at 23 °C for

3–7 days until points of infection appeared (Linderman and Zeitoun, 1977). For the second, Granny Smith apples were used as baits. Four perpendicular perforations of 1 cm^2 in diameter were made around an apple previously disinfected with 96% alcohol. In each perforation, a previously moistened soil subsample was added and covered with a plastic tape, to maintain humidity. The apples were incubated at room temperature for 3–7 days (Erwin and Ribeiro, 1996). The leaves and apples that presented necrotic or watery lesions were cut into 1–2 mm pieces and seeded in Petri dishes with CMA-PARPB and CMA-PARPBH media, then incubated at 25 °C for one week, checking daily for the development of oomycete-like colonies. Pure cultures were transferred to PDA as described above. The oomycete isolates were stored in an incubator at 10 °C, in plates with oatmeal agar, in the dark, at Instituto Agroforestal Mediterráneo in Universitat Politècnica de València (IAM-UPV; Valencia, Spain).

Figure 5.2 Map showing the location of sampled provinces in Spain. The sampled provinces are highlighted in light gray.

DNA Isolation, Amplification and Sequencing of Oomycetes. Genomic DNA was obtained from a one-week-old pure culture of each sample to be analyzed,

grown on PDA at 25 °C in the dark. For DNA extraction, the method of Collado-Romero et al. (2006) was used, with a slight variation, whereby the mycelium was scraped with a sterile pipette tip and placed in a 0.2 mL polymerase chain reaction (PCR) tube with 20 µL of 25 mM NaOH at pH 12. The tubes with the samples were incubated in a PTC 200 thermal cycler (MJ Research Inc., Waltham, MA, USA) with a DNA denaturation program (100 °C for 15 min and 4 °C for 5 min). Subsequently, 40 µL of 40 mM Tris-HCl was added at pH 5. The ITS region of the ribosomal DNA of the isolates was amplified, using universal primers ITS-6 (5' GAA GGT GAA GTC GTA ACA AGG 3') (Cooke et al., 2000) and ITS-4 (5' TCC TCC GCT TAT TGA TATGC 3') (White et al., 1990). Each PCR tube contained water (13.3 µL), Canvax Buffer B (x) (2.5 µL), Canvax $MgCl_2$ (nM) (2.5 µL), Canvax dNTPs (nM) (2.5 µL), Canvax Horse Power Taq polymerase (U/µL) (0.2 µL), primers (1 µL of each) and genomic DNA (2 µL), for a total of 25 µL. PCR amplification was performed in the same thermocycler mentioned above, using the following program: initial denaturation of 1 cycle at 94 °C for 3 min; 35 cycles of denaturation, annealing and extension at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s, respectively; and a final amplification cycle at 72 °C for 10 min. The PCR products were electrophoresed (140 V) on 1.5% agarose gel (1.5% agarose dissolved in TAE buffer (Tris-acetate-EDTA) 40 mM Tris-acetate, 1 mM EDTA) 1X; the staining of nucleic acids was made with RedSafe (20,000×). To identify the size of the bands, a molecular marker (GeneRuler T.M. 100 bp Plus DNA Ladder; Thermo Scientific, Waltham, MA, USA) was loaded into the first well of the gel. The presence of the bands was observed with the aid of a UV light transilluminator. The PCR products were sent to Macrogen (Madrid, Spain) for sequencing. The sequences obtained were subjected to a search in NCBI BLAST (http: //https://blast.ncbi.nlm.nih.gov/Blast.cgi; access on 15 October 2021), identifying the isolates at the species level.

Pathogenicity Tests. The inoculum was prepared in 1 L glass flasks with a mix of 200 mL of vermiculite, 20 mL of oat grains and 175 mL of eight vegetables (V8) broth (200 mL/L of V8 juice, 800 mL/L of demineralized water and 2 g/L of $CaCO_3$) (Jung et al, 1996). The glass flasks were sterilized three times for 20 min at 120 °C in an autoclave.

The flasks were inoculated separately with four isolates of *Ph. niederhauserii* (PAL-21, PAL-62, PAL74 and PAL-100) and one isolate of *Pp. vexans* (PAL-98), previously grown on V8 media (V8A). Inoculated flaks were incubated for six weeks in the dark at room temperature (Pérez-Sierra et al.,

2013). After this time, the inoculum mixture was rinsed with demineralized water to remove excess nutrients, before inoculations.

The pathogenicity tests were conducted on one-year-old almond seedlings of 'Garnem' rootstock. Seedlings were selected based on morphological homogeneity and healthy appearance. For inoculation, 20 g of inoculum was added to plastic pots with 200 g of potting mix that contained peat, vermiculite and sand (1:1:1, v/v/v), autoclaved three times prior to use; then, the rootstocks were planted. Control plants were inoculated with a non-infested inoculum mixture. In total, 5 plants were inoculated for each isolate and control and the experiment was repeated. All plants were randomly distributed in a growth chamber under a 12 h photoperiod at 23 °C. All seedlings were watered the day before the inoculation. Immediately after inoculation, the seedlings were flooded for 48 h and the flooding was repeated every two weeks to stimulate the formation of zoosporangia, as previously described by Pérez-Sierra et al. (2013).

The experiment was completed 58 days after inoculation. Seedlings were uprooted and the root system was washed carefully under running water to remove the substrate. Re-isolations from seedlings were performed by plating symptomatic fine root fragments in CMA-PARPBH to confirm Koch's postulates.

Severity evaluations for each plant started ten days after inoculation; then, they were performed every four days until the end of the experiment. Severity was evaluated using the following scale: 0 = symptom-free plant; 1 = foliar chlorosis; 2 = wilting, dieback and defoliation; and 3 = dead plant (Jönson et al., 2003). With the severity values of each plant over time, the area under the disease-progress curve (AUDPC) was calculated by the trapezoidal integration method (Campbell and Madden, 1990).

The dry weight of the root biomass was measured. For this purpose, roots were separated from the main stem and shoots by cutting at the root crown, then placed into paper bags and dried for five days in an oven at 35 °C. The dry weight of the roots was recorded.

Data Analysis. An analysis of variance (ANOVA) was performed for the dry weight of the root biomass and AUDPC. The assumptions of normality and homoscedasticity of the ANOVA were previously verified. Those data that did not meet the assumptions were transformed to the reciprocal value (1/×). Mean values were compared using the least significant difference (LSD) test at the 99% confidence level. All calculations were performed using Statgraphics Centurion XVI (Statgraphics Technologies, Inc., The Plains, VA, USA).

RESULTS

Occurrence of Oomycetes in Almond Samples. A total of 104 oomycete isolates was obtained (Table 5.1). Lleida was the province in which the most isolates were obtained, 41, representing 39.4% of the total, followed by Valencia, with 29 (27.8%). Fewer isolates were obtained from the Huelva, Toledo, Sevilla and Córdoba provinces, having 12 (11.5%), 11 (10.5%), 7 (6.7%) and 4 (3.8%), respectively.

Table 5.1 Origin and GenBank accession numbers of *Globisporangium*, *Phytophthora*, *Phytopythium* and *Pythium* species recovered from almond tree samples showing root and crown rot symptoms in six provinces of Spain.

Species	Code	Origin	Source	Rootstock/Scion	Acc. No.
Globisporangium carolinianum	PAL-106	Valencia	Soil		MZ921970
G. carolinianum	PAL-13	Lleida	Soil		MZ921972
G. carolinianum	PAL-69	Valencia	Soil		MZ922023
G. echinulatum	PAL-44	Toledo	Soil		MZ921949
G. glomeratum	PAL-107	Valencia	Soil		MZ921971
G. glomeratum	PAL-14	Lleida	Soil		MZ921973
G. glomeratum	PAL-41	Lleida	Soil		MZ922001
G. heterothallicum	PAL-42	Toledo	Soil		MZ922002
G. hypogynum	PAL-68	Valencia	Soil		MZ921955
G. irregulare	PAL-45	Toledo	Soil		MZ922003
G. irregulare	PAL-46	Toledo	Soil		MZ922004
G. irregulare	PAL-47	Toledo	Soil		MZ922005
G. irregulare	PAL-48	Toledo	Soil		MZ922006
G. irregulare	PAL-49	Toledo	Soil		MZ922007
G. irregulare	PAL-51	Toledo	Soil		MZ922009
G. irregulare	PAL-52	Toledo	Soil		MZ922010
G. irregulare	PAL-90	Huelva	Soil		MZ922042
G. irregulare	PAL-91	Sevilla	Root	'Rootpac-40′/nd	MZ922043
G. middletonii	PAL-15	Lleida	Soil		MZ921965
G. speculum	PAL-50	Toledo	Soil		MZ921950
G. ultimum var. *ultimum*	PAL-17	Lleida	Soil		MZ921974
G. ultimum var. *ultimum*	PAL-58	Valencia	Root	'GF-677′/'Lauranne'	MZ922015
G. ultimum var. *ultimum*	PAL-61	Valencia	Root	'GF-677′/'Lauranne'	MZ922017
G. ultimum var. *ultimum*	PAL-63	Valencia	Soil		MZ922018
G. ultimum var. *ultimum*	PAL-79	Huelva	Soil		MZ922030
G. ultimum var. *ultimum*	PAL-84	Huelva	Soil		MZ922035
G. ultimum var. *ultimum*	PAL-92	Sevilla	Soil		MZ922044
G. ultimum var. *ultimum*	PAL-94	Sevilla	Soil		MZ922046
Phytophthora cactorum	PAL-10	Lleida	Soil		MZ921975
Ph. cactorum	PAL-12	Lleida	Soil		MZ921977

Continued Table 5.1

Species	Code	Origin	Source	Rootstock/Scion	Acc. No.
Ph. cactorum	PAL-24	Lleida	Soil		MZ921983
Ph. cactorum	PAL-28	Lleida	Soil		MZ921984
Ph. cactorum	PAL-35	Lleida	Soil		MZ921994
Ph. cactorum	PAL-38	Lleida	Soil		MZ921997
Ph. cactorum	PAL-4	Lleida	Soil		MZ921999
Ph. citrophthora	PAL-16	Lleida	Soil		MZ921978
Ph. citrophthora	PAL-18	Lleida	Soil		MZ921979
Ph. citrophthora	PAL-19	Lleida	Soil		MZ921980
Ph. citrophthora	PAL-2	Lleida	Soil		MZ921981
Ph. citrophthora	PAL-20	Lleida	Soil		MZ921982
Ph. humicola/condilina	PAL-5	Lleida	Soil		MZ922008
Ph. nicotianae	PAL-57	Valencia	Soil		MZ922014
Ph. nicotianae	PAL-71	Valencia	Soil		MZ922025
Ph. niederhauserii	PAL-11	Lleida	Soil		MZ921964
Ph. niederhauserii	PAL-34	Lleida	Soil		MZ921969
Ph. niederhauserii	PAL-60	Valencia	Root	'GF-677'/'Lauranne'	MZ921953
Ph. niederhauserii	PAL-62	Valencia	Root	'GF-677'/'Lauranne'	MZ921954
Ph. niederhauserii	PAL-1	Lleida	Soil		MZ921961
Ph. niederhauserii	PAL-100	Córdoba	Root	'GF-677'/nd	MZ921962
Ph. niederhauserii	PAL-105	Valencia	Root	'Garnem/'Lauranne'	MZ921963
Ph. niederhauserii	PAL-21	Lleida	Soil		MZ921966
Ph. niederhauserii	PAL-26	Lleida	Soil		MZ921967
Ph. niederhauserii	PAL-3	Lleida	Soil		MZ921968
Ph. niederhauserii	PAL-6	Lleida	Soil		MZ921952
Ph. niederhauserii	PAL-7	Lleida	Soil		MZ921956
Ph. niederhauserii	PAL-74	Valencia	Soil		MZ921957
Ph. niederhauserii	PAL-77	Valencia	Soil		MZ921958
Ph. niederhauserii	PAL-78	Valencia	Root	'GF-677'/nd	MZ921959
Ph. niederhauserii	PAL-8	Lleida	Soil		MZ921960
Ph. palmivora	PAL-66	Valencia	Soil		MZ922021
Ph. palmivora	PAL-72	Valencia	Soil		MZ922026
Ph. tropicalis	PAL-101	Córdoba	Soil		MZ921976
Phytopythium chamaehyphon	PAL-99	Córdoba	Soil		MZ922051
Pp. cucurbitacearum	PAL-53	Valencia	Root	'Garnem'/nd	MZ921951
Pp. helicoides	PAL-76	Valencia	Root	'Garnem'/'Penta'	MZ922029
Pp. helicoides	PAL-96	Sevilla	Root	'GF-677'/nd	MZ922048
Pp. mercuriale	PAL-86	Huelva	Soil		MZ922037
Pp. mercuriale	PAL-87	Huelva	Soil		MZ922038
Pp. vexans	PAL-22	Lleida	Soil		MZ921985
Pp. vexans	PAL-23	Lleida	Root	'GF-677'/'Constantí	MZ921986
Pp. vexans	PAL-25	Lleida	Root	'GF-677'/'Vairo'	MZ921987
Pp. vexans	PAL-27	Lleida	Root	'GF-677'/'Marinada'	MZ921988
Pp. vexans	PAL-29	Lleida	Root	'Garnem'/'Soleta'	MZ921989
Pp. vexans	PAL-32	Lleida	Soil		MZ921992
Pp. vexans	PAL-33	Lleida	Soil		MZ921993

Continued Table 5.1

Species	Code	Origin	Source	Rootstock/Scion	Acc. No.
Pp. vexans	PAL-36	Lleida	Soil		MZ921995
Pp. vexans	PAL-37	Lleida	Soil		MZ921996
Pp. vexans	PAL-40	Lleida	Soil		MZ922000
Pp. vexans	PAL-59	Valencia	Root	'GF-677'/'Lauranne'	MZ922016
Pp. vexans	PAL-70	Valencia	Soil		MZ922024
Pp. vexans	PAL-73	Valencia	Soil		MZ922027
Pp. vexans	PAL-75	Valencia	Soil		MZ922028
Pp. vexans	PAL-82	Huelva	Soil		MZ922033
Pp. vexans	PAL-83	Huelva	Soil		MZ922034
Pp. vexans	PAL-85	Huelva	Soil		MZ922036
Pp. vexans	PAL-88	Huelva	Soil		MZ922039
Pp. vexans	PAL-89	Huelva	Soil		MZ922040
Pp. vexans	PAL-9	Lleida	Soil		MZ922041
Pp. vexans	PAL-93	Sevilla	Soil		MZ922045
Pp. vexans	PAL-98	Córdoba	Root	'GF-677'/nd	MZ922050
Pythium aphanidermatum	PAL-39	Lleida	Soil		MZ921998
Py. dissotocum	PAL-30	Lleida	Soil		MZ921990
Py. dissotocum	PAL-31	Lleida	Soil		MZ921991
Py. dissotocum	PAL-54	Valencia	Soil		MZ922011
Py. dissotocum	PAL-55	Valencia	Soil		MZ922012
Py. dissotocum	PAL-65	Valencia	Soil		MZ922020
Py. dissotocum	PAL-80	Huelva	Soil		MZ922031
Py. dissotocum	PAL-81	Huelva	Soil		MZ922032
Py. dissotocum	PAL-95	Sevilla	Soil		MZ922047
Py. dissotocum	PAL-97	Sevilla	Soil		MZ922049
Py. nodosum	PAL-56	Valencia	Soil		MZ922013
Py. nodosum	PAL-64	Valencia	Soil		MZ922019
Py. nodosum	PAL-67	Valencia	Soil		MZ922022
Py. pachycaule	PAL-43	Toledo	Soil		MZ921948

The most frequent genus was *Phytophthora*, representing 32.7% of isolates, followed by *Globisporangium* and *Phytopythium* both with 26.9% and *Pythium* with 13.4% of the total isolates. Regarding the species frequency (Figure 5.3), the most frequent was *Pp. vexans*, which represented 21.2% of the isolates, followed by *Ph. niederhauserii* (15.4%). Less frequent species were *G. irregulare* and *Py. dissotocum* (8.7%), *G. ultimum* var. *ultimum* (7.7%), *Ph. cactorum* (6.7%) and *Ph. citrophthora* (4.8%). The remaining species had percentages below 3%. Regarding the source of isolation, 83.6% of the oomycetes were isolated from soil samples with baiting techniques and only 16.3% were isolated directly from the roots.

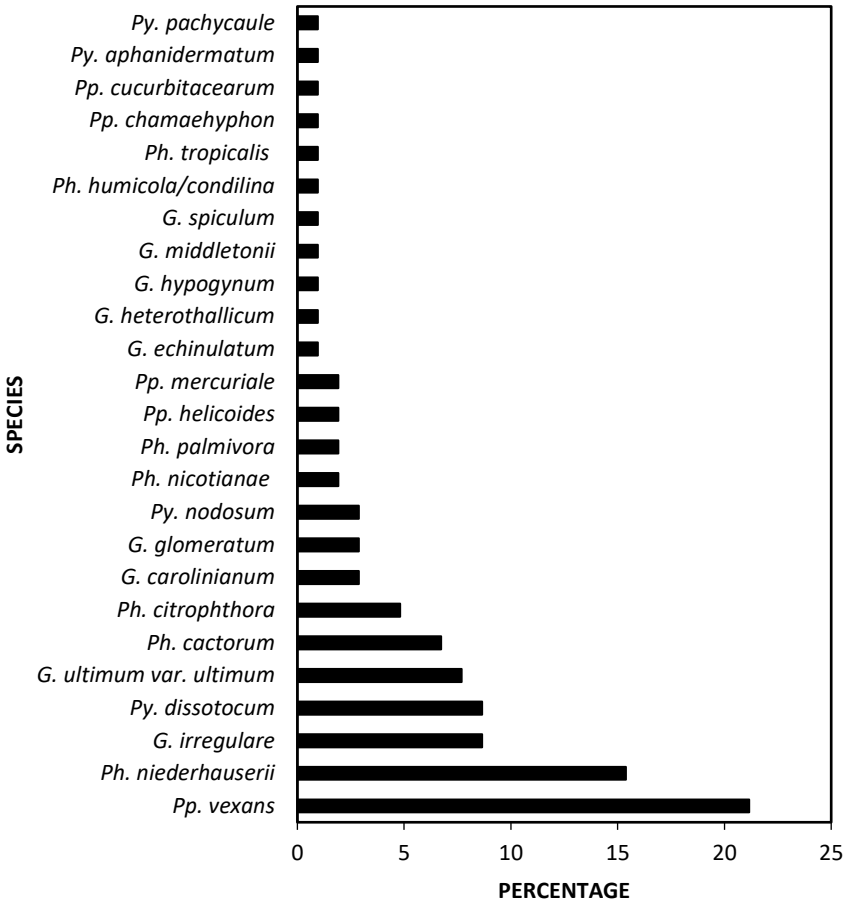

Figure 5.3 Frequency of occurrence of *Globisporangium*, *Phytophthora*, *Phytopythium* and *Pythium* species recovered from almond orchards showing root and crown rot symptoms in six provinces of Spain (n = 104).

Pathogenicity Tests. All 'Garnem' seedlings inoculated with *Ph. niederhauserii* and *Pp. vexans* isolates showed root symptoms (small dark necrotic lesions, root cankers, loss of fine roots and tap root rot), as well as aerial symptoms (chlorosis, wilting and defoliation), and some plants died. The re-isolations from symptomatic roots confirmed Koch's postulates. In contrast, control treatment seedlings remained healthy and it was not possible to re-isolate *Ph. niederhauserii* and *Pp. vexans* from the roots.

The severity of the infections and the AUDPC calculated for each isolate of *Ph. niederhauserii* and *Pp. vexans* are shown in Figures 5.4 and 5.5. In

general, the symptoms appeared earlier in the rootstocks inoculated with the isolate PAL-98 of *Pp. vexans* (10 days post-inoculation), where the disease progressed rapidly, reaching an AUDPC of 62.8 at day 58 after inoculation. The symptoms caused by isolates PAL-62, PAL-74 and PAL-100 of *Ph. niederhauserii* started on day 26 after inoculation for the three isolates, reaching an AUDPC value of 53, 64.6 and 62.8, respectively, at 58 days after inoculation. The latest isolate in the expression of symptoms was PAL-21, which started 30 days after inoculation, reaching a value of 40.4 of AUDPC, this being the lowest value obtained for all the inoculated isolates. The ANOVA analysis showed statistically significant differences in the AUDPC mean values ($P \leq 0.01$) between the PAL-21 isolate and the other inoculated species. There were no statistically significant differences in the AUDPC of the isolates PAL-62, PAL-74, PAL-98 and PAL-100.

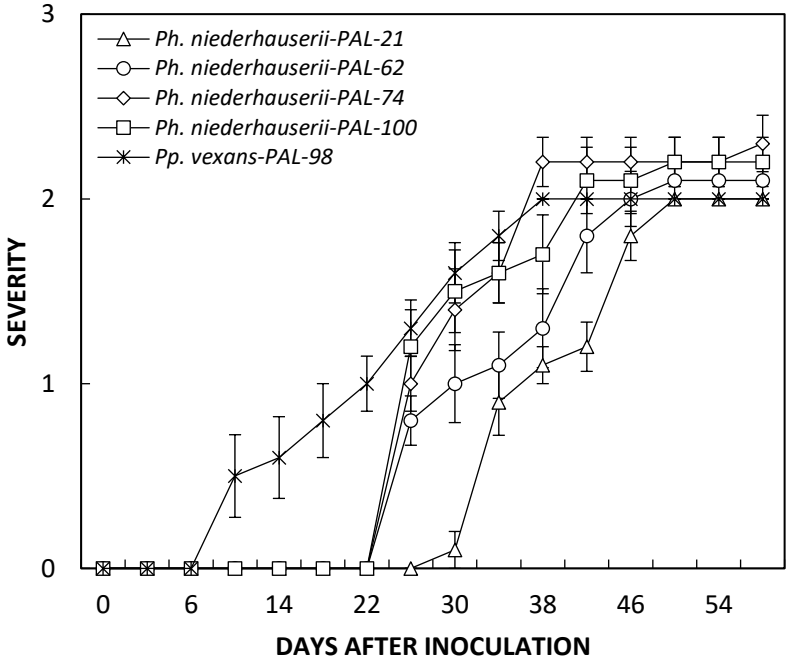

Figure 5.4 Results of severity of four isolates of *Ph. niederhauserii* and one of *Pp. vexans* to 'Garnem' rootstock 58 days after inoculation. Severity was evaluated using the following scale: 0 = symptom-free plant; 1 = foliar chlorosis; 2 = wilting, dieback and defoliation; and 3 = dead plant. Values are the mean of 10 almond seedlings. The vertical bars represent the standard error of the mean.

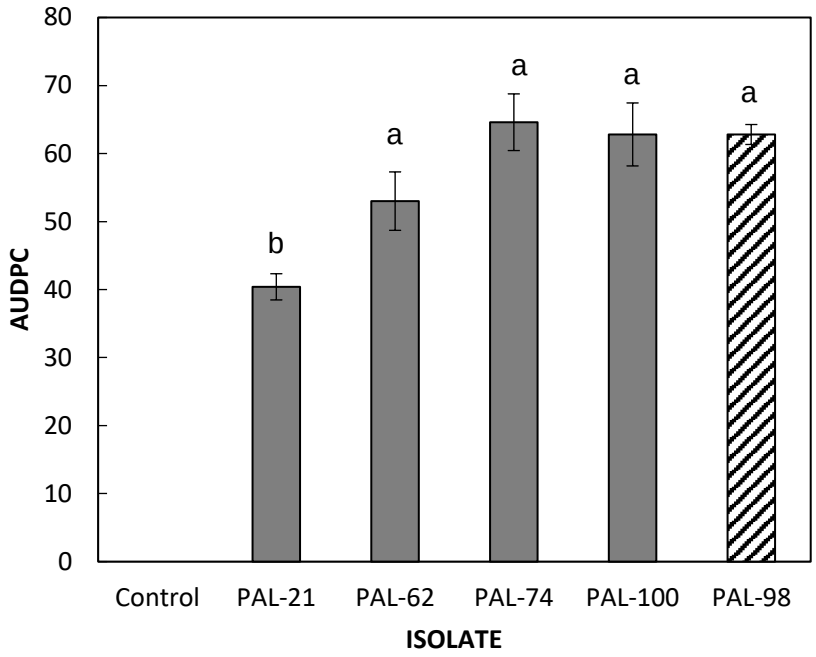

Figure 5.5 Results of area under the disease progress curve AUDPC of four isolates of *Ph. niederhauserii* and one of *Pp. vexans* to 'Garnem' rootstock 58 days after inoculation. The vertical bars represent the standard error of the mean. The letters indicate significant differences (LSD; $P \leq 0.01$) among the means. The white column represents the control, gray columns represent the isolates of *Ph. niederhauserii* and the striped column represents the isolate of *Pp. vexans.*

The root dry-weight results are shown in Figure 5.6. There were statistically significant differences ($P \leq 0.01$) between the root dry weight of the control and the isolates PAL-74 and PAL-100 of *Ph. niederhauserii*, which caused a 33% and a 31% reduction in root dry weight when compared with the un-inoculated control, respectively. There were no statistically significant differences between the control and the isolates PAL-21 and PAL-62 of *Ph. niederhauserii* and the isolate PAL-98 of *Pp. vexans*. Although, in the isolates PAL-21 and PAL-62, there were no statistically significant differences with respect to the control, a reduction in the mean weight of 22% and 18%, respectively, was observed when they were compared with the control.

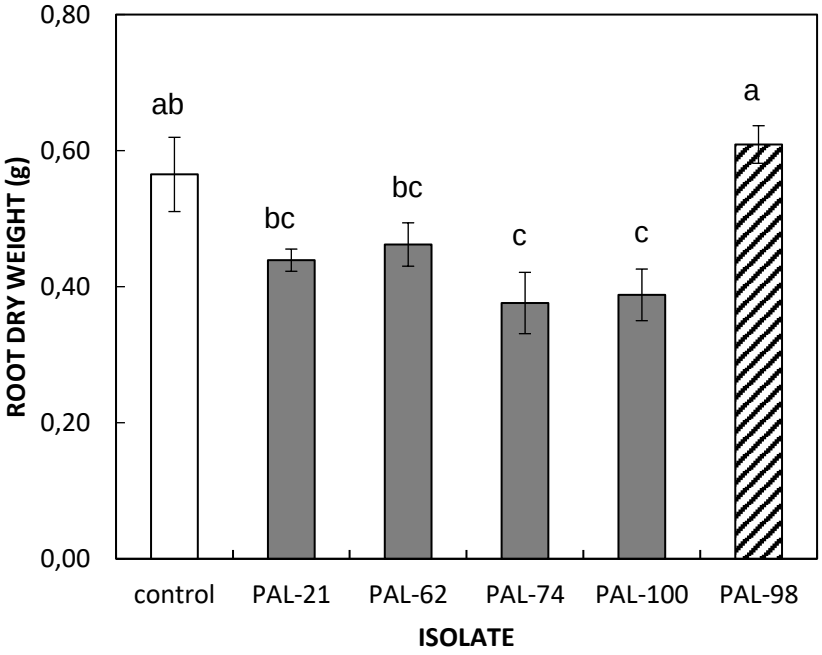

Figure 5.6 Results of root dry weight of four isolates of *Ph. niederhauserii* and one of *Pp. vexans* to 'Garnem' rootstock 58 days after inoculation. The vertical bars represent the standard error of the mean. The letters indicate significant differences (LSD; $P \leq 0.01$) among the isolates' means. The white column represents the control, the gray columns represent the isolates of *Ph. niederhauserii* and the striped column represents the isolate of *Pp. vexans.*

DISCUSSION

This study presents the results of a three-year survey of oomycetes associated with root and crown rot of almond trees in Spain. We found a great diversity of oomycete species belonging to the genera *Globisporangium*, *Phytophthora*, *Phytopythium* and *Pythium* present in root and soil samples, as previously described in almond crops in Iran (Azizi et al., 2013) and California (Browne et al., 2015; Browne et al., 2019). *Phytopythium vexans* and *Ph. niederhauserii* were the most frequent species isolated; thus, its pathogenicity to almond rootstock 'Garnem', widely used in Spain due to its high vigor and high resistance to *Meloidogyne* spp., chlorosis and drought (Bielsa and Rubio-Cabetas, 2018), was investigated. Moreover, most of the oomycetes were

isolated from the soil samples with baiting techniques. It is well known that most oomycetes are difficult to isolate from necrotic tissues because they often harbor many secondary pathogens; therefore, performing the baiting of soil samples taken from affected trees is the best approach (Erwin and Ribeiro, 1996; Drenth and Sendall, 2001).

The high frequency of *Pp. vexans* agrees with the results from a study conducted in peach-orchard replanting soil in California by Yang et al. (2012), who reported a 65% prevalence of this species in peach trees, showing a dramatic reduction in plant growth, vigor and yield, with a shortened production life. *Phytopythium vexans* was considered a saprophytic species, rapidly colonizing dead roots in peach trees, playing an invasive role in respect to secondary decaying rootlets (Mircetich, 1971). However, its pathogenicity has recently been confirmed on fruit hosts such as grapevine in South Africa (Langenhoven et al., 2018), avocado in Spain (Rodríguez-Padrón et al., 2018) and apple trees in Morocco (Jabiri et al., 2020). Regarding the inoculation of *Pp*. *vexans* on the 'Garnem' rootstock, our results show a high severity of symptoms at the late stage of the pathogenicity test, which corresponded with advanced defoliation, wilting and dieback symptoms, leading to the plant death of some seedlings. On the contrary, the dry weight of the root did not reduce compared with the control. Similar results were obtained by Ivors et al. (2008), who indicated that the root rot indices and the maximum fresh weight of Fraser fir plants (*Abies fraseri* (Pursh) Poir) inoculated with *Pp*. *vexans* were not significantly different from the weight of the controls. To our knowledge, there are no previous works that reported the pathogenicity of this species on almonds; thus, our study is the first report of *Pp*. *vexans* as an almond pathogen.

The second species with the highest frequency of isolation was *Ph*. *niederhauserii*. Previous studies conducted in Spain reported it for the first time to be affecting almond trees in nurseries (Pérez-Sierra et al., 2010), but our results confirm that this species is currently present in almond orchards in diverse Spanish provinces. Later, this oomycete was found to affect almonds in Turkey (Kurbetli and Yilmaz, 2016) and California (Browne et al., 2015). Regarding its pathogenicity to 'Garnem' rootstock, the four isolates included in our study caused severe symptoms in the aerial part of the seedlings at the end of the experiment, although only the isolates PAL-74 and PAL-100 significantly reduced the dry weight of the roots compared with the control. Root dry-weight reduction could possibly be the consequence of the reduction in the lateral roots, due to the necrosis caused by *Ph*. *niederhauserii* infection. This effect was already described by Rodríguez-Padrón et al. (2018) and Kurbetli et al. (2020), when these authors inoculated *Ph*. *niederhauserii* to avocado and pomegranate, respectively. Furthermore, both research works evidenced that infection by this

species was more virulent than that caused by other *Phytophthora* species evaluated. Although attacks of *Ph*. *niederhauserii* have been reported in other crops, mainly ornamental hosts (Abad et al., 2014; Moralejo et al., 2009; Prigigallo et al., 2015), its worldwide geographical distribution in almond crops is still limited.

Globisporangium irregulare and *G*. *ultimum* var. *ultimum* presented an isolation frequency of less than 10%. Mircetich (1971) indicated that *G. irregulare* and *G. ultimum* var. *ultimum* were isolated with high frequency in peach orchards, but there was no relationship between the decay of the trees and the frequency of appearance of these species in the roots. For this reason, this author suggested that both species may be saprophytes on the roots of the peach tree. Although the pathogenicity of *G. irregulare* and *G. ultimum* var. *ultimum* was not investigated in our study, it has been previously studied in America. In Chile, Pinto de Torres and Mircetich (1976) reported trunk rot at the crown area of almond trees caused by *G. ultimum*. Later, in Michigan, USA, Smither and Jones (1989) isolated *G. irregulare* from the ground, in sour cherry orchards, with symptoms of discolored and necrotic roots, in which *Phytophthora* spp. are commonly found. In greenhouse trials, increased root discoloration, necrosis and reductions in root and shoot growth occurred on sour cherry seedlings inoculated with *G. irregulare*. These authors concluded that, despite causing these symptoms, *G. irregulare* was not able to kill this host. For this reason, they suggested that this species was not the cause of the decline in and death of cherry trees in the evaluated orchards but may contribute to a reduction in the growth of cherry trees planted in heavy soils. Coinciding with our study, Yang et al. (2012) reported a low isolation frequency of *G. irregulare* in replanting soils of peach orchards in California; around 8% of the isolates of total oomycetes recovered. Regarding the pathogenicity of these species, in California, Schmidt and Browne (2013) detected the presence of *Pythium* sp. associated with Prunus Replant Disease (PRD), a little-known disease caused by a complex of soil microorganisms. These authors carried out pathogenicity tests in the greenhouse, with grafted peach trees on 'Nemaguard' rootstock, inoculating *G. irregulare* and *G. ultimum*, among others. In this study, *G. irregulare* and *G. ultimum* significantly reduced the fresh weight of the 'Nemaguard' rootstock and caused various levels of necrosis of the root cortex, confirming that these species contribute to the development of PRD.

The isolation frequency of *Py. dissotocum* was also less than 10%. This oomycete has been described mainly as a pathogen of vegetables grown in hydroponic conditions such as lettuce in the USA (McGehee et al., 2018), sweet

peppers in Canada (Owen-Going et al., 2003) and spinach in China (Huo et al., 2020), but not on fruit crops.

Phytophthora cactorum and *Ph. citrophthora* were isolated less than 7%. These results match with previous works performed by Wicks and Lee (1986) and Browne and Viveros (1997), who reported the isolation of these pathogens from almond cankers and soil, without proving their pathogenicity. Later, in Iran, Sahragard and Banihashemi (2006) confirmed the pathogenicity of *Ph. cactorum* in a local almond tree rootstock, which increased mortality rate and decreased root weight. Despite this, the literature is not conclusive in associating specific *Phytophthora* species with root rot and cankers on the root crown, trunk, or scaffolds, observed in almond trees (Browne et al., 2015). Multiple species, such as *Ph. cambivora*, *Ph. cryptogea* (Wicks, 1989), *Ph. citricola* (Browne and Viveros, 1997), *Ph. megasperma* (Kurbetli and Yilmaz, 2016), *Ph. plurivora* (Çiftçi et al., 2016) and *Ph. chlamydospora* (Browne et al., 2020; Türkölmez et al., 2016), have been associated with almond crops, but they were not found in our survey.

The remaining oomycete species were found with a very low frequency and were occasionally isolated from a few samples. Most of these species are pathogens of ornamentals, cereals, vegetables and some fruit trees and some have also been described as saprophytes. However, *Pp. helicoides* has been reported as a pathogen in *Prunus* in California, causing root rot and roots of stunted seedlings of 'Nemaguard' peach rootstock (Browne et al., 2019).

Disease severity, AUDPC and root dry weight were useful parameters to evaluate the pathogenicity of *Pp. vexans* and *Ph. niederhauserii* to almond rootstock 'Garnem'. The application of standard protocols for pathogenicity testing and disease assessment in plants facilitates inter-study comparisons, thus improving accuracy (Bhunjun et al., 2021). Moreover, further studies about pathogenicity testing of oomycetes to *Prunus* rootstocks would improve the understanding of their infections mechanisms and identifying methods to provide durable resistance, which are major research goals for rootstock breeding programs (El Hadrami et al., 2012; Fawke et al., 2015).

The results of our research study provide new information about the pathogenic oomycete species present in almond crops in Spain, which represent an emerging threat for almond and other *Prunus* spp. production. They also highlight the importance of carrying out frequent phytosanitary surveys in fruit nurseries and orchards for a better knowledge of potential risks posed by soil-borne pathogens, including the pathogenicity evaluation of the species found.

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References

Abad, Z. G., Abad, J. A., Cacciola, S. O., Pane, A., Faedda, R., Moralejo, E., Perez-Sierra, A., Abad-Campos, P., Alvarez-Bernaola, L. A., Bakonyi, J., Jozsa, A., Herrero, M. L., Burgess, T. I., Cunnington, J. H., Smith, I. W., Balci, Y., Blomquist, C., Henricot, B., Denton, G., Spies, C., Mcleod, A., Belbahri, L., Cooke, D., Kageyama, K., Uematsu, S., Kurbetli, I. and De irmenci, K. 2014. *Phytophthora niederhauserii* sp. nov., a polyphagous species associated with ornamentals, fruit trees and native plants in 13 countries. Mycologia 106:431-447.

Azizi, Z., Amini, J., Shaykh-Eslami, M. and Abasi, S. 2013. Pathogenicity of some isolates of *Pythium* and *Phytophthora* on detached shoots and seedlings of almond. Iran. J. Plant. Pathol. 49:33-39.

Bhunjun, C., Phillips, A., Jayawardena, R., Promputtha, I. and Hyde, K. 2021. Importance of molecular data to identify fungal plant pathogens and guidelines for pathogenicity testing based on Koch's Postulates. Pathogens 10:1096.

Bielsa, B., Rubio-Cabetas, M. 2018. La elección del patrón en la almendricultura moderna. Rev. Frut. 65:92-113.

Browne, G. and Viveros, M. 1997. Diverse symptoms and tree losses caused by *Phytophthora* spp. in California almonds. Acta Hortic. 470:570-575.

Browne, G., Ott, N. and Fichtner, E. 2019. First report of *Phytopythium helicoides* causing root rot on peach rootstock in California. Plant. Dis. 103:2968.

Browne, G., Ott, N., Forbes, H., Yaghmour, M. and Milliron, L. 2020. First report of *Phytophthora chlamydospora* causing crown and root rot on almond in California. Plant. Dis. 104:2033-2033.

Browne, G., Schmidt, L. and Brar, G. 2015. First report of *Phytophthora niederhauserii* causing crown rot of almond (*Prunus dulcis*) in California. Plant. Dis. 99:1863.

Campbell, C. and Madden, L. Introduction to plant disease epidemiology, 1st ed; John Wiley & Sons: New York, NY, USA, 1990, pp. 192–194.

Çiftçi, O., Türkölmez, Ş., Derviş, S. and Serçe, Ç. 2016. First report of canker and root rot of almond caused by *Phytophthora plurivora* in Turkey. Plant. Dis. 100:1507.

Collado-Romero, M., Mercado-Blanco, J., Olivares-García, C., Valverde-Corredor, A. and Jiménez-Díaz, R. 2006. Molecular variability within and among Verticillium dahliae vegetative compatibility groups determined by fluorescent amplified fragment length polymorphism and polymerase chain reaction markers. Phytopathology 96:485-495.

Cooke, D., Drenth, A., Duncan, J., Wagels, G. and Brasier, C. 2000. A molecular phylogeny of *Phytophthora* and related oomycetes. Fungal Genet. Biol. 30:17-32.

Drenth, A. and Sendall, B. Practical Guide to Detection and Identification of *Phytophthora*, CRC for Tropical Plant Protection: Brisbane, Australia, 2001, 41 pp.

El Hadrami, A., El-Bebany, A., Yao, Z., Adam, L., El Hadrami, I. and Daayf, F. 2012. Plants versus fungi and oomycetes: Pathogenesis, defense and counter-defense in the proteomics era. Int. J. Mol. Sci. 13:7237-7259.

Erwin, D. and Ribeiro, O. 1996. *Phytophthora* Disease Worldwide, American Phytopathology Society Press: Saint Paul, MN, USA,, 562 pp.

Fawke, S. and Doumane, M., Schornack, S. 2015. Oomycete interactions with plants: Infection strategies and resistance principles. Microbiol. Mol. Biol. Rev. 79:263-280.

Felipe, A. El Almendro I: El Material Vegetal, 1st ed., Mira Editores: Zaragoza, Spain, 2000, 461 pp.

Gradziel, T., Curtis, R. and Socias i Company, R. 2017. Production and Growing Regions. In: Almonds, botany, production and uses. R. Socias i Company and T. M. Gradziel, Eds., CAB International, Boston, MA. pp. 70-86.

Huo, C., Cao, J., Wu, K., Chen, Y. and Zhao, Z. 2020. First report of *Pythium dissotocum* causing root rot on hydroponically grown spinach in China. Plant. Dis. 104:3084.

Iglesias, I. and Carbó, J. 2006. Situació actual, característiques i comportament agronòmic dels portaempelts de presseguer. Dossier Tècnic. 17:3-18.

Ivors, K., Abad, Z. and Benson, D. 2008. Evaluating the pathogenicity of *Pythium vexans* isolates from Fraser fir in North Carolina. Plant. Health Prog. 9:8.

Jabiri, S., Lahlali, R., Bahra, C., Amraoui, M., Tahiri, A. and Amiri, S. 2020. First report of *Phytopythium vexans* associated with dieback disease of apple trees in Morocco. J. Plant. Pathol. 102:1319.

Javadi, N. and Sharifnabi, B. 2016. *Phytopythium litorale*, the causal agent of almond root and crown rot in Iran. In: Proceedings of 22nd Iranian Plant Protection Congress, Karaj, Iran, 27–30 August, College of Agriculture and Natural Resources: Karaj, Iran, pp. 27-30.

Jeffers, S. and Martin, S. 1986. Comparison of two media selective for *Phytophthora* and *Pythium* species. Plant. Dis. 70:1038-1043.

Jönsson, U., Jung, T., Rosengren, U., Nihlgård, B. and Sonesson, K. 2003. Pathogenicity of Swedish isolates of *Phytophthora quercina* to *Quercus* robur in two different soils. New Phytol. 158:355-364.

Jung, T., Blaschke, H. and Neumann, P. 1996. Isolation, identification and pathogenicity of *Phytophthora* species from declining oak stands. Eur. J. For. Pathol. 26:253-272.

Kurbetli, I. and Değirmenci, K. 2011. First report of *Phytophthora* taxon *niederhauserii* causing decline of almond in Turkey. New Dis. Rep. 23:14.

Kurbetli, İ. and Yılmaz, A. 2016. Almond decline caused by *Phytophthora megasperma* in southeastern Anatolian region of Turkey. J. Turk. Phytopathol. 45:13-20.

Kurbetli, İ., Karaca, G., Aydoğdu, M. and Sülü, G. 2020. *Phytophthora* species causing root and collar rot of pomegranate in Turkey. Eur. J. Plant. Pathol. 157:485-496.

Langenhoven, S., Halleen, F., Spies, C., Stempien, E. and Mostert, L. 2018. Detection and quantification of black foot and crown and root rot pathogens in grapevine nursery soils in the Western Cape of South Africa. Phytopathol. Mediter. 57:519-537.

Linderman, R. and Zeitoun, F. 1977. *Phytophthora cinnamomi* causing root rot and wilt of nursery-grown native western azalea and salal. Plant. Dis. Report. 61:1045-1048.

McGehee, C., Raudales, R. and Elmer, W. 2018. First report of *Pythium dissotocum* causing Pythium root rot on hydroponically grown lettuce in Connecticut. Plant. Dis. 102:2043.

Miarnau, X., Torguet, L., Batlle, I. and Alegre, S. 2016. El cultivo del almendro en alta densidad. Rev. Frutic. 49:68-87.

Mircetich, S. 1971. The role of *Pythium* in feeder roots of diseased and symptomless peach trees. Phytopathology 61:357-360.

Moralejo, E., Pérez-Sierra, A., Álvarez, L., Belbahri, L., Lefort, F. and Descals, E. 2009. Multiple alien *Phytophthora* taxa discovered on diseased ornamental plants in Spain. Plant. Pathol. 58:100-110.

Owen-Going, N., Sutton, J. and Grodzinski, B. 2003. Relationships of *Pythium* isolates and sweet pepper plants in single-plant hydroponic units. Can. J. Plant. Pathol. 25:155-167.

Pérez-Sierra, A., León, M., Álvarez, L., Alaniz, S., Berbegal, M., García-Jiménez, J. and Abad-Campos, P. 2010. Outbreak of a new *Phytophthora* sp. associated with severe decline of almond trees in eastern Spain. Plant. Dis. 94:534-541.

Pérez-Sierra, A., López-García, C., León, M., García-Jiménez, J., Abad-Campos, P. and Jung, T. 2013. Previously unrecorded low-temperature *Phytophthora* species associated with *Quercus* decline in a Mediterranean forest in eastern Spain. For. Pathol. 43:331-339.

Pérez-Sierra, A., Mora-Sala, B., León-Santana, M., García-Jiménez, J. and Abad-Campos, P. 2012. Enfermedades causadas por *Phytophthora* en viveros de plantas ornamentales. Bol. Sanid. Veg. Plagas. 38:143-156.

Pinochet, J. 2010. 'Replantpac' (Rootpac® R), a plum–almond hybrid rootstock for replant situations. HortScience. 45:299-301,

Pinto de Torres, A. and Mircetich, S. 1976. *Pythium ultimun* Trow causante de pudrición del tronco en plántulas de frutales de carozo y en almendros y damascos de dos años. Agric. Téc. 36:171-173.

Prigigallo, M., Mosca, S., Cacciola, S., Cooke, D. and Schena, L. 2015. Molecular analysis of *Phytophthora* diversity in nursery-grown ornamental and fruit plants. Plant. Pathol. 64:1308-1319.

Rodríguez-Padrón, C., Siverio, F., Pérez-Sierra, A. and Rodríguez, A. 2018. Isolation and pathogenicity of *Phytophthora* species and *Phytopythium vexans* recovered from avocado orchards in the Canary Islands, including *Phytophthora niederhauserii* as a new pathogen of avocado. Phytopathol. Mediterr. 57:89-106.

Rubio-Cabetas, M., Felipe, A. and Reighard. G. Rootstock Development. In: Almonds, botany, production and uses. R. Socias i Company and T. M. Gradziel, Eds., CAB International, Boston, MA. pp. 209–227.

Sahragard, N. and Banihashemi, Z. 2006. Evaluation of resistance of some almond genotypes and cultivars to *Phytophthora cactorum*. Iran. J. Plant. Pathol. 42:97-99.

Schmidt, L. and Browne, G. 2013. Characterization of *Pythium* species associated with Prunus replant disease. In: Phytopathology, The American Phytopathological Society: Saint Paul, MN, USA,, Volume 103, p. 128.

Smither, M. and Jones, A. 1989. *Pythium* species associated with sour cherry and the effect of *P. irregulare* on the growth of mahaleb cherry. Can. J. Plant. Pathol. 11:1-8.

Teviotdale, B., Michailides, T. and Pscheidt, J. 2002. Compendium of nut crop diseases in temperate zones. American Phytopathological Society: Portland, OR, USA. pp. 18–429.

Torguet, L., Maldonado, M. and Miarnau, X. 2019. Importancia y control de las enfermedades en el cultivo del almendro. Agri. Rev. Agropec. Gan. 1026:72-77.

Türkölmez, Ş., Derviş, S., Çiftçi, O. and Ulubaş Serçe, Ç. 2016. First report of *Phytophthora chlamydospora* causing root and crown rot on almond (*Prunus dulcis*) trees in Turkey. Plant. Dis. 100:1796.

Wang, Y., Tyler, B. and Wang, Y. 2019. Defense and counterdefense during plant-pathogenic oomycete infection. Annu. Rev. Microbiol. 73:667-696.

White, T., Bruns, T., Lee, S. and Taylor, J. 1990. Amplification and direct sequencing of fungi ribosomal RNA genes for phylogenetics. In: PCR Protocols. A guide to methods and applications. Innis, M., Gelfand, D., Sninsky, J., White, T., Eds., Academic Press: Millbrae, CA, USA, pp. 315–322.

Wicks, T. 1989. Susceptibility of almond and cherry rootstocks and scions to *Phytophthora* species. Aust. J. Exp. Agric. 29:103-109.

Wicks, T. and Lee, T. 1986. *Phytophthora* crown rot of almond trees. Aust. J. Agric. Res. 37:277-287.

Yang, J., Ruegger, P., McKenry, M., Becker, J. and Borneman, J. 2012. Correlations between root-associated microorganisms and peach replant disease symptoms in a California Soil. PLoS ONE, 7:e46420.

Chapter 6

Chapter 6

Pathogenicity of oomycete species to different *Prunus* hybrid rootstocks

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Abstract. Diseases caused by soil-borne oomycetes are a limiting factor for the cultivation of *Prunus* spp., which makes the choice of a suitable rootstock a key factor. The objective of this study was to evaluate the pathogenicity of 12 oomycete species belonging to the genera *Globisporangium*, *Phytophthora* (*Ph.*) and *Phytopythium* (*Pp.*) to three *Prunus* hybrid rootstocks: 'Garnem', 'GF-677' and 'Rootpac-40'. These three rootstocks are widely used to grow stone fruit and almond in the Mediterranean Basin. Pathogenicity tests were conducted using 15 oomycete isolates and 1-year-old rootstock seedlings. Ninety days after inoculation, disease symptoms were evaluated on a severity scale, and the Area Under the Disease Progression Curve and the survival probability of the inoculated seedlings were calculated. Moreover, root dry weight was recorded. All the isolates included in the pathogenicity tests were pathogenic on the rootstock seedlings and were re-isolated from root lesions. Large differences in virulence were detected among the different oomycete species and isolates of *Ph. niederhauserii* for each rootstock. *Phytophthora multivora* and *Pp. helicoides* were generally the most virulent species. The results of the present research offer substantial contribution to increase our knowledge about the pathogenicity of several oomycete species that are frequently isolated in *Prunus* orchards, and the potential risks that they pose for *Prunus* spp. crops.

INTRODUCTION

In the last 5 years, a sustained increase has taken place in the cultivation of *Prunus* spp., including crops like almonds [*P. dulcis* (Miller.) D.A. Webb], apricots (*P. armeniaca* L.), cherries (*P. avium* L.), peaches and nectarines [*P. persica* (L.) Batsch], plums (*P. domestica* L.), sloes (*P. spinosa* L.) and sour cherries (*P. cerasus* L.), with a total world production 50 million tons in 2019

(FAOSTAT, 2021). This was because planted areas have extended, together with new cultivar releases from breeding programs for *Prunus* spp., which have allowed higher yields. These breeding programs have focused mainly on optimizing different tree agronomic aspects, such as floral self-compatibility, blooming and ripening times, productivity, and resistance to pests and diseases (Bielsa and Rubio-Cabetas, 2018).

Significant progress has been made in developing rootstock breeding programs. Rootstocks are essential components in modern fruit production for their ability to adapt a cultivar of commercial interest to several environmental conditions and cultural practices. These capacities are currently being evaluated with the following objectives: shorten or prolong fruit ripening, improve fruit yield and quality, control vigor, compatibility with many cultivars, good tolerance to hypoxia, high water-use efficiency, tolerance to water or soil salinity, and resistance or tolerance to soil-borne pathogens (Gainza et al., 2015).

Most *Prunus* crops grown on their own roots, which were formerly used to cultivate stone fruit and almond, are very susceptible to diseases caused by the genus *Phytophthora* (*Ph.*), such as *Ph. cactorum* [(Lebert and Cohn) Schröter], *Ph. cambivora* [Petri (Buisman)], *Ph. cryptogea* (Pethybr. and Lafferty), *Ph. megasperma* (Drechsler) and *Ph. syringae* [(Kleb.) Kleb.] (Browne and Mircetich, 1995), especially in soils with a fine texture, high bulk density or poor drainage (Reighard and Loreti, 2008). This has been a limiting factor for *Prunus* spp. production, which makes the choice of a suitable rootstock a key factor (Tsipouridis et al., 2005). Therefore, in the 1970s, trees growing on their own roots were progressively replaced by interspecific *Prunus* hybrid rootstocks (Bielsa and Rubio-Cabetas, 2018) to improve qualities of adaptation or tolerance to heavy soils, waterlogging, alkalinity and drought, and to control vigor and tolerance against soil-borne pathogens (Bielsa and Rubio-Cabetas, 2018; Reighard and Loreti, 2008).

Currently, the most popular *Prunus* hybrid rootstocks in the Mediterranean Basin are 'GF-677' and 'Garnem' (*P. dulcis* x *P. persica*), being used for almond and peach trees (Reighard and Loreti, 2008; Rubio-Cabetas et al., 2017; Thomidis, 2003b). In the last decade, less vigorous rootstocks have also been developed, and adapted to high-density crops and mechanized harvesting (Yahmed et al., 2016). Examples of such are 'Rootpac-40' (complex almond x peach hybrid), which induces a high yield per *Prunus* tree, similar to that of more vigorous rootstocks like 'Garnem' and 'GF-677' (Jiménez et al., 2011).

Despite the positive horticultural qualities conferred by hybrid rootstocks during *Prunus* spp. cultivation, current production intensification is characterized by incorporating irrigation systems together with exploring several soil types. This

has increased problems related to waterlogging in association with root asphyxia (Felipe, 2009; Rubio-Cabetas et al., 2017), and the proliferation of diseases caused by oomycetes, to which hybrid rootstocks like 'GF- 677' and 'Garnem' are still susceptible (Reighard and Loreti, 2008; Rubio-Cabetas et al., 2017).

Several previous studies have investigated the susceptibility of *Prunus* rootstocks to soil-borne pathogens such as *Phytophthora* spp. and *Phytopythium* spp. In Australia, Wicks (1989) evaluated the susceptibility of 'Nemaguard' [*P. persica* x *P. davidiana* (Franch., Pl. David.)], 'Titan' (*P. dulcis* x *P. persica*), and the hybrid 'Nemaguard' x *P. dulcis* to *Ph cambivora*, which were rated as resistant, moderately resistant and susceptible, respectively. In Greece, Elena and Tsipouridis (2000) reported the susceptibility of rootstocks 'GF-677' and 'J01-ADAFUEL' (both hybrids of *P. dulcis* x *P. persica*) to crown rot caused by *Ph. cactorum, Ph. citrophthora* [(R.E.Sm. and E.H.Sm.) Leonian], and *Ph. megasperma*. In the same country, Thomidis (2000) indicated the susceptibility of peach trees grafted onto rootstocks 'GF-677' and 'KID-I' to *Ph. cactorum*, *Ph. citrophthora*, and *Ph. syringae*. New research into rootstock 'GF-677' showed susceptibility when inoculated with *Ph. cactorum* and *Ph. megasperma* under laboratory conditions (Thomidis, 2003b; Thomidis et al., 2001). Different results were reported by Mehri et al. (2009), who indicated that rootstock 'GF-677' was resistant to two different *Ph. cactorum* strains when inoculated in tests done on excised twigs and plants cultured *in vitro*. In California, Yang et al. (2012) reported a high incidence of *Phytopythium* (*Pp.*) *vexans* [(de Bary) Abad, de Cock, Bala, Robideau, A.M. Lodhi and Lévesque] in a field location with peach trees grafted onto 'Nemaguard' showing replant disease symptoms. *Phytophthora chlamydospora* (Brasier and E.M. Hansen) was reported to attack almond trees grafted onto rootstock 'GF-677' in Turkey (Türkölmez et al., 2016), and almond seedlings grafted onto 'Nemaguard' and peach trees grafted onto 'Lovell' (*P. persica*) in California (Browne et al., 2020). Also in California, Browne (2017) concluded that hybrid rootstocks (almond x peach), such as 'Bright Hybrid-5', 'Bright Hybrid-106', 'Hansen-536' and 'Garnem', were susceptible to *Ph. niederhauserii* (Z.G. Abad et J.A. Abad, sp. nov).

Regarding the genus *Globisporangium*, information about its pathogenicity to *Prunus* rootstocks is scarce. Smither and Jones (1989) reported a low virulence of *G. irregulare* [(Buisman) Uzuhashi, Tojo and Kakish.] in *P. mahaleb* seedlings. However, Schmidt and Browne (2013) indicated a high virulence of *G. irregulare* in peach seedlings grafted onto 'Nemaguard' roostock.

Recent reports about new oomycete pathogens to *Prunus* crops grafted onto hybrid rootstocks, such as *Ph*. *niederhauserii* on almond trees (Browne et al., 2015; Pérez-Sierra et al., 2010) and *Pp*. *helicoides* (Drechsler) on peach

trees (Browne et al., 2019), together with the potential banning of some fungicides currently used for the chemical control of many oomycetes, are of much concern (Thomidis, 2003a). This fact reinforces the need to make new pathogenicity evaluations to obtain updated information about the susceptibility or tolerance of the most widely used rootstocks to well-known and newly described oomycete species.

Thus, the objective of this study was to evaluate the pathogenicity of 12 oomycete species belonging to the genera *Globisporangium*, *Phytophthora* and *Phytopythium* to three *Prunus* hybrid rootstocks, 'Garnem', 'GF-677' and 'Rootpac-40', which are widely used to cultivate stone fruit and almond in the Mediterranean Basin.

MATERIAL AND METHODS

***Oomycete species and isolates*.** Fifteen isolates representing 12 oomycete species were used in the pathogenicity tests (Table 6.1). They were obtained from extensive surveys of *P. dulcis* plantations and nurseries, which were carried out during the 2018-2021 period by Beluzán et al. (2022) in five Spanish provinces: Córdoba, Huelva, Lérida, Sevilla and Valencia, and identified by Internal Transcribed Spacer (ITS) sequencing.

For this study their identity was also confirmed by sequencing cytochrome c oxidase subunit 1 (*cox*1). For this purpose, genomic DNA was extracted from pure cultures grown on PDA at 25 ºC in the dark for 7 days. For DNA extraction, the method of Collado-Romero et al. (2006) was followed, with a slight modification, namely the mycelium was scraped with a sterile pipette tip and placed in a 0.2 mL PCR tube with 20 µL of 25mM NaOH at pH 12. The tubes with the samples were incubated in a PTC 200 thermal cycler (MJ Research Inc., Waltham, MA, USA) following a DNA denaturation program (100 ºC for 15 minutes and 4 ºC for 5 minutes). Subsequently, 40 µL of 40mM Tris-HCl were added at pH 5. The *cox*1 amplification for *Phytopythium* and *Globisporangium* isolates was performed using the forward primer OomCOI-Lev-up (5' TCA WCW MGA TGG CTT TTT TCA AC 3') (Bala et al., 2010) and the reverse primer FM85mod (5' AAC TTG ACT RAT AAT ACC AAA 3') modified from FM85 of Martin and Tooley (2003). For *Phytophthora*, the *cox*1 primers were FM84 (5' TTT AAT TTT TAG TGC TTT TGC 3') and FM50rev (5' CAT CTA AAC CAA CAG TAA AC 3'), the reverse complementary sequence of the FM50 (Martin and Tooley, 2003). Each PCR reaction tube contained water (13.3 µL), Canvax Buffer B (2.5 µL), Canvax $MgCl_2$ (25nM) (2.5 µL), Canvax dNTPs (8nM) (2.5 µL), Canvax Horse Power Taq polymerase (U/µL) (0.2 µL), primers (1 µL of each) and genomic DNA (2 µL), totaling 25 µL (Canvax Biotech SL, Córdoba, Spain).

PCR amplification was performed in the same aforementioned thermal cycler by the following program: initial denaturation of 1 cycle at 94 ºC for 3 minutes, 35 cycles of denaturation, annealing and extension at 94 ºC for 30 seconds, 54 ºC for 30 seconds and 72 ºC for 45 seconds, respectively, and a final amplification cycle at 72 ºC for 10 minutes. PCR products were separated by electrophoresis (140 V) on 1.5% agarose gel [1.5% agarose (Conda, Madrid, Spain) dissolved in TAE buffer; Tris-acetate-EDTA, 40mM Tris-acetate, 1mM EDTA]. Nucleic acids staining was done with RedSafe (20000x). To observe band size, a molecular marker (GeneRuler T.M. 100 bp Plus DNA Ladder, Thermo Scientific) was loaded into the first gel well. Presence of bands was observed using a UV light transilluminator. PCR products were sent to the Instituto de Biología Molecular y Celular de Plantas (IBMCP) (Valencia, Spain) for sequencing. The obtained sequences were subjected to a search in NCBI BLAST (http: //https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify isolates at the species level.

All isolates were stored in the oomycete collection of the Instituto Agroforestal Mediterráneo (Valencia, Spain) in soil solution extract (Mora-Sala et al., 2022) and tubes of oat agar and potato-carrot agar at 10 ºC.

***Rootstocks*.** Pathogenicity tests were conducted using 1-year-old seedlings (≈25 cm height) of three different *Prunus* hybrid rootstocks: 'Garnem', 'GF-677' and 'Rootpac-40' (Table 6.2). Seedlings were provided by a commercial nursery and, prior to inoculations, they were selected based on morphological homogeneity and healthy appearance.

***Inoculum production and inoculation*.** The inoculum of oomycete isolates was prepared in 500 mL glass flasks with a mixture of 250 mL of vermiculite, 20 mL of oat grains and 175 mL of V8 broth medium (200 mL/L V8 juice, 800 mL/L demineralized water and 3 g/L of $CaCO_3$) (Jung et al., 1996). Glass flasks were autoclaved 3 times for 20 min at 120 ºC. Flasks were inoculated separately with each isolate, which was previously grown on V8 juice agar medium (V8A). Then flasks were incubated for 6 weeks in the dark at room temperature (Pérez-Sierra et al., 2013). After this time, the inoculum mixture was rinsed with demineralized water to remove nutrient excess before inoculations.

Table 6.1 Oomycete species and isolates used for the pathogenicity tests.

Species	Isolate	Source	Location	ITS[1]			*cox1*[2]		
				Identity (%)	Reference Isolate[3]	GenBank Accession[4]	Identity (%)	Reference Isolate[3]	GenBank Accession[4]
Globisporangium heterothallicum	PAL-42	Soil	Lérida	98.0	HQ643559	MZ922002	100	HQ708603	OP067002
G. irregulare	PAL-90	Soil	Huelva	99.9	HQ643596	MZ922042	98.1	HQ708640	OP067007
G. ultimum var. *ultimum*	PAL-58	'GF-677' rootstock	Valencia	99.2	HQ643869	MZ922015	99.7	HQ708910	OP067000
Phytophthora cactorum	PAL-4	Soil	Lérida	100	MG783385	MZ921999	99.7	MH136858	OP067004
Ph. citrophthora	PAL-16	Soil	Lérida	99.9	MG865476	MZ921978	99.0	MH136872	OP067006
Ph. multivora	PAL-103	Irrigation water	Valencia	100	MG865546	ON159753	99.2	MH136939	OP067005
Ph. nicotianae	PAL-71	Soil	Valencia	99.7	MG865550	MZ922025	99.9	MH136943	OP067003
Ph. niederhauserii	PAL-21	Soil	Lérida	100	MG865552	MZ921966	100	MH136944	OP066996
Ph. niederhauserii	PAL-62	'GF-677' rootstock	Valencia	100	MG865552	MZ921954	99.9	MH136944	OP066997
Ph. niederhauserii	PAL-74	Soil	Valencia	99.9	MG865552	MZ921957	99.9	MH136944	OP066998
Ph. niederhauserii	PAL-100	'GF-677' rootstock	Córdoba	100	MG865552	MZ921962	99.9	MH136944	OP066999
Ph. tropicalis	PAL-101	Soil	Córdoba	100	MG865596	MZ921976	98.3	MH136987	OP067001
Phytopythium chamaehyphon	PAL-99	Soil	Córdoba	99.3	HQ643374	MZ922051	100	HQ708421	OP066994
Pp. helicoides	PAL-96	'GF-677' rootstock	Sevilla	99.4	HQ643383	MZ922048	99.6	HQ708430	OP066993
Pp. vexans	PAL-98	'GF-677' rootstock	Córdoba	99.5	HQ643400	MZ922050	99.9	HQ708447	OP066995

[1] Isolates identified by ITS (internal transcribed spacer).
[2] Isolates identified by *cox*1 (cytochrome c oxidase subunit 1).
[3] Reference isolates taken from Abad et al. (2022) and Robideau et al. (2011).
[4] Accession number of amplified sequences of ITS and *cox*1 deposited in GenBank.

Table 6.2 Rootstocks used in the pathogenicity tests, and the breeding programs from which they were obtained (Bielsa and Rubio-Cabetas, 2018).

Rootstock	Species	Genetic background	Breeding programs
'Garnem'	*P. dulcis* x *P. persica*	'Garfi' x 'Nemared'	CITA[1], Spain
'GF-677'	*P. dulcis* x *P. persica*	Open-pollinated	INRAE[2], France
'Rootpac-40'	(*P. dulcis* x *P. persica*) x (*P. dulcis* x *P. persica*)	('Marcona' x 'Nemaguard') x 'Felinem'	Agromillora Ibérica, Spain

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[2]Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement.

For inoculation, 20 g of inoculum homogenized with 200 g of potting mix that contained peat, vermiculite and sand (1:1:1, v/v/v), and equally autoclaved 3 times prior to use, were added to 350 mL pots. Rootstocks were planted by making a cavity in the pot previously filled with the mixture, then covering the plant with potting mix until the root was completely covered. The control plants were inoculated with a non-infected inoculum mixture. Five plants were inoculated for each isolate plus the negative control making a total of 16 inocula x 3 rootstocks x 5 replicates = 240 plants, and the experiment was repeated twice. All the plants were randomly distributed in a growth chamber with a 12-hour day and night photoperiod at 23 ºC. All the seedlings were watered the day before inoculation. Immediately after inoculation, seedlings were flooded for 48 h and flooding repeated every 2 weeks to stimulate zoosporangial formation, as previously described by Pérez-Sierra et al. (2013).

***Pathogenicity evaluation*.** Disease severity evaluations for each plant started 10 days after inoculation, and were then performed every 4 days until the experiment ended 90 days after inoculation. At each evaluation, disease severity was evaluated on this following scale: 0 = asymptomatic, 1 = foliar yellowing, 2 = wilting, dieback and defoliation, 3 = dead plant (Jönsson et al., 2003). With the severity values of each plant over time, the Area Under the Disease Progress Curve (AUDPC) was calculated by the trapezoidal integration method (Campbell and Madden, 1990).

At the end of the experiment, seedlings were uprooted, and the root system carefully washed under running water to remove substrate. Re-isolations were done from one symptomatic seedling showing wilting and defoliation (rating scale 2) per oomycete isolate to confirm Koch's postulates. For this purpose, small segments from the primary and secondary roots showing browning and necrosis symptoms were cut. These plant tissue fragments were disinfested with

70% ethanol for 30 s and then rinsed with sterile distilled water and allowed to dry on sterile absorbent paper. Pieces (1-2 mm long) were cut in a laminar flow chamber and plated onto Petri dishes with CMA-PARPB medium and CMA-PARPBH (CMA-PARPB amended with 0.069 g liter^{-1} Hymexazol) and then incubated at 25 °C for 72 h (Jeffers and Martin, 1986). Pure cultures were obtained when small pieces from the margins of colonies were transferred to potato dextrose agar medium (PDA, Biokar-Diagnostics, Beauvais, France) for their subsequent identification (Erwin and Ribeiro, 1996).

For isolate identification, the ITS region of the ribosomal DNA of the isolates was amplified using universal primers ITS-6 (5' GAA GGT GAA GTC GTA ACA AGG 3') (Cooke et al., 2000) and ITS-4 (5' TCC TCC GCT TAT TGA TAT GC 3') (White et al., 1990). Genomic DNA extraction, PCR reactions and sequencing was performed as described before for *cox*1.

The root dry weight of the root biomass of each seedling was measured. For this purpose, roots were separated from the main stem and shoots by cutting at the root crown. Then they were placed inside paper bags and dried for 5 days in an oven at 35 ºC. Root dry weight was recorded.

***Statistical analyses*.** A non-parametric Kruskal-Wallis analysis was performed for the AUDPC and the root dry weight. When there were statistically significant differences, medians were classified into homogeneous groups by Dunn's test at the 95% confidence level. All the calculations were performed using the InfoStat-2004 statistical software.

The survival probability of the seedlings inoculated with the different oomycete isolates was studied with the Kaplan-Meier estimate (Goel et al., 2010). To test for statistical differences in the inoculated plants' survival probabilities, the log-rank test was used (Collett, 2003). This non parametric analysis was carried out using STATGRAPHICS Centurion XVI.

RESULTS

All the rootstock seedlings inoculated with the 12 oomycetes species showed root symptoms, such as dark necrotic lesions and loss of fine roots (Figure 6.1). These symptoms were associated with a general aerial decline in seedlings including yellowing, wilting and defoliation, which resulted in variable disease severity values. Re-isolations from symptomatic roots confirmed pathogenicity for all the inoculated species. In contrast, the control seedlings remained healthy and it was not possible to isolate oomycetes from their roots.

Figure 6.1 Examples of the root symptoms observed during the pathogenicity tests conducted on three different *Prunus* hybrid rootstocks. (a) Control rootstock 'Garnem'; (b) *Pp. helicoides* (PAL-96) on 'Garnem'; (c) *Pp. helicoides* (PAL-96) on 'Rootpac-40'; (d) *Ph. multivora* (PAL-103) on 'GF-677'; (e) *Ph. multivora* (PAL-103) on 'Garnem'; (f) *Ph. niederhauserii* (PAL-100) on 'Garnem'; (g) *Ph. niederhauserii* (PAL-100) on 'GF-677'; (h) *Ph. nicotianae* (PAL-71) on 'Rootpac-40'; and (i) *G. ultimum* var. *ultimum* (PAL-58) on 'GF-677'.

The progress of the mean disease severity values obtained throughout a 90-day period after inoculation is shown in Figure 6.2. The 'Rootpac-40' seedlings generally obtained higher mean disease severity values than those observed for rootstocks 'Garnem' and 'GF-677'. Severe disease symptoms (between 2 and 3 according to the rating scale) started sooner in 'Rootpac-40' than in the other rootstocks, specifically 1 month after inoculation, and in most inoculated isolates.

The isolate PAL-103 of *Ph. multivora* (P.M. Scott and T. Jung) was particularly virulent. Seedlings began to show symptoms 10 days after inoculation, with mean severity values of 0.6 and 0.7 in 'Garnem' and 'GF-677', respectively, and 1.6 in rootstock 'Rootpac-40'. This resulted in all the seedlings dying 22 days after inoculation in the three evaluated rootstocks. The second species to reach the maximum mean severity was *Pp. helicoides,* in which all the seedlings died 50 days after inoculation, and also for the three rootstocks. For the remaining isolates, the inoculated seedlings generally reached high mean disease severity values (over 2). Isolates of *Ph. niederhauserii* showed intraspecific variability. Isolates PAL-21 and PAL-62 reached lower mean disease severity values than isolates PAL-74 and PAL-100 in rootstocks 'Garnem' and 'GF-677', but the highest mean disease severity value in rootstock 'Rootpac-40' was obtained for isolate PAL-62.

The mean AUDPC values are shown in Figure 6.3. There was wide variability among the different isolates, as well as statistically significant differences ($P \leq 0.05$). The mean AUDPC values varied from 28.2 obtained by isolate PAL-21 of *Ph. niederhauserii* in rootstock 'GF-677' to 235.2 obtained by isolate PAL-103 of *Ph. multivora* in rootstock 'Rootpac-40'. In the three evaluated rootstocks, isolates PAL-21 of *Ph. niederhauserii*, PAL-16 of *Ph. citrophthora* and PAL-99 of *Pp. chamaehyphon* [(Sideris) Abad, de Cock, Bala, Robideau, A.M. Lodhi and Lévesque] obtained low mean AUDPC values, and showed statistically significant differences ($P \leq 0.05$) with isolate PAL-103 of *Ph. multivora*, which had the highest AUDPC for the three evaluated rootstocks.

The mean root dry weight results appear in Figure 6.4, which also displays wide variability between oomycete isolates. In the three rootstocks, there were statistically significant differences ($P \leq 0.05$) for some isolates compared to the control. In rootstock 'GF-677', mean root dry weight of the uninoculated control was lower than the values obtained for some of the isolates inoculated, but only statistically significant compared with isolate PAL-16 of *Ph. citrophthora*. In rootstocks 'Garnem' and 'GF-677', the lowest mean root dry weight value went to seedlings inoculated with isolate PAL-103 of *Ph. multivora*, with a value of 0.07 g and 0.31 g, respectively, followed by those seedlings inoculated with isolate PAL-96 of *Pp. helicoides* with a value of 0.14 g and 0.36

g, respectively. In these two rootstocks, these differences represented a root dry weight reduction of 92.4% and 57.5% in relation to the uninoculated control for isolate PAL-103 of *Ph. multivora*, and of 84.9% and 50.6% for isolate PAL-96 of *Pp. helicoides*, both respectively. For rootstock 'Rootpac-40', the lowest mean root dry weight value was caused by isolate PAL-71 of *Ph. nicotianae* (Breda de Haan) (0.64 g), which represented a 49.2% reduction in root dry weight compared to the control.

Plots showing the survival probabilities of the inoculated seedlings are in Figure 6.5. According to the *P*-value of the log-Rank test ($P \leq 0.05$), there were statistically significant differences among the survival curves of the three inoculated rootstocks. In the evaluated three rootstocks, isolates PAL-103 of *Ph. multivora* and PAL-96 of *Pp. helicoides* displayed a 100% plant mortality at the end of the trial, with the lowest survival probability 50 days after inoculation. On the contrary, on rootstocks 'Garnem' and 'GF-677', isolates PAL-4 of *Ph. cactorum*, PAL-16 of *Ph. citrophthora* and PAL-21 and PAL-62 of *Ph. niederhauserii*, and on rootstock 'Rootpac-40', isolate PAL-101 of *Ph. tropicalis* (Aragaki & J.Y. Uchida) caused no plant mortality at the end of the trial. Hence, the survival probability of these isolates/rootstocks combinations was the highest throughout the trial.

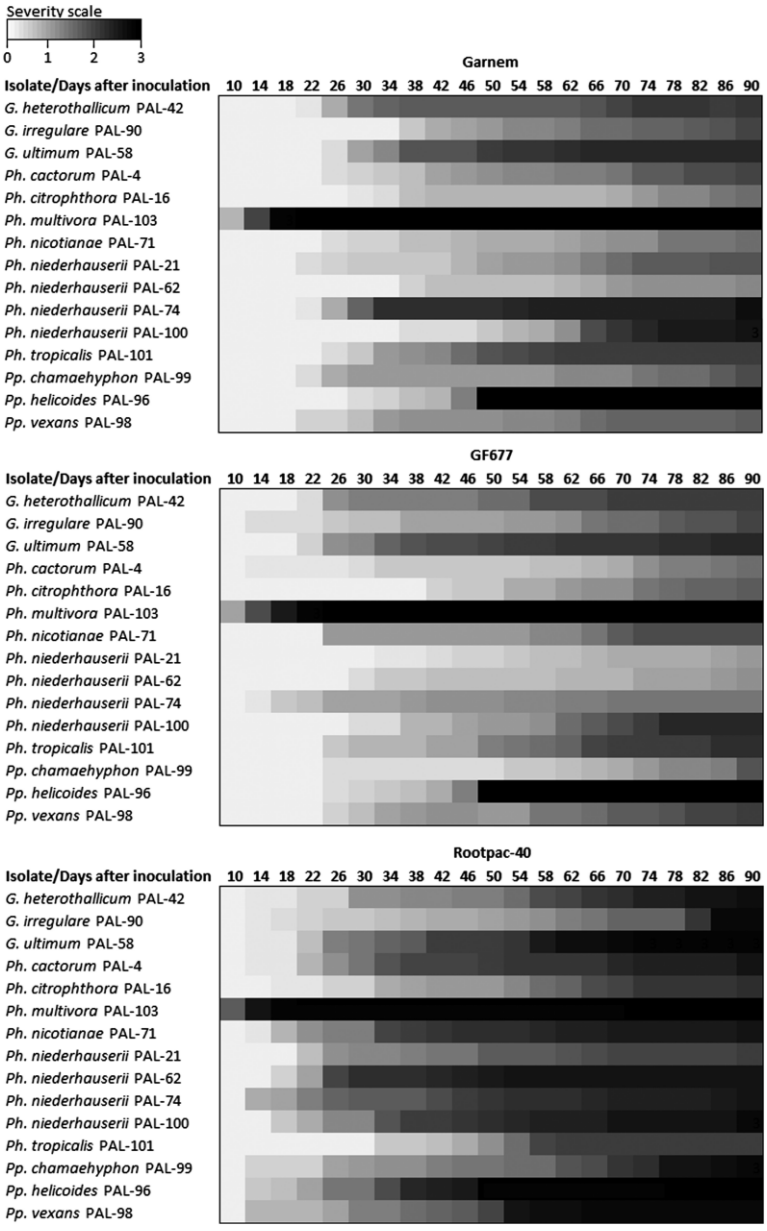

Figure 6.2 Heatmap showing the progress of the mean disease severity values obtained over a 90-day period after the inoculation of three *Prunus* hybrids rootstocks with 15 isolates of 12 oomycete species. Disease severity was evaluated using a 0-3 rating scale (lighter squares represent the lowest disease severity, and darker squares denote the highest disease severity).

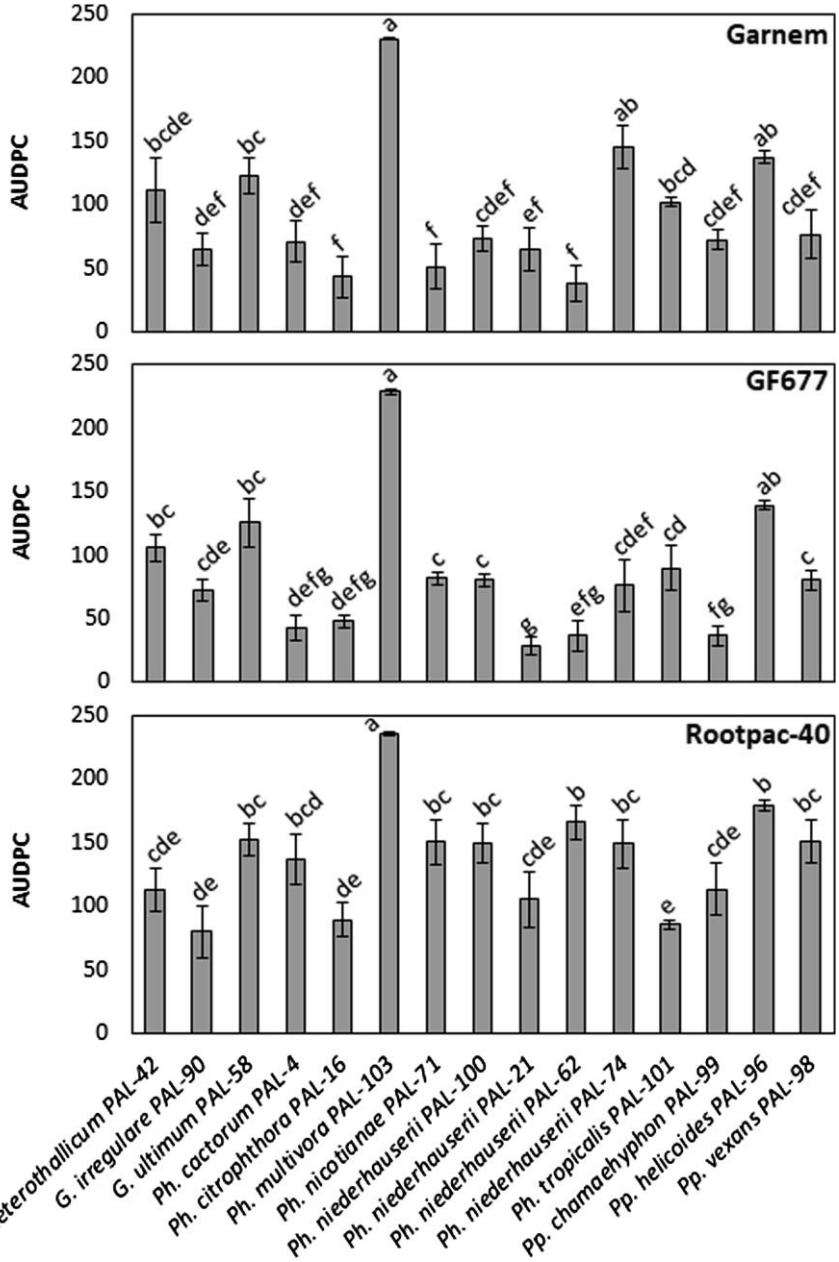

Figure 6.3 Area Under the Disease Progress Curve (AUDPC) of rootstocks 'Garnem', 'GF-677' and 'Rootpac-40' inoculated with 15 different oomycete isolates. Bars with different letters indicate statistically significant differences according to Dunn's test ($P \leq 0.05$).

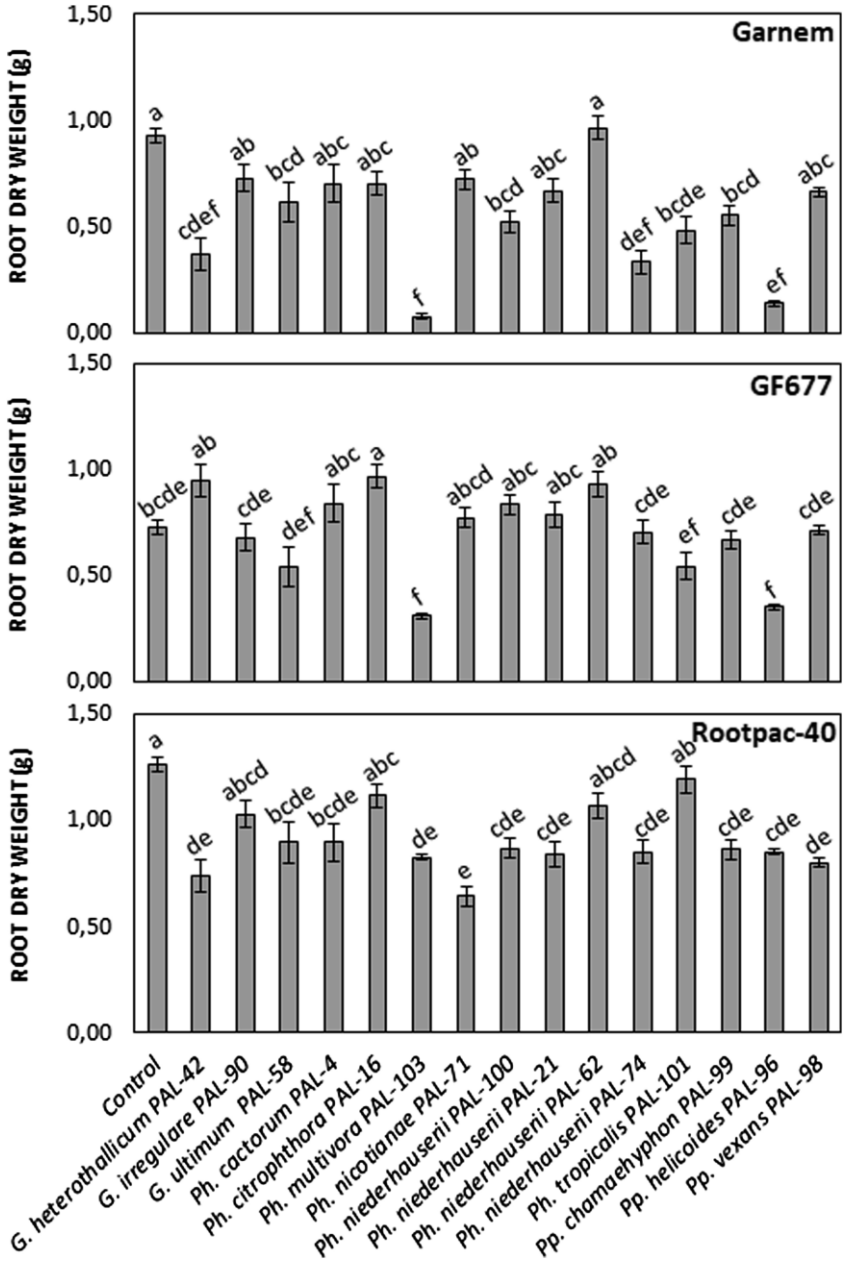

Figure 6.4 Mean root dry weight (± standard error) of hybrid rootstocks 'Garnem', 'GF-677', and 'Rootpac-40' inoculated with 15 different oomycete isolates, and the uninoculated control. Bars with different letters represent statistically significant differences according to Dunn's test ($P \leq 0.05$).

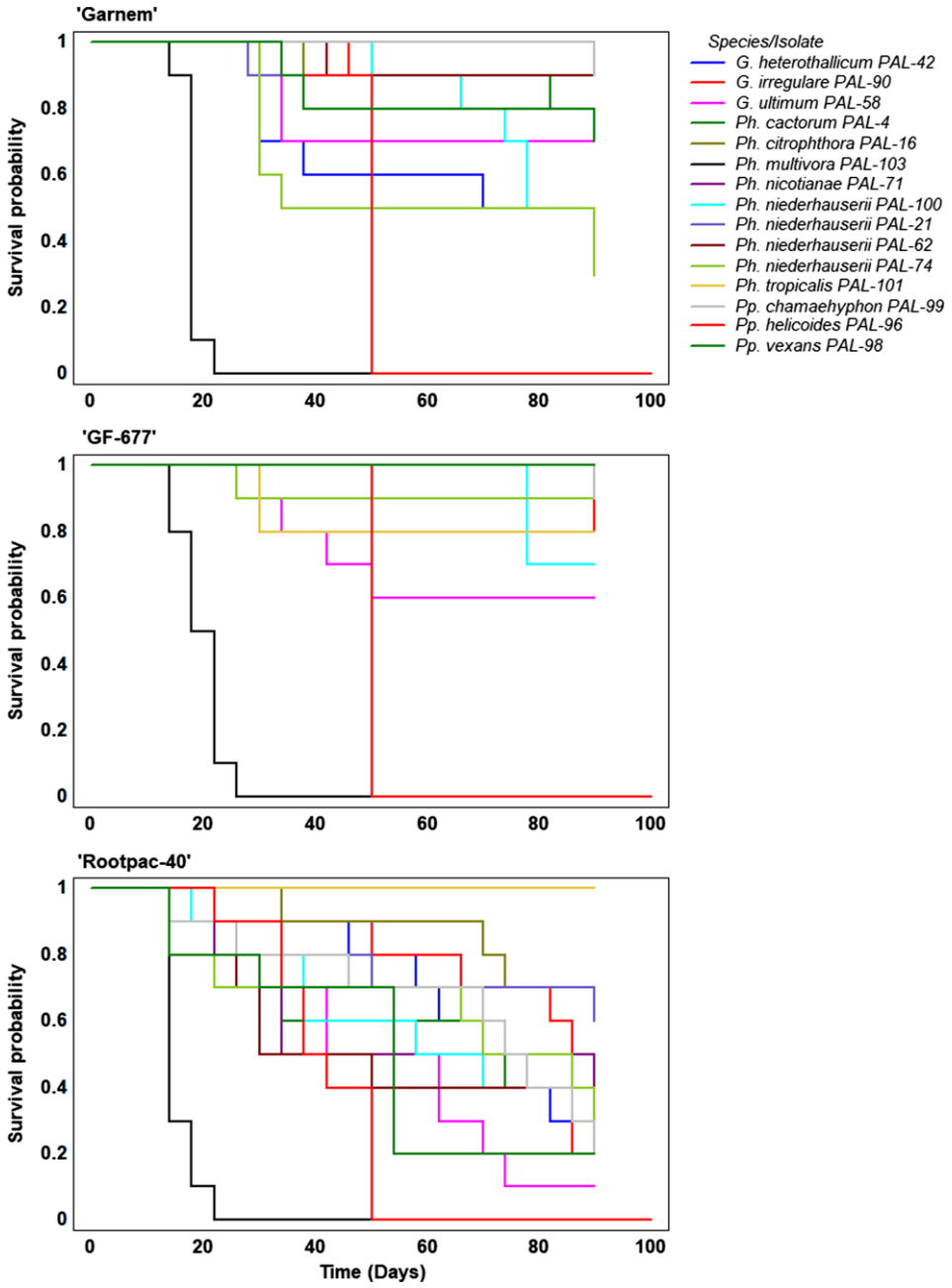

Figure 6.5 Survival probabilities plot obtained using the Kaplan-Meier estimate ($P \leq 0.00$) of the survival function for rootstocks 'Garnem', 'GF-677' and 'Rootpac-40' inoculated with 15 different oomycete isolates.

DISCUSSION

All the isolates included in the pathogenicity tests were pathogenic to the rootstock seedlings and were re-isolated from root lesions. Nevertheless, large differences in virulence were detected between the different oomycete species and *Ph. niederhauserii* isolates for each rootstock evaluated for disease severity, AUDPC, root dry weight and survival probability.

In this study isolate PAL-103 of *Ph. multivora* was the most virulent. This species is currently considered as an important emerging pathogen because of its worldwide distribution, being associated with a wide range of hosts in nurseries, woody plants in urban environments and natural ecosystems (Migliorini et al., 2019), such as citrus (Meitz-Hopkins et al., 2014), avocado (Rodríguez-Padrón et al., 2018), and macadamia (Jeff-Ego et al., 2021). According to our results, this pathogen significantly reduced the root dry weight of rootstocks in relation to the control. This effect has been previously shown on inoculations of *Ph. multivora* on *Eucalyptus gomphocephala* (DC.) (Scott et al., 2012), *Corymbia calophylla* [(Lindl.) K.D. Hill and L.A.S. Johnson] (Mrázková et al., 2013), and *E. marginata* (Sm.) (Belhaj et al., 2018). In Western Australia, *Ph. multivora* geographical distribution is considerable, and it is active on calcareous soils (Migliorini et al., 2019), which are characteristic of the main Mediterranean stone fruit and almond production areas in Spain. Jung and Burgess (2009) indicated that this pathogen is better adapted to dry climates due to its oospores being thick walled. As far as we know, *Ph. multivora* has not been described to cause infections in stone fruit or almond trees orchards, but our results indicate that this species could represent a serious potential threat to the rootstocks used in these crops.

Isolate PAL-96 of *Pp. helicoides* also performed high virulence. This species has a polyphagous characteristic because it has been reported in fruit crops like kiwifruit and mandarin (Chen et al., 2016; Wang et al., 2015), strawberry (Ishiguro et al., 2014; Marin et al., 2019; Zhan et al., 2020), pistachio (Fichtner et al., 2016), cereals like maize (Xie et al., 2021) and ornamental plants (Chen et al., 2021; Yang et al., 2013), but its worldwide distribution is still limited or unknown. Although its pathogenicity has been widely documented in China and the United States, information on *Prunus* crops is very scarce. Only the recent studies by Browne et al. (2019) have studied the pathogenicity of *Pp. helicoides* on the 'Nemaguard' rootstock seedlings in California, which caused a fresh root weight reduction and significant root cortex necrosis levels. Subsequently, Beluzán et al. (2022) isolated *Pp. helicoides* from soil (using baiting techniques) in almond orchards in Spain without determining its pathogenicity.

The four evaluated isolates of *Ph. niederhauserii* performed different degrees of virulence. Although intraspecific variability has not been studied in *Ph. niederhauserii*, it has been previously mentioned in the literature for other *Phytophthora* species (Kurbetli et al., 2020; Tian and Babadoost, 2004). Root dry weight reduction and decreased plant survival probability could have been caused by the reduction in lateral roots due to the necrosis produced by this oomycete. This symptom has been previously reported by Rodríguez-Padrón et al. (2018) and Kurbetli et al. (2020) for avocado and pomegranate, respectively. These researches have positioned *Ph. niederhauserii* as a pathogen with more or equal virulence than *Ph. nicotianae*, which coincides with our results in *Prunus* rootstocks.

Another oomycete with high virulence was isolate PAL-58 of *G. ultimum* var. *ultimum* [(Trow) Uzuhashi, Tojo & Kakish]. This pathogen, along with *Pythium* spp. and some fungi, are known to cause damping-off in more than 300 hosts (Toribio et al., 2021). More specifically, *G. ultimum* and *G. irregulare,* together with other *Pythium* species, have been described to cause Prunus Replant Disease (PRD) (Schmidt and Browne, 2013). The authors of this study indicated that the fresh weight of the 'Nemaguard' rootstock is significantly affected when inoculated with *G. ultimum* and *G. irregulare* due to the necrosis produced by both root pathogens. Their results partially agree with ours. Although *G. ultimum* var. *ultimum* was able to significantly reduce root dry weight in rootstocks 'Garnem' and 'Rootpac-40', isolate PAL-90 of *G. irregulare* was unable to significantly reduce it in the three evaluated rootstocks. Research carried out in the 1980s by Smither and Jones (1989) reported low virulence of *G. irregulare* when inoculated into *P. mahaleb* (L.) seedlings. Regarding plant mortality caused by *G. irregulare*, our results partially differ from those obtained by Smither and Jones (1989), because we found that *G. irregulare* caused high mortality in 'Rootpac-40'.

Regarding isolate PAL-42 of *G. heterothallicum* [(W.A. Campb. and F.F. Hendrix) Uzuhashi, Tojo and Kakish.], in the last decade this species has been associated with grapevine (Spies et al., 2011) and soybean (Rojas et al., 2017), and its pathogenicity has been confirmed in pepper (Derviş et al., 2020) and kiwifruit (Türkkan et al., 2022), but there are no reports about its pathogenicity to stone fruit trees. As to how the disease caused by *G. heterothallicum* progresses, Derviş et al. (2020) showed that pepper plants inoculated with this oomycete withered in the first week and died within 3 weeks after inoculation due to severe root and crown rot, as it happened in our pathogenicity test. A very recent study by Türkkan et al. (2022) in kiwifruit indicated *G. heterothallicum* as a pathogen with a significantly lower virulence than that caused by *Pp. vexans*. These results

disagree with our results, as both pathogens did not present statistically significant differences in severity according to their AUDPC values. It is important to note that *G. heterothallicum* abundance increases in alkaline soils and at high temperatures (Rojas et al., 2017), such as those used for stone fruit and almond production in the Mediterranean Basin of Spain.

For isolate PAL-98 of *Pp. vexans*, the pathogenicity tests showed a statistically significant reduction in root dry weight in 'Rootpac-40' and high plant mortality. *Phytopythium vexans* has been considered a secondary pathogen in peach trees (Mircetich, 1971). Nevertheless, this oomycete has been confirmed as the main causal agent of root diseases in apple trees (Jabiri et al., 2020), avocado (Rodríguez-Padrón et al., 2018), citrus (Benfradj et al., 2017; Noireung et al., 2020), grapevine (Langenhoven et al., 2018), kiwifruit (Polat et al., 2017; Prencipe et al., 2020; Türkkan et al., 2022) and potato (Santika et al., 2021). Pathogenicity studies of *Pp. vexans* in *Prunus* are scarce, but there has been a recent report about it causing root and crown rot in flowering cherry [*P. serrulata* (Lindl.)] in a nursery in Tennessee, USA (Baysal-Gurel et al., 2021), and also in commercial almond orchards and nurseries (Beluzán et al., 2022).

Isolate PAL-99 of *Pp. chamaehyphon* performed medium virulence compared to the other evaluated oomycetes. This oomycete has been isolated from diverse crops of different botanical families worldwide (Rai et al., 2020), but its pathogenicity has not been widely documented in fruit trees. Recently, a study by Savian et al. (2021) reported the pathogenicity of *Pp. chamaehyphon* to be the cause of Kiwifruit Vine Decline Syndrome (KVDS) in Italy, which causes leaf wilt and root rot in greenhouse trials and commercial orchards.

For isolate PAL-71 of *Ph. nicotianae*, the highest virulence was found in rootstock 'Rootpac-40', showing severe defoliation. Similar results were obtained by Pane et al. (2009), who previously reported that this pathogen affected 1-year-old apricot seedlings in a nursery in Italy by producing leaf yellowing, wilting and defoliation associated with root and crown rot.

Infections caused by *Ph. tropicalis* have been reported in several ornamental crops and in some fruit trees (Uchida and Kadooka, 2013). The only report of this pathogen on *Prunus* was on 1-year-old plants in apricot nurseries in southern Italy, where it caused symptoms of leaf yellowing, wilting and defoliation, which were associated with root and crown rot in these plants (Pane et al., 2009). These symptoms are consistent with those obtained in our study for isolate PAL-101, which caused a statistically significant root dry weight reduction in rootstock 'Garnem'.

The pathogenicity of *Ph. cactorum* and *Ph. citrophthora* has already been reported on stone fruit trees (Browne, 2017; Thomidis, 2000; Thomidis, 2001; Thomidis, 2003c; Thomidis et al., 2008). In our study, isolate PAL-4 (*Ph.*

cactorum) significantly reduced root dry weight only in rootstock 'Rootpac-40' and PAL-16 (*Ph. citrophthora*) only in rootstock 'GF-677'. However, for this last isolate, this difference is due to the fact that the uninoculated control presented an unusually low mean root dry weight value. Although the rootstock seedlings were selected with a homogeneous height, perhaps some of them had less developed roots, affecting the mean root dry weight value of the control and, moreover, PAL-16 isolate was not very virulent. In both cases, the severity in 'Garnem' and 'GF-677' was medium and plant mortality was low. Opposite results were obtained by Thomidis (2003c), who pointed out that species *Ph. cactorum* and *Ph. citrophthora* are highly virulent in stone fruit trees. Subsequent research by Thomidis et al. (2008) concluded that *Ph. cactorum* was the most virulent species in 30 different genotypes of cherries.

The results of the present research offer substantial contribution to increase our knowledge about the pathogenicity of several oomycete species that are frequently isolated in *Prunus* orchards, and the potential risks that they pose for *Prunus* spp. crops. Our results and the methodology we used, complemented with additional field trials, could also have immediate implications for the selection of rootstocks for almond and stone fruit cultivation.

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References

Abad, G., Burgess, T., Redford, A., Bienapfl, J., Mathew, R., Srivastava, S. and Jennings, K. 2022. IDphy: An international online resource for molecular and morphological identification of *Phytophthora*. Plant Dis., (ja).

Bala, K., Robideau, G., Désaulniers, N., De Cock, A. and Lévesque, C. 2010. Taxonomy, DNA barcoding and phylogeny of three new species of *Pythium* from Canada. Pers.: Mol. Phylogeny Evol. Fungi. 25:22-31.

Baysal-Gurel, F., Liyanapathiranage, P., Panth, M., Avin, F. and Simmons, T. 2021. First report of *Phytopythium vexans* causing root and crown rot on flowering cherry in Tennessee. Plant Dis. 105:232.

Belhaj, R., McComb, J., Burgess, T. and Hardy, G. 2018. Pathogenicity of 21 newly described *Phytophthora* species against seven Western Australian native plant species. Plant Pathol. 67:1140-1149.

Beluzán, F., Miarnau, X., Torguet, L., Armengol, J. and Abad-Campos, P. 2022. Survey of oomycetes associated with root and crown rot of almond in Spain and pathogenicity of *Phytophthora niederhauserii* and *Phytopythium vexans* to 'Garnem' rootstock. Agriculture 12:294.

Benfradj, N., Migliorini, D., Luchi, N., Santini, A. and Boughalleb-M'Hamdi, N. 2017. Occurrence of *Pythium* and *Phytopythium* species isolated from citrus trees infected with gummosis disease in Tunisia. Arch. Phytopathol. Pflanzenschutz 50:286-302.

Bielsa, B. and Rubio-Cabetas, M. 2018. La elección del patrón en la almendricultura moderna. Rev. frutic. 65:92-113.

Browne, G. 2017. Resistance to *Phytophthora* species among rootstocks for cultivated *Prunus* species. HortScience, 52:1471-1476.

Browne, G. and Mircetich, S. 1995. *Phytophthora* root and crown rots. In: Compendium of stone fruit diseases; Ogawa, J., Zehr, E., Bird, G., Ritchie, D., Uriu, K. and Uyemoto, J., Eds.; The American Phytopathological Society, 1995 pp. 38-40.

Browne, G., Ott, N. and Fichtner, E. 2019. First report of *Phytopythium helicoides* causing root rot on peach rootstock in California. Plant Dis. 103:2968.

Browne, G., Ott, N., Forbes, H., Yaghmour, M. and Milliron, L. 2020. First report of *Phytophthora chlamydospora* causing crown and root rot on almond in California. Plant Dis. 104:2033-2033.

Browne, G., Schmidt, L. and Brar, G. 2015. First report of *Phytophthora niederhauserii* causing crown rot of almond (*Prunus dulcis*) in California. Plant Dis. 99:1863-1863.

Campbell, C. and Madden, L. 1990. Introduction to Plant Disease Epidemiology. New York, NY: John Wiley and Sons.

Chen, X., Liu, B., Xing, Y., Cheng, B., Liu, M., Tong and Xu, J. 2016. Identification and characterization of *Phytopythium helicoides* causing stem rot of Shatangju mandarin seedlings in China. Eur. J. Plant Pathol. 146:715-727.

Chen, Z., Yang, X., Xu, J., Jiao, B., Li, Y., Xu, Y. and Dai, T. 2021. First report of *Phytopythium helicoides* causing crown and root rot of *Rhododendron pulchrum* in China. Plant Dis. 105:713-713.

Collado-Romero, M., Mercado-Blanco, J., Olivares-García, C., Valverde-Corredor, A. and Jiménez-Díaz, R. 2006. Molecular variability within and among *Verticillium dahliae* vegetative compatibility groups determined by fluorescent amplified fragment length polymorphism and polymerase chain reaction markers. Phytopathology 96:485-495.

Collett, D. 2003. Modelling survival data in medical research. Boca Raton: Chapman and Hall/CRC.

Cooke, D., Drenth, A., Duncan, J., Wagels, G. and Brasier, C. 2000. A molecular phylogeny of *Phytophthora* and related oomycetes. Fungal Genet. Biol. 30:17-32.

Derviş, S., Özer, G., Türkölmez, Ş. and Çiftçi, O. 2020. First report of *Globisporangium heterothallicum* causing root and crown rot of pepper in Turkey. New Dis. Rep. 41:36.

Elena, K. and Tsipouridis, K. 2000. Evaluation of resistance of stone fruit rootstocks to *Phytophthora* crown rot. J. Phytopathol. 148:365-369.

Erwin, D. and Ribeiro, O. 1996. *Phytophthora* diseases worldwide. American Phytopathological Society (APS Press), 562 pp.

FAOSTAT, Food and Agriculture Organization of the United Nations. 2021. Available online: <https://www.fao.org/faostat/es/#data/QCL> (accessed on 26 October 2021).

Felipe, A. 2009. 'Felinem', 'Garnem' and 'Monegro' almond × peach hybrid rootstocks. HortScience 44: 196-197.

Fichtner, E., Browne, G., Mortaz, M., Ferguson, L. and Blomquist, C. 2016. First report of root rot caused by *Phytopythium helicoides* on pistachio rootstock in California. Plant Dis. 100:2337-2337.

Gainza, F., Opazo, I., Guajardo, V., Meza, P., Ortiz, M., Pinochet, J. and Muñoz, C. 2015. Rootstock breeding in *Prunus* species: Ongoing efforts and new challenges. Chil. J. Agric. Res. 75:6-16.

Goel, M., Khanna, P. and Kishore, J. 2010. Understanding survival analysis: Kaplan-Meier estimate. Int. J. Ayurveda Res. 1:274.

Ishiguro, Y., Otsubo, K., Watanabe, H., Suzuki, M., Nakayama, K., Fukuda, T. Fujinaga, M., Suga, H. and Kageyama, K. 2014. Root and crown rot of strawberry caused by *Pythium helicoides* and its distribution in strawberry production areas of Japan. J. Gen. Plant Pathol. 80:423-429.

Jabiri, S., Lahlali, R., Bahra, C., Bendriss Amraoui, M., Tahiri, A. and Amiri, S. 2020. First report of *Phytopythium vexans* associated with dieback disease of apple trees in Morocco. J. Plant Pathol. 102:1319-1319.

Jeff-Ego, O., Topp, B., Drenth, A., Henderson, J. and Akinsanmi, O. 2021. Resistance in wild macadamia germplasm to *Phytophthora cinnamomi* and *Phytophthora multivora*. Ann. Appl. Biol. 178:519-526.

Jeffers, S. and Martin, S. 1986. Comparison of two media selective for *Phytophthora* and *Pythium* species. Plant Dis. 70:1038-1043.

Jiménez, S., Pinochet, J., Romero, J., Gogorcena, Y., Moreno, M. and Espada, J. 2011. Performance of peach and plum based rootstocks of different vigour on a late peach cultivar in replant and calcareous conditions. Sci. Hortic. 129:58-63.

Jönsson, U., Jung, T., Rosengren, U., Nihlgård, B. and Sonesson, K. 2003. Pathogenicity of Swedish isolates of *Phytophthora quercina* to *Quercus robur* in two different soils. New Phytol. 158:355-364.

Jung, T. and Burgess, T. 2009. Re-evaluation of *Phytophthora citricola* isolates from multiple woody hosts in Europe and North America reveals a new species, *Phytophthora plurivora* sp. nov. Pers.: Mol. Phylogeny Evol. Fungi. 22:95-110.

Jung, T., Blaschke, H. and Neumann, P. 1996. Isolation, identification and pathogenicity of *Phytophthora* species from declining oak stands. Eur J. Forest Pathol. 26:253-272.

Kurbetli, İ., Karaca, G., Aydoğdu, M. and Sülü, G. 2020. *Phytophthora* species causing root and collar rot of pomegranate in Turkey. Eur. J. Plant Pathol. 157:485-496.

Langenhoven, S. D., Halleen, F., Spies, C. F., Stempien, E. and Mostert, L. 2018. Detection and quantification of black foot and crown and root rot pathogens in grapevine nursery soils in the Western Cape of South Africa. Phytopathol. Mediterr. 57:519-537.

Marin, M., Seijo, T., Mertely, J. and Peres, N. 2019. First report of crown rot caused by *Phytopythium helicoides* on strawberry in the Americas. Plant Dis. 103:2696.

Martin, F. and Tooley, P. 2003. Phylogenetic relationships among *Phytophthora* species inferred from sequence analysis of mitochondrially encoded cytochrome oxidase I and II genes. Mycologia. 95:269-284.

Mehri, H., Mehri-Kamoun, R. and Hibar, K. 2009. In Vitro evaluation of resistance of *Pyrus syriaca*, a pear-tree rootstock, to *Phytophthora* crown rot. Afr. J. Plant Sci. Biotechnol. 3:41-43.

Meitz-Hopkins, J., Pretorius, M., Spies, C., Huisman, L., Botha, W., Langenhoven, S. and McLeod, A. 2014. *Phytophthora* species distribution in South African citrus production regions. Eur. J. Plant Pathol. 138:733-749.

Migliorini, D., Khdiar, M., Rodríguez-Padrón, C., Vivas, M., Barber, P., Hardy, G. and Burgess, T. 2019. Extending the host range of *Phytophthora multivora*, a pathogen of woody plants in horticulture, nurseries, urban environments and natural ecosystems. Urban For. Urban Green. 46:126460.

Mircetich, S. 1971. The Role of *Pythium* in feeder roots of diseased and symptomless peach trees. Pathology 61:357-360.

Mora-Sala, B., León, M., Pérez-Sierra, A. and Abad-Campos, P. 2022 New reports of *Phytophthora* species in plant nurseries in Spain. Pathogens 11:826.

Mrázková, M., Černý, K., Tomšovský, M., Strnadová, V., Gregorová, B., Holub, V., Pánek, M., Havrdová, L. and Hejná, M. 2013. Occurrence of *Phytophthora multivora* and *Phytophthora plurivora* in the Czech Republic. Plant Prot. Sci. 49:155-164.

Noireung, P., Intaparn, P., Maumoon, R., Wongwan, T. and To–anun, C. 2020. First Record of *Phytopythium vexans* causing root rot on Mandarin (*Citrus reticulate* L. cv. Sainampueng) in Thailand. Plant Pathol Quar. 10:85-90.

Pane, A., Cacciola, S., Scibetta, S., Bentivenga, G. and Magnano di San Lio, G. 2009. Four *Phytophthora* species causing foot and root rot of apricot in Italy. Plant Dis. 93:844-844.

Pérez-Sierra, A., León, M., Álvarez, L., Alaniz, S., Berbegal, M., García-Jiménez, J. and Abad-Campos, P. 2010. Outbreak of a new *Phytophthora* sp. associated with severe decline of almond trees in eastern Spain. Plant Dis. 94:534-541.

Pérez-Sierra, A., López-García, C., León, M., García-Jiménez, J., Abad-Campos, P. and Jung, T. 2013. Previously unrecorded low-temperature *Phytophthora* species associated with *Quercus* decline in a Mediterranean forest in eastern Spain. For. Pathol. 43:331-339.

Polat, Z., Awan, Q., Hussain, M. and Akgül, D. 2017. First report of *Phytopythium vexans* causing root and collar rot of kiwifruit in Turkey. Plant Dis. 101:1058.

Prencipe, S., Savian, F., Nari, L., Ermacora, P., Spadaro, D. and Martini, M. 2020. First report of *Phytopythium vexans* causing decline syndrome of *Actinidia deliciosa* 'Hayward' in Italy. Plant Dis. 104:2032.

Rai, M., Abd-Elsalam, K. and Ingle, A. 2020. Pythium: diagnosis, diseases and management. CRC Press.

Reighard, G. and Loreti, F. 2008. Rootstock development. In: Peach, botany, production and uses. Layne, D. and Bassi, D., Eds. CAB International, 2008; pp. 193-220.

Robideau, G., De Cock, A., Coffey, M., Voglmayr, H., Brouwer, H., Bala, K., Chitty, D., Désaulniers, N., Eggertson, Q., Gachon, C., Hu, C., Küpper, F., Rintoul, T., Sarhan, E., Verstappen, E., Zhang, Y., Bonants, P., Ristaino, J. and Lévesque, C. 2011. DNA barcoding of oomycetes with cytochrome c oxidase subunit I and internal transcribed spacer. Mol. 11:1002-1011.

Rodríguez-Padrón, C., Siverio, F., Pérez-Sierra, A. and Rodríguez, A. 2018. Isolation and pathogenicity of *Phytophthora* species and *Phytopythium vexans* recovered from avocado orchards in the Canary Islands, including *Phytophthora niederhauserii* as a new pathogen of avocado. Phytopathol. Mediterr. 57:89-106.

Rojas, J., Jacobs, J., Napieralski, S., Karaj, B., Bradley, C., Chase, T., Esker, P., Giesler, L., Jardine, D., Malvick, D., Markell, S., Nelson, B., Robertson, A., Rupe, J., Smith, D., Sweets, L., Tenuta, A., Wise, K. and Chilvers, M. 2017. Oomycete species associated with soybean seedlings in North America Part II: Diversity and ecology in relation to environmental and edaphic factors. Phytopathology, 107:293-304.

Rubio-Cabetas, M., Felipe, A. and Reighard. G. 2017. Rootstock development. In: Almonds, botany, production and uses; Socias i Company, R. and Gradziel, T., Eds.; CAB International, Boston, MA, 2017; pp. 209-227.

Santika, I., Widiastuti, A. and Wibowo, A. 2021. First report of *Phytopythium vexans* (de Barry) Abad, de Cock, Bala, Robideau, Lodhi and Lévesque causing potato tuber rot in Indonesia. Jurnal Perlindungan Tanaman Indonesia 25:173-181.

Savian, F., Prencipe, S., Filippini, N., Nari, L., Martini, M., Ermacora, P. and Spadaro, D. 2021. Pathogenicity of *Phytopythium chamaehyphon*: A New Player in Kiwifruit Vine Decline Syndrome of *Actinidia chinensis* var. *deliciosa* 'Hayward' in Italy. Plant Dis. 105:2781-2784.

Schmidt, L. and Browne, G. 2013 Characterization of *Pythium* species associated with Prunus replant disease. (Abstr.) Phytopathology 103:S2.128

Scott, P., Jung, T., Shearer, B., Barber, P., Calver, M. and Hardy, G. 2012. Pathogenicity of *Phytophthora multivora* to *Eucalyptus gomphocephala* and *Eucalyptus marginata*. For. Pathol. 42:289-298.

Smither, M. and Jones, A. 1989. *Pythium* species associated with sour cherry and the effect of *P. irregulare* on the growth of mahaleb cherry. Can. J. Plant Pathol. 11:1-8.

Spies, C., Mazzola, M. and McLeod, A. 2011. Characterisation and detection of *Pythium* and *Phytophthora* species associated with grapevines in South Africa. Eur. J. Plant Pathol. 131:103-119.

Thomidis, T. 2000. Seasonal variation in crown rot of GF677 and KID I peach rootstocks by *Phytophthora cactorum, P. citrophthora* and *P. syringae*. Phytopathol. Mediterr. 39:396-403.

Thomidis, T. 2001. Testing variability in pathogenicity of *Phytophthora cactorum*, *P. citrophthora* and *P. syringae* to apple, pear, peach, cherry and plum rootstocks. Phytoparasitica 29:47-49.

Thomidis, T. 2003a. The *Phytophthora* diseases of stone fruit trees in Greece. Arch. Phytopathol. Pflanzenschutz. 36:69-80.

Thomidis, T. 2003b. Variability in pathogenicity among Greek isolates of *Phytophthora cactorum* to four peach rootstocks. Aust. J. Exp. Agric. 43:99-103.

Thomidis, T. 2003c. Influence of temperature and bark injuries on the development of *Phytophthora cactorum* and *P. citrophthora* on peach trees. Sci. Hortic. 98:347-355.

Thomidis, T., Cullum, J., Elena, K. and Jeffers, S. 2001. Relative resistance of four peach rootstocks to *Phytophthora cactorum* and *Phytophthora megasperma*. J. Phytopathol. 149:599-604.

Thomidis, T., Karayiannis, I. and Tsipouridis, C. 2008. Susceptibility of thirty cherry genotypes on *Phytophthora cactorum*, *P. citrophthora, P. citricola* and *P. parasitica*. J. Phytopathol. 156:446-451.

Tian, D. and Babadoost, M. 2004. Host range of *Phytophthora capsici* from pumpkin and pathogenicity of isolates. Plant Dis. 88:485-489.

Toribio, A., Jurado, M., Suárez-Estrella, F., López, M., López-González, J. and Moreno, J. 2021. Seed biopriming with cyanobacterial extracts as an eco-friendly strategy to control damping off caused by *Pythium ultimum* in seedbeds. Microbiol. Res. 248:126766.

Tsipouridis, C., Thomidis, T., Elena, K. and Isaakidis, A. 2005. Effect of peach cultivars, rootstocks and *Phytophthora* on iron chlorosis. WJAS. 1:137-142.

Türkkan, M., Özer, G., Karaca, G., Erper, İ. and Derviş, S. 2022. Characterization and pathogenicity of *Pythium*-like species associated with root and collar rot of kiwifruit in Turkey. Plant Dis. 106:854-863.

Türkölmez, Ş., Derviş, S., Çiftçi, O. and Ulubaş Serçe, Ç. 2016. First report of *Phytophthora chlamydospora* causing root and crown rot on almond (*Prunus dulcis*) trees in Turkey. Plant Dis. 100:1796.

Uchida, J. and Kadooka, C. 2013. Distribution and biology of *Phytophthora tropicalis,* In: *Phytophthora:* a global perspective; Lamour, K., Ed.; CABI Plant Protection Series, 2013; pp. 178-186.

Wang, K., Xie, Y., Yuan, G., Li, Q. and Lin, W. 2015. First report of root and collar rot caused by *Phytopythium helicoides* on kiwifruit (*Actinidia chinensis*). Plant Dis. 99:725.

White, T., Bruns, T., Lee, S. and Taylor, J. 1990. Amplification and direct sequencing of fungi ribosomal RNA genes for phylogenetics, In: *PCR* Protocols. A Guide to Methods and Applications; Innis, M., Gelfand, D., Sninsky, J. and White, T., Eds.; Academic Press, San Diego, CA, 1990; pp. 315-322.

Wicks, T. 1989. Susceptibility of almond and cherry rootstocks and scions to *Phytophthora* species. Aust. J. Exp. Agric. 29:103-109.

Xie, X., Zhou, H., Fan, S. and Zhang, X. 2021. First report of *Phytopythium helicoides* causing stalk rot on corn in China. Plant Dis. 105:3766.

Yahmed, J., Ghrab, M. and Mimoun, M. 2016. Eco-physiological evaluation of different scion-rootstock combinations of almond grown in Mediterranean conditions. Fruits, 71:185-193.

Yang J., Ruegger, P., McKenry, M., Becker, J. and Borneman, J. 2012 Correlations between root-associated microorganisms and peach replant disease symptoms in a California Soil. PloS One 7:e46420.

Yang, X., Richardson, P., Olson, H. and Hong, C. 2013. Root and stem rot of begonia caused by *Phytopythium helicoides* in Virginia. Plant Dis. 97:1385-1385.

Zhan, Y., Peng, W., Xu, Z., Xu, H. and Feng, X. 2020. First report of *Phytopythium helicoides* causing root and crown rot on strawberry in China. Plant Dis. 104:2528.

Chapter 7

Chapter 7

General discussion

The studies presented in this PhD Thesis are divided into two different groups. The first two studies focus on the pathogen *D. amygdali*, a fungus that causes twig canker and shoot blight in almond and peach trees (Chapters 3 and 4). The following two studies are focus on pathogenic oomycetes, which are associated with root and crown rot in *Prunus* hybrid rootstocks used for almond cultivation (chapter 5 and 6). In this way, a holistic vision of some of the main phytosanitary problems currently threatening almond cultivation in Spain is proposed. This research has been developed from a practical approach and with the general objective of increasing the current knowledge of these diseases in order to meet the needs of field technicians and farmers. They require specific tools for both the diagnosis of diseases caused by these microorganisms and for the selection of plant material (cultivars and rootstocks), which are suitable for maintaining an adequate health of the almond tree crop in conditions of optimal productivity.

Regarding the studies on the epidemiology of *D. amygdali* (chapter 3), first of all the role of weather conditions on the dispersal of *D. amygdali* inoculum on almond crops in Mediterranean conditions was investigated. A two-part model's approach was used to study the relationships between DNA concentration of *D. amygdali* inoculum obtained from spore traps and weather variables that affect the probability of detecting and quantifying it. A two-part Hurdle models used in this study include a qualitative part (Bernoulli), with a binary response, that is, it detects the presence or absence of DNA, and a quantitative part (Gamma) that estimates the average DNA concentration of *D. amygdali*, for each growing season. The results showed that some weather variables affected positively and others negatively, on both parts of the model.

The temperature effect seems to originate from the greater or lesser daily thermal amplitude. When considering a greater thermal amplitude during the day, a negative effect was observed mainly in the Gamma part of the model, in both growing seasons, decreasing the average DNA concentration of *D. amygdali*. This effect may be due to extreme temperatures such as daily minimum and maximum temperatures, which are less favorable for canker development in peach trees than 20°C (Udin and Reynolds, 1995). Both the formation of cirrus and the production of conidia were observed from 0 to 37°C, with an optimum at 22°C, but decreased considerably as temperatures approached the extremes (Lalancette et al., 2003). On the contrary, when considering a lower thermal

amplitude, it increased the probability of detecting *D. amygdali* DNA during the 2019-2020 growing season, because this fungus increases its sporulation rate between 10 and 25°C, thus increasing also the probability of detecting *D. amygdali* DNA.

Regarding relative humidity, the number of days with average relative humidity above 80% had a negative effect on the concentration of *D. amygdali* DNA in both parts of the model (2020-2021). It is important to note that both relative humidity and temperature should not be considered separately in the models, but rather together, in addition to the time of exposure to these favorable conditions. Similar effects are reported in other species of *Diaporthe*, as is the case of *D. citri*, the infection by this fungus was very low with 4 h of leaf humidity and reached maximum levels at 24 h of leaf humidity, at 22°C, when it was inoculated into lemon seedlings (Agostini et al., 2003). A study conducted by Anco et al. (2013) on the epidemiology of *D. neoviticola* in grapevines, indicated that sporulation of this fungus increased significantly after at least 47 h of continuous humidity, when temperature conditions were between 18 and 25°C. In contrast, it was observed that sporulation decreased considerably when the duration of humidity was less than 12 h. These findings suggest that for sporulation it is essential that humidity conditions must be maintained for a prolonged period of time, hence days with 80% average relative humidity (as in our study) may be insufficient for the production and dispersal of *D. amygdali* conidia, negatively affecting both parts of the model.

Regarding rainfall, it was observed that it had a positive effect. When a raindrop hits with mature pycnidia, thousands of droplets are produced that carry conidia. These droplets can initiate new infections by being thrown onto uninfected plants adjacent to the inoculum source (Agostini et al., 2003; Linders et al., 1996). This effect has recently been studied in hazelnuts, indicating that increase in the amount of rainfall during the crop growing season was associated with a higher incidence of infections caused by *Diaporthe* spp. (Battilani et al., 2018).

Finally, regarding wind speed, when this was maximum, negatively influenced the quantitative part of the model (2020-2021). High wind speed can reduce relative humidity, affecting the sporulation of pycnidia, which require high relative humidity for prolonged periods of time (Anco et al. 2013; Lalancette et al. 2003). Furthermore, spores that grow on fruiting bodies (such as the conidia of *D. amygdali*) are moist air spores and their release depends on relative humidity and rainfall, but not on wind speed (Troutt and Levetin, 2001). As for days with wind speed less than the maximum of the day, positively influence in the qualitative part (2019-2020) and the quantitative part in both growing seasons. The effect of wind speed on disease development has been also associated to

the physical damage caused to the plant organs (Agrios 2005), which benefits *D. amygdali*, since requires wounds to infect (Adaskaveg et al. 2008; Lalancette and Robison 2001; Lalancette et al. 2003).

This epidemiological study provides a valuable contribution to the understanding of the relationships among different weather conditions and the inoculum dispersal of *D. amygdali*, which is essential for twig canker and shoot blight management on almond in Mediterranean conditions.

Second, the susceptibility of almond cultivars to twig canker and shoot blight caused by *D. amygdali* was investigated (Chapter 4). The results evidenced the susceptibility of all the cultivars evaluated to all the inoculated isolates of *D. amygdali*. Lesion length measurements showed a wide range of variation between cultivars in all experiments, in addition to intraspecific variation in virulence between *D. amygdali* isolates, previously reported by Diogo et al. (2010) and León et al. (2020) in 'Ferragnès' and 'Vairo', respectively. When carrying out the classification of the susceptibility of the cultivars in susceptible or very susceptible according to a cluster analysis, the second group showed a 30% increase in the average length of the lesions in comparison with the susceptible ones. Previous works had already studied the susceptibility of almond cultivars to *D. amygdali*, in which the cultivar 'Ferragnès' is the most mentioned. Investigations carried out by Cabrita et al. (2004) and Diogo et al. (2010), confirmed part of our results, indicating that the cultivar 'Ferragnès' presents a high susceptibility to species of the genus *Diaporthe*. On the contrary, Vargas and Miarnau (2011) when evaluating susceptibility to *D. amygdali* in 70 naturally infected almond cultivars, pointed out cultivars 'Masbovera' and 'Tarraco' as very tolerant, cultivars that according to our results are considered very susceptible. These discrepancies in the results can be explained by the different methods of inoculation of the fungus in the plant that can be differentiated from the natural infection, directly influencing the results, as indicated by Mathew et al. (2018) and Ghimire et al. (2019), for different species of *Diaporthe* in sunflower and soybean, respectively.

When studying the influence of some agronomic traits and the susceptibility of the species to *D. amygdali*, we found that desired characteristics in breeding programs such as late blooming and early ripening (Batlle et al., 2017), negatively influenced the susceptibility to this fungus, being the cultivars 'Constantí', 'Lauranne', 'Penta' and 'Tardona', highly susceptible. This may be because these four cultivars have a common ancestor, the 'Tuono' cultivar, which was shown as susceptible in this study and in previous studies (Martins et al., 2005; Vargas and Miarnau, 2011). Regarding vigor, the average size of the lesions caused by *D. amygdali* was generally lower in high-vigor cultivars. This

may be due to the fact that, in addition to understanding that high vigor gives plants greater qualities to face a disease, cultural practices aimed at improving plant vigor often help increase its tolerance to pathogens (Agrios 2005; Raines 1922).

Regarding the studies about oomycetes affecting rootstocks used for almond trees cultivation, these have focused on field diagnosis and *in planta* pathogenicity and virulence studies.

Chapter 5 presents a three-year survey (2018, 2019 and 2020) conducted in Spanish almond plantations, in which root and crown rot symptoms were prevalent. Although in this study the pathogenicity of *Pp. vexans* and *Ph. niederhauserii* was evaluated, this will be discussed later, when addressing the fourth study of this doctoral thesis, which focuses on the pathogenicity of several oomycete species on *Prunus* hybrid rootstocks.

A great diversity of oomycetes belonging to the genera *Globisporangium*, *Phytophthora*, *Phytopythium* and *Pythium*, were isolated and identified in root and soil samples, as in previous studies from other countries (Azizi et al., 2013, Browne et al., 2015; Browne et al., 2019). *Phytopythium vexans* and *Ph. niederhauserii* were the species with the highest frequency of isolation, so it was decided to perform a pathogenicity test on the 'Garnem' rootstock. The high frequency of isolation of *Pp. vexans* in *Prunus* soils has been previously reported, causing reduced growth and yield of infected plants (Yang et al., 2012). This species has been considered saprophytic (Mircetich, 1971), but its pathogenicity has currently been proven in some fruit trees in several countries (Jabiri et al., 2020; Langenhoven et al., 2018; Rodríguez-Padrón et al., 2018). Currently there are no reports of this species infecting the almond tree; therefore, our study is the first report of *Pp. vexans* as a pathogen in this crop. The second most frequently isolated species was *Ph. niederhauserii*, reported for the first time in almond trees in Spain a decade ago (Pérez-Sierra et al., 2010). According to our results, this species is currently present in several Spanish provinces. Moreover, *Ph. niederhauserii* has also been described affecting almonds in Turkey (Kurbetli and Yilmaz, 2016) and California (Browne et al., 2015). However, the literature is not conclusive in associating a specific species of *Phytophthora* with the symptoms of root rot and crown cankers in almond trees (Browne et al., 2015). Multiple *Phytophthora* species have been associated with almond but were not found in our study (Browne et al., 2020; Browne and Viveros, 1997; Çiftçi et al., 2016; Kurbetli and Yilmaz, 2016; Türkölmez et al., 2016; Wicks, 1989). *Globisporangium irregulare* and *G. ultimum* var. *ultimum* presented an isolation frequency of less than 10%. Mircetich (1971) indicated that these oomycetes are isolated with high frequency in peach crops, but they are not related to tree root rot, acting as saprophytic organisms in peach trees.

Phytophthora cactorum and *Ph. citrophthora* were isolated with a frequency of less than 7%, coinciding with previous works by Wicks and Lee (1986) and Browne and Viveros (1997), who reported the isolation of these pathogens from almond and soil cankers. The remaining oomycete species were found with very low frequency and were occasionally isolated from some samples. Most of these species are pathogenic of ornamental plants, cereals, vegetables and some fruit trees, some have also been described as saprophytes. However, *Pp. helicoides* has been reported as a pathogen on *Prunus* in California, causing root rot and stunted roots of seedlings of 'Nemaguard' peach rootstocks (Browne et al., 2019).

Regarding the study of pathogenicity and virulence of oomycetes in *Prunus* hybrid rootstocks: 'Garnem', 'GF-677' and 'Rootpac-40' (Chapter 6), all the isolates were pathogenic, and a wide range of virulence was evidenced even in different isolates of the same species.

The species of the genus *Phytophthora* included in the pathogenicity test showed high virulence, as is the case of *Ph. multivora*, an emerging pathogen associated with multiple hosts in diverse ecosystems (Jeff-Ego et al., 2021; Meitz-Hopkins et al., 2014; Migliorini et al., 2019; Rodríguez-Padrón et al., 2018). This oomycete significantly reduced the dry weight of the root, an effect previously documented in forest species (Belhaj et al., 2018; Mrázková et al., 2013; Scott et al., 2012) and its presence in Spanish almond trees, compromises the health of the almond cultivation, due to its adaptability to calcareous soils (Migliorini et al., 2019) and dry climates (Jung and Burgess, 2009). The *Ph. niederhauserii* isolates presented intraspecific virulence variability, described in other species of the genus (Kurbetli et al., 2020; Tian and Babadoost, 2004). Root necrosis produced by *Ph. niederhauserii* reduced root dry weight, a symptom described by other authors in avocado and pomegranate (Kurbetli et al., 2020; Rodríguez-Padrón et al., 2018). In the case of *Ph. nicotianae*, severely affected rootstock 'Rootpac--40', causing symptoms similar to those mentioned by Pane et al. (2009), in young apricot plants. With lower virulence in our pathogenicity test, *Ph. tropicalis, Ph. cactorum* and *Ph. citrophthora* species reduced root dry weight, but with low plant mortality. Meanwhile, there is only one report of *Ph. tropicalis* affecting young apricot nurseries (Pane et al., 2009), the pathogenicity and virulence of *Ph. cactorum* and *Ph. citrophthora* in stone fruit trees has been widely documented, evidencing the high pathogenicity in *Prunus* of these oomycetes (Thomidis, 2003; Thomidis et al., 2008).

In the case of the species of the genus *Phytopythium*, *Pp. helicoides*, already reported in California, affecting 'Nemaguard' rootstocks (Browne et al., 2019), presented the highest virulence, with similar ranges of severity and root

weight reduction than *Phytophthora* species. This species has been reported infecting fruit trees, cereals and ornamentals (Chen et al., 2016; Chen et al., 2021; Fichtner et al., 2016; Ishiguro et al., 2014; Marin et al., 2019; Wang et al., 2015; Xie et al., 2021; Yang et al., 2013; Zhan et al., 2020), but its global distribution is still limited or unknown. Similarly, *Pp. vexans*, considered for many years as a secondary pathogen in peach trees (Mircetich, 1971), showed high virulence, significantly affecting root dry weight and producing high mortality in plants. The symptoms produced by *Pp. vexans* in our study have been widely documented in other fruit trees (Benfradj et al., 2017; Jabiri et al., 2020; Langenhoven et al., 2018; Noireung et al., 2020; Polat et al., 2017; Prencipe et al., 2020; Rodríguez-Padrón et al., 2018; Türkkan et al., 2022) and scarcely in *Prunus* crops (Baysal-Gurel et al., 2021; Beluzán et al., 2022).

The last genus evaluated was *Globisporangium*, whose species *G. ultimum* var. *ultimum* and *G. irregulare* presented differences in virulence in the evaluated rootstocks. Previous studies by Smither and Jones (1989) indicate a low virulence of *G. irregulare* when inoculated into *P. mahaleb* seedlings. Later, Schmidt and Browne (2013) indicated that *G. ultimum* var. *ultimum* and *G. irregulare* were able to decrease the fresh weight of 'Nemaguard' plants, when they were inoculated, contributing to the development of Prunus replanting disease (PRD). However, in our study *G. irregulare* was not able to reduce root dry weight in the three rootstocks evaluated. Regarding *G. heterothallicum*, this species has been reported in different cultivated agricultural species, although it has not been informed of its pathogenicity in *Prunus* plants (Derviş et al., 2020; Rojas et al., 2017; Spies et al., 2011; Türkkan et al., 2022). However, in our study *G. heterothallicum* demonstrated medium virulence, significantly reducing root dry weight in two evaluated rootstocks.

The results of our research studies provide new information on the biology and epidemiology of *D. amygdali* and on the diagnosis and pathogenicity of oomycete species present in almond crops in Spain, offering a holistic vision of the phytosanitary problems of the crop.

References

Adaskaveg, J., Schnabel, G., and Förster, H. 2008. Diseases of peach caused by fungi and fungal-like organisms: biology, epidemiology and management. In: The Peach: botany, production and uses; Layne D. R. and Bassi D., Eds.; CAB International, Wallingford, Oxfordshire, UK; pp. 352-406

Agostini, J., Bushong, P., Bhatia, A. and Timmer, L. 2003. Influence of environmental factors on severity of citrus scab and melanose. Plant Dis. 87:1102-1106.

Agrios, G. 2005. Plant Pathology 5th Ed. Elsewier Academic Press, Burlington, MA 01803, USA: 922 pp.

Anco, D., Madden, L. and Ellis, M. 2013. Effects of temperature and wetness duration on the sporulation rate of *Phomopsis viticola* on infected grape canes. Plant Dis. 97:579-589.

Azizi, Z., Amini, J., Shaykh-Eslami, M. and Abasi, S. 2013. Pathogenicity of some isolates of *Pythium* and *Phytophthora* on detached shoots and seedlings of almond. Iran. J. Plant. Pathol. 49:33-39.

Batlle, I., Dicenta, F., Socias i Company, R., Gradziel, T., Wirthensohn, M., Duval, H. and Vargas, F. 2017. Classical genetics and breeding. In: Almonds, botany, production and uses; R. Socias i Company and T. M. Gradziel, Eds.; CAB International, Boston, MA; pp. 111–148.

Battilani, P., Chiusa, G., Arciuolo, R., Somenzi, M., Fontana, M., Castello, G. and Spigolon, N. 2018. *Diaporthe* as the main cause of hazelnut defects in the Caucasus region. Phytopathol. Mediterr. 57:320-333.

Baysal-Gurel, F., Liyanapathiranage, P., Panth, M., Avin, F. and Simmons, T. 2021. First report of *Phytopythium vexans* causing root and crown rot on flowering cherry in Tennessee. Plant Dis. 105:232.

Belhaj, R., McComb, J., Burgess, T. and Hardy, G. 2018. Pathogenicity of 21 newly described *Phytophthora* species against seven Western Australian native plant species. Plant Pathol. 67:1140-1149.

Beluzán, F., Miarnau, X., Torguet, L., Armengol, J. and Abad-Campos, P. 2022. Survey of oomycetes associated with root and crown rot of almond in Spain and pathogenicity of *Phytophthora niederhauserii* and *Phytopythium vexans* to 'Garnem' rootstock. Agriculture 12:294.

Benfradj, N., Migliorini, D., Luchi, N., Santini, A. and Boughalleb-M'Hamdi, N. 2017. Occurrence of *Pythium* and *Phytopythium* species isolated from citrus trees infected with gummosis disease in Tunisia. Arch. Phytopathol. Pflanzenschutz 50:286-302.

Browne, G. and Viveros, M. 1997. Diverse symptoms and tree losses caused by *Phytophthora* spp. in California almonds. Acta Hortic. 470:570-575.

Browne, G., Ott, N. and Fichtner, E. 2019. First report of *Phytopythium helicoides* causing root rot on peach rootstock in California. Plant. Dis. 103:2968.

Browne, G., Ott, N., Forbes, H., Yaghmour, M. and Milliron, L. 2020. First report of *Phytophthora chlamydospora* causing crown and root rot on almond in California. Plant. Dis. 104:2033-2033.

Browne, G., Schmidt, L. and Brar, G. 2015. First report of *Phytophthora niederhauserii* causing crown rot of almond (Prunus dulcis) in California. Plant. Dis. 99:1863.

Cabrita, L., Neves, A. and Leitão, J. 2004. Evaluation of resistance to *Phomopsis amygdali* in almond. In Proceedings of the XIth Eucarpia symposium on fruit breeding and genetics, Vols 1 and 2 (Vol. 663, pp. 235-238). International Society for Horticultural Science.

Chen, X., Liu, B., Xing, Y., Cheng, B., Liu, M., Tong and Xu, J. 2016. Identification and characterization of *Phytopythium helicoides* causing stem rot of Shatangju mandarin seedlings in China. Eur. J. Plant Pathol. 146:715-727.

Chen, Z., Yang, X., Xu, J., Jiao, B., Li, Y., Xu, Y. and Dai, T. 2021. First report of *Phytopythium helicoides* causing crown and root rot of *Rhododendron pulchrum* in China. Plant Dis. 105:713-713.

Çiftçi, O., Türkölmez, Ş., Derviş, S. and Serçe, Ç. 2016. First report of canker and root rot of almond caused by *Phytophthora plurivora* in Turkey. Plant. Dis. 100:1507.

Derviş, S., Özer, G., Türkölmez, Ş. and Çiftçi, O. 2020. First report of *Globisporangium heterothallicum* causing root and crown rot of pepper in Turkey. New Dis. Rep. 41:36-36.

Diogo, E., Santos, J. and Phillips, A. 2010. Phylogeny, morphology and pathogenicity of *Diaporthe* and *Phomopsis* species on almond in Portugal. Fungal Divers. 44:107-115.

Fichtner, E., Browne, G., Mortaz, M., Ferguson, L. and Blomquist, C. 2016. First report of root rot caused by *Phytopythium helicoides* on pistachio rootstock in California. Plant Dis. 100:2337-2337.

Ghimire, K., Petrović, K., Kontz, B., Bradley, C., Chilvers, M., Mueller, D., Smith, D., Wise, K. and Mathew, F. 2019. Inoculation method impacts symptom development associated with *Diaporthe aspalathi*, *D. caulivora*, and *D. longicolla* on soybean (Glycine max). Plant Dis. 103:677-684.

Ishiguro, Y., Otsubo, K., Watanabe, H., Suzuki, M., Nakayama, K., Fukuda, T. Fujinaga, M., Suga, H. and Kageyama, K. 2014. Root and crown rot of strawberry caused by *Pythium helicoides* and its distribution in strawberry production areas of Japan. J. Gen. Plant Pathol. 80:423-429.

Jabiri, S., Lahlali, R., Bahra, C., Amraoui, M., Tahiri, A. and Amiri, S. 2020. First report of *Phytopythium vexans* associated with dieback disease of apple trees in Morocco. J. Plant. Pathol. 102:1319.

Jeff-Ego, O., Topp, B., Drenth, A., Henderson, J. and Akinsanmi, O. 2021. Resistance in wild macadamia germplasm to *Phytophthora cinnamomi* and *Phytophthora multivora*. Ann. Appl. Biol. 178:519-526.

Jung, T. and Burgess, T. 2009. Re-evaluation of *Phytophthora citricola* isolates from multiple woody hosts in Europe and North America reveals a new species, *Phytophthora plurivora* sp. nov. Pers. Mol. Phylogeny Evol. Fungi. 22:95-110.

Kurbetli, İ. and Yılmaz, A. 2016. Almond decline caused by *Phytophthora megasperma* in southeastern Anatolian region of Turkey. J. Turk. Phytopathol. 45:13-20.

Kurbetli, İ., Karaca, G., Aydoğdu, M. and Sülü, G. 2020. *Phytophthora* species causing root and collar rot of pomegranate in Turkey. Eur. J. Plant Pathol. 157:485-496.

Lalancette, N., and Robison, D. 2001. Seasonal availability of inoculum for constriction canker of peach in New Jersey. Phytopathology 91:1109-1115.

Lalancette, N., Foster, K., and Robison, D. 2003. Quantitative models for describing temperature and moisture effects on sporulation *of Phomopsis amygdali* on peach. Phytopathology 93:1165-1172.

Langenhoven, S. D., Halleen, F., Spies, C. F., Stempien, E. and Mostert, L. 2018. Detection and quantification of black foot and crown and root rot pathogens in grapevine nursery soils in the Western Cape of South Africa. Phytopathol. Mediterr. 57:519-537.

León, M., Berbegal, M., Rodríguez-Reina, J. M., Elena, G., Abad-Campos, P., Ramón-Albalat, A., Olmo, D., Vicent, A., Luque, J., Miarnau, X., Agustí-Brisach, C., Trapero, A., Capote, N., Arroyo, F., Avilés, M., Gramaje, D., Andrés-Sodupe, M. and Armengol, J. 2020. Identification and characterization of *Diaporthe* spp. associated with twig cankers and shoot blight of almonds in Spain. Agronomy 10:1062.

Linders, E., Van Damme, J. and Zadoks, J. 1996. Transmission and overseasoning of *Diaporthe adunca* on *Plantago lanceolata*. Plant Pathol. 45:59-69.

Marin, M., Seijo, T., Mertely, J. and Peres, N. 2019. First report of crown rot caused by *Phytopythium helicoides* on strawberry in the Americas. Plant Dis. 103:2696.

Martins, M., Sarmento, D., Batlle, I., Vargas, F. and Oliveira, M. 2005. Development of SCAR/CAPS markers linked to tolerance/sensitivity to *Fusicoccum* in almond. Options Méditerranéennes CIHEAM/IAMZ 63:187-191.

Mathew, F., Jordahl, J., Gulya, T. and Markell, S. 2018. Comparison of greenhouse-based inoculation methods to study aggressiveness of *Diaporthe helianthi* isolates causing Phomopsis stem canker of sunflower (*Helianthus annuus*). Plant Health Prog. 19:92-96.

Meitz-Hopkins, J., Pretorius, M., Spies, C., Huisman, L., Botha, W., Langenhoven, S. and McLeod, A. 2014. *Phytophthora* species distribution in South African citrus production regions. Eur. J. Plant Pathol. 138:733-749.

Migliorini, D., Khdiar, M., Rodríguez-Padrón, C., Vivas, M., Barber, P., Hardy, G. and Burgess, T. 2019. Extending the host range of *Phytophthora multivora*, a pathogen of woody plants in horticulture, nurseries, urban environments and natural ecosystems. Urban For. Urban Green. 46:126460.

Mircetich, S. 1971. The role of *Pythium* in feeder roots of diseased and symptomless peach trees. Phytopathology 61:357-360.

Mrázková, M., Černý, K., Tomšovský, M., Strnadová, V., Gregorová, B., Holub, V., Pánek, M., Havrdová, L. and Hejná, M. 2013. Occurrence of *Phytophthora multivora* and *Phytophthora plurivora* in the Czech Republic. Plant Prot. Sci. 49:155-164.

Noireung, P., Intaparn, P., Maumoon, R., Wongwan, T. and To–anun, C. 2020. First Record of *Phytopythium vexans* causing root rot on Mandarin (*Citrus reticulate* L. cv. *Sainampueng*) in Thailand. Plant Pathol Quar. 10:85-90.

Pane, A., Cacciola, S., Scibetta, S., Bentivenga, G. and Magnano di San Lio, G. 2009. Four *Phytophthora* species causing foot and root rot of apricot in Italy. Plant Dis. 93, 844-844.

Pérez-Sierra, A., León, M., Álvarez, L. A., Alaniz, S., Berbegal, M., García-Jiménez, J. and Abad-Campos, P. 2010. Outbreak of a new *Phytophthora* sp. associated with severe decline of almond trees in eastern Spain. Plant. Dis. 94:534-541.

Polat, Z., Awan, Q., Hussain, M. and Akgül, D. 2017. First report of *Phytopythium vexans* causing root and collar rot of kiwifruit in Turkey. Plant Dis. 101:1058.

Prencipe, S., Savian, F., Nari, L., Ermacora, P., Spadaro, D. and Martini, M. 2020. First report of *Phytopythium vexans* causing decline syndrome of Actinidia deliciosa 'Hayward' in Italy. Plant Dis. 104:2032.

Raines M. 1922. Vegetative vigor of the host as a factor influencing susceptibility and resistance to certain rust diseases of the higher plants. Am. J. Bot. 9:183-203.

Rodríguez-Padrón, C., Siverio, F., Pérez-Sierra, A. and Rodríguez, A. 2018. Isolation and pathogenicity of *Phytophthora* species and *Phytopythium vexans* recovered from avocado orchards in the Canary Islands, including *Phytophthora niederhauserii* as a new pathogen of avocado. Phytopathol. Mediterr. 57:89-106.

Rojas, J., Jacobs, J., Napieralski, S., Karaj, B., Bradley, C., Chase, T., Esker, P., Giesler, L., Jardine, D., Malvick, D., Markell, S., Nelson, B., Robertson, A., Rupe, J., Smith, D., Sweets, L., Tenuta, A., Wise, K. and Chilvers, M. 2017. Oomycete species associated with soybean seedlings in North America Part II: Diversity and ecology in relation to environmental and edaphic factors. Phytopathology 107:293-304.

Schmidt, L. and Browne, G. 2013 Characterization of *Pythium* species associated with Prunus replant disease. (Abstr.) Phytopathology 103:S2.128

Scott, P., Jung, T., Shearer, B., Barber, P., Calver, M. and Hardy, G. 2012. Pathogenicity of *Phytophthora multivora* to *Eucalyptus gomphocephala* and *Eucalyptus marginata*. For. Pathol. 42:289-298.

Smither, M. and Jones, A. 1989. *Pythium* species associated with sour cherry and the effect of *P. irregulare* on the growth of mahaleb cherry. Can. J. Plant Pathol. 11:1-8.

Spies, C., Mazzola, M. and McLeod, A. 2011. Characterisation and detection of *Pythium* and *Phytophthora* species associated with grapevines in South Africa. Eur. J. Plant Pathol. 131:103-119.

Thomidis, T. 2003. Influence of temperature and bark injuries on the development of *Phytophthora cactorum* and *P. citrophthora* on peach trees. Sci. Hortic. 98:347-355.

Thomidis, T., Karayiannis, I. and Tsipouridis, C. 2008. Susceptibility of thirty cherry genotypes on *Phytophthora cactorum, P. citrophthora, P. citricola* and *P. parasitica*. J. Phytopathol. 156:446-451.

Tian, D. and Babadoost, M. 2004. Host range of *Phytophthora capsici* from pumpkin and pathogenicity of isolates. Plant Dis. 88:485-489.

Troutt, C. and Levetin, E. 2001. Correlation of spring spore concentrations and meteorological conditions in Tulsa, Oklahoma. Int. J. Biometeorol., 45:64-74.

Türkkan, M., Özer, G., Karaca, G., Erper, İ. and Derviş, S. 2022. Characterization and pathogenicity of *Pythium*-like species associated with root and collar rot of kiwifruit in Turkey. Plant Dis. 106:854-863.

Türkölmez, Ş., Derviş, S., Çiftçi, O. and Ulubaş Serçe, Ç. 2016. First report of *Phytophthora chlamydospora* causing root and crown rot on almond (*Prunus dulcis*) trees in Turkey. Plant. Dis. 100:1796.

Uddin, W. and Reynolds, K. L. 1995. *Phomopsis* twig blight of peach: Pathogenicity and influence of temperature on disease development. (Abstr.) Phytopathology 85:1127.

Vargas, F. and Miarnau, X. 2011. Field susceptibility to *fusicoccum* canker of almond cultivars. Acta Hortic. 912:751-755.

Wang, K., Xie, Y., Yuan, G., Li, Q. and Lin, W. 2015. First report of root and collar rot caused by *Phytopythium helicoides* on kiwifruit (Actinidia chinensis). Plant Dis. 99:725.

Wicks, T. 1989. Susceptibility of almond and cherry rootstocks and scions to *Phytophthora* species. Aust. J. Exp. Agric. 29:103-109.

Wicks, T. and Lee, T. 1986. *Phytophthora* crown rot of almond trees. Aust. J. Agric. Res. 37:277-287.

Xie, X., Zhou, H., Fan, S. and Zhang, X. 2021. First report of *Phytopythium helicoides* causing stalk rot on corn in China. Plant Dis. 105:3766.

Yang, J., Ruegger, P., McKenry, M., Becker, J. and Borneman, J. 2012. Correlations between root-associated microorganisms and peach replant disease symptoms in a California Soil. PLoS ONE 7:e46420.

Yang, X., Richardson, P., Olson, H. and Hong, C. 2013. Root and stem rot of begonia caused by *Phytopythium helicoides* in Virginia. Plant Dis. 97:1385-1385.

Zhan, Y., Peng, W., Xu, Z., Xu, H. and Feng, X. 2020. First report of *Phytopythium helicoides* causing root and crown rot on strawberry in China. Plant Dis. 104:2528.

Chapter 8

Chapter 8

Conclusions

In the case of the studies related to *Diaporthe amygdali* presented in this doctoral thesis, it can be concluded that:

a) The two-part Hurdle models elaborated in this PhD Thesis using empirical data (DNA concentration) obtained from spore traps by a qPCR protocol, allowed a deep understanding of the fundamental role that weather conditions play in the inoculum dispersal of *D. amygdali* on almond crops under Mediterranean conditions. The models showed both the positive and negative influence of various weather variables, such as temperature, relative humidity, rainfall and wind speed. The temperature effect was related to the greater or lesser daily thermal amplitude; wider thermal ranges reduced DNA concentration, while narrower thermal ranges increased DNA detection. Regarding relative humidity, days with average relative humidity above 80% had a negative effect on the concentration of *D. amygdali* DNA. Rainfall had a positive influence on the quantitative and qualitative parts of the models, confirming the contribution of precipitation in the dispersal of inoculum. Finally, in general wind speed variable also positively influenced both part of the models in both growing seasons.

b) Inoculation of 25 European and American almond cultivars with *D. amygdali* confirmed that all of them were susceptible to infection by this pathogen. Lesion length measurements showed a wide range of variation between cultivars, which were classified as susceptible (including 'Antoñeta', 'Desmayo Largueta', 'Ferragnès', 'Marcona', 'Mardía', and 'Marta') or very susceptible (which included 'Belona', 'Constantí', 'Ferraduel', 'Glorieta', 'Guara', 'Lauranne', 'Marinada', 'Masbovera', 'Penta', 'Soleta', 'Tardona', 'Tuono', 'Vairo', 'Francolí', and 'Tarraco') according to a cluster analysis. The relationship between some agronomic traits and cultivar susceptibility showed that blooming and ripening times were relevant variables to explain cultivars performance related to *D. amygdali* susceptibility. Late and very late blooming, and early and medium ripening cultivars were highly susceptible to *D. amygdali.*

Regarding the studies related to oomycetes, it can be concluded that:

a) A total of 104 oomycete isolates were obtained from plant and soils samples in a three-year survey (2018, 2019 and 2020) conducted in Spanish almond plantations, in which root and crown rot symptoms were prevalent. Diverse oomycete species belonging to the genera *Globisporangium*, *Phytophthora*, *Phytopythium* and *Pythium* were found, being *Phytopythium vexans* and *Phytophthora niederhauserii* the most frequent. The pathogenicity of these two species to one-year-old almond seedlings of 'Garnem' rootstock confirmed their pathogenicity, showing severe symptoms at the late stage of the pathogenicity test (defoliation, wilting and dieback), and several plants died. Some isolates of *Ph. niederhauserii* significantly reduced the dry weight of the roots compared to the non-inoculated control, but this effect was not observed on seedlings inoculated with *Pp. vexans*.

b) The pathogenicity of 12 oomycete species belonging to the genera *Globisporangium*, *Phytophthora* and *Phytopythium* to three *Prunus* hybrid rootstocks: 'Garnem', 'GF-677' and 'Rootpac-40' was evaluated through pathogenicity tests conducted using 15 oomycete isolates and 1-year-old rootstock seedlings. All the isolates included in the pathogenicity tests were pathogenic on the rootstock seedlings and were re-isolated from root lesions. Large differences in virulence were detected among the different oomycete species and isolates of *Ph. niederhauserii* for each rootstock. *Phytophthora multivora* and *Pp. helicoides* were generally the most virulent species.