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TESIS DOCTORAL

"ETIOLOGÍA, EPIDEMIOLOGÍA Y CONTROL DEL MILDIU DE LA ADORMIDERA"

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Córdoba, Diciembre de 2008

TITULO: *ETIOLOGÍA, EPIDEMIOLOGÍA Y CONTROL DE MILDIU DE LA ADORMIDERA*

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"ETIOLOGÍA, EPIDEMIOLOGÍA Y CONTROL DEL MILDIU DE LA ADORMIDERA"

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Córdoba, Diciembre de 2008

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INFORMA: Que el trabajo **"Etiología, epidemiología y control del Mildiu de la adormidera"**, que ha llevado a cabo el Ingeniero Agrónomo D. Miguel Montes Borrego bajo la dirección del Prof. Dr. D. Rafael M. Jiménez Díaz, Catedrático de Patología Vegetal del Departamento de Agronomía de la Universidad de Córdoba, y la Dra. Dña. Blanca B. Landa del Castillo, Científico Titular del Departamento de Protección de Cultivos del Instituto de Agricultura Sostenible, CSIC, fue aprobado por el Consejo de Departamento celebrado el día 23 de septiembre de 2008, por lo que puede ser presentado para su exposición y defensa como Tesis Doctoral en el Departamento de Agronomía de la Universidad de Córdoba.

Córdoba, Diciembre de 2008

Esteban Alcántara Vara

DEPARTAMENTO DE AGRONOMÍA

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DEPARTAMENTO DE PROTECCIÓN DE CULTIVOS

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A mis padres

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BIOGRAFÍA

Miguel Montes Borrego nació en Marchena, provincia de Sevilla, el 9 de Septiembre de 1979. Realizó sus estudios de E.G.B en el C.P. Nuestro Padre Jesús Nazareno de Marchena. Realizó los estudios de B.U.P y C.O.U. en el Instituto Isidro de Arcenegui y Carmona de Marchena. Estudios simultaneados con su afición al baloncesto, al campo, a la agricultura en general y en especial a la horticultura, el olivar y otros frutales.

En Septiembre de 1997 inició sus estudios de Ingeniero Agrónomo en la Escuela Técnica Superior de Ingenieros Agrónomos y Montes de la Universidad de Córdoba, obteniendo el título en 2003 tras defender su Trabajo Profesional Fin de Carrera "Respuestas a la aplicación de la proteína Harpin (Messenger®) en el cultivo del tomate" realizado con la concesión de una beca en el Departamento de Química Agrícola dirigido por el Prof. Rafael de Prado en la ETSIAM en el curso académico 2002/2003. Durante este período comenzó su formación investigadora colaborando en otros estudios de la proteína Harpin y en la evaluación de la resistencia de malas hierbas a diferentes formulaciones de herbicidas. En 2003 le fue concedida una estancia de cinco meses en la Universidad de California, Davis como "Visiting Research Scholar" en el Departamento "Vegetable Crops" donde continúo su formación investigadora con el Prof. Albert Fischer estudiando la actividad de la enzima acetolactactato sintasa en *Cyperus difformis* y *Scirpus mucronatus* frente a diferentes familias de inhibidores y en donde le fue ofrecido continuar su especialización científica por dos años más, y que no pudo disfrutar por falta de acoplamiento con los cursos de Tercer ciclo en España.

Desde 2004 hasta día de hoy viene desempeñando su actividad investigadora en el Grupo de Sanidad Vegetal AGR136 del Dpto. de Agronomía de la Universidad de Córdoba y el Departamento de Protección de Cultivos del Instituto de Agricultura Sostenible de Córdoba gracias a la concesión de la becas y contratos de Postgrado del CSIC, la cofinanciación de Alcaliber, S.A. y el proyecto PET2006-0444 del 'Ministerio de Ciencia e Innovación' de España. Durante este período ha participado en diferentes Proyectos de investigación sobre enfermedades que han dado lugar a participaciones en congresos nacionales e internacionales, así como diferentes publicaciones en revistas incluidas en SCI y a tres patentes nacionales con extensión internacional. Además de participar en una Acción integrada francoespañola realizando una estancia en el UMR Microbiologie du Sol et de l'Environnement del INRA en Dijon, Francia.

RESUMEN

La adormidera (*Papaver somniferun* L.) es uno de los cultivos más importantes para la industria farmacéutica en España porque constituye la única fuente de morfina, codeína y tebaína. España es el 5º productor europeo de semilla y paja de adormidera, con cerca de 7.500 hectáreas cultivadas en Andalucía, Castilla-la Mancha, y Castilla y León. Durante los últimos años, los rendimientos del cultivo de adormidera han venido disminuyendo como consecuencia de ataques de una nueva enfermedad, cuya extensión y severidad se han incrementado notablemente a medida que el cultivo ha ocupado nuevas zonas frescas y húmedas en España o se ha incorporado al regadío a fin de mejorar el rendimiento de las cosechas. El objetivo finalista de esta Tesis Doctoral ha sido el obtener nuevos conocimientos sobre la etiología, biología y epidemiología del Mildiu de la adormidera, así como sobre la caracterización y detección molecular específica del agente causal, que permitan establecer las bases científicas para el desarrollo de estrategias eficientes para el control integrado de la enfermedad.

En primer lugar se determinó mediante técnicas tradicionales (observaciones microscópicas y ensayos de patogenicidad) y moleculares (análisis de secuenciación de la región espaciadora interna transcrita (ITS del ADN ribosómico (ITS1 e ITS2) y región 5,8S del ADN ribosómico, ADNr) la etiología del Mildiu de la adormidera en cultivos comerciales en España y su agente causal como el oomiceto biotrofo obligado *Peronospora arborescens* (Berk) de Bary.

Una vez determinada la etiología del Mildiu de la adormidera se procedió a establecer la composición y diversidad genética existente en las poblaciones del patógeno y sus relaciones filogenéticas basadas en el análisis de las secuencias de la región ITS1-5,8S-ITS2 del ADNr de *P. arborescens* y *P. cristata* de diversos orígenes geográficos. Los resultados obtenidos en el estudio sugirieron que solo *P. arborescens* está presente en los cultivos de *Pap. somniferun* en España. Además, los análisis filogenéticos de la región ITS1-5,8S-ITS2 del ADNr de 27 muestras de tejidos de adormideras infectadas por Mildiu de todas las regiones de cultivo en España y de Francia demostraron que la diversidad genética en las poblaciones de *P. arborescens* es escasa y que éstas son filogenéticamente diferentes de *P. cristata*, el agente causal del Mildiu de cultivos comerciales de adormidera en Tasmania (Australia).

Posteriormente, nuevos análisis filogenéticos donde se incluyeron ejemplares de diferentes especies de *Papaver* silvestres (*Pap. dubium, Pap. hybridum, Pap. rhoeas*, y *Pap. somniferum*) procedentes de colecciones de herbario y de campo con diferentes orígenes geográficos indicaron por primera vez que podría existir algún grado de especialización del huésped en las poblaciones de *P. arborescens* sugiriendo que las *Papaver* spp. silvestres pueden no actuar necesariamente como fuente de inóculo primario importante de la enfermedad.

A partir de secuencias de las regiones ITS1-5,8S-ITS2 del ADNr se desarrollaron tres pares de cebadores específicos P3Pa2fw/P3Pa2rv (P2), OMPac1fw/OMPac1rv (P3) y OMPac7fw/OMPac7rv (P6) de *P. arborescens* y un protocolo basado en PCR, el cual ha sido empleado para responder de forma eficiente a diferentes cuestiones sobre la biología de este patosistema. Los cebadores específicos desarrollados diferenciaron eficientemente *P. arborescens* de *P. cristata* y permitieron detectar *P. arborescens* en diferentes tejidos sintomáticos y asintomáticos de la planta. El uso del protocolo de detección y de los cebadores desarrollados permitió demostrar que *P. arborescens* puede ser trasmitido por semillas. Este método ha quedado recogido en la patente española Nº P200603319 y sus dos extensiones P200803261 y P200803262, y ha sido extendida internacionalmente con la aplicación Nº PCT/ES2007/000781.

Asimismo, utilizando los pares de iniciadores P3 y P6 se desarrolló una metodología de PCR anidada o 'nested'-PCR, que mejoró la detección in planta de DNA de *P. arborescens*. El nuevo protocolo representó un incremento en la sensibilidad de x100 a x1000 del oomiceto en tejidos de adormidera en comparación con el límite de detección obtenido con el protocolo de PCR específica simple y los pares de iniciadores P3 y P6, respectivamente. El nuevo protocolo permitió la amplificación de 5 a 0.5 fg de ADN de *P. arborescens* mezclado con ADN de *Pap. somniferun*. La utilización de esta técnica permitió amplificar *P. arborescens* en muestras de herbario de hasta 98 años de antigüedad, y demostrar que *P. arborescens* puede generar infecciones sistémicas asintomáticas tanto en adormidera cultivada como en *Papaver* spp. arvenses, y estar presente con alta frecuencia en lotes de semillas comerciales en España.

Siguiendo con el desarrollo de técnicas para la detección de *P. arborescens* en adormidera, se desarrolló un protocolo de PCR cuantitativa (qPCR) en tiempo real para identificar y cuantificar ADN de *P. arborescens* en diferentes tejidos de adormidera.

Este método es fiable, innovador, rápido y económico y posee las mismas ventajas de especificidad, sensibilidad, y versatilidad que los protocolos de PCR simple y aninada. El protocolo puesto a punto con el par de cebadores específicos P6 y el fluoróforo Syber Green I alcanzó alta reproducibilidad, buenas eficiencias y linealidad en seis órdenes de magnitud entre los valores de C_T y el Log (DNA) de *P. arborescens*, con altos valores (>0.99) del coeficiente de determinación, alcanzando límites de detección de hasta 10 fg en un fondo de ADN de tejidos de adormidera. Las propiedades de reproducibilidad, eficiencias y valores del coeficiente de determinación se mantuvieron en valores similares cuando se utilizó ADN plasmídico con el inserto diana de la región ITS del ADNr de *P. arborescens*. En base a estos resultados fue posible desarrollar un modelo robusto y universal para su uso como control de la cuantificación de ADN de *P. arborescens* entre diferentes muestras, experimentos, y/o laboratorios, etc. El modelo está basado en los modelos lineales que establecen las relaciones numéricas entre el C_T y el Log (DNA) en la línea estándar obtenida con ADN plasmídico y la línea estándar obtenida con ADN genómico de *P. arborescens*. Las aplicaciones realizadas (en tallos de adormidera, lotes comerciales de semillas y semillas procedentes de cápsulas con diferente incidencia de la enfermedad) de dicho modelo utilizando los iniciadores y protocolos diseñados, muestran resultados de gran interés ya que han permitido cuantificar el patógeno en infecciones sistémicas asintomáticas y en semillas procedentes de cápsulas sin síntomas.

Finalmente, en esta Tesis Doctoral se abordó el estudio de la biología de la interacción *Pap. somniferum*/*P. arborescens* determinando la naturaleza de la(s) fuente(s) de inóculo primario y el/los tipo(s) de infección determinantes del desarrollo de epidemias de Mildiu. Dicho estudio se llevó a cabo mediante un abordaje experimental integrador que ha combinado (i) observaciones de campo, (ii) ensayos de patogenicidad en condiciones controladas y campo, y (iii) desarrollo de técnicas moleculares (PCR específica simple, PCR anidada y qPCR) para la detección y cuantificación del patógeno en tejidos de adormidera sintomáticos y asintomáticos. Los resultados de la presente Tesis Doctoral han permitido determinar los siguientes aspectos clave del ciclo de patogénesis de la enfermedad: Se ha demostrado la transmisión del patógeno en semillas infectadas/infestadas y su papel como inóculo primario contribuyente a la extensión de la enfermedad a campos o áreas geográficas libres de ésta. Las semillas en la cápsula pueden ser infectadas/infestadas debido a

infecciones sistémicas primarias por oosporas así como por infecciones secundarias por esporangios, que a su vez pueden ser sistémicas o no, y sintomáticas o asintomáticas. Los esporangios desarrollados en plantas infectadas en condiciones ambientales favorables son eficientes en producir nuevas infecciones secundarias que diseminan la enfermedad a plantas asintomáticas. A su vez, estas infecciones pueden ser sistémicas o no. Las oosporas contenidas en restos de cosecha infestados o en suelo infestado son eficientes en originar infecciones en los órganos subterráneos de plántulas de adormidera durante el establecimiento del cultivo y originar infecciones sistémicas sintomáticas o asintomáticas.

Esta Tesis Doctoral ha permitido por tanto desarrollar nuevas tecnologías que permitirán establecer medidas efectivas de control de la enfermedad, fundamentalmente basadas en los principios de exclusión y erradicación, aplicables fundamentalmente sobre lotes de semilla infectados o sobre cultivos de adormidera comerciales. Dichas medidas pueden ser practicadas en las condiciones agronómicas en que tiene lugar el cultivo directamente por el agricultor con la asistencia técnica correspondiente por Alcaliber S.A. Además, la presente Tesis Doctoral ha establecido las bases científicas y tecnológicas para el desarrollo de (i) programas de certificación sanitaria de semilla de adormidera, (ii) evaluación de material vegetal en programas de mejora genética de variedades resistentes/tolerantes, y (iii) estudios sobre la epidemiología y dispersión del agente causal que actualmente se están llevando a cabo y que son aspectos fundamentales para el control de la enfermedad.

SUMMARY

Oppium poppy (*Papaver somniferun* L.) is one of the most important crops for the pharmaceutical industry in Spain because it is the only source of the alkaloid drugs morphine, codeine and thebaine. In Spain, opium poppy is grown annually on approximately 7,500 ha, primarily in the southern (Andalucía) and central (Castilla-La Mancha and Castilla y León) regions of the country. During the last few years, attacks by a new, undiagnosed Downy mildew disease have lead to a steady decrease in yields of opium poppy crops in Spain. Also, the incidence and severity of those attacks have increased steadily due mainly to expansion of the crop to new cooler and irrigated areas in central Spain for improving harvest yields. The main objectives of this Ph Thesis research were: (i) to develop new knowledge about the etiology, biology and epidemiology of the Downy mildew of opium poppy; (ii) the molecular characterization and specific molecular detection of the causal agent of the disease; and (iii) to develop knowledge-based disease management strategies that would help to protect yields of opium poppy crops in Spain and elsewhere.

Firstly, we determined the etiology of the Downy mildew of oppium poppy in comercial crops in Spain, and identified the causal agent as the obligated biotrophic Oomycete *Peronospora arborescens* (Berk) de Bary, using conventional (microscopic observations and pathogenicity assays) and molecular (sequence analyses of the ITS1-5,8S-ITS2 region of the ribosomal DNA, rDNA) techniques. Thereafter, we determined the composition, genetic diversity and phylogenetic relationships in populations of *P. arborescens* based on the sequence analyses of the ITS1-5,8S-ITS2 region of rDNA using isolates of *P. arborescens* and *P. cristata* from diverse geographic origins. Results suggested that only *P. arborescens* occurs in cultivated *Pap. somniferum* in Spain. Moreover, analyses of the ITS1-5,8S-ITS2 region of rDNA from 27 samples of Downy mildew-affected plants from all opium-poppy-growing regions in Spain showed that low genetic diversity exists within *P. arborescens* populations in Spain and France, as well as that they are phylogenetically distinct from *P. cristata,* the Downy mildew pathogen of opium poppy crops in Tasmania (Australia). Moreover, a detailed phylogenetic analysis of *P. arborescens* populations, using Downy mildew-affected tissues from different species of *Papaver* (*Pap. dubium, Pap. hybridum, Pap. rhoeas*, y *Pap. somniferum*) from herbarium

collections of different geographical locations, indicated for the first time that a degree of host specificity may exist within *P. arborescens* populations.

The ITS1-5,8S-ITS2 rDNA sequences have been proved useful for the development of three *P. arborescens*-specific primers pairs P3Pa2fw/P3Pa2rv (P2), OMPac1fw/OMPac1rv (P3) and OMPac7fw/OMPac7rv (P6), as well as of a PCR-based protocol that was used to answer several lingering questions in the *Pap. somniferum*/*P. arborescens* pathosystem. The species-specific primers and the PCR assay protocol developed allowed to differentiated *P. arborescens* from *P. cristata* and proved useful for the detection of *P. arborescens* in symptomatic and asymptomatic opium poppy plant tissues. Use of these primers demonstrated that *P. arborescens* can be seed borne and seed transmitted and that commercial seed stocks collected from crops with high incidence of the disease were frequently infected by the pathogen. This detection protocol has been registered as a Spanish patent application Nº P200603319 (with two extensions P200803261 y P200803262) and it has been internationally extended with the number PCT/ES2007/000781.

Additionally, the *P. arborescens*-specific primer pairs P3 and P6 were used to develop a sensitive nested-PCR protocol that improves the in-planta detection of *P. arborescens* DNA. The new protocol provides an increase in sensitivity of 100- to 1,000-fold in the detection of the oomycete in opium poppy tissue compared with the detection limit from single PCR using the same primer pairs, respectively. The new protocol allowed amplification of 5 to 0.5 fg of *P. arborescens* DNA mixed with *Pap. somniferum* DNA. The protocol proved useful for amplifying *P. arborescens* DNA from 96-year-old herbarium specimens of *Papaver* spp. and to demonstrate that asymptomatic, systemic infections by *P. arborescens* can occur in wild *Papaver* spp. as well as in cultivated opium poppy. Also, the increase in sensitivity of the protocol made it possible to demonstrate a high frequency of seedborne *P. arborescens* in commercial opium poppy seed stocks in Spain, which posses a threat for pathogen spread to new cropping areas.

An additional further step was then made to develop a reliable, innovative, quick, and low-cost real-time quantitative PCR (qPCR) assay that, while keeping the advantages of specificity, sensitivity and versatility described for the simple- and nested-PCR protocols, would be useful in the identification and quantification of *P. arborescens* DNA in different opium poppy tissues. The use of primer pair P6 and the Syber Green

I fluorescent dye in the new protocol resulted in an assay that showed highly reproducible and efficient, as well as good linear relationship over six orders between C_T and Log (DNA) of *P. arborescens* values with highly significant coefficient of determination (R^2>0.99), reaching a detection limit of up to 10 fg of *P. arborescens* DNA within a background host DNA. Furthermore, the reproducibility, efficiency and level of correlation remained at levels similar to those referred above when plasmidic DNA containing the target DNA as a cloned insert were used as template for the assays. On basis of those results, we developed a robust and universal model to be used as a control in the quantification of *P. arborescens* DNA among different samples, assays, laboratories, etc. This model is based on the numerical relationship that was established between C_T and Log (DNA) of *P. arborescens* for the standard line obtained for DNA extracted from the plasmid with the target DNA as cloned insert and the standard line obtained for genomic *P. arborescens* DNA. To validate the developed model, we carried out assays using the designed primers pairs and protocols and stem of oppium poppy plant, commercial seed stocks, and seeds harvested from capsules with different levels of disease incidence. Results showed that the newly developed quantification protocol and model would be of much interest for the quantification of the pathogen amount in systemically infected, asymptomatic opium poppy plants as well as seeds from asymptomatic capsules.

Finally, in the research we aimed to provide better understanding of the interaction between *Pap. somniferum* and *P. arborescens* by determining the nature and source(s) of primary inoculum and infection types for the development of Downy mildew epidemics. That was carried out by means of an integrative experimental approach that combined: (i) field observations, (ii) pathogenicity assays in growth chambers and field microplots and (iii) molecular detection assays of *P. arborescens* infection using the species-specific simple-, nested-PCR, and real-time qPCR protocols for the detection and quantification of the pathogen in oppium poppy symptomatic or asymptomatic tissues. Results revealed key aspects of this pathosystem in relation to the cycle of pathogenesis of the disease. Thus, our results demonstrate that infected/infested seeds are a major source of primary inoculum of the pathogen, and suggested that seeds harbouring the pathogen may have contributed to the spread of the disease to fields or geograohic areas free from *P. arborescens*. Furthermore, oospores harbored in infested crop debris or in infested soil are efficient in originating infections

of underground plant tissues early during poppy seedling growth and to give rise to systemic, symptomatic or asymptomatic infections. Therefore; infested soil as well as overwintering debris from infected crops can also serve as source of primary inoculum for the disease.

We demonstrated that opium poppy seeds can be infected/infested as a consequence of systemic primary infections by oospores, as well as either systemic or non-systemic secondary infections by airborne sporangia, and that both kinds of infection types can take place without symptom expression in the plant. The sporangia formed on infected plants in favorable environmental conditions are effective in producing new secondary local infections that may spread the disease to asymptomatic plants. These new secondary local infections can be systemic or non-systemic and symptomatic or asymptomatic.

We conclude that the technologies and knowledge developed in this PhD research will help to establish effective control measures of opium poppy Downy mildew, mainly based on the principles of exclusion and eradication and aimed mainly to commercial seed stocks and commercial poppy crops. These technologies can be practiced under the agronomic conditions currently practiced for the crop in Spain, and can be easily practiced by the farmer with the proper technical assistance from Alcaliber S.A. staff. In addition we have established the scientific and technological basis for implementing and enhancing: (i) current programs of sanitary seed certification; (ii) breeding programs for the development of opium poppy varieties resistant/tolerant varieties to Downy mildew; and (iii) studies on the epidemiology of the disease and airborne dispersion of the causal agent, that are currently taken place.

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CAPÍTULO I

Introducción General y Objetivos

I.1

INTRODUCCIÓN GENERAL

Durante las últimas décadas está teniendo lugar un envejecimiento progresivo de la población y notable aumento de la prevalencia de algunas enfermedades crónicas, al cual puede haber contribuido el incremento en la supervivencia de muchos pacientes por los avances en el tratamiento de patologías como el cáncer. Sin embargo, la mejora de calidad de vida de los pacientes durante las fases avanzadas de dichas enfermedades depende fundamentalmente del esmero en los cuidados paliativos y el tratamiento integral del paciente terminal. Los fármacos opiáceos son cada vez más necesarios en la sanidad de la población.

En 1986, la magnitud del problema del tratamiento insuficiente del dolor oncológico llevó a la Organización Mundial de la salud (OMS) a publicar las directrices para el control del dolor en pacientes afectados de cáncer (World Health Organization, 1986) basado en el uso de fármacos opioides como piedra angular, lo cual supone que más del 80% de los pacientes pueden ser controlados utilizando este sistema (Zech et al., 1995). Por ello, el papel de las sustancias opioides, fundamentalmente morfina, como medicamentos para el dolor oncológico terminal determinó su inclusión en la lista de medicamentos esenciales de la OMS. En España, una media anual de cerca de 90.000 personas fallecen al año debido a tumores malignos. Esto supone que las enfermedades comúnmente denominadas como cáncer son la primera causa de muerte en nuestro país (i.e., una de cada cuatro muertes es atribuible a esta enfermedad) (Regidor et al., 1998). El 30-40% de los pacientes con cáncer sometidos a tratamiento activo contra esta enfermedad refieren al dolor como el síntoma más temido e incapacitante para el paciente, y este porcentaje llega a ser del 70 al 90% en estados avanzados de esta enfermed (Cleeland et al., 1994; Llobera et al., 1998), lo cual hace imprescindible que sea aliviado mediante la administración de opiáceos.

El tratamiento del dolor neuropático sigue siendo uno de los problemas pendientes de resolver de una manera satisfactoria, por la gran variedad clínica y fisiopatólogica de los síndromes dolorosos que explica la gran variedad de fármacos y técnicas empleadas. La utilización de opioides en el dolor neuropático es un tema controvertido. Clásicamente no se admitía la utilización en base a la probabilidad de producir escasos efectos analgésicos y la posibilidad de aparición de tolerancia y dependencia física. Sin embargo desde los años 80 y debido a la experiencia acumulada a largo plazo en el tratamiento del dolor oncológico con opioides se empezó a cuestionar dicho planteamiento. Existen estudios favorables de la administración de

opioides en pacientes con dolor neuropático con buenos resultados tanto en alivio del dolor como en la mejoría del estado funcional sin observarse problemas graves de tolerancia y adicción (Herrera Silva, 2001). Otros artículos hacen referencias a la prioridad del manejo del dolor crónico en los pacientes y al conveniente uso de opioides para tratar el dolor crónico no producido por cáncer, aunque siempre con previas evaluaciones del paciente (American Academy of Pain Medicine and the American Pain Society, 1997).

En España, se comercializan en la actualidad varios opioides con fines farmacológicos, entre los que se encuentran los alcaloides morfina, codeína y tebaína, que son ampliamente utilizados en medicina como analgésicos, antitusivos y antiespasmódicos (Bryant, 1988; Hansen, 1994; Heeger y Poetheke, 1947). La industria farmacéutica española consume anualmente alrededor de 5.500 kg de morfina, y existe una tendencia al incremento de esta cantidad, entre otros motivos, por el aumento de la esperanza de vida de los pacientes oncológicos y el envejecimiento de la población española.

De hecho, se puede concluir que en la actualidad nos encontramos ante una tendencia creciente y generalizada respecto de la utilización de opioides naturales o semisintéticos, tanto para el tratamiento de dolores en pacientes terminales como en el tratamiento del dolor crónico no maligno, lo cual requiere que la estabilidad de provisión de la materia prima para la obtención de estos opioides deba estar garantizada a nivel mundial.

I.1.1 EL CULTIVO DE LA ADORMIDERA

La adormidera (*Papaver somniferum* L.) es una de las plantas cultivadas más importantes para la industria farmacéutica, ya que constituye la única fuente de alcaloides como la morfina, codeína y tebaína.

La adormidera es una planta herbácea, anual, con una fase vegetativa distintiva caracterizada por grandes y numerosas hojas pinnadas que se distribuyen en forma de roseta, y una etapa reproductiva durante la que florecen los brotes de los tallos. La raíz es pivotante y profunda, y el tallo erguido, robusto, poco ramificado y de hasta 1,60 m de altura. Las hojas abrazan el tallo por su base y son alternas, sentadas, grandes, lobuladas, lampiñas, de color verde claro y con nervaduras prominentes en el envés.

Las flores son terminales y solitarias, compuestas de un cáliz de dos sépalos lampiños que se desprenden al abrirse la flor, y por una corola de cuatro pétalos en forma de cruz con numerosos estambres y con un pistilo de forma redondeada u ovoidea con varios estigmas. Los pétalos son generalmente blancos, aunque suelen tener tonalidades rosas o violáceas, pero siempre con una mancha oscura en la base. Sus frutos son cápsulas globosas y deprimidas, rematadas por una corona estigmática lobulada. La pared de la cápsula presenta de ocho a 10 láminas placentarias que dividen la cápsula internamente en celdillas incompletas que contienen las semillas. Éstas son muy numerosas y pequeñas, de aproximadamente 1 mm de longitud y 0,7 mm de anchura, reniformes y de superficie reticulada, y color blanco a pardo oscuro (Heeger y Poetheke, 1947; Singh, 1982) (Fig. I.1.1).

La historia divergente y larga de la domesticación y cultivo de *P. somniferum* ha dado lugar al desarrollo de varias razas, variedades quimiotipo, y cultivares adaptados a usos específicos y condiciones climáticas particulares. El cultivo de la planta cubre un área geográfica amplia, que en el hemisferio Norte abarca desde Bombay en la India hasta Moscú en Rusia, y Tasmania en el hemisferio Sur (Krikorian y Ledbetter, 1975).

Figura I.1.1. A. Planta de adormidera. B. Flor multipétala roja. 1. Estructura de la flor. 2. Estambres. 3. Grano de polen. 4. Cápsula en desarrollo. 5. Sección transversal de una cápsula. 6. Cápsula madura. 7. Semillas. 8. Sección longitudinal de una semilla. 9. Sección transversal de una semilla (Fuente: www.botanical.com).

I.1.1.1 Importancia del cultivo de adormidera a nivel mundial

En países de la zona templada, el cultivo de la adormidera se realiza con dos propósitos: 1) para la obtención de paja, importante materia prima para la obtención de productos farmacéuticos; y 2) para obtención de semilla y aceite, ambos utilizados en alimentación y otros procesos de producción industrial. En algunos países, la semilla es el único producto del cultivo local de adormidera que se comercializa (ej., Alemania y Austria). Sin embargo, tanto la paja como la semilla tienen un importante uso y valor de mercado que contribuyen a la rentabilidad del cultivo. La semilla, que es considerada como un subproducto, se utiliza también en alimentación y está catalogada como una buena fuente de aceite y proteínas (Maza et al., 1988).

Las plantas de adormidera también tienen una importancia menor, aunque creciente, como especie decorativa y las cápsulas secas se usan en las composiciones de flores secas, lo que ha dado lugar al desarrollo de cultivares específicos para este fin.

En la adormidera, la maduración de las cápsulas coincide con una elevada concentración de alcaloides en éstas. En los países de clima tropical (ej., India y China), la adormidera se cultiva principalmente para la obtención de opio (también denominado opio bruto) que es el látex seco que se obtiene al practicar incisiones en las cápsulas verdes de la planta de adormidera (Singh et al., 1982).

El opio bruto y la paja de adormidera son las materias primas de las que se extraen los alcaloides morfina, codeína, tebaína, noscapina, oripavina, papaverina y narceína para su utilización en la industria farmacéutica.

El concentrado de adormidera que se obtiene en el proceso de extracción de alcaloides de la paja de adormidera está sometido a fiscalización como estupefaciente separado en virtud de la Convención de 1961 por la Junta Internacional de Fiscalización de Estupefacientes (INBC, siglas en inglés). La morfina y la codeína están sujetas a fiscalización internacional debido a los riesgos de abuso que conllevan, y la tebaína porque se puede transformar en opioides que son objeto de abuso. La noscapina, oripavina, papaverina y narceína no están sometidas a fiscalización internacional. La morfina es el prototipo de los opiáceos naturales y de muchos opioides y, debido a su gran poder analgésico, se utiliza como parámetro de referencia para hacer comparaciones.

La demanda de alcaloides ha aumentado en los últimos 20 años y de las dos materias primas que se utilizan para la obtención de éstos, el opio y la paja de adormidera, es esta última la que más se ha utilizado a lo largo de ese período para atender dicha demanda (INCB, 2006). Sin embargo, debido a la peculiaridad del cultivo (lícito e ilícito) de adormidera, resulta difícil cuantificar el área real de cultivo en el mundo, aunque ésta se estima en más de 250.000 ha.

Por paja de adormidera se entienden todas las partes de la planta de la adormidera después de cortada, excepto las semillas. La morfina es el alcaloide que predomina en las variedades de paja de adormidera cultivadas en la mayoría de los países productores. El cultivo comercial de paja de adormidera con un alto contenido de tebaína comenzó en la segunda mitad del decenio de 1990 en respuesta al acentuado aumento de la demanda de ese alcaloide. La paja de adormidera obtenida de variedades de adormidera rica en morfina se denomina "paja de adormidera (M)" y la paja de adormidera obtenida de variedades de la adormidera ricas en tebaína se denomina "paja de adormidera (T)". Además de un alcaloide principal (morfina o tebaína), algunas de esas variedades contienen adicionalmente otros alcaloides que también se pueden extraer.

En 2005, alrededor del 80% de la morfina y más del 93% de la tebaína fabricada a nivel mundial se obtuvieron a partir de la paja de adormidera, y el resto se extrajo del opio.

La concentración de alcaloides en la paja de adormidera varía considerablemente de un país productor a otro. La comparación de los volúmenes de producción de paja de adormidera de los distintos países y la determinación de las tendencias mundiales de la producción sólo son posibles mediante la utilización de un denominador común, que es el volumen equivalente de morfina o tebaína de la cantidad de paja de adormidera producida en cada país.

En la Tabla I.1.1 se muestra la evolución de la superficie cultivada de adormidera, el volumen de la paja de adormidera (M) y (T) cosechada, y los rendimientos de cultivo obtenidos en los principales países productores a nivel mundial, y en la Tabla I.1.2 se presentan los datos anteriores a nivel mundial obtenidos a partir de la media de todos los países productores.

A lo largo del último decenio, los principales países productores de paja de adormidera han sido Australia, Francia, España, Hungría y Turquía, que suman más

del 90% del total mundial. En 2005, Australia lideraba la producción de adormidera con 112 t, que representaban el 32% de la producción mundial, seguida por Francia (96 t, 27% del total mundial), Turquía (64,4 t, 18% del total mundial), España (36 t, 10% del total mundial) y Hungría (15,3 t 4% del total mundial) (Tablas I.1.1 y I.1.2).

La producción mundial de paja de adormidera (M) expresada en la cantidad equivalente de morfina ha fluctuado ampliamente en los últimos dos decenios debido a las condiciones climáticas y a la demanda en los países productores, pero ha seguido en general una tendencia ascendente. La producción mundial alcanzó el nivel máximo hasta la fecha en el 2003, cifrándose en el equivalente de 451 t de morfina. En 2005, la producción mundial comunicada de paja de adormidera (M) ascendió al equivalente de 355 t de morfina.

La cantidad de paja de adormidera (M) utilizada en 2005 para la extracción de alcaloides ascendió a 17.508 t en Turquía, 5.598 t en Australia, 5.540 t en Eslovaquia, 4.945 t en Francia, 3.665 t en Hungría, 3.509 t en España y 1.203 t en China (NCBI, 2006).

Finalmente, la producción ilícita de opio también es practicada por grupos étnicos del este y sudeste de Asia, en particular en países como Afganistán, Irán, Líbano y Pakistán. Dado su carácter ilícito, se dispone de muy poca información disponible sobre la producción en estos países; no obstante, se estima que la superficie total de cultivo de adormidera de opio en estas regiones pudo alcanzar a principios de los años 90 las 30.000 ha, lo que supone aproximadamente el 15% de la superficie total dedicada al cultivo de adormidera en el mundo (Gordon, 1994).

Tabla I.1.1. Cultivo de *Papaver somniferum* con fines distintos de la producción de opio por principales países productores en los años 2001-2007.

		Paja de adormidera (M)[a]				Paja de adormidera (T)[b]			
País	Año[c]	Superficie (ha)	Cantidad cosechada (kg)	Rendimiento/ hectárea (kg)	Export. (kg)	Superficie (ha)	Cantidad cosechada (kg)	Rendimiento/ hectárea (kg)	Export (kg)
	2001	8.925	5.691.000	638	25	10.369	7.211.000	695	—
Australia	2002	11.701	12.639.000	1.080	—	7.865	9.146.000	1.163	—
	2003	9.811	8.518.000	868	—	7.637	7.274.000	952	—
	2004	6.644	5.768.000	868	—	5.578	4.373.000	784	—
	2005	6.599	5.900.000	894	2	4.633	4.266.000	921	—
	2006	*4.900*				*5.300*			
	2007	*4.710*				*3.210*			
Austria	2001	. . [d]		28.516					
	2002	1.547 [d]		20.795					
	2003	1.740 [d]		33.642					
	2004	1.707 [d]		32.130					
	2005	3.092 [d]		17.278					
	2006	*1.700* [d]							
	2007	*1 900* [d]							
República	2001	33.235	4.174.560	126	4.174.560				
Checa	2002	29.637	4.202.260	142	4.202.260				
	2003	21.045	5.090.050	242	5.090.050				
	2004	16.030	4.563.360	285	4.563.360				
	2005	39.944	4.480.940	112	4.480.940				
	2006	*38.000*							
	2007	*56.000*							
Francia	2001	5.402	2.691.000	498	—	2.157	1.248.000	579	—
	2002	6.451	5.723.000	887	50	2.533	2.553.000	1.008	—
	2003	7.919	5.428.270	685	125.005	1.499	1.144.540	764	2.000
	2004	8.312	8.289.160	997	216.000	1.007	568.040	564	—
	2005	8.841	8.680.740	982	528.000	524	339.180	647	—
	2006	*9.100*				*1.000*			
	2007	*5.500*				*1.000*			
Hungria	2001	6.911	2.269.820	326	23.616				
	2002	9.924	3.250.988	328	33.824				
	2003	2.937	882.109	300	30.039	. .			
	2004	7.084	4.297.868	607	49.043	. .			
	2005	5.106	2.189.772	429	189.494	16	740	46	
	2006	*12.000*				*16*			
	2007	*13.000*				*16*			
España	2001	5.536	3.400.000	614	1.088.240	—	—	—	—
	2002	7.912	6.212.552	785	1.415.311	—	—	—	—
	2003	5.732	3.500.000	611	306.460	—	—	—	—
	2004	5.986	4.961.290	829	1.828.520	996	832.120	835	—
	2005	4.802	3.405.000	709	75.000	490	834.000	1.702	—
	2006	*6.002*				*1.000*			
	2007	*7.600*				—			
Turquía	2001	45.836	21.436.000	468	—				
	2002	50.741	17.529.000	345	—				
	2003	99.430	47.618.000	479	—				
	2004	30.343	16.190.360	534	—				
	2005	25.335	12.403.000	490	—				
	2006	*70.000*							
	2007	*70.000*							
Ucrania	2001	5.625[d]		—					
	2002	6.649[d]		—					
	2003	1.348[d]		—					
	2004	5.985[d]		—					
	2005	12.564[d]		—					
	2006	*7.800*[d]							
	2007	13.540[d]							

[a] Paja de adormidera producida a partir de adormidera *(P. somniferum)* rica en morfina.

[b] Paja de adormidera producida a partir de adormidera *(P. somniferum)* rica en tebaína.

[c] Los datos de los años en cursivas corresponden a estimaciones de cultivo.

[d] Cultivos para fines culinarios y decorativos.

Tabla I.1.2. Cultivo de *Papaver somniferum* con fines distintos de la producción de opio a nivel mundial en los años 2001-2007.

		Paja de adormidera (M)[a]				Paja de adormidera (T)[b]			
País	Año[c]	Superficie (ha)	Cantidad cosechada (kg)	Rendimiento/ hectárea (kg)	Export. (kg)	Superficie (ha)	Cantidad cosechada (kg)	Rendimiento/ hectárea (kg)	Export (kg)
Total	2001	116.009	40.630.839	350	5.317.026	12.526	8.459.000	675	
	2002	126.670	50.619.979	400	5.804.568	10.405	11.702.014	1.125	
	2003	153.420	73.222.010	477	5.592.800	9.170	8.434.503	920	2.000
	2004	86.030	46.341.811	539	6.689.739	7.581	5.773.160	762	
	2005	108.439	41.907.760	386	5.292.628	5.667	5.441.620	960	
	2006	*157.322*				*7.366*			
	2007	*179.688*				*4.276*			

[a] Paja de adormidera producida a partir de adormidera *(P. somniferum)* rica en morfina.
[b] Paja de adormidera producida a partir de adormidera *(P. somniferum)* rica en tebaína.
[c] Los datos de los años en cursivas corresponden a estimaciones de cultivo.

I.1.1.2 Importancia del cultivo en España

La industria farmacéutica española consume anualmente alrededor de 5.500 kg de morfina. La adormidera es por tanto uno de los cultivos más importantes para esta industria en España, ya que los alcaloides morfina, codeína y tebaína se obtienen únicamente a partir de la paja de adormidera.

España es el cuarto productor mundial y el tercer productor europeo de semilla y paja de adormidera (M) y (T) (Tabla I.1.1; NCBI, 2006). Respecto a la producción de alcaloides, España es el tercer productor representando actualmente el 12,3 % de la producción mundial de alcaloides (NCBI, 2006). En nuestro país, en el 2007 el cultivo de la adormidera ocupó una extensión de 7.160 ha y una producción total de 11.733 t (6.686 t semilla + 5.047 t cápsula) (Alcaliber, S.A., 2007), que se distribuyeron en Andalucía (2.626 ha), Castilla-La Mancha (4.429 ha), y Castilla y León (105 ha), lo cual supone aproximadamente el 4 % de la superficie mundial (Fig. I.1.2). Para el 2008 existen unas previsiones de cultivo de aproximadamente unas 10.000 has, que se distribuirán en unas 4.000 ha en Andalucía, 5.500 ha en Castilla-La Mancha, y 500 ha en Castilla y León (F.J. Muñoz Ledesma, comunicación personal). Estos niveles de producción convierten a nuestro país en una de las áreas cultivadoras de adormidera más importantes del oeste de Europa.

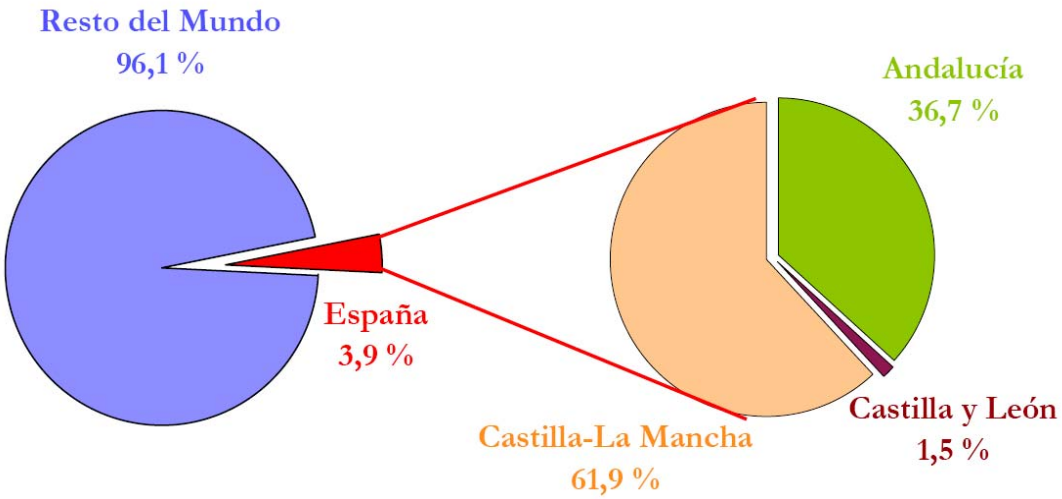

Figura I.1.2. Superficie cultivada de paja de adormidera en España por Comunidades Autónomas, respecto del total mundial en el año 2007.

La adormidera está considerada actualmente como un cultivo estratégico en España, por lo cual está sujeto a un estricto control de su superficie y producción por el Ministerio de Sanidad y Consumo en base a las directivas de la INCB auspiciada y regulada por la Organización de las Naciones Unidas (ONU). En nuestro país Alcaliber S.A. es la única empresa autorizada al cultivo de adormidera desde 1973, con vistas a su transformación en concentrado de paja de adormidera y posterior extracción química de sus alcaloides destinados exclusivamente a usos medicinales.

I.1.2 PRINCIPALES ENFERMEDADES DE LA ADORMIDERA

Las enfermedades constituyen una de las principales limitaciones para la producción eficiente de la adormidera. Existen numerosas enfermedades de muy diversa etiología que pueden afectar a la adormidera en diferentes partes del mundo. Hasta ahora, se han descrito 168 microorganismos (en su mayoría patógenos) aislados de plantas de adormidera, incluyendo 82 especies de hongos, cinco especies de bacterias, 70 especies de nematodos y 11 virus, algunos de ellos específicos de papaveráceas (Muñoz-Lesdesma, 2002). No obstante, sólo algunas de estas enfermedades han sido reconocidas como restrictivas económicamente para la producción de adormidera a nivel mundial (Schmitt, 1975). Entre estas últimas, destacan por su importancia la Necrosis de raíz y cuello causada por *Pleospora papaveracea* (Not.) Sacc. (Sivanesan y Holliday, 1982), el Mildiu causado por *Peronospora*

arborescens (Berk.) Bary (Nolte, 1951; Pape-Kiel, 1944; Yossifovith, 1928), la Podredumbre del tallo causada por *Sclerotinia sclerotiorum* (Lib.) Bary (Laughlin y Munro, 1983) y el Carbón foliar causado por *Entyloma fuscum* Schroet. (Laughlin y Beattie, 1987; Savile, 1946; Wiberg, 1990).

En España la Necrosis de raíz y cuello causada por *Pleospora papaveracea* (de Not.) Sacc. fue descrita en 2002 (Muñoz-Lesdesma, 2002). Recientemente, ha sido descrita sobre el cultivo de adormidera una enfermedad de etiología bacteriana causando podredumbre blanda en plantas de adormidera y cuyo agente causal ha sido descrito como *Pectobacterium carotovorum* (Jones) Waldee (Aranda, 2008).

I.1.3 EL MILDIU DE LA ADORMIDERA

I.1.3.1 Oomicetos y su clasificación taxonómica

Introducción

Las enfermedades de las plantas causan miles de millones de dólares de pérdidas en cultivos agrícolas cada año (Rossman y Palm, 2006). Uno de los grupos de organismos que causan importantes enfermedades en las plantas son los conocidos durante mucho tiempo como Oomicota u oomycetos. Hasta recientemente este grupo estaba clasificado como hongos no verdaderos. En las últimas tres décadas, el conocimiento sobre las relaciones filogenéticas entre grupos de hongos se ha incrementando ampliamente, en tanto que muchos de los agrupamientos taxonómicos tradicionales no reflejaban en gran medida las relaciones filogenéticas descubiertas actualmente.

Los Oomicetos son un grupo diverso de microorganismos del Reino Straminopiles que comprenden más de 800 especies (Greenville-Briggs y van West, 2005) e incluyen miembros saprofitos así como patógenos de gran variedad de organismos entre los que se encuentran plantas, insectos, peces, nematodos, vertebrados y varios microorganismos incluyendo otros Oomicetos. Los Oomicetos fitopatógenos infectan un amplio espectro de plantas, incluyendo plantas cultivadas y no cultivadas, además de plantas y árboles ornamentales.

Aunque esta clase de organismos ha sido tradicionalmente conocida como los mohos de agua, las familias de la clase Oomicetos exhiben una clara tendencia evolutiva hacia una menor dependencia del ambiente acuático y algunos miembros del orden Peronosporales, como las especies del género *Hyaloperonospora*, no presentan ya una fase zoospórica en su ciclo de vida (Koch y Slusarenko, 1990).

Las enfermedades de plantas causadas por Oomicetos han tenido una fuerte influencia en el desarrollo de la historia de la humanidad; el caso más dramático y quizás conocido de enfermedades de plantas cultivadas es el Tizón tardío o Mildiu causado por *Phytophthora infestans* (Mont.) Bary, que dio como resultado la hambruna en Irlanda. A consecuencia de esta enfermedad, aproximadamente un millón de personas murieron de hambre y otros 1.5 millones tuvieron que emigrar a EE. UU. y otros países (Alexopoulos, et al., 1997). Otras enfermedades de las plantas causadas por especies de *Phytophthora* incluyen la Muerte Súbita del roble causada por *Phytophthora ramorum* Werres, la Podredumbre Negra del cacao causada por *Phytophthora megakarya* Brasier y Griffin y otras muchas enfermedades relacionadas con la podredumbre de raíces. Otros importantes miembros fitopatógenos de este grupo incluyen a *Pythium aphanidermatum* (Edson) Fitzp., y *Pythium ultimum* Throw, que causan Podredumbre de semillas, Muerte de plántulas en pre- y post emergencia, y Necrosis radicales en multitud de cultivos; *Peronosporai tabacina* Adam agente causal del Mildiu del tabaco (*Nicotiana tabacum* L.); *Plasmopara viticola* Berl. y De Toni agente causal del Mildiu de la vid (*Vitis vinifera* L.); y *Plasmopara halstedii* (Halsted) Berl. y De Toni agente causal del Mildiu del girasol (*Helianthus annuus* L.); entre muchos otros.

Durante años, los Oomicetos ha sido considerados hongos, con la particularidad de haber sido diferenciados como "hongos no verdaderos" por multitud de características. Actualmente hay suficientes evidencias para separar los Oomicetos del reino de los hongos verdaderos (Baldauf et al., 2000; Hall, 1996; Kamoun, 2003; Margulis et al., 2000). Estudios de su metabolismo (Pfyffer et al., 1990) y secuencias del ARN ribosómico (ARNr) (Cooke et al., 2000; Forster et al., 1990) han reclasificado a los Oomicetos con las Crysophytas y las algas marrones en el reino Chromista o Straminopila (Fig. I.1.3).

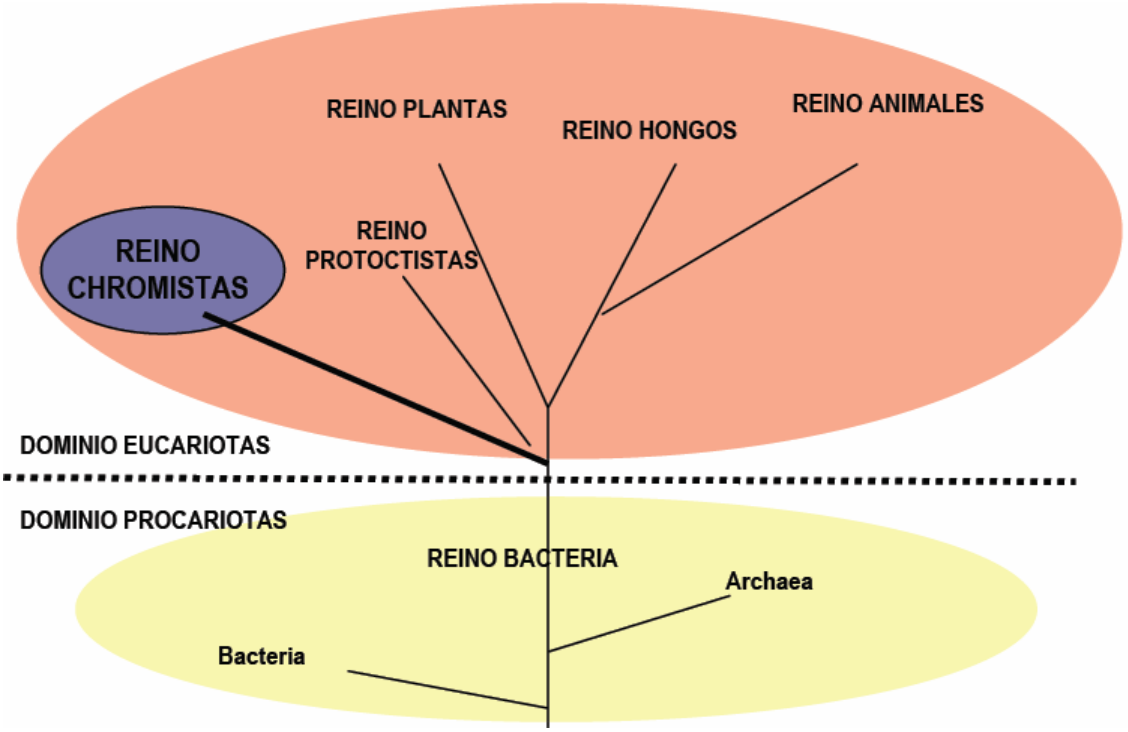

Figura I.1.3. Clasificación de los seres vivos según Cavalier-Smith, 2004.

En la Tabla I.1.3 (Rossman y Palm, 2006) se recogen las diferencias principales entre Oomicetos y los hongos verdaderos recopiladas a partir de los estudios anteriormente referidos.

Tabla I.1.3. Principales diferencias entre los Omycetos Cromistas y los hongos verdaderos (Chytridiomycetos, Glomeromycetos, Zygomycetos, Ascomycetos, Basidiomycetos) (Fuente: Rossman y Palm, 2006).

Característica	Clase Oomicetos	Hongos Verdaderos
Reproducción Sexual	Heterogametos. La fertilización del núcleo de la oosfera por el anteridio da lugar a la oospora	No producen oosporas. La reproducción sexual produce zigosporas, ascosporas o basidiosporas
Núcleo del micelio vegetativo	Diploide	Haploide o Dicariótico
Composición de la pared celular	ß-glucanos, celulasa	Quitina, celulasa raramente presente
Tipo de flagelo en las zoosporas (si es que éste es producido)	Flagelos, de dos tipos, uno en forma de látigo, situado posteriormente, el otro fibroso, ciliado, y situado anteriormente	Si se produce flagelo, normalmente este es de un único tipo, posterior y en forma de látigo.
Mitocondria	Con crestas tubulares	Con crestas planas

Relaciones entre Oomicetos

Los Oomicetos fitopatógenos más importantes se encuadran dentro de dos órdenes: Saprolegniales y Peronosporales. Dentro del Saprolegniales, sólo el género *Aphanomyces* se considera que contiene fitopatógenos importantes. Las especies de *Aphanomyces* son patógenos necrotróficos, y son agentes causales de Podredumbres de raíces en una amplia gama de plantas anuales, incluyendo cultivos como guisante y remolacha (Grenville-Briggs y van West, 2005).

El orden Peronosporales contiene un gran número de patógenos que causan importantes pérdidas económicas en las plantas cultivadas. En este orden se incluyen los géneros *Phytophthora*, qué contiene más de 60 especies patogénicas causantes de Marchiteces, Necrosis y Podredumbres de raíces; *Pythium,* que causa Podredumbres de semillas y raíces; *Albugo*, que causa la Roya blanca; los géneros *Bremia*, *Peronospora*, *Hyaloperonospora, Plasmopara* y *Pseudoperonospora* cuyas especies causan enfermedades comúnmente denominadas como Mildiu (Grenville-Briggs y van West, 2005)

Recientemente se han llevado a cabo numerosos y diversos estudios filogenéticos de secuencias del ADN ribosómico (ADNr), para resolver, no sin discrepancias, las relaciones filogéneticas existentes entre los miembros de la clase Peronosporomycetes (antiguos Oomicetos) (Cooke et al., 2000; Dick et al., 1999a; Förster et al., 2000; Leclerc et al., 2000; Matsumoto et al., 1999; Petersen y Rosendahl, 2000; Riethmüller et al., 1999).

Algunos de los géneros de la familia Peronosporaceae más avanzados evolutivamente son reconocidos como los patógenos económicamente más importantes; y constituyen un grupo interesante para su estudio en relación al proceso de su evolución (Riethmuller et al. 2002). Su éxito evolutivo se refleja por una rápida radiación en su árbol filogenético que probablemente es el resultado de la especialización parasítica sobre las angiospermas y de la transición a la dispersión más eficaz de sus propágulos por el viento, con la formación de esporangióforos. Estas tendencias evolutivas han ejercido una notable influencia sobre las hipótesis filogenéticas del grupo completo.

Generalmente se ha aceptado que los géneros más evolucionados son los agentes causales de los Mildius y Royas blancas, que tienen su origen en antepasados en los géneros *Pythium-Phytophthora* más primitivos (Barr, 1983; Dick et al., 1989;

Gaümann, 1964; Shaw, 1978, 1981). Como resultado, la clasificación supragenérica de los Peronosporomycetidae (antigua familia Peronosporaceae) ha permanecido estable durante años.

En las clasificaciones actuales, como la de Hawksworth et al. (1995) y Dick (2001a), basadas en la interpretación del biotrofismo obligado, el crecimiento determinado del esporangióforo, la producción de conidias, y el carácter apomíctico, los Peronosporomycetidae contienen dos órdenes, el orden Pythiales y el orden Peronosporales. Se considera que los Pythiales representan un linaje más primitivo y consta de dos familias: La pequeña y poco conocida familia Pythiogetonaceae y la mas ampliamente conocida Pythiaceae con los géneros *Pythium* y *Phytophthora* al frente, ambos importantes parásitos necrotrofos facultativos de plantas. El segundo orden, Peronosporales, comprende patógenos obligados de la parte aérea de las plantas e incluye dos familias: Albuginaceae (Royas blancas) y Peronosporaceae (Mildius), la última de las cuales contiene actualmente ocho géneros fitopatógenos: *Benua*, *Bremia*, *Bremiella*, *Paraperonospora*, *Peronospora*, *Plasmopara*, y *Pseudoperonospora* (Riethmüler et al, 2002). Recientemente en un estudio basado en caracteres fenotípicos y datos moleculares (usando secuencias de la región ITS, Región Espaciadora Interna del ADNr) se ha introducido el género *Hyaloperonospora* para acomodar las especies de *Peronospora* que infectan especies de *Brassicaceae* (Constantinescu y Fatehi, 2002) como un nuevo género fitopatógeno de importancia en la familia Peronosporaceae.

Según Dick (2001a) la familia Peronosporaceae es la más grande de todas las comprendidas en los Oomicetos, con aproximadamente 600 especies en total. Sin embargo, los datos de análisis de diversidad genética más recientes (Cooke et al., 2000; Petersen y Rosendahl, 2000; Riethmüller et al., 1999; 2002) han suscitado algunas dudas sobre esta interpretación. En estos estudios, como el de Riethmuller et al. (2002) basado fundamentalmente en las relaciones de los Mildius verdaderos con grupos cercanos a partir de secuencias de ADNr, se ha dejado entrever una nueva situación para los géneros *Pythium* y *Phytophthora* tal como ya habían adelantado Cooke (2000) y Hall (1996).

Riethmuller et al. (2002) establecen que los árboles filogenéticos generados en su estudio basado en secuencias de ADNr son compatibles con la división basal de Oomicetos (Peronosporaceae y Saprolegniomycetidae) que propusieron Dick et al. (1984) y que dicha interpretación es consistente con los resultados de investigaciones

anteriores basadas en el análisis de secuencias de la subunidad grande ["large subunit" LSU (Petersen y Rosendahl, 2000)] y la subunidad pequeña ["small subunit" SSU (Dick et al., 1999b)] del ADNr, y de secuencias del gen mitocondrial ciclooxygenasa-2 (COX2) (Hudspeth et al., 2000).

Dentro de Peronosporomycetidae, los árboles filogenéticos generados por Riethmuller et al. (2002) no son totalmente compatibles con la clasificación jerárquica más aceptada actualmente (Dick 2001a; Hawksworth et. al., 1995), donde Pythiales y Peronosporales parecen ser órdenes polifiléticos. El género *Phytophthora*, asumido hasta ahora como un miembro del orden Pythiales, se relaciona más estrechamente con la familia Peronosporaceae que con la Pythiaceae, que ocupa una posición basal en el orden Peronosporomycetes. Además, estos resultados concuerdan con los análisis filogenéticos realizados por otros autores (Cooke et al., 2000; Petersen y Rosendahl, 2000; Riethmüller et al., 1999).

Sin embargo, en la literatura más reciente se continúa discutiendo sobre estos aspectos. Así, Göker et al. (2007) concluyen que las relaciones filogenéticas entre los géneros que causan Mildius y *Phytophthora* no pueden ser clarificadas a fecha de hoy. De hecho, los análisis realizados por Göker et al. (2007) basados en un alto número de genes conservados, de los biotrofos obligados más representativos, confirman con un soporte estadístico altamente significativo los resultados de Cooke et al. (2000 y 2002) y Voglmayr (2003), en los que se indica que *Phytophthora* es parafilético y que existen grupos (Clades) de *Phytophthora* más cercanos a los biotrofos obligados que otros.

En todos los trabajos anteriormente referidos el orden Peronosporales se considera como polifilético. Sin embargo, esta consideración se cuestiona por primera vez en el trabajo de Göker et al. (2007), en el que contando con substanciales evidencias filogenéticas se considerara a los Mildius como un grupo monofilético. Mientras, el género *Albugo* aparece en situación basal con el resto de los Peronosporaceae (Riethmüller et al., 2002).

Para la clasificación supragenérica, a falta de completar los estudios filogenéticos con nuevas regiones del genoma y con más géneros, Riethmüller et al. (2002) sugieren la utilización de una clasificación ligeramente modificada de la de Waterhouse (1973), con las siguientes características:

En los Peronosporomycetidae o Peronosporales se aceptaría la existencia de tres familias (que podrían ser elevadas a órdenes), con los siguientes cambios

destacables: el género *Lagenidium* formaría parte de la familia Pythiaceae; y los géneros *Phytophthora* y *Peronophythora* serían transferidos de la familia Pythiaceae a la Peronosporaceae. Similarmente, Greenville-Briggs y van West (2005) incluyen dentro del orden Peronosporales a la familia Phythiaceae (con los géneros *Pythium* y *Phytophthora*) mientras que Cooke (2000) y otros investigadores consideran a Pythiales como orden independiente, y dentro de éste incluyen a la familia Phythiaceae con el género *Pythium*.

Tal como decíamos anteriormente, la taxonomía tradicional de los Oomicetos (por ejemplo, Dick, 2001b, 2002; Waterhouse, 1973) se ha ido revelando como insatisfactoria en los últimos años. Al igual que con las familias Pythiaceae y Peronosporaceae, los estudios moleculares (Hudspeth et al., 2003; Riethmüller et al., 2002; Thines y Spring, 2005) también han repercutido sobre la ubicación taxonómica de la familia Albuginaceae, respecto de la cual se cuestiona ahora que deba ser considerada como miembro de Peronosporales (como por ejemplo en Dick, 2001b, 2002). Por lo tanto, en el orden Peronosporales podría permanecer una sola familia, Peronosporaceae, que sería considerada sinónimo del orden. Sin embargo, en este momento no hay soporte molecular suficiente para considerar a los Mildius como monofiléticos (Göker el al., 2007).

Basado en los resultados de análisis filogenéticos, Goker et al. (2007) sugieren que de aquí en adelante "Peronosporaceae" se restrinja a los Mildius, es decir, a los biotrofos obligados dentro de los Peronosporales. Hasta ahora, éstos incluyen a los géneros *Basidiophora, Benua, Bremia, Graminivora, Hyaloperonospora, Paraperonospora, Perofascia, Peronosclerospora, Peronospora, Plasmopara, Plasmoverna, Protobremia, Sclerospora,* y *Viennotia. Phytophthora* debe asignarse a Peronosporales, pero no a Peronosporaceae, y el género *Peronophythora* debería desaparecer e incluirse dentro de *Phytophthora.* Además son necesarios estudios adicionales para lograr una taxonomía mas satisfactoria del género *Phytophthora* (Göker et al., 2007).

En la Figura I.1.4 se presenta la clasificación taxonómica que tomamos como válida a partir de la bibliografía científica más actual consultada.

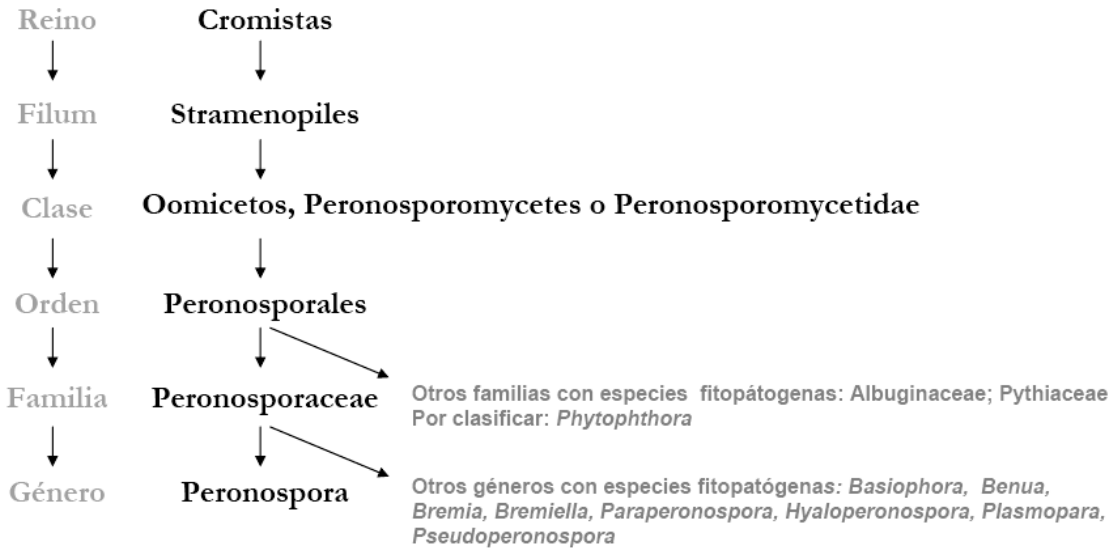

Figura I.1.4. Clasificación taxonómica del género Peronospora (Fuente: Constantinescu y Fatehi, 2002; Göker et al, 2007; Riethmüller et al., 2002; Voglmayr, 2003).

El género *Peronospora* Corda

Introducción

El género fúngico objeto de la presente Tesis Doctoral, *Peronospora*, se engloba dentro de un grupo de Oomicetos parásitos obligados a los que generalmente se conocen como Mildius, si bien tienen poco en común a excepción de la proliferación y fructificación de micelio en la superficie de los tejidos vegetales. No obstante, los Mildius constituyen un grupo especial de agentes fitopatógenos tanto por sus características morfológicas como las epidemias que originan en cultivos. Varias de las especies de este genero son patógenos económicamente muy importantes tanto en cultivos como plantas ornamentales (Agrios, 2005). Así, las especies de *Peronospora* ocasionan enfermedades severas en cultivos de considerable importancia económica y social como alfalfa, cebollas, girasol, judías, sorgo, tabaco, etc. Por ejemplo, en los últimos años, el Mildiu del sorgo ha aparecido y se ha extendido con gran rapidez en los Estados Unidos, lo que ha generado cierta inquietud ante la eventualidad de que tengan lugar futuras introducciones de otros Mildius de cultivos de cereales que ya existen en Asia y África (Agrios, 2005). Otro ejemplo de devastación debido a epidemias causadas por especies de *Peronospora* ocurrió con el Mildiu del tabaco en

1979, que fue detectado en Florida y rápidamente se extendió hacia Nueva Inglaterra y Canadá, causando pérdidas a los productores por valor de cientos de millones de dólares (Agrios, 2005).

El género *Peronospora* fue establecido por Corda en 1837 (*Icones Fungorum hucusque Cognitorum* 1: 20; Fig. I.1.5). Con respecto al número de especies dentro de este género existe cierta problemática debido a la especificidad de huésped de la mayoría de las especies de este género. Dependiendo de la definición de especie, el género *Peronospora* puede comprender entre 75 y 460 especies (Dick 2001c). La especie tipo de este género fúngico es *Peronospora rumicis* Corda (Fig. I.1.5).

Figura I.1.5. Descripción inicial del género *Peronospora* y su especia tipo *Peronospora rumicis* realizado por Corda (Fuente: Corda, 1837).

Peronospora puede ser considerado como el género más evolucionado y exitoso de su orden dada su biodiversidad y distribución (Voglmayr, 2003). Las especies de este género infectan principalmente a un amplio número de plantas dicotiledóneas herbáceas de clima templado, e incluso una monocotiledónea de la familia Alliaceae (Dick, 1988, 2001c), tal es el caso de *Peronospora destructor* (Berk.) Casp. agente causal del Mildiu de la cebolla (*Allium cepa* L.).

Dentro de la familia Peronosporaceae existen secuencias obvias de su evolución que comprenden desde la germinación zoosporangial inicial a la germinación como una verdadera conidia. El que los propágulos asexuales de los

Mildius sean esporangios o conidias puede ser determinado no sólo mediante observación de su germinación, sino también mediante análisis morfológicos de las esporas. Concretamente, *Peronospora* ha perdido la capacidad de producir zoosporas; siendo ésta una característica diferenciadora respecto a los otros géneros de la familia. El propágalo asexual o conidia de *Peronospora* tiene una pared uniforme, sin ápice papilado, y germina siempre mediante la formación de un tubo germinativo que emerge de cualquier lugar de la superficie de aquélla.

Peronospora representa la culminación de la evolución filogenética dentro de este grupo sobre plantas dicotiledóneas, con la excepción de *P. destructor* que infecta *Allium* spp. Este grupo de patógenos, al ser biotrofos obligados, no pueden ser cultivados artificialmente en medios de cultivo y se ha de recurrir para ello a realizar inoculaciones artificiales sobre plantas o tejidos vegetales vivos. Estas inoculaciones pueden hacerse siguiendo diversas metodologías, que siempre incluyen condiciones de alta humedad y temperaturas que oscilan entre los 10 y los 24 ºC según las especies. Algunos de los procedimientos que se siguen con este género para su estudio en laboratorio son: extracción de oosporas de suelos infestados o tejidos vegetales infectados; bioensayos de germinabilidad y supervivencia de oosporas y esporas; pulverización de plantas con solución de esporangios o conidias a concentraciones predeterminadas; inoculaciones de hojas de la planta huésped realizadas en cámaras húmedas; siembra o transplante de semillas o plantas en suelo infestado por oosporas; y finalmente la conservación de inóculo a – 80 ºC. En los últimos cuatro puntos, y según la especie concreta de *Peronospora*, se encuentran multitud de variantes específicos para asegurar el éxito del procedimiento.

El género *Peronospora* se caracteriza por la producción de esporas sexuales denominadas oosporas. Los criterios para diferenciar las oosporas del género son entre otros: la posición del anteridio con respecto al oogonio, y si la oospora es plerótica o aplerótica, según ocupe completamente o no el lumen del oogonio, respectivamente. Por otro lado, las especies de este género producen esporas asexuales denominados esporangios o conidias, que se desarrollan sobre esterigmas de hifas especializadas denominadas esporangióforos. Los esporangióforos de *Peronospora* son ramificados y de crecimiento determinado, y junto con los de *Bremia* ramifican dicotómicamente a diferencia de *Basidiophora*, *Plasmopara* y *Sclerospora*, en cuyos esporangióforos la ramificación es monocotómica. *Peronospora* y *Bremia* se pueden

diferenciar entre sí por la forma de los ápices de los esporangióforos, que en *Peronospora* son agudos y en *Bremia* tienen forma de disco del cual derivan cuatro esterigmas.

Como se ha descrito anteriormente, los modernos métodos de análisis sistemáticos basados en técnicas moleculares han abierto nuevos caminos en los criterios taxonómicos para la clasificación a niveles infragenéricos y por tanto son de gran utilidad en la separación de géneros afines.

Hasta recientemente, el carácter en el que se había basado la separación de especies afines de *Peronospora* era la naturaleza del huésped infectado, ya que una de las características principales de las especies que comprenden este género es la naturaleza huésped-específica de su parasitismo. Sin embargo, en 1977, Dickinson y Greenhalgh propusieron que para la separación de especies afines se utilizaran medidas biométricas del esporangióforo, de la conidia, y de las oosporas. Las aproximaciones existentes al concepto de especie en este grupo (morfométrica, especie biológica de Gaumann´s y conceptos eco-fisio-fenéticos de Skalicý) han sido examinados recientemente y se han encontrado inadecuados, de ahí el énfasis en la utilización de análisis moleculares para diferenciar los taxones (Hall, 1996).

Biología y Ecología

Hábitat y distribución

Actualmente se acepta de forma generalizada que los modelos de distribución geográfica de las enfermedades de plantas y sus agentes causales no solo vienen determinado por la distribución geográfica de sus plantas huéspedes, sino por multitud de factores. Entre ellos, los factores ambientales son igualmente significativos para la ocurrencia de una enfermedad. Como el patógeno es dependiente del huésped, pero el huésped no depende del patógeno, el conjunto de las condiciones medioambientales en las que el huésped se desarrolla determina el área potencial máxima donde un patógeno puede causar enfermedad. Si el patógeno es endémico de la zona, o ha sido introducido con el huésped, éste podría encontrarse esporádicamente donde quiera que el huésped crezca, pero su importancia económica puede ser o no significativa, dependiendo de los factores ambientales y de la planta huésped.

Los Mildius tienen un factor común en relación a sus necesidades de condiciones ambientales concretas para el desarrollo de la enfermedad, y es que todos ellos requieren que exista agua libre sobre la superficie de la planta (eventualmente determinada por humedad relativa elevada y temperaturas moderadas o bajas) para que la infección conidial tenga lugar (Barrau y Romero, 1996; Weltzien, 1981). La humectación nocturna (rocío) siendo adecuado para la infección, no permite en cambio la propagación del inóculo y además sólo contribuye al desarrollo de la epidemia en casos excepcionales (Weltzien, 1981). De esta manera, la lluvia, o en algunas casos el riego por aspersión puede considerarse el factor limitante para la mayoría de las epidemias de Mildiu basadas en infecciones conidiales. Si el periodo vegetativo del huésped se encuentra dentro de una estación seca la enfermedad no suele alcanzar proporciones elevadas. Otros factores que influyen en la distribución de los Midius son las altas temperaturas y los golpes de calor, que resultan perjudiciales para su desarrollo, mientras que la alta humedad relativa y la oscuridad favorecen la esporulación de las especies de este género (Weltzien, 1981).

La distribución geográfica de este género, por tanto, va asociada a la ocurrencia de dos factores fundamentales: presencia del huésped al ser biotrofos obligados y condiciones ambientales adecuadas; es decir, alta humedad relativa y temperaturas suaves. Las especies del género *Peronospora* no están restringidas a ninguna parte específica del globo terrestre, excepto por áreas muy secas, sin embargo cada especie tiene requerimientos ambientales específicos.

Ciclo biológico

En la mayoría de los Mildius se conoce que el agente causal puede ser introducido en la semilla o bulbos teniendo lugar la infección en los estadíos jóvenes de la planta. En la fase siguiente el patógeno causa infección sistémica en el brote del huésped tal como ocurre con *P. halstedii* en girasol (*H. annuus*) (Cohen and Sackston, 1974), *Peronosclerospora sorghi* (Weston y Uppal) Shaw, en sorgo (*Sorghum bicolor* L.) y maíz (*Zea mays* L.), y *Sclerospora graminicola* (Sacc.) Schröt en mijo (*Pennisetum glaucum* L.) (Jeger et al., 1998). Cuando la infección se produce, en las parte maduras de la planta se desarrollan infecciones localizadas en áreas pequeñas o grandes, que en condiciones favorables pueden extenderse por los tejidos de la planta y convertirse de infección local en infección sistémica generalizada (Agrios, 2005).

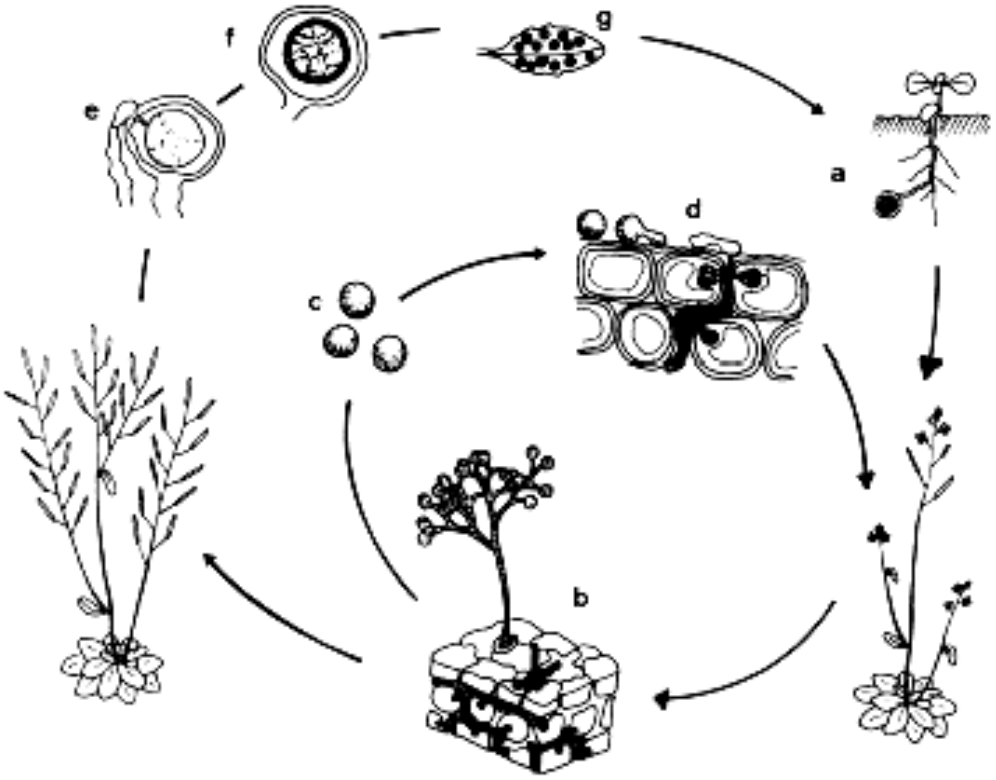

Figura I.1.6. Diagrama esquemático del ciclo biológico general de las especies de *Peronospora* adaptado del ciclo de vida de *Peronospora parasitica.* (Fuente: Slusarenko y Schlaich, 2003). **a.** La infección inicial de plantas ocurre mediante las estructuras de supervivencia del hongo, las oosporas. Según la especie esta fuente de inóculo primario podría provenir en la misma semilla o encontrarse en el suelo tras haberse formado en cultivos anteriores. **b.** Las plantas son colonizadas por el crecimiento intercelular del micelio, el cual da lugar a amarillamientos y abullanomientos en las hojas. Las hifas forman haustorios intracelulares. Después de un periodo variable de crecimiento (1-2 semanas) se forman, conidióforos externamente sobre los tejidos vegetales infectados y sobre ellos las esporas o conidias esferoidales e hialinas. El crecimiento de los conidióforos hacia el exterior de la hoja (esporulación) suele ocurrir a través del estoma. **c.** Las conidias son liberadas y diseminadas. Suele ocurrir durante la mañana como consecuencia de procesos de desecación y rehumectación de los conidióforos. Estas conidias pueden ser transportadas por el viento hasta varios kilómetros y en algunos casos tienen viabilidad de hasta tres días (a diferencia de los Oomicetos con germinación zoosporángial, cuyos esporangios son lábiles en ambientes aéreos). **d.** Si las condiciones ambientales son las adecuadas estas conidias pueden germinan y dan lugar a nuevos ciclos de infección (ciclos secundarios). **e.** El oogonio, órgano sexual femenino, contiene una oosfera, que es fertilizada por el anteridio masculino mediante un tubo que atraviesa su pared. **f.** Las oosferas fertilizadas desarrollaran en su interior una oospora madura**. g.** En las hojas infectadas suelen encontrarse un gran número de oosporas.

Patogenicidad

Entre las características generales de la patogenicidad de especie del género *Peronospora* destaca una alta especialización fisiológica (Renfro y Shankara-Bhat, 1981). La gama de plantas que infectan son dicotiledóneas (a excepción de *P. destructor*, como se indicó con anterioridad) y entre los géneros de plantas huéspedes más importantes se encuentran cultivos de considerable importancia económica y social como adormidera, alfalfa, cebolla, clavel, espinaca, girasol, guisante, judías, remolacha, rosal y tabaco, entre otros.

La sintomatología de las enfermedades causadas por especies del género *Peronospora*, comúnmente conocidas como Mildius, es similar con independencia de la especie del género *Peronospora* y el huésped que infecte. Los síntomas generales de los mildius son amarillamiento y deformaciones de los tejidos afectados, que progresan hasta ocasionar necrosis generaliza de los tejidos afectados y finalmente la muerte de la planta en casos de alta incidencia (Agrios, 2005; Populer, 1981). Además, en condiciones óptimas de humedad se produce una profusa esporulación, visible a simple vista en el envés de las hojas de las plantas infectadas.

Los Mildius causan a menudo pérdidas severas y muy rápidas fundamentalmente cuando ocurren en los estadios jóvenes del cultivo. En condiciones favorables para su desarrollo, estas enfermedades pueden destruir entre el 40 al 90% de las plantas jóvenes, causando pérdidas de cosecha muy importantes o incluso la pérdida total de ésta. La magnitud de las pérdidas depende de la duración de condiciones óptimas para la enfermedad, i.e., la presencia de humedad elevada, y temperaturas suaves durante la esporulación del patógeno, ya que bajo estas condiciones ambientales el patógeno puede causar infecciones numerosas y nuevas, propagándose rápidamente por la planta provocando necrosis importantes en los tejidos jóvenes más suculentos. Es por tanto que en determinadas condiciones de alta humedad y temperaturas suaves los Mildius puede llegar a ser a menudo incontrolable sólo pudiendo ser restringido su desarrollo cuando suben las temperaturas y baja de manera considerable la humedad ambiental. Desde el descubrimiento de los fungicidas sistémicos, la posibilidad de combatir estas enfermedades mejoró considerablemente, aunque la lucha contra los Mildius es todavía especialmente difícil fundamentalmente

porque cuando los síntomas aparecen (esporulación) puede ser demasiado tarde para el tratamiento, ya que se han podido producir infecciones secundarias (Agrios, 2005).

Peronospora arborescens **(Berk.) de Bary**

Peronospora arborescens produce esporangióforos con ramificaciones dicotómicas, de 5 a ocho veces. Las esporas, de forma elíptica, varían de tamaño según el autor que realiza la descripción desde los 18 hasta los 26 µm en su parte más amplia y desde los 16 hasta los 20 µm en la parte más estrecha. Las oosporas tienen un diámetro de 42-48 µm, son de color marrón y poseen una pared exterior irregular que varía entre los 4-10 µm. (Fig. I.1.7).

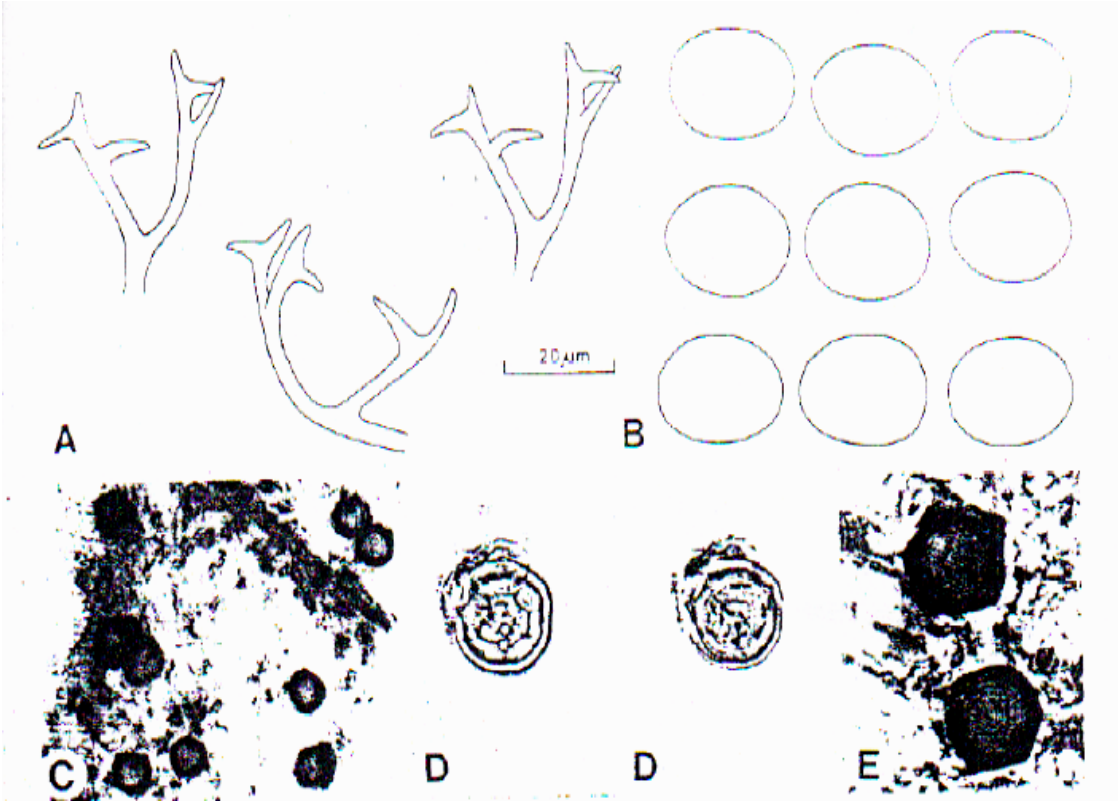

Figura I.1.7. *Peronospora arborescens.* A: Ramificaciones finales de los conidioforos; B: Conidias; C: Oosporas x 125; D: Oogonio con anteridio x 650; E: Oosporas x 650 (Fuente: Francis, 1981).

Los huéspedes en los que se ha descrito *P. arborescens* son: *Argemone mexicana* (Maiti y Chattopadhyay, 1986), varias especies de *Meconopsis* spp. incluyendo *M. betonicifolia* Franch*, M. cambrica* (L.) Vig.*, M. latifolia* Prain*, M. napaulensis* D.C.*, M. polyanthemos* (Fedde) Ownbey y *M. simplicifolia* Vig. (Alcock, 1933; Cotton, 1929; Farr et al., 2006; Reid, 1969;), varias especies de *Papaver* incluyendo *Pap. alpinum* L.*, Pap. argemone* L.*, Pap. Caucasicum* Bieb.*, Pap. dubium* L.*, Pap. hybridum* L.*, Pap. Lecoqii* Lamotte*, Pap. Litwinowii* (Bornm) Fedde*, Pap. nudicaule* L., *Pap. orientale* L.*, Pap. Pavonimumm* Fisch y May*, Pap. rhoeas* L.*, Pap. Setigerum* D.C. y *Pap. Somniferum* L. (Alcock, 1933; Behr, 1956; Berkeley, 1846; Cotteril y Pascoe, 1998; Cotton, 1929; Farr et al., 2006; Francis, 1981; García-Blázquez et al., 2007; Gustavsson, 1959, 1991; Savulescu, 1948).

P. arborescens se encuentra distribuida en zonas donde se encuentran especies huéspedes, como Norte de África, países Asiáticos donde se cultiva adormidera (Japón, Pakistán, Rusia, Turquía, etc.), Australia, Europa y Sur de América.

P. arborescens causa el Mildiu en la adormidera como especie de interés económico, con las características sintomatológicas comunes a los Mildius que ya se han descrito con anterioridad, como amarillamientos, deformaciones del limbo foliar, abullonamientos, clorosis y necrosis de las hojas, y enanismo (Fig. I.1.8. A, B, C, D, E, F, H). Además, y tal como describía Fokin (1922) *P. arborescens* causa hipertrofia y curvatura de tallos y escapos florales (Fig. I.1.8. C, D, J, K, L, M). Los tallos y ramificaciones e incluso las cápsulas pueden ser atacadas resultando en una muerte prematura de la planta (Fig. I.1.8. F, G, H, I, J, K), y pérdidas muy significativas del rendimiento (Fig. I.1.8. L, M).

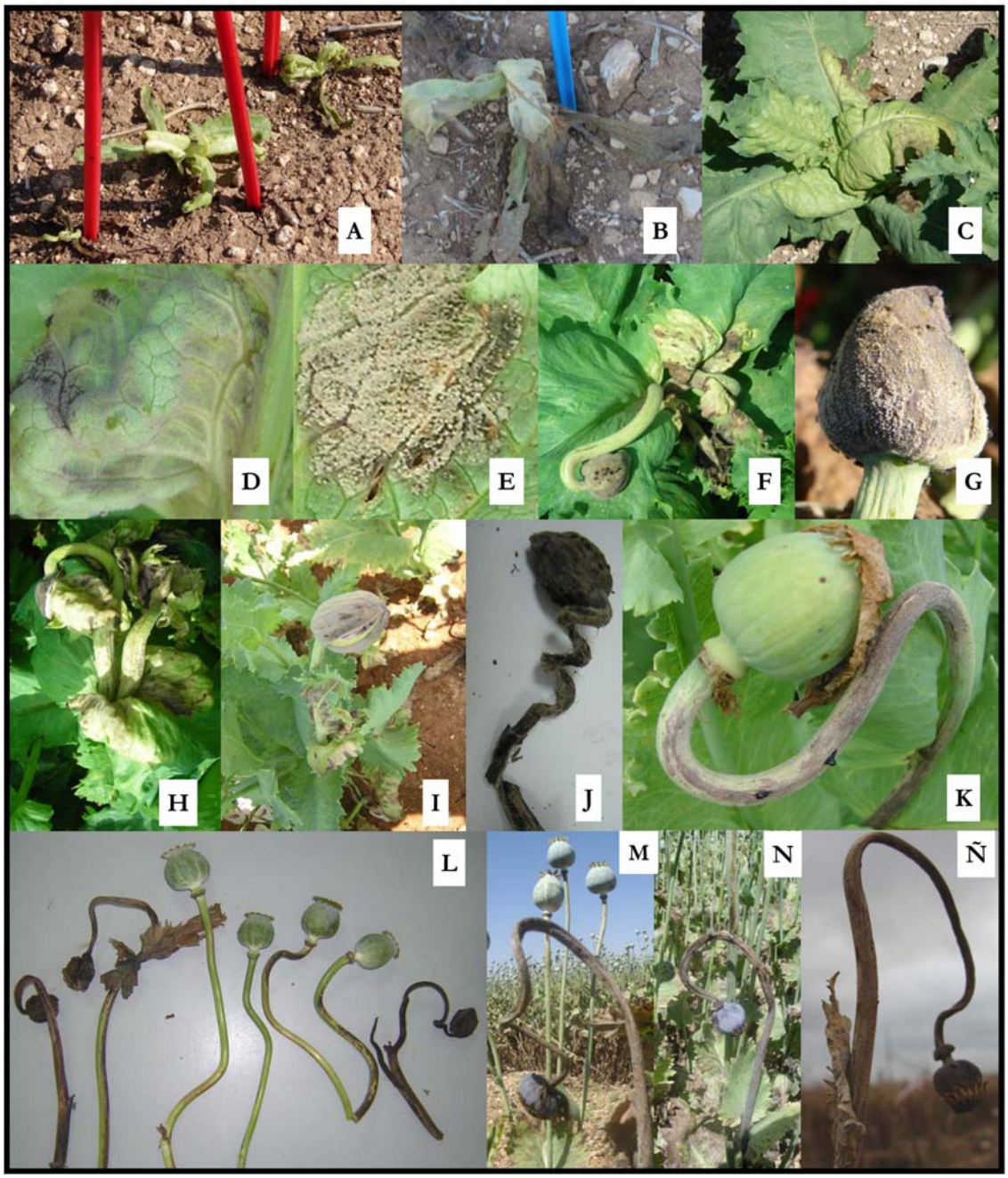

Figura I.1.8. Síntomas del Mildiu de la adormidera en diferentes estadíos de la planta (Fuente: M. Montes-Borrego). A y B: Necrosis en estadío de roseta. C y D: Clorosis generalizada y abunllonamientos. E: Esporulación profusa en limbo foliar. F, G, H, I: Deformaciones, esporulación y necrosis del botón floral. K,L, M, N, Ñ: Deformaciones, esporulación y necrosis de la cápsula.

I.1.3.2 Importancia y distribución del mildiu de la adormidera

El Mildiu de la adormidera es una de las enfermedades más importantes de *Pap. somniferum* (Kapoor, 1995; Khristov, 1943; Yossifovith, 1929) habiendo sido descrita en la mayoría de zonas cultivadoras de dicha planta en el mundo. En caso de que las zonas de cultivo se encuentren en zonas húmedas los daños son intensos y extensivos en todo el cultivo (Pietkiewicz, 1958). Así, esta enfermedad puede llegar incluso a causar la devastación completa del cultivo en ambientes lluviosos o de humedad elevada (Yossifovitch, 1929).

El conocimiento científico específico que se posee sobre el Mildiu de la adormidera a nivel mundial es muy escaso, fragmentario, y nulo a nivel europeo. Estudios recientes indican que el Mildiu se ha convertido en la enfermedad más preocupante para la industria de adormidera en Tasmania, Australia (primer productor mundial), desde que se diagnosticara por vez primera en dicha zona en 1996. De hecho, la enfermedad se desarrolla con carácter epidémico y devastador en esta región cada año, obligando a abordar su control mediante tratamientos fungicidas debido a la magnitud y consistencia de las pérdidas que ocasiona (Scott et al., 2003). Los ataques de Mildiu pueden causar la devastación completa del cultivo en ambientes lluviosos o de humedad elevada con una incidencia de enfermedad de hasta el 90% en el cultivo y con una severidad de ataque a las plantas individuales significativamente alta (Cotteril y Pascoe, 1998).

En la India la enfermedad aparece, cada año, en las áreas de cultivo desde los estadíos de cotiledones hasta los estadíos de madurez de cápsula. La formación de la cápsula se ve afectada adversamente; además el rendimiento de opio es reducido significativamente (Hussain y Sharma, 1983).

En Europa existen referencias antiguas sobre el potencial de la enfermedad para destruir completamente el cultivo (Yossifovith, 1929). Ciertos estudios señalan que los ataques de Mildiu comienzan con una distribución agregada cuando el cultivo de adormidera comienza a cerrarse, y se extienden con rapidez hasta alcanzar el 100% de incidencia en unos 40 días, hacia el final de la floración. La interpretación más plausible de estos hechos es que el desarrollo de las epidemias depende fundamentalmente del establecimiento de focos primarios desde los cuales se produce

la dispersión subsiguiente del patógeno al resto del cultivo mediante ciclos secundarios.

El Mildiu de la adormidera ha sido diagnosticado en los siguientes países: Alemania (Gustavsson, 1959; Harrison y Schmitt, 1967), Argentina (Gustavsson, 1959; Harrison y Schmitt, 1967), Australia (Cotterill y Pascoe, 1998; Harrison y Schmitt, 1967), Austria (Gustavsson, 1959; Harrison y Schmitt, 1967), Bulgaria (Harrison y Schmitt, 1967; Vanev et al., 1993), Canadá (Tewari y Skoropad, 1981), China (Harrison y Schmitt, 1967; Zhuang, 2005), Corea (Cho y Shin, 2004), Dinamarca (Gustavsson, 1959; Harrison y Schmitt, 1967), EE.UU. (French, 1989; Harrison y Schmitt, 1967), Egipto (Gustavsson, 1959; Harrison y Schmitt, 1967), Francia (Gustavsson, 1959; Harrison y Schmitt, 1967), Finlandia (Gustavsson, 1959; Harrison y Schmitt, 1967), Gran Bretaña (Francis y Waterhouse, 1988; Gustavsson, 1959; Harrison y Schmitt, 1967), Holanda (Gustavsson, 1959; Harrison y Schmitt, 1967), Hungría (Gustavsson 1959; Harrison y Schmitt, 1967), India (Bajpal et al., 1999; Gustavsson, 1959; Harrison y Schmitt, 1967; Kothari y Prasad, 1970), Irán (Gustavsson, 1959; Harrison y Schmitt, 1967; Sharif, 1970), Irlanda (Harrison y Schmitt, 1967), Israel (Harrison y Schmitt, 1967), Italia (Gustavsson, 1959; Harrison y Schmitt, 1967), Japón (Gustavsson, 1959; Harrison y Schmitt, 1967), Portugal (Gustavsson, 1991), Rumania (Gustavsson, 1959; Harrison y Schmitt, 1967), República Checa (Gustavsson, 1959; Harrison y Schmitt, 1967), Sudafrica (Crous et al., 2000; Gorter, 1977), Suiza (Gustavsson, 1959, Harrison y Schmitt, 1967), Suecia (Gustavsson, 1959; Harrison y Schmitt, 1967), Ex Unión Soviética (Gustavsson, 1959; Harrison y Schmitt, 1967; Melnik y Pystina, 1995), Uruguay (Harrison y Schmitt, 1967) y Yugoslavia (Harrison y Schmitt, 1967; Yossifovith, 1928).

Aunque *P. arborescens* fue descrito como agente causal de Mildiu de *Papaver* spp. en Europa ya en 1929, recientes estudios de diagnóstico molecular en Australia identificaron a *P. cristata* como agente causal de la enfermedad en Tasmania. *P. cristata* también se ha descrito como agente causal del Mildiu en distintas plantas silvestres de la familia Papaveraceae (*Papaver* spp. y *Meconopsis* spp.), concretamente *M. betonicifolia* (Muskett y Malone, 1984), *M. cambrica* (Reid, 1969), *Pap. argemone, Pap. hybridum,* y *Pap. rhoeas* (Constantinescu, 1991; Gaumann, 1923; Gustavsson, 1959; Reid, 1969). Por tanto, la etiología de esta enfermedad puede ser más compleja de lo que inicialmente se ha considerado.

I.1.4 NATURALEZA DEL PROBLEMA QUE SE PLANTEA EN LA PRESENTE TESIS DOCTORAL

En España, durante los últimos años, los rendimientos del cultivo de adormidera han venido disminuyendo como consecuencia de ataques de diversas enfermedades, algunas de las cuales son de etiología aún desconocida. Durante las campañas agrícolas 2001/2002 a 2004/2005 se observaron ataques severos de una nueva enfermedad de etiología fúngica, diferente de la Necrosis de raíz y cuello causada por *P. papaveracea,* hasta ahora considerada la enfermedad más importante de la adormidera en España (Muñoz-Lesdesma, 2002). Esta nueva enfermedad se denominó Mildiu tanto por los síntomas como por los signos desarrollados en las plantas enfermas. En el curso de las campañas agrícolas referidas (4 años), la incidencia y severidad en los ataques de esta nueva enfermedad se incrementaron de manera notable, fundamentalmente en la primavera de 2004, donde se desarrollaron epidemias severas en las distintas regiones cultivadoras de España. Esta primavera se caracterizó por condiciones excepcionales de humedad elevada y temperaturas suaves, particularmente el sur de España. Así la lluvia mensual media acumulada fue mayor (46-182 mm) y la temperatura media menor (10-17 ºC) que las medias normales registradas entre los años 1971 y 2000 de 31-55 mm de lluvia y 12-19 ºC de temperatura, para esta estación (INM, 2006).

El Mildiu de la adormidera ha podido haber estado presente con baja incidencia en España desde que la adormidera se estableció a principios de los 1970`s en las áreas más secas de Andalucía. Sin embargo, a medida que el cultivo se ha extendido a nuevas zonas frescas y húmedas en España o se ha incorporado el regadío al cultivo a fin de mejorar el rendimiento de las cosechas (Muñoz-Ledesma, 2002), se han producido situaciones de ambientes más favorables para el desarrollo de la enfermedad, lo cual ha contribuido a que la incidencia y severidad de los ataques de Mildiu se hayan incrementado.

En España, las primeras hipótesis señalan que el inóculo del hongo puede acceder con facilidad al cultivo, y/o puede estar presente en los campos en que el cultivo se establece, de manera que la medida en que el ambiente favorece el desarrollo de la enfermedad es determinante de la severidad de los ataques. Sin embargo, los medios por los que tiene lugar dicho acceso no son conocidos. La extensión

geográfica de la enfermedad, en las últimas campañas agrícolas, unida a la distribución agregada de las plantas afectadas, sugiere que el inóculo del patógeno puede ser introducido en los nuevos cultivos en semillas infectadas. Esto plantea la necesidad de disponer de una técnica rápida, precisa y sensible de detección del patógeno en lotes de semilla que serán utilizados para siembra. Como ya hemos indicado con anterioridad, además de la reproducción asexual en forma de conidias, *P. arborescens* se reproduce sexualmente en forma de oosporas (Francis, 1981), que pueden actuar como estructuras de supervivencia del patógeno en restos de cosecha y/o en el suelo, y que además pueden actuar como inóculo primario.

El reto que desde el punto de vista fitopatológico constituye el problema planteado y las características que presenta en lo concerniente a la disciplina científica que desarrollamos, acentúan el interés de esta Tesis Doctoral en cuanto a la necesidad de dar respuestas a los interrogantes pendientes de responder en la patogénesis tan poco conocida de esta enfermedad, que hacen que la actuaciones para su control, por el momento, sólo puedan baserse en el conocimiento sobre otros patosistemas, i.e., *P. halstedii*/*H. annus* (Barrau y Romero, 1996; Sackston, 1981), *P. tabacina*/*N. tabacum* (Schiltz, 1981), *Peronospora sparsa* Berk./*Rosa sp.* (Xu y Ridout, 2001).

I.1.5 HIPÓTESIS EXPERIMENTALES

Como en todo proceso de investigación las hipótesis experimentales representan un elemento fundamental ya que permiten derivar los objetivos del estudio constituyéndose en la base de los procedimientos de investigación. En nuestro caso, y tras recopilar y discutir los conocimientos preliminares sobre el patosistema enunciamos una serie de hipótesis, las cuales orientaron esta Tesis doctoral y permitieron llegar a conclusiones concretas del presente proyecto de investigación.

Se establecieron las **hipótesis experimentales** que se refieren a continuación y que **no son mutuamente excluyentes**. Estas hipótesis corresponden a las siguientes preguntas:

1) ¿Cuál es el **origen del primer inóculo** con el que se inician las epidemias de Mildiu en cultivos de dormidera?;

2) ¿Qué **tipos de infección** tienen lugar en el patosistema?;

3) ¿Cuál es la composición de las poblaciones del patógeno en los cultivos afectados en relación con las dos especies que han sido citada sobre adormidera (*P. arborescens, P. cristata*), y que **diversidad genética** puede existir en ellas?.

Origen del inóculo primario

A. El patógeno accede a los campos de adormidera en semillas infectadas (y consecuentemente la enfermedad puede ser controlada mediante certificación de semilla de siembra, o el tratamiento fungicida de semilla no certificada).

B. El patógeno se encuentra establecido en el suelo de siembra mediante oosporas que se produjeron en plantas infectadas en cultivos anteriores y son efectivas en originar infección (y consecuentemente la elección de parcelas de siembra, rotación de cultivos no huésped, y el tratamiento de la semilla para protegerla de la infección primaria pueden ser eficaces para el control de la enfermedad).

C. El patógeno se encuentra establecido en poblaciones de papaveráceas arvenses próximas a las parcelas de siembra, en las que se reproduce y desde las que se dispersa para originar infecciones primarias y /o secundarias (y consecuentemente la reducción o eliminación de la flora papaverácea arvense puede ser una medida de control).

Tipos de infección

A. Las oosporas contenidas en restos de cosecha infestados son activas en originar infecciones en los órganos subterráneos de las plántulas de adormidera durante el establecimiento del cultivo.

B. Los esporangios desarrollados en tejidos aéreos de plantas de adormidera infectados son activos en originar nuevas infecciones, y éstas pueden ser de naturaleza sistémica.

Diversidad genética del patógeno

A. En ausencia de información sobre la naturaleza taxonómica del agente causal, la información de carácter molecular sobre Peronosporales disponible en Internet puede permitir diseñar protocolos moleculares (iniciadores, ensayos PCR, etc) para la identificación del patógeno mediante análisis de su ADN genómico.

B. Se pueden diseñar ensayos de PCR y condiciones de reacción útiles para la detección del patógeno en lotes de semilla de siembra.

C. El análisis de las secuencias amplificadas del ADN del patógeno puede ser de utilidad para determinar la diversidad molecular en sus poblaciones, sugerentes de la diversidad genética y fenotípica existente en ellas.

I.2

OBJETIVOS

El objetivo general de esta Tesis Doctoral es obtener nuevos conocimientos sobre la etiología, biología y epidemiología del Mildiu de la adormidera, así como sobre la caracterización y detección molecular específica del agente causal, que permitan establecer las bases científicas para el desarrollo de estrategias eficientes para el control integrado de la enfermedad.

Los objetivos concretos son:

1. Determinar la naturaleza, distribución, y agente(s) causal(es) del Mildiu de la adormidera en cultivos comerciales en España y Francia.

2. Determinar la distribución y diversidad genética existente en las poblaciones del oomyceto biotrofo obligado *Peronospora arborescens* (Berk) de Bary y sus relaciones filogenéticas basadas en el análisis de las secuencias de la región ITS del ADN ribosómico (ADNr).

3. Desarrollar procedimientos de detección molecular del agente causal en tejidos sintomáticos y asintomáticos de adormidera y papaveráceas arvenses, mediante el diseño de iniciadores específicos, y protocolos de PCR simple o anidada.

4. Desarrollar un protocolo de PCR en tiempo real para la cuantificación del nivel de infección/infestación de semillas de adormidera y plantas sintomáticas y asintomáticas por el patógeno, y correlacionar dicho nivel con el desarrollo de enfermedad y la sintomatología de ésta.

5. Establecer las bases biológicas para el desarrollo del Mildiu de la adormidera, determinando las fuentes de inóculo primario, y los factores que influyen en la epidemiología de la enfermedad.

Los resultados relativos al objetivo primero se recoge en los manuscritos "First Report of Downy Mildew of Opium Poppy Caused by *Peronospora arborescens* in Spain, ***Plant Disease* 89: 338**" y "Downy Mildew of Commercial Opium Poppy Crops in France is caused by *Peronospora arborescens,* ***Plant Disease* 92: 317**" del Capítulo II. Los resultados concernientes a los objetivos segundo y tercero se recogen en los manuscritos "Phylogenetic analysis of downy mildew pathogens of opium poppy and PCR-based in-planta and seed detection of *Peronospora arborescens*, ***Phytopathology*, 97: 1380-1390**" y "A nested-PCR protocol for the detection and population biology studies of *Peronospora arborescens*, the downy mildew pathogen of opium poppy, using herbarium specimens and asymptomatic fresh plant tissues, ***Phytopathology*, 99: 73-81**" del Capítulo III. Los resultados sobre el objetivo cuarto se recoge en el manuscrito "A Robust, Universal Real-Time Quantitative PCR Assay for the in planta Detection and Quantification of the Opium Poppy Downy Mildew pathogen *Peronospora arborescens* of Use for Resistance Screening and Certification Schemes", ***Applied and Environmental Microbiology*, en preparación**" del Capítulo IV; y finalmente, los resultados relativos al objetivo quinto se recogen en el manuscrito "Sources of Primary Inoculum for Epidemics of Downy Mildew Caused by *Peronospora arborescens* in Opium Poppy Crops in Spain. ***Phytopathology*, en revisión**" del Capítulo V.

Finalmente, es de destacar que el desarrollo de esta Tesis Doctoral ha tenido lugar en el marco de una colaboración estrecha entre una empresa privada (Alcaliber, S. A.) y dos organismos públicos de investigación (Universidad de Córdoba y Consejo Superior de Investigaciones Científicas). Además, esta Tesis Doctoral ofrece una oportunidad destacable de evidenciar el beneficio de la transmisión directa y eficaz al sector privado del conocimiento y de la tecnología científica generada en la misma. El conocimiento generado en la presente Tesis Doctoral, permitirá establecer las bases y desarrollar medidas de control de esta enfermedad en adormidera, cuyos ataques comprometen la estabilidad y extensión de este cultivo de reciente introducción y demostrada productividad y rentabilidad, que cuenta con el respaldo de la empresa privada y con la aceptación de los agricultores.

CAPÍTULO II

Naturaleza, distribución, y agente(s) causal(es) del Mildiu de la adormidera en cultivos comerciales en España y Francia.

En este capítulo se recogen los resultados relativos al objetivo primero de esta Tesis Doctoral, en el cual se pretendía "Determinar la naturaleza, distribución, y agente(s) causal(es) del Mildiu de la adormidera en cultivos comerciales en España y Francia".

Los resultados obtenidos se recogen en dos manuscritos, los cuales corresponden a los puntos II.1 y II.2 de este capítulo, recogidos en la revista Plant Disease de la Sociedad Americana de Fitopatología, y que llevan por títulos:

"First Report of Downy Mildew of Opium Poppy Caused by *Peronospora arborescens* in Spain". *Plant Disease 89: 338*

"Downy Mildew of Commercial Opium Poppy Crops in France is caused by *Peronospora arborescens". Plant Disease 92: 317*

II.1

First Report of Downy Mildew of Opium Poppy Caused by *Peronospora arborescens* in Spain

Blanca B. Landa**, Miguel Montes-Borrego**, Francisco J. Muñoz-Ledesma and Rafael M. Jiménez-Díaz

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Plant Disease Mar 2005, Volume 89, Number 3: 338

Opium poppy (*Papaver somniferum*) is an economically important pharmaceutical crop in Spain with approximately 7,400 ha cultivated annually. In the spring of 2004, severe attacks by a new foliar disease were observed approximately 500 km apart in commercial opium poppy fields in the Castilla-La Mancha and Andalusia regions of central and southern Spain, respectively. The incidence of affected fields ranged from 40 to 50%, and incidence of diseased plants ranged from 20 to 30%. Initial disease symptoms included irregularly shaped, chlorotic-to-light yellow leaf lesions (ranging in size from 0.5 to 4 cm) (Fig. II.1.1C, F). Affected tissues curled, thickened, and became deformed and necrotic as disease developed. Lesions expanded in size and often coalesced, eventually giving rise to large necrotic areas in leaves or death of entire leaves (Fig. II.1.1I). In wet weather or conditions of high relative humidity, a dense felt of sporangiophores with sporangia was produced on the abaxial leaf surface (Fig. II.1.1A, C) and occasionally on the adaxial surface. Microscopic observations revealed sporangiophores (Fig. II.1.1A, B) branching dichotomically at least four to six times, ending with sterigmata bearing single sporangia (Fig. II.1.1B). Sporangia (Fig. II.1.1D) were hyaline, elliptical to spherical in shape, and measured 18 to 24 × 14 to 18 µm (average 19 ± 1.2 × 15 ± 1.6 µm). Occasionally, oospores formed in necrotic leaf tissues (Fig. II.1.1E, G, H). Oospores were dark brown (the surface was irregularly ridged) and measured 36 to 46 µm in diameter (average 39 ± 4.4 µm). The oospore wall was 3 to 11 µm thick. On the basis of the observed morphological features of six symptomatic plant samples from fields at Castilla-La Mancha and Andalusia regions, we identified the pathogen as *Peronospora arborescens* (1). Pathogenicity was confirmed by inoculating 4- to 6-week-old opium poppy plants (cv. nigrum) with an isolate collected from a field in Ecija, Andalusia. Seed of test plants was surface disinfested and germinated under sterile conditions. Plants were sprayed with a suspension of 1 to 5×10^5 sporangia per ml in sterile distilled water. Plants sprayed with sterile water served as controls. There were five replicate plants per treatment. Plants were enclosed in sealed plastic bags and kept in the dark for 24 h. This was followed by incubation in a growth chamber at 21°C, 60 to 90% relative humidity, and a 12-h photoperiod (fluorescent light: 360 $\mu E \cdot m^{-2} \cdot s^{-1}$). After 5 to 7 days, typical downy mildew symptoms developed in inoculated plants (Fig. II.1.2A-H). All control plants remained symptomless. Sporulation by the pathogen on symptomatic leaves occurred when affected plants were sprayed with water, enclosed in sealed plastic bags, and

incubated at 21°C in the dark for 24 h. To our knowledge, this is the first report of *P. arborescens* infecting opium poppy in Spain. Infestations of poppy weeds (*Papaver rhoeas*) and wild *Papaver somniferum* were also observed in affected opium poppy fields, which may bear importance in the epidemiology of the disease as alternative hosts for inoculum increase and survival of *P. arborescens* under field conditions.

References: (1) S. M. Francis. No. 686 in: Descriptions of Pathogenic Fungi and Bacteria. CMI, Kew, Surrey, UK, 1981.

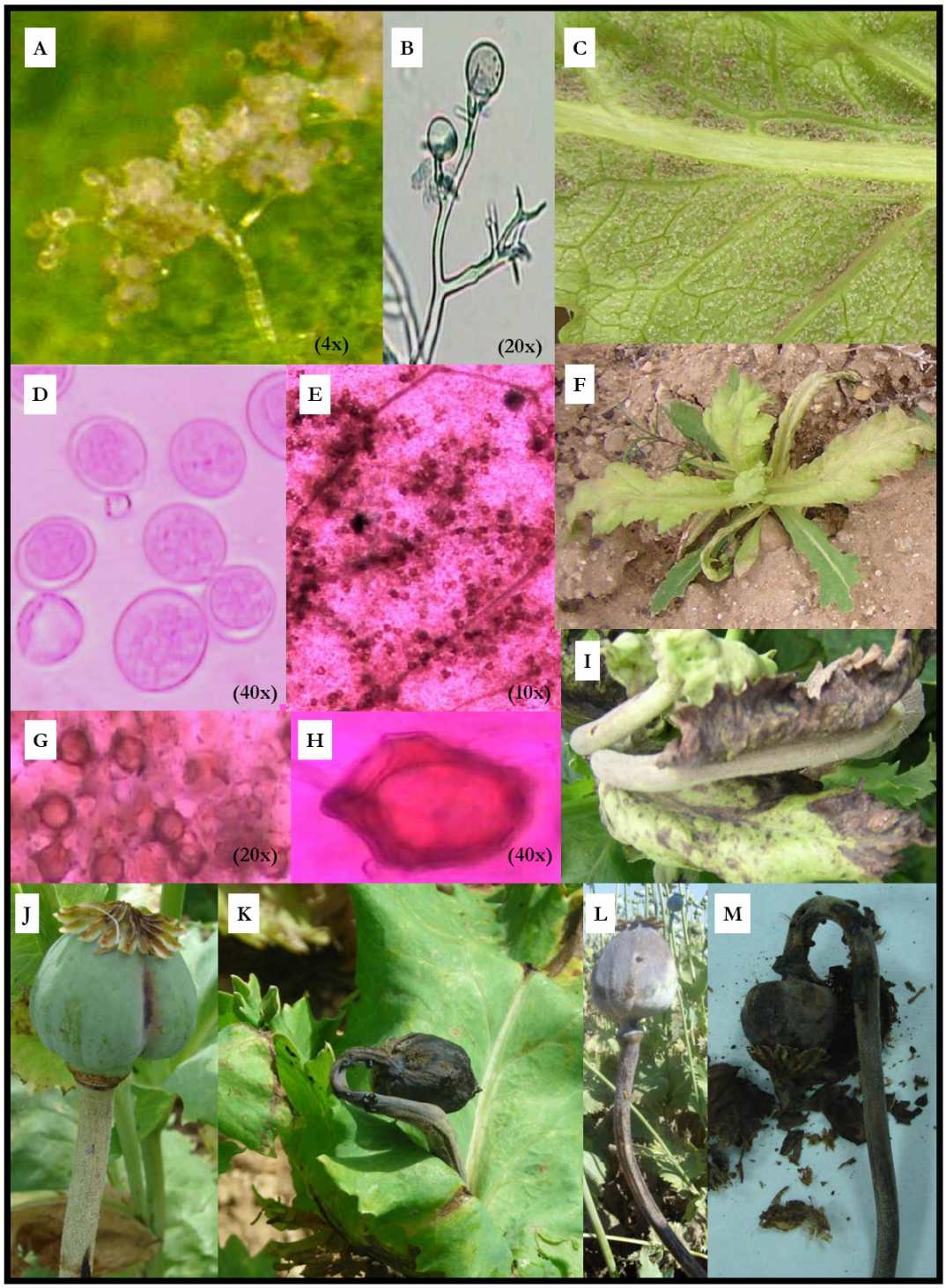

Figure II.1.1. Microscopic observations of *P. arborescens*. A-B: Sporangiophores with sporangia; D: Sporangia; E, G, H. Oospores. Symptoms of downy mildew on opium poppy developed in commercial crops in Spain (C, F, I-M) (Fuente: M. Montes-Borrego)

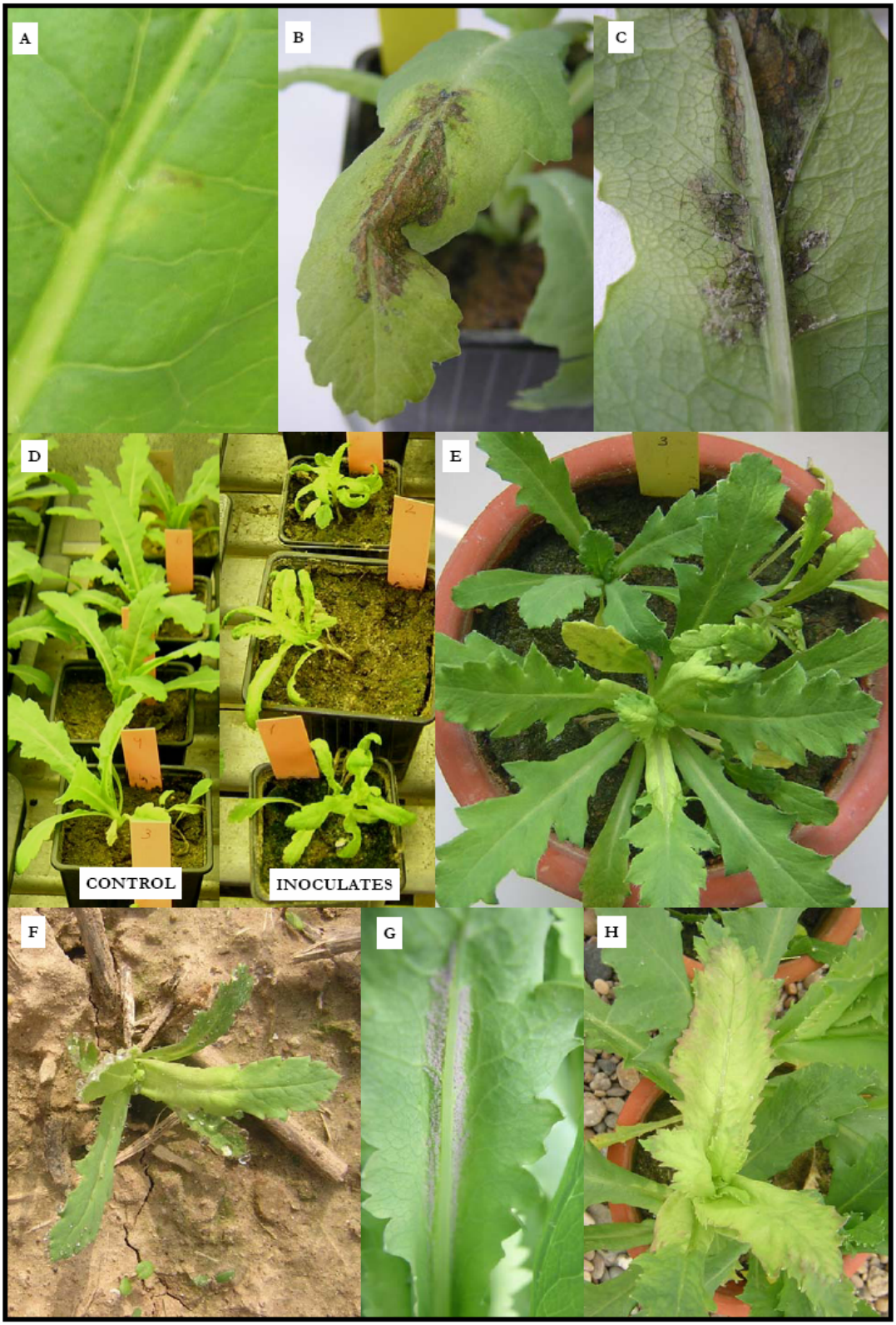

Figure II.1.2. Symptoms of downy mildew on opium poppy developed after artificial inoculations using sporangia (A-H) of *Peronospora arborescens* as inoculum (Fuente: M. Montes-Borrego).

II.2

Peronospora arborescens Causes Downy Mildew Disease in Commercial Opium Poppy Crops in France

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Plant Disease May 2008, Volume 92, Number 5: 834

Opium poppy is a strategic crop for the pharmaceutical industry because it is the only source of morphine, codeine, and thebaine alkaloid drugs. Approximately 7,360 ha (average from 2001 through 2007) of opium poppy (*Papaver somniferum*) are grown annually in France, mainly in the Northern-East (Champagne-Ardenne) and Centre-West (Centre and Poitou-Charentes) regions of the country. This acreage accounts for nearly 5.6% of the legally cultivated opium poppies worldwide. Disease symptoms resembling those of downy mildew (2) have been observed frequently in those opium-poppy-growing areas, especially in the Charente-Maritime, Cher, Loiret, and Loir et Cher departments. Disease symptoms included chlorotic to light yellow lesions on the leaf blade, curling and thickening of affected tissues, and expanding necrotic lesions that coalesced, eventually giving rise to large necrotic areas or death of the entire leaf tissues and the plant (Fig. II.1.1F, I, J-M). With wet weather or high relative humidity, sporangiophores with sporangia were produced frequently on the abaxial leaf surface and occasionally on the adaxial side (Fig. II.1.1A-D). *Peronospora arborescens* and *P. cristata* have been demonstrated as causal agents of opium poppy downy mildew disease and both have been reported in Europe (1–3); however, the specific identity causal agent in commercial opium poppy crops in France has not yet been determined. Microscopic observations of affected leaves in symptomatic opium poppy leaves sampled from three commercial fields in Loiret Department revealed dichotomously branching sporangiophores bearing single sporangia and oospores of shape and measurements similar to those reported for *P. arborescens* and *P. cristata* (1,3). Sporangia dimensions of *P. arborescens* and *P. cristata* overlapped, making it difficult to differentiate between the two species based solely on morphological characters (3). A species-specific PCR assay protocol (2) that differentiated *P. arborescens* from *P. cristata* was used to diagnose the pathogen. Also, the sequence of the complete 5.8S ribosomal DNA gene and internal transcribed spacers (ITS) 1 and 2 were determined and maximum parsimony analysis was performed with the *Peronospora* spp. data set described by Landa et al. (2). Both species-specific PCR and phylogenetic analyses of ITS sequences showed that *P. arborescens* was the only *Peronospora* species associated with the three samples of downy-mildew-affected leaves analyzed. Thus, DNA fragments of 545, 594, and 456 bp were amplified using total DNA extracted from the sampled leaves and P2, P3, and P6 primer pairs (2), respectively. ITS sequences of all three samples showed 100% homology (GenBank Accession No. EU295529).

Phylogenetic analyses using Neighbor Joining of those sequences placed the infecting *Peronospora* sp. in a clade (100% support) that included all *P. arborescens* sequences from the GenBank database with 99.2 to 99.9% homology among sequences (2,3). To our knowledge, this is the first report and molecular evidence that *P. arborescens* causes downy mildew disease in commercial opium poppy crops in France.

References: (1) S. M. Francis. No. 686 in: Descriptions of Pathogenic Fungi and Bacteria. CMI, Kew, Surrey, UK, 1981. (2) B. B. Landa et al. Phytopathology 97:1380, 2007. (3) J. B. Scott et al. Phytopathology 93:752, 2003

CAPÍTULO III

Distribución y diversidad genética existente en las poblaciones del oomiceto biotrofo obligado *Peronospora arborescens*

Procedimientos de detección molecular del agente causal

En este capítulo se recogen los resultados relativos al objetivo segundo y tercero de esta Tesis Doctoral en el cual se pretendía:

En primer lugar, determinar la distribución y diversidad genética existente en las poblaciones del oomiceto biotrofo obligado *Peronospora arborescens* (Berk) de Bary y sus relaciones filogenéticas basadas en el análisis de las secuencias de la región ITS del ADN ribosómico (ADNr).

Y en segundo lugar, desarrollar procedimientos de detección molecular del agente causal en tejidos sintomáticos y asintomáticos de adormidera y papaveráceas arvenses, mediante el diseño de iniciadores específicos, y protocolos de PCR simple o anidada.

Los resultados obtenidos se recogen en dos manuscritos, los cuales corresponden a los puntos III.1 y III.2 de este capítulo, publicados en la revista *Phytopathology* de la Sociedad Americana de Fitopatología, y que llevan por títulos:

"Phylogenetic analysis of downy mildew pathogens of opium poppy and PCR-based in planta and seed detection of *Peronospora arborescens". Phytopathology 97: 1380-1390*

"A nested-PCR protocol for detection and population biology studies of *Peronospora arborescens*, the downy mildew pathogen of opium poppy, using herbarium specimens and asymptomatic, fresh plant tissues". *Phytopathology 99: 73-81*

III.1

Phylogenetic analysis of downy mildew pathogens of opium poppy and PCR-Based in planta and seed detection of *Peronospora arborescens*

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III.1.1 ABSTRACT

Severe downy mildew diseases of opium poppy (*Papaver somniferum*) can be caused by *Peronospora arborescens* and *P. cristata*, but differentiating between the two pathogens is difficult because they share morphological features and a similar host range. In Spain, where severe epidemics of downy mildew of opium poppy have occurred recently, the pathogen was identified as *P. arborescens* on the basis of morphological traits. In this current study, sequence homology and phylogenetic analyses of the internal transcribed spacer regions (ITS) of the ribosomal DNA (rDNA) were carried out with DNA from *P. arborescens* and *P. cristata* from diverse geographic origins, which suggested that only *P. arborescens* occurs in cultivated *Papaver somniferum* in Spain. Moreover, analyses of the rDNA ITS region from 27 samples of downy-mildew-affected tissues from all opium-poppy-growing regions in Spain showed that genetic diversity exists within *P. arborescens* populations in Spain and that these are phylogenetically distinct from *P. cristata*. *P. cristata* instead shares a more recent, common ancestor with a range of *Peronospora* species that includes those found on host plants that are not members of the Papaveraceae. Species-specific primers and a PCR assay protocol were developed that differentiated *P. arborescens* and *P. cristata* and proved useful for the detection of *P. arborescens* in symptomatic and asymptomatic opium poppy plant parts. Use of these primers demonstrated that *P. arborescens* can be transmitted in seeds and that commercial seed stocks collected from crops with high incidence of the disease were frequently infected. Field experiments conducted in microplots free from *P. arborescens* using seed stocks harvested from infected capsules further demonstrated that transmission from seedborne *P. arborescens* to opium poppy plants can occur. Therefore, the specific-PCR detection protocol developed in this study can be of use for epidemiological studies and diagnosing the pathogen in commercial seed stocks; thus facilitating the sanitary control of the disease and avoidance of the pathogen distribution in seeds.

Additional keywords: genetic diversity, *Papaver* spp., *Peronospora cristata*, seedborne transmission.

III.1.2 INTRODUCTION

The exact characterization and understanding of the genetic relationships among taxonomically related plant pathogenic species are key elements for the design of disease management strategies. Opium poppy (*Papaver somniferum* L.), the only source of the alkaloid drugs (morphine, codeine, and thebaine) for the pharmaceutical industry, can be severely affected worldwide by downy mildew diseases caused by *Peronospora arborescens* and *P. cristata* (11,36,37,45). These two *Peronospora* species were first differentiated on the basis of the average conidium dimensions (33). However, conidium dimensions of the two species were later found to overlap, making it difficult to differentiate between them solely on the basis of morphological characters (36). A more accurate differentiation of the two species was accomplished on the basis of sequence homology and phylogenetic analyses of the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) (36).

In addition to overlapping morphological characters, *P. arborescens* and *P. cristata* also exhibit an overlap in their host range. *P. arborescens* has been reported to infect *Argemone mexicana* (29), as well as several *Meconopsis* spp., including *M. betonicifolia*, *M. cambrica*, *M. latifolia*, *M. napaulensis*, *M. polyanthemos*, and *M. simplicifolia* (2,16,18,33), and *Papaver* spp., including *Papaver alpinum*, *Papaver argemone*, *Papaver caucasicum*, *Papaver dubium*, *Papaver hybridum*, *Papaver lecoqii*, *Papaver litwinowii*, *Papaver nudicaule*, *Papaver orientale*, *Papaver pavonimumm*, *Papaver rhoeas*, *Papaver Setigerum*, and *Papaver somniferum* (2,4,6,15,16,18,19,23,24,34). Similarly, *P. cristata* was reported to infect *M. betonicifolia* (31), *M. cambrica* (33), *Papaver argemone*, *Papaver hybridum*, and *Papaver rhoeas* (11,21,23,33), and recently, *Papaver somniferum* (36). Interestingly, *P. cristata* has only been reported on host species that are also recorded hosts of *P. arborescens.*

In Spain, opium poppy is grown annually on approximately 7,500 ha, primarily in the southern (Andalucia) and central (Castilla-La Mancha and Castilla-León) regions of the country. This acreage accounts for at least 5% of the legally cultivated opium poppy worldwide, making Spain the fifth largest European producer of poppy seeds and straw (28,30,32). During the last few years, yields of opium poppy crops in Spain have decreased as a consequence of losses from several diseases, including poppy fire caused by *Pleospora papaveracea* (anamorph = *Dendryphion penicillatum*) and others of unknown etiology (30). In the spring of 2004, severe epidemics of downy-mildew-affected commercial opium poppy crops in different growing regions across the coun-

try. This time period was characterized by exceptionally wet and mild weather, particularly in southern Spain; the mean monthly rainfall was higher (46 to 182 mm) and the temperature was lower (10 to 17°C) than the means of 31 to 55 mm and 12 to 19°C, respectively, recorded between 1971 and 2000 (26). At that time, the pathogen was identified as *P. arborescens* based on morphological features (28). Although this was the first report of this pathogen in Spain, opium poppy growers had previously observed symptoms of downy mildew (irregularly shaped, chlorotic lesion on upper poppy leaves and brown, angular necrotic spots on the lowermost ones) (F. J. Muñoz-Ledesma, *personal communication*). Therefore, this downy mildew disease may have occurred in Spain with low prevalence and incidence since opium poppy was first established as a crop in the drier areas of Andalucía in the early 1970s. If this is the same disease, then it has now spread and increased in incidence and severity as production of the crop has expanded to new cooler and more humid irrigated areas in central Spain for improving harvest yields (30). This geographical extension of the downy mildew disease to areas where opium poppy had not been cropped before, together with the patchy aggregation of affected plants often observed (15,36; M. Montes-Borrego, B. B. Landa, F. J. Muñoz-Ledesma, and R. M. Jiménez-Díaz, *unpublished data*), suggested that primary inoculum for the disease may have been seedborne and/or distributed from infected wild poppy plants. Additionally, the risk of *P. cristata* infection resulting from indigenous inoculum should be considered since *P. arborescens* and *P. cristata* have been reported in Europe and several of their common hosts are present in Spain (i.e., *M. cambrica*, *Papaver argemone*, *Papaver hybridum*, and *Papaver rhoeas*) (8). However, the role that *P. cristata* may play in epidemics of opium poppy downy mildew in Spain still remains to be investigated.

The specific objectives of this research were to: (i) confirm the taxonomic identity of the main opium poppy downy mildew pathogen in Spain; (ii) determine the phylogenetic relationships that exists between and within *P. arborescens* and *P. cristata* populations and with related genera within the Peronosporales on the basis of analysis of ITS rDNA sequences; (iii) develop a polymerase chain reaction (PCR) assay for the specific identification and detection of *P. arborescens*; and (iv) determine if *P. arborescens* can be seedborne in opium poppy seeds.

III.1.1 MATERIALS AND METHODS

Sample collections

Diseased and healthy opium poppy plants were sampled in the spring (April–June) of 2004–2006 from commercial crops in all growing areas of the country, including the provinces of Córdoba, Málaga, and Sevilla in Andalucía, southern Spain and Albacete and Toledo in the Castilla-La Mancha Region of central Spain (Table III.1.1). Samples included roots and symptomatic leaves, stems, and capsules (showing pathogen sporulation, irregularly shaped chlorotic lesions, and brown, angular necrotic spots) from diseased plants, as well as asymptomatic tissues from infected and healthy plants. DNA extracted from samples of *M. cambrica* (United Kingdom) and *Papaver somniferum* (Australia) infected by *P. cristata*, *Allium cepa* infected by *Peronospora destructor*, *Lactuca sativa* infected by *Bremia lactucae*, *Helianthus annuus* infected by *Plasmopara halstedii*, and from other fungi isolated from opium poppy tissues (*Beauveria bassiana*, *Fusarium* spp., and *Pleospora papaveracea*) were included in the study (Table III.1.1).

DNA extraction and quantification

Total genomic DNA was extracted from the oomycetes mycelia and sporangia scrapped from sporulating leaves, as well as from symptomatic and asymptomatic plant tissues with the Fast DNA kit (Qbiogene, Madrid, Spain). This latter system was selected as the most suitable for DNA extraction in the study because of the: (i) high number of samples that could be processed per operator; (ii) consistency in the amount of total DNA extracted; (iii) quality and stability of the extracted DNA; and (iv) total DNA extracted can be used directly for PCR assays.

The obligate biotrophy of *P. arborescens* makes it difficult to obtain DNA of PCR quality of the oomycete that is also free from contaminant DNAs (mainly bacterial and plant DNA). For this reason, pathogen DNA was extracted from sporangiophores bearing sporangia that were carefully removed from naturally or artificially induced sporulating leaves. These structures were collected with a sterile needle and placed in 1.5-ml microcentrifuge tubes containing 1,000 µl of sterile distilled water and a drop of Tween 20 (Sigma-Aldrich, Madrid, Spain). The

suspension was vortexed for a few seconds, filtered through a 0.8-μM Millipore filter, and the filter was washed three times with ultrapure, sterile water (40). Sporangia and sporangiophores retained on the filter were resuspended in 500 μl of sterile water and centrifuged at 12,000 × *g* for 2 min and the pellet was finally resuspended in 200 μl of ultrapure, sterile water. For plant material, fresh tissues were cut into small pieces with a sterile blade and used for DNA extraction. Approximately 100 to 200 mg of plant tissue or sporangiophores and sporangia suspensions were placed in a 1.5-ml Fast DNA tube containing lysing matrix A, 800 μl of CLS-VF solution, and 200 μl of protein precipitation solution (PPS) for plant material, and 1,000 μl CLS-Y solution for suspensions of pathogen structures. Cells were mechanically disrupted in a Fast Prep System Bio 101 (Qbiogene) by reciprocal shaking of the samples for 30 s at a 5.5 speed, twice. Samples were incubated on ice for 2 min between successive homogenizations. Then, the supernatant was collected by centrifugation (10 min at 12000 × *g*) and processed with the Fast DNA kit according to the manufacturer's instructions.

For seed assays, approximately 400 to 500 opium poppy seeds (average weight = 130 to 150 mg) were placed in a 1.5-ml Fast DNA tube containing lysing matrix A, 800 μl of buffer CLS-VF and 200 μl of PPS, and allowed to soften for 1 h before processing as described above. Thereafter, the DNA pellet was resuspended in ultrapure, sterile water, quantified with the Quant-iT DNA Assay Kit Broad Range fluorometric assay (Molecular Probes Inc., Leiden, the Netherlands) and a Tecan Safire fluorospectrometer (Tecan Spain, Barcelona, Spain) according to the manufacturer's instructions, diluted with ultrapure, sterile water, and used for PCR assays.

Table III.1.1. List of species of *Peronospora* spp. and specimens for which PCR amplicons were sequenced in the study, and fungi and oomycetes used to determine specificity of the species-specific primers.

Pathogen species	Host species, sampled tissue	Geographic origin	Collection code	Year	GenBank accession nº
Peronospora spp.					
P. arborescens	*Pap. somniferum,* mycelium	"Casa Arriba Los Llanos", Albacete, Spain	P13ACALL	2005	DQ885367
	Pap. somniferum, leaves	"Casilla San José", Écija , Sevilla, Spain	P2ESJ	2004	AY695805
			P5ESJ	2004	AY695807
	Pap. somniferum, mycelium	"Casilla San José", Écija, Sevilla, Spain	P38ESJ	2004	DQ886487
			P6ESJ	2005	DQ885363
	Pap. somniferum, leaves	"San Rafael", Écija, Sevilla, Spain	P1ESR	2004	AY695804
			P4ESR	2004	AY695806
	Pap. somniferum, mycelium	"San Rafael", Écija, Sevilla, Spain	P37ESR	2005	DQ885384
	Pap. somniferum, leaves	"Ruidero", Écija, Sevilla, Spain	M2ERUI	2004	DQ885362
	Pap. somniferum, mycelium	"Viso alto", Écija, Sevilla, Spain	P8EVA	2005	DQ885365
	Pap. somniferum, leaves		P7EVA	2005	DQ885364
	Pap. somniferum, leaves	"La Estrella", Écija, Sevilla, Spain	P9ELE	2005	DQ885366
	Pap. somniferum, mycelium	"Monteluna", Antequera, Málaga, Spain	P29MAM	2006	DQ885378
			P30MAM	2006	DQ885379
	Pap. somniferum, mycelium	"Valsequillo", Antequera, Málaga, Spain	P27MAV	2006	DQ885376
	Pap. somniferum, leaves	"Valsequillo", Antequera, Málaga, Spain	P28MAV	2006	DQ885377
	Pap. somniferum, mycelium	"El Pontón", Antequera, Málaga, Spain	P31MEP	2006	DQ885380
	Pap. somniferum, mycelium	"Cortijo del Río", Antequera, Málaga, Spain	P32MACR	2006	DQ885381
			P33MACR	2006	DQ885382
			P34MACR	2006	DQ885383
	Pap. somniferum, leaves	"Hormigos", Malpica de Tajo, Toledo, Spain	TO	2004	AY702098
			M1THO	2005	DQ886488
	Pap. somniferum, mycelium	"Valdemerino", Malpica de Tajo, Toledo, Spain	P16TVMT	2005	DQ885368
			P17TVMT	2005	DQ885369
			P19TVMT	2005	DQ885371
	Pap. somniferum, mycelium	"El Torrejón", Malpica de Tajo, Toledo, Spain	P18TTMT	2005	DQ885370
			P20TTMT	2005	DQ885372
	Pap. somniferum, stem	"El Torrejón", Malpica de Tajo, Toledo, Spain	P21TTMT	2005	DQ885373
P. cristata[a]	*Meconopsis cambrica,* leaves	England, UK	P22MC	2005	DQ885374
			P24MC	2005	DQ885375
	Pap. somniferum, leaves	Tasmania, Australia (F. S. Hay)		2005	Np[b]
	Pap. somniferum, leaves	Tasmania, Australia (P. J. Cotterill)		2005	Np
P. destructor[a]	*Allium cepa,* leaves	Norway	P46N	2005	DQ885385
Other fungi and Oomycetes					
Fusarium sp.	*Pap. somniferum,* leaves	Écija, Sevilla, Spain	FVM	2004	DQ885387
Fusarium sp.	*Pap. somniferum,* leaves	Écija, Sevilla, Spain	FVS3	2004	DQ885388
Beauveria bassiana	*Pap. somniferum,* leaves	Carmona, Sevilla, Spain	EaBb 04/01	2004	DQ364698
Pleospora papaveracea	*Pap. somniferum,* seeds	Carmona, Sevilla, Spain	D2	2004	DQ885386
Bremia lactucae[a]	*Lactuca sativa*	Córdoba, Spain		2006	Np
Plasmopara halstedii[a]	*Helianthus annuus*	Córdoba, Spain		2004	Np
------	*Pap. somniferum* var. *nigrum*	ALCALIBER, S.A., Carmona, Sevilla, Spain		2004	DQ364699

[a] Samples of *M. cambrica* infected by *P. cristata, Pap. somniferum* infected by *P. cristata, P. destructor, Pl. halstedii* and *B. lactucae* were kindly provided by D. E. L. Cooke (Scottish Crop Research Institute, Invergowrie, Dundee, Scotland, UK), F. S. Hay (Tasmanian Institute of Agricultural Research, University of Tasmania, Burnie, Australia), P. J. Cotterill (GlaxoSmithKline, Latrobe, Tasmania, Australia), B. Nordskog (Plante forsk, The Norwegian Crop Research Institute, Norway), L. Molinero (IAS-CSIC, Córdoba, Spain), and J.A. Navas-Cortés (IAS-CSIC, Córdoba, Spain), respectively.

[b] Np= not performed.

PCR protocols using universal primers

The ITS1-5.8S-ITS2 regions of the rDNA of different species of Peronosporaceae, fungi, and plants used in the study (Table III.1.1) were amplified in PCR assays using universal primers (44) as well as primer DC6 (7) specific for species in the orders Pythiales and Peronosporales of the Oomycota.

ITS5/ITS4 proved the most effective universal primers in a reaction mix (final volume of 50 μl) of 5 μl of 10× reaction buffer (166 mM [NH4]2SO4, 670 mM Tris-HCl [pH 8.0, 25°C], Tween 20), 1 μM of each primer, 200 μM of each dNTP, 2 units of *EcoTaq* DNA polymerase (EcoGen, Madrid, Spain), 1.5 mM MgCl2, and 1 μl of template DNA (5 to 20 ng of DNA). Amplifications were performed in Perkin-Elmer 9600 (Perkin-Elmer, Norwalk, CT) and PTC 100 (MJ Research Inc., Watertown, MA) thermocyclers. The cycling program included an initial denaturation step of 4 min at 95°C, followed by 35 cycles of 1 min denaturation at 95°C, 1 min annealing at 56°C, 2 min extension at 72°C, and a final 10 min extension step at 72°C followed by a 4°C soak. The DC6/ITS4 primer pair was used for the selective amplification of the complete ITS region of rDNA together with a portion of the 18S rDNA. PCR reactions were conducted in 50-μl volumes as described before, except that 0.6 μM of each primer and 60 μM of each dNTP were used. PCR conditions were initial denaturation of 5 min at 95°C, followed by 30 cycles of 1 min denaturation at 95°C, 1 min annealing at 62°C, 2 min extension at 72°C, and a final extension of 10 min at 72°C followed by a 4°C soak.

All reactions were repeated at least twice and always included a positive control (*P. arborescens* DNA obtained from sporangiophores with sporangia scraped from sporulating leaves) and negative controls (*Papaver somniferum* DNA or no DNA). Amplification products were separated by electrophoresis in 1.5% agarose gels in 1× TAE buffer for 60 to 120 min at 80V, stained with ethidium bromide, and visualized under UV light. The GeneRuler DNA ladder mix (Fermentas, St Leon-Rot, Germany) was used for electrophoresis.

Sequencing of amplified products and phylogenetic analysis

PCR products amplified with primer pairs ITS5/ITS4, DC6/ITS4, or the primer pair pdm3/pdm4 (36; see below) were purified with a gel extraction kit (Geneclean turbo, Qbiogene, Illkirch, France), quantified with the Quant-iT DNA Assay Kit Broad Range fluorometric assay as described before, and used for direct DNA sequencing or for cloning. Purified PCR products were ligated into pGEM-T easy vector system II using *Escherichia coli* strain JM109 for transformation according to the manufacturer's procedure (Promega, Madison, WI). Transformed clones were cultured and the plasmid DNA extracted from them (two clones per PCR amplicon) with the Wizard Plus SV Mini-preps DNA purification system (Promega) was used for sequencing. PCR amplicons and cloned inserts were sequenced in both directions with a terminator cycle sequencing ready reaction kit (BigDye; Perkin-Elmer Applied Biosystems, Madrid, Spain) using DC6, ITS4, and ITS5 primers (PCR amplicons) or universal primers (M13-20fw/M13rev) (cloned inserts), according to the manufacturer's instructions. The resulting products were purified and run on a DNA multicapilar sequencer (ABI Prism 3100 genetic analyzer; Perkin-Elmer Applied Biosystems) at the University of Córdoba sequencing facilities. All ITS1-5.8S-ITS2 sequences obtained from *P. arborescens* collections in the study were deposited in GenBank (Table III.1.1). In addition, ITS1-5.8S-ITS2 sequences from *Papaver somniferum*, *Fusarium* spp., and *Pleospora papaveracea* isolated from opium poppy, *P. destructor* infecting onion, and *P. cristata* infecting *M. cambrica* were obtained (Table III.1.1).

The ITS sequences of *P. arborescens* (27 sequences), *P. destructor* (1 sequence), and *P. cristata* (2 sequences) obtained in this study were aligned with 41 published DNA sequences of Oomycete species belonging to the Peronosporaceae (Table III.1.2), including the genera *Hyaloperonospora*, *Perofascia*, *Peronospora*, and *Pseudoperonospora*. The *Peronospora* and *Pseudoperonospora* spp. were selected because they: (i) are closely related to *P. arborescens* (36,43); (ii) have their hosts within the Ranunculales (Papaveraceae and Fumariaceae) (17); or (iii) have been recorded in Spain (24). Sequences were initially aligned with the ClustalX 1.83 software (41) with default options. Then, the Bionumerics 4.5 software (Applied Maths, Kortrijk, Belgium) was used to generate phylogenetic trees with the neighbor-joining (NJ) and

maximum-parsimony (MP) methods and UPGMA cluster analysis. The phylograms were bootstrapped 1,000 times to assess the degree of support for the phylogenetic branching indicated by the optimal trees. Trees were rooted with *Phytophthora infestans* AF266779 ITS1-5.8S-ITS2 gene sequence as an outgroup (43).

Table III.1.2. List of Peronosporales taxa used in the present study, with reference to their host species and GenBank accession numbers.

Species	Host species	Geographic origin	GenBank accession nos. (reference)
Perofascia lepidii	*Lepidium virginicum*	Korea	AY211013 (11)
P. lepidii	*Capsella bursa-pastoris*	Romania	AF465760 (14)
Peronospora spp.			
P. alpicola	*Ranunculus aconitifolius*	Germany	AY198271 (53)
P. aparines	*Galium aparine*	Austria	AY198300 (53)
P. arborescens	*Papaver rhoeas*	Austria	AY198292 (53)
P. arborescens	*Papaver rhoeas*	Romania	AY465761 (14)
P. bulbocapni	*Corydalis cava*	Austria	AY198272 (53)
P. conglomerata	*Geranium molle*	Austria	AY919304 (5)
P. conglomerata	*Geranium molle*	Austria	AY198246 (53)
P. cristata	*Meconopsis cambrica*	England, UK	AY374984 (D.E.L. Cooke and N.A. Williams, *unpublished results*)
P. cristata	*Papaver somniferum*	Tasmania, Australia	AY225472-AY225482, AY225484 (47)
P. chenopodi	*Chenopodium album*	Austria	AY198285 (53)
P. corydalis	*Corydalis ocotensis*	Korea	AY211015 (11)
P. corydalis	*Corydalis speciosa*	Korea	AY211016 (11)
P. destructor	*Allium cepa*	Tasmania, Australia	AY225469 (47)
P. dicentrae	*Dicentra canadensis*	Tennessee, USA	AY198273 (53)
P. farinose	*Chenopodium album*	Romania	AF465762 (14)
P. farinose	*Chenopodium album*	Korea	AY211017 (11)
P. farinose	*Chenopodium serotinum*	Korea	AY211018 (11)
P. manshurica	*Glycine soja*	Korea	AY211019 (11)
P. manshurica	*Glycine max*	Unknown	AB021711 (M. Saito, *unpublished results*)
P. ranunculi	*Ranunculus acris*	Austria	AY198267 (53)
P. sparsa	*Rosa* sp.	England, U.K.	AF266783 (15)
P. sparsa	*Rosa* sp.	Tasmania, Australia	AY225470 (47)
P. tabacina	*Nicotiana alata*	Austria	AY198289 (53)
P. viciae	*Pisum sativa*	Tasmania, Australia	AY225471 (47)
P. viciae	*Vicia angustifolia*	Austria	AY198230 (53)
Hyaloperonospora spp.			
H. parasitica	*Thlaspi arvense*	Romania	AF465759 (14)
H. parasitica	*Capsella bursa-pastoris*	Austria	AY198254 (53)
Pseudoperonospora spp.			
Ps. cubensis	*Cucumis sativa*	Austria	AY198306 (53)
Ps. humuli	*Humulus lupulus*	Unknown	AF448225 (Patzak, J., *unpublished results*)
Ps. humuli	*Humulus lupulus*	Austria	AY198304, AY198305 (53)
Ps. urticae	*Urtica dioica*	Austria	AY198307 (53)

Design of *P. arborescens* specific primers

A subset of nonredundant ITS1-5.8S-ITS2 sequences of *P. arborescens* and *P. cristata* selected from GenBank, as well as sequences obtained in this study, were aligned using the ClustalX 1.83 software and utilized for designing *P. arborescens*-specific PCR primers (Table III.1.3). Primers were designed with Primer3 (http://www.ge-nome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and Beacon Designer (http://www.premierbiosoft.com/netprimer/netprimer.html) software to ensure that at least one of the primers contain less than 85% homology and includes one nucleotide insertion as compared with *P. cristata* ITS1-5.8S-ITS2 sequences and ensure lack of homology with ITS1-5.8S-ITS2 sequences of *Papaver somniferum* or fungi commonly isolated from this plant (see below). An in silico test of primer specificity was conducted by running the primer sequences against the nonredundant GenBank dataset with parameters set for the identification of short, nearly exact matches.

Table III.1.3. PCR primers designed in this study, their sequences, origins and specificities.

Primer name[a]	Sense	Primer set name	Detects	Source	Secuence (5'- 3')	Melting temperature (ºC)	Amplicon size (bp)
P3Pa1fw[b]	Forward	P1	*P. arborescens*	ITS2	GCTATGGCGATAATGGAGGA	60,0	200
P3Pa1rv[b]	Reverse		Peronosporales	ITS2	CAAATTTCCCAAATGGGTTG	60,0	
P3Pa2fw	Forward	P2	*Peronospora* spp.	ITS1	TGATCTCGGTCGGAGCTAGT	60,0	545
P3Pa2rv[b]	Reverse		*Peronospora* spp.	ITS2	TCACCAGTTATACCGCCACA	60.0	
OMPac1fw	Forward	P3	Oomycetes	ITS1	CCACACCTAAAAACTTTCC	52.3	594
OMPa1rv[b]	Reverse		*P. arborescens*	ITS2	AACACTCCTCCATTATCG	50.5	
OMPac7fw	Forward	P6	Oomycetes	5.8S	GAACGCATATTGCACTTCC	56.7	456
OMPa7rv[b]	Reverse		*P. arborescens*	ITS2	CGCACAACACAAATTTCC	55.9	

[a] The use of the sequences described for a diagnostic test for *P. arborescens* is covered by a Spanish patent application (number assignation P200603319) owned by University of Córdoba and ALCALIBER S.A..
[b] Primers include a nucleotide that is a deletion in the ITS1-5.8S-ITS2 sequence of *P. cristata.*

Specific PCR assays

Reaction conditions such as annealing temperature and MgCl2 and primer concentrations were adjusted experimentally to optimize the amplification with each primer pair (Table III.1.3). Optimized PCR reactions were (final volume of 25 µl) 2.5 µl of 10× reaction buffer, 0.75 µM of each primer, 50 µM of each dNTP, 0.75 unit of *EcoTaq* DNA polymerase (EcoGen), 1 mM MgCl2, and 1 µl of the template DNA.

The cycling program consisted of an initial denaturation step of 3 min at 95°C, followed by 30 cycles of 1 min denaturation at 95°C, 1 min annealing at 61°C, 1 min at 72°C, and a final extension step of 5 min at 72°C. Gel electrophoresis was performed as described for universal primers.

Specificity of the designed primer pairs was tested by including the following controls in PCR assays: (i) *P. arborescens* DNA extracted from sporangiophores and sporangia scraped from sporulating opium poppy leaves; (ii) total DNA extracted directly from opium poppy leaves infected with *P. arborescens*; (iii) DNA extracted from healthy *Papaver somniferum*; (iv) total DNA extracted from tissues of *Papaver somniferum* and *M. cambrica* infected with *P. cristata*; (v) DNA extracted from fungi isolated from opium poppy tissues (i.e., *B. bassiana*, *Fusarium* spp., and *Pleospora papaveracea*); (vi) DNA extracted from other oomycetes within the Peronosporaceae (i.e., *B. lactucae*, *P. destructor*, and *Plasmopara halstedii*); and (vii) no DNA as a negative control.

Primers pdm3 and pdm4, reported as specific for the identification and detection of *P. cristata* (36), were also used as described to test for their specificity against populations of *P. arborescens*.

Sensitivity of the diagnostic PCR was assessed by determining the minimum amount of *P. arborescens* DNA that could be detected in PCR reactions. For this purpose, *P. arborescens* DNA (10 ng/µl) was diluted (1:2, 1:10, 1:20, $1:10^2$, $1:10^3$, $1:10^4$, and $1:10^5$) in ultrapure, sterile water, as well as in *Papaver somniferum* DNA extracted from healthy opium poppy leaves (10 ng/µl) or seeds (20 ng/µl) to account for any possible influence that the host DNA might have on the amplification of pathogen DNA. Special care was taken to get accurate pathogen and host DNA concentrations. For this objective, both the stock DNA and its dilutions were quantified fluorimetrically in triplicate in two independent plates using the Quant-iT DNA Assay kit as described above. The host DNA concentrations used were selected as representative of DNA amounts yielded with the Fast DNA protocol described above from 100 mg of leaf tissue and 100 seeds of opium poppy, respectively. All PCR reactions were repeated at least three times by independent operators using the conditions referred to as above and always included *Papaver somniferum* DNA or no DNA as negative controls.

Species-specific PCR assays using samples from opium poppy crops.

During the spring of 2005 and 2006, plants were sampled from a commercial opium poppy field with a high incidence of the downy mildew disease in Écija, Seville Province of Andalucía. Samples were representative of severely through mildly affected tissues as well as asymptomatic tissue. Some affected plants showed dwarfing and rosetting, with the uppermost leaves showing light chlorosis, suggesting systemic infection of the plants. All plant tissues were surface sterilized (1% NaOCl for 3 min) prior to DNA extraction. Total DNA was extracted from complete stem samples, the epidermal and cortex tissues of stems, capsules, and leaves affected with necrosis, light chlorosis, or no symptoms. PCR reactions were conducted using three selected specific primer sets (P2, P3, and P6; Table III.1.3).

Detection and transmission of *P. arborescens* in opium poppy seeds.

Artificially infested and naturally infected or infested seed samples were used to test the reliability of the developed assay for detecting *P. arborescens* DNA in or on seed. For artificial infestation, healthy seeds (i.e., that gave no amplification for *P. arborescens* in previous assays using the species-specific PCR protocol) were surface sterilized with 1% NaOCl for 3 min, rinsed with distilled, sterile water, dipped in 1 ml of sporangium suspensions adjusted to get 1×10^4, 5×10^3, 2.5×10^3, 2.5×10^2, and 25 sporangia per 100 seeds, and vortexed. The treated seeds were allowed to dry at room temperature in a Vacufuge TM Concentrator 5301 system (Eppendorf Ibérica S.L., Madrid, Spain).

Total DNA was extracted from the artificially infested seeds as described above. Stocks of naturally infected or infested seeds were obtained from capsules of diseased plants sampled from opium poppy fields with high incidence of downy mildew. These capsules showed a range of disease symptoms, from nonsymptomatic but having light sporulation on the capsule peduncle to the entire capsule showing severe necrosis. Seeds were obtained from capsules through a hole at the base of a capsule made by aseptically removing its peduncle at the site of junction and emptying the seeds into a sterile eppendorf tube. Four samples of 500 seeds each were processed for DNA extraction as described above. PCR assays using DNA extracted

from either artificially infested seeds or naturally infected or infested seeds sampled from infected capsules were conducted with the three specific primer sets as described before.

To further investigate whether seedborne *P. arborescens* can give rise to infected opium poppy plants, we conducted an experiment in nonirrigated field microplots that had never been cropped with *Papaver somniferum* (sandy loam soil, pH 8.5, 1.4% organic matter) at the Alameda del Obispo Research Station near Córdoba (37.5°N, 4.8°W, altitude 110 m). The microplots (1.25 × 1.25 m^2, 50-cm depth) were sown with seed stocks numbers 471, SR, 432, and 431 of the commercial opium poppy cv. Nigrum on 3 January 2005. Seeds of these seed stocks were provided by ALCALIBER S.A. (Carmona, Sevilla, Spain), the only enterprise officially authorized for opium poppy cultivation in Spain, and originated from fields severely affected by downy mildew. Seeds of stock number 451, which had been washed with 1% NaOCl for 3 min and further treated with metalaxyl (Apron XL; Syngenta Agro, Madrid, Spain), were used as a control. Each microplot consisted of four furrows, 0.2 m apart and 0.2 m from the closest microplot edge barrier (a 25 cm tall, 0.5 cm width fiber cement sheet). A microplot was hand sown by carefully spreading 100 seeds per furrow. There were four replicated microplots per seed stock in a completely randomized design. Weeds (especially *Papaver* spp.) that developed inside or outside of the microplots were removed by hand as soon as they developed. Daily mean temperature and rainfall were recorded in a weather station located at the experimental site. After emergence, plants were observed at weekly intervals for development of symptoms of opium poppy downy mildew.

III.1.4 RESULTS

PCR assays using universal primers

Amplification of DNA extracted from *P. arborescens* sporangiophores and sporangia scraped from downy-mildew-affected opium poppy with primers ITS4 and ITS5 yielded fragments of approximately 900 bp. However, amplification of total DNA from symptomatic leaves yielded the 900-bp band and an approximately 800-bp product similar in size to that amplified using DNA extracted from healthy *Papaver*

somniferum in a previous study (32).

Amplification of DNA extracted from *P. arborescens* sporangiophores and sporangia with primers DC6 and ITS4 yielded a single PCR amplicon of approximately 1,200 bp. There was no amplification when those two primers were used with DNA extracted from tissues of healthy opium poppy plants.

PCR assays using *P. cristata*-specific primers

Amplification of total DNA extracted from samples of *M. cambrica* and opium poppy infected with *P. cristata* with primer pair pdm3/pdm4 (reportedly specific for *P. cristata*; 36) (Table III.1.1) yielded a single band of approximately 389 bp. Interestingly, when primers pdm3/pdm4 were used for PCR assays of more than 200 samples of diseased opium poppy from Spain, 15% of samples amplified DNA from *P. arborescens* (see below).

Sequencing of amplified products and phylogenetic analysis

Sequence homology and phylogenetic analysis of the ITS1-5.8S-ITS2 regions of rDNA amplified from samples of the downy mildew pathogen confirmed that *P. arborescens* was the sole causal agent of the disease affecting opium poppy in Spain (Fig. III.1.1). Sequencing and phylogenetic analysis confirmed that all pathogen samples that had previously yielded a 389-bp amplicon with primers pdm3/pdm4 were *P. arborescens* rather than *P. cristata*. The 27 collections of the downy mildew pathogen recovered from *Papaver somniferum* in Spain showed high sequence homology (99.8 to 100%) and were differentiated into four ITS sequence types on the basis of sequence differences in a total number of eight nucleotide positions in the ITS1, 5.8S, and ITS2 regions. These differences were not related to geographical origin (region, province, or field) of isolates (Table III.1.1). ITS sequence homology between collections of *P. arborescens* from opium poppy in Spain and *P. arborescens* AF465761 from Romania (12) and AY198292 from Austria (43) ranged from 99.3 to 99.6%. Sequence homology between *P. arborescens* from Spain and *P. cristata* AY225472–AY225482 (36) from Tasmania and *P. cristata* AY374984 from Europe (D. E. L. Cooke and N. A. Williams, *unpublished data*) ranged from 80.9 to 93.7% and 93.6 to 94%, respectively.

The topology of the phylogenetic trees produced with the NJ (Fig. III.1.1) and MP method were almost identical (data not shown).

P. arborescens from *Papaver rhoeas* in Romania (AF465761) and Austria (AY198292) grouped with all poppy downy mildew collections from Spain, with bootstrapping values of 100% both for NJ and MP analyses. The two methods of analysis indicated that *P. arborescens* from *Papaver somniferum* and *Papaver rhoeas* formed a single, highly supported clade, phylogenetically distinct to the clade comprising *P. cristata*. Interestingly, *P. cristata* from *Papaver somniferum* and *M. cambrica* is more closely related to other *Peronospora* spp. that have their host in the Ranunculales (*P. corydalis* and *P. cristata* [Papaveraceae], *P. alpicola* and *P. ranunculi* [Ranunculaceae], and *P. bulbocapni* and *P. dicentrae* [Fumariaceae]) (Fig. III.1.1).

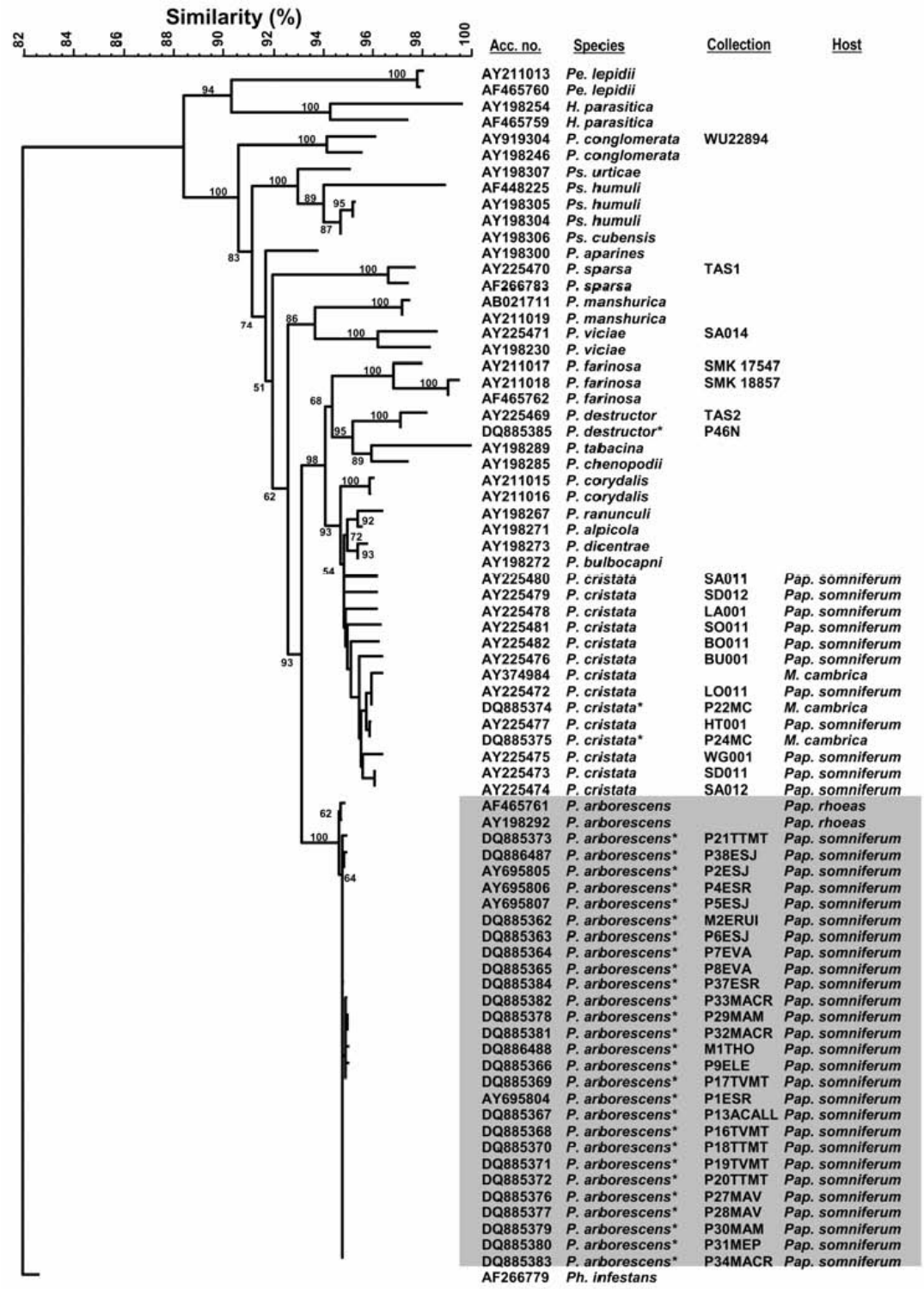

Fig. III.1.1. Phylogenetic tree inferred from neighbor-joining analysis of the complete ITS region (ITS1, 5.8S rDNA, and ITS2) indicating relationships between downy mildew species. Bootstrap supports of more than 50% are given for appropriate main order clades. Asterisks (*) show taxa whose data were obtained in this study. H. = *Hyaloperonospora*, Pe. = *Perofascia*, P. = *Peronospora*, Ps. = *Pseudoperonospora*, and Ph. = *Phytophthora*.

Design of *P. arborescens*-specific primers

Four primer pairs were designed with the criteria described above, namely P3Pa1fw/P3Pa1rv, P3Pa2fw/P3Pa12rv, OMPac1fw/OMPa1rv, and OMPac7fw /OMPac7rv that were designated P1, P2, P3, and P6 primer sets, respectively. The primer sequences (Table III.1.3) were compared with the NCBI nonredundant nucleotide database (May 7, 2006) using BLAST 2.2.14 (3). Sets P1, P2, P3, and P6 were predicted to be specific for *P. arborescens.*

Specificity of the newly developed *P. arborescens*-specific primers

Primer sets P1, P2, P3, and P6 reproducibly amplified DNA fragments of 200, 545, 594, and 456 bp, respectively, in PCR assays with DNA extracted from all collections of *P. arborescens* sporangiophores and sporangia (Fig. III.1.2) as well as from infected *Papaver somniferum* tissue and samples of *Papaver somniferum* that previously had yielded the 389-bp band (potentially specific for *P. cristata*) with the pdm3/pdm4 primer pair (Fig. 3). No cross amplifications were observed when the tested primer sets were used with DNA extracted from plant samples infected with *P. cristata* either from the United Kingdom or Tasmania or DNA from healthy plants (Fig. 3). Likewise, no cross amplification occurred with DNA of other fungi and Oomycetes tested (data not shown). Use of primer set P1 also yielded a DNA band larger than 200 bp, but of much lower intensity, in addition to the predicted 200-bp amplicon (Fig. III.1.2). Attempts were made to increase the specificity of this primer set with no success; consequently, P1 primer set was disregarded for further studies.

PCR quality of all DNA samples, including samples containing *P. cristata* DNA, was confirmed with the universal ITS5/ITS4 or DC6/ITS4 primer pairs.

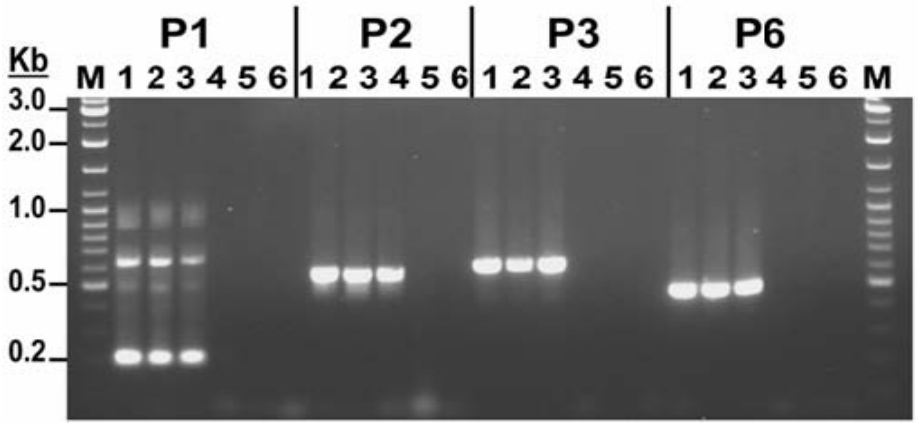

Fig. III.1.2. Specificity of *Peronospora arborescens*-specific primers P3Pa1fw/ P3Pa1rv (P1), P3Pa2fw/P3Pa12rv (P2), OMPac1fw/OMPa1rv (P3), and OMPac7fw/OMPac7rv (P6). M, GeneRuler DNA ladder mix (Fermentas, St Leon-Rot, Germany); lane 1, *P. arborescens* sporangiophore and sporangia from sporulating opium poppy leaves (positive control); lane 2, *P. arbores-cens*-infected leaves of *Papaver somniferum*; lane 3, *P. arborescens*-infected stem of *Papaver somniferum*; lane 4, healthy leaves of *Papaver somniferum*; lane 5, healthy stem of *Papaver somniferum*; and lane 6, no DNA template.

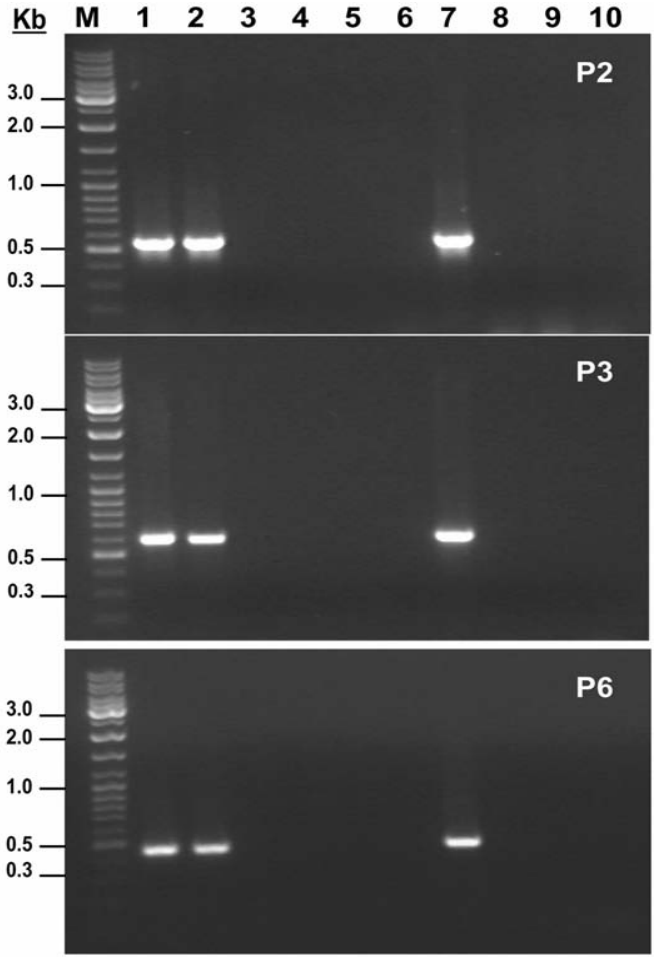

Fig. III.1.3. Specificity of *Peronospora arborescens*-specific primers P3Pa2fw/ P3Pa12rv (P2), OMPac1fw/OMPa1rv (P3), and OMPac7fw/OMPac7rv (P6). M, GeneRuler DNA ladder mix (Fermentas, St Leon-Rot, Germany); lane 1, *P. arborescens* sporangiophores and sporangia from sporulating opium poppy leaves (positive control); lane 2, *P. arborescens*-infected leaves of *Papaver somniferum*; lanes 3–5, *P. cristata*-infected leaves of *Papaver somniferum* from Australia; lane 6, *P. cristata*-infected leaves of *Meconopis cambrica* from the United Kingdom; lane 7, *P. arborescens*-infected leaves of *Papaver somniferum* that also yielded amplification with primers pdm3/pdm4 (described as specific for *P. cristata,* 37); lane 8, healthy leaves of *Papaver somniferum*; lane 9, healthy, surface-sterilized seeds of *Papaver somniferum*; and lane 10, no DNA template.

Sensitivity of *P. arborescens*-specific primers

Adding DNA from opium poppy in the PCR reaction mix did not influence sensitivity of the specific PCR assays (Table III.1.4). Use of reaction mixes containing 10 ng of opium poppy DNA extracted from healthy leaves resulted in a PCR detection limit of 10, 1 to 10, and 1 to 10 pg of *P. arborescens* DNA for primer sets P2, P3, and P6, respectively (Table III.1.4). Interestingly, the detection limit for each of the primer sets was increased when DNA from surface-sterilized, healthy seeds rather than from leaves was used for dilutions. This occurred even though the background plant DNA concentration was double for seed DNA and both sets of assays were performed with the same stock solutions of *P. arborescens* DNA. Therefore, using reaction mixes containing 20 ng of opium poppy DNA extracted from seeds allowed a detection limit of PCR assays of 1, 1 to 0.1, and 1 pg (Fig. III.1.4) of *P. arborescens* DNA for primer sets P2, P3, and P6, respectively. Adding DNA from poppy seeds or leaves to PCR reaction mixes using primer pair DC6/ITS4 resulted in a detection limit of 100 pg of *P. arborescens* DNA, irrespective of the source of plant DNA (Table III.1.4). Use of the detection method by different operators did not influence reproducibility and consistency of results.

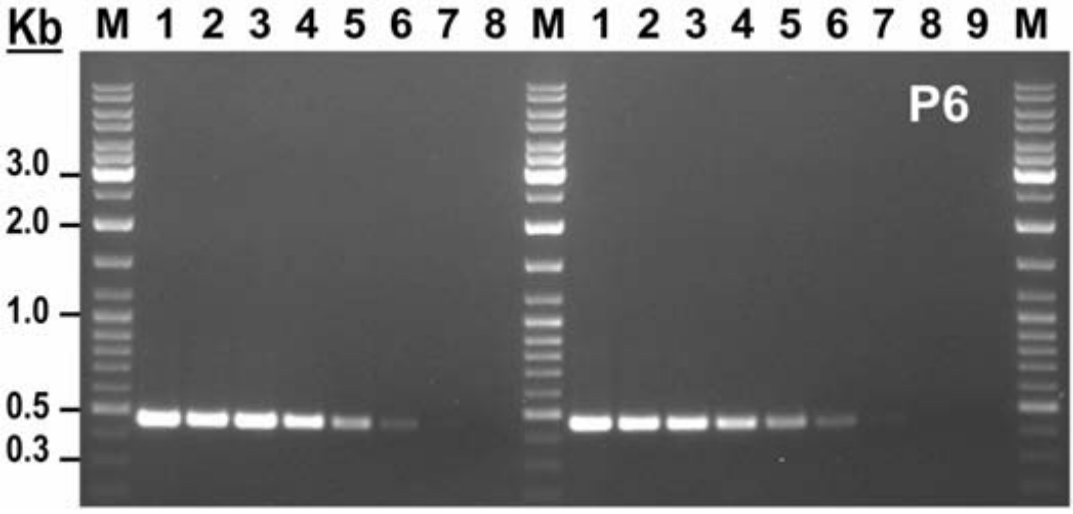

Fig. III.1.4. Sensitivity of *Peronospora arborescens*-specific primers OMPac7fw/ OMPac7rv (P6) with *P. arborescens* DNA (10 ng/µl) diluted in water (H2O Series) or in DNA of *Papaver somniferum* (20 ng/µl) extracted from seeds. M, GeneRuler DNA ladder mix (Fermentas, St Leon-Rot, Germany); lanes 1–7, DNA dilution series 1:2 (lane 1), 1:10 (lane 2), 1:20 (lane 3), $1:10^2$ (lane 4), $1:10^3$ (lane 5), $1:10^4$ (lane 6), and $1:10^5$ (lane 7); lane 8, no DNA template; and lane 9, healthy, surface-sterilized seeds of *Papaver somniferum*.

Table III.1.4. Sensitivity of *Peronospora arborescens*-specific primers designed in this study and Oomycetes universal primers.

Primer pair	Dilution Series[a]	Background DNA (ng/ul)[a]	DNA of *Peronospora arborescens* (ng/µl)[a] 5	1	0.5	0.1	0.01	0.001	0.0001	Neg. control	H_2O Control
P3Pa2fw/P3Pa12rv	I	0	+/+/+[b]	+/+/+	+/+/+	+/+/+	-/+/+	-/-/-	-/-/-		-/-/-
		10	+/+/+	+/+/+	+/+/+	+/+/+	±/+/+	-/-/-	-/-/-	-/-/-	-/-/-
	II	0	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	-/+/±	-/-/-		-/-/-
		20	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	-/+/±	-/-/-	-/-/-	-/-/-
OMPac1fw/OMPa1rv	I	0	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	-/±/+	-/± /±		-/-/-
		10	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	-/±/+	-/± /±	-/-/-	-/-/-
	II	0	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	+/+/-	±/±/-		-/-/-
		20	+/+/+	+/+/+	+/+/+	+/+/+	+/+/-	±/±/+	± /±/-	-/-/-	-/-/-
OMPac7fw/OMPac7rv	I	0	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	±/±/-	-/-/-		-/-/-
		10	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	+/±/+	-/-/-	-/-/-	-/-/-
	II[c]	0	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	+/±/+	-/-/-		-/-/-
		20	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	+/±/-	-/-/-	-/-/-	-/-/-
DC6/ITS4[d]	I	0	+/+	+/+	+/+	+/	±/+	-/-	-/-		-/-
		10	+/+	+/+	+/+	+/	-/-	-/-	-/-	-/-	-/-
	II	0	+/+	+/+	+/+	+/	+/±	-/-	-/-		-/-
		20	+/+	+/+	+/+	+/	-/-	-/-	-/-	-/-	-/-

[a] *P. arborescens* DNA (10 ng/µl) was serially diluted to obtain 5 to 0.0001 ng of pathogen per microliter of PCR reaction in distilled water (0 ng background DNA) or in *Pap. somniferum* DNA extracted from leaves (10 ng/µl background DNA; Dilution series I) or seeds (20 ng/µl background DNA; Dilution series II) to investigate any possible influence of host DNA on amplification of pathogen DNA.

[b] Results presented were obtained by three independent operators with same dilution series. + = Positive amplification; ± = Positive weak amplification; - = no amplification.

[c] Representative results from this dilution series experiment are shown in Figure 4.

[d] This primer pair combination was assessed only in to independent PCR reactions.

Species-specific PCR assays with samples from opium poppy crops

PCR assays using P2, P3, and P6 primer sets and DNA extracted from leaf, stems, and capsules of diseased opium poppy plants, as well as from roots of plants with dwarfing and resetting symptoms, resulted in nearly 100% positive detection of

P. arborescens in the infected tissues (Fig. III.1.5). There were no amplification products in PCR assays with DNA extracted from leaves and capsules of apparently healthy opium poppy plants. When DNA was extracted from infected plants showing very light chlorosis or no symptoms, detection of the pathogen on leaves and epidermal stem tissues was more efficient using primer set P3 compared with use of primer sets P2 and P6 (Fig. III.1.5).

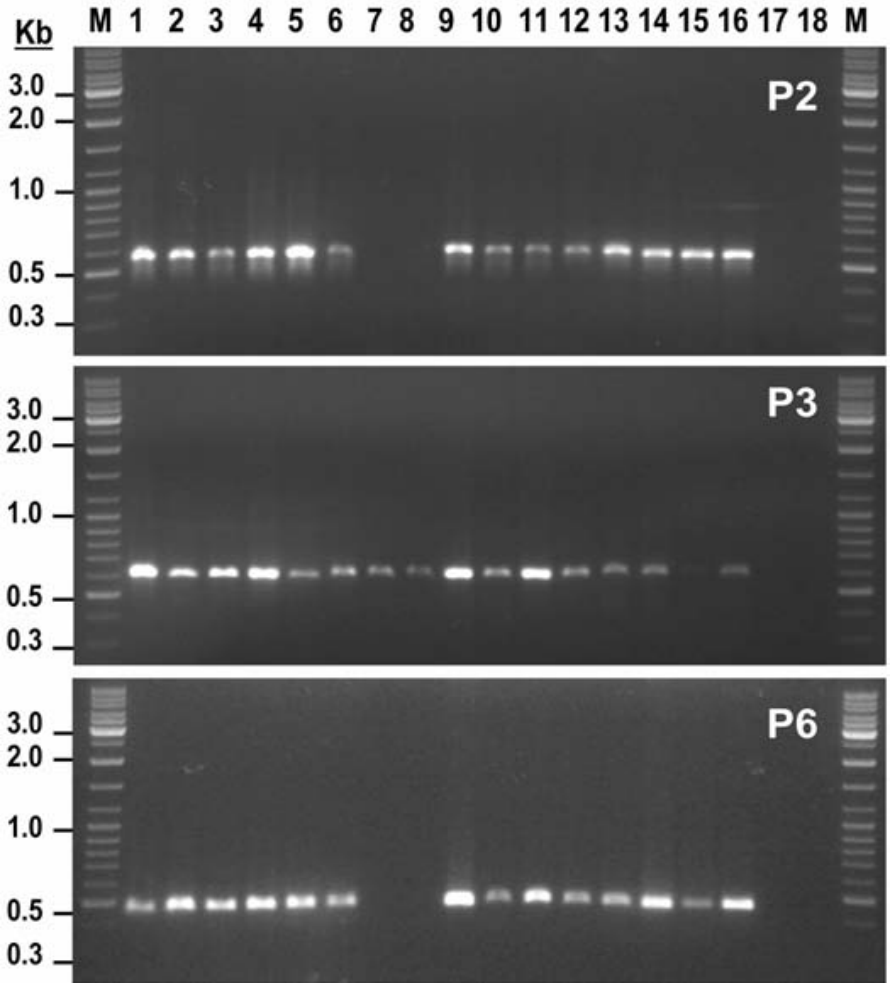

Fig. III.1.5. Detection of *Peronospora arborescens* in naturally infected opium poppy tissues with specific primers P3Pa2fw/P3Pa12rv (P2), OMPac1fw/ OMPa1rv (P3), and OMPac7fw/OMPac7rv (P6). M, GeneRuler DNA ladder mix (Fermentas, St Leon-Rot, Germany); lanes 1–2, *P. arborescens* sporangiophores and sporangia from sporulating opium poppy leaves (positive control); lanes 3–7, *P. arborescens*-infected leaves with different severity of symptoms (necrosis, pathogen sporulation, chlorosis, light chlorosis, and no symptoms, lanes 3 to 7, respectively); lanes 8–9, surface-sterilized, symptomatic stem (epidermal and cortex tissue, lanes 8 and 9, respectively); lane 10, peduncles of infected capsule; lanes 11–12, surface-sterilized root tissue from plants showing dwarfing and rosseting; lane 13–14, seeds from symptomatic capsules; lanes 15–16, capsule tissue (with light symptoms or pathogen sporulation, lanes 15 and 16, respectively); lane 17 healthy *Papaver somniferum* seeds; and lane 18, no DNA template.

Detection and transmission of *P. arborescens* in opium poppy seeds

PCR assays with primer sets P2, P3, or P6 were equally effective in the detection of *P. arborescens* in artificially infested seeds and allowed detection of up to 25 sporangia per 100 seeds with any of the primers (data not shown). Similarly, each of the three primer sets was effective in the detection of seedborne *P. arborescens* in seed stocks collected from infected capsules harvested from affected plants (Fig. III.1.5). Conversely, *P. arborescens* was not detected on seed stocks collected from asymptomatic capsules (data not shown).

Seed stocks collected from fields severely affected by downy mildew and used for field experiments resulted in positive amplification for *P. arborescens* in 75% of samples using the species-specific primers. Seedlings in the field microplots emerged 30 to 40 days after sowing. Typical symptoms of downy mildew (i.e., chlorotic foliar lesions on lower leaves that later became brown, angular spots) appeared on the opium poppy plants in microplots approximately 40 to 60 days after seedling emergence. All symptomatic leaves showed sporulation of the pathogen. In general, there was a low incidence of downy mildew for all seed stocks tested. No disease developed in microplots sown to control seeds. The disease occurred in 25 to 100% of microplots sown to an infected seed stock, with 25 to 50% of rows of plants within a microplot showing affected plants and only a few plants within these rows showing disease symptoms. Usually, affected plants in a microplot were aggregated (three to six plants showing symptoms clustered in a patch). Unusual warm and dry weather conditions occurred during the year of experiment, which caused symptomatic plants to become necrotic and die very early in the season (7 to 14 days after pathogen sporulation). These conditions were characterized by monthly average mean maximum and minimum temperatures from March through May that were 1.8 to 5.8°C higher than those occurring in regular years. Monthly average rainfall and mean relative humidity from January through May were 7 to 64 mm and 2 to 17% lower than normal, respectively (26). The unusually warm and dry weather probably accounted for the low incidence of diseased plants and reduced pathogen spread within a microplot. The presence of *P. arborescens* on symptomatic plants was confirmed by the species-specific PCR assay (data not shown).

III.1.4 DISCUSSION

Phylogenetic analysis of the internal transcribed spacer (ITS) regions of the ribosomal DNA (rDNA) demonstrated that *P. arborescens* is the downy mildew pathogen of *Papaver somniferum* in Spain. We also documented that there is a low degree of genetic diversity within *P. arborescens* populations occurring in Spain. *P. arborescens* has been reported in different countries throughout the world, including Afghanistan, Algeria, Argentina, Australia, Austria, Azerbaijan, Belgium, Bulgaria, Canada, China, Egypt, Finland, Germany, Greece, India, Iran, Italy, Japan, Korea, Libya, Pakistan, Poland, Romania, South Africa, Sweden, Thailand, Turkey, United Kingdom, United States, and Uzbekistan. Comparatively, the geographic range of *P. cristata* appears to be more reduced, since this species has been reported only in Australia, Bulgaria, England, and Ireland (18,19). In the Iberian Peninsula, *P. arborescens* has been reported to be infecting *Papaver dubium*, *Papaver hybridum*, and *Papaver rhoeas* in Spain, and infecting wild *Papaver somniferum* and *Papaver rhoeas* in Portugal (24). Since *P. arborescens* and *P. cristata* overlap in morphological characters (33,36) and host ranges, and most of the reports referred to above were based on symptoms on the hosts and morphology of the pathogen only, it is possible that some of the descriptions attributed to one of the two species in a country might in fact correspond to the other. In the same way, the possibility that *P. cristata* might be present in Spain on hosts where it has been reported from other countries, such as *Meconopsis cambrica*, wild poppies *Papaver argemone*, *Papaver hybridum*, and *Papaver rhoeas*, wild *Papaver somniferum*, or other *Papaver* spp. (8), should not be ignored since our study focused on cultivated *Papaver somniferum* only. In fact, this was the scenario that occurred in Australia where downy mildew of *Papaver nudicaule*, *Papaver hybridum*, and *Papaver dubium* in different states of the country and downy mildew of commercial opium poppy in Tasmania were initially attributed to *P. arbores-cens* (15,37). However, a detailed phylogenetic analysis of the pathogen population demonstrated that *P. cristata* rather that *P. arborescens* was the causal agent of *Papaver somniferum* downy mildew in Tasmania (36). Since the Tasmanian poppy industry was founded from England (36), it would be noteworthy to determine whether *P. cristata* and/or *P. arborescens* are the causal agents of downy mildew of cultivated *Papaver somniferum* in the United Kingdom. Future studies are worth conducting to determine if *P. cristata* and *P. arborescens* differ in virulence on *Papaver somniferum*, host specialization on *Papaver* spp. or *Meconopsis* spp.,

and worldwide geographic distribution.

The phylogenetic analysis also revealed that *P. cristata* is more distantly related to a clade that comprises *P. arborescens* infecting *Papaver somniferum* and *Papaver rhoeas* than to other *Peronospora* spp., whose hosts are outside the Papaveraceae. Interestingly, all *Peronospora* spp. infecting species in the order Ranunculales grouped together in the same clade (with the exception of *P. arborescens*). This clade was more closely related to a clade containing *Peronospora* spp. infecting species within the Caryophyllales than to all *P. arborescens* collections, which may indicate host jumping between distantly related host families (43). Similar results were obtained by Scott et al. (36) for *P. arbore-cens* and *P. cristata* with a data set for phylogenetic analysis smaller than that used in the current study.

In addition to use for phylogenetic studies, ITS rDNA sequences have been proved useful in the development of species-specific PCR protocols for in planta detection and identification of plant pathogenic oomycetes (1,7,9,36,38). We employed a similar method to develop *P. arborescens*-specific primers and developed a PCR-based protocol that was employed to answer several lingering questions in this pathosystem. First, we were able to efficiently differentiate *P. arborescens* from *P. cristata* and demonstrate that only *P. arborescens* occurs in cultivated *Papaver somniferum* in Spain. Second, the assay was used to detect *P. arborescens* in symptomatic and asymptomatic opium poppy plant parts and, third, we documented that *P. arborescens* can be seed-borne in seeds from infected opium poppy plants. Because of the obligate biotrophy of Peronosporales, availability of molecular protocols for their in planta and seed detection and identification is particularly important for the implementation of health certification schemes. Such a diagnostic technology should be both sensitive enough to detect small amounts of pathogen before evident symptom expression on the host, as well as specific to avoid cross-reaction problems with other related pathogens. The spe-cies-specific PCR protocol developed in this study satisfies those desirable characteristics because it shows: (i) considerable flexibility, since it can be applied to different plant samples including capsules, leaves, roots, seeds, and stems; (ii) high sensitivity, since the pathogen can be detected in symptomless plant tissues and seed stocks where the pathogen may be present at very low concentrations, i.e., as little as 0.1 to 10 pg of *P. arborescens* DNA (depending upon the primer set) against a background of 10 to 20 ng of opium poppy DNA; and more important (iii) high

specificity, since no cross amplification occurred with other closely related pathogens, especially with *P. cristata*-infected opium poppy tissues.

In similar studies, it was usually necessary to develop a nested-PCR protocol to increase flexibility and sensitivity in the amplification of pathogen DNA from infected plant samples (38) or concentrations of template pathogen DNA similar to that amplified with the single-PCR protocol developed in our work (7,38). Also, in contrast to other studies (e.g., 38) the presence of plant DNA did not reduce the sensitivity of the PCR protocol. Interestingly, the detection limit of the single round-PCR assay that we developed is rather similar to that reported for quantitative real-time PCR assays developed for quantifying *Plasmopara viticola* on leaves of *Vitis vinifera* (42), different *Phytophthora* spp. on decaying leaves (35), and *Alternaria brassicae* in cruciferous seeds (22). In those latter studies, the standard deviations of replicates increased greatly at a concentration of pathogen DNA <1 pg or when host DNA was present in the reaction mix, thus reducing the accuracy of the quantitative technique.

A final advantage of the three primer sets designed in this study concerns their high specificity for *P. arborescens*. In our study, we aimed for an identification and detection protocol that would avoid cross amplification of *P. cristata* DNA because we found that the *P. cristata*-specific primers designed by Scott et al. (36), pdm3/pdm4, yielded cross reaction when used for PCR assays of some *P. arborescens* samples from Spain. These authors had to base the specificity of the pdm3/pdm4 primer pair only on sequence comparison and BLAST searches on GenBank database, since apparently they could not test plant samples infected with *P. arborescens*. In our study, the comparison of pdm3/pdm4 sequences with all the *P. arborescens* sequences that we used identified only one or two single nucleotide difference for both primers, which may account for the cross amplification of *P. arborescens* DNA. While designing our primer sets, we ensured specificity by including one nucleotide that was a deletion in the ITS sequence of *P. cristata* and/or 100% homology in its sequence with *P. arborescens* in at least one of the primers of each set. This theoretical specificity was experimentally demonstrated with DNA extracted from samples of *P. cristata*-infected leaves from Australia and the United Kingdom.

Although the *P. arborescens*-specific primer sets and PCR protocol developed in this study was not aimed at quantitative assays, we believe that it may be of great use in opium poppy production to avoid the introduction of *P. arborescens*-contaminated

seeds in areas free from the pathogen and when introduc- ing seeds of germplasm for plant breeding purposes. Also, this protocol can be of use for epidemiological studies of the disease, helping to demonstrate the occurrence of asymptomatic, systemic infections, or to choose appropriate timing of fungicide treatments for chemical control of the disease before symptom expression.

Downy mildew of opium poppy is one of the most destructive diseases of this crop worldwide (27,36,37). Consequently, efforts should be made to avoid dispersal of the pathogen to opium-poppy-growing areas free from *P. arborescens*. In Spain, the rapid spread of the opium poppy downy mildew to areas where opium poppy had not been cropped before, together with the progressive increase in the incidence and severity of the disease during the last few years, suggested seedborne transmission of the pathogen. Results from this study confirm the potential of this hypothesis. Thus, by using the species-specific PCR assay developed, we demonstrated that *P. arborescens* was seedborne on commercial seed stocks, and further, that seedborne inoculum can give rise to infected plants under field conditions. To our knowledge, this is the first demonstration that *P. arborescens* can be seed transmitted. Further research is needed to determine location of the pathogen in seed tissues as well as factors that determine efficacy of transmission. Thin- and thick-walled mycelia and/or oospores of different *Peronospora* spp. (i.e., *P. ducomati*, *P. effusa*, *P. farinosa*, *P. manshurica*, and *P. viciae*) have been detected on the seed surface, as well as in infected seed coat (pericarp and spermoderm layer), persistent calyx, and pods of their respective hosts (25,39,46). In our study, seedborne transmission of *P. arborescens* occurred with rather low frequency, perhaps because of the unusual warm and dry weather conditions that took place during the year of the field experiment. The day/night temperatures and relative humidity that occurred at the site during the field experiment have been reported unfavorable for sporulation, germination of sporangia, appresorium formation, and infection for other *Peronospora* spp. (14,20). Nevertheless, the efficacy of seed transmission might also be low in nature, as shown for *P. lactucae*, with a level of infection that reached 0.3 to 2.9% in commercial seed stocks of *Lactuca sativa* (25). In contrast, sporangia of *Peronospora* spp. from infected sporulated leaves can be readily dispersed by wind and rain splashing, and consequently, infected leaves could be an important source of inoculum (14,20). In our field study, we are confident that the possibility of external aerial inoculum giving rise to plant infection did not occur, since

the field microplots of the study were: (i) located 60 to 120 km apart from the closest cultivated opium poppy fields; (ii) surrounded by fields within the research station that were kept free of weeds (including *Papaver* spp.) by conventional tillage or herbicide treatment; and (iii) that the disease did not show up in the control plots planted close to plots planted with infected seeds.

An additional important finding of this study was the detection of putative systemic infection of opium poppy by *P. arborescens*, since the pathogen DNA was amplified from roots of plants showing dwarfing and rosetting in the field with no other evident symptom of downy mildew. The systemic infection of these plants may have taken place from infected seeds or by soilborne oospores of the pathogen during seed germination and/or seedling growth. Systemic infection of buckwheat and soybean seedlings from infected seeds and oospores has been demonstrated for *P. ducomati* and *P. manshurica*, respectively (39,46). Systemic infections of opium poppy by *P. arborescens* may be of significance in the epidemiology of downy mildew if, as reported for sunflower downy mildew caused by *Plasmopara halstedii* (10), asymptomatic systemic infections give rise to viable, infected seeds.

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III.1.7 LITERATURE CITED

1. Aegerter, B. J., Nuñez, J. J., and Davis, R. M. 2002. Detection and management of downy mildew in rose rootstock. Plant Dis. 86:1363-1368.
2. Alcock, N. L. 1933. Downy mildew of *Meconopsis*. New Flora Silva 5:279-282.
3. Altschul, S. F., Madden, T.L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res. 25:3389-3402.
4. Behr, L. 1956. Der falsche mehltau am mohn (*Peronospora arborescens* (Berk.) de Bary). Untersuchungen zur biologie des erregers. J. Phytopathol. 27:289-334.
5. Belbahri, L., Calmin, G., Pawlowski, J., and Lefort, F. 2005. Phylogenetic analysis and real time PCR detection of a presumbably undescribed *Peronospora* species on sweet basil and sage. Mycol. Res. 109:1276-1287.
6. Berkeley, M. J. 1846. Observations, botanical and physiological on the potato murrain. J. Royal Hortic. Soc. 1:9-34.
7. Bonants, P., De Weerdt, M. H., Van Gent Pelzer, M., Lacourt, I., Cooke, D., and Duncan, J. 1997. Detection and identification of *Phytophthora fragariae* by the polymerase chain reaction. Eur. J. Plant Pathol. 103:345-355.
8. Castroviejo, S., Lainz, M., López, G., Monserrat, P., Muñoz-Garmendia, F., Paiva, J., and Villar, L. 1986. Flora Ibérica. Plantas vasculares de la Península Ibérica e Islas Baleares. Vol. I. Real Jardín Botánico, CSIC, Madrid, Spain.
9. Choi, Y.-J., Hong, S.-B., and Shin, H.-D. 2003. Diversity of the *Hyaloperonospora parasitica* complex from core brassicaceous hosts based on ITS rDNA sequences. Mycol. Res. 107:1314-1322.
10. Cohen, Y., and Sackston, W. E. 1974. Seed infection and latent infection of sunflowers by *Plasmopara halstedii*. Can. J. Bot. 52:231-238.
11. Constantinescu, O. 1991. An annotated list of Peronospora names. Thunbergia 15:1-110.
12. Constantinescu, O., and Fatehi, J. 2002. Peronospora-like fungi (Chromista, Peronosporales) parasitic on Brassicaceae and related hosts. Nova Hedwigia 74:291-338.
13. Cooke, D. E. L., Drenth, A., Duncan, J. M., Wagels, G., and Brasier, C. M. 2000. A molecular phylogeny of *Phytophthora* and related oomycetes. Fungal Genet. Biol. 30:17-32.

14. Correll, J. C., Morelock, T. E., Black, M. C., Koike, S. T., Brandenberger, L. P., and Dainello, F. J. 1994. Economically important diseases of spinach. Plant Dis. 78:653-660. 15.
15. Cotterill, P. J., and Pascoe, I. G. 1998. Downy mildew of *Papaver somniferum* in Tasmania. Australas. Plant Pathol. 27:263-264.
16. Cotton, A. D. 1929. A fungus disease of *Meconopsis*. Gard. Chron. 85:143-144.
17. Dick, M. W. 2002. Binomials in the Peronosporales, Sclerosporales and Pythiales. Pages 225-266 in: Advances in Downy Mildew Research. P. T. N. Spencer, U. Gisi, and A. Lebeda, eds. Kluwer Academic Publishers, Dordrecht, the Netherlands.
18. Farr, D. F., Rossman, A. Y., Palm, M. E., and McCray, E. B. Fungal Databases, Systematic Botany and Mycology Laboratory. Online publication. ARS, USDA, 2006.
19. Francis, S. M. 1981. *Peronospora arborescens*. No. 686 in: Descriptions of Pathogenic Fungi and Bacteria. CMI, Kew, Surrey, UK,
20. Friedrich, S., Leinhos, G. M. E., and Löpmeier, F.-J. 2003. Development of ZWIPERO, a model forecasting sporulation and infection periods of onion downy mildew based on meteorological data. Eur. J. Plant Pathol. 109:35-45.
21. Gaumann, E. 1923. Beitrage zu einer monograph der gattung *Peronospora* Corda. Beitr. Kryptogamenflora Schweiz 5:1-360.
22. Guillemette, T., Iacomi-Vasilescu, B., and Simoneau, P. 2004. Conventional and real-time PCR-based assay for detecting pathogenic *Alternaria brassicae* in cruciferous seed. Plant Dis. 88:490-496.
23. Gustavsson, A. 1959. Studies on nordic Peronosporas I. Taxonomic revision. Opera Bot. 3 (Suppl.):1-270.
24. Gustavsson, A. 1991. The genus *Peronospora* in the Iberian peninsula, especially in northern Spain and Andorra. An. Jard. Bot. Madr. 49:3-38.
25. Inava, T., Takahashi, K., and Morinaka, T. 1983. Seed transmission of spinach downy mildew. Plant Dis. 67:1139-1141.
26. Instituto Nacional de Metereología. 2006. Ministerio de Medio Ambiente. Valores Climatológicos normales. Online publication.
27. Kapoor, L. D. 1995. Opium Poppy: Botany, Chemistry and Pharmacology. Food Products Press, New York.

28. Landa, B. B., Montes-Borrego, M., Muñoz-Ledesma, F. J., and Jiménez-Díaz, R. M. 2005. First report of downy mildew of opium poppy caused by *Peronospora arborescens* in Spain. Plant Dis. 89:338.
29. Maiti, S., and Chattopadhyay, S. B. 1986. Studies on diseases of Indian medicinal plants. Indian J. Mycol. Res. 24:21-27.
30. Muñoz-Ledesma, F. J. 2002. Etiología y control de la necrosis de la adormidera *Papaver somniferum* L. Ph.D. thesis. University of Córdoba, Córdoba, Spain.
31. Muskett, A., and Malone, J. 1984. Catalogue of Irish fungi-V. Mastigomycotina and Zygomycotina. Proc. R. Ir. Acad. Sect. B. 84:83-102.
32. Quesada-Moraga, E., Landa, B. B., Muñoz-Ledesma, F. J., Jiménez-Díaz, R. M., and Santiago-Álvarez, C. 2006. Endophytic colonisation of opium poppy, *Papaver somniferum*, by an entomopathogenic *Beauveria bassiana* strain. Mycopathologia 161:323-329.
33. Reid, D. A. 1969. New or interesting British plant diseases. Trans. Br. Mycol. Soc. 52:19-38.
34. Savulescu, T. 1948. Les especes de *Peronospora* Corda de Roumanie. Sydowia 2:255-307.
35. Schena, L., Hughes, K. J. D., and Cooke, D. E. L. 2006. Detection and quantification of *Phytophthora ramorum*, *P. kernoviae*, *P. citricola* and *P. quercina* in symptomatic leaves by multiplex real-time PCR. Mol. Plant Pathol. 7:365-379.
36. Scott, J. B., Hay, F. S., and Wilson, C. R. 2004. Phylogenetic analysis of the downy mildew pathogen of oilseed poppy in Tasmania, and its detection by PCR. Mycol. Res. 108:198-295.
37. Scott, J. B., Hay, F. S., Wilson, C. R., Cotterill, P. J., and Fist, A. J. 2003. Spatiotemporal analysis of epiphytotics of downy mildew of oilseed poppy in Tasmania, Australia. Phytopathology 93:752-757.
38. Silvar, C., Duncan, J. M., Cooke, D. E. L., Williams, N. A., Díaz, J., and Merino, F. 2005. Development of specific PCR primers for identification and detection of *Phytophthora capsici* Leon. Eur. J. Plant Pathol. 112:43-52.
39. Singh, D., and Mathur, S. B. 2004. Location of fungal hyphae in seeds. Pages 101-168 in: Histopathology of Seed-Borne Infections. CRC Press, Boca Raton, FL.

40. Sukno, S. A., Taylor, A. M., and Farmar, M. 2002. Development of contamination-free restriction fragment length polymorphism probes for the obligate biotroph *Peronospora tabacina*, an oomycete causing blue mold of tobacco. Phytopathology 92:1227-1235.
41. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. 1997.The ClustalX windows interface: Fexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24:4876-4882.
42. Valsesia, G., Gobbin, D., Patocchi, A., Vecchione, A., Pertot, I., and Gessler, C. 2005. Development of a high-throughput method for quantification of *Plasmopara viticola* DNA in grapevine leaves by means of quantitative real-time polymerase chain reaction. Phytopathology 95:672-678.
43. Voglmayr, H. 2003. Phylogenetic relationships of *Peronospora* and related genera based on nuclear ribosomal ITS sequences. Mycol. Res. 107:1132-1142.
44. White, T. J., Bruns, T., Lee S., and Taylor, J. W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: PCR Protocols: A Guide to Methods and Applications. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. T. White, eds. Academic Press Inc., New York.
45. Yossifovitch, M. 1929. *Peronospora arborescens* (Berk.) de Bary, parasite tres important de *Papaver somniferum* en Yougoslavie. Rev. Pathol. Entomol. Agric. 16:235-270.
46. Zimmer, R. C., McKeen, W. E., and Campbell, C. G. 1992. Location of oospores in buckwheat seed and probable roles of oospores and conidia of *Peronospora ducometti* in the disease cycle on buckwheat. J. Phytopathol. 46:183-186.

III.2

A nested-PCR protocol for detection and population biology studies of *Peronospora arborescens*, the downy mildew pathogen of opium poppy, using herbarium specimens and asymptomatic, fresh plant tissues

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III.2.1 ABSTRACT

A sensitive nested-PCR protocol was developed using either of two primer pairs that improves the in-planta detection of *Peronospora arborescens* DNA. The new protocol represented an increase in sensitivity of 100- to 1,000-fold of detection of the oomycete in opium poppy tissue compared with the detection limit of single PCR using the same primer pairs. The new protocol allowed amplification of 5 to 0.5 fg of *P. arborescens* DNA mixed with *Papaver somniferum* DNA. The protocol proved useful for amplifying *P. arborescens* DNA from 96-year-old herbarium specimens of *Papaver* spp. and to demonstrate that asymptomatic, systemic infections by *P. arborescens* can occur in wild *Papaver* spp. as well as in cultivated opium poppy. Also, the increase in sensitivity of the protocol made possible the detection of seedborne *P. arborescens* in commercial opium poppy seed stocks in Spain with a high frequency, which poses a threat for pathogen spread. Direct sequencing of purified amplicons allowed alignment of a *P. arborescens* Internal Transcribed Spacer (ITS) rDNA sequence up to 730-bp long when combining the sequences obtained with the two primer sets. Maximum parsimony analysis of amplified *P. arborescens* ITS rDNA sequences from specimens of *Pap. dubium, Pap. hybridum, Pap. rhoeas*, and *Pap. somniferum* from different countries indicated for the first time that a degree of host specificity may exist within populations of *P. arborescens*. The reported protocol will be useful for epidemiological and biogeographical studies of downy mildew diseases as well as to unravel misclassification of *P. arborescens* and *P. cristata,* the reported causal agents of the opium poppy downy mildew disease.

Additional Keywords: Ancient DNA, biogeography, PCR sensitivity, seed detection, ribosomal DNA.

III.2.2 INTRODUCTION

Opium poppy (*Papaver somniferum*) is the only source of morphine, codeine and thebaine alkaloids for the pharmaceutical industry. Opium poppy production is curtailed by downy mildew, one of the most destructive diseases of the crop worldwide (11,23,25,36,43). Two *Peronospora* species*, P. arborescens* and *P. cristata*, are causal agents of the opium poppy downy mildew disease. They overlap in morphological characters, such as sporangia dimensions, and host range (25,36). This makes it difficult to differentiate between the two pathogens and this may have lead to their misclassification in the past.

Historically, herbarium specimens have been an important source for confirmation or re-description of misidentified plant pathogens. Herbarium specimens have also played a paramount role for better understanding plant disease epidemics, especially the biogeography, origin, and dispersal of plant pathogens (e.g., 26,33,34). Use of current molecular techniques for the study of herbarium specimens has provided accurate diagnostic tools as an alternative to conventional methods for the identification of plants and their pathogens. For example, use of herbarium specimens from four continents allowed the construction of phylogenetic trees of the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) within *Peronospora* accessions that solved the taxonomic status of the spinach downy mildew and lead to reinstatement of *Peronospora effusa* as the pathogen on spinach (*Spinacea oleracea*) (7).

Some downy mildew pathogens have the ability to cause asymptomatic, systemic infections on a wide range of herbaceous crops (e.g., 13,44), and some evidence was provided that this phenomenon also occurs in the *P. arborescens*/opium poppy pathosystem (25). Systemic infections by *P. arborescens* may be of significance in the epidemiology of downy mildew if asymptomatic systemic infections in commercial opium poppy fields give rise to viable infected seeds, or if infected wild poppy plants eventually give rise to conidia on leaves that serve as inoculum for opium poppy fields.

In Spain, the rapid spread of the opium poppy downy mildew to areas where opium poppy had not been cropped before, together with the progressive increase in the incidence and severity of the disease during the past 5 years, suggested seedborne transmission of the pathogen. Recent research (25) confirmed this hypothesis and demonstrated that: (i) *P. arborescens* can be seedborne on commercial opium poppy

seed stocks, and (ii) seedborne inoculum can give rise to infected plants under field conditions. For opium poppy downy mildew, a few primary infections by the pathogen can give rise to several secondary cycles of infection that result in rapid epidemic development of the pathogen within a field (36). Consequently, efforts should be made to avoid introduction of the pathogen to opium poppy growing areas or fields free from *P. arborescens*. The ability to detect low amounts of pathogen inoculum on commercial seed stocks is an important disease management tactic.

Species-specific PCR protocols have been developed that allow detection of *P. arborescens* with primer pairs OMPac1fw/OMPac1rv and OMPac7fw/OMPac7rv (Spanish patent application P200603319, international patent application PCT/ES2007/000781; 25), and *P. cristata* with primer pair pmd3/pmd4 (37), using total DNA extracted from symptomatic and/or the pathogen as it sporulats on tissues of opium poppy plants. However, use of the *P. arborescens*-detection protocol developed by Landa et al. (25) was not sensitive enough for pathogen detection in herbarium specimens or commercial seed stocks (B. B. Landa et al., *unpublished results*). The specific objectives of this study were: (i) to improve the sensitivity of the single-PCR protocol developed by Landa et al. (25) by designing a new nested-PCR protocol that allows amplification of *P. arborescens* DNA from old herbarium specimens, as well as from asymptomatic, infected plants of *Papaver* spp. And commercial opium poppy seed stocks; (ii) to determine the occurrence of asymptomatic systemic infections of *P. arborescens* in wild *Papaver* spp. and commercial opium poppy plants; and (iii) to study the genetic diversity within *P. arborescens* populations from different geographical origins and host plants.

III.2.3 MATERIALS AND METHODS

Plant materials and herbarium specimens

Six herbarium specimens were provided by 'Real Jardín Botánico', Spanish Council for Scientific Research (CSIC), Madrid, Spain (Table III.2.1). Some of the samples, especially ancient ones, showed no appearance of sporangia or oospores as determined by microscopic observations. Leaf samples from symptomatic and

asymptomatic *Papaver* spp. plants collected from opium poppy growing areas in Spain were also used for the study (Table III.2.1).

Table III.2.1. Host source, geographic origin, collection number and ITS rDNA GenBank accessions from *Peronospora arborescens* samples used in the study.

Host source	Sample tissue	Geographic origin	Collection number	Year	GenBank
Papaver dubium	Leaves, herbarium specimen	Puerto San Glorio, León, Spain[a]	27841(*)	1990	EU570198
Papaver dubium	Asymptomatic leaves, fresh tissue	Marchena, Sevilla, Spain	B30	2006	EU570202
Papaver hybridum	Asymptomatic leaves, fresh tissue	Antequera, Málaga, Spain	R74	2004	EU570208
Papaver rhoeas	Sporulated leaves, fresh tissue	Malpica de Tajo, Toledo, Spain	R1	2004	EU570203
Papaver rhoeas	Asymptomatic capsule, fresh tissue	Antequera, Málaga, Spain	R76	2004	EU570205
Papaver rhoeas	Asymptomatic leaves, fresh tissue	Antequera, Málaga, Spain	R75	2004	EU570204
Papaver rhoeas	Leaves, herbarium specimen	Algeria, Africa[a].	15749	1912	EU570196
Papaver rhoeas	Leaves, herbarium specimen	Puerto de Canales, Burgos, Spain[a]	27844 (*)	1990	EU570197
Papaver rhoeas	Leaves, herbarium specimen	Naroba, Cantabria, Spain[a]	27843 (*)	1990	EU570199
Papaver rhoeas	Leaves, herbarium specimen	Constanta, Hagieni, Romania[a]	9164	1977	EU570201
Papaver somniferum	Leaves, herbarium specimen	Lepola, Pornainen, Finland[a]	28507	1916	EU570200
Papaver somniferum	Asymptomatic stem, fresh tissue	Growth chamber experiments	D175	2007	EU570206
Papaver somniferum	Seeds from commercial seed stocks, fresh tissue	ALCALIBER S.A.	D120	2007	EU570207

[a] Samples provided by Herbarium Real Jardin Botanico, CSIC, Madrid (29). (*) Samples collected by Arne Gustavsson and referenced in Gustavsson (18).

To study the occurrence of asymptomatic systemic infections, 75 opium poppy plants grown under controlled conditions in a soil naturally infested by *P. arborescens* were sampled when plants had formed a capsule in the main stem. This soil was collected from a commercial opium poppy field plot with high incidence of downy mildew and was proven to contain oospores of the pathogen (M. Montes-Borrego, B. B. Landa, J. A. Navas, F. J. Muñoz-Ledesma and R. M. Jiménez-Díaz, *unpublished results*). For that purpose a 4 to 5-cm-long piece of the basal stem was cut from each plant with sterile scissors, washed with sterile distilled water and peeled off with a sterile scalpel. Sampled tissues were ground into 0.3-0.5 g pieces, placed in 1.5 ml Eppendorf tubes and frozen immediately in liquid nitrogen before DNA extraction.

Additionally, to validate the utility of the nested-PCR protocol for detection of the pathogen in opium poppy seeds, 18 commercial seed stocks, provided by ALCALIBER S.A. (Carmona, Sevilla, Spain) were analyzed to determine the presence of *P. arborescens* in seed. Seed stocks derived from commercial fields in the main opium-poppy growing areas of Andalucia and Castilla-La Mancha regions of Spain were obtained by ALCALIBER S.A following the conventional industrial processes. These seed stocks were collected by the company to be sown in commercial fields the

following year. Therefore, the fields from which the commercial seed stocks were collected were selected from among those where the downy mildew disease either was not apparent or disease symptoms were rated low (0 to 1 symptomatic plant per m^2; F. J. Muñoz-Ledesma, *personal communication*). In addition, seven seed stocks originating from opium poppy crops in France, for which there was no additional information, were used in the study to include opium poppy cultivars different from those cropped in Spain. Three samples from each seed stock were processed for DNA extraction as described below.

DNA extraction and quantification

Genomic DNA was extracted using the Spin Fast DNA kit (Qbiogene, Madrid, Spain) as described (25). Pieces of fresh leaf tissue (approximately 2 cm^2, 30 to 80 mg) from symptomatic and asymptomatic *Papaver* spp. plants (Table III.2.1) were placed in a 1.5-ml Fast DNA tube containing lysing matrix A, 800 µl of CLS-VF solution, and 200 µl of Protein Precipitation Solution (PPS). Seed samples (approximately 500 seeds, 150-200 mg), stem samples (approximately 50 to 100 mg), and pieces of dry herbarium specimens (approximately 2 cm^2, 3 to 8 mg) (Table III.2.1) were processed as described above but allowed to soften in the buffer for 1 h before processing. Cells were mechanically disrupted in a Fast Prep System Bio 101 (Qbiogene) by reciprocal shaking of samples for 30 s at 5.5 speed. Subsequently, the supernatant was collected by centrifugation (10 min at 12000 x *g*) and processed with the Fast DNA kit according to the manufacturer's instructions.

Extracted DNA was quantified using the Quant-iT DNA Assay Kit Broad Range fluorometric assay (Molecular Probes Inc., Leiden, The Netherlands) and a Tecan Safire fluorospectrometer (Tecan Spain, Barcelona, Spain) according to manufacturer's instructions, and diluted to 20 ng/µl with ultrapure sterile water (USW).

Nested specific-PCR protocol

Primer DC6, specific for Pythiales and Peronosporales oomycete species (3), and the universal ITS4 primer (42), were used for the first round of amplifications in

the nested-PCR protocol using 1 µl of DNA from tissues of either symptomatic or asymptomatic poppy plants in 25-µl reaction volumes as described (25). Purified *P. arborescens* DNA (1 ng) and USW were used as positive and negative controls, respectively. The amplified product was diluted 1:1, 1:5, 1:10, 1:50 and 1:100 with USW to optimize sensitivity and decrease background effects of the first PCR on the second PCR and subsequent direct sequencing quality. One µl of this diluted PCR product was used for the second round of amplification in the nested-PCR protocol using primer pairs OMPac1fw (5'-CCACACCTAAAAACTTTCC-3')/OMPac1rv (5'-AACACTCCTCCATTATCG-3') (set P3) or OMPac7fw (5'-GAACGCATATTGCACTTCC-3')/OMPac7rv (5'-CGCACAACACAAATTTCC-3') (set P6) and PCR conditions described by Landa et al. (25).

All reactions (both first round- and second round-PCR) were repeated at least twice and always included a positive control (*P. arborescens* DNA obtained from sporangiophores with sporangia scraped from sporulating leaves) and negative controls including (i) four *Pap. somniferum* DNA samples from sterile disinfested seeds (2 samples) and from seedlings grown under sterile conditions (2 samples) that consistently tested negative in previous experiments, and (ii) four reactions with no DNA (water blanks). None of the negative controls yielded the PCR product, indicating that positive assays were genuine and were not due to cross-contaminations with templates. Additionally, to ensure the validity of the nested-PCR results, all amplicons from herbarium specimens and *Papaver* spp. samples, and 25% of amplicons from plant and seed stock samples were sequenced (see below).

Amplification products were separated by electrophoresis in 1.5% agarose gels in 1× TAE buffer for 60-120 min at 80 V, stained with ethidium bromide, and visualized under UV light. The Gene-ruler™ DNA ladder mix (Fermentas, St Leon-Rot, Germany) was used for electrophoresis.

Sensitivity of the nested-PCR protocol

Sensitivity of the nested-PCR protocol was assessed and compared with the single PCR assay described by Landa et al. (25). *P. arborescens* DNA was diluted in USW as well as in *Pap. somniferum* DNA extracted from healthy opium poppy leaves (25 or 50 ng/µl) to reach DNA concentrations ranging from 50 pg to 0.005 fg (Table

III.2.2). Special care was taken to get accurate pathogen and host DNA concentrations by fluorimetric quantification using the Quant-iT DNA Assay Kit as described above in triplicate and in two independent plates. All PCR reactions were repeated twice by independent operators using the conditions above and always included *Pap. somniferum* DNA or no DNA as negative controls.

DNA sequencing and phylogenetic analysis

Products of the second-round PCR were purified using a Geneclean turbo kit (Q-BIOgene, Illkirch, France), quantified fluorometrically, and used for direct DNA sequencing with the same primers used in each second-round amplification. DNA sequencing was done on a multicapilar sequencer (ABI PRISM 3100 genetic analyzer, Applied Biosystems) at the University of Córdoba sequencing facilities. All amplifications and DNA sequencing were performed twice to assess reproducibility of chromatograms. All ITS1-5.8S-ITS2 sequences obtained from *P. arborescens* collections in the study were deposited in GenBank with Accession numbers recorded in Table III.2.1. Sequences generated in this study were aligned with the *Peronospora* spp. data set described by Landa et al. (25). A maximum parsimony (MP) analysis was performed (Bionumerics 5.1 software; Applied Maths, Kortrijk, Belgium) using either all *Peronospora* spp. sequences or only *P. arboresces* sequences. The phylograms were bootstrapped 1,000 times to assess the degree of support for the phylogenetic branching indicated by the optimal trees.

III.2.4 RESULTS

Nested-PCR protocol

Best amplification results of the nested-PCR protocol were achieved using a 1:10 dilution of the first PCR product as template for the second round (Fig. III.2.1A). The 1:10 dilution allowed similar levels of sensitivity as compared to using 1:1 and 1:5 dilutions, with the benefit that background effects were lower and the chromatogram signal after direct sequencing was clean; thus avoiding an intermediate need for cloning the fragment (see below).

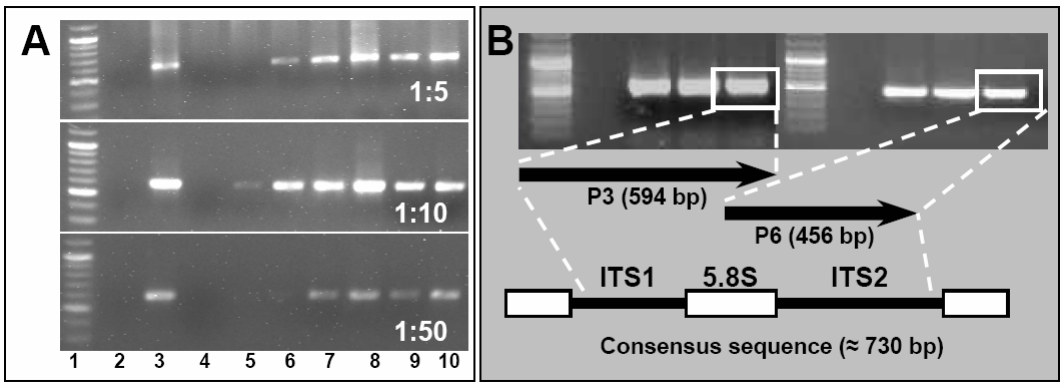

Fig. III.2.1. (A) Gel electrophoresis of DNA amplicons from nested PCR assays using primers DC6/ITS4 in the first round and primers OMPac1fw/OMPac1rv in the second round. Dilution of products of the first-round of amplification was 1:5 (upper gel), 1:10 (middle gel) and 1:50 (lower gel). Lanes: 1, Gene-ruler ™ DNA ladder mix (Fermentas, St Leon-Rot, Germany); 2, negative control (water); 3, positive control (*P. arborescens*-infected leaves of *Papaver somniferum*); 4 negative control (pathogen-free *Papaver somniferum* leaves); 5, asymptomatic leaves from *Pap. hybridum*; 6-10, herbarium specimens 27841, 15749, 27844, 27843, and 28507 from Table III.2.1. (B) Diagrammatic representation of products size (bp) of the ribosomal DNA region amplified with primers OMPac1fw/OMPac1rv (set P3) and OMPac7fw/OMPac7rv (set P6) and the approximate size of the sequence obtained after direct sequencing and alignment of amplified products. Figures are not to scale.

The PCR detection limit for single-PCR assays was similar to that reported previously [1 to 10 pg of *P. arborescens* DNA for primer pairs OMPac1fw/OMPac1rv (set P3) or OMPac7fw/OMPac7rv (set P6)] (25). Conversely, the detection limit for nested-PCR was 0.5 and 0.05 fg of *P. arborescens* DNA using either primer set P3 or P6, respectively. This represented an increase in sensitivity of 100- to 1,000-fold for primer sets P3 and P6, respectively compared with the detection limit of single PCR using the same primer pairs (Table III.2.3; Fig. III.2.2). In general, the detection limit was similar when the pathogen DNA was diluted with ultrapure, sterile water or plant DNA (25 ng or 50 ng of host DNA). However, there was a trend to higher sensitivity when host DNA was present in the reaction (Fig. III.2.2). Use of the detection protocol by different operators did no influence reproducibility and consistency of results (Table III.2.2).

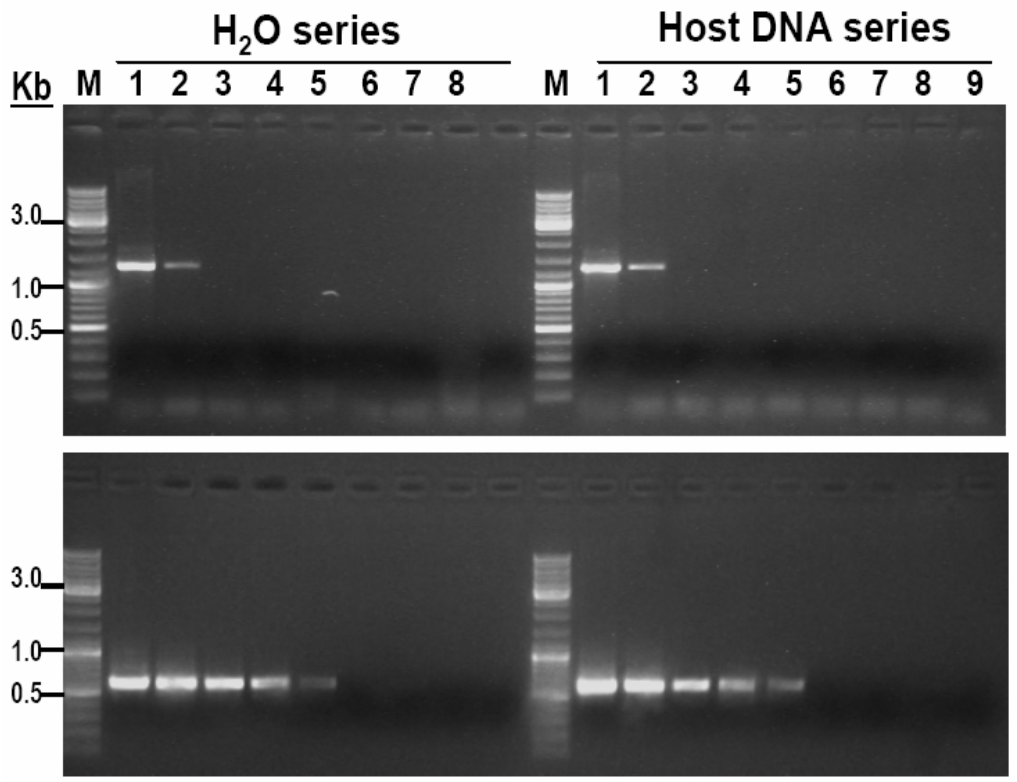

Fig. III.2.2. Sensitivity of *Peronospora arborescens*-specific primers OMPac7fw/ OMPac7rv (set P6) in single- (upper panel) and nested-PCR (lower panel) assays using *P. arborescens* DNA (50 pg/µl) diluted in water (H_2O Series) or in DNA of *Papaver somniferum* (50 ng/µl) extracted from leaves (Host DNA Series). M, Generuler ™ DNA ladder mix (Fermentas, St Leon-Rot, Germany); lanes 1-7, DNA dilution series 1:10 (lane 1), 1: 10^2 (lane 2), 1: 10^3 (lane 3), 1:10^4 (lane 4), 1:10^5 (lane 5), 1:10^6 (lane 6), and 1:10^7 (lane 7); lane 8, no DNA template; lane 9, pathogen-free, disinfested leaves of *P. somniferum*.

Table III.2.2. Sensitivity of *Peronospora arborescens*-specific primers used in single-and nested-PCR protocols and of Oomycete universal primers using purified DNA of *P. arborescens* singly or mixed with host DNA.

		DNA	DNA of *Peronospora arborescens* (fg/µl)[b]								Negative	H_2O
Primer pair[a]	DS[b]	ng/ul[b]	50,000	5,000	500	50	5	0.5	0.05	0.005	Control	Control
DC6/ITS4	I	0	+/+[c]	-/	-/-	-/-	-/-	-/-	-/-	-/-		-/-
		25	+/+	-/	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	II	0	+/+	±/-	-/-	-/-	-/-	-/-	-/-	-/-		-/-
		50	+/+	±/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
OMPac1fw/OMPa1rv (P3)	I	0	+/+	+/+	±/-	-/-	-/-	-/-	-/-	-/-		-/-
		25	+/+	+/+	+/±	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	II	0	+/+	+/+	±/-	-/-	-/-	-/-	-/-	-/-		-/-
		50	+/+	+/+	±/±	-/-	-/-	-/-	-/-	-/-	-/-	-/-
OMPac7fw/OMPac7rv (P6)	I	0	+/+	+/+	+/+	-/-	-/-	-/-	-/-	-/-		-/-
		25	+/+	+/+	+/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	II	0	+/+	+/+	+/+	-/-	-/-	-/-	-/-	-/-		-/-
		50	+/+	+/+	+/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-
DC6/ITS4 (1st PCR)	I	0	+/+	+/+	+/+	+/+	±/-	-/-	-/-	-/-		-/-
+		25	+/+	+/+	+/+	+/+	+/+	-/-	-/-	-/-	-/-	-/-
P3 (2nd PCR)	II	0	+/	+/	+/	+/	+/+	-/-	-/-	-/-		-/-
		50	+/	+/	+/	+/	+/+	-/-	-/-	-/-	-/-	-/-
DC6/ITS4 (1st PCR)	I	0	+/+	+/+	+/+	+/+	+/±	+/±	-/-	-/-		-/-
+		25	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-	-/-	-/-
P6 (2nd PCR)	II	0	+/+	+/+	+/+	+/+	±/+	±/-	-/-	-/-		-/-
		50	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-	-/-	-/-

[a] Primers OMPac1fw/OMPa1rv (set P3) and OMPac7fw/OMPac7rv (set P6) are protected under Spanish patent application P200603319.

[b] *P. arborescens* DNA (10 ng/µl) was serially diluted to obtain 50 pg to 0.005 fg of pathogen DNA per microliter of PCR reaction in sterile distilled water (0 ng background DNA) or in *Pap. somniferum* DNA (25 ng per µl, Dilution series (DS) I, or 50 ng per µl background DNA, DS II) to investigate any possible influence of host DNA on amplification of pathogen DNA.

[c] Results were obtained by two independent operators with the same dilution series. + = Positive amplification; ± = Positive weak amplification; - = no amplification.

Occurrence of asymptomatic infections by *Peronospora arborescens*

Use of the newly developed nested-PCR protocol allowed detection of *P. arborescens* DNA from *Papaver* spp. specimens stored in herbarium from 18 up to 96 years (e.g., *Pap. rhoeas* sampled from Africa in 1912) (Fig. III.2.1A). Also, positive detection of *P. arborescens* was achieved with leaves and capsules of asymptomatic *Pap. dubium, Pap. hybridum* (Fig. III.2.1A), *Pap. rhoeas* and *Pap. somniferum* plants (Table III.2.1) grown in commercial opium poppy fields in Andalucia and Castilla-La Mancha regions of Spain (M. Montes Borrego et al., *unpublished results*). This indicated the

potential occurrence of asymptomatic systemic infections. No amplification or weak amplification signals were obtained when those samples were assayed by single PCR using primer sets P3 and P6 (*data not shown*).

The nested-PCR protocol was more efficacious and sensitive in the detection of asymptomatic systemic infections compared with the single-PCR protocol. Thus, use of the nested PCR protocol determined that 22 out of 75 plants (22.3%) grown under controlled conditions in a soil naturally infested with *P. arborescens* oospores were infected by the pathogen, whereas the single PCR protocol with the same plants indicated an incidence of only 6 out of 75 (8%). In general, primer set P6 was more effective than P3 in detecting infection by the pathogen when used either in single or nested-PCR assays (*data not shown*).

Occurrence of *Peronospora arborescens* in commercial seed stocks

A high percentage of commercial opium poppy seed stocks were infected by the pathogen. This was especially evident for seeds harvested from crops in the Castilla-La Mancha region, for which all seed stocks showed positive amplification with primer sets P3 and P6 even when they were used in the single-PCR assay. Primer set P6 was more effective than primer set P3 in the detection of seedborne *P. arborescens* in commercial seed stocks used either in single- or nested-PCR assays (Table III.2.3). Thus, use of primer set P3 in single- and nested-PCR allowed detection of the pathogen in 37.0% and 57.4% of seed samples, respectively; whereas incidence in similar assays using primer set P6 was 55.6% (single-PCR) and 79.7% (nested-PCR) of samples scored positive. Use of the nested-PCR protocol increased the sensitivity of detection and allowed positive diagnosis of infections in commercial opium poppy seed stocks that would have given rise to false negatives if assayed using the single-PCR protocol. This fact was particularly evident for seeds stocks derived from crops in France (Table III.2.3), for which all seed stocks with the exception of one were negative in single-PCR assays using primer sets P3 and P6, respectively. Conversely, nested-PCR assays using primer set P3 and P6 indicated seedborne inoculum of *P. arborescens* in four and five of the seven seed stocks, respectively.

Table III.2.3. Detection of *Peronospora arborescens* in naturally-infected opium poppy commercial seed stocks using either single- or nested- specific-PCR assays.

Seed code	Cultivar, Origin, Year	Single-PCR[a]			Nested-PCR [a]	
		DC6/ITS4	P3	P6	P3	P6
Andalucia Region, Spain						
919	Madrigal, Castillón invernadero, 2007	-/-/-[b]	-/-/±	-/±/±	-/+/+	-/++/++
922	Madrigal, Castillón Huerto, 2007	±/-/-	+/±/-	+/+/+	++/+/+	++/++/++
923	Madrigal, Martín Delgado, 2007	-/-/-	-/±/+	+/++/+	+/+/++	++/++/++
925	Madrigal, Las Coronas, 2007	-/-/-	±/-/+	+/+/++	+/+/++	++/++/++
927	Madrigal, Barbarrota, 2007	-/-/-	-/-/-	-/-/-	-/-/-	±/+/+
931	Madrigal, Molino gallego, 2007	-/-/-	-/-/+	±/±/+	-/±/++	++/+/++
916	Madrigal, Viso alto, 2007	-/-/-	-/-/-	+/±/-	-/-/-	++/++/+
Castilla La Mancha Region, Spain						
9145	Madrigal, Casa del Alcalde, 2007	±/-/±	±/+/+	±/+/+	++/++/++	++/++/++
9147	Madrigal, Casablanca, 2007	±/-/-	++/±/+	+/±/++	++/++/++	++/++/++
9149	Madrigal, Casa Nueva, 2007	+/+/-	+/+/+	++/++/+	++/++/++	++/++/++
9151	Madrigal, Los Machorros, 2007	-/-/-	+/±/±	+/+/±	++/++/++	++/++/++
France						
7519	V9, 2007	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
7521	Marianne, 2007	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
7522	V40, 2006	-/-/-	-/-/-	+/+/-	+/±/-	++/+/-
7523	V40, 2006	-/-/-	-/-/-	-/-/-	-/±/-	-/+/+
7524	V40, 2007	-/-/-	-/-/-	-/-/-	+/+/-	++/++/-
7526	V9, 2006	-/-/-	-/-/-	-/-/-	+/-/-	++/++/-
7527	Marianne, 2007	-/-/-	-/-/-	-/-/-	-/-/-	±/±/±

[a] *Peronospora arborescens*-specific primers OMPac1fw/OMPa1rv (set P3) and OMPac7fw/OMPac7rv (set P6) were used in nested- and single-PCR assays (25). Primer DC6, specific for Pythiales and Peronosporales oomycetes (3), and the universal ITS4 primer (42), were used for the first round of amplifications in the nested-PCR protocol. Then, 1 µl of a 1/10 dilution of the first PCR product was used for the second round of amplification in that protocol.

[b] Results of DNA amplification from three seed samples. ++= Positive amplification (very intense); + = Positive amplification; ± = Positive weak amplification; - = no amplification.

Sequencing and Phylogenetic analysis

Direct sequencing using primers OMPac1fw or OMPac7fw of purified amplicons from nested-PCR assays with primer sets P3 and P6, allowed alignment of an ITS1-5.8S-ITS2 rDNA sequence up to 730-bp long when both sequences were combined (Table III.2.1, Fig. III.2.1B). There were no differences in the chromatograms obtained from independent nested-PCR assays when using the same sample or when using either forward or reverse primers (*data not shown*).

Maximum parsimony analysis using data from all *Peronospora* spp. in the GenBank database placed all downy mildew specimens in this study in a single clade

(100% support; 99.0 to 99.9 % homology among sequences) which included all *P. arborescens* sequences from GenBank (*data not shown*). Interestingly, MP analysis of the *P. arborescens* dataset alone indicated that a degree of host specialization and geographical isolation may exist within *P. arborescens* populations (Fig. III.2.3). Thus, all sequences of *P. arborescens* from crops of *Pap. somniferum* in Spain and France formed a single population together with a specimen from each of *Pap. rhoeas* and *Pap. hybridum* from Spain. *P. arborescens* from a herbarium specimen of *Pap. somniferum* collected in Finland on 1916 was located in a different clade. On the other hand, *P. arborescens* isolates from *Pap. rhoeas* showed higher diversity, with fresh and herbarium specimens from northern Europe and Spain forming a clade different from that including specimens from southern Spain and the one collected in Algeria in 1912. The two isolates from specimens of *Pap. dubium* formed a third, independent clade (Fig. III.2.3).

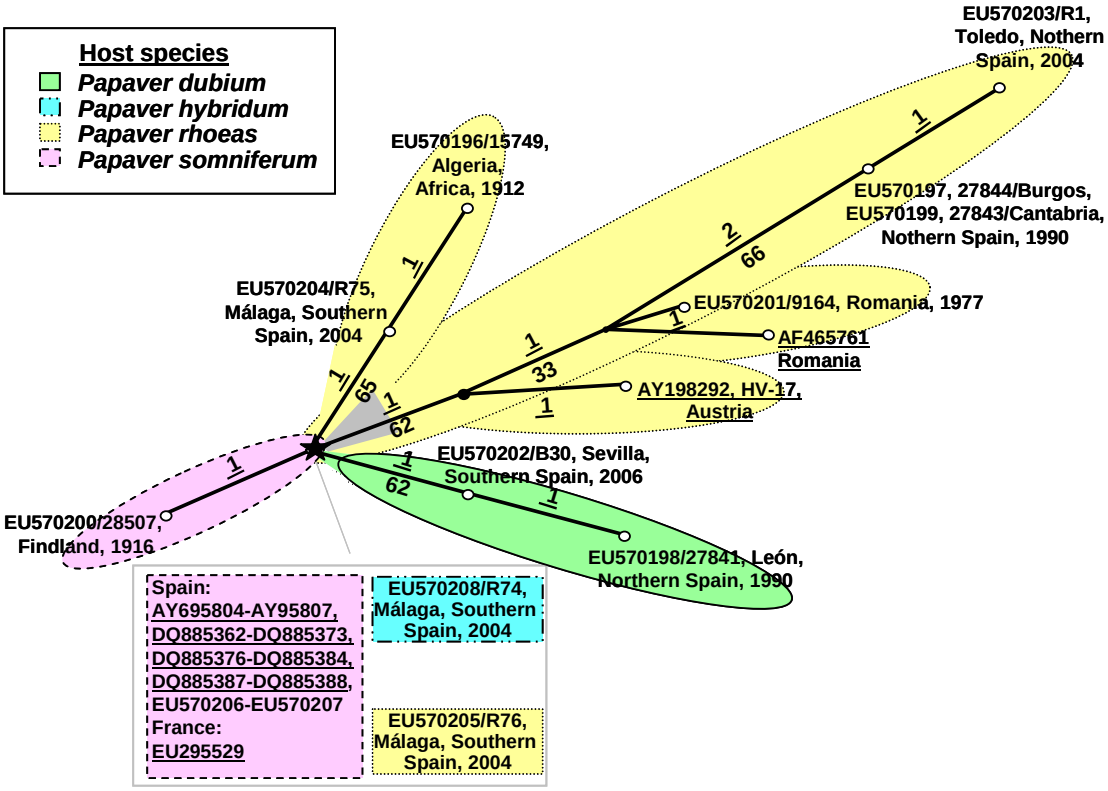

Fig. III.2.3. Maximum parsimony tree from sequences of the ITS region (ITS1, 5.8S rDNA, and ITS2) of *P. arborescens* isolates from samples of herbarium specimens and *Papaver* spp. (Table III.2.1) and all *P. arborescens* sequences deposited in the GenBank database (underlined accession numbers). The absolute numbers of nucleotide differences between specimens (underlined numbers) and bootstrap values (%) are indicated in the branches. The accession number, herbarium code, region, country and year of collection of specimens are indicated. Host source of *P. arborescens* isolates is marked in different colors as shown in the legend. (★) = All *P. arborescens* isolates included in this node are shown in the frame.

III.2.5 DISCUSSION

In this study, a *P. arborescens*-specific nested-PCR protocol was developed that: (i) strengthens previous findings that only *P. arborescens* occurs in opium poppy crops and other *Papaver* spp. In Spain (24,25); (ii) amplifies *P. arborescens* in ancient, up to 96-year-old, herbarium specimens of different *Papaver* spp.; and (iii) demonstrates that *P. arborescens* can generate asymptomatic, systemic infections both in wild *Papaver* spp. And cultivated opium poppy, and infects seed stocks harvested from crops with low or no symptoms of the disease. Furthermore, phylogenetic analyses of the ITS rDNA indicate that: (i) genetic diversity exists within *P. arborescens* populations occurring worldwide; and (ii) a degree of host specificity exists within *P. arborescens* infecting *Papaver* spp., with isolates infecting wild poppy being more diverse than those infecting cultivated *Pap. Somniferum* (Fig. III.2.3).

Taxonomic concepts in downy mildew taxa are now considered inadequate and potentially misleading because the taxonomic and evolutionary studies done in the past were based mainly on few morphological characters and host preference (5,9,19). This has caused much confusion concerning species definition and delimitation, especially when following Gaümann's concept of biological species 'one host – one parasitic species' (12,19,41). Recently, phylogenetic analyses of rDNA sequences in Peronosporales have helped to resolve taxonomic and phylogenetic relationships among closely related species within *Peronospora* and allied genera (e.g., 5, 6, 7, 10, 17, 32, 41). For instance, *P. sparsa* from *Rosa* spp. and *P. rubi* from *Rubus* spp. share identical ITS sequences, as do *P. viciae* from faba bean (*Vicia faba*) and *P. viciae* (*Pisum sativum*) from pea, which suggest conspecificity (4, 10). Conversely, *Hyaloperonospora parasitica* from *Arabidopsis* spp. Differs in ITS sequences from the pathogen that infects *Brassica oleracea*, which suggests reproductive isolation of the two populations (30).

Previous phylogenetic analyses and species-specific PCR assays of the ITS rDNA sequence demonstrated that *P. arborescens* is the sole causal agent of opium poppy downy mildew in commercial crops in France and Spain (24, 25, 27). Similar studies demonstrated that *P. cristata*, but not *P. arborescens*, is the downy mildew pathogen in opium poppy crops in Tasmania (Australia) (36). *P. cristata* has been reported in different countries, including Australia, Bulgaria, England, Ireland, Portugal, Ukraine and the former USSR (14). Comparatively, the geographic range of

P. arborescens appears to be more widespread, since this species has been reported in Afghanistan, Algeria, Andorra, Argentina, Australia, Austria, Azerbaijan, Belgium, Bulgaria, Canada, Central Asia, China, Egypt, Finland, Germany, Greece, India, Iran, Italy, Japan, Korea, Libya, Mongolia, Pakistan, Poland, Portugal, Romania, South Africa, Sweden, Thailand, Turkey, UK, USA and Uzbekistan (14,15). In the Iberian Peninsula, *P. arborescens* has been reported infecting *Pap. dubium*, *Pap. hybridum,* and *Pap. rhoeas* in Spain, and infecting wild *Pap. somniferum* and *Pap. rhoeas* in Portugal. Conversely, *P. cristata* has been reported only in Portugal and infecting *Pap. hybridum* (16,18). *P. arborescens* and *P. cristata* overlap in morphological characters (15,31,37) and host range, and since most previous reports were based on symptoms on the hosts and morphology of the pathogen only, it is possible that some of the descriptions attributed to one of the two species in a country might in fact correspond to the other.

The low degree of morphological diversity in biotrophic Oomycetes lead to numerous taxa being differentiated according to their host plants rather than by diagnostic characters of the parasites themselves. The ability to trace material that has been stored for a long term (e.g., herbarium specimens) could help to close the gap between traditional, phenetic systematics and recent molecular-based systematics (39). In a recent study using herbarium specimens, Choi et al. (7) showed that *P. farinosa* from *S. oleracea* is distinct from downy mildew of other chenopodiaceous hosts, which lead to reinstatement of *P. effusa* as the pathogen on spinach. Results of this present work also indicate the usefulness of herbarium specimens for clarifying systematics in *Peronospora* spp. Infecting *Papaver* spp. Thus, the newly developed nested-PCR protocol confirmed that only *P. arborescens* is present in herbarium specimens and fresh asymptomatic tissue sampled from different *Papaver* spp. in Spain, as well as in herbarium specimens of *Papaver* spp. from Algeria, Finland and Romania. Ancient herbarium specimens have been scarcely used for phylogenetic studies of downy mildew pathogens, and when used, a clonation step of DNA amplicons was generally required before DNA sequencing (e.g., 6). The protocol developed in this present research allowed direct sequencing of ancient DNA (up to 96 year old) from an obligate biotroph infecting herbarium specimens, thus avoiding the need of a clonation step. Therefore, this nested-PCR protocol may be useful for elucidating identity of downy mildew pathogen in herbarium specimens of opium poppy and other *Papaver* spp. In different areas of the world, as well as to unravel possible

misclassification of *P. arborescens* with *P. cristata,* the reported causal agents of the opium poppy downy mildew disease.

To the best of our knowledge, MP analysis of amplified ITS rDNA sequences of *P. arborescens* infecting *Pap. dubium, Pap. hybridum, Pap. rhoeas*, and *Pap. somniferum* specimens from different countries indicated for the first time that a degree of host specificity may exist within *P. arborescens* populations. Interestingly, all sequences of *P. arborescens* from commercial opium poppy crops in Spain and France formed a single population together with those of isolates from *Pap. rhoeas* and *Pap. hybridum* from Spain that were sampled in commercial opium poppy fields. However, ITS rDNA sequences from *P. arborescens* infecting wild poppies were genetically more diverse among themselves compared with those of *P. arborescens* isolates from cultivated *Pap. somniferum*. It would be productive future work to determine if *P. arborescens* isolates infecting opium poppy cultivars in different areas of the world harbor genetic diversity, which would tentatively indicate selection of pathogen genotypes by host genotypes, co-migration with the host plant and/or reproductive isolation. Further studies should be done to determine if *P. arborescens* populations differing in ITS sequences or host source also differ in host specialization and virulence on *Pap. somniferum* and *Papaver* spp., as established for other downy mildews (e.g., *Chenopodium quinoa/P. farinosa* f. sp. *chenopodii* (28); *S. oleracea/P. farinosa* f. sp. *Spinaciae* (22). Ongoing investigations in our laboratory, based in phylogenetic analysis of ITS rDNA sequences, indicate that a downy mildew disease in *Pap. hybridum* in the field may be caused by a *Peronospora* spp. different from *P. arborescens* and *P. cristata* (M. Montes-Borrego, J. A. Navas-Cortés, R. M. Jiménez-Díaz and B. B. Landa, *unpublished results*). This would suggest that pathogen diversity in *Papaver* spp./downy mildew pathosystems might be greater than initially thought.

Because of the obligate biotrophy of phytopathogenic Peronosporales, availability of molecular protocols for their identification, as well as in-planta and seed detection, would be of importance for the implementation of health certification schemes. In previous work (25) we developed a *P. arborescens*-specific PCR protocol that: (i) can be used for pathogen detection in capsules, leaves, roots, seeds, and stems of plants; (ii) is sensitive enough to detect 0.1 to 10 pg of *P. arborescens* DNA; and (iii) has high specificity since no cross-amplification occurs with other closely related pathogens, especially *P. cristata* in infected opium poppy tissues. The newly developed

nested-PCR protocol has greater sensitivity for the in-planta detection of *P. arborescens* DNA increasing by x100 to x1000 fold the detection limit (depending of the primer set) compared with that of the single-PCR assay using the same primers (25), and maintains the above referred properties of this latter assay. The increased sensitivity of the nested-PCR protocol allowed detection of as little as 5 to 0.5 fg of *P. arborescens* DNA against a background of 25 to 50 ng of opium poppy DNA. Nested-PCR protocols already developed for the detection of other oomycetes (e.g., 21,35,38,40) showed detection limits (1 pg to 100 fg of different *Phytophthora* spp. and of *P. tabacina*) greater than those achieved in our study. Also, the presence of host DNA in our protocol did not influence the sensitivity of our single-PCR (25) nor the nested-PCR protocols in contrast to other studies where sensitivity was decreased one to two fold (e.g., 38).

The new nested-PCR protocol was effective in detecting pathogen DNA when the single-PCR assay either failed to detect it, or was less efficacious. This was the case of pathogen detection in ancient herbarium specimens, which demonstrated the occurrence of asymptomatic, systemic infections of *Papaver* spp. by *P. arborescens*. Thus, the pathogen was detected in leaves of wild *Papaver* spp. Grown in fields that did not show morphologic symptoms of downy mildew. In previous work, we reported the occurrence of putative systemic infections of opium poppy by *P. arborescens* in field-grown plants showing dwarfing and rosetting but no other obvious symptom of downy mildew (25). In this present study, we demonstrate that the nested-PCR protocol is more efficient than the single-PCR assay in the detection of asymptomatic, systemic infections of plants grown under controlled conditions, and is more useful for studies on the biology of opium poppy downy mildew. These infections were caused by soilborne inoculum during seed germination and/or seedling growth (M. Montes-Borrego, B. B. Landa, J. A. Navas, F. J. Muñoz-Ledesma and R. M. Jiménez-Díaz, *unpublished results*). Research is ongoing in our laboratory to determine the role of oospores as primary inoculum for development of downy mildew epidemics of opium poppy using the nested-PCR protocol to demonstrate root infection by the pathogen.

Downy mildews are among the most destructive diseases of crops worldwide and can be seedborne. Consequently, efforts should be made to avoid introduction of infected seeds into areas where the pathogen does not occur. For instance, specific-

PCR assays of 11 commercial sweet basil seed batches of unknown origin (2) indicated the presence of *Peronospora* sp. in nine of them. Similarly, oospores of *P. effuse* were demonstrated in seeds of six out of 11 commercial seed lots of spinach (20) and three out of seven maize seed lots were shown to harbor and transmit *Peronosclerospora sorghi* (1). Recently, we demonstrated that *P. arborescens* can be seed transmitted and that commercial seed stocks harvested from opium poppy crops with high incidence of the downy mildew disease were frequently infected by the pathogen (25). Use of the nested-PCR protocol developed in this study demonstrated further that the pathogen can be detected in commercial seed stocks harvested from opium poppy crops that have either no evidence or low incidence of the disease. Some of those seed stocks would have given rise to false negatives for presence of the pathogen if the single-PCR assay had been used for detection assays. This fact is particularly important for companies like ALCALIBER S. A., the only enterprise officially authorized for opium poppy cultivation in Spain that aims to select seed stocks for the next year from those fields that have not shown any symptoms of disease. Using the best methods available as practiced by this company, the downy mildew disease has nevertheless spread rapidly to large areas where opium poppy had not been cropped before. This suggests seedborne transmission of *P. arborescens* since isolation of the sampled crops from other opium poppy crops within a range of several hundreds of kilometres would make it unlikely that distant plantings could serve as a source of airborne, primary inoculum. Studies are in progress to ascertain the biology of systemic infections by *P. arborescens* and to determine whether or not low inoculum levels of the pathogen on seeds may give rise to a high number of infected plants as well as if asymptomatic systemic infection of a plant give rise to viable, infected seeds as demonstrated for other downy mildew pathogens (e.g., *Plasmopara halstedii/Helianthus annuus* (8); *Peronospora ducometi/Fagopyrum sculentum* (44). In the meantime, the nested-PCR protocol is being currently used by ALCALIBER S.A. to assure use of seed stocks free of infection by the pathogen, discarding infected ones, and to avoid dispersal of the pathogen in seed to opium poppy growing areas free from *P. arborescens.*

In conclusion, results from this study demonstrate the usefulness of the nested-PCR protocol developed for biogeographic, diagnostic, and epidemiological studies of the opium poppy disease.

III.2.6 ACKNOWLEDGEMENTS

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III.2.7 LITERATURE CITED

1. Adenle, V. O., and Cardwell, K. F. 2000. Seed transmission of maize downy mildew (*Peronosclerospora sorghi*) in Nigeria. Plant Pathol. 49:628-634.
2. Belbahri, L., Calmin, G., Pawlowski, J., Lefort, F. 2005. Phylogenetic analysis and real time PCR detection of a presumably undescribed *Peronospora* species on sweet basil and sage. Mycol Res. 109:1276-87.
3. Bonants, P., De Weerdt, M. H., Van Gent Pelzer, M., Lacourt, I., Cooke, D., and Duncan, J. 1997. Detection and identification of *Phytophthora fragariae* by the polymerase chain reaction. Eur. J. Plant Pathol. 103: 345-355.
4. Breese, W. A., Schattock, R. C., Williamson, B., and Hackett, C. 1994. In vitro spore germination and infection of cultivars of *Rubus* and *Rosa* by downy mildews from both hosts. Ann. Appl. Biol. 125:73-85.
5. Choi, Y.-J., Hong, S.-B., and Shin, H.-D. 2003. Diversity of the *Hyaloperonospora parasitica* complex from core brassicaceous hosts based on ITS rDNA sequences. Mycol. Res. 107:1314-1322.
6. Choi, Y.-J., Hong, S.-B., and Shin, H.-D. 2005. A re-consideration of *Pseudoperonospora cubensis* and *P. humuli* based on molecular and morphological data. Mycol. Res. 109:841-848.

7. Choi Y.-J., Hong, S.-B., and Shin, H.-D. 2007. Re-consideration of *Peronospora farinosa* infecting *Spinacia oleracea* as distinct species, *Peronospora effusa*. Mycol. Res. 111:381-391.
8. Cohen, Y., and Sackston, W. E. 1974. Seed infection and latent infection of sunflowers by *Plasmopara halstedii*. Can. J. Bot. 52:231-238.
9. Constantinescu, O., and Fatehi, J. 2002. *Peronospora*-like fungi (Chromista, Peronosporales) parasitic on Brassicaceae and related hosts. Nova Hedwigia 74:291–338.
10. Cooke, D. E. L., Williams, N. A., Williamson, B., and Duncan, J. M. 2002. An ITS-based phylogenetic analysis of the relationships between *Peronospora* and *Phytophthora*. Pages 161–165 in: Advances in Downy Mildew Research. P. T. N. Spencer, U. Gisi and A. Lebeda, eds. Kluwer Academic Publishers, Dordrecht, The Netherlands.
11. Cotterill, P. J., and Pascoe, I. G. 1998. Downy mildew of *Papaver somniferum* in Tasmania. Australasian Plant Pathol. 27:263–264.
12. Dick, M. W. 2002. Towards an understanding of the evolution of the downy mildews. Pages 1–57 in: Advances in Downy Mildew Research. P. T. N. Spencer, U. Gisi and A. Lebeda, eds. Kluwer Academic Publishers, Dordrecht, The Netherlands.
13. Döken, M. T. 1986. The nature of systemic invasion of stem and leaves of sunflowers by *Plasmopara helianthi* Novot. var. *helianthi* Novot. with mechanism of sporulation and zoospore release. J. Phytopathol. 117:270-275.
14. Farr, D. F., Rossman, A. Y., Palm, M. E., and McCray, E. B. Fungal Databases, Systematic Botany & Mycology Laboratory, ARS, USDA. Online publication Retrieved June 2, 2008, from http://nt.ars-grin.gov/fungaldatabases/
15. Francis, S. M. 1981. *Peronospora arborescens*. CMI Descriptions of Pathogenic Fungi and Bacteria 686:1–2.
16. Garcia-Blazquez, G., Constantinescu, O., Telleria, M. T., and Martin, M. P. 2006. Preliminary checklist of Albuginales and Peronosporales (Chromista) reported from the Iberian Peninsula and Balearic Islands. Mycotaxon 98:185-188.
17. Göker, M., Voglmayr, H., Riethmüller, A.,Weiß, M., and Oberwinkler, F. 2003. Taxonomic aspects of Peronosporaceae inferred from Bayesian molecular phylogenetics. Can. J. Bot. 81:672-683.

18. Gustavsson, A. 1991. The genus *Peronospora* in the Iberian peninsula, especially in northern Spain and Andorra. Anales Jard. Bot. Madrid 49:3-38.
19. Hall, G. S. 1996. Modern approaches to species concepts in downy mildews. Plant Pathol. 45:1009-1026.
20. Inava, T., Takahashi, K., and Morinaka, T. 1983. Seed transmission of spinach downy mildew. Plant Dis. 67:1139-1141.
21. Ippolito, A., Schena, L., and Nigro, F. 2002. Detection of *Phytophthora nicotianae* and *P. citrophthora* in citrus roots and soils by nested PCR. Eur. J. Plant Pathol. 108:855–868.
22. Irish, B. M., Correll, J. C., Koike, S. T., and Morelock, T. E. 2007. Three new races of the spinach downy mildew pathogen identified by a modified set of spinach differentials. Plant Dis. 91:1392-1396.
23. Kapoor, L. D. 1995. Opium poppy: botany, chemistry and pharmacology. Food products Press, New York
24. Landa, B. B., Montes-Borrego, M., Muñoz-Ledesma, F. J., and Jiménez-Díaz, R. M. 2005. First report of downy mildew of opium poppy caused by *Peronospora arborescens* in Spain. Plant Dis. 89:338.
25. Landa, B. B., Montes-Borrego, M., Muñoz-Ledesma, F. J., and Jiménez-Díaz, R. M. 2007. Phylogenetic analysis of Downy Mildew pathogens of opium poppy and PCR-based in-planta and seed detection of *Peronospora arborescens*. Phytopathology 97:1380-1390.
26. Li, W., Brlansky, R. H., and Hartung, J. S. 2006. Amplification of DNA of *Xanthomonas axonopodis* pv. *citri* from historic citrus canker herbarium specimens. *J. Microbiol. Meth.* 65**:**237-246.
27. Montes-Borrego, M., Muñoz-Ledesma, F. J., Jiménez-Díaz, R. M., and Landa, B. B. 2008. Downy Mildew of commercial opium poppy crops in France is caused by *Peronospora arborescens*. Plant Dis. 92:834.
28. Ochoa, J., Frinking, H. D., and Jacobs T. H.. 2005. Postulation of virulence groups and resistance factors in the quinoa/downy mildew pathosystem using material from Ecuador. Plant Pathol. 48:425-430.
29. Pando, F. 2000. Bases de datos en línea de las colecciones de criptógamas del Herbario MA. http://www.rjb.csic.es/colecciones_herbario_cripto_consult.php.

30. Rehmany, A. P., Lynn, J. R., Tör, M., Holub, E. B., and Beynon, J. L. 2000. A comparison of *Peronospora parasitica* (downy mildew) isolates from *Arabidopsis thaliana* and *Brassica oleracea* using amplified fragment length polymorphism and internal transcribed spacer 1 (ITS1) sequence analyses. Fungal Genet. Biol. 30: 95-103.
31. Reid, D. A. 1969. New or interesting British plant diseases. Trans. Br. Mycol. Soc. 52:19-38.
32. Riethmuller, A., Voglmayr, H., Göker, M., Weiß, M., and Oberwinkler, F. 2002. Phylogenetic relationships of the downy mildews (Peronosporales) and related groups based on nuclear large subunit ribosomal DNA sequences. Mycologia 94:834-849.
33. Ristaino, J. B. 1998. The importance of archival and herbarium materials in understanding the role of oospores in late blight epidemics of the past. Phytopathology 88:1120- 1130.
34. Ristaino, J. B., Groves, C. T., and Parra, G. R. 2001. PCR amplification of the Irish potato famine pathogen from historic specimens. Nature 411:695-697.
35. Schena, L., Duncan, J. M., and Cooke, D. E. L. 2008. Development and application of a PCR-based 'molecular tool box' for the identification of *Phytophthora* species damaging forests and natural ecosystems. Plant Pathol. 57:64-75.
36. Scott, J. B., Hay, F. S., Wilson, C. R., Cotterill, P. J., and Fist, A. J. 2003. Spatiotemporal analysis of epiphytotics of downy mildew of oilseed poppy in Tasmania, Australia. Phytopathology 93:752-757.
37. Scott, J. B., Hay, F. S., Wilson, C. R. 2004. Phylogenetic analysis of the downy mildew pathogen of oilseed poppy in Tasmania, and its detection by PCR. Mycol. Res. 108:98-295.
38. Silvar, C., Duncan, J. M., Cooke, D. E. L., Williams, N. A., Díaz, J., and Merino, F. 2005. Development of specific PCR primers for identification and detection of *Phytophthora capsici* Leon. Eur. J. Plant Pathol. 112:3-52.
39. Spring, O., and Thines, M. 2004. On the necessity of new characters for classification and systematics of biotrophic Peronosporomycetes. Planta 219:910-914.

40. Tsay, J.-G., Chu, C., Chuang, Y.-H., and Chen, R.-S. 2006. Specific detection of *Peronospora tabacina* by PCR-amplified rDNA Sequences. Plant Pathol. J. 5:78-382.
41. Voglmayr, H. 2003. Phylogenetic relationships of *Peronospora* and related genera based on nuclear ribosomal ITS sequences. Mycol. Res. 107:1132-1142.
42. White, T. J., Bruns, T., Lee S., and Taylor, J. W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: PCR protocols: A guide to methods and applications. M. A. Innis, D. H. Gelfand, J. J. Sninsky and T. T. White, eds. Academic Press Inc., New York.
43. Yossifovitch, M. 1929. *Peronospora arborescens* (Berk.) de Bary, parasite tres important de *Papaver somniferum* en Yougoslavie. Rev. Pathol. Entomol. Agric. 16:235-270.
44. Zimmer, R. C., McKeen, W.E., and Campbell, C. G. 1992. Location of oospores in buckwheat seed and probable roles of oospores and conidia of *Peronospora ducometi* in the disease cycle on buckwheat. J. Phytopathol. 135:217-223.

CAPÍTULO IV

Detección molecular cuantitativa del agente causal

En este capítulo se recogen los resultados relativos al cuarto objetivo de esta Tesis Doctoral en el cual se pretendía desarrollar un protocolo de PCR en tiempo real para la cuantificación del nivel de infección/infestación de semillas de adormidera y plantas sintomáticas y asintomáticas por el patógeno, y correlacionar dicho nivel con el desarrollo de enfermedad y la sintomatología de ésta.

Los resultados obtenidos se recogen en el capítulo IV.1 el cual se encuentra en preparación para su envío a la revista *Applied and Environmental Microbiology* de la Sociedad Americana de Microbiología, y que lleva por título:

"A Robust, Universal Real-Time Quantitative PCR Assay for the in planta Detection and Quantification of the Opium Poppy Downy Mildew pathogen *Peronospora arborescens* of Use for Resistance Screening and Certification Schemes". *Applied and Environmental Microbiology,* En preparación.

IV.1

A Robust, Universal Real-Time Quantitative PCR Assay for the in planta Detection and Quantification of the Opium Poppy Downy Mildew pathogen *Peronospora arborescens* of Use for Resistance Screening and Certification Schemes

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IV.1.1 ABSTRACT

Downy mildew caused by *Peronospora arborescens* is one of the main yield-limiting factors of opium poppy crops worldwide. In-depth understanding of the biology of the pathogen and the epidemiology and management of the disease is curtailed by obligate biotrophic nature of the pathogen that makes it difficult its proper in planta identification and quantification. In this study, we developed a new, reliable, innovative, and quick real-time quantitative polymerase chain reaction (qPCR) assay that allows the exact identification and accurate quantification of *P. arborescens* in different opium poppy tissues and seed stocks of use by opium poppy producers and researchers for field-scale studies at a viable, economic cost. Moreover, we developed a standard curve based on DNA extracted from a plasmid with the target pathogen DNA as cloned insert, and established a robust numerical relationship with a standard curve based on *P. arborescens* genomic DNA that may facilitate the use of the new protocol on a large scale and by different laboratories. Our protocol was highly reproducible and the pathogen DNA could be accurately quantified up to 10 fg in opium poppy leaf and seed DNA backgrounds without loss of accuracy and efficiency. Additionally, we demonstrated the feasibility of the technique to quantify pathogen biomass in complex, naturally infested tissue samples, including seeds from asymptomatic capsules and commercial seed stocks as well as from asymptomatic opium poppy plants systemically infected by *P. arborescens*. This may be of much importance for advancing our understanding of the epidemiology and management of this downy mildew disease, and facilitate the knowledge-based implementation of appropriate control strategies such as quarantine and certification schemes as well as selection and use of host resistance.

IV.1.2 INTRODUCTION

Opium poppy (*Papaver somniferum*) is the only source of pharmaceutical morphine, codeine, and thebaine, which are key drugs for alleviation of chronic pain associated with cancer diseases. In Spain, opium poppy is grown annually on approximately 9,500 ha, primarily in the southern (Andalucía) and central (Castilla-La Mancha and Castilla-León) regions of the country (27,35). This acreage accounts for at least 5% of the legally cultivated opium poppy worldwide, making Spain the third and fourth largest European and world producer of poppy straw rich in morphine, respectively (22).

Commercial opium poppy crops in Spain can be affected by several diseases of diverse etiology (4,26,27). However, during the last 5 years downy mildew has become the main yield-limiting factor for this crop in the country (35). Nowadays, downy mildew attacks occurs throughout all opium poppy-growing areas in Spain and their incidence and severity has increased steadily since the disease was first recorded (26). In Spain and France, downy mildew in opium poppy commercial fields is caused by the obligate biotrophic oomycete *Peronospora arborescens* (26,35), as compared to Australia where *P. cristata* has been reported as the causal agent of the disease (43).

The exact detection and proper identification of the causal agent is essential for the effective control of a plant disease. During the last 15 years, molecular techniques based mainly on conventional polymerase-chain-reaction (PCR) assays have emerged as a major tool for diagnosing and identifying phytopathogenic fungi and have contributed to relieve some of the problems associated with the detection, control, and containment of plant pathogens (16,32). We have used that technology and recently made considerable progress in the development of molecular protocols for detection of *P. arborescens* in different plant tissues (27,35). In a recent work (27) we developed a *P. arborescens*-specific single-PCR protocol using either of two sets of primer pairs (OMPac1fw/OMPac1rv and OMPac7fw/OMPac7rv) that: (i) can be used for pathogen detection in capsules, leaves, roots, seeds, and stems of opium poppy plants; (ii) is sensitive enough to detect 0.1 to 10 pg of *P. arborescens* DNA; and (iii) is highly specific since no cross-amplification occurs with other closely related pathogens in infected opium poppy tissues, especially of *P. cristata.* Later on, a significant improvement for the in planta detection of *P. arborescens* DNA was achieved

with a newly developed nested-PCR protocol (35) which has a sensitivity 2- to 3-orders of magnitude (depending of the primer set) higher compared with that of the single-PCR assay using the same primers while maintaining the properties of this latter assay. That increase in sensitivity of the nested-PCR protocol allowed detection of as little as 5 to 0.5 fg of *P. arborescens* DNA against a background of 25 to 50 ng of opium poppy DNA.

Since *P. arborescens* is a non-culturable, strictly biotrophic plant pathogen, availability of those *P. arborescens*-specific PCR protocols (simple and nested) has been of great importance for making significant progress in our knowledge of the biology of this pathosystem. Thus, they have allowed to demonstrate important aspects in the cycle of pathogenesis of the downy mildew disease including the main role of oospores as primary inoculum and the occurrence of asymptomatic, systemic infections that can give rise to seed infection and pathogen transmission (27,35, M. Montes-Borrego, B. B. Landa, J. A. Navas-Cortés, F.J. Muñoz-Ledesma, and R.M. Jiménez-Diaz, *unpublished results*). One of the limitations of the developed PCR protocols is that they do not allow quantifying the level of infection in the plant by the pathogen. A quantitative and sensitive PCR method would be particularly useful for epidemiological studies, quarantine and disease control schemes, particularly to: (i) establish the existence of resistant or tolerant opium poppy germplasm by quantifying the pathogen in symptomatic and asymptomatic infections; (ii) to establish a correlation between the amount of disease that may develop from the use of commercial seed stocks with quantified amount of pathogen in it, which would help to avoid the use of seed stocks that pose a risk for epidemic development; and (iii) to correlate the amount of pathogen in infected plant tissues with the subsequent level of disease development and/or symptom expression following the use of disease control measures (biological, chemical o physical).

Quantitative real-time (qPCR) was introduced in the monitoring of plant pathogens directly from plant tissues at the end of 1990s, and since then has been adapted for use in many plant-pathogen systems, including several plant pathogenic viruses, nematodes, bacteria, fungi and oomycetes (39,38,41). In the case of oomycetes, most of qPCR assays have been developed for different species of *Pythium* (2,31,42) and *Phytophthora* (2,5,7,8,20,24,40,44,46,49,50). Also, some qPCR assays have been developed for the strictly biotrophic oomycetes *Plasmopara viticola* (48) and some

Peronospora spp. including *P. sparsa* (21), *P. parasitica* (9) and an unidentified *Peronospora* spp. (6).

Real-time qPCR chemistries used for the detection and study phytopathogenic microorganisms can be grouped into amplicon sequence non-specific methods (SYBR Green) (42,44) and sequence-specific methods (TaqMan, Molecular Beacons, Scorpion-PCR, etc.) (38,41,48). Sequence-specific chemistries are more specific but increase the cost of analysis when those methods are used in a generalized and practical way. Use of SYBR Green I fluorescent dye for detection of amplicons is a good and reliable method if specific primers are well-designed; but more importantly, it is probably the most economical option as compared to other chemistries thus allowing to perform higher number of qPCR assays for practical applications in field studies.

In real-time qPCR, each reaction is characterized by a specific cycle number, the threshold cycle (C_T), at which a statistically significant increase in fluorescence over the baseline can be detected. The C_T is inversely proportional to the log of target sequence concentration. This means that the more template is present in a PCR sample, the lower is the number of amplification cycles needed to reach the point where fluorescence is detectable above the baseline (30,36). The analysis methods for real-time qPCR achieved by comparing the C_T values of the test samples to a standard curve originated from samples of known concentration are commonly known as absolute quantification. For absolute quantification, the accuracy of quantification of unknown samples depends entirely on the accuracy of the standards. Therefore, much care is needed for the design, synthesis, purification and calibration of DNA standards (47). In the case of strictly biotrophic organisms, such as *P. arborescens,* DNA samples to obtain standard curves can be obtained from mycelia and sporangia scrapped from sporulating leaves (21,27,35,48). Also, more recently, absolute standard curves have been constructed from plasmids containing the target DNA as a cloned insert (21).

In this study, we report the development of a reliable, innovative, quick and economical real-time qPCR assay to identify and quantify DNA of *P. arborescens* in different opium poppy tissues. We chose the use of SYBR Green I as a fluorescent dye for detection to provide ALCALIBER S.A. (Carmona, Sevilla province, Spain), the only enterprise officially authorized for opium poppy cultivation in Spain, with a cost-viable technology that can be used on a practical basis. We evaluated the effects

of different host plant tissues in the amplification, reproducibility and sensitivity of the assays, and present data on detection limits comparing the sensitivity of the real-time qPCR protocol with *P. arborescens*-specific single- and nested-PCR protocols previously developed (27,35). Moreover, we developed a standard curve based on DNA extracted from a plasmid with the target DNA as cloned insert, and established a robust numerical relationship with a standard curve based on *P. arborescens* genomic DNA to facilitate their use in a larger scale and by different laboratories. This allowed us to establish a universal protocol to quantify *P. arborescens* in opium poppy tissue which: (i) it does not depend of obtaining mycelium of this strictly obligate biotroph to be applied; (ii) it is reproducible and easy to use by different operators in different laboratories; and (iii) the initial DNA sample to make the standard curve and the standard curve itself can be stored and conserved for a long-time period without loosing accuracy in the quantification. Finally, the robustness of the newly developed assay was addressed and demonstrated by quantifying the amount of *P. arborescens* in naturally infested samples from multiple origins, including seeds from asymptomatic capsules and commercial seed stocks as well as from asymptomatic opium poppy plants systemically infected by *P. arborescens*.

IV.1.3 MATERIALS AND METHODS

Plant material

Seeds of commercial opium poppy cv. Nigrum provided by Alcaliber S.A were used throughout the study. Seeds were assayed healthy by negative amplification for *P. arborescens* using a nested species-specific PCR protocol (34). Seeds were surface-disinfested in 1% NaOCl for 5 min, rinsed twice with ultrapure, sterile water (USW), dried up in a flow hood and stored in sterile conditions until used for experiments. Seedlings to obtain mature leaf tissue were grown from seeds germinated on sterile layers of filter paper moistened with 2 ml of sterile 50 % Hoagland solution (19) in 9-cm-diameter petri dishes at 20°C for 1 week. Seedlings were then transplanted into a pasteurized (70ºC, 60 min) soil mixture (clay loam/peat, 2:1, vol/vol) in pots and incubated in a growth chamber (Sanyo MLR-350 H, Sanyo Electric Co., Ltd. Japan) at 20ºC 70/90% relative humidity (RH) and a 12-h photoperiod of fluorescent light at 360 $\mu E \cdot m^{-2} \cdot s^{-1}$.

Peronospora arborescens material

Samples of *P. arborescens* were obtained from fresh, sporulating leaves from plants used for artificial inoculation experiments in growth chamber (M. Montes-Borrego, B. B. Landa, J. A. Navas-Cortés, F.J. Muñoz-Ledesma, and R.M. Jiménez-Diaz, *unpublished results*), and from leaves of naturally-infected opium poppy plants in commercial crops at "Ruidero" and "Casilla San José" farms, Écija, Sevilla province, Spain.

Construction of control plasmids

The ITS and 5.8S regions of rDNA from two different specimens of *P. arborescens* (P38ESJ and M2ERUI, 27) of our culture collection was amplified using primer pairs DC6/ITS4 and ITS5/ITS4. Amplicons were purified and ligated into pGEM-T Easy Vector System I (Promega, Madison, WI, USA) using *Escherichia coli* strain JM109 for transformation as described before (27). The correct cloning was confirmed by sequencing cloned inserts (two from each PCR product) in both directions using a terminator cycle sequencing ready reaction kit (BigDye, Perkin-Elmer Applied Biosystems, Madrid, Spain) and primers DC6, ITS4 and ITS5, and universal M13-20fw M13rev. The resulting products were purified and run on a DNA multicapilar sequencer (ABI Prism 3100 genetic analyzer, Applied Biosystems) at the University of Córdoba sequencing facilities. Transformed clones were stored in glycerol (20%) at -80ºC and cultured when needed.

DNA extraction and quantification

Genomic DNA of *P. arborescens* and opium poppy seeds and leaves was extracted using the 'G-SpinTM IIp Plant Genomic DNA extraction kit' (Intron Biotechnology, Korea) and the Fast Prep System Bio 101 (Qbiogene, Madrid, Spain) according to (27).

Plasmid DNA was extracted from cultures of *E. coli* strain JM109 grown overnight at 37°C in Luria-Bertani (LB) broth (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, 1 liter of H2O [pH 7.2]) supplemented with ampicillin (100 μg/ml) using

the QuickGene Plasmid kit (Fujifilm Corporation, Tokyo, Japan) and QuickGene-Mini80 device (Fujifilm Corporation, Tokyo, Japan) according to the manufacturer's instructions.

DNA quality was assessed by gel electrophoresis and ethidium bromide staining. All DNA samples were accurately quantified using the Quant-iT DNA Assay Kit Broad Range fluorometric assay (Molecular Probes Inc., Leiden, The Netherlands) and a Tecan Safire fluorospectrometer (Tecan Spain, Barcelona, Spain) (27). Special care was taken to get accurate concentrations of plasmid, pathogen, and host DNAs by quantifying each DNA sample in triplicate, in two independent microplates. To have an internal control of DNA quantification, opium poppy and *P. arborescens* DNA samples of known concentration were included in each quantification plate. Genomic and Plasmid DNA were diluted with USW as appropriate.

DNA standard curves

DNA standard curves were obtained from 10-fold dilutions of *P. arborescens* genomic DNA (G-series) or of plasmid DNA (P-series). For this purpose, *P. arborescens* genomic DNA (1 ng/µl) or plasmid DNA (1 ng/µl; equivalent to 2.33 x 10^8 copies/µl) were serially diluted [1:1, 1:10, 1:10^2, 1:10^3, 1:10^4, 1:10^5, and 1:10^6 (only for P-series)] in USW (Curves G_W and P_W) as well as in a fixed background of *Pap. somniferum* DNA (40 ng/µl) extracted from healthy opium poppy leaves (Curves G_L and P_L) and seeds (Curves G_S and P_S). Each standard curve always included *Pap. somniferum* DNA and/or no DNA as negative controls. Ten independent DNA standard curves (five for G-series, named G1-5, and five for P-series, named P1-5) were obtained using different plant and pathogen DNA sources (Table 1), i.e.: opium poppy leaves of different age (mature and cotyledonary leaves), leaves and seeds from different origin, *P. arborescens* sporangia from sporulating leaves in the field or growth chamber, different plasmid cultures, etc. Finally, after building the G1-5_W and P1-5_W DNA standard curves each dilution point was quantified in triplicate by using the fluorometric Quant-iT High Sensitivity DNA assay kit (Molecular Probes Inc.), assuming the same pathogen DNA in the G1-5_L G1-5_S and P1-5_L, and P1-5_S DNA standard curves.

Table IV.1.1. Sources of *Peronospora arborescens* and *Papaver somniferum* DNA used to built up the different series of DNA standard curves used in this study

Source of DNA[a]	DNA Series[b] 1	2	3	4	5
Peronospora arborescens					
Genomic	Fresh DNA extracted from frozen (-80ºC) mycelium, specimen P98CSJ-2004, "Casilla San José" Écija, Sevilla, Spain	Fresh DNA extracted from fresh mycelium, specimen C79CHO-2008, "Ruidero" Écija, Sevilla, Spain	Frozen (-20ºC) DNA from specimen C85EX-2007, growth chamber experiments	Fresh DNA extracted from fresh mycelium, specimen C168EX-2008, growth chamber experiments	Frozen (-20ºC) DNA from specimen C85EX-2007 from series 3
Plasmid	Fresh DNA extracted from clone EC3_P38ESJ in June 2008	Frozen (-20ºC) plasmid DNA from series 1	Fresh DNA extracted from clone EC3_P38ESJ in September 2008	Frozen (-20ºC) plasmid DNA from series 3	Frozen (-20ºC) plasmid DNA from series 1
***Papaver somniferum* cv. Nigrum**					
Leaves	Fresh DNA extracted from seedling leaves germinated in Petri dishes, seed stock L1	Frozen (-20ºC) DNA from seedlings from series 1	Fresh DNA extracted from mature leaves of plants in growth chamber experiments, seed stock L2	Frozen (-20ºC) DNA from mature leaves from series 3	Frozen (-20ºC) DNA from seedlings from series 1
Seeds	Fresh DNA extracted from commercial seed stock S2, ALCALIBER S.A., 2007	Fresh DNA extracted from commercial seed stock S2, ALCALIBER S.A., 2007	Frozen (-20ºC) DNA from seed stock D200 from growth chamber experiment, 2007	Frozen (-20ºC) DNA from seed stock D205 from growth chamber experiment, 2007	Frozen (-20ºC) DNA from seed stock D205 from growth chamber experiment, 2007

[a] Genomic DNA of *P. arborescens* and opium poppy seeds and leaves was extracted using the 'G-SpinTM IIp Plant Genomic DNA extraction kit' and the Fast Prep System Bio 101 according to Landa et al. (27). Plasmid DNA from *E. coli* strain JM109 was extracted with QuickGene Plasmid kit using the QuickGene-Mini80 device according to the manufacturer's instructions.

[b] Five series of DNA standard curves were obtained from 10-fold dilutions [1:1, 1:10, $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$, and $1:10^6$]of *P. arborescens* genomic DNA (1 ng/µl) or of plasmid DNA (1 ng/µl; equivalent to 2.33×10^8 copies/µl) in USW (Curves G_W and P_W) as well as in a fixed background of *Pap. somniferum* DNA (40 ng/µl) extracted from healthy opium poppy leaves (Curves G_L and P_L) or seeds (Curves G_S and P_S).

Optimization, reproducibility and sensitivity of the real-time qPCR protocol

All real time PCR amplifications were performed using the iQ SYBR Green Supermix (BioRad, Madrid, Spain) and the iCycler IQ apparatus (BioRad). In the initial stages of optimization of the real-time PCR assay, we evaluated the cycling conditions and primer pairs P3Pa2fw/P3Pa12rv (set P2), OMPac1fw/OMPac1rv (set P3) and OMPac7fw/OMPac7rv (set P6) using DNA standard curves $G1_W$-$G1_S$. These primer pairs had been designed to specifically amplify a fragment of the *P. arborescens* ITS region developed by Landa et al. (27). For this experiment, an initial concentration of 10 ng, but not the lowest pathogen concentration of 100 fg, were included. Reaction conditions such as volume of reaction, annealing temperature, primer concentration and temperature to measure the fluorescence signal of the amplicon were adjusted experimentally to optimize the real-time qPCR protocol. After the final amplification cycle, a melting curve temperature profile was obtained by heating to 95°C, cooling to 72°C, and slowly heating to 95°C at 0.5°C every 10 s with continuous measurement of fluorescence at 520 nm. All reactions were analyzed by gel electrophoresis to confirm that only one PCR product was amplified in the samples with genomic DNA of *P. arborescens* and no amplification product was obtained in the negative controls.

To investigate any possible influence of host DNA on the accuracy of amplification and quantification of target DNA (genomic or plasmid), two DNA standard curves $G1_{W,L,S}$-$G2_{W,L,S}$ and $P1_{W,L,S}$-$P2_{W,L,S}$ and primer pair OMPac7fw/OMPac7rv (set P6) were used. Real time reactions for each DNA standard curve were performed independently. All DNA backgrounds were performed in the same 96-well PCR plate, with each experimental combination being replicated twice and in two independent experiments (different PCR plates and operators).

To further evaluate the robustness and efficiency of the newly developed qPCR protocol, the five series of DNA standard curves obtained with genomic *P. arborescens* DNA ($G1\text{-}5_L$ and $G1\text{-}5_S$, background DNA from leaves and seeds, respectively) and or DNA from the selected plasmid EC3_P38ESJ ($P1\text{-}5_L$ and $P1\text{-}5_S$, background DNA from leaves and seeds, respectively) were assessed in series of two-by-two ($G1_{L,S}$-$P1_{L,S}$, $G2_{L,S}$-$P2_{L,S}$, $G3_{L,S}$-$P3_{L,S}$, $G4_{L,S}$-$P4_{L,S}$ and $G5_{L,S}$-$P5_{L,S}$) in the same

PCR plate. Quantitative PCR amplifications of each series of DNA standard curves included two replications per plate and were repeated twice in independent experiments (PCR plates and operators).

Reproducibility of the real-time qPCR assay was determined by comparing C_T results and parameters (intercept and slopes) of the standard regression lines obtained with G1-$5_{L,S}$ and P1-$5_{L,S}$ DNA standard curves that were obtained in independent experiments by different operators (see data analysis below). These standard curves derived from DNA samples obtained from opium poppy leaves of different age, and leaves and seeds from different origin, after up to 10 frost (-20ºC)/defrost cycles.

Also, to determine the sensitivity of the real-time qPCR protocol using primer set P6 two series of DNA standard curves (G1-$2_{W,S,L}$ and P1-$2_{W,S,L}$) were used for comparing the new species-specific real-time qPCR protocol with the single- and nested-PCR protocols previously developed for in planta *P. arborescens* detection (27, 35).

Analysis of *Peronospora arborescens*-infected opium poppy plant and seeds by real-time qPCR

To validate the utility of the real-time qPCR technique for evaluating resistance or tolerance to *P. arborescens* in opium poppy germplasm, we used 50 opium poppy plants from a previous study (35) that were systemically infected by *P. arborescens* (as determined by the nested-PCR detection assay) but did not show any symptoms of disease. Those plants grew under controlled conditions in a soil naturally infested by *P. arborescens,* and were sampled when they had formed a capsule in the main stem. DNA was extracted from a 4 to 5-cm-long piece of the basal stem of each plant as previously described (35) and used to detect and quantify the level of *P. arborescens* infection. In this real-time qPCR assays DNA standard curves $G4_L$ and $P4_L$ were used and included in each of three independent plates, with two replications per each DNA sample.

To validate the utility of the real-time qPCR protocol for quantifying *P. arborescens* inoculum in seeds, we used DNA samples from a previous study (35) obtained from 11 commercial seed stocks (three replications/seed stock) provided by ALCALIBER S.A. Those seed stocks had been selected by the company for

commercial sowings from previous year commercial fields where downy mildew either was not apparent or the disease attack was rated low (0 to 1 affected plant per m^2) (35). Also, DNA was extracted as described before (27) from seeds obtained from capsules of 30 randomly-chosen opium poppy plants grown in a field with a disease incidence of *ca.* 5%. Of those 30 capsules, 19 showed downy mildew symptoms of different severity and 11 were asymptomatic. In these real-time qPCR assays, DNA standard curves $G4_S$ and $P4_S$ were used and included in each of two independent plates, with two replications per each DNA sample.

DNA from opium poppy plant or seed was 1/2- or 1/5-fold diluted in USW to obtain a DNA concentration in a range similar to that used in the DNA standard curve. Then, DNA samples were accurately quantified in triplicate using the Quant-iT DNA Assay Kit Broad Range fluorimetric assay as described above and used to normalize *P. arborescens* DNA to total plant DNA present in each reaction. Results from qPCR of unknown (seeds and stems) samples were compared to those obtained previously with single- and nested-PCR assays using primer set P6 (35). Finally, the amount of pathogen DNA in unknown samples was estimated from the universal G-series regression lines, once it was proven that $P4_S$ standard regression lines did not differed statistically ($P \geq 0.05$) from universal P-series regression lines (see below).

Data analyses

The cycle threshold (C_T) values for each reaction were first calculated by determining the PCR cycle number at which the fluorescence signal exceeded background using the default estimation criteria in the iCycler IQ software version 3.0a (Bio-Rad). Then, in order to compare and establish relationships between different DNA standard curves generated from different treatments, the threshold position was manually defined and fixed at the same position for all treatments and experiments to appropriately compare all standard curves (47). Amplification efficiency (AE), were calculated from the slopes of the standard curves using the equation $AE = 10^{(1/slope)} - 1$ (1,18).

Linear regressions of the natural logarithm of known concentrations of the target DNA versus the C_T values were performed for each DNA standard curve by using Statistix 8.0 (Analytical Software, Tallahassee, USA). Standard regression lines

from each plate chosen as reference curves were used for transforming the experimental C_T values into amounts of pathogen DNA (nanograms). Statistical differences in C_T, and amplification efficiency (AE) between and among standard regression lines obtained for G-curves and P-curves in different backgrounds were determined by one-way analysis of variance (ANOVA). Also, all standard regression lines obtained for *P. arborescesns* genomic DNA (G-curves) and plasmid DNA (P-curves) in different backgrounds (W, L, and S) were statistically compared for homogeneicity ($P \geq 0.05$) of variance (Bartlett's test) and for equality of slopes and intercepts using a F test at $P < 0.05$.

IV.1.4 RESULTS

Real-time qPCR optimization

Optimization of real-time qPCR involved step-by-step fine tuning of the following PCR parameters: primer set selection (P2 *vs* P3 *vs* P6), primer concentration, annealing temperature, and final volume of PCR reactions. This was performed using DNA standard curves ($G1_W$ and $G1_S$), in two independent experiments for each primer set.

Reducing the standard reaction volume from 50 to 20 µl had no effect on the accuracy of the assay (data not shown). Consequently, a final volume of 20 µl was chosen to minimize the cost per real-time qPCR assay. Occasionally in the melting curve analysis, we observed that weak peaks occurred between 60-75 ºC (e.g., Fig. 1.B,D) Theoretically, these peaks are reported as unspecific products or primer dimmers. In our assays, these unspecific amplifications were not detected by electrophoresis in a 1.2 % (wt/vol) agarose gel (data not shown). To avoid measuring fluorescence signal emitted by this unspecific amplifications, fluorescence of the target amplicon [melting temperature (Tm) = 86.5°C] was detected at 83°C (51). No fluorescence of the target amplicon occurred for negative controls using any of the three primer sets at 83°C; but occasionally, negative controls also showed weak peaks between 60-80ºC (e.g., Fig. 1.B,D).

Optimized PCR reaction mixture was similar for each of primer sets and contained (final volume of 20µl): 1 µl of DNA sample, 1× iQ SYBR Green Supermix

(BioRad), and 0.3 µM of each primer. The thermal cycling conditions consisted of an initial denaturation at 95°C for 2 min followed by 35 cycles of 1 min at 95°C, 1 min at 63°C, 2 min at 72°C, and 83°C for 15 s. A final extension step at 72°C for 10 min was added. Fluorescence of the target amplicon was detected at 83°C. A threshold position of 400 was fixed for all experiments to determine C_T values.

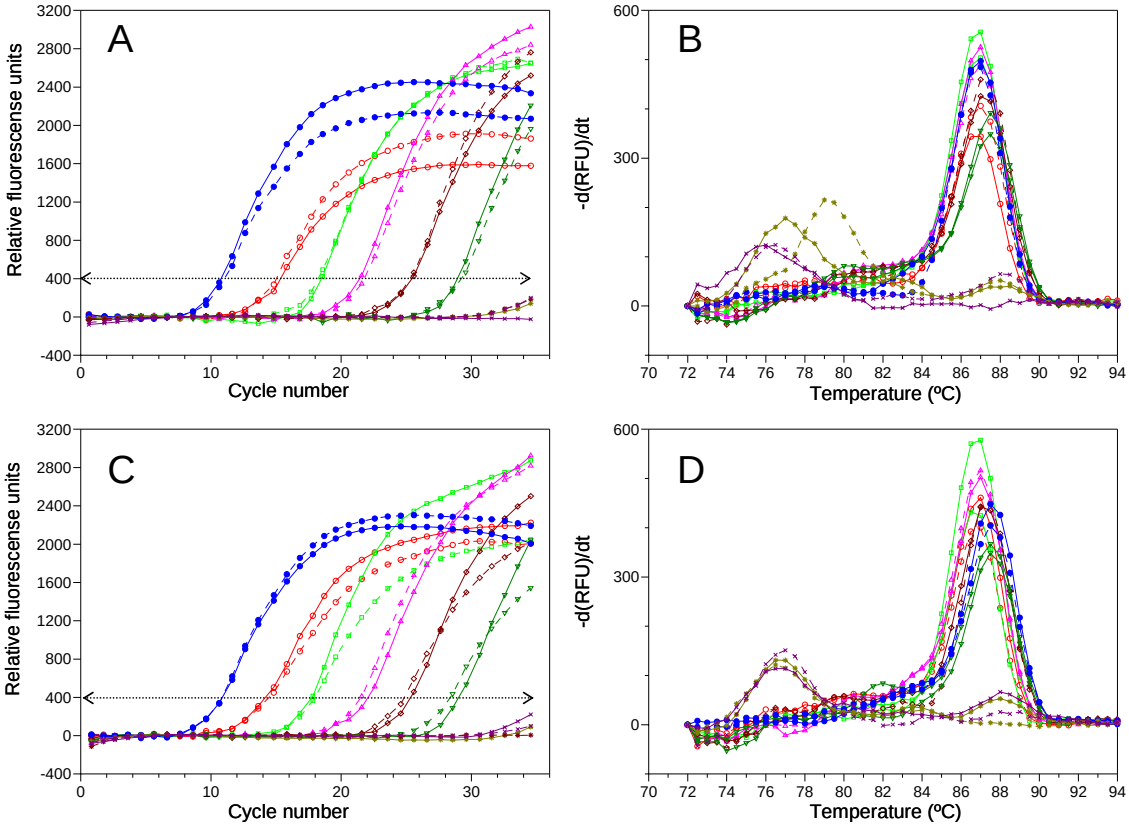

Fig. IV.1.1. Real time PCR amplification profiles generated from cycle-by-cycle collection of *P. arborescens* genomic DNA (10 ng/µl) serially diluted [1:1 (●), 1:10 (○), $1:10^2$ (□), $1:10^3$(△), $1:10^4$(◇), and $1:10^5$(▽)] in: (**A**) ultrapure, sterile water (G_W); and (**C**) a fixed background of *Papaver somniferum* DNA (40 ng/µl) extracted from healthy opium poppy seeds (G_S). The corresponding melting curves analysis for G_W (**B**) and G_S (**D**) are shown as the negative first derivate of the relative fluorescense units -d(RFU)/dt plotted *vs* temperature. (∗) Water (G_W) or *P. somniferum* DNA from pathogen-free, disinfested opium poppy tissues (G_S); (×) no DNA template. Threshold position to determine the PCR cycle number was manually fixed to 400. Each dilution series was replicated twice (solid line and broken line).

After optimizing real-time qPCR assays, standard regression lines were generated for each primer set using a range of DNA from 10 ng to 100 fg. The highest reproducibility of amplifications was obtained with primer set P6 (Fig. 1A,C), for which an elevated efficiency was measured over six orders of magnitude of DNA concentration exhibiting a linear dynamic range of amplification. Also, no significant differences ($P \geq 0.05$) for C_T values occurred with primer set P6 for both DNA backgrounds ($G1_W$ and $G1_S$) and independent experiments; whereas significant differences ($P < 0.0019$) in C_T values were found for primer sets 2 and 3 between independent experiments at least in one of the $G1_W$ and $G1_S$ DNA curves. Standard regression lines obtained for primer set P6 showed very reproducible amplifications, better efficiencies and higher coefficient of determination values (AE = 90.6-100.3%; R^2 = 0.990-0.992) as compared to primer sets P2 (AE = 92.2-112.3%; R^2 =0.778-0.955) and P3 (AE = 83.3-107.8%; R^2 =0.979-0.980) (Fig. 2). Consequently, primer set P6 was selected for subsequent experiments.

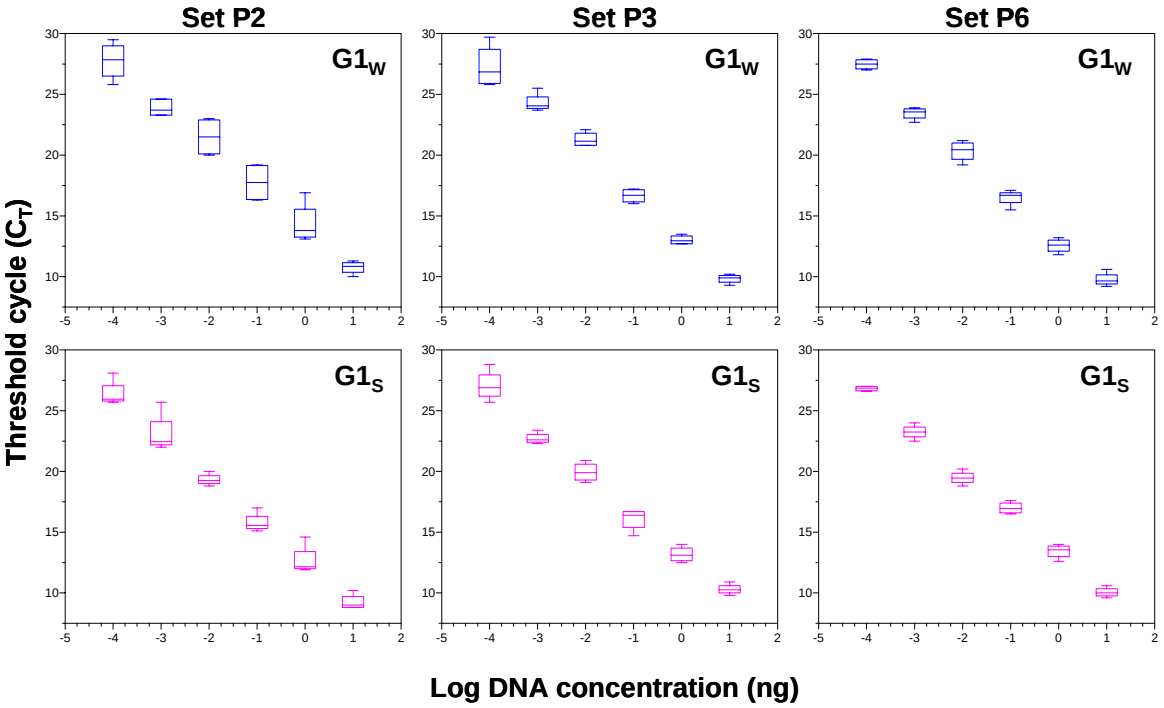

Fig. IV.1.2. Box plots showing the reproducibility of qPCR assays using primer pairs P3Pa2fw/P3Pa2rv (set P2), OMPac1fw/OMPac1rv (set P3) and OMPac7fw/OMPac7rv (set P6). Threshold cycles (C_T) were plotted against the log of *Peronospora arborescens* genomic DNA (from 10 ng to 100 fg) standard curves diluted in ultrapure, sterile water ($G1_W$ graphs) or in *Papaver somniferum* DNA (40 ng) extracted from seeds ($G1_S$ graphs). Each DNA concentration in each background DNA was tested using all three primer sets in the same plate in duplicate and the experiment was repeated twice.

Real-time quantitative PCR reproducibility, efficiency and sensitivity

One-way ANOVA analysis of C_T values derived from $G1_{W,L,S}$-$G2_{W,L,S}$ DNA standard curves indicated homogeneicity of variances ($P \geq 0.05$) and that neither the origin of the DNA sample (G1 *vs* G2 DNA series) ($P = 0.9330$) nor the operator ($P = 0.8898$) or background DNA (W, L and S) ($P = 0.8831$) significantly influenced real-time qPCR results. Consequently, the four series of real-time qPCR data obtained in independent assays were pooled for further analyses (Table 2). The efficiency and reproducibility of real-time qPCR assays of G-series and P-series DNA standard curves was positively influenced by the presence of the source of host DNA (i.e., leaf and seed tissues). Thus, for G-series, accuracy and AE of real-time PCR assays was higher ($0.2<SD<0.6$; $R^2 > 0.993$; AE = 93.0-93.1%) when *P. arborescens* DNA was diluted in leaf (G_L) or seed (G_S) DNA compared to that when DNA was diluted in water (G_W) ($0.5<SD<0.8$; $R^2 = 0.978$; AE = 90.5%) (Table 2; Fig. 3A). Statistical comparisons indicated that the standard regression lines of the three curves differ significantly in the intercept ($P < 0.0001$) but not in the slopes ($P = 0.7664$). Indeed, the intercept of G_W standard regression line was significantly higher than that of G_L and G_S standard regression lines, which indicate lower sensitivity o the former. Thus, *P. arborescens* DNA was not accurately quantified at concentrations lower than 1 pg if diluted in water (G_W series) and the linear dynamic range of amplification was lost at this pathogen concentration. Conversely, *P. arborescens* DNA could be quantified up to 0.1 pg when diluted in host leaf or seed DNA, and standard deviations of replicated did not increase for G_L and G_S curves at such a lower concentration (Table 2; Fig. 3A).

As found for G-series, P_W standard regression lines showed lower AE and reproducibility of amplifications ($0.3<SD<1.9$; $R^2 > 0.964$; AE > 96.1%) compared with P_L and P_S curves ($0.2<SD<0.5$; $R^2 > 0.990$; AE = 109.1-110.2%). Also, the target DNA could not be quantified consistently in some replications at the highest concentration of 10 ng due to inhibition of the amplification reaction. Comparison of standard regression lines indicated that both the slope and intercept of the P_W standard regression line differed significantly ($P < 0.001$) from those of P_L and P_S regression lines. No significant differences ($P \geq 0.05$) occurred between P_L and P_S regression lines (Table 2; Fig. 3B).

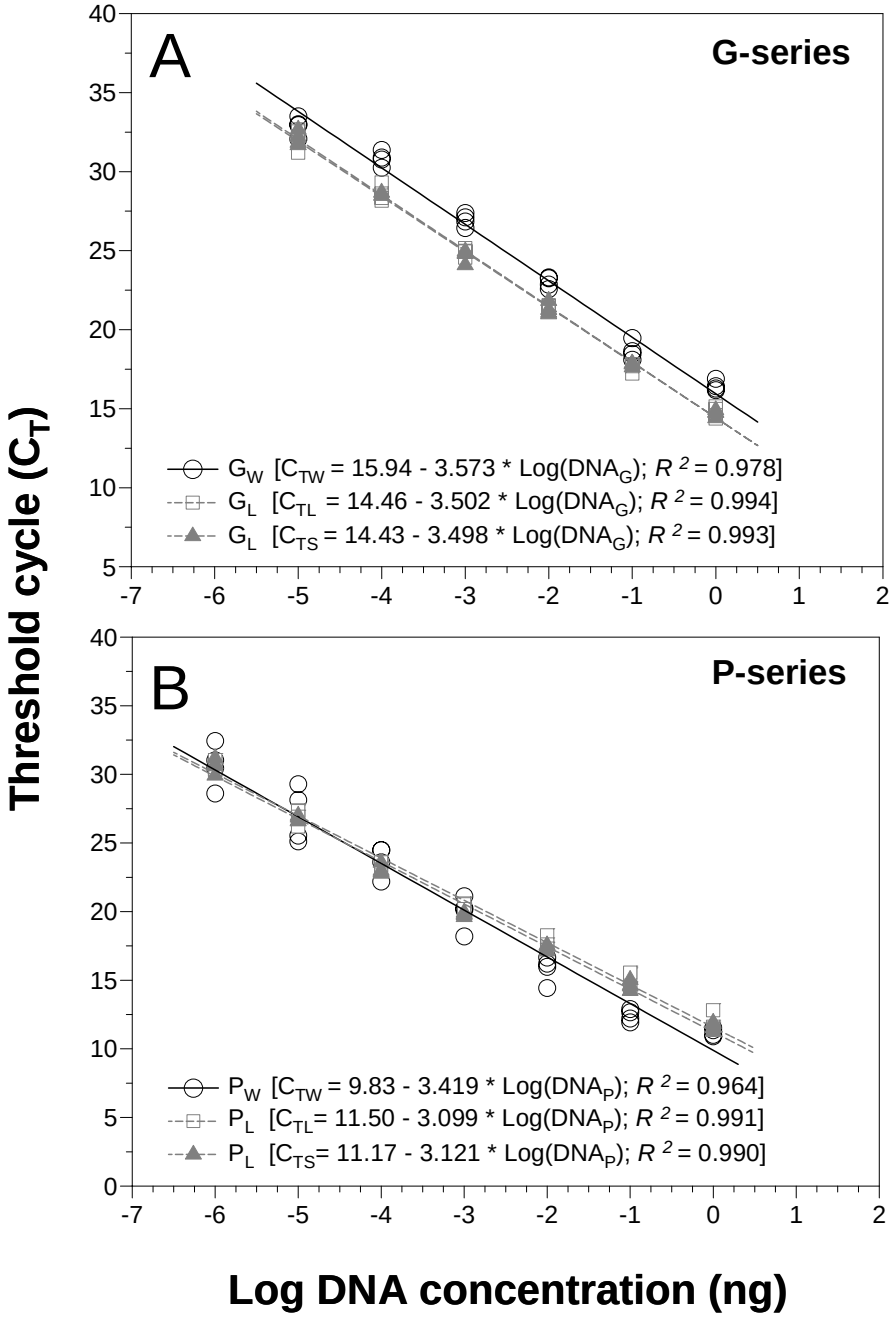

Fig. IV.1.3. Standard regression lines of: (**A**) a five-point 10-fold serial dilution of *Peronospora arborescens* DNA (1 ng/µl; G-series); and (**B**) six-point 10-fold serial dilution of plasmid EC3_P38ESJ DNA (1 ng/µl; P-series) containing the ITS region of the pathogen. DNA was diluted in ultrapure sterile water (G_W and P_W), or in *Papaver somniferum* DNA (40 ng) extracted from opium poppy leaves (G_L and P_L) or seeds (G_S and P_S). Threshold cycles (C_T) were plotted against the log of genomic DNA standard curves of known concentrations. Data shown are from two independent DNA standard curves and two independent operators, and each data point represents the mean of two qPCR replications.

Table IV.1.2. Sensitivity of *Peronospora arborescens*-specific primers used in single-, nested-PCR assays, and the newly developed quantitative-PCR (qPCR) protocols using purified DNA of *P. arborescens* singly or mixed with host DNA.

PCR	DNA	DNA	DNA of *Peronospora arborescens* (pg/µl)[b]							Negative
Protocol[a]	SC[b]	ng/µl[b]	1,000	100	10	1	0.1	0.01	0.001	Control
Single	G_W	0	+/+	+/+	±/±	-/-	-/-	-/-	np	-/-
	G_L	40	+/+	+/+	+/+	+/±	-/-	-/-	np	-/-
	G_S	40	+/+	+/+	+/+	±/±	-/-	-/-	np	-/-
Quantitative	G_W	0	+/+ 16.1±0.8	+/+ 18.8±0.5	+/+ 22.8±0.6	±/- 26.7±0.7	±/- 30.6±0.8	-/- NA	np	-/- na
	G_L	40	+/+ 14.8±0.3	+/+ 17.9±0.5	+/+ 21.6±0.5	+/+ 25.0±0.4	+/+ 28.5±0.6	±/± 31.9±0.4	np	-/- na
	G_S	40	+/+ 14.8±0.3	+/+ 17.9±0.4	+/+ 21.5±0.4	+/+ 24.8±0.5	+/+ 28.5±0.6	±/± 31.6±0.2	np	-/- na
	P_W	0	+/+ 11.1±0.3	+/+ 12.3±0.4 0.0.84	+/+ 15.6±0.9	+/+ 19.5±1.1	+/+ 23.2±1.1	±/- 27.1±1.9	-/- 30.8±1.5	-/- na
	P_L	40	+/+ 11.4±0.3	+/+ 14.4±0.5	+/+ 17.2±0.4	+/+ 20.2±0.3	+/± 23.3±0.5	+/+ 26.7±0.4	±/- 30.7±0.4	-/- na
	P_S	40	+/+ 11.6±0.3	+/+ 14.3±0.3	+/+ 17.4±0.2	+/+ 19.8±0. 3	+/+ 23.0±0.4	+/+ 26.5±0.4	±/- 30.6±0.5	-/- na
Nested	G_W	0	+/+	+/+	+/+	+/+	+/±	+/±	np	-/-
	G_L	40	+/+	+/+	+/+	+/+	+/+	+/+	np	-/-
	G_S	40	+/+	+/+	+/+	+/+	+/+	+/+	np	-/-

[a] Primers OMPac7fw/OMPac7rv (set P6) were used in single and nested-PCR protocols as previously described (27, 35) or in a quantitative PCR assay (This study).

[b] Four independent DNA standard curves (SC) were obtained by serially diluting *P. arborescens* DNA (1 ng/µl; G-series) or plasmid EC3_P38ESJ DNA (1 ng/µl; P-series) containing the ITS region of the pathogen, to obtain from 1 ng to 10 fg of DNA per qPCR reaction in sterile distilled water (G_W and P_W; 0 ng background DNA) or in *Pap. somniferum* DNA extracted from leaves (G_L and P_L; 40 ng background DNA) or from seeds (G_L and P_L; 40 ng background DNA) to investigate any possible influence of host DNA on amplification of pathogen DNA. Results shown were obtained for two series and by two independent operators with the same dilution series. + = Positive amplification; ± = Positive weak amplification; - = no signal; na = no amplification (i.e., C_T values were under a threshold fluorescence value of 400); np = not performed. Numbers shown in the quantitative PCR assays correspond to eight C_T values ± standard deviation from two independent SC, each performed by two independent operators and two replications within each plate.

Since use of host DNA background provided results highly reproducible and amplification efficiency values were within ideal values for real-time qPCR assays, we choose to use G_L, G_S, P_L and P_S DNA standard curves for subsequent experiments.

The sensitivity of the real-time qPCR protocol using primer set P6 was determined for two series of DNA standard curves (G1-$2_{W,S,L}$ and P1-$2_{W,S,I}$) and results were compared to those obtained by gel electrophoresis using the single- and nested-PCR protocols previously developed (27,35). When using G1-$2_{W,S,L}$ curves, the PCR detection limits for single- and nested-PCR assays were similar to those reported previously (27,35); with nested-PCR amplifying up to the lowest amount of pathogen DNA assayed of 0.01 pg (Fig. 4, Table 2). The detection limit of the real-time qPCR assay using *P. arborescens* DNA diluted in USW (G_W series) was greater than that using host leaf DNA (G_L series) or seed DNA (G_S series). Thus, there was a trend for increased detection and sensitivity when host DNA was present in the amplification reaction (Fig. 4, Table 2) as shown previously for the single- and nested-PCR protocols (27,35). The detection limit of real-time qPCR was 0.10 pg of *P. arborescens* DNA for G1-2_W and 0.01 pg for G1-$2_{S,L}$ curves, as determined by the fluorescence signal of the amplicon, but the amplified DNA band was scarcely discernable by gel electrophoresis at those pathogen DNA concentrations (Fig. 4, Table 2). Use of the real-time qPCR detection protocol with independently-built DNA standard curves or by different operators did no influence reproducibility and consistency of results (Table 2). When using plasmid DNA P1-$2_{W,S,L}$ curves, the detection limit for de real-time qPCR assay increased by two (P_W series) or three ($P_{L,S}$ series) orders of magnitude compared to those obtained with their respective DNA G-series curves. In all cases, up to 0.001 pg of plasmid DNA (approximately 233 copies of insert) could be accurately quantified, although P_W series showed less reproducible results (SD > 1.9) (Table 2).

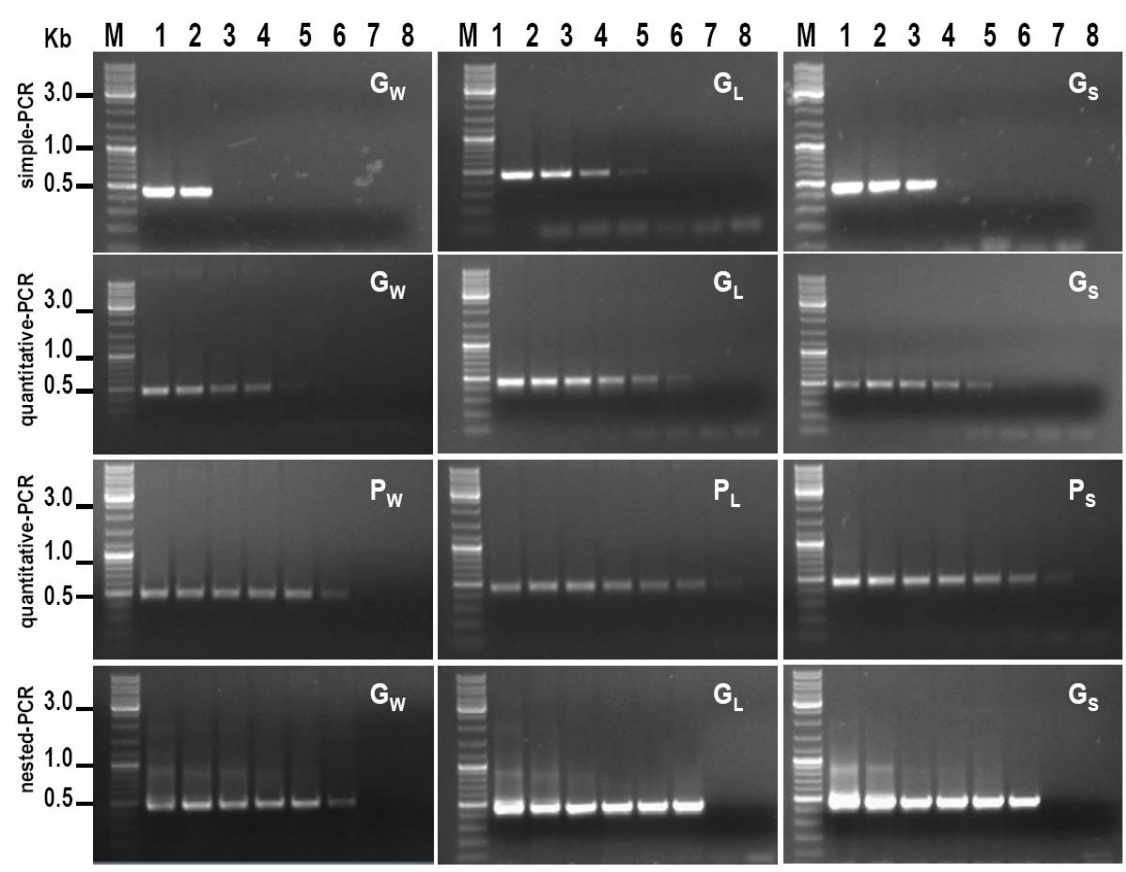

Fig. IV.1.4. Sensitivity in agarosa gel electrophoresis of *Peronospora arborescens*-specific primers OMPac7fw/OMPac7rv (set P6) in single-, quantitative- and nested-PCR assays using *P. arborescens* DNA (1 ng/µl; G-series) or plasmid EC3_P38ESJ DNA (1 ng/µl; P-series). DNA was diluted in ultrapure sterile water (G_W and P_W), or in *Papaver somniferum* DNA (40 ng) extracted from opium poppy leaves (G_L and P_L) or seeds (G_S and P_S). M, Gene-ruler TM DNA ladder mix (Fermentas, St Leon-Rot, Germany); DNA dilution series 1:1 (lane 1); 1: 10^1 (lane 2); 1:10^2 (lane 3); 1:10^3 (lane 4); 1:10^4 (lane 5); 1:10^5 (lane 6); ultrapure, sterile water (G_W), pathogen-free DNA of *P. somniferum* ($G_{L,S}$) or 1:10^6 DNA dilution serie ($P_{W,L,S}$) (lane 7); no DNA template (lane 8).

Development of a universal model for quantifying *P. arborescens* DNA in plant tissues

The real-time qPCR protocol newly developed is highly reproducible and precise as indicated by one-way ANOVA analysis of C_T values derived from 10 independent experiments for each of G- or P-series [five independent DNA standard curves (Table 1) each processed twice by independent operators]. Thus, there was homogeneicity of variances ($P \geq 0.05$) and neither the origin of the DNA sample ($P = 0.9990$ and $P = 0.9724$ for G- or P-series, respectively) nor the operator ($P = 0.9759$ and $P = 0.9467$ for G- or P-series, respectively) or the background DNA (L and S) ($P = 0.7934$ and $P = 0.8558$ for G- or P-series, respectively) significantly influenced results of real-time qPCR assays. All standard regression lines showed high efficiency and reproducibility of amplifications, with AE values ranging from 89.6 to 99.6% for G-series, and from 95.7 to 111.1% for P-series, and standard deviations of C_Ts lower than 0.5 in almost all cases (Fig 5A). Also, comparison of the 10 standard regression lines for each of G- or P- showed no significant differences among slopes ($P = 0.3243$ and $P = 0.1962$ for G-series and P-series, respectively) and intercepts ($P = 0.0998$ and $P = 0.3366$ for G-series and P-series, respectively) of regression lines. Consequently, to obtain a robust and universal regression model, the 10 series of real-time qPCR data obtained independently for each G- or P-DNA series were pooled and used to fit a single regression model for each of G- or P-series (Fig 5B), i.e., G-standard regression model and P-standard regression model, respectively. With this regression equation model, data from each experiment or plate can be validated using the P-series DNA standard curve only. Therefore, it can be now statistically determined if the slopes and intercepts of the P-standard regression line for each experiment do not differ significantly ($P \geq 0.05$) from those of the P-standard regression equation model, and assume then that same would occur for a putative G-standard regression line *versus* the G-standard regression equation model. To validate this, we also included a G-series DNA standard curve in each experiment and compared the two regression lines. Then, the log (DNA_G) concentrations can be estimated by substituting directly the C_T values into the G-standard regression equation model (see below).

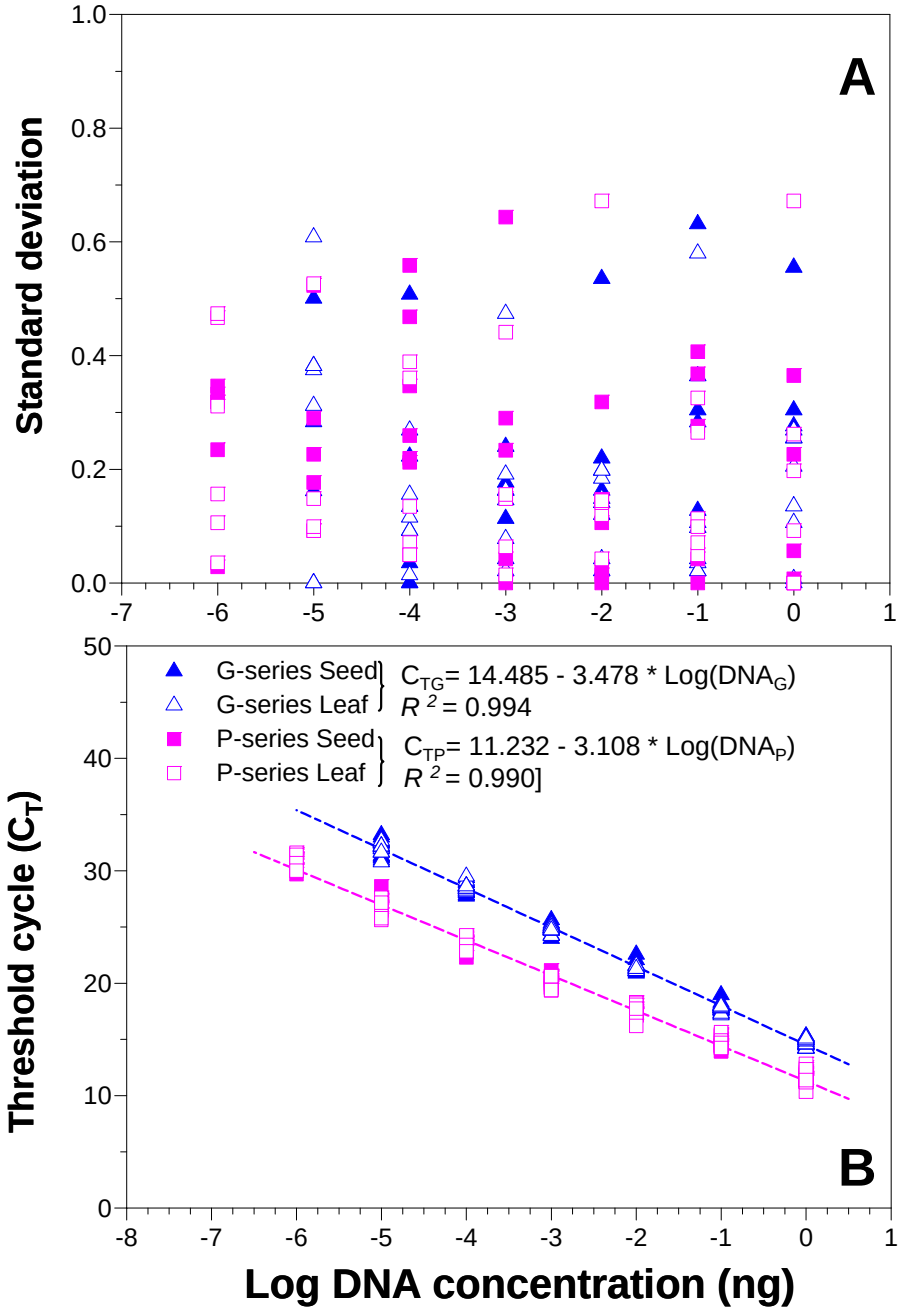

Fig. IV.1.5. (**A**) Standard deviations, and (**B**) standard regression lines of a five-point 10-fold serial dilution of *Peronospora arborescens* DNA (1 ng/µl; G-series) or a six-point 10-fold serial dilution of plasmid EC3_P38ESJ DNA (1 ng/µl; P-series) containing the ITS region of the pathogen. DNA was diluted in *Papaver somniferum* DNA (40 ng) extracted from opium poppy leaves (G_L and P_L) or seeds (G_S and P_S). Threshold cycles (C_T) were plotted against the log of genomic DNA standard curves of known concentrations. Data shown are from five independent DNA standard curves and two independent operators, and each data point represents the mean of two qPCR replications.

Validation of the regression model and quantification of *P. arborescens* DNA in plant tissues

There were not significant differences ($P > 0.405$) among slopes and intercepts of the $G4_L$, $G4_S$ and $P4_L$, $P4_S$ regression lines for the independent PCR plates used for quantification of *P. arborescens* DNA in plant tissues in each of the different experiments performed. Consequently, real-time qPCR data obtained in independent plates were joined and single regression lines were fitted for $G4_L$, $G4_S$ and $P4_L$, $P4_S$ series within each experiment (Fig.6A, Fig.7A). Comparison of $P4_L$ and $P4_S$ regression lines *versus* the universal P-series regression equation model indicated that there were not significant differences between slopes ($P > 0.2061$) and intercepts ($P > 0.1035$). Therefore, we could hypothesize that similar results would have been obtained for $G4_L$ and $G4_S$ regression lines *versus* the universal G-series regression equation model if genomic DNA of *P. arborescens* were not available to built up a G-series DNA standard curve. That was further demonstrated by lack of significant differences between slopes ($P > 0.0905$) and intercepts ($P > 0.0771$) when $G4_L$ and $G4_S$ regression lines were statistically compared with the universal G-series regression equation model.
Therefore, the usefulness of using the universal P-series and G-series regression equation models to quantify *P. arborescens* DNA in plant tissues is now validated, and the log (DNA_G) concentrations can be estimated by substituting directly the C_T values into the G-standard regression equation model once the experiment is validated with a P-series DNA standard curve and its correspondent regression model.

Use of the real-time qPCR protocol allowed detection of *P. arborescens* DNA in 68.2% of stem samples from 22 asymptomatic opium poppy plants that were proved systemically infected with *P. arborescens* by nested-PCR assays in a previous work (Fig. 6); (35). The pathogen could not be quantified with the real-time qPCR assay in those stem samples that showed a weak amplification in the nested-PCR assay (*data not shown*). Amount of *P. arborescens* DNA in samples of stem DNA from asymptomatic plants were very variable, ranging from 0.110 ppm up to 5,557 ppm (5.6‰) (Fig. 6B).

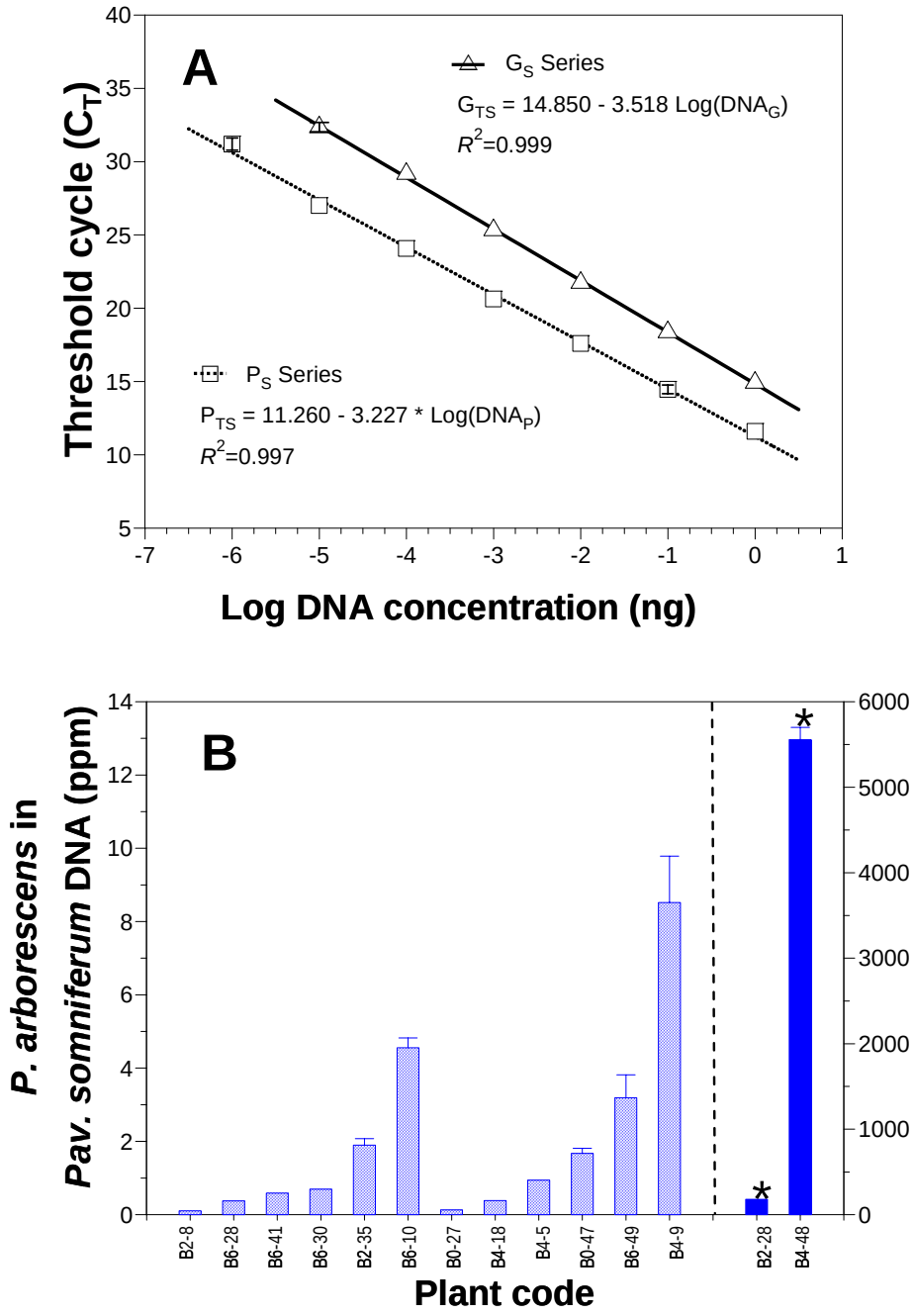

Fig. IV.1.6. Quantification of *Peronospora arborescens* DNA in stem of asymptomatically infected *Papaver somniferum* plants. (**A**) Standard regression lines of a six-point 10-fold serial dilution of *P. arborescens* DNA (1 ng/µl; G_L-series) or a seven-point 10-fold serial dilution of plasmid EC3_P38ESJ DNA (1 ng/µl; P_L -series) containing the ITS region of the pathogen. DNA was diluted in *Pap. somniferum* DNA (40 ng) extracted from opium poppy leaves and used in the qPCR assays. Data shown are the mean of three independent PCR plates and two qPCR replications ±standard deviation. **B**. Relative amounts of *P. arborescens* DNA in *Pap. somniferum* DNA (ppm) quantified in each individual plant. Data shown are the mean of two qPCR replications ± standard deviation. (*) Scale for this two stem samples is on the right Y-axis.

Use of the real-time qPCR protocol allowed detection *P. arborescens* DNA in 100% of commercial seed stocks and 97.0% of seed samples analyzed, and to quantify a pathogen biomass as low as 1.2 pg of target DNA per microgram of seed DNA (Fig.7B), that corresponds to 0.0013‰ of *P. arborescens* in *Pap. somniferum* DNA. The level of detection of the pathogen in seed samples by real-time qPCR assays correlated to that found in previous detection assays of those seed samples using single- or nested-PCR assays (27, 35). The quantity of *P. arborescens* DNA ranged from 0.074 to 0.0013‰ (Fig. 7B), 0.0004 to 0.091‰ (Fig. 7C), and 0.05 to 1.275‰ (Fig. 7D) in seed samples that produced, respectively, null, weak, or positive amplifications in single-PCR assay; and weak, positive, or positive amplifications in nested-PCR assays. In highly-infested seed stocks (e.g., number 923, Fig. 7D) *P. arborescens* amounted as much as 0.077 % in *Pap. somniferum* DNA which represents approximately 0.256 mg of *P. arborescens* DNA per kg of seed. The amount of pathogen within a seed stock varied among the three independent samples assayed which indicates the need of performing several replicates per seed stock to get accurate pathogen quantification (Fig. 7).

Finally, use of the real-time qPCR protocol for assay of seed samples from 30 individual capsules (11 asymptomatic, 6 with mild downy mildew symptoms, and 13 with evident pathogen sporulation) of field-grown opium poppy allowed detection of *P. arborescens* in 70.0% (21/30) of all samples assayed and 36.4% (4/11) of asymptomatic capsules. In seed samples from asymptomatic capsules in which the pathogen could be quantified, *P. arborescens* amounted 0.0003-0.007‰ in *Pap. somniferum* DNA. On the other hand, in symptomatic capsules, *P. arborescens* amounted 0.006-0.61‰ (capsules with mild symptoms) or 0.12-2.53% (capsules with pathogen sporulation) in *Pap. somniferum* DNA (*data not shown*).

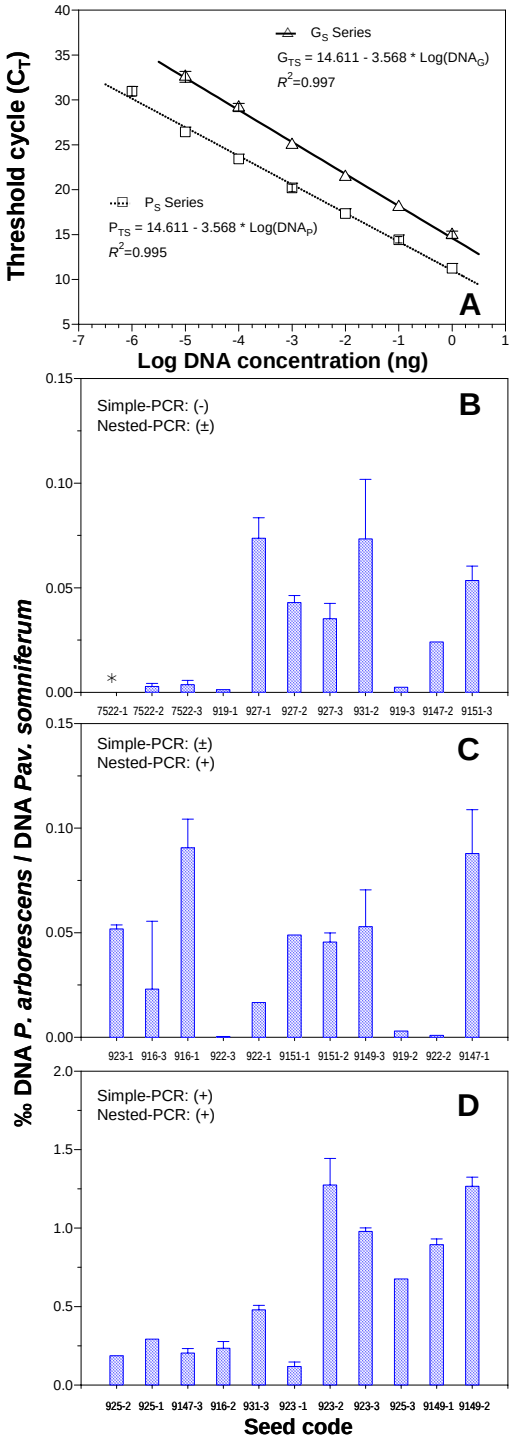

Fig. IV.1.7. Quantification of *Peronospora arborescens* DNA in commercial seed stock samples of *Papaver somniferum*. **A.** Standard regression lines of a six-point 10-fold serial dilution of *P. arborescens* DNA (1 ng/µl; G_L-series) or a seven-point 10-fold serial dilution of plasmid EC3_P38ESJ DNA (1 ng/µl; P_L -series) containing the ITS region of the pathogen. DNA was diluted in *Pap. somniferum* DNA extracted from opium poppy seeds and used in the qPCR assays. Data shown are the mean of two independent PCR plates and two qPCR replications ±standard deviation. **B**, C, D. Relative amounts of *P. arborescens* DNA in *Pap. somniferum* DNA (‰) quantified in each seed sample (300 mg). Data shown are the mean of two qPCR replications ± standard deviation. (+), (±) and (-) indicate positive, weak or no amplification, respectively, after simple or nested-PCR assays using same DNA samples as shown in Montes-Borrego et al. (35). (*) This sample was below the detection limit of the qPCR assay.

IV.1.5 DISCUSSION

The exact in planta identification and proper quantification of obligate biotrophic plant pathogens would be much useful for epidemiological studies and control strategies of the diseases they cause. In this study, we developed a new, reliable, innovative, and quick real-time qPCR assay that allows the exact identification and accurate quantification of *P. arborescens* in asymptomatic opium poppy tissues and seed stocks of use by opium poppy producers and researchers for field-scale studies at a viable, economic cost. Although the, real-time qPCR technology is becoming widely used for the detection and quantification of fungal plant pathogens (reviewed by e.g. 38, 39, 41), its use for oomycetes has been mainly for *Phytophthora* spp. and *Pythium* spp. (e.g., 2,5,7,8,20,24,31,40,42,44,46,49,50). To the best of our knowledge, only three studies dealt with the development of real-time qPCR protocols for the identification or quantification of *Peronospora* spp. causing downy mildew diseases (9,21,48). Although species-specific PCR protocols using primers targeting the ITS rDNA region were available for the diagnosis of either *P. arborescens* or *P. cristata*, the two downy mildew pathogens of opium poppy described in the literature, those protocols are of qualitative but not of quantitative nature (27,35,43).

In our research, we paid careful considerations to each of stages of the experimental protocol, starting from the laboratory setup and proceeding through sample acquisition, template preparation (both test samples and DNA standard curves), and finally the real-time qPCR step. Each of those stages was properly validated, which made it possible to obtain very reliable quantitative data and a robust real-time qPCR assay. Accurate and reliable detection and quantification of a target sequence by qPCR assays is influenced by a number of factors, including linearity, precision, specificity, sensitivity, amplification efficiency, quality of genomic DNA to be quantified, and accuracy of DNA standard curves among others (47,52). Therefore, all processes in the assay must be subjected to strict optimization. In our research, we made a strong case of that requirement being satisfied in the course of the study as it will be described below.

An additional caveat in our study was that the technology developed could be used at a cost economically assumable by opium poppy agronomists, breeders, pathologists, etc., on a practical basis at a field scale for field studies. For that reason, we selected SYBR Green I for the qPCR assay which reaction cost is lower compared

with that of real-time PCR assays that depend on hybridization of labeled-sequence specific probes (41,44). However, as a disadvantage, the SYBR Green I does not discriminate between different double-stranded (ds) DNA molecules but bind to any of them, such as nonspecific products and primer dimmers, that may disturb the results (52). In our assay, this potential problem was solved by using a highly specific primer set (i.e., primer P6) developed in earlier studies (27,35), as well as by quantifying the target amplicon at a Tm of 83°C at which the potential nonspecific products would be denatured. That assured that only the fluorogenic emission from the target amplicon was quantified (44,51). Moreover, that was done without reducing the amplification efficiency of our assay as has been described in other experimental scenarios (51). Additionally, after the final amplification cycle, the analyses of the melting curve temperature profile and gel electrophoresis products confirmed that only one PCR product was amplified from templates with genomic DNA of *P. arborescens* and no amplification product was obtained in the negative controls (host DNA or water).

For absolute DNA quantification in real-time qPCR assays, the accuracy of quantification of unknown samples depends entirely on the accuracy of the DNA standard curves (47,52). In the development of real-time qPCR protocols, the technique most commonly used for measuring nucleic acid concentration is the assessment of absorbance at 260 nm because it is easy to perform, inexpensive and allows rapidly checking of DNA purity (260/280nm ratio). The major disadvantages of this method are the relative lower sensitivity of the assay and the interference with RNA, single-stranded DNA, or single nucleotides and contaminants that may contribute to the signal (37). As an alternative, some of fluorescent dyes (as for example the one present in the kit used in our study) are highly selective for dsDNA over RNA, and provide a fluorescence signal which is unaffected by many common contaminants as well as an increase in sensitivity extending the detection range significantly as compared to absorbance determination (15,37,45); a disadvantage is the increased cost of the technique.

We believe that one of the reasons for high reproducibility, precision and accuracy of the real-time qPCR assay newly developed in this work was that special care was taken to get accurate concentrations of plasmid and pathogen DNAs to build up the DNA standard curves. This was accomplished by quantifying each of the DNA

samples with the Quant-iT DNA assay kit in triplicate, in two independent microplates, and using internal controls of DNA quantification in each assay. Similar results could be obtained using other fluorescent intercalating DNA dyes and kits after appropriate adjustments (37). An additional advantage of the kit used in our research was that it allowed for a final normalization step in our assay since the ratio of pathogen DNA (quantified by the real-time qPCR assay) was referred to host DNA in each test sample (previously quantified by fluorescence). That facilitated adjusting the variation introduced by sample to sample differences in DNA extraction and avoided the cost of quantifying the host DNA by additional qPCR assays (13,48,52). Another possible reason for high reproducibility, precision and accuracy of the new real-time qPCR assay was the way by which we calculated the threshold cycles (C_{Ts}). In most real-time qPCR assays developed for the quantification of plant pathogens, either no information is given regarding threshold position calculation or that is normally calculated by the equipment's software. Nowadays, many instruments automatically calculate the best baseline for each plot individually thus providing the most accurate C_{Ts} for each plate. C_{Ts} are the basis of any real-time qPCR assay as they are used to determine copy numbers and are defined on the basis of the threshold position that is not a constant or absolute value since background fluorescence it is influenced by changing reaction conditions. As a consequence; we believe that calculating a different threshold for each treatment or plate may result in an error specially when trying to combine different plates for a single experiment (10). In this present work, a DNA standard curve was included in each microplate when performing a real-time qPCR assay. Also, the threshold position was manually defined and fixed after appropriate validation at the same position for all treatments and experiments, which made it possible to compare independent plates and pool results after adequate verification of them being statistically similar ($P \geq 0.05$). The position was chosen on the basis of establishing a compromise between detection of the C_Ts in the exponential phase of the reaction and at the same time avoidance of background fluorescence (10,47).

In our assay, the amplification efficiency (AE), sensitivity and reproducibility of qPCR assays of both G-series and P-series DNA standard curves was influenced positively by the presence of DNA from both opium poppy leaf and seed tissues. Thus, there was a trend toward higher sensitivity when host DNA was present in the reaction which also occurred in the simple- and nested-PCR protocols designed

previously (27,35). Other authors found that AE, accuracy and detection limit either were reduced (28,29,40,48), or did not vary (2,48), when a background DNA of host plant tissues rather that water was used in the qPCR assays. However, most studies did not asses the influence of host DNA in those parameters of the qPCR assay (e.g., 21,40,42,44). It has been reported that low concentrations of target DNA favor the formation of primer-dimers thus lowering the sensitivity and producing skews of the standard curves. Also, DNA may adhere to the plastic ware and addition of some host or foreign matrix DNA to the qPCR mix can minimize the loss of target DNA (2,17,21).

Because experimental variation is unavoidable, it is important that results of a qPCR assay are validated by measuring intra- and inter-assay variation. Although most studies estimate such variations using C_T values only, this may not be the most appropriate procedure since these values are logarithmic units and will misrepresent true variability (52). In our study, we made statistical comparisons of slopes and intercepts of the standard regression lines obtained for both *P. arborescens* genomic DNA (G-series) and plasmid DNA (P-series) standard curves as well as for leaf and seed background DNA. A similar approach to this validation procedure by comparisons of either one or the two regression line parameters was recently used by Atallah et al. (2) and McNeil et al. (33). In our research, one-way ANOVA analysis of C_T values and the statistical comparisons among slopes and intercepts of standard regression lines derived from 10 independent experiments indicated high precision and reproducibility of our results which demonstrated high reliability of the newly developed real-time qPCR assay. All standard regression lines showed high efficiency and reproducibility of amplifications, with low standard deviations of C_{Ts}, AE values ranging from 89.6 to 111.1% for G- and P-series, and a linear dynamic range over 6 orders of magnitude with R^2 values exceeding 0.99. Furthermore, statistical analysis of the 10 independent DNA standard curves obtained using different plant and pathogen DNA sources (Table 1) indicated that neither the origin of the DNA sample, nor the background DNA or the operator performing the experiment significantly influenced results of real-time qPCR assays, which again validated the robustness of the protocol developed. This allowed fitting a single regression equation models for each of G- or P-series (Fig 5B) with which it is now possible to validate data from experiments using only the P-series DNA standard curve based on the plasmid DNA containing the *P.*

arborescens target gene. With that, a universal protocol has been established for the quantification of *P. arborescens* in opium poppy tissues, since the P-series DNA standard curve can be built and stored for a long-time period without loss of accuracy. More importantly, the P-series DNA standard curve can be interchanged among laboratories and facilitate the use of the newly our real-time qPCR protocol on a large scale and by different laboratories.

To the best of our knowledge, an optimization process of quantifying the amount of plant pathogens in host tissues by real-time qPCR assays as strict as that carried out in the present study has not been done before. Conversely, most of studies reported in the literature dealt with a single or few experiments for optimization of the real-time qPCR assay and show results from just a single standard regression line for pathogen DNA diluted in water or host DNA (e.g., 2,9,21,40,44). Possibly, as indicated by some authors (10), it will worthwhile and necessary to begin introducing more standard analysis and reporting procedures for real-time qPCR assays, as has been done for microarray technology in the establishment of the MIAME guidelines (www.mged.org/miame). Certainly, in the absence of such standards for real-time qPCR, it remains with authors to ensure that results of experimental work using this technology are appropriately validated, and that conclusions are rigorously supported by the actual data in order to be reproduced in other laboratories.

One of the main features of our real-time qPCR assay using primer set P6 was its high sensitivity as it was able to readily detect up to 10 fg of *P. arborescens* DNA in 40 ng of host DNA without loss of accuracy. Comparatively, the sensitivity of the newly developed qPCR procedure using that primer set was in-between the single-PCR (10 pg) and the nested-PCR (1 fg) protocols developed previously (27,35) using the same primer set. Interestingly, our qPCR assay showed sensitivity higher than that reported for other qPCR assays using Taq Man probes for quantifying *Plasmopara viticola* on leaves of grapevine (*Vitis vinifera*) (48) or four species of *Phytophthora* (*Phytophthora ramorum, Ph. kernoviae*, *Ph. quercina* and *Ph. citricola*) in different plant species (40), as well as SYBR Green I to quantify *P. sparsa* in *Rubus* spp. (21), *Alternaria brassicae* in cruciferous seeds (14), *Ph. capsici* in pepper (*Capsicum annuus*) genotypes (44) and *Verticillium dahliae* in potato lines (*Solanum tuberosum*) (2), or scorpion primers to quantify *Ph. nicotianae* and *Ph. citrophthora* in citrus (*Citrus sinensis*) roots (24). Such a higher level of sensitivity may be due to the fact that the primer set P6 was developed

from the ITS regions of the rDNA, that occurs in multicopies (e.g., 414 ± 12 copies per haploid genome in *Ph. infestans*) (25). However, some of the studies refereed to above used primers targeting the same ITS regions (24,44).

One of main advantages from the high sensitivity of the newly developed real-time qPCR assay is that use of the protocol allows detection and quantification of the pathogen biomass in complex, naturally infested tissues of diverse origin, including seeds from asymptomatic capsules and commercial seed stocks as well as from asymptomatic opium poppy plants systemically infected by *P. arborescens*. Downy mildews are among the most destructive diseases of crops worldwide and can be seedborne. Consequently, efforts should be made to avoid introduction of infected seeds into areas where the pathogen does not occur. Although the presence of different *Peronospora* spp. on commercial seed lots have been documented by using traditional or specific-PCR assays (6,23), no studies had developed so far real-time qPCR assays to quantify *Peronospora* spp. on seeds as it has been done for several plant pathogenic fungi (11,14,33). Recently, we demonstrated that *P. arborescens* can be seed transmitted (27) and that the pathogen can be detected in commercial seed stocks harvested from opium poppy crops that have either no evidence or low incidence of the disease (35). In this present research we demonstrated that the quantity of *P. arborescens* DNA in commercial seed stocks can range from 0.0013‰ to 1.275‰ in *Pap. somniferum* DNA, and amounts as much as 0.256 mg of *P. arborescens* DNA per kg of opium poppy seed. We also demonstrated that *P. arborescens* within the range of 0.006-0.61‰ of *Pap. somniferum* DNA can be detected in seeds from asymptomatic capsules. This fact is particularly important for companies officially authorized for opium poppy cultivation, like ALCALIBER S. A., and may explain that the downy mildew disease has spread rapidly to large areas within Spain where opium poppy had not been cropped before even though seed stocks for the next year sowings were selected from fields free from symptoms of disease.. Studies are in progress to determine whether or not low inoculum levels of the pathogen on seeds such as those quantified in asymptomatic capsules or capsules with mild symptoms may give rise to a high number of downy mildew-infected plants.

In a previous work, use of a nested-PCR protocol lead to demonstrate the occurrence of asymptomatic, systemic infections of plants grown under controlled conditions (35). More importantly, we recently verified that primary systemic

infections by *P. arborescens* soilborne oospores as well as systemic secondary infections by airborne sporangia may give rise to viable, infected seeds in opium plants cv. Nigrum free from symptoms (M. Montes-Borrego, B. B. Landa, J. A. Navas, F. J. Muñoz-Ledesma and R. M. Jiménez-Díaz, *unpublished results*), as it has been demonstrated for other downy mildew pathogens (e.g., *Plasmopara halstedii/Helianthus annuus* (12); *Peronospora ducometi/Fagopyrum sculentum* (53). Those results are of particular importance for establishing disease control strategies based in use of pathogen-free seed stocks, since the absence of symptoms in a plant does not guarantee production of healthy seeds. In our study we have quantified highly variable amount of *P. arborescens* DNA in stem samples from asymptomatic plants, ranging from 0.110 ppm to up to 5,557 ppm (5.6‰). It would worthwhile to establish a relationship between the amount of *P. arborescens* DNA present in the stem of an asymptomatic plant and the subsequent success of seed infection.

Most of real-time qPCR protocols developed for in planta quantification of plant pathogens were aimed to use with symptomatic host tissues (21,31,40,48), but only a for a few of them there have been reports on the quantification of pathogens in tissues free from symptoms or affected with low disease severity (44,49). The opium poppy cv. Nigrum in which asymptomatic systemic infections by *P. arborescens* were demonstrated is considered moderately resistant to downy mildew (F. J. Muñoz-Ledesma, *personal communication*). Consequently, results of this present research would be of much interest for the assessment of plant-pathogen interactions in this pathosystem based on the direct measurement of tissue colonization by the pathogen since lack of symptoms does not correlate with lack of infection. Indeed, several opium poppy lines and accessions have identified in the ALCALIBER S.A. breeding program that show no downy mildew symptoms under high level of disease pressure and *P. arborescens* soilborne inoculum in the field. Therefore, determining whether or not the asymptomatic reactions in those varieties are due to resistance or tolerance to pathogen infection will be of great value for identifying sources of resistance of use in the breeding of highly resistant, commercial opium poppy varieties through hybridization.

In summary, we consider that the real-time qPCR protocol developed in this study for the in planta identification and quantification of *P. arborescens* in opium poppy plant tissues and seed stocks will be of much importance for advancing our

understanding of the epidemiology and management of this downy mildew disease, and facilitate the knowledge-based implementation of appropriate control strategies such as quarantine and certification schemes as well as use of host resistance.

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IV.1.7 LITERATURE CITED

1. **Adams, P. S.** 2006. Data analysis and reporting, p. 39-62. In: M. Tevfik Dorak (ed.), Real-Time PCR. Taylor & Francis, New York.
2. **Atallah, Z. K., and W. R. Stevenson.** 2006. A methodology to detect and quantify five pathogens causing potato tuber decay using real-time quantitative polymerase chain reaction. Phytopathology **96:**1037-1045.
3. **Atallah, Z. K., J. Bae, S. H. Jansky, D. I. Rouse, and W. R. Stevenson.** 2007. Multiplex real-time quantitative PCR to detect and quantify *Verticillium dahliae* colonization in potato lines that differ in response to *Verticillium* wilt. Phytopathology **97:**865-872.
4. **Aranda, S., M. Montes-Borrego, F. J. Muñoz-Ledesma, R. M. Jiménez-Díaz, and B. B. Landa.** 2008. First Report of *Pectobacterium carotovorum* causing soft rot of opium poppy in Spain. Plant Dis. **2:**317.
5. **Avrova, A., E. Venter, P. R. J. Birch, and S. C. Whissona.** 2003. Profiling and quantifying differential gene transcription in *Phytophthora infestans* prior to and during the early stages of potato infection. Fung. Genet. Biol. **40:**4-14.
6. **Belbahri, L., G. Calmin, J. Pawlowski, and F. Lefort.** 2005. Phylogenetic analysis and Real Time PCR detection of a presumbably undescribed *Peronospora* species on sweet basil and sage. Mycol. Res. **109:**1276-1287.

7. **Bilodeau, G. J., C. A. Lévesque, A. W. A. M. de Cock, C. Duchaine, S. Brière, P. Uribe, F. N. Martin, and R. C. Hamelin.** 2007. Molecular detection of *Phytophthora ramorum* by real-time polymerase chain reaction using TaqMan, SYBR Green, and molecular beacons. Phytopathology **97:**632-642.
8. **Böhm, J., A. Hahn, R. Schubert, G. Bahnweg, N. Adler, J. Nechwatal, R. Oehlmann, and W. Oβwald.** 1999. Real-time quantitative PCR: DNA determination in isolated spores of the mycorrhizal fungus *Glomus mosseae* and monitoring of *Phytophthora infestans* and *Phytophthora citricola* in their respective host plants. J. Phytopathol. **147:**409-416.
9. **Brouwer, M., B. Lievens, W. Van Hemelrijck, G. Van den Ackerveken, B.P.A Cammue, and B. P. H. J. Thomma.** 2003. Quantification of disease progression of several microbial pathogens on *Arabidopsis thaliana* using real-time fluorescence PCR. FEMS Microbiol. Lett. **228:**241-248.
10. **Bustin, S. A., and Nolan, T.** 2004. Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. J. Biomol. Tech. **15:**155-166.
11. **Chilvers, M. I., L. J. du Toit, H. Akamatsu, and T. L. Peever.** 2007. A real-time, quantitative PCR seed assay for *Botrytis* spp. that cause neck rot of onion. Plant Dis. **91:**599-608.
12. **Cohen, Y., and W. E., Sackston.** 1974. Seed infection and latent infection of sunflowers by *Plasmopara halstedii*. Can. J. Bot. **52:**231-238.
13. **Gachon, C., and P. Saindrenan.** 2004. Real-time PCR monitoring of fungal development in Arabidopsis thaliana infected by Alternaria brassicicola and Botrytis cinerea. Plant Physiol. Biochem. **42:**367–371.
14. **Guillemette, T., B. Iacomi-Vasilescu, and P. Simoneau.** 2004. Conventional and real-time PCR based assay for detecting pathogenic *Alternaria brassicae* in cruciferous seed. Plant Dis. **88:**490-496.
15. **Haugland R. P.** 2005. The Handbook. A Guide to Fluorescent Probes and Labeling Technologies, 10th ed., Molecular Probes, Inc., Eugene, Oregon, USA.
16. **Henson, J.M., and R. French.** 1993. The polymerase chain reaction and plant disease diagnosis. Annu. Rev. Phytopathol. **31:**81-109.
17. **Heuser, T., and Zimmer, W.** 2002. Quantitative analysis of phytopathogenic ascomycota on leaves of pedunculate oaks (*Quercus robur* L.) by real-time PCR. FEMS Microbiol. Letters **209:**295-299.

18. **Higuchi, R., C. Fockler, G. Dollinger, and R. Watson. 1993.** Kinetic PCR analysis: real time monitoring of DNA amplification reactions. Biotechnology **11:**1026-1030.
19. **Hoagland, D. R., and D. I. Arnon.** 1950. The water culture method for growing plants without soil. Calif. Agric. Exp. Stn. Circ. No. 347.
20. **Hughes, K. J. D., J. A. Tomlinson, R. L. Griffin, N. Boonham, A. J. Inman, and C. R. Lane.** 2006. Development of a one-step real-time polymerase chain reaction assay for diagnosis of *Phytophthora ramorum*. Phytopathology **96:**975-981.
21. **Hukkanen, A., L. Pietikäinen, S. Kärenlampi, and H. Kokko.** 2006. Quantification of downy mildew (*Peronospora sparsa*) in *Rubus* species using real-time PCR. Eur. J. Plant Pathol. **116:**225-235.
22. **INCB** (International Narcotics Control Board). 2006. Narcotic drugs. Estimated World Requirements for 2007. Statistics for 2005. New York (Vienna), UN.
23. **Inava, T., K. Takahashi, and T. Morinaka.** 1983. Seed transmission of spinach downy mildew. Plant Dis. **67:**1139-1141.
24. **Ippolito, A., L. Schena, F. Nigro, V.-S. Ligorio, and T. Yaseen.** 2004. Real-time detection of *Phytophthora nicotianae* and *P. citrophthora* in citrus roots and soil. Eur. J. Plant Pathol. **110:**833-843.
25. **Judelson, H. S., and T. A. Randall.** 1998. Families of repeated DNA in the oomycete Phytophthora infestans and their distribution within the genus. Genome **41:**605-615.
26. **Landa, B. B., M. Montes-Borrego, F. J. Muñoz-Ledesma, and R. M. Jiménez-Díaz.** 2005. First report of downy mildew of opium poppy caused by *Peronospora arborescens* in Spain. Plant Dis. **89:**338.
27. **Landa, B. B., M. Montes-Borrego, F. J. Muñoz-Ledesma, and R. M. Jiménez-Díaz.** 2007. Phylogenetic analysis of Downy Mildew pathogens of opium poppy and PCR-based in planta and seed detection of *Peronospora arborescens*. Phytopathology **97:**1380-1390.
28. **Li, W., J. S. Hartung, and L. Levy**. 2007. Evaluation of DNA amplification methods for improved detection of "*Candidatus* Liberibacter species" associated with citrus huanglongbing. Plant Dis. **91**:51-58.

29. **Li, W, D. Li, E. Twieg, J. S. Hartung, L. and Levy**. 2008. Optimized quantification of unculturable *Candidatus* Liberibacter spp. in host plants using real-time PCR. Plant Dis. **92**:854-861.
30. **Lie, Y. S., and C. J. Petropoulos.** 1998. Advances in quantitative PCR technology: 5' nuclease assays. Curr. Opin. Biotechnol. **9:**43-48.
31. **Lievens, B., M. Brouwer, A. C. R. C. Vanachter, B. P. A. Cammue, and B. P. H. J. Thomma.** 2006. Real-time PCR for detection and quantification of fungal and oomycete tomato pathogens in plant and soil samples. Plant Sci. **171:**155-165.
32. **Martin, R. R., D. James, and C. A. Lévesque.** 2000. Impact of molecular diagnostic technologies on plant disease management. Annu. Rev. Phytopathol. **38:**207-239.
33. **McNeil, M., A. M. I. Roberts, V. Cockerell, and V. Mulholland.** 2004. Real-time PCR assay for quantification of *Tilletia caries* contamination of UK wheat seed. Plant Pathol. **53:**741-750.
34. **Montes-Borrego, M., F. J. Muñoz-Ledesma, R. M. Jiménez-Díaz, and B. B. Landa.** 2008. Downy Mildew of commercial opium poppy crops in France is caused by *Peronospora arborescens*. Plant Dis. **92:**834.
35. **Montes-Borrego, M., F. J. Muñoz-Ledesma, R. M. Jiménez-Díaz, and B. B. Landa.** 2009. A Nested-PCR Protocol for detection and population biology studies of *Peronospora arborescens*, the downy mildew pathogen of opium poppy, using herbarium specimens and asymptomatic, fresh plant tissues. Phytopathology **99:**73-81.
36. **Qi, M., and Y. Yang.** 2002. Quantification of *Magnaporthe grisea* during infection of rice plants using real-time polymerase chain reaction and northern blot/phosphoimaging analyses. Phytopathology **92:**870-876.
37. **Rengarajan, K., S. M. Cristol, M. Mehta, J. M. Nickerson.** 2002. Quantifying DNA concentrations using fluorometry: A comparison of fluorophores. Mol. Vision **8**:416-21
38. **Schaad, N. W., R. D. Frederick, J. Shaw, W. L. Schneider, R. Hickson, M. D. Petrillo, and D. G. Luster.** 2003. Advances in molecular-based diagnostics in meeting crop biosecurity and phytosanitary issues. Annu. Rev. Phytopathol. **41:**305-324.

39. **Schaad, N. W., and R. D. Frederick.** 2002. Real-time PCR and its application for rapid plant disease diagnostics. Can. J. Plant Pathol. **24:**250-258.
40. **Schena, L., K. J. D. Hughes, and D. E. L. Cooke.** 2006. Detection and quantification of *Phytophthora ramorum*, *P. kernoviae* , *P. citricola* and *P. quercina* in symptomatic leaves by multiplex real-time PCR. Mol Plant Pathol. **7:**365-379.
41. **Schena, L., F. Nigro, A. Ippolito, and D. Gallitelli.** 2004. Real-time quantitative PCR: a new technology to detect and study phytopathogenic and antagonistic fungi. Eur. J. Plant Pathol. **110:** 893-908.
42. **Schroeder, K. L., P. A. Okubara, J. T. Tambong, C. A. Lévesque, and T. C. Paulitz.** 2006. Identification and quantification of pathogenic *Pythium* spp. from soils in eastern Washington using real-time polymerase chain reaction. Phytopathology **96:**637-647.
43. **Scott, J. B., F. S. Hay, and C. R. Wilson.** 2004. Phylogenetic analysis of the downy mildew pathogen of oilseed poppy in Tasmania, and its detection by PCR. Mycol. Res. **108:**198-295.
44. **Silvar, C., J. Díaz, and F. Merino.** 2005. Real-Time Polymerase Chain Reaction Quantification of *Phytophthora capsici* in different pepper genotypes. Phytopathology **95:**1423-1429.
45. **Singer, V.L., L. J. Jones, S.T. Yue, and R. P Haugland.** 1997. Characterization of Picogreen reagent and development of a fluorescence-based solution assay for double stranded DNA quantitation. Anal Biochem **249:**228-238.
46. **Tooley, P. W., F. N. Martin, M. M. Carras, and R. D. Frederick,** 2006. Real-time fluorescent polymerase chain reaction detection of *Phytophthora ramorum* and *Phytophthora pseudosyringae* using mitochondrial gene regions. Phytopathology **96:**336-345.
47. **Vaerman, J. L., P. Sausssoy, and I. Ingargiola.** 2004. Evaluation of real-time PCR data. J. Biol. Regul. Homeost. Agents **18:** 212-214.
48. **Valsesia, G., D. Gobbin, A. Patocchi, A. Vecchione, I. Pertot, and C. Gessler.** 2005. Development of a high-throughput method for quantification of *Plasmopara viticola* DNA in grapevine leaves by means of quantitative real-time polymerase chain reaction. Phytopathology **95:**672-678.

49. **Vandemark, G. J., and B. M. Barker.** 2003. Quantifying *Phytophthora medicaginis* in susceptible and resistant alfalfa with a real-time fluorescent PCR assay. J. Phytopathol. **151:**577-583.
50. **Wang, Y., W. Zhang, Y. Wang, and X. Zheng.** 2006. Rapid and sensitive detection of *Phytophthora sojae* in soil and infected soybeans by species-specific polymerase chain reaction assays. Phytopathology **96:**1315-1321.
51. **Weng, T., N. Jin, and L. Liu.** 2005. Differentiation between amplicon polymerization and nonspecific products in SYBR green I real-time polymerase chain reaction. Anal. Biochem. **342:**167–169.
52. **Wong, M. L., and J. F. Medrano.** 2005. Real-time PCR for mRNA quantitation. BioTechniques **39:**75-85.
53. **Zimmer, R. C., W.E. McKeen, and C. G., Campbell.** 1992. Location of oospores in buckwheat seed and probable roles of oospores and conidia of *Peronospora ducometi* in the disease cycle on buckwheat. J. Phytopathol. **135:**217-223.

CAPÍTULO V

Bases biológicas del Mildiu de la adormidera

En este capítulo se recogen los resultados relativos al quinto objetivo de esta Tesis Doctoral en el cual se pretendía establecer las bases biológicas del Mildiu de la adormidera, determinando las fuentes de inóculo primario, y los factores que influyen en la epidemiología de la enfermedad

Los resultados obtenidos se recogen en el manuscrito correspondiente al punto V.1, el cual se encuentra en revisión en la revista *Phytopathology* de la Sociedad Americana de Fitopatología, y que lleva por título:

"Sources of primary inoculum for epidemics of downy mildew Caused by *Peronospora arborescens* in opium poppy crops in Spain". *Phytopathology, en revisión.*

V.1

Sources of Primary Inoculum for Epidemics of Downy Mildew Caused by *Peronospora arborescens* in Opium Poppy Crops in Spain

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Phytopathology, en revisión

V.1.1 ABSTRACT

Downy mildew of opium poppy (*Papaver somniferum* L.) caused by *Peronospora arborescens* is one of the most destructive diseases of this crop in Spain and worldwide. In Spain, the pathogen has spread to new areas and attacks by the disease have caused significant decrease in yields during the last few years. However, little was known about sources of primary inoculum for disease development which knowledge can help in the control of the disease. In this study, disease surveys indicated that *P. arborescens* is now widespread throughout the two major opium poppy growing areas of southern and central Spain and that plants are affected by either an early, generalized chorosis and stunting syndrome or localized chlorotic spots with pathogen sporulation that form at later stages of crop development. Also, we determined the nature and source(s) of primary inoculum and infection types for the disease in Spain by means of an integrative experimental approach that combined pathogencity assays in growth chambers and field microplots together with molecular detection assays of *P. arborescens* infection using a specific nested-PCR. Results demonstrate that oospores in infested soil or leaf debris are effective inoculum giving rise to pathogen ingress through underground plant tissues early during poppy seedling growth which result in systemic infections that reproduced the stunting, chorotic syndrome. Infection of underground tissues of older plants by oospore inoculum can remain asymptomatic. Results also indicated that sporangia formed on infected plants are effective in producing secondary local infections that may or may not become latter systemic and either symptomatic or asymptomatic. Finally and more importantly, those late symptomatic or asymptomatic, systemic infections as well as secondary local infections of capsules can give rise to production of infected seeds. Our research on the biology of *P. arborescens* on poppy plants and epidemiology of downy mildew may help to develop knowledge-based disease management strategies of use in the protection of yields of opium poppy crops in Spain and elsewhere.

Additional Keywords: Pathogen biology, overwintering oospores, wild *Papaver* spp., seedborne inoculum, secondary infections

V.1.2 INTRODUCTION

Knowledge about the nature and location of the sources of primary inoculum may be the single most important information for disease management. For downy mildew of opium poppy (*Papaver somniferum* L.) caused by either *Peronospora arborescens* or *P. cristata* (11,12,13,19,27,30), one of the most destructive diseases of this crop worldwide (18,29), little is known about sources of primary inoculum, except that *P. arborescens* was demonstrated seedborne and seed transmitted (20).

Opium poppy is the only source of morphine, codeine, and thebaine drugs for the pharmaceutical industry, which are key components for alleviation of chronic pain associated with cancer diseases. In Spain, opium poppy is grown annually on approximately 9,500 ha, primarily in the southern (Andalucía) and central (Castilla-La Mancha and Castilla-León) regions of the country (20, F.J. Múñoz-Ledesma, *unpublished data*). This acreage accounts for at least 5% of the legally cultivated opium poppy worldwide, making Spain the third and fourth largest European and world producer of poppy straw rich in morphine, respectively (16). During the last decade, yields of the opium poppy crop in Spain have decreased as consequence of attacks from several diseases, including poppy fire caused by *Pleospora papaveracea* (anamorph = *Dendryphion penicillatum*) and downy mildew caused by *P. arborescens* (19,20,24). Until recently, poppy fire was considered the most important disease of the crop (24) but efficient management of this disease has made downy mildew to take over as the main yield limiting factor for poppy crops in Spain (F.J. Múñoz-Ledesma, *unpublished data*). In fact, severe downy mildew attacks occur now throughout all opium poppy-growing areas at Castilla-La Mancha and Andalucía regions of central and southern Spain, respectively. Also, the incidence and severity of those attacks by the disease have increased steadily since the disease was first recorded (19); also the pathogen has spread rapidly into new cropping areas. Changes in agricultural practices, but mainly the expansion of the crop to new cooler and irrigated areas in central Spain for improving harvest yields situates the crop under more conducive environmental conditions, and could be responsible for this dramatic increase in disease incidence and prevalence. This new scenario supposes a serious threat for the stability of opium poppy cultivation in areas where the crop has been grown traditionally. Besides Spain, severe yield losses in opium poppy crops have been reported in other European

countries (22,35) and Tasmania (Australia) (9,30) due to infections by *P. arborescens* and *P. cristata*, respectively.

Downy mildew pathogens can develop local or systemic infections in plants and produce oospores and large numbers of fragile, short-lived sporangia which are believed to disseminate only a few hundred metres by air currents in viable conditions (28). Thus, the main primary inoculum source should be diseased plant parts or soil carrying mycelia and oospores. Despite the importance of opium poppy downy mildew worldwide, little was known in Spain and elsewhere concerning the epidemiology of the disease and how local environmental conditions could influence disease development and severity. Published research on opium downy mildew is scarce and has focused mainly on resistance to the pathogen in varieties of *Pap. somniferum* (4), spatiotemporal analysis of epidemics in Tasmania (29), and molecular detection and genetic diversity of the pathogen (20,30). As a consequence, the biology of the pathogen and epidemiology of the disease are poorly understood. In particular, much information is lacking on the sources and infectivity of *P. arborescens* inoculum. Infection by this pathogen gives rise to abundant production of oospores and sporangia in the plant (12,35). Results from recent research demonstrated that *P. arborescens* can be seedborne and be transmitted from seeds to opium poppy plants, and also suggested that asymptomatic systemic infection of the plant might occur (20). However, the relative importance of oospores for overwintering of the pathogen and of both, oospores and sporangia as primary inocula for the disease has not been assessed yet. Consequently, there was a need of research on the biology of *P. arborescens* on poppy plants and epidemiology of downy mildew to develop knowledge-based disease management strategies that would protect yields of opium poppy crops in Spain and elsewhere.

The objectives of this research were to determine: (i) if *P. arborescens* sporangia from infected plants, and oospores in soil and infected tissues, are effective inocula for the development of opium poppy downy mildew; (ii) if *P. arborescens* can establish systemic infection in the plant that give rise to disease; and (iii) the effects of temperature and plant age on infection and sporulation by the pathogen.

V.1.3 MATERIALS AND METHODS

Disease surveys

Systematic disease surveys and observations on the development of the downy mildew disease under field conditions were carried out during 2003/2004 and 2004/2005 in the main opium poppy growing areas in Spain, including Málaga and Seville provinces of Andalucía region, southern Spain; and Albacete and Toledo provinces of Castilla-La Mancha region, central Spain (Fig. V.1.1). Climatic conditions prevailing during the crop season vary between those two regions which account for differences in the crop cycle. Thus, the crop is sown in late autumn in Andalucía region whereas in Castilla-La Mancha sowings are delayed to late winter or early spring (Fig. V.1.2). Three field plots at Écija (Seville province; Fig. V.1.1) differing in their history of poppy crops in previous years, namely 'Casilla San José' (2-year crop rotation with opium poppy, regular sprinkler irrigation), 'Viso Alto' (no history of opium poppy, no irrigation), and 'La Estrella' (opium poppy being grown 2 years ago, no irrigation), were thoroughly inspected for development of downy mildew at 2-weeks intervals from seedling emergence in early March throughout harvesting in early July 2005. Additionally, 17 commercial crops were inspected at different growth stages from rosette to capsule filling at Antequera (Malaga province), Carmona and Écija (Seville, province), Albacete (Albacete province) and Malpica (Toledo province) during the spring time of 2004 and 2005 (Fig. V.1.1).

In each of the inspected fields, samples from symptomatic and asymptomatic leaves, stems, buds, and capsules of opium poppy plants were randomly collected at different stages of crop phenology. In addition, *Pavaper* spp. weeds in the proximity of inspected fields that have never been grown to opium poppy were collected. Plant samples were observed under the stereoscope 100 x for sporulation of *P. arborescens* and used for specific polymerase-chain-reaction (PCR) assays (described below) to determine infection by the pathogen.

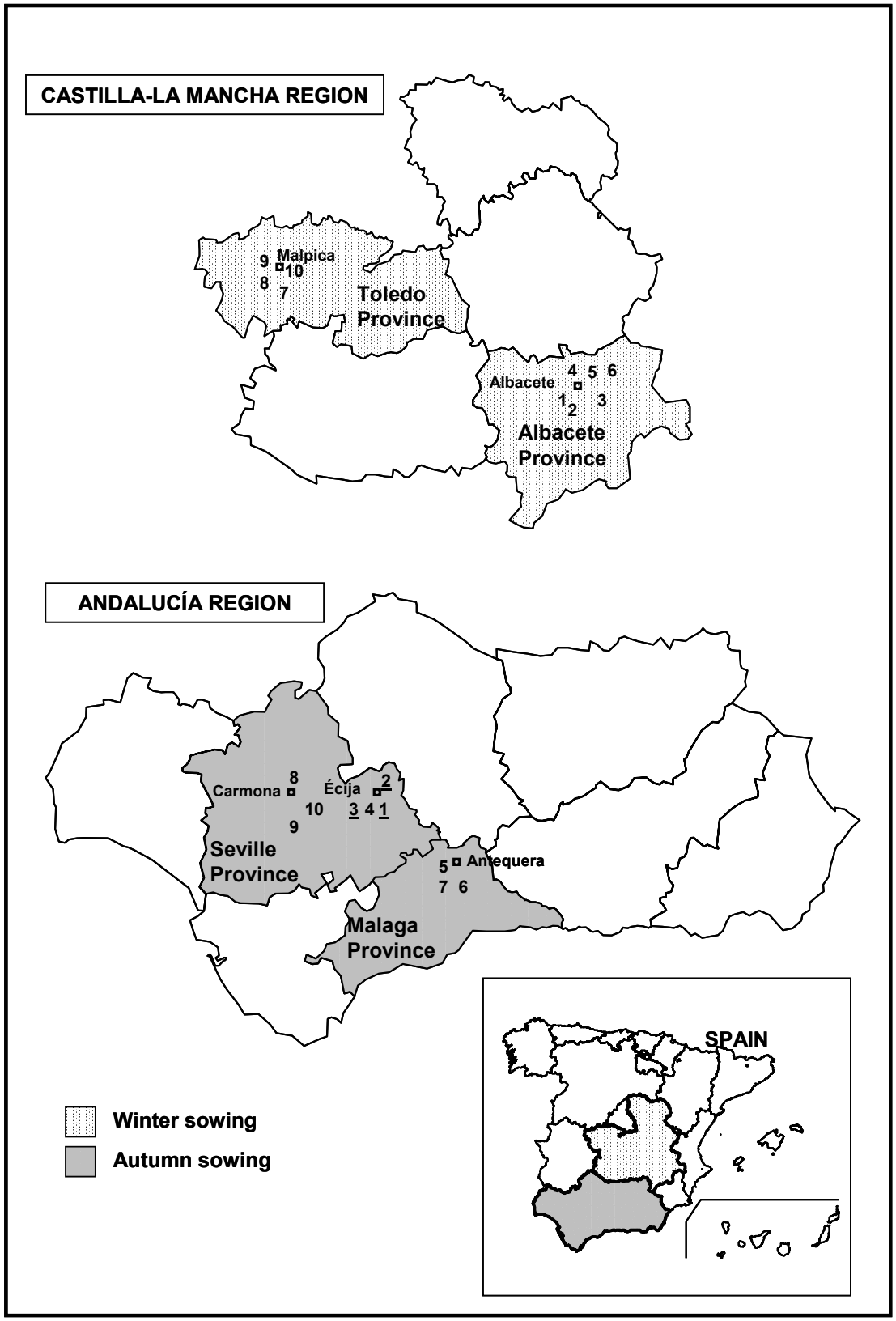

Fig. V.1.1. Location of main areas of opium poppy cultivation in Spain and field crops surveyed: Castilla-La Mancha Region: 1, Grajuela; 2, Santa Paula; 3, Casa Don Pedro; 4, Villar Pozo Rubio; 5, Los Llanos; 6, La Choriza; 7, Valdemerino; 8, Hormigos; 9, Torrejón; 10, Corralejo. Andalucía Region: 1, Casilla San José; 2, La Estrella; 3, Viso Alto; 4, Vacas; 5, Monteluna; 6, Cortijo Río; 7, El Pontón; 8, Rosalino; 9, Santa Clara; 10, Marchena. Underlined field plots in Écija were surveyed systematically for disease development.

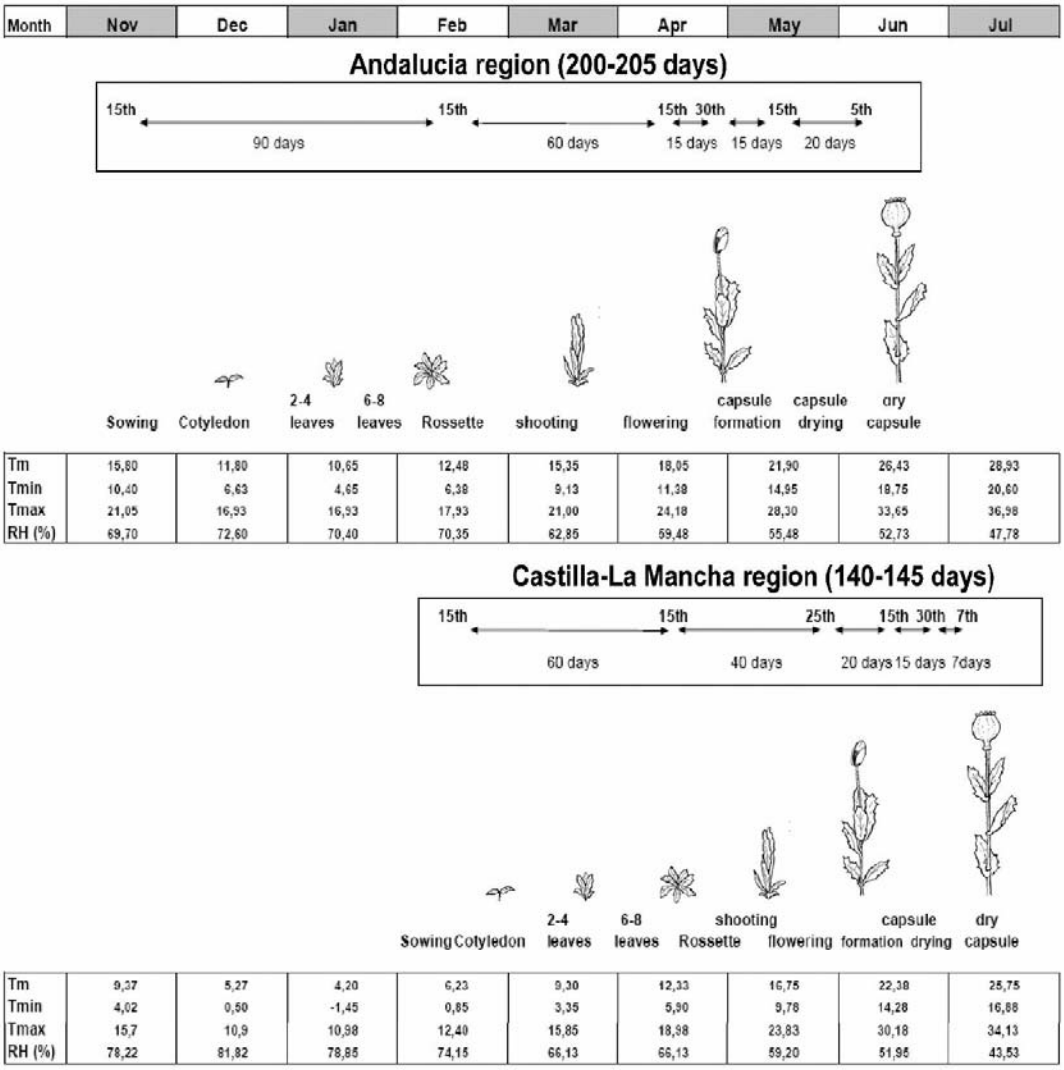

Fig. V.1.2. Stages of opium poppy crop development and climatic conditions [Tm, Tmin, and Tmax = mean, minimum, and maximum, respectively, monthly average temperatures (ºC); RH= relative humidity (%)] in the main areas of opium poppy cultivation in Spain. The approximate duration and dates to reach each growth stage is indicated.

Opium poppy plants

Seeds of opium poppy cv. Nigrun provided by Alcaliber S.A, Carmona (Sevilla province) were used for all experiments. Seeds were surface-disinfested in 1% NaOCl for 5 min, rinsed twice in sterile water, dried up in a flow hood and stored in sterile conditions until used for experiments. Seeds [Growth stage (GS) 0-weeks; GS-0] and plants at growth stages GS-1, -2, -4, and -6 (see below) were used for experiments. Seeds were germinated on sterile layers of filter paper moistened with 2 ml of sterile 50 % Hoagland solution (14) in 9-cm-diameter petri dishes at 20°C for 1 week, and seedlings developed (GS-1) were used for experiments. Alternatively, seeds were sown in a pasteurized (70ºC, 60 min) soil mixture (clay loam/peat, 2:1, vol/vol) in pots (7 x 7 x 6.5 cm) and incubated in a growth chamber (Sanyo MLR-350 H, Sanyo Electric Co., Ltd. Japan) adjusted to 20±1°C, 70/90% relative humidity (RH) and a 12-h photoperiod of fluorescent light at 360 μE $\cdot m^{-2} \cdot s^{-1}$ for 2 (GS-2), 4 (GS-4), or 6 (GS-6) weeks. Plants were watered daily as needed and fertilized every week with 50 ml of Hoagland solution.

Infection of opium poppy using sporangia as inoculum

Different inoculation procedures were tested in the growth chamber using sporangia of *P. arborescens* as inoculum. Sporangia were collected from sporulating opium poppy leaves that were sampled in a commercial field and kept in plastic bags at 4ºC for up to 2 days. Sporangia scrapped off with a sterile needle were placed in 1 ml of ultrapure sterile (US) water with a drop of Tween 20 (Sigma-Aldrich, Madrid, Spain) in 1.5-ml microcentrifuge tubes. Sporangia suspensions were vortexed for a few seconds, filtered through a 0.8-μM Millipore filter that was washed three times with US water (20). Sporangia and sporangiophores retained onto filters were suspended in 1 ml of US water and their concentration in the suspensions was adjusted with US water to 10^3 and 10^6 sporangia/ml using a hemacytometer. Before use, sporangia in the suspensions were observed under the compound microscope 400x to check for morphology and viability.

Four-week-old (GS-4) plants with 6 to 8 true leaves were inoculated by spraying the plant with a sporangia suspension, or placing four drops of the

suspension onto or injecting the suspension into selected leaves. Plants were sprayed 2 to 3 times with sporangia suspensions adjusted to 10^3 and 10^6 sporangia/ml until run off. For injection inoculation, plants were moistened by spraying sterile distilled water onto them and 0.1 ml of the 10^3 and 10^6 sporangia/ml suspensions was then injected into the petiole of three arbitrarily selected leaves using a hypodermic syringe. For the 'drop' inoculation method, plants were moistened as above, then one 25-µl-drop of each of the sporangia suspensions was placed carefully onto the adaxial surface of each of four leaves or at the junction of a leaf petiole with the stem, using a micropipette. Plants similarly treated but using sterile distilled water without sporangia served as controls for the experiments.

After inoculation, plants were sprayed with sterile distilled water and placed inside a moistened bag of transparent polyethylene. Bags were then sealed to facilitate a confined environment of high RH over the inoculated plants. Inoculated and control plants were incubated at 17±1°C in the darkness for 24 hr, and at the same temperature under a 10-h photoperiod of fluorescent light at 360 $\mu E \cdot m^{-2} \cdot s^{-1}$ and 70/90% RH thereafter. Bags were removed three days after inoculation and plants were allowed to dry. There were 30 replicated pots per inoculation treatment (one plant per pot) arranged in a completely randomized design, and the experiment was repeated three times. Plants were watered daily as needed and fertilized every week with 50 ml of Hoagland solution. The experiment lasted for 60 days after the inoculation.

Infection of opium poppy using oospores as inoculum

Four experiments (I to IV) were carried out to determine the ability as inoculum of *P. arborescens* oospores in infested soil and infected tissues as inoculum. Experiment I, II and IV were conducted in growth chambers. Experiment III was done in a shelter under natural environmental conditions. *P. arborescens*-infested soil was sampled from opium poppy crops at 'Casilla San José' and 'Vacas' field plots (Écija, Seville province) in November 2005 and 2006, respectively. Both field plots were selected because of their history of the disease in the previous season when incidence of downy mildew was higher than 10% and 30%, respectively. Four soil samples, each of approximately 500 g, were taken at a soil depth of 0- to 25-cm-from

each plot and oospores of *P. arborescens* were extracted from the soil samples according to Van de Gaag and Frinking (33). Briefly, soil samples were air dried at room temperature (24 to 28 ºC) for 1 week and then sieved through 1x1-cm mesh. The sieved soil (200 g) was homogenized in 2 L of sterile distilled water for 15 min with a magnetic stirrer. Then, the soil suspension was sonicated (Ultrasons, JP Selecta SA, Barcelona, Spain) for 10 min to remove oospores from adhering soil particles and sieved consecutively through 70-, 50- and 30-μm mesh, respectively. The final soil residue was suspended in 10 ml of US water, centrifuged (10,000 x *g*) and the pellet was resuspended in 3 ml of US water. To verify that the extracted oospores were of *P. arborescens,* three samples of oospore suspension from each soil, each of approximately 1 ml, were assayed using the nested *P. arborescens*-specific PCR protocol described below and the amplified product was sequenced as described before (20) to verify pathogen identity.

Additionally, opium poppy plants severely affected by downy mildew and showing different degree of decay were collected on April to June, 2005 and 2006 from the same plots of soil sampling as an alternative source of oospore inoculum. Leaf pieces from sampled plant were dried between sterilized filter paper at room temperature (15 to 35 ºC) from 6 to 7 months Samples of dried tissues were then grinded and observed under compound microscope at 400x for the presence of *P. arborescens* oospores. The dried poppy tissues were grinded to a fine powder using a mill and the powder was stored at 4ºC until use. To verify that oospores in the sampled tissues were of *P. arborescens,* five sets of 50 mg of the powdered tissue were assayed using the *P. arborescens*-specific PCR protocol described below.

For experiment I, 1-week-old (GS-1) opium poppy seedlings plants selected for uniformity were transplanted into 12-cm-diameter clay pots (1.2-L) filled with 1 kg of the pasteurized soil mixture infested with 100 mg of powdered, infested opium poppy leaf tissues containing oospores of *P. arborescens* (treatment A1). Seedlings transplanted into uninfested pasteurized soil mixture served as controls (treatment E1). Plants were grown in a growth chamber (Sanyo MLR-350 H, Sanyo Electric Co., Ltd. Japan) adjusted to 16±1ºC, 70/90% RH and a 10-h photoperiod of fluorescent light at 360 $\mu E \cdot m^{-2} \cdot s^{-1}$. There were 25 replicated pots (three plants per pot) for each treatment in a completely randomized design. Plants were watered daily as needed and fertilized every week with 50 ml of Hoagland solution. The experiment lasted for 120

days after inoculation when plants had formed a capsule in the main stem and lateral buds started flowering.

Experiment II was conducted to determine the effect of temperature (16ºC and 20ºC) and plant growth stage (GS-1 and GS-4) on the ability of *P. arborescens* oospores to establish root infections. Seedlings at GS-1 and GS-4 stages were used. Soil adhered to roots of GS-4 seedlings was removed before transplanting by carefully shaking and dipping the root system in sterile distilled water. Seedlings representative of each of growth stage were transplanted into 15-cm-diameter clay pots (1.2 L) filled with 1.1 kg of soil corresponding to the following treatments: A2, a 5-cm depth layer of a mixture of the pasteurized soil (500 g) and powdered, infested opium poppy leaf tissues (18 g) were placed on top of 600 g of the pasteurized soil; B2, the naturally infested soil sampled from 'Casilla San José' field plot; C2, same as for treatment A2 except that the infested, powdered leaf tissue was sterilized (20 min, 121ºC, two times) previously; D2, same as treatment B2 except that soil was sterilized (40 min, 121ºC, three times) previously; and E2, uninfested pasteurized soil as used in treatment A2. The experiment consisted of a factorial treatment design with four pots (four plants per pot) per treatment combination replicated in a completely randomized design. In summary, the experimental set of treatments was (soil treatment/plant growth stage): A2/GS1, A2/GS-4, B2/GS-1, B2/GS-4, C2/GS-1, C2/GS-4, D2/GS-1, D2/GS-4, E2/GS-1 and E2/GS-4. The experiment was replicated at 16°C and 20°C. Plants were grown in growth chambers (Sanyo Gallencamp PLC, Fitotron™ Leicester, England) adjusted to either 16±1°C or 20±1°C, 70/90% RH and a 10-h photoperiod of fluorescent light at 360 $\mu E \cdot m^{-2} \cdot s^{-1}$. Plants were watered and fertilized as described before. The experiment lasted for 120 days after inoculation when plants had formed a capsule in the main stem and lateral buds started flowering.

Experiment III consisted of same set of experimental treatments and design that Experiment II except that plants were grown in a shelter under natural environmental conditions from middle January to middle June, 2006. The experimental set of treatments in the experiment was: A3/GS1, A3/GS-4, B3/GS-1, B3/GS-4, C3/GS-1, C3/GS-4, D3/GS-1, D3/GS-4, E3/GS-1 and E3/GS-4. Daily mean temperature and rainfall data were recorded in a weather station placed at the experimental site. The experiment consisted of a factorial treatment design with five pots per treatment (four plants per pot) and was arranged in a randomized complete

block design with four replications. Plants were watered and fertilized as described before. The experiment lasted for 150 days after inoculation when plants had formed capsules in the main and lateral buds.

Experiment IV was conducted to confirm results from the previous experiments (I through III). Ungerminated seeds (GS-0) and seedlings at growth stages GS-2, -4, and -6 were used. Sowing of disinfested seeds were sown, and transplanting of seedlings were done as before in 12-cm-diameter clay pots (1.2 L) filled with 1 kg of soil corresponding to the following treatments: A4, a mixture of pasteurized soil where 18 g of powdered, infested opium poppy leaf tissues were placed over and around the seeds or the seedling roots; B4, the naturally infested soil sampled from 'Vacas' plot ; C4, same as for treatment A4 except that the infested powered tissue was previously sterilized; D4, same as treatment B4 except that soil was previously sterilized; and E4, uninfested pasteurized soil as used in treatment A4. In summary, the experimental set of treatments was (soil treatment/plant growth stage): A4/GS-0, A4/GS-2, A4/GS-4, A4/GS-6, B4/GS-0, B4/GS-2, B4/GS-4, B4/GS-6, C4/GS-0, C4/GS-2, C4/GS-4, C4/GS-6, D4/GS-0, D4/GS-2, D4/GS-4, D4/GS-6, E4/GS-0, E4/GS-2, E4/GS-4, E4/GS-6.

Plants were grown in a growth chamber adjusted to 16±1ºC, 60/80% RH and a 10-h photoperiod of fluorescent light at 360 $\mu E \cdot m^{-2} \cdot s^{-1}$.The experiment consisted of a factorial treatment design replicated in a randomized complete blocks with seven replications. There were five pots per treatment (one plant per pot) for treatments A4 and B4, and two pots per treatment (one plants per pot) for treatments C4, D4 and E4. Plants were watered and fertilized as described before. The experiment lasted 150 days after inoculation, when plants had formed capsules in the main and lateral buds.

Microplots experiments

Two experiments (V and VI) were conducted in microplots (1.25 × 1.25 m, 50-cm depth) at two field sites with a sandy loam soil (pH 8.5, 1.4% organic matter) at the Alameda del Obispo Research Station near Córdoba, Spain (latitude 38º north, longitude 5º east). These fields had never been sown to opium poppy before.

In Experiment V, we assayed soils sampled from six downy mildew-affected opium poppy fields at Écija (Seville province) as source of inoculum for the disease

under field conditions. The year before the experiment, disease incidence in those fields was rated low (0 to 1 affected plant per m^2, field 'Ruidero'); moderate (1 to 5 affected plants per m^2, fields 'La Estrella 1' and 'La Proveedora'); and high (> 5 affected plants per m^2, fields 'Casilla San José', 'La Estrella 2', and 'San Rafael'). One hundred liters (approximately 140 kg) of soil was collected at 0- to 25-cm depth in each of the sampled fields in June 2005, just before harvest. The soil was stored at room temperature until used. In October 2005, 1 month before sowing, the collected soil samples were thoroughly mixed with the uppermost 10-cm-depth soil layer of microplots using a MultiSystem with bolo tines accessory (Andreas Stihl, S.A., STIHL Vertriebszentrale AG & Co. KG Dieburg, Germany).

Experiment VI aimed to assay debris of downy mildew-affected opium poppy crops as source of inoculum for the disease under field conditions. Microplots for this experiment were located 2 km away from those of experiment V. In October 2005, 1 month before sowing, 300 g of powdered, infested opium poppy leaf tissues were mixed with the uppermost 10-cm-depth soil layer of microplots as described above.

Microplots which soil had never been cultivated to opium poppy were used as control in both experiments. Microplots were sown to a density of 500 to 600 opium poppy seeds per m^2 (similar to that used in commercial crops) in November 2005. Seeds were surface-disinfested with 0.5% NaClO for 1 min and proved free from *P. arborescens* by means of a *P. arborescens*-specific PCR assay (20). Microplots were fertilized with 100 g "Nitrophoska® Azul Especial" (12% N, 12% P_2O_5, 17% K_2O, 1.2% MgO, 6% S and microelements; Compo Agricultura, Barcelona, Spain) twice; before sowing and before blooming of the crop. Microplots were sprinkler-irrigated as needed. There were three replicated microplots for each treatment arranged in a completely randomized design. All microplots in experiments V and VI were sown again in November 2006 using a seed stock free from *P. arborescens* as determined by the *P. arborescens*-specific PCR assay. Microplots were sown and managed as previously described. Daily mean temperature and rainfall data were recorded in a weather station located at the experimental site.

Disease score and plant sampling

Plants in growth chamber and field experiments were observed at 1- to 2-day intervals until appearance of downy mildew symptoms and signs (i.e., chlorosis, necrosis, and/or sporulation) and at weekly intervals thereafter. Plants showing initial sporulation were removed immediately in Experiments I, II and IV to avoid that airborne sporangia could cause secondary infections that would mask infections by oospores. Conversely, plants that showed sporulation in experiment III were not removed, but allowed for capsules to form as well as secondary and systemic infections to develop that would facilitate to determine if capsule and seed infection may occur.

Five plants from all treatments in experiment III were arbitrarily chosen as representative of those having capsules necrotic or sporulated, or capsules free from necrosis and sporulation but showing sporulation in other plant parts. Seeds were obtained from two capsules necrotic or sporulated and three capsules free from symptoms formed on sporulated plants. Seeds from the capsules were assayed for detection of *P. arborescens* using the nested specific-PCR protocol described below. Similarly, 15 asymptomatic plants of treatments A4, and B4 in experiment IV, and five of treatments C4, D4 and E4, were arbitrarily chosen for in planta detection of *P. arborescens* on basal stem, main stem, and seeds that would indicate asymptomatic systemic infection. For that purpose, a piece of tissue were cut with a sterile scissors from the collar and the fourth and sixth stem internodes of a plant, washed with sterile distilled water and peeled off with a sterile scalpel. Then, sampled tissues were grinded into 0.3- to 0.5-g pieces, placed in 1.5-ml Eppendorf tubes and immediately frozen before DNA extraction. Seeds were collected from a capsule after aseptically removing the peduncle at its bottom. Four samples of approximately 500 seeds each were frozen before DNA extraction.

DNA extraction and specific-PCR assays

Genomic DNA was extracted from symptomatic and asymptomatic opium poppy tissues, seeds, and *P. arborescens* oospores extracted from soil or plant tissues, using the Fast DNA kit (Qbiogene, Madrid, Spain) according to Landa et al. (20).

DNA was quantified using the Quant-iT DNA Assay Kit Broad Range fluorometric assay (Molecular Probes Inc., Leiden, The Netherlands) (20) and a Nanodrop spectrophotometer ND-1000 (Nanodrop Technologies, Wilmington, DE). DNA quality was assessed by staining with ethidium bromide. DNA was diluted with US water to 50 ng/µl.

A newly developed nested-PCR protocol with improved sensibility for in planta detection of *P. arborescens* in asymptomatic opium poppy tissues (23) was used to confirm the presence of *P. arborescen*s in symptomatic and asymptomatic tissues of plants sampled during experiments. Briefly, primer DC6 specific for species in the orders Pythiales and Peronosporales of the Oomycota (6) and the universal ITS4 primer (34) were used in the first round of a nested-PCR protocol. Amplifications were performed as described by Landa et al. (20) in a PTC 100 (MJ Research Inc., Watertown, MA) thermocycler. The resulting PCR product was diluted 1:10 and used as a template for a second round of amplification using primers OMPac7fw/OMPac7rv and the PCR conditions described by Landa et al. (20). Primers OMPac7fw/OMPac7rv were used in the second round PCR instead of primers OMPac1fw/OMPac1rv of Landa et al. (20) because they showed higher sensitivity (23). Sterile distilled water and 50 ng of opium poppy DNA or 1 ng of purified *P. arborescens* DNA were used as negative and positive controls, respectively, in each round of amplification. Twenty microliters of each PCR reaction product were electrophoresed through 1% agarose gel and stained with ethidium bromide to verify amplification. In addition, oospores extracted from soil and seeds from asymptomatic capsules of the growth chamber experiment IV were assayed with the nested-PCR protocol. The amplified products from the second round PCR of these assays were partially sequenced using primer OMPac7fw (20) as described before (23) to verify pathogen identity; aligned sequences were deposited in the Genbank database.

Data analysis

Disease incidence data were analyzed with the GENMOD procedure using the binomial distribution and the logit as link function in SAS (version 9.1, SAS Institute Inc., Cary, NC). A likelihood ratio test was used to determine whether the treatment and/or plant growth stage significantly affected ($P < 0.05$) disease incidence. Statistical

significance ($P < 0.05$) of the likelihood ratio was determined by chi-square test and the contrast statement was used to determine significant differences ($P < 0.05$) between treatments and/or growth stages (2).

V.1.4 RESULTS

Disease surveys

Disease development in the three selected field plots at Écija (Seville province) varied with the history of opium poppy in them. In 'Casilla San Jose' plot (2 year crop rotation with opium poppy, sprinkler irrigation), average incidence of downy mildew ranged from 10 to 15%. Diseased plants were in small patches randomly distributed, each with three to 10 plants (Fig. V.1.3E). First symptoms of downy mildew appeared in plants at the young rosette stage (4 to 6 leaves) by 3 months after sowing in mid November (Fig. V.1.3E). Location of these plants in the field was mapped. Affected plants were dwarf and remained so thereafter, their leaves showing extensive chlorotic lesions or complete chlorosis that started at the base of the blade (Fig. V.1.3A,C,E) and profuse sporulation mainly on the abaxial surface (Fig. V.1.3D). Chlorotic lesions eventually became necrotic. Coalescing lesions gave rise to large necrotic areas on the blade or death of entire leaf (Fig. V.1.3B). Under conducive weather conditions, the entire affected plant died within 2 to 3 weeks after appearance of first symptoms. In the necrotic tissues, numerous oospores were observed while inspecting leaf blades under the compound microscope 400 x (Fig. V.1.3H). Two to 4 weeks after death of those plants, new plants at the rosette stage in their close proximity showed symptoms similar to those described (Fig. V.1.3C) but usually did not die. Later on, after canopy closure, patches of diseased plants were not evident due to disappearance of plants that died at early stages of crop development or to falling of diseased leaves in surviving plants. By late March, at the end of the rosette or shooting stages, wet weather favored the development of small, irregularly shaped, chlorotic to light-yellow leaf lesions (ranging from 0.5 to 3 cm in size) with intense sporulation on the abaxial surface on plants that showed no other symptoms. Those plants were randomly distributed throughout the field. Disease symptoms during the flowering stage were similar to those observed in the remaining field surveyed (see below).

In 'Viso Alto' plot (dry land, no history of opium poppy before), the incidence of downy mildew was lower than 1%. Diseased plants were aggregated in some small patches within the field. First disease symptoms were observed in plants at advanced rosette stage, with 12-14 leaves, by 4 months after sowing in mid November. At this time plants stopped to grow further and became dwarf. Leaves of affected plants showed extensive chlorosis developing from the base of the blade and abundant sporulation. New diseased plants did not appeared at advanced crop growth stages and those previously affected died as temperature increased during the spring season.

In 'La Estrella' plot (dry land, opium poppy had been grown 2 years ago) there was 5% incidence of downy mildew; with affected plants being distributed as those in 'Viso Alto' plot and showing symptoms similar to those described above.

All 17 additional opium poppy fields surveyed in two regions of Spain during springtime of years 2004 and 2005 were affected by downy mildew. Disease incidence and severity varied according to crop history and phenology, which ranged from advanced rosette and blooming to capsule crop stages. Of the 17 fields, seven were located in Antequera (3), Carmona (3) and Écija (1), in Andalucía region, and 10 were in Albacete (6) and Toledo (4), in Castilla-La Mancha region (Fig. V.1.1). In both regions, severe downy mildew symptoms were observed during the advanced rosette stage by mid March in Andalucía and mid May in Castilla-La Mancha. Symptoms were similar to those described above for 'Casilla San Jose' field plot. Also, new disease symptoms developed at the blooming stage and drying of capsules. However a clear difference in disease incidence was observed between the two cropping regions. Incidence of downy mildew in Albacete and Toledo ranged from 15 to 30 % and in general was higher than that in fields located in Andalucía region (ranging from 5 to 15 %).

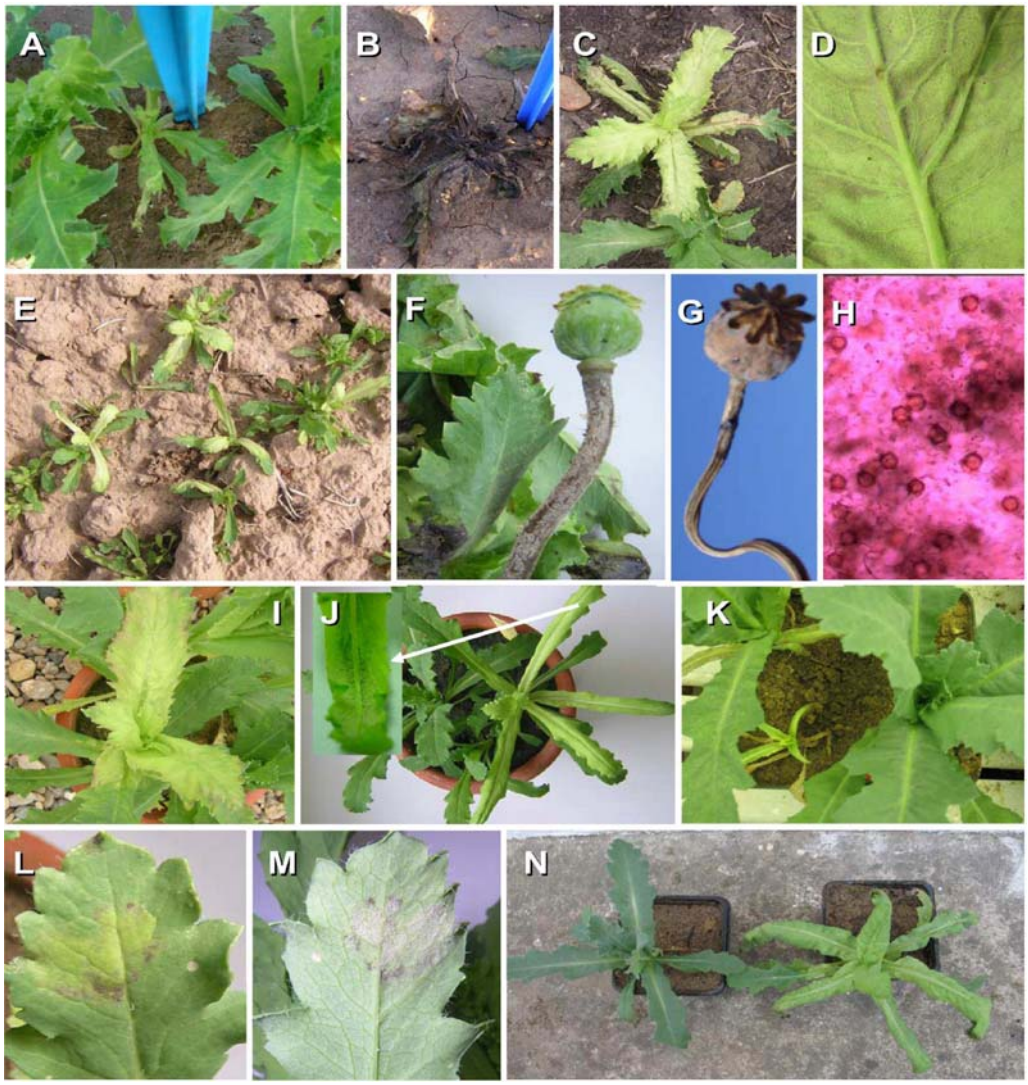

Fig. V.1.3. Symptoms of downy mildew on opium poppy developed in commercial crops in Spain (A-G), or after artificial inoculations using oospores and sporangia of *Peronospora arborescens* as inoculum (H-M). **A,** Plant at the rossete stage (6 to 8 leaves) with chlorosis and stunting. **B,** Dead plant that previously showed symptoms described for A. **C,** Plant at the rossete stage (10 to 12 leaves formed) with generalized chlorosis. **D,** Profuse sporulation on the abaxial side of a chorotic leaf. **E,** Patch of stunted opium poppy plants with generalized chlorosis**. F,** Green capsule formed on the main plant stem that shows profuse sporulation of *P. arborescens*. **G,** Capsule of an infected opium poppy plant which already dried up and shows stunting and little development. **H,** Oospores of *P. arborescens* formed in necrotic leaf tissues (X 10); **I,** Plant at initial shooting stage from Experiment I grown in soil naturally infested with oospores of *P. arborescens*. Note the intensive leaf chlorosis developed. **J,** Plant at the rossete stage (6 to 8 leaves formed) from Experiment II grown in soil infested with oospores of *P. arborescens* formed in naturally infected leaves showing chlorosis and profuse sporulation on the abaxial leaf side (arrow). **K,** Plant grown in soil infested with oospores of *P. arborescens* formed in naturally infected leaves showing extensive leaf yellowish and stunting. **L,** Chlorotic spot and initial necrosis on the adaxial leaf surface of control plants caused by secondary infections by *P. arborescens* sporangia in Experiment III. **M,** *P. arborescens* sporulation on the abaxial side of a corresponding chlorotic spot on the adaxial side as shown in figure L. **N,** Control (left) and systemically infected (right) opium poppy plants which leaves were inoculated by the drop inoculation method using a suspension of *P. arborescens* sporangia.

In the two regions symptoms could be differentiated into two syndromes. The first syndrome occurred on plants at the flowering stage or with mature capsules and included dwarfing of the plant (Fig. V.1.3G) together with generalized chlorosis, curling and deformation of the leaf blade, and sporulation on the abaxial leaf surface (Fig. V.1.3D). The second syndrome was less severe; the plant growth was not affected but downy mildew symptoms presumably due to secondary infections occurred on leaf blades including deformation and chlorotic lesions of variable size (ranging from 2-3 cm^2 up to the entire leaf surface). Abundant sporulation was observed on the abaxial side of chlorotic leaf lesions, the main stem, and less frequently on capsules (Fig. V.1.3F). In general, sporulation on the stem and capsules were less frequent on plants affected in fields at Andalucía region than on those at Castilla-La Mancha, and when present it covered <5% of the tissue surface compared to up 25% in the latter region. Frequently in this region, plants also showed deformations and curling of the main flower peduncle and capsule stalk (Fig. V.1.3G). Interestingly, these symptoms were rarely observed in crops in Andalucía region.

Coincident with the flowering of the crop, high number of wild *Papaver* spp. (mainly *Pap. rhoeas*) were observed within or nearby each of the 20 surveyed fields. Out of 10 *Papaver* sp. plants sampled per field surveyed in Andalucía region, only two of *Pap. rhoeas* at a field located in Monteluna, Antequera, Málaga were infected by *P. arborescens,* as indicated by the nested PCR assay. These plants showed light leaf chlorosis. Neither downy mildew symptoms nor sporulation were observed on other *Papaver* sp. plants. Conversely, some plants of *Pap. rhoeas* sampled from three field plots at Toledo (Castilla-La Mancha region) showed typical symptoms and sporulation of downy mildew. Subsequent nested-PCR assays of affected tissues and sequencing of amplified products confirmed infection by *P. arborescens* (23).

Infection of opium poppy by *P. arborescens* sporangia as inoculum

No symptoms developed on control plants**.** Symptoms of downy mildew developed on inoculated leaves regardless the inoculation method but efficiency of infection varied accordingly with methods. Among the three methods tested, the highest incidence of disease was achieved with the drop inoculation method. Using this method, the first symptoms of downy mildew appeared 10 days after inoculation

and a maximum disease incidence of 20 to 30% (depending of the experiment) was reached 20 days later. Two different types of symptoms were observed on inoculated plants. In the first one, chlorotic areas of approximately of 1 to 3 cm^2 with a small necrotic lesion at their center developed at the site where sporangia were placed (Fig. V.1.3L). Eventually sporulation formed on the abaxial surface of those areas (Fig. V.1.3M) but no further symptoms developed. The second type of symptoms consisted of a generalized chlorosis, leaf curling, and deformation similar to those observed under field conditions (Fig. V.1.3N). These symptoms developed on inoculated leaves following the localized chlorosis described above, as well as in uninoculated leaves of the same plant. Growth of these plants was reduced or eventually ended, and affected leaves showed moderate sporulation and became necrotic within 20-30 days after appearance of first chlorotic lesions. Petioles of leaves affected with generalized chlorosis and deformation as well as internodes separating inoculated from uninoculated leaves of symptomatic plants were sampled and assayed for in-planta detection of the pathogen using the nested-PCR protocol. Results indicated development of systemic infection following formation of chlorotic lesions on inoculated leaf blades (*data not shown*).

The other inoculation methods tested spraying the leaves with a sporangia suspension and injecting the suspension into a leaf petiole) induced symptoms, with much lesser efficiency and gave rise to 3-4% incidence only. When the leaves were sprayed with sporangia the sympthoms were similar to those described for the 'drop' inoculation method, although extension and severity of symptoms was lower. When the sporangia were injected in leaves, a necrosis developed in the inoculation point; then normally no further symptoms developed, and when they appeared, plants showed a generalized plant chlorosis and very rarely sporulation on the abaxial surface of leaves.

Infection of opium poppy by *P. arborescens* oospores as inoculum

Results of the nested, species-specific PCR assays confirmed that *P. arborescens* DNA was present in the powdered leaf tissues and natural soil used as source of inoculum for experiments (*data not shown*). Moreover, partial sequencing of the

amplificon produced in assays using from extracted from oospores as template DNA confirmed the pathogen identity (GenBank Acc. Number EU871705).

In Experiment I, symptoms of downy mildew developed on six out of 75 plants (8.0% of plants) in six out of 25 pots (24.0% of pots).No symptoms developed on control plants. Symptoms developed 3 (two plants, Fig. V.1.3K) or 8 (four plants, Fig. V.1.3J) weeks after transplanting 1-week-old opium poppy seedlings into the infested soil. Symptoms consisted on extensive leaf chlorosis and dwarfing of the plant. Sporulation of the pathogen developed by 1 to 2 weeks after symptoms appearance. These results indicate that *P. arborescens* oospores in debris of infected leaves can serve as primary inoculum for infection of opium poppy early during crop development. .

In Experiment II, both the plant growth stage (GS) and temperature influenced downy mildew development using either powdered, infested leaf tissues or naturally infested soil as source of inoculum. No symptoms developed on control plants. Symptoms developed only in plants transplanted at the GS-1 (1-week old seedlings), regardless incubation temperature (16ºC *vs.* 20ºC). At 20ºC, only one out of 16 plants (6.2 % of plants) transplanted to soil infested with powdered tissues was affected by downy mildew. Symptoms developed by 2 weeks after transplanting and consisted of generalized chlorosis and fast decay of the entire plant, but limited sporulation of the pathogen occurred. At 16ºC, three out of 16 plants grown if soil infested with powdered leaf tissues (18.7% of plants) and one of 16 ones grown in soil infested with natural soil (6.2 % of plants) showed disease symptoms. In both cases, symptoms developed by 4 weeks after transplanting, when plants had formed 6 to 10 leaves. Symptoms consisted on generalized leaf yellowing, curling and deformation of affected tissues. On those tissues, profuse sporulation developed on the abaxial surface (Fig. V.1.3I).

Experiment III consisted of the same set of experimental treatments than Experiment II except for plants were incubated in a shelter under natural environmental conditions. The first downy mildew symptoms appeared on plants at the rosette stage in mid February, 2 months after transplanting. Symptoms were characteristic of those due to systemic infections, including generalized yellowing, bulky plant growth, tissue deformation, and abundant sporulation of the pathogen) (Fig. V.1.3I). Symptoms developed under natural environment were delayed by 3

weeks compared with those that developed in plants similarly inoculated but incubated at a constant temperature of 16ºC in the growth chamber (Experiment II). Later on, symptoms continued to appear until the flower stalk started to form, by 1 month after appearance of first symptoms. The plant growth stage at inoculation influenced both the time to symptom appearance and disease incidence, regardless the type of source of inoculum used. Thus, symptoms developed 59 and 73 days after inoculation of plants at GS-1 using grinded, infested leaves or naturally infested soil as source of inoculum, respectively; but they took additional 14 and 20 days to develop in plants inoculated at GS-4 (4 week old) with that same inoculum, respectively (Table V.1.1). Overall, the incidence of infection was about twice with oospore inoculum in powdered opium poppy leaves compared with that in naturally-infested soil, but the difference was not statistically significant ($P = 0.1071$).

Later on during the course of the experiment, a number of chlorotic areas similar to those formed on leaves inoculated with drops of sporangia suspensions (Fig. V.1.3L-M) developed in plants regardless experimental treatment, including those that served as controls (i.e., treatments C3, D3, and E3). These symptoms probably originated from secondary infections caused by sporangia of *P. arborescens* that formed on plants infected by soilborne inoculum of treatments A3 and B3. Sporulation of the pathogen on leaf tissue might have been favored by environmental conditions prevailing in the shelter during the experiment, (mean temperatures of 8 to 25ºC, relative humidity of 60 to 85%, and cumulative rain of 250 mm). At the end of the experiment, by 150 days after inoculation, total disease incidence resulting from primary and secondary infections in treatments A3 and B3 ranged from 34.6 to 58.8% but they did not differ significantly ($P = 0.896$) (Table V.1.1). Secondary infections in plants of control treatments C3, D3, and E3 resulted in 12.5 to 45.1% final disease incidence that was significant lower ($P < 0.001$) than that reached in plants inoculated with soilborne inoculum (treatments A3 and B3). There were no significant differences between plant growth stage at inoculation ($P = 0.310$) (Table V.1.1).

Table V.1.1. Effect of plant age and *Peronospora arborescens* oospores in soil or leaf debris in the development of systemic infections of opium poppy plants incubated under a shelter and natural environmental conditions in experiment III.

Treatment [a]	Time to first symptom [b]	Symptomatic plants (%) [c]		Number of seed samples infected by *Peronospora arborescens* [d]		
		Primary infections	Primary + secondary infections	Symptomatic capsules	Asymptomatic capsules	Total (%)
A3/GS-1	59	6/62 (9.7)	36/62 (58.1)	2/2	1/3	60.0
A3/GS-4	73	3/65 (4.6)	24/65 (36.9)	2/2	2/3	80.0
B3/GS-1	73	2/52 (3.8)	18/52 (34.6)	2/2	3/3	100.0
B3/GS-4	93	1/68 (1.5)	40/68 (58.8)	2/2	3/3	100.0
C3/GS-1	93	0/51 (0.0)	23/51 (45.1)	2/2	2/3	100.0
C3/GS-4	100	0/52 (0.0)	14/52 (26.9)	2/2	1/3	60.0
D3/GS-1	100	0/55 (0.0)	10/55 (18.2)	2/2	2/3	80.0
D3/GS-4	107	0/64 (0.0)	8/64 (12.5)	2/2	2/3	80.0
E3/GS-1	100	0/67 (0.0)	12/67 (17.9)	2/2	1/3	60.0
E3/GS-4	107	0/65 (0.0)	13/65 (20.0)	2/2	2/3	80.0

Source[e]	df	Chi-square	*P* > Chi-square	df	Chi-square	*P* > Chi-square
Soil treatment (ST)	1	2.60	0.1071	4	51.74	<0.0001
Growth stage (GS)	1	1.64	0.2005	1	1.04	0.3079
ST*GS	1	0.02	0.8939	4	16.37	0.0026
Contrasts: By treatment						
(A3,B3) vs (C3,D3,E3)		-----	-----	1	37.80	<0.0001
A3 vs B3		-----	-----	1	0.02	0.8955

[a] Seedlings at GS-1 (1 week old) and GS-4 (4-week old) stages were transplanted into 15-cm-diameter clay pots (1.2 L) filled with 1,100 g of soil corresponding to the following treatments: A3, a 5-cm depth layer of a mixture of pasteurized soil (500 g) and powered, infested opium poppy leaf tissues (18 g) were placed on top of 600 g of noninfested pasteurized soil; B3, naturally infested soil sampled from 'Casilla San José' plot; C3, same as for treatment A3 except that the infested powered plant tissue was previously sterilized; D3, same as treatment B3 except that soil was previously sterilized; and E3, uninfested pasteurized soil as used in treatment A3.

[b] Days after inoculation and first appearance of symptoms, determined by visual assessment of leaf chlorosis and confirmed by development of pathogen sporulation.

[c] Assessed as the number of plants showing symptoms of downy mildew 93 days after inoculation referred to the total number of plants within a treatment (at this time period, only plants growing in soil mixtures containing inoculum of *P. arborescens* showed symptoms and they were considered to result from primary infections by oospore inoculum); or 150 days after transplant when plants grown in sterilizer soil mixtures showed symptoms as a result of secondary infections.

[d] Determined by the number of samples showing positive amplification for the pathogen DNA using a nested PCR protocol (23) referred to the total number of seed samples.

[e] Likelihood ratio statistics for the effects of plant age and source of *P. arborescens* oospores as inoculum source in the development of primary and secondary infections of opium poppy plants.

Table V.1.2. Effect of plant age and *Peronospora arborescens* oospores in soil or leaf debris in the development of symptomatic and asymptomatic infections of opium plants incubated under controlled conditions in experiment IV.

Treatment[a]	Symptomatic plants (%)[b]	Number of plants with asymptomatic systemic infection by *P. arborescens* (% of assayed)[c]		Total estimated (%)[d]
		Basal stem	4th-6th stem internode	
A4/GS-0	5/35 (14.3)	5/15 (33.3)	4/15 (26.7; 80.0)	42.9
A4/GS-2	7/35 (20.0)	10/15 (66.7)	6/15 (40.0; 60.0)	74.3
A4/GS-4	4/35 (11.4)	7/15 (46.7)	5/15 (33.3; 71.4)	51.4
A4/GS-6	3/35 (8.6)	6/15 (40.0)	4/15 (26.7; 66.7)	45.7
B4/GS-0	2/35 (5.7)	4/15 (26.7)	3/15 (20.0; 75.0)	31.4
B4/GS-2	3/35 (8.6)	6/15 (40.0)	3/15 (20.0; 50.0)	45.7
B4/GS-4	3/35 (8.6)	9/15 (60.0)	5/15 (33.3; 55.6)	62.9
B4/GS-6	2/35 (5.7)	7/15 (46.7)	3/15 (20.0; 42.9)	48.6
C4/GS-0	0/14 (0.0)	0/5 (0.0)	0/5 (0.0; 0.0)	0.0
C4/GS-2	0/14 (0.0)	0/5 (0.0)	0/5 (0.0; 0.0)	0.0
C4/GS-4	0/14 (0.0)	0/5 (0.0)	0/5 (0.0; 0.0)	0.0
C4/GS-6	0/14 (0.0)	0/5 (0.0)	0/5 (0.0; 0.0)	0.0
D4/GS-0	0/14 (0.0)	0/5 (0.0)	0/5 (0.0; 0.0)	0.0
D4/GS-2	0/14 (0.0)	0/5 (0.0)	0/5 (0.0; 0.0)	0.0
D4/GS-4	0/14 (0.0)	0/5 (0.0)	0/5 (0.0; 0.0)	0.0
D4/GS-6	0/14 (0.0)	0/5 (0.0)	0/5 (0.0; 0.0)	0.0
E4/GS-0	0/14 (0.0)	0/5 (0.0)	0/5 (0.0; 0.0)	0.0
E4/GS-2	0/14 (0.0)	0/5 (0.0)	0/5 (0.0; 0.0)	0.0
E4/GS-4	0/14 (0.0)	0/5 (0.0)	0/5 (0.0; 0.0)	0.0
E4/GS-6	0/14 (0.0)	0/5 (0.0)	0/5 (0.0; 0.0)	0.0

Source[e]	df[e]	Chi-square	*F* > Chi-square	Chi-square	*P* > Chi-square	Chi-square	*P* > Chi-square	Chi-square[e]	*P* > Chi-square
Soil treatment (ST)	1	2.76	0.0969	0.16	0.6899	1.08	0.2995	1.33	0.2480
Growth stage (GS)	3	1.48	0.6864	4.55	0.2081	1.10	0.7782	9.49	0.0234
ST*GS	3	0.57	0.9022	2.86	0.4134	0.77	0.8578	6.83	0.0774
Contrast: By GS									
GS0 vs (GS2,GS4,GS6)	1	------	------	------	------	------	------	6.85	0.0089
GS2 vs (GS4,GS6)	1	------	------	------	------	------	------	1.35	0.2459
GS4 vs GS6	1	------	------	------	------	------	------	1.42	0.2330

[a] Seeds (GS-0) or seedlings at GS-2 (2 week old), GS-4 (4 week old) and GS-6 (6 week old) stages were transplanted into 12-cm-diameter clay pots (1.2 L) filled with 1,000 g of soil corresponding to the following treatments: A4, a mixture of pasteurized soil where 18 g of powered, infested opium poppy leaf tissues were placed over and around the seeds or the seedling roots; B4, naturally infested soil sampled from 'Vacas' plot ; C4, same as for treatment A4 except that the infested powered tissue was previously sterilized; D4, same as treatment B4 except that soil was previously sterilized; and E4, uninfested pasteurized soil as used in treatment A4.

[b] Number of plants showing downy mildew symptoms at the end of the experiment (150 days) referred to the total number of plants within a treatment.

[c] The number of asymptomatic systemically infected plants by *P. arborescens* was determined by nested, species-specific PCR assays (23) using DNA extracted from the basal stem of plants or from the 4th to 6th stem internodes of them. For data corresponding to the 4th to 6th stem internodes, the first percentage is the total number of plants assayed, and the second one is the number of plants that showed positive infection of basal stem.

[d] Determined as the sum of symptomatic plants and asymptomatic ones estimated as infected according to the percentage of plants that showed positive molecular detection.

[e] Maximum likelihood analysis of variance from log-linear analyses for the effects of plant age and source of *P. arborescens* oospores as inoculum in the development of symptomatic and asymptomatic infections of opium poppy plants.

At the end of the experiment, seeds were sampled from capsules (five capsules per treatment) that either showed external sporulation (two capsules) or were asymptomatic (three capsules) and assayed for infection by *P. arborescens* using the nested-PCR protocol. Results demonstrated that both primary and secondary *P. arborescens* infections may result in infection of capsules and give rise to infected seeds. Thus, all seed samples from sporulating capsules were positive for the detection of *P. arborescens* (Table V.1.1). Similarly, 60 to 100% of seed samples from asymptomatic capsules of plants in treatments A3 and B3 infected from both primary and secondary inocula were positive for the detection of *P. arborescens* (Table V.1.1). Interestingly, similar incidences of infection (60 to 80%) were detected in seed samples from asymptomatic capsules developed in control plants exposed only to airborne inoculum (treatments C3, D3, and E3).

In Experiment IV, both the powdered leaf tissues and natural soil harboring *P. arborescens* oospores were equally effective in the development of symptomatic ($P = 0.097$) and asymptomatic infected plants ($P = 0.690$), as well as in originating infection regardless symptoms development ($P = 0.248$) (Table V.1.2). There were no symptoms developing on control plants. The incidence of symptomatic and asymptomatic plants was not affected by the plant growth stage ($P \geq 0.05$), but a significantly higher total number of estimated infected plants developed from seeds sown directly in infested soil (GS-0) regardless the inoculum source ($P = 0.009$). However, no significant differences resulted when seedlings of different ages (i.e., GS-2, GS-4 or GS-6) were transplanted into infested soil ($P \geq 0.233$) (Table V.1.2). The basal stem and the 4th to six internodes of symptomatic and asymptomatic plants were sampled and assayed for in-planta detection of the pathogen using the nested-PCR protocol. Results of the molecular assay indicated a high sensitivity of the nested-PCR protocol in the detection of systemic infections by *P. arborescens* in symptomatic as well as asymptomatic plants. This protocol allowed detection of 26.7 to 66.7% infection of asymptomatic plants by assaying tissues of the basal stem and 20.0 to 40.0% infection of those plants using tissues of the 4th to 6th stem internodes (Table V.1.2). Of the asymptomatic plants positive for infection at the basal stem, 42.9 to 80% were also infected at the 4th to 6th stem internodes. Only six of 54 seeds samples from asymptomatic plants assayed with the detection protocol were infected by the pathogen. Of the six positive samples, three corresponded to treatment A4-GS2 and one to each of treatments A4-GS6, B4-GS0 and B4-GS2.

Microplots experiments

Seedlings in the microplots emerged by 20 to 30 days after sowing in November of years 2005 and 2006. Typical symptoms of downy mildew developed on opium poppy plants in 100% of microplots which soil had been infested with either soil from an affected opium poppy field or powdered leaf tissue from affected plants harbouring *P. arborescens* oospores. Symptoms appeared by 40 to 60 days after seedling emergence in 2005 and 2006, respectively. Most of symptomatic plants showed sporulation of the pathogen on the abaxial leaf surface. In general, there was a low incidence of downy mildew in all treatments. As found in growth chamber and shelter experiments, oospores in grinded opium poppy leaf tissues were most successful in originating infection of a plant and gave rise to an average 20% disease incidence. In contrast, 1 to 6% disease incidence was observed when naturally infested soil was used as source of inoculum. In these later cases, the level of disease incidence tended to correlate with the level of disease recorded in the previous season (Table V.1.3). Thus, incidence of downy mildew was similar ($P = 0.734$) in microplots infested with field soils which had moderate or high incidence of disease the year before. The highest levels ($P < 0.001$) of disease incidence occurred in microplots infested with soil sampled from 'La Estrella 2' field plot.

Severe downy mildew symptoms developed in plants at the 4- to 8-leaf stage by late February, which was coincident with occurrence of mild temperatures (10 to 15ºC) and high relative humidity (75 to 90%). These symptoms were similar to those observed in commercial field plots at comparable plant growth stage. In some cases, a few plants in control microplots containing noninfested soil showed downy mildew symptoms (localized chlorotic lesions with sporulation in the abaxial leaf side) late in the season (end of April middle of May). This was likely due to infections caused by airborne sporangia produced on infected tissues of diseased plants growing in artificially-infested microplots. In the second year of the experiment, mean disease incidence also correlated with the level of disease developed in the first year of sowing, and as occurred in that year there was some disease (< 0 to 1 plant per m^2) developing in noninfested microplots that served as control (Table V.1.3).

Table V.1.3. Effect of sources of *Peronospora arborescens* oospores in development of opium poppy downy mildew in microplots experiments under natural environmental conditions.

Microplot treatment	Inoculum source	Previous disease incidence in the field plot [a]	Number of infected plants (1st year of sowing)[b]				Mean disease incidence (2nd year of sowing)[c]
			I	II	III	Average (%)	
Naturally infested soil (Experiment V) [d]							
1	Initial soil/Control	-------	0	1	0	0	Low
2	Estrella 1	Moderate	17	15	12	2 %	Moderate
4	Estrella 2	High	35	32	38	6 %	High
6	Initial soil/Control	-------	0	0	0	0	Low
8	C. San José	High	14	32	29	3 %	High
11	San Rafael	High	7	2	3	< 1 %	High
14	La Proveedora	Moderate	19	13	13	2 %	Moderate
15	Initial soil/Control	-------	0	0	0	0	-
18	Ruidero	Low	0	0	1	0	Low
Infested opium poppy leaf tissues (Experiment VI) [d]							
D	Initial soil/tissues	-------	147	112	89	20%	High
G	Initial soil/Control	-------	0	2	0	0	Low

Source [e]	df	Chi-square	*P* > Chi-square
Treatment	5	711.71	<0.0001
Contrast: By treatment			
Naturally infested soil vs Soil infected with leaf tissues	1	656.40	<0.0001
Infested soil: Moderate vs High [Previous disease incidence(PDI)]	1	0.12	0.7343
Moderate PDI: La Estrella 1 vs La Proveedora	1	0.01	0.9130
High PDI: La Estrella 2 vs (C. San José, San Rafael)	1	59.48	<0.0001
High PDI: C. San José vs San Rafael	1	53.61	<0.0001

[a] The incidence of downy mildew in those fields the year before to the experiment was rated low (0 to 1 affected plant per m^2), moderate (1 to 5 affected plants per m^2) and high (> 5 affected plants per m^2).
[b] Number of individual plants showing downy mildew symptoms 80 days after sowing, and percentage of the total number of plants in a microplot that were symptomatic. For each treatment data are the mean of three replications.
[c] Mean disease incidence in microplots the second year of experiments, scored 80 days after sowing.
[d] In October 2005, 1 month before sowing, 300 g of powered, infested opium poppy leaf tissues were mixed with the uppermost 10-cm depth soil layer of microplots. Microplots which soil had never been cultivated to opium poppy were used as control in both experiments. Microplots were sown to a density of 500 to 600 opium poppy seeds per m^2 in November 2005 and 2006, using surface-disinfested seeds free of *P. arborescens*.
[e] Maximum likelihood analysis of variance from log-linear analyses for the effects of plant age and source of *P. arborescens* oospores as inoculum in the development of symptomatic and asymptomatic infections of opium poppy plants.

V.1.5 DISCUSSION

The main goal of this research was to determine the nature and source(s) of primary inoculum and infection type that determine development of opium poppy downy mildew in Spain. For that purpose, we used an integrative experimental approach that combined pathogencity assays under controlled and field conditions and molecular detection of the pathogen in symptomatic and asymptomatic poppy plants. Our results demonstrate that: (i) oospores in infested soil or leaf debris are effective inoculum giving rise to pathogen ingress through underground plant tissues early during poppy seedling growth that result in symptomatic or asymptomatic systemic infections; (ii) sporangia formed on infected plants are effective in producing secondary local infections that may latter become systemic; and (iii) primary systemic infections by soilborne oospores, and secondary infections by airborne sporangia, either systemic or non-systemic and symptomatic or asymptomatic, can give rise to production of infected seeds.

Downy mildew affected all opium poppy fields surveyed in our study indicating that *P. arborescens* is widespread throughout the two major opium poppy growing areas of southern and central Spain (Fig V.1.1). Two main disease syndromes were differentiated in affected fields during the surveys. Firstly, when infection occurred at early stages of crop development plants were severely stunted, showed generalized chlorosis, and usually died before formation of capsules and seeds (up to 12 to 14 leaves formed). Artificial inoculation experiments successfully reproduced this stunting syndrome, and in-planta detection of the pathogen by means of nested-PCR assays demonstrated that stunting results from systemic infections of underground young tissues by *P. arborescens* oospores in soil or leaf debris. Secondly, localized chlorotic spots were observed on leaves developed at later stages of crop development, with the corresponding abaxial side of those spots showing sporulation of the pathogen. The formation of those chlorotic spots must be due to secondary infections by airborne *P. arborescens* sporangia, as suggested by symptoms developed after artificial inoculation of poppy leaves with drops of a suspension of sporangia collected from naturally infected opium poppy leaves, or by spraying plants with the suspension. Interestingly, these localized infections allowed the pathogen to systemically colonize plant parts distant from the sites of inoculation and produce

additional secondary inoculum. Thus, pathogenesis in the opium poppy downy mildew disease appears to be similar to that caused by other soilborne downy mildew pathogens, such *Pl. halstedii* on sunflower (*Helianthus annuus*) (8), as well as *Peronosclerospora sorghi* on sorghum (*Sorghum bicolor*) and maize (*Zea mays*), and *Sclerospora graminicola* on pearl millet (*Pennisetum glaucum*) (17). Such a pathogenesis characterizes by root infection of young seedlings by overwintering oospores in soil as the primary mode of infection, which results in systemic infection that usually gives rise to either seedling death or an extremely stunted plant with minimal seed yield.

While artificial inoculation experiments demonstrated that *P. arborescens* oospores in soil or leaf debris are effective as inoculum for development of systemic infection of opium poppy plants, results from the field microplot experiments provided further evidence that naturally infested soil is an important source of primary inoculum for the development of epidemics of the opium poppy downy mildew disease. Oospore inoculum in soil may easily increase over the years as infected leaf debris decomposes and release oospores formed in them. In our study, we have observed that leaves on severely affected plants become necrotic, dried, eventually shrivel and contain numerous oospores that hereby have been proved effective as inoculum. Several factors may influence the efficiency of oospores in soil and leaf debris for infection and disease development. In this work, we have addressed the effects of plant age and air temperature on disease development. Overall, *P. arborescens* oospores were more efficient in causing systemic infections of opium poppy seedlings 1-week-old or younger compared to older growth stages, but plants up to 6-week-old plants were infected when transplanted to infested soil. Infection of older plants remained mostly asymptomatic but seeds formed in them were infected by the pathogen. A similar behavior in the plant-pathogen relationships have been reported for other systemic downy mildew diseases (e.g., sunflower downy mildew caused by *Pl. halstedii*, 5,8). Also, a decrease of infection efficiency by sporangial inoculum with increasing plant age was reported for other downy mildew pathogens characterized by local infections of aerial plant parts, such as *P. tabacina* on tobacco (29), *Bremia lactucae* on lettuce seedlings (10) and *P. viciae* on pea (21).

Air temperature also influenced infection and further development of systemic infections by oospores of *P. arborescens*. A constant temperature of 16ºC was more favorable for seedling infection compared with 20ºC. Temperature was shown to be

an important factor in the onset and spread of sunflower downy mildew, the average air temperatures most favourable for infection being in the range of 10 to 15ºC, whereas temperatures above 17ºC were detrimental for oospore germination and development of mycelia within the plant tissue (15). For other downy mildews that characterize by systemic infections, temperature can be more restrictive for systemic infection and colonization by soilborne oospores than for the subsequent sporulation of the pathogen on leaves or for leaf infection and colonization by airborne sporangia in secondary infection cycles (e.g., 1,17). Airborne sporangia of downy mildews are ephemeral and may die within hours after take off from sporulating lesions; however, they can give rise to successful and frequent secondary infections because of the high numbers formed on leaves and that the detrimental effects of suboptimal temperatures for germination and infection can be complemented by longer wetness periods (1,3,17). This may explain that the incidence and severity of symptoms developed on plants incubated under a shelter (Experiment III) or in field microplots under natural environmental conditions, where secondary infections took place, where higher than those occurring in the growth chamber experiments (I, II, and IV), where secondary infections were avoided.

Besides infested soil, infected seeds can be a source of primary inoculum for opium poppy downy mildew. In a recent study, we demonstrated that *P. arborescens* is seed borne and transmitted to newly developed poppy seedlings and that commercial seed stocks harvested from opium poppy crops with high incidence of the downy mildew disease were frequently infected by the pathogen (20). In addition, alternative hosts (i.e., wild *Papaver* spp.) might be potential sources of primary inoculum for the disease. Observations in this present study suggest that wild poppy plants do not play a significant role as a source of primary inoculum for opium poppy downy mildew. Thus, only a few *Pap. rhoeas* plants were found infected by *P. arborescens* in a single field in Andalucía that lacked both symptoms and sporulation of the pathogen. Similarly, only a few wild poppy plants in three fields at Toledo province of Castilla-La Mancha region, central Spain, were found infected by *P. arborescens* though they showed symptoms and sporulation similar to that occurring in cultivated opium poppy. These differences in the infection of wild poppy plants between the two regions might be due to differences in environmental conduciveness for infection and/or host specialization of the pathogen to *Pap. rhoeas* populations present in them. In a recent

study (23), maximum parsimony analysis of ITS rDNA sequences of *P. arborescens* from fresh and herbarium specimens of *Pap. dubium, Pap. hybridum, Pap. rhoeas*, and *Pap. somniferum* from different countries showed that a degree of host specificity may exist within populations of *P. arborescens*. Thus, *P. arborescens* isolates infecting wild poppy were more diverse than those infecting cultivated *Pap. somniferum*. In any case, even if infection of wild poppies can infest opium poppy crops, we have no evidence yet that oospores formed in any of the *Papaver* spp. plants sampled so far. Therefore, *Papaver* spp. do not seem to play an important role in the provision of oospores as primary inoculum for downy mildew epidemics in opium poppy crops and it is doubtful that sporangia produced in wild poppies might significantly contribute to disseminating the pathogen within those crops in springtime in some areas. Similarly to our results, oospores of *P. cristata*, the other downy mildew opium poppy pathogen, were not detected in any of the wild poppy specimens examined in the Derwent Valley of Tasmania (9).

In consequence, considering the limited role, if any, of alternative hosts as a source of primary inoculum, our results about the ability of airborne *P. arborescens* sporangia from diseased opium poppy plants to cause local or systemic infections of capsules that give rise to infected seeds are of much importance for understanding the pathogenesis of the disease. Moreover, research in progress utilizing the same nested-PCR protocol used in this present work have shown that the pathogen can be detected in commercial seed stocks harvested from opium poppy crops that have either no evidence or low incidence of downy mildew (23). In this present study, we have demonstrated that opium poppy seeds can be infected as a consequence of systemic primary infections by oospores, as well as either systemic or non-systemic secondary infections by sporangia, and that both kinds of infection types can take place without symptom expression in the plant. This fact is particularly important since ALCALIBER S. A., the only enterprise officially authorized for opium poppy cultivation in Spain, has practiced an strategy of selecting seed stocks for next year sowings from fields that have not showed any sign of disease. Even with this caution, the downy mildew disease has spread rapidly to large areas where opium poppy had not been cropped before. Our results help to identify that seedborne transmission of *P. arborescens* in commercial seed stocks may have contributed to that disease spread. Seed infection appears to be a general feature of downy mildew pathogens, and it has

been demonstrated in many cases; for example, *P. ducometi* in buckwheat *Fagopyrum esculentum*) (36), *P. manshurica* in soybean (*Glycine max*) (26), *P. tabacina* in tobacco (*Nicotiana tabacum*) (7), *Pl. halstedii* in sunflower (8), and *S. graminicola* in pearl millet (32), but the relative importance of infected seeds as a source of primary inoculum depends upon the specific pathogen and geographic area.

In the opium poppy/*P. arborescens* pathosystem, repeated secondary cycles of infection are thought to drive epidemics under favourable environment once sporangia of the pathogen form on the abaxial leaf side of systemically colonized plants following primary infection events, or development of local secondary foliar lesions. Research in Tasmania demonstrated that airborne sporangia of *P. cristata* are a major factor for downy mildew epidemics and determine the incidence and severity of the diseases due to the rapid spread of the pathogen throughout opium poppy crops (29). Preliminary studies in southern Spain also suggest a similar pattern of pathogen spread and disease development (25). Moreover, oospores formed in infected leaves can eventually contribute to built up of inoculum in soil as they become released following decomposition of affected tissues and .are effective as inoculum just the next crop season of their production on the plant and after incorporation into soil.

In our study, we found that incidence and severity of downy mildew attacks were always higher in opium poppy crops in Castilla-La Mancha region at central Spain compared with those in Andalucía at southern Spain. This could be explained by environmental conditions being more conducive to disease in the former region (Fig. 2), but also be due to differences in cultural practices. While sprinkler irrigation is a common agricultural practice in Castilla-La Mancha, opium poppy crops in Andalucía are mainly rain fed. In fact, opium poppy plants grown in Castilla-La Mancha develop wider canopies where microclimate conducive to disease may easily occur that facilitate the spread of sporangia as well as their survival, germination and infection. This likely result in the higher downy mildew incidence and severity that we observed in this region. Furthermore, disease conduciveness of weather in that region is of particular importance during the flowering and capsule formation stages of the crop because it can increase the risk of seed infection. On the contrary, conducive weather for development of downy mildew in Andalucía occurs only during the earlier growth stages of crop development when canopy closure has not taken place yet. Conversely, the weather conditions (higher temperatures and lower humidity) prevailing later

during the crop growth are less conducive to downy mildew, and therefore secondary infection cycles and particularly seed infection should be less frequent in that area at southern Spain. Since geographical areas in Spain suitable for the crop and yet free of the pathogen share the agro-ecological characteristics prevailing in Castilla-La Mancha region, the disease may likely establish in them if the pathogen spread is not properly controlled. In fact, downy mildew has been recently detected in opium poppy crops in areas at Castilla-Leon region, north-central Spain, where opium poppy had not been grown before (F.J. Muñoz-Ledesma, *personal communication*). The source of *P. arborescens* inoculum in those later areas has not been determined yet.

In summary, the results referred above indicated that in Spain both infected and infested soil are the major sources of primary inoculum for opium poppy downy mildew caused by *P. arborescens*. Consequently, efforts should be taken to avoid used of pathogen infected seed or soils with history of the disease. In this sense, the use of the nested-PCR protocol developed by Montes-Borrego et al. (23) has proved of great use for diagnosing the pathogen in commercial seed stocks and it is being currently used by ALCALIBER S.A. for selecting commercial seed stocks to be used in next year sowings. Research is currently ongoing to adapt that protocol for the detection of the pathogen in soil.

V.1.6 ACKNOWLEDGEMENTS

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V.1.7 LITERATURE CITED

1. Aegerter, B. J., Nuñez, J. J., and Davis, R. M. 2003. Environmental factors affecting rose downy mildew and development of a forecasting model for a nursery production system. Plant Dis. 87: 732-738.
2. Agresti, A. 2002. Categorical Data analysis, 2nd ed. John Wiley & Sons, Inc., Hoboken, New Jersey, 732 pp.
3. Bains, S. S., Jhooty, J. S. 1976. Overwintering of *Pseudoperonospora cubensis* causing downy mildew of muskmelon. Indian Phytopathol. 29:213–214.
4. Bajpal, S., Gupta, M. M., and Kumar, S. 1999. Identification of Indian landraces of opium poppy *Papaver somniferum* resistant to damping-off and downy Mildew fungal diseases. J. Phytopathol. 147: 535-538.
5. Baldini, M., Danuso, F., Turi, M., Sandra, M., and Raranciuc, S. 2008. Main factors influencing downy mildew (*Plasmopara halstedii*) infection in high-oleic sunflower hybrids in northern Italy. Crop Prot. 27: 590-599.
6. Bonants, P., De Weerdt, M. H., Van Gent Pelzer, M., Lacourt, I., Cooke, D., and Duncan, J. 1997. Detection and identification of *Phytophthora fragariae* by the polymerase chain reaction. Eur. J. Plant Pathol. 103: 345-355.
7. Borovskaya, M. 1965. Trasmission of Peronospora tabacina by seeds. Russ. Zascher rash. Med. Boleznei 3:44.
8. Cohen, Y., and Sackston, W. E. 1974. Seed infection and latent infection of sunflowers by *Plasmopara halstedii*. Can. J. Bot. 52: 231-238.
9. Cotterill, P. J. and Pascoe, I. G. 1998. Downy mildew of *Papaver somniferum* in Tasmania. Australas. Plant Pathol. 27: 263-264.
10. Dickinson, C. H., and Crute, J. R. 1974. The influence of seedling age and development on the infection of lettuce by *Bremia lactucae*. Ann. Appl. Biol. 76:49-61.
11. Farr, D. F., Rossman, A. Y., Palm, M. E., and McCray, E. B. 2008. Fungal Databases, Systematic Botany & Mycology Laboratory, ARS, USDA. Online publication Retrieved July 04, 2008, from http://nt.ars-grin.gov/fungaldatabases/
12. Francis, S. M. 1981. *Peronospora arborescens*. CMI Descriptions of Pathogenic Fungi and Bacteria 686:1–2.

13. Gustavsson, A. 1991. The genus *Peronospora* in the Iberian peninsula, especially in northern Spain and Andorra. Anales Jard. Bot. Madrid 49: 3-38.
14. Hoagland, D. R., and Arnon, D. I. 1950. The water culture method for growing plants without soil. Calif. Agric. Exp. Stn. Circ. No. 347.
15. Iliescu, H., Vranceanu, A.V., Pirvu, N., 1977. Unele aspecte privind etiologia manei florii-soarelui si diseminarea ciupercii *Plasmopara helianthi* Novot. An. ICCPT 42: 353-361.
16. INCB (International Narcotics Control Board). 2006. Narcotic drugs. Estimated World Requirements for 2007. Statistics for 2005. New York (Vienna), UN.
17. Jeger, M. J., Gilijamse, E., Bock, C. H., and Frinking, H. D. 1998. The epidemiology, variability and control of the downy mildews of pearl millet and sorghum, with particular reference to Africa. Plant Pathol. 47: 544-569.
18. Kappor, L. D. 1995. Opium Poppy: Botany, Chemistry, and Pharmacology. Haworth Press, Inc., NY.
19. Landa, B. B., Montes-Borrego, M., Muñoz-Ledesma, F. J., and Jiménez-Díaz, R. M. 2005. First report of downy mildew of opium poppy caused by *Peronospora arborescens* in Spain. Plant Dis. 89: 338.
20. Landa, B. B., Montes-Borrego, M., Muñoz-Ledesma, F. J., and Jiménez-Díaz, R. M. 2007. Phylogenetic analysis of downy mildew pathogens of opium poppy and PCR-based in-planta and seed detection of *Peronospora arborescens*. Phytopathology 97: 1380-1390.
21. Mence, M. J., and Pegg, G. F. 1971. The biology of *Peronospora viciae* on pea: Factors affecting the susceptibility of plants to local infection and systemic colonization. Ann. Appl. Biol. 97:297-308.
22. Montes-Borrego, M., Muñoz-Ledesma, F. J., Jiménez-Díaz, R. M. and Landa, B. B. 2008a. Downy mildew of commercial opium poppy crops in France is caused by *Peronospora arborescens*. Plant Dis. 92: 834.
23. Montes-Borrego, M., Muñoz-Ledesma, F. J., Jiménez-Díaz, R. M. and Landa, B. B. 2008b. An improved nested-PCR protocol for the detection of *Peronospora arborescens*, the downy mildew pathogen of opium poppy, from herbarium specimens and asymptomatic tissues useful for population biology studies. Phytopathology 99:73-81.

24. Muñoz-Ledesma, F. J. 2002. Etiología y Control de la Necrosis de la Adormidera *Papaver somniferum* L. University of Córdoba. PhD Thesis. Córdoba, Spain.
25. Navas-Cortés, J. A., Montes-Borrego, M., Muñoz-Ledesma, F. J., Jiménez-Díaz, R. M., and Landa, B. B. 2008. Dinámica espacio-temporal de epidemias de Mildiu (*Peronospora arborescens*) y sus ciclos de patogénesis en adormidera en Andalucía. XIV Congreso de la Sociedad Española de Fitopatología, Septiembre 2008, Lugo, Spain.
26. Pathak, V. K., Mathur, S. B., and Neergaard, P. 1978. Detection of *Peronospora manshurica* (Naum.) Syd. in seeds of soybean, *Glycine max*. EPPO Bull. 8: 21-28.
27. Reid, D. A. 1969. New or interesting British plant diseases. Trans. Br. Mycol. Soc. 52: 19-38.
28. Renfro, B. L. and Shankara Bhat, S. 1981. Role of wild hosts in downy mildew diseases. Pages 107-119 in: The Downy Mildews. Spencer, D.M., ed. Academic Press, London.
29. Reuveni, M., Tuzun, S., Cole, J. S., Siegel, M. R., and Kuc, J. 1987. The effects of plant age and leaf position on the susceptibility of tobacco to blue mold caused by *Peronospora tabacina*. Phytopathology 79: 455-458.
30. Scott, J. B., Hay, F. S., Wilson, C. R., Cotterill, P.J., and Fist, A. J. 2003. Spatiotemporal analysis of epiphytotics of downy mildew of oilseed poppy in Tasmania, Australia. Phytopathology 93: 725-757.
31. Scott, J. B., Hay, F. S., and Wilson, C. R. 2004. Phylogenetic analysis of the downy mildew pathogen of oilseed poppy in Tasmania, and its detection by PCR. Mycol. Res. 108: 198-295.
32. Shetty, H. S., Khanzada, A. K., Mathur, S. B., and Neergaard, P. 1978. Procedures for detecting seed-borne inoculum of *Sclerospora graminicola* in pearl millet (*Pennisetum typhoides*). Seed Sci. Technol. 6: 935–941.
33. Van Der Gaag, D. J. and Frinnking, H. D. 1997b. Extraction of oospores of *Peronospora viciae* from soil. Plant Pathol. 46: 675-679.
34. White, T. J., Bruns, T., Lee S., and Taylor, J. W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: PCR protocols: A guide to methods and Applications. M. A. Innis, D. H. Gelfand, J. J. Sninsky and T. T. White, eds. Academic Press Inc., NY.

35. Yossifovith, M. 1929. *Peronospora arborescens* (Berk.) de Bary parasite tres important de *Papaver somniferum* en Yougoslavie. Rev. Pathol. Entomol. Agric. 16: 235-270.

36. Zimmer, R. C., McKeen, W. E., and Campbell, C. G. 1992. Location of oospores in buckwheat seed and probable roles of oospores and conidia of *Peronospora ducometi* in the disease cycle on buchwheat. J. Phytopathol. 135: 217-223.

CAPÍTULO VI

Discusión General y Conclusiones

VI.1

DISCUSIÓN GENERAL

Las enfermedades constituyen un factor de importancia para los rendimientos del cultivo de adormidera y en consecuencia pueden repercutir negativamente sobre el papel que éste desempeña en la industria farmacológica y la sanidad humana.

Durante las últimas décadas se está acentuando el envejecimiento progresivo de la población y paralelamente se está produciendo un aumento manifiesto de la prevalencia de algunas enfermedades crónicas en ella. A ello han contribuido los avances en el tratamiento de patologías oncológicas, que han aumentado la supervivencia de muchos pacientes. Sin embargo, la mejora de la calidad de vida de éstos durante las fases avanzadas de dichas enfermedades depende fundamentalmente del esmero en los cuidados paliativos y el tratamiento integral del paciente terminal. En 1986, la magnitud del problema del tratamiento insuficiente del dolor oncológico llevó a la Organización Mundial de la salud (OMS) a publicar las directrices para el control del dolor en pacientes afectados de cáncer de diversa naturaleza (World Health Organization 1986), basado en el uso de fármacos opioides como piedra angular, y supone que más del 80% de los pacientes pueden ser atendidos utilizando este sistema (Zech et al., 1995).

El papel de las sustancias opioides, fundamentalmente morfina, como medicamento para el dolor oncológico terminal determinó su inclusión en la lista de medicamentos esenciales de la OMS. En España, alrededor de 90.000 personas fallecen cada año debido a tumores malignos. Esto supone que diversos tipos de cáncer constituyen la primera causa de muerte en nuestro país, a la cual se atribuyen una de cada cuatro muertes (Regidor et al., 1998). El 30-40% de los pacientes con cáncer sometidos a tratamiento activo contra la enfermedad refieren al dolor como el síntoma más temido y discapacitante para el paciente canceroso, y en enfermedad avanzada este porcentaje llega a ser del 70 al 90% (Cleeland et al., 1994; Llobera et. al., 1998). Ello determina la necesidad de aliviar el dolor en dichos pacientes como medida paliativa en ausencia de curación.

La adormidera (*Papaver somniferum* L.) es uno de los cultivos más importantes para la industria farmacéutica en España, ya que constituye la única fuente de los alcaloides morfina, codeína y tebaína ampliamente utilizados en medicina, bien directamente o como fuente para otros derivados semisintéticos, como analgésicos, antitusivos y antiespasmódicos. La industria farmacéutica española consume anualmente alrededor de 5.500 kg de morfina, con una tendencia al incremento, entre

otros motivos, por el aumento de la esperanza de vida de los pacientes oncológicos y el envejecimiento de la población española.

España es el quinto productor europeo de semilla y paja de adormidera con una superficie de cultivo de aproximadamente 7.500 hectáreas repartidas entre las Comunidades Autónomas de Andalucía, Castilla- la Mancha, y Castilla y León, lo cual supone casi el 4 % de la superficie mundial cultivada de adormidera. Durante los últimos años, los rendimientos del cultivo de adormidera han venido disminuyendo como consecuencia de ataques de diversas enfermedades, algunas de las cuales son de etiología aún desconocida. Durante las campañas agrícolas 2001/2002 a 2005/2006 se observaron ataques severos de una nueva enfermedad de aparente etiología fúngica, diferente por sus características sintomatológicas de la Necrosis de raíz y cuello causada por *Pleospora papaveracea* hasta ahora considerada la enfermedad más importante de la adormidera (Muñoz-Ledesma, 2002). En el curso de las cinco campañas referidas, la incidencia y severidad en los ataques de la nueva enfermedad se han incrementado de manera notable. Esta nueva enfermedad, inicialmente denominada como Mildiu por su sintomatología, puede haber estado presente en España con baja incidencia desde que la adormidera se estableció a principios de los 1970`s en las áreas más secas de Andalucía. Sin embargo, a medida que el cultivo se ha extendido a nuevas zonas geográficas más frescas y húmedas en España (i.e., Castilla-la Mancha, y Castilla y León) o se ha incorporado al regadío a fin de mejorar el rendimiento de las cosechas (i.e., Andalucía; Castilla-la Mancha) (Muñoz-Ledesma, 2002), se le ha ubicado aparentemente en ambientes más favorables para el desarrollo de enfermedades que dependen de temperaturas moderadas y abundante pluviometría/humedad relativa, como son los Mildius, y ello ha podido determinar que la incidencia y severidad de los ataques de esta nueva enfermedad se haya incrementado en el tiempo.

El incremento en la incidencia y severidad del Mildiu en cultivos de adormidera en España y la rápida dispersión del agente causal a las diferentes áreas de cultivo en los últimos años, han obligado a Alcaliber, S.A. (única empresa que desde 1973 está autorizada en España a su cultivo con vistas a la obtención de concentrado de paja de adormidera y posterior extracción química de sus alcaloides con fines exclusivamente medicinales) a promover acciones de investigación sobre el patosistema *P. arboresens-Pap. somniferum* para establecer las bases etiológicas y

epidemiológicas que permitan establecer estrategias y medidas de control de la enfermedad para la protección del rendimiento y garantía de la producción de adormidera en la agricultura española.

El conocimiento científico que se posee sobre el Mildiu de la adormidera es muy escaso y fragmentario a nivel mundial, y nulo a nivel europeo. Estudios recientes indican que el Mildiu se ha convertido en la enfermedad de mayor importancia para la industria de adormidera en Tasmania (Australia; primer productor mundial), desde que se diagnosticara por vez primera en dicha zona en 1996. De hecho, esta enfermedad puede causar la devastación completa del cultivo en dicha zona en ambientes lluviosos o de humedad elevada (Cotterill y Pascoe, 1998), y se desarrolla con carácter epidémico y devastador en ella cada año obligando a abordar su control mediante tratamientos fungicidas debido a la magnitud y consistencia de las pérdidas que ocasiona (Scott et al., 2003).

Esta Tesis Doctoral se planteó con el objetivo finalista de obtener nuevos conocimientos sobre la etiología, biología y epidemiología del Mildiu de la adormidera, incluyendo la caracterización y detección molecular específica del agente causal, que hicieran posible establecer las bases científicas para el desarrollo de estrategias eficientes para el control integrado de la enfermedad. Como resultado de las investigaciones llevadas a cabo en la presente Tesis doctoral, se ha determinado que el **agente causal del Mildiu de la adormidera en cultivos comerciales en España es el oomyceto biotrofo obligado *Peronospora arborescens* (Berk) de Bary** (Capítulo II.1; Landa et al., 2005). En una primera aproximación, la sintomatología de la enfermedad, el análisis morfológico de las estructuras del agente, y los ensayos de patogenicidad así como los análisis de secuencias de la región ITS1-5,8S-ITS2 del ADN ribosómico del patógeno, confirmaron al agente causal del Mildiu de la adormidera en España como *P. arborescens*. Posteriormente, mediante la utilización de técnicas convencionales y moleculares descritas y puestas a punto en esta Tesis Doctoral (Capítulo III.1; Landa et al., 2007), se pudo confirmar a *P. arborescens* como agente causal del Mildiu en Francia, otro de los principales países productores de adormidera en Europa (Capítulo II.2; Montes-Borrego et al., 2008). *P. arborecens* fue descrito por primera vez como agente causal de Mildiu de *Papaver* spp. en Europa en 1929 (Yossifovith, 1929). Posteriormente, *P. arborecens* ha sido descrita como agente del Mildiu de *Papaver* spp. en otros países, incluyendo Alemania, Argentina, Australia, Austria, Bulgaria, Canadá, China,

Dinamarca, EE.UU., Egipto, Francia, Finlandia, Gran Bretaña, Holanda, Hungría, India, Irán, Irlanda, Israel, Italia, Japón, República Checa, Rumania, Suecia, Suiza, Ex Unión Soviética, Uruguay, y Yugoslavia (Capítulo I.1). Sin embargo, la identificación del agente causal del Mildiu de la adormidera en Tasmania no tuvo lugar hasta el año 2004, y la caracterización molecular del mismo lo identificó como *P. cristata*, una especie morfológicamente indistinguible de *P. arborescens* (Scott et al., 2004).

Posteriormente, una vez caracterizada la identidad del agente causal del Mildiu de la adormidera procedimos a establecer **la composición y diversidad genética existente en las poblaciones de éste y sus relaciones filogenéticas basadas en el análisis de las secuencias de la región ITS del ADN ribosómico (ADNr)** (Capítulo III.1; Landa et al., 2007). Para ello, se han utilizado secuencias de *Peronospora* spp. depositadas en las bases de datos del Genbank y nuevas secuencias obtenidas en el curso de la investigación a partir de muestras de tejidos frescos o desecados de *Meconopsis cambrica*, diversas *Papaver* spp., y raíz, tallo, hojas y semillas de *Pap. somniferum* cultivadas y silvestres. Los resultados del estudio demuestran que *Pap. somniferum* puede ser infectada por dos especies distintas de *Peronospora*; y que a diferencia de lo encontrado en Tasmania (Australia) *P. arborescens* en lugar de *P. cristata* es el único agente causal del Mildiu de adormidera en España y Francia, confirmando con ello trabajos anteriores (Yossifovith, 1929; Landa et al., 2005).

Los análisis filogenéticos iniciales demostraron que las poblaciones de *P. arborescens* procedentes de diferentes áreas de cultivo de las tres principales regiones productoras de adormidera en España y Francia presentan un bajo nivel de diversidad genética (Capítulo III.1 y II.2; Landa et al., 2007, Montes-Borrego et al., 2008). Sin embargo, nuevos análisis filogenéticos para los que se incluyeron diferentes especies de *Papaver* (*Pap. dubium, Pap. hybridum, Pap. rhoeas*, y *Pap. somniferum*) procedentes de colecciones de herbario y con diferentes orígenes geográficos (Capítulo III.2; Montes-Borrego et al., 2009), indicaron por primera vez que en las poblaciones de *P. arborescens* podría existir cierto grado de especialización parasítica sobre huéspedes determinados. En resumen, el nuevo análisis filogenético de las secuencias ITS del ADNr indicó que (Capítulo III.2; Montes-Borrego et al., 2009):

(i) existe diversidad genética en las poblaciones de *P. arborescens* existentes en diferentes lugares del mundo; y

(ii) que existe un cierto grado de especialización parasítica en las poblaciones de *P. arborescens* que infectan *Papaver* spp., siendo las poblaciones del agente que infectan las *Papaver* spp. arvenses más diversas que los que se encuentran infectando cultivos de *Pap. somniferum.*

Actualmente, los conceptos taxonómicos en la clasificación de los Mildius se consideran inadecuados y potencialmente erróneos, ya que los estudios previos de taxonomía y evolución se basaron principalmente en unos pocos caracteres morfológicos y en la naturaleza botánica del huésped de origen (Choi et al., 2003; Constantinescu y Fatehi, 2002; Hall, 1996). La mayoría de los estudios previos en *P. arborescens* y *P. cristata* se han realizado basándose en características morfológicas del patógeno y la sintomatología del huésped infectado. Puesto que tales características son muy similares en ambos patógenos (Francis, 1981; Reid, 1969; Scott et al., 2004), es posible que algunas de las descripciones atribuidas a una de las dos especies en un país determinado pudieran corresponder a la otra especie. Los protocolos puestos a punto en los dos trabajos incluidos en el Capítulo III de esta Tesis Doctoral serán posiblemente de gran utilidad en trabajos futuros sobre epidemiología y biogeografía de los Mildius, así como para aclarar posibles identificaciones erróneas en el pasado de *P. arborescens* y *P. cristata*, los dos agentes causales descritos del Mildiu de la adormidera.

Además de su uso para estudios filogenéticos, las secuencias de las regiones espaciadoras internas (ITS) de ADN ribosómico (ADNr) han demostrado ser útiles en el **desarrollo de protocolos de PCR especie-específicos para la detección e identificación** *in planta* de hongos fitopatógenos (Aegerter et al., 2002; Bonants et al., 1997; Choi et al., 2003; Scott et al., 2004; Silvar et al., 2005). En los trabajos realizados en esta Tesis Doctoral se empleó una metodología similar para desarrollar tres pares de iniciadores específicos de *P. arborescens* y un protocolo basado en PCR, que se han demostrado eficaces para responder de forma precisa a diferentes cuestiones sobre este patosistema. Este método ha quedado recogido en la patente española Nº P200603319, y sus dos extensiones P200803261 y P200803262, y ha sido extendida internacionalmente con la aplicación Nº PCT/ES2007/000781.

Mediante el uso del método para la identificación y detección de *P. arborescens* desarrollado en esta Tesis Doctoral (Capítulo III.1; Landa et al., 2007) es posible:

(i) Identificar y diferenciar sin ambigüedad a *P. arborescens* de *P. cristata*. Además, permitió corroborar a lo largo del desarrollo de este trabajo que *P. arborescens* es el

único agente causal del Mildiu de la adormidera en los cultivo de *Pap. somniferum* en España.

(ii) Detectar de forma específica *P. arborescens* en tejidos de plantas de adormidera infectadas sintomática o asintomáticamente; y

(iii) Detectar y confirmar la presencia de *P. arborescens* en semillas de adormidera; lo cual hace a la tecnología desarrollada útil para llevar a cabo acciones que impidan la diseminación el patógeno entre cultivos y zonas geográficas exentas de éste.

Debido a la naturaleza biotrófica obligada de los Peronosporales, la disponibilidad de protocolos moleculares para su detección e identificación *in planta*, incluida la semilla, es particularmente importante para la puesta en práctica de programas de certificación sanitaria de la planta y el cultivo. Tal tecnología de diagnóstico debe ser, por tanto, lo suficientemente sensible como para detectar pequeñas cantidades de estructuras del patógeno antes de la expresión evidente de los síntomas en el huésped, y lo suficientemente específica como para evitar problemas de reacción cruzada de otros patógenos con los que pudiera estar relacionado.

Por ello, en el desarrollo en esta Tesis Doctoral del referido método de análisis para identificar a *P. arborescens* se ha puesto particular énfasis en asegurar que presenta entre otras las siguientes capacidades:

(i) Alta *especificidad*: puesto que no se produce amplificación cruzada con otros patógenos relacionados, especialmente cuando se utilizan tejidos de adormidera infectados por *P. cristata*.

(ii) Alta *sensibilidad*: puesto que el patógeno puede ser detectado en tejidos de plantas sin síntomas y en lotes de semillas en los que el patógeno puede estar presente en concentraciones muy bajas; y

(iii) Considerable *versatilidad*: puesto que puede ser aplicado a diferentes muestras vegetales de diversas especies de *Papaver*, incluyendo cápsulas, hojas, raíces, semillas y tallos;

Los resultados obtenidos demuestran sin ambigüedad la ***especificidad*** de los iniciadores elegidos y la capacidad de éstos de diferenciar entre si a las dos especies causantes de Mildiu en adormidera, *P. arborescens y P. cristata.* Así, sólo se obtuvieron productos de PCR del tamaño pronosticado cuando se realizaban amplificaciones de

muestras que contenían ADN de *P. arborescens*; y no se produjo amplificación cruzada cuando se utilizó ADN de otros hongos y oomycetos sometidos a ensayo.

Más importante aún es el hecho de que el patógeno puede ser detectado a muy bajas concentraciones. Así, la ***sensibilidad*** o límite de detección para cada uno de los pares de iniciadores varió entre 0,1- 1,0 pg de ADN de *P. arborescens* (dependiendo de la par de iniciadores). Esta sensibilidad no se vio afectada cuando el ADN de *P. arborescens* se encuentra diluido en un fondo de ADN adormidera de hasta 20 ng, e incluso la sensibilidad aumentó cuando para las diluciones se utilizó ADN de semillas sanas, desinfestadas, en lugar de hojas. La cantidad de ADN de *P. arborescens* que podría ser detectada utilizando el par de iniciadores DC6/ITS4, descritos como universales para Oomycetos, fue de 2x a 3x superior (i.e., menor sensibilidad) comparada con la detectada usando los iniciadores específicos diseñados en este estudio.

La ***versatilidad*** de la técnica ha quedado suficientemente contrastada a lo largo del desarrollo de los trabajos de investigación realizados en esta Tesis Doctoral, donde esta técnica ha sido utilizada con éxito en análisis con ADN extraído de todos las partes de plantas, y en estados de crecimiento que van desde semilla hasta de planta madura una vez completado el ciclo del cultivo, e incluso cuando hemos utilizado muestras de ADN procedentes de suelos de campos de adormidera (Capítulo V.1; Montes-Borrego et al., en revisión).

En trabajos futuros de investigación, sería necesario desarrollar nuevos iniciadores y protocolos con los que sea posible garantizar la diferenciación entre *P. arborescens y P. cristata* durante los procesos de detección *in planta*, con el fin de faciltar la puesta en práctica eficiente de acciones de exclusión y erradicación (Maloy, 1993). La sensibilidad, especificidad y robustez de dichos iniciadores y protocolos deben ser, en razón de lo anteriormente descrito, suficientes para obviar los inconvenientes que se han señalado para otros métodos de detección molecular (Guillemette et al., 2004; Silvar et al., 2005).

El avance en la investigación sobre protocolos para la detección e identificación molecular de *P. arborescens* mediante PCR específica simple, y subsiguiente análisis de su diversidad genética, sugirió que se podían introducir mejoras sobre la sensibilidad de detección mediante la tecnología de PCR anidada o 'nested'-PCR, debido al empleo como molde de ADN amplificado en una primera

reacción PCR y al uso de iniciadores que reconocen secuencias internas del amplicón producido en ésta. En el procedimiento desarrollado en esta Tesis Doctoral (Capítulo III.2; Montes-Borrego et al., 2009), se utilizan un par de iniciadores específicos para Oomycetos (i.e., DC6/ITS4) para la primera ronda de amplificación y los pares de iniciadores específicos de *P. arborescens* P3 y P6 desarrollados y patentados (Capítulo III.1; Landa et al., 2007), en la segunda.

La mejora en la sensibilidad de la detección alcanzada mediante el nuevo procedimiento fue especialmente importante para el análisis de muestras en las que la cantidad de patógeno, y por tanto el ADN de él extraíble, puedan ser muy bajos en relación con el ADN extraído del tejido vegetal. El protocolo de 'nested'-PCR especifico de *P. arborescens* permitió:

(i) Corroborar que *P. arborescens* es el único agente causal del Mildiu en cultivos de adormidera y en diferentes *Papaver spp.* en España procedentes de diversas zonas geográficas

(ii) Amplificar *P. arborescens* en especímenes de herbario de *Papaver* spp. de hasta 96 años de antigüedad, cuya amplificación había resultado fallida con el protocolo de PCR simple.

(iii) Demostrar que *P. arborescens* puede generar tanto en adormidera cultivada como en *Papaver* spp. arvenses infecciones sistémicas asintomáticas, e infectar lotes de semillas recogidas de cultivos con incidencia de la enfermedad baja o difícilmente perceptible.

Comparado con la metodología de PCR simple, el nuevo protocolo 'nested'-PCR desarrollado proporciona un incremento en el límite de detección de 2x a 3x órdenes de magnitud cuando se utiliza con tejidos vegetales frescos y semilla (dependiendo de la pareja de iniciadores), que es de gran importancia para su aplicación a procesos de certificación de semillas, especialmente cuando la detección mediante PCR simple es poco eficiente o negativa. Este incremento de 2x a 3x órdenes de magnitud en el límite de detección permite detectar hasta 5,0-0,5 fg de ADN de *P. arborescens* en una mezcla de ADN de la planta en comparación con los límites de detección (1 pg a 100 fg) de otros protocolos para otros oomycetos como *Phytophthora* spp. y *P. tabacina* descritos en la literatura (e.g., Ippolito et al., 2002; Schena et al., 2008; Tsay et al., 2006), lo cual refrenda la alta sensibilidad de los iniciadores diseñados y el acierto en el protocolo original de PCR simple.

El uso de la técnica 'nested'-PCR demostró que *P. arborescens* puede estar presente en lotes de semillas cosechados en cultivos de adormidera con escasa o imperceptible incidencia de Mildiu, al igual que en plantas aparentemente sanas. Estos resultados podría explicar la extensa y rápida extensión del patógeno y la enfermedad a nuevas zonas de cultivo, debido a la potencial presencia de *P. arborescens* en semillas de cultivos aparentemente libres de infección. La disponibilidad de la nueva y mejorada de detección es particularmente importante para Alcaliber S.A. y otras compañías implicadas en el cultivo controlado de adormidera, a fin de asegurar que los lotes de semilla proporcionados a los agricultores se encuentran libres del patógeno.

Las técnicas de detección e identificación molecular cualitativa descritas (Capítulos III.1 y III.2: Landa et al., 2007, Montes-Borrego et al., 2009) han contribuido a resolver problemas del patosistema *P. arborescens-Pap. somniferum* asociados tanto con la identificación y detección del patógeno, como al mejor conocimiento de la biología y epidemiología de la enfermedad, y son especialmente importantes en el caso de agentes microbianos biotrofos estrictamente obligados como *P. arborescens*.

Sin embargo, dichas técnicas presentan la gran limitación de no ser de utilidad para cuantificar el nivel de infección/infestación por el patógeno en los substratos analizados. Por tanto, era necesario **desarrollar un método cuantitativo y basado en las técnicas de detección e identificación molecular**, que dada la naturaleza del patógeno y las características asociadas con la infección de la planta y modos de transmisión, permitiera establecer niveles de resistencia/tolerancia/susceptibilidad en germoplasma de *Papaver* spp. disponible con fines de mejora genética del cultivo, así como establecer correlaciones entre niveles de infección en semilla y planta antes y después de aplicar diferentes estrategias de control, ya fuesen éstas de naturaleza biológica, química o física.

La Reacción en Cadena de Polimerasa cuantitativa (qPCR) en tiempo real está siendo cada vez más utilizada en Fitopatología para la detección y cuantificación de los agentes fitopatógenos, incluso a niveles de infección incipiente (Winton et al., 2002; Zhang et al., 2005). Esta técnica permite la cuantificación de ácidos nucleicos en muestras con cantidades desconocidas de éstos mediante comparación directa con muestras estándar amplificadas en reacciones paralelas (Heid et al., 1996; Wong y Medrano, 2005). En el caso del Mildiu de la adormidera, los ensayos qPCR podrían

además tener el valor añadido de ser un procedimiento útil para monitorizar la infección en el tiempo de cualquier tejido de la planta por el patógeno, ya que puede facilitar la cuantificación exacta de la cantidad de ADN de *P. arborescens* presente en aquél, contrarrestando con ello la dificultad que presenta dicha monitorización por métodos microbiológicos en razón de la naturaleza biotrófica obligada del agente. Dicha monitorización permitiría, por ejemplo, evaluar el fenotipo (resistente/tolerante/susceptible) de genotipos de adormidera en cuanto a su capacidad de reducir la infección y/o colonización de la planta por el patógeno; y establecer relaciones entre la cantidad de dicho agente en la semilla o en suelo infestado (indicada por el ADN de *P. arborescens* cuantificado en el substrato) y el desarrollo posterior de la enfermedad.

En esta Tesis Doctoral se ha desarrollado un protocolo qPCR (Capítulo IV.1 Montes-Borrego et al., en preparación) que posee las mismas ventajas de *especificidad*, *sensibilidad*, y *versatilidad* que el protocolo de PCR-simple antes descrito (Capítulo III.1; Landa et al., 2007). Además, el protocolo qPCR desarrollado presenta como ganancia neta la rapidez, precisión y sencillez en la detección y cuantificación de *P. arborescens* en semillas y diversos tejidos de adormidera. Las aplicaciones que hemos realizado de dicho procedimiento, utilizando los iniciadores y protocolos diseñados en los estudios anteriores, han proporcionado resultados de gran interés que son fundamentalmente consecuencia de la calidad del procedimiento para la detección molecular cualitativa que habíamos desarrollado con anterioridad (Capítulo III.1; Landa et al., 2007). El procedimiento de cuantificación molecular de *P. arborescens* basado en la qPCR puesto a punto ha permitido ahora, y auspicia posibilidades adicionales en el futuro:

(i) Certificar la sanidad de la semilla de adormidera que es distribuida a los agricultores.

ii) Tomar decisiones sobre la necesidad de desinfección/desinfestación de la semilla o de realizar tratamientos fungicidas de lotes de semillas infectadas.

iii) Evaluar la eficiencia del proceso de desinfección/desinfestación de lotes de semillas.

iv) Evaluar la resistencia o tolerancia a *P. arborescens* en germoplasma de adormidera de interés mediante la monitorización y cuantificación de la infección y colonización de la planta por el patógeno.

v) Establecer relaciones entre la densidad de inóculo de *P. arborescens* en el suelo y la cantidad de Mildiu que se desarrolla posteriormente en cultivos de adormidera, que tengan valor predictivo de utilidad para la toma de decisiones en estrategias de control integrado de la enfermedad.

Desde el comienzo de los trabajos de investigación sobre Mildiu de la adormidera a principios del 2004, y en base a una serie de hipótesis experimentales establecidas en esta Tesis Doctoral (Capítulo I.1.5), se abordó el **estudio de la biología de la interacción *Pap. somniferum/P. arborescens* determinando la naturaleza de la(s) fuente(s) de inóculo primario y el/los tipo(s) de infección determinantes del desarrollo de epidemias de Mildiu.** Para dicho estudio se adoptó un ***abordaje experimental integrador*** que combina (i) observaciones de campo; (ii) ensayos de patogenicidad en condiciones controladas y campo; y (iii) utilización de las tecnologías moleculares (simple-PCR específica, nested-PCR y qPCR) para la detección y cuantificación del patógeno en tejidos de adormidera sintomáticos y asintomáticos. Esta metodología integradora descrita, ha dado respuesta a las cuestiones planteadas en las hipótesis experimentales iniciales, de tal forma que el ciclo de patogénesis en el patosistema es ahora mejor comprendido para abordar nuevos retos en el futuro que permitan integrar medidas de control de la enfermedad que sean sostenibles con la protección del medio ambiente.

Así los resultados obtenidos en la presente Tesis Doctoral (Capítulo V.1 Montes-Borrego et al., en revisión) han demostrado que el Mildiu de la adormidera esta presente en todos los campos de cultivos comerciales de adormidera en España que han sido prospectados, y que la sintomatología de la enfermedad en condiciones naturales incluye dos síndromes, como se describe en el Capítulo V.1. Ambos tipos de síndromes han sido reproducidos en los ensayos de patogenicidad realizados en el desarrollo de experimentos bajo condiciones controladas en esta Tesis Doctoral.

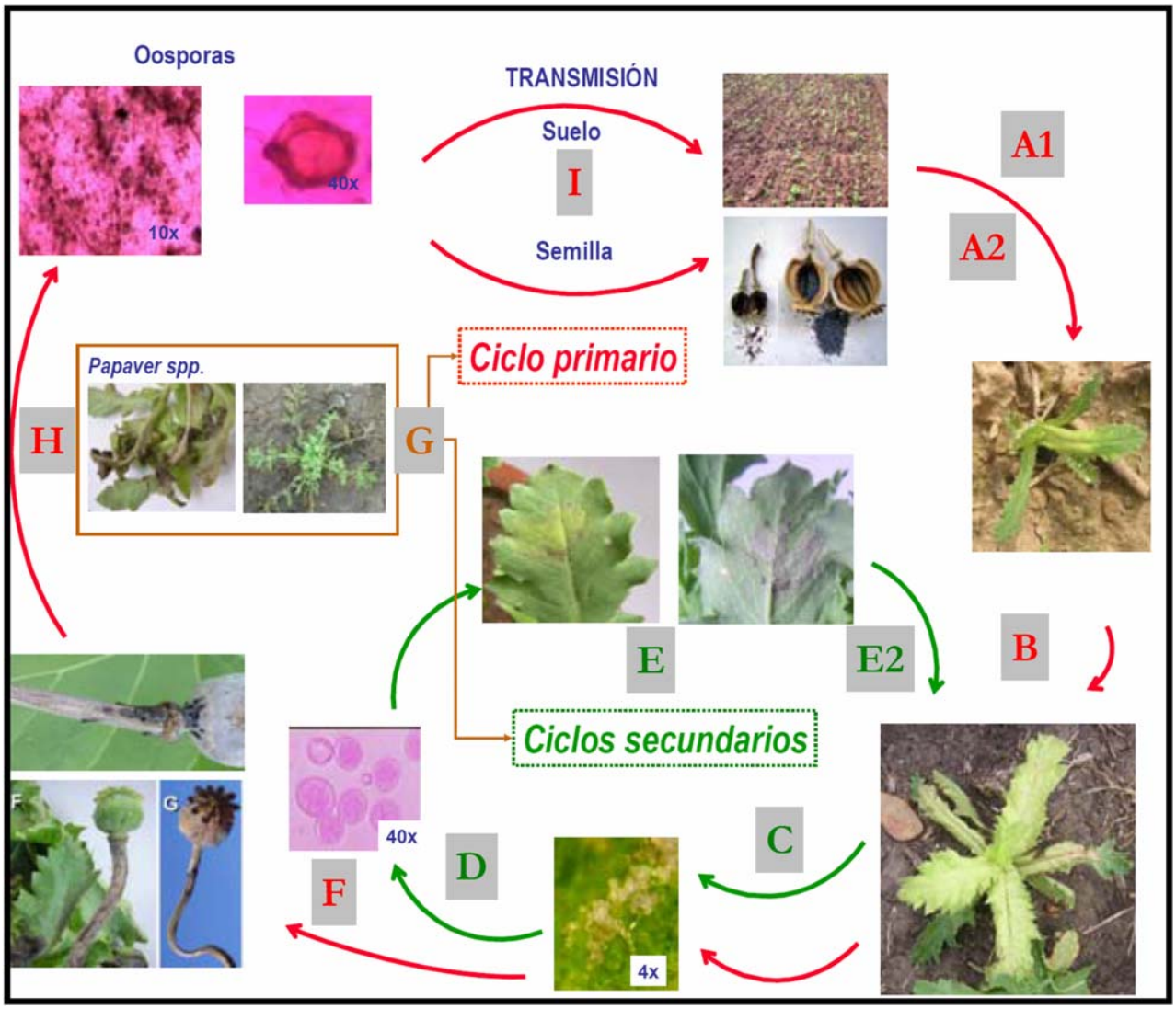
Hasta el momento de inicio de esta Tesis Doctoral, el ciclo de patogénesis del Mildiu de la adormidera era escasamente conocido, y en la mayoría de los casos su interpretación se basaba en los síntomas de infecciones foliares que se observadan en campo y en la información *ad hoc* sobre dicha patogénesis existente sobre patosistemas similares. Este conocimiento era manifiestamente insuficiente sobre aspectos claves del patosistema necesarios para el control de la enfermedad, y consecuentemente dificultaba el desarrollo de estrategias para el control eficiente del Mildiu. Los

resultados obtenidos es este trabajo demuestran, que tanto las oosporas que se encuentran en suelos o en restos de cultivos infectados, como los esporangios desarrollados en tejidos de plantas infectadas bajo condiciones naturales, son inóculos eficientes para el desarrollo de la enfermedad (Figura VI.1.1). Además, factores como edad de la planta y las condiciones ambientales, fundamentalmente la temperatura, influyen significativamente sobre el desarrollo de la infección y expresión de la sintomatología característica del Mildiu, como se describe en el Capítulo V.1. De esta forma, ha quedado demostrado que la infección primaria de adormidera por *P. arborescens* ocurre tras la invasión de tejidos radicales durante la germinación y establecimiento de plántulas jóvenes, que tiene lugar a partir de oosporas contenidas en el suelo o en restos de tejidos infectados, así como a partir de propágulos infectivos del patógeno (de naturaleza aún no determinada) contenidos en semillas infectadas/infestadas. Nuestros resultados sugieren además que el papel de las *Papaver* spp. arvenses como fuente de inóculo primario o secundario para originar epidemias de Mildiu de adormidera puede no ser lo determinante que podría presumirse, ya que en contadas ocasiones se han encontrado plantas de *Papaver* spp. mostrando esporulación del patógeno en campo, aunque el ciclo de crecimiento de éstas coincide con el de la adormidera cultivada (Capítulo V.1; Montes-Borrego et al., en revisión). Adicionalmente, el análisis de Máxima Parsimonia de aislados de *P. arborescens* de plantas y especímenes de herbario de papaveráceas silvestres y plantas de adormidera de cultivos comerciales en España, Francia y otros países, indican que las poblaciones de *P. arborescens* procedentes de papaveráceas silvestres difieren marcadamente y son más heterogéneas que las procedentes de *P. somniferum* cultivada, sugiriendo que puede existir cierta diversidad genética y patogénica entre poblaciones de *P. arborescens* según el huésped de procedencia de los aislados, como se demostró en el Capítulo III.2 (Montes-Borrego et al 2009). Esta posibilidad debería ser contrastad mediante investigaciones más extensas, que incluyan ensayos de patogenicidad de los distintos genotipos de *P. arborescens* identificados sobre diferentes especies de *Papaver* spp.

Nuestros resultados han demostrado la transmisión de *P. arborescens* en semillas de adormidera infectadas/infestadas y su papel como inoculo primario (Capítulo III.1; Landa et al., 2007), lo cual tiene el potencial de contribuir significativamente a la extensión del agente y la enfermedad a campos o áreas geográficas libres de ésta. Además, estas semillas pueden ser infectadas/infestadas debido a infecciones

sistémicas primarias por oosporas, así como por infecciones secundarias por esporangios, que a su vez pueden ser sistémicas o no y sintomáticas o asintomáticas Capítulo V.1 (Montes-Borrego et al., en revisión). Tal como se expone en el Capítulo V.1, los esporangios desarrollados en plantas infectadas en condiciones ambientales favorables para el desarrollo de Mildiu son eficientes en producir nuevas infecciones secundarias que diseminan la enfermedad a nuevas plantas originado infecciones y éstas ser sistémicas o localizadas. Ambos tipo de infecciones secundarias pueden estar relacionadas con las diferencias en la incidencia y severidad del Mildiu que hemos observado en las dos principales áreas de cultivo de adormidera en España. Así, mientras que las altas temperaturas y baja humedad que se dan en el sur de España desde el entallado de la planta hasta formación de cápsula no favorecen el desarrollo de nuevos ciclos secundarios de la enfermedad; en la zona central de la Península prevalecen durante la floración temperaturas más suaves y humedad más alta (fundamentalmente debido al riego por aspersión) que pueden favorecer que se produzcan gran número de infecciones secundarias en los estadios de floración y formación de cápsula. Estas infecciones pueden potencialmente incrementar el riesgo de infección/infestación de semillas, tanto como consecuencia de la colonización sistémica por el patógeno como por las invasiones de cápsulas en proceso de formación de semillas. En la Figura VI.1.1 queda recogido a modo de resumen los resultados obtenidos sobre el ciclo de patogénesis de *P. arborescens* en adormidera durante el desarrollo de esta Tesis Doctoral.

Figura VI. 1.1 Ciclo de patogénesis de *Peronospora arborescens* en adormidera (*Papaver somniferum*), con indicación del tipo(s) de infección que pueden contribuir de forma determinante al desarrollo de la enfermedad



A.1 Las **oosporas** contenidas en restos de cosecha infestados o en **suelo** infestado son eficientes en originar infecciones en los órganos subterráneos de plántulas de adormidera durante el establecimiento del cultivo y originar infecciones sistémicas sintomáticas o asintomáticas.
A.2 Las semillas de adormidera pueden ser infectadas/infestadas a partir de infecciones primarias sistémicas y secundarias sistémicas o no, que a su vez pueden ser sintomáticas o asintomáticas. Las **semillas infectadas** son efectivas como fuente de inóculo primario.
B. Las plantas son colonizadas sistémicamente a partir de la infección por oosporas en el suelo o de semillas infectadas, dando lugar a síntomas típicos de Mildiu asociados con el crecimiento intercelular del micelio del patógeno en el parénquima de las hojas sintomáticas.
C. Tras la colonización extensa del tejido, y cuando se dan las condiciones ambientales de elevada humedad relativa, se producen esporangióforos y esporangios característicos de la reproducción asexual del oomyceto.
D. Los esporangios formados sobre las hojas infectadas son liberados en condiciones de agua libre y viento y transportados sobre nuevos tejidos sanos.
E1. La germinación de los esporangios dispersados por el viento y/o agua sobre la superficie de las hojas da lugar a infecciones localizadas en estás y el desarrollo de **ciclos secundarios de patogénesis.**
E2. Las infecciones localizadas originadas en los ciclos secundarios de patogénesis pueden llegar a ser de naturaleza sistémica**.**
F. Según el estado de desarrollo de la planta en el momento de la infección y la severidad de ésta, la planta infectada crece escasamente y muere, o puede continuar su desarrollo hasta alcanzar el estado reproductivo y producirse en ella la formación de esporangios en hojas y cápsulas conteniendo semillas.
G. El papel de las *Papaver* spp. arvenses como fuente de inóculo primario o secundario para originar epidemias no parece ser determinante, ya que en contadas ocasiones se han encontrado plantas de *Papaver* spp. con esporulación del patógeno en el campo aunque su el ciclo de crecimiento coincide con el de la adormidera cultivada (Capítulo V.1).
H. Los tejidos infectados (hojas, tallos, cápsulas y potencialmente semillas) contienen un elevado de oosporas de *P. arborescens,* que pueden ser incorporadas al suelo tras la degradación de los restos de aquéllos tras la cosecha y consecuentemente incrementar la densidad de inóculo primario para siguientes infecciones.
I. El **ciclo primario de patogénesis** se completa con la transmisión del agente causal en semilla y suelo. Estos resultados indican que para el control eficiente del Mildiu de la adormidera deben ser consideradas conjuntamente estrategias que aseguren la sanidad de la semilla y además se evite el uso de suelos con historia de adormidera, y en particular de la enfermedad

El reto que desde el punto de vista fitopatológico constituyó el problema planteado, y las características que presentaba en lo concerniente a la disciplina científica -Patología Vegetal- que desarrollamos, acentúan el interés de esta Tesis Doctoral en cuanto a las respuestas que su desarrollo ha proporcionado a los interrogantes existentes sobre el Mildiu de la adormidera. La falta de adecuado de conocimiento específico respecto de la patogénesis de la enfermedad obligaba hasta ahora a que las actuaciones para su control hubieran de ser basadas en proyectar sobre ella el conocimiento disponible sobre patosistemas similares, que se caracterizan por infecciones exclusivamente foliares en los que la semilla infectada y el suelo infestado no juegan un papel determinante para su desarrollo. Por ello, los resultados obtenidos en esta Tesis Doctoral auspician a desarrollar nuevas tecnologías que permitan establecer estrategias y medidas efectivas y ambientalmente respetuosas de control de la enfermedad, fundamentalmente basadas en los principios de exclusión y erradicación, aplicables particularmente a semillas infectadas o sobre cultivos de adormidera comerciales, y practicables en las condiciones agronómicas propias del cultivo y directamente por el parte del agricultor con la asistencia técnica correspondiente por Alcaliber S.A.

Finalmente, queremos destacar que el desarrollo de esta Tesis Doctoral es ejemplo de colaboración estrecha y exitosa entre el sector empresarial (a través de Alcaliber S.A.) y la Universidad (a través de la UCO) u Organismos Públicos de Investigación (a través del IAS-CSIC). Tal colaboración ofrece una oportunidad destacable para resaltar el potencial de beneficio mutuo que puede alcanzarse para la transmisión directa y eficaz de la tecnología científica generada por el sector público al sector privado, perjudicado por la incidencia de elementos negativos (como las enfermedades) sobre su actividad productiva, así como para mejorar la formación especializada de técnicos superiores en materias científicas de repercusión inmediata sobre el uso eficiente de recursos agrícolas limitados.

VI.2

CONCLUSIONES

Las conclusiones generales de esta Tesis Doctoral son las siguientes:

1. El agente causal del Mildiu en cultivos comerciales de adormidera en España y Francia es el oomiceto biotrofo obligado *Peronospora arborescens* (Berk) de Bary; y su distribución en España es extensa, incluyendo todas las zonas de dicho cultivo en el área Peninsular.
2. El análisis filogenético mediante máxima parsimonia de secuencias de la región ITS del ADNr de *P. arborescens*, realizado con muestras del patógeno procedentes de plantas de adormidera de cultivos comerciales en España, Francia y otros países, discrimina a *P. arborescens* de *Peronospora cristata*, el otro agente causal de esta enfermedad descrito hasta ahora en Australia. Dichos análisis indican también que las poblaciones de *P. arborescens* que infectan *Papaver somniferum* son diferentes y más homogéneas entre sí comparadas con las procedentes de papaveráceas silvestres, sugiriendo que puede existir algún nivel de especialización patogénica sobre huéspedes determinados y que los huéspedes alternativos no actúan necesariamente como fuente de inóculo primario de relevancia para las epidemias de Mildiu en adormidera cultivada.
3. Mediante el uso de protocolos de PCR simple, PCR anidada, y PCR cuantitativa en tiempo real desarrollados y optimizados en esta Tesis Doctoral, es posible la detección eficiente, identificación precisa y cuantificación de *P. arborescens*, en plantas de adormidera infectadas, sintomáticas o asintomáticas, así como en lotes de semilla de siembra. La tecnología de detección molecular desarrollada está siendo utilizada para la certificación de lotes comerciales de semilla de siembra libres del patógeno, y puede ser de utilidad para investigaciones sobre la biología de poblaciones de *P. arborescens*, la epidemiología del Mildiu de la adormidera y la resistencia al patógeno en cultivares de ésta.
4. Las semillas de adormidera pueden ser infestadas o infectadas por *P. arborescens* a partir de infecciones primarias sistémicas de tejidos subterráneos, y secundarias sistémicas o no de tejidos aéreos, que a su vez pueden ser sintomáticas o asintomáticas. Las semillas infestadas o infectadas son efectivas como fuente de inóculo primario para originar epidemias de Mildiu.

5. La utilización de la tecnología de detección molecular para el análisis de lotes de semilla ha demostrado que existe una alta frecuencia de infección por *P. arborescens* en lotes comerciales de semilla de adormidera en España y Francia. El uso de lotes de semilla infestados para la siembra de cultivos comerciales podría explicar la rápida extensión del patógeno a nuevas zonas de cultivo en España.
6. Las oosporas de *P. arborescens* contenidas en restos de cosecha infestados, o existentes en suelos de cultivo de adormidera, son eficientes para originar infecciones de los órganos subterráneos en plántulas de adormidera durante el establecimiento del cultivo, y que dan lugar a la colonización sistémica, sintomática o asintomática de la planta.
7. Los esporangios de *P. arborescens* que se desarrollan en tejidos aéreos infectados son eficientes como inóculo secundario para originar nuevas infecciones, y éstas pueden ser de naturaleza sistémica.

BIBLIOGRAFÍA

Aegerter, B.J., Nuñez, J.J., and Davis, R.M. 2002. Detection and management of downy mildew in rose rootstock. Plant Dis. 86:1363-1368.

Agrios, G.N. 2005. Plant pathology. Fifth edition. Elsevier Academic San Diego Press.

Alcock, N.L. 1933. Downy mildew of *Meconopsis*. New Flora and Silva 5: 279-282.

Alexopoulos, C.J., Mims C.W., and Blackwell M. 1997. Introductory Mycology. John Wiley and Sons, Inc., New York.

American Academy of Pain Medicine and the American Pain Society. 1997. The use of opioids for the Treatment of Chronic Pain. The Clinical Journal of Pain 13:6-8.

Aranda, S., Montes-Borrego, M., Muñoz-Ledesma, F.J., Jiménez-Díaz, R.M., and Landa, B.B. 2008. First Report of *Pectobacterium carotovorum* causing soft rot of opium poppy in Spain. Plant Dis. 2: 317.

Bajpal, S., Gupta, M.M., and Kumar, S. 1999. Identification of Indian landraces of opium poppy *Papaver somniferum* resistant to damping-off and downy mildew fungal diseases. J. Phytopathol. 147: 535-538.

Baldauf, S.L., Roger, A.J., Wenk-Siefert I., and Doolittle W.F. 2000. A kingdom-level phylogeny of eukaryotes based on combined protein data. Science 290: 972-977.

Barr, D.J.S. 1983. The zoosporic groupings of plant pathogens. Entity or non-entity? Págs. 43-83 en: Zoosporic Plant Pathogens: A modern perspective. Ed: S.T. Buczacki. Academic Press, London.

Barrau, C., y Romero, F. 1996. Mildius. Págs. 805-820 en: Patología Vegetal, Tomo II. Eds: G. LLácer, M.M. López, A. Trapero y A. Bello. SEF Phytoma, España.

Behr, L. 1956. Der falsche mehltau am mohn (*Peronospora arborescens* (Berk.) de Bary). Untersuchungen zur biologie des erregers. J. Phytopathol. 27: 289-334.

Berkeley, M.J. 1846. Observations, botanical and physiological on the potato murrain. J. Roy. Hort. Soc. 1: 9-34.

Bonants, P., De Weerdt, M.H., Van Gent Pelzer, M., Lacourt, I., Cooke, D., and Duncan, J. 1997. Detection and identification of *Phytophthora fragariae* by the polymerase chain reaction. Eur. J. Plant Pathol. 103: 345-355.

Bryant, R.J. 1988. The manufacture of medicinal alkaloids from the opium poppy a review of a traditional biotechnology. Chemistry and Industry 7: 146-153.

Cavalier-Smith, T. 2004. Only six kingdoms of life. Proc. R. Soc. Lond. B 271: 1251-1262

Cho, W.D., and Shin, H.D. 2004. List of plant diseases in Korea. Fourth edition. Korean Society of Plant Pathology.

Choi, Y.J., Hong, S.B., and Shin, H.D. 2003. Diversity of the *Hyaloperonospora parasitica* complex from core brassicaceous hosts based on ITS rDNA sequences. Mycol. Res. 107:1314-1322.

Cleeland, C.S., Gonin R., Hartfield, A.K., Edmonson, J.H., Blum, R.H., Stewart, J.A., and Pandya, K.J. 1994. Pain and its treatment in outpatients with metastatic cancer. New England Journal of Medicine 330: 592-596.

Cohen, Y., and Sackston, W.E. 1974. Seed infection and latent infection of sunflowers by *Plasmopara halstedii*. Can. J. Bot. 52: 231-238.

Constantinescu, O. 1991. An annotated list of Peronospora names. Thunbergia 15:1-110.

Constantinescu, O., and Fatehi, J. 2002. *Peronospora*-like fungi (Chromista, Peronosporales) parasitic on Brassicaceae and related hosts. Nova Hedwigia 74:291-338.

Cooke, D.E.L., Drenth, A., Duncan, J.M., Wagesl, G., and Brasier, C. M. 2000. A molecular phylogeny of *Phytophthora* and related oomycetes. Fung. Genet. Biol. 30: 17–32.

Cooke, D.E.L., Williams, N.A., Williamson, B., and Duncan, J.M. 2002. An ITS-based phylogenetic analysis of the relationships between *Peronospora* and *Phytophthora*. Págs. 161-165 en: Advances in Downy Mildew Research. Eds: P. T. N. Spencer, U. Gisi and A. Lebeda. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Corda, A.C.I. 1837. Icones fungorum hucusque cognitorum 1: 20. Cramer, Weinhein, Germany

Cotterill, P.J., and Pascoe, I.G. 1998. Downy mildew of *Papaver somniferum* in Tasmania. Australas. Plant Pathol. 27: 263-264.

Cotton, A. D. 1929. A fungus disease of *Meconopsis*. The Gardeners Chronicle 85: 143–144.

Crous, P.W., Phillips, A.J.L., and Baxter, A.P. 2000. Phytopathogenic fungi from South Africa. University of Stellenbosch, Department of Plant Pathology Press, 358 p.

Dick, M.W. 1988. Coevolution in the heterokont fungi (with emphasis on the downy mildews and their angiosperm hosts). Págs. 31-62 en: Coevolution of fungi with plants and animals. Eds: D.L. Hawksworth, and K.A. Pirozynski. Academic Press, London.

Dick, M.W. 1999. Classification of the Peronosporomycetes. Pages 871-882 in: Encyclopedia of food microbiology. Vol. 2. R. Robinson, C. Batt, P. Patel, eds. Academic Press, London

Dick, M.W. 2001a. The Peronosporomycetes. Pages 39-72 in: The Mycota. Vol. VII A. D.J. McLaughlin, E.G. McLaughlin, P.A. Lemke, eds. Springer, Berlin.

Dick, M.W. 2001b. Straminipilous Fungi, Systematics of the Peronosporomycetes, Including Accounts of the Marine Straminipilous Protists, the Plasmodiophorids and Similar Organisms. Kluwer Academic Publishers, Dordrecht 670 p.

Dick, M.W. 2001c. Systematics of the Peronosporomycetes including accounts of the marine straminipilous protists, the plasmodiophorids and similar organisms. Kluwer Academic Publishers, Dordrecht.

Dick, M.W. 2002. Towards an understanding of the evolution of the downy mildews. Págs 1-57 en: Advances in Downy Mildew Research. Eds. P. T. N. Spencer, U. Gisi and A. Lebeda. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Dick, M.W., Croft, B.J., Magarey, R.C., De Cock, A.W.A.M., and Clark, G. 1989. A new genus of the Verrucalvaceae (Oomycetes). Botanical Journal of the Linnean Society 99: 97-113.

Dick M.W., Vick M.C., Gibbings J.G., Hedderson T.A., and Lopez Lastra, C.C. 1999. 18S rDNA for species of Leptolegnia and other Peronosporomycetes: justification of the subclass taxa Saprolegniomycetidae and Peronosporomycetidae and division of the Saprolegniaceae sensu lato into the Leptolegniaceae and Saprolegniaceae. Mycol. Res. 103: 1119-1125.

Dick, M.W., Wong P.T.W., and Clark, G. 1984. The identity of the oomycete causing 'Kikuyu Yellows', with a reclassification of the downy mildews. Botanical Journal of the Linnean Society 89:171-197.

Dickinson, C.H. and Greenhalgh, J.R. 1977. Host range and taxonomy of *Peronospora* on crucifers. Transactions of the British Mycological Society 69: 111-116.

Farr, D.F., Rossman, A.Y., Palm, M.E., and McCray, E.B. 2006. Fungal Databases, Systematic Botany & Mycology Laboratory, ARS, USDA. Online publication. http://nt.ars-grin.gov/fungaldatabases/

Fokin, A.D. 1922. Diseases and injuries of cultivated plants observed during the summer of 1922 in the Government of Vyatka. Transactions fourth all russian entomo-phytopathological Congresses. Moscow, 8-14 Dec, pag 108.

Förster, H., Coffey, M. D., Elwood, H., and Sogin, M. L. 1990. Sequence analysis of the small subunit ribosomal RNAs of three zoosporic fungi and implications for fungal evolution. Mycologia 82: 306–312.

Förster, H., Cummings, M.P., and Coffey, M.D. 2000. Phylogenetic relationship of *Phytophthora* species based on ribosomal ITS I DNA sequence analysis with emphasis on Waterhouse groups V and VI. Mycol. Res. 104: 1055–1061.

Francis, S.M. 1981. *Peronospora arborescens*. CMI Descriptions of Pathogenic Fungi and Bacteria 686: 1-2.

Francis, S.M., and Waterhouse, G. 1988. List of Peronosporaceae reported from the British Isles. Transactions of the British Mycological Society 91: 1-62.

French, A.M. **1989.** California Plant Disease Host Index. Calif. Dept. Food Agric., Sacramento, 394 pages

García-Blázquez, G., Constantinescu, O., Tellería, M.T., and Martín, M.P. 2007 Preliminary check list of *Albuginales* and *Peronosporales* (*Chromista*) reported from the Iberian Peninsula and Balearic Islands. Mycotaxon 98: 185-188.

Gaümann. 1923. Beiträge zu einer Monographie der Gattung *Peronospora* Corda. Beitr. Kryptogamenflora Schweiz 5: 1-360.

Gaümann. 1964. Die Pilze. Basel: Birkhaüser. 541 p.

Göker, M., Voglmayr, H., Riethmüller, A., and Oberwinkler, F. 2007. How do obligate parasites evolve? A multi-gene phylogenetic analysis of downy mildews. Fung. Genet. Biol 44: 105-122.

Gordon, J. 1994. Illicit opium production. Compendium of data. Grand River Informatics Inc., Fergus. 320 pp.

Gorter, G.J.M.A. 1977. Index of plant pathogens and the diseases they cause in cultivated plants in South Africa. Republic South Africa Dept. Agric. Technical Service, Plant Protection Research Institute Science Bulletin 392: 1-177.

Grenville-Briggs, L.J. and van West, P. 2005. The biotrophic stages of Oomycete-Plant Interactions. Págs 217-243 en: Advances in Applied Microbiolgy Volume 57. Eds: A. I. Laskin, J. W. Bennett, and G. M. Gadd.

Guillemette, T., Iacomi-Vasilescu, B., and Simoneau, P. 2004. Conventional and real-time PCR-based assay for detecting pathogenic *Alternaria brassicae* in cruciferous seed. Plant Dis. 88:490-496.

Gustavsson, A. 1959. Studies on nordic *Peronosporas* I. Taxonomic revision. Opera Botanica 3: 1-270.

Gustavsson, A. 1991. The genus *Peronospora* in the Iberian Peninsula, especially in Northern Spain and Andorra. Anales del Jardín Botánico de Madrid 49: 1-38.

Hall, G.S. 1996. Modern approaches to species concepts in downy mildews. Plant Pathol. 45: 1009–1026.

Hansen, H.R. 1944. Industriplanter. [Industrial plants]. [Denmark] Statens Plantepatologiske Forsog Overs. Plantesgydomme 270: 41-59.

Harrison, W.C., and Schmitt, C.G. 1967. Diseases of the opium poppy (*Papaver somniferum*): A check list. Miscellaneous Publication 27. Department of the Army (Fort Detrick). Frederick, Maryland.

Hawksworth, D.L., Kirk, P.M., Sutton, B.C., Pegler, D.N. 1995. Ainsworth & Bisby's Dictionary of the Fungi. Eighth edition. Wallingford, UK: CAB International. 616 p.

Heeger, E.F., and Poethke, W. 1947. *Papaver somniferum* L. der Mohn; Anbau, Chemie, Verwendung. [*P. somniferum* L. the poppy; cultivation, chemistry and use]. Pharmazie 4 Beih. 1 Erganzungsband: 235-340.

Heid, C.A., Stevens, J., Livak, K.J. and Williams, P.M. 1996. Real time quantitative PCR. Genome Research 6: 986-994.

Herrera Silva, J. 2001. Utilidad de los opioides orales en el dolor neuropático. Aparición de nuevas formulaciones de tramadol y morfina. V Reunión Científica de la sociedad del Dolor. Granada.

Hudspeth, D.S.S., Nadler, S.A., and Hudspeth, M.E.S. 2000. A COX2 molecular phylogeny of the Peronosporomycetes. Mycologia 92:674–684.

Husain, A., and Sharma J.R. 1983. The Opium Poppy. A Monograph, Central Institute of Medicinal and Aromatic Plants, Lucknow, India.

INCB (International Narcotics Control Board) 2006. Narcotic drugs. Estimated World Requirements for 2007. Statistics for 2005. New York (Vienna), UN.

INM: Instituto Nacional de Metereología. 2006. Ministerio de Medio Ambiente. Valores Climatológicos normales. Online publication.

Ippolito, A., Schena, L., and Nigro, F. 2002. Detection of *Phytophthora nicotianae* and *P. citrophthora* in citrus roots and soils by nested PCR. Eur. J. Plant Pathol. 108:855–868.

Jeger, M.J., Gilijamse, E., Bock, C.H., and Frinking, H.D. 1998. The epidemiology, variability and control of the downy mildews of pearl millet and sorghum, with particular reference to Africa. Plant Pathol. 47: 544-569.

Kamoun, S. 2003. Molecular genetics of pathogenic oomycetes. Euk. Cell. 2: 191-199.

Kapoor, L. D. 1995. Opium poppy: botany, chemistry and pharmacology. Food products Press, New York. 326pp.

Khristov, A. 1943. Fungi casusing spot on the balls and moulding the seed of opium poppy. Journ. Agr. Exp. Sta. Bulgaria 13: 13-19.

Koch, E. and Slusarenko, A. 1990. Arabidopsis is susceptible to Infection by a downy mildew fungus. Plant Cell 2: 437-445.

Kothari, K.L., and Prasad, N. 1970. Downy mildew of opium poppy in Rajasthan. Indian Phytopathol. 23: 674-687.

Krikorian, A.D., and Ledbetter, M.C. 1975. Some observations on the cultivation of opium poppy (*Papaver somniferum* L.) for its latex. The Botanical Review 41: 30-102.

Landa, B.B., Montes-Borrego, M., Muñoz-Ledesma, F.J., and Jiménez-Díaz, R.M. 2005. First Report of Downy Mildew of Opium Poppy Caused by *Peronospora arborescens* in Spain. Plant Dis. 89: 338.

Landa, B.B., Montes-Borrego, M., Muñoz-Ledesma, F.J., and Jiménez-Díaz, R.M. 2007. Phylogenetic analysis of Downy Mildew pathogens of opium poppy and PCR-based in-planta and seed detection of *Peronospora arborescens*. Phytopathology 97: 1380-1390.

Laughlin, J.C., and Beattie, B.M. 1987. Defoliation effect on the yield components of poppies (*Papaver somniferum* L.). En: Proceedings of the First Princess

Chulabhorn Science Congress, International Congress on Natural Products, Bangkok (Reutrakul, V., ed.). 31 pp.

Laughlin, J.C., and Munro, D. 1983. The effect of *Sclerotinia* stem infection on morphine production and distribution in poppy (*Papaver somniferum* L.) plants. J. Agric. Sci. Cambridge 100: 299-303.

Leclerc, M.C., Guillot, J., and Deville, M. 2000. Taxonomic and phylogenetic analysis of Saprolegniaceae (Oomycetes) inferred from LSU rDNA and ITS sequence comparisons. Antonie van Leeuwenhoek 77: 369–377.

Llobera-Cánaves, J., Benito-Oliver, E., Esteva-Cantó, M., Terrasa-Pons, J., Pons-Sureda, O., y Rojas-Sanna, C. 1998. La enfermedad oncológica: frecuencia de síntomas en el curso de la fase terminal. Revista de Medicina Familiar y Comunitaria 8: 90-99.

Maiti, S., and Chattopadhyay, S.B. 1986. Studies on diseases of Indian medicinal plants. J. Mycol. Res. 24: 21-27.

Maloy, O.C. 1993. Plant Disease Control. Principles and Practice. John Wiley & Sons. New York, EEUU.

Margulis, L., Dolan, M.F., and Guerrero, R. 2000. The chimeric eukaryote: Origin of thenucleus from the karyomastigont in amitochondriate protists. Proceedings of the National Academy Sciences USA 97: 6954–6959.

Matsumoto, C., Kageyama K., Suga, H., and Hyakumachi, M. 1999. Phylogenetic relationships of *Pythium* species based on ITS and 5, 8S sequences of the ribosomal DNA. Mycoscience 40: 321–331

Maza, M.P., Millán, F., Alaiz, M., Zamora, R., Hidalgo, F.J. y Vioque, E. 1988. Adormidera (*Papaver somniferum* L.), estudio de su aceite y de la harina residual en semillas de producción nacional. Grasas y Aceites 39: 102-104.

Melnik, V.A., and Pystina, K.A. 1995. Novitates de micromycetibus reservati Svirensis inferioris. Novosti Sist. Nizsh. Rast. 30: 29-36.

Montes-Borrego, M., Muñoz-Ledesma F.J., Jiménez-Díaz R.M., and Landa B.B. 2009. An improved nested-PCR protocol for the detection of *Peronospora arborescens*, the downy mildew pathogen of opium poppy, from herbarium specimens and asymptomatic tissues useful for population biology studies. Phytopathology 99: 73-81.

Montes-Borrego, M., Muñoz-Ledesma, F. J., Jiménez-Díaz, R. M., and Landa, B. B. 2008. Downy Mildew of commercial opium poppy crops in France is caused by *Peronospora arborescens*. Plant Dis. 92: 834.

Muñoz-Ledesma. 2002. Etiología y Control de la Necrosis de la adormidera *Papaver somniferum* L. Tesis Doctoral. Universidad de Córdoba.

Muskett, A., and Malone, J. 1984. Catalogue of Irish fungi - V. Mastigomycotina and Zygomycotina. Proceedings of the Royal Society of London B 84: 83-102.

Nolte, H.W. 1951. Krankheiten und Schädlinge der Olfrüchte. [Diseases and pests of oil crops]. Radebeul and Berlin, 64 pp.

Pape-Kiel, H. 1944. Die wichtigsten Ölmohn krankheiten und Schädlingen und ihre Bekämpfung. [The most important poppy diseases and pests and their control]. Deut. Landw. Mitt. 59: 734-736.

Petersen, A.B. and Rosendahl, S. 2000. Phylogeny of the Peronosporomycetes (Oomycota) based on partial sequences of the large ribosomal subunit (LSU rDNA). Mycol. Res. 104: 1295-1303.

Pfyffer, G., Borashi-Gaia, E., Weber, B., Hoesch, L., Orpin, C.G. and Rast, D. 1990. A further report on the occurrence of acylic sugar alcohols in fungi. Mycol. Res. 92: 219-222.

Pietkiewicz, T.A. 1958. From studies on diseases of oleaginous plants. Roczn. Nunk. Rol. 78: 199.

Populer, C. 1981. Epidemiology of downy mildews. Págs 45-105 en: The Downy Mildews. Ed: D.M. Spencer. Academic Press, London.

Regidor, E., Gutiérrez-Fisac J.L., Calle, M.E. y Otero, A. 1998. Patrón de mortalidad en España. Medicina Clínica 118: 13-5.

Reid, D.A. 1969. New or interesting British plant diseases. Transactions of the British Mycological Society 52: 19–38.

Renfro, B.L. and Shankara-Bhat S. 1981. Role of Wild Host in Downy Mildew Diseases. Págs 107-119 en: The Downy Mildews. Ed: D.M. Spencer. Academic Press, London.

Riethmüller, A., Voglmayr H., Göker, M., Weiß, M., and Oberwinkler, F. 2002. Phylogenetic relationships of the downy mildews (Peronosporales) and related groups based on nuclear large subunit ribosomal DNA sequences. Mycologia, 94: 834–849.

Riethmüller, A., Weiß, M., Oberwinkler, F. 1999. Phylogenetic studies of Saprolegniomycetidae and related groups based on nuclear large subunit DNA sequences. Can. J. Bot. 77: 1790-1800.

Rossman, A.Y., and Palm, M.E. 2006. Why are Phytophthora and other Oomycota not true Fungi? http://www.apsnet.org/online/feature/oomycetes/

Sackston, W.E. 1981. Downy mildew of sunflower. Págs 546-572 en: The Downy Mildews. Ed: D.M. Spencer. Academic Press, London.

Savile, D.B.O. 1946. Entyloma fuscum and related smuts attacking *Papaveraceae*. Canadian Journal Res. Sect. C. 24: 109-114.

Savulescu, T. 1948. Les especes de *Peronospora* Corda de Roumanie. Sydowia 2: 255-307.

Schena, L., Duncan, J.M., and Cooke, D.E L. 2008. Development and application of a PCR-based 'molecular tool box' for the identification of *Phytophthora* species damaging forests and natural ecosystems. Plant Pathol. 57:64-75.

Schiltz, P. 1981. Downy mildew of tobacco. Págs 577-599 en: The Downy Mildews. Ed: D.M. Spencer. Academic Press, London.

Schmitt, C.G., and Lipscomb, B. 1975. Pathogens of selected members of the *Papaveraceae*, an annotated bibliography. (Washington). Series title: U. S. Agricultural Research Service. Northeastern Region. ARS-NE-62, 186 pp.

Scott, J.B., Hay, F.S., and Wilson, C.R. 2004. Phylogenetic analysis of the downy mildew pathogen of oilseed poppy in Tasmania, and its detection by PCR. Mycol. Res. 108:98-295.

Scott, J.B., Hay, F.S., Wilson, C.R., Cotterill, P.J., and Fist, A.J. 2003. Spatiotemporal analysis of epiphytotics of downy mildew of oilseed poppy in Tasmania, Australia. Phytopathology 93:725-757.

Sharif, 1970. Downy mildew of opium poppy in Iran, *Peronsopora arborescens* (Berk.) de Bary. Iranian J. Plant Pathol. 6:1-16

Shaw, C.G. 1978. *Peronosclerospora* species and other downy mildews of the Gramineae. Mycologia 70: 594–604.

Shaw, C.G. 1981. Taxonomy and evolution. Págs 17-29 en: The Downy Mildews. Ed: D.M. Spencer. Academic Press, London.

Silvar, C., Duncan, J.M., Cooke, D. E. L., Williams, N.A., Díaz, J., and Merino, F. 2005. Development of specific PCR primers for identification and detection of *Phytophthora capsici* Leon. Eur. J. Plant Pathol. 112:3-52

Singh, H.G. 1982. Cultivation of opium poppy. Págs 120-138 en: Cultivation and utilization of medicinal plants. Eds: C.K. Atal and B.M. Kapur. Regional Research Laboratory Council of Scientific and Industrial Research, Jammu-Tawi.

Sivanesan, A., and Holliday, P. 1982. Commonwealth Mycological Institute. CMI Descriptions of Pathogenic Fungi and Bacteria Nº 730. *Pleospora papaveracea.*

Skalický, V. 1966. Taxonomie der Gattungen der Familie Peronosporaceae. Preslia 38: 117-129.

Slusarenko, A.J., and Schlaich, N.L. 2003. Downy mildew of *Arabidopsis thaliana* caused by *Hyaloperonospora parasitica* (formerly *Peronospora parasitica*). Mol. Plant Pathol. 4: 159-170.

Tewari, J.P., and Skoropad, W.P. 1981. A new disease of common poppy in Canada caused by a downy mildew. Can. Plant Dis. Survey 61: 37-38

Thines, M., and Spring, O. 2005. A revision of *Albugo* (Chromista, Peronosporomycetes). Mycotaxon **92**: 443-458.

Tsay, J.G., Chu, C., Chuang, Y.H., and Chen, R.S. 2006. Specific detection of *Peronospora tabacina* by PCR-amplified rDNA Sequences. Plant Pathol. J. 5:78-382.

Vanev, S.G., Dimitrova, E.G., and Ilieva, E.I. 1993. Order Peronosporales. Fungi Bulgaricae 2: 1-195.

Voglmayr, H. 2003. Phylogenetic relationships of *Peronospora* and related genera based on nuclear ribosomal ITS sequences. Mycol. Res. 107: 1132-1142

Waterhouse, G.M. 1973. Peronosporales. Págs 165-183 en: The fungi-an advanced treatise. Eds: G.C. Ainsworth, F.K. Sparrow, and A.S. Sussman. New York: Academic Press.

Weltzien, H.C. 1981. Geographical Distribution of Downy Mildews. Págs 31-43 en: The Downy Mildews. Ed: D.M. Spencer, Academic Press, London.

Wiberg, L. 1990. Diseases of oil poppy. Farm Note Nº 221. Tasmanian Department of Primary Industry, 69 pp.

Winton, L.M., Stone, J.K., Watrud, L.S. and Hansen, E.M. 2002. Simultaneous one-tube quantification of host and pathogen DNA with real-time polymerase chain reaction. Phytopathology 92: 112-116.

Wong, M.L., and Medrano, J.F. 2005. Real-time PCR mRNA quantification. Biotechniques 39:75-85

World Health Organization. 1986. Cancer Pain Relief. Geneva: Worl Health Organization.

Xu, X.M. and Ridout, M.S. 2001. Effects of prevailing wind direction on spatial statistics of plant disease epidemics. J. of Phytopathol. 149: 155-166

Yossifovith, M. 1928. Kingdom of the Serbs, Croats, and Slovenes. A destructive disease of the poppy in Southern Serbia. Int. Bul. Plant Prot. 2 (2): 210-211.

Yossifovith, M. 1929. Peronospora arborescens(Berk.) de Bary parasite tres important de Papaver somniferum en Yougoslavie. Review. Pathological Entomological. Agricola. 16: 235-270.

Zech, D.F.J., Grond, S., Lynch, J., Hertel D. and Lehman, K. A. 1995. Validation of World Health Organization guidelines for cancer pain relief. A 10 year prospective study. Pain 63:65-7

Zhang, Z., Zhang, J., Wang, Y. and Zheng, X. 2005. Molecular detection of *Fusarium oxysporum* f. sp. *niveum* and *Mycosphaerella melonis* in infected plant tissues and soil. FEMS Microbiology Letters 249: 39-47.

Zhuang, W.Y. 2005. Fungi of northwestern China. Mycotaxon, Ltd., Ithaca, NY, 430 pages.