UNIVERSIDAD DE CÓRDOBA
ESCUELA TÉCNICA SUPERIOR DE INGENIEROS AGRÓNOMOS Y MONTES

TESIS DOCTORAL

CONTROL DE LA PODREDUMBRE RADICAL CAUSADA POR *Phytophthora cinnamomi* EN DEHESAS MEDIANTE BIOFUMIGACIÓN CON *Brassica* spp.

Pedro Ríos Castaño
Córdoba, 2017

ETSIAM

TITULO: *Control de la podredumbre radical causada por Phytophthora cinnamomi en dehesas mediante biofumigación con Brassica spp.*

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ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA AGRONÓMICA Y DE MONTES

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BRASSICACEAE BIOFUMIGANTS FOR CONTROL OF THE OAK ROOT ROT CAUSED BY *PHYTOPHTHORA CINNAMOMI* IN OPEN WOODLANDS

PEDRO RÍOS CASTAÑO

Directores:

Dra. María Esperanza Sánchez Hernández

Dr. Antonio de Haro Bailón

Dra. María del Perpetuo Socorro Serrano Moral

Córdoba, junio de dos mil diecisiete

ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA AGRONÓMICA Y DE MONTES

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Memoria redactada para optar al grado de Doctor con Mención Internacional por la Universidad de Córdoba, por el Ingeniero de Montes:

Pedro Ríos Castaño

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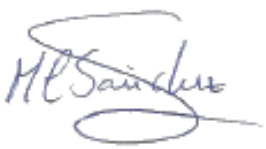		
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Este trabajo ha sido realizado en el Grupo PAIDI AGR- 216 (*Patología Agroforestal*) del Departamento de Agronomía de la Escuela Técnica Superior de Ingeniería Agronómica y Montes (ETSIAM) de la Universidad de Córdoba a través de un contrato laboral predoctoral financiado por la Consejería de Economía, Innovación y Ciencia de la Junta de Andalucía mediante el proyecto de excelencia *"Control de la podredumbre radical de la encina en las dehesas: biofumigación, fertilización y tolerancia natural e inducida"* (P10-AGR-6501). Asimismo, el proyecto LIFE+ "*Dehesa ecosystems: development of policies and tools for biodiversity conservation and management*" de la Unión Europea (LIFE11 BIO/ES/000726) y el proyecto INIA "*La Seca de la encina y el alcornoque en la dehesa. Seguimiento temporal de su impacto y alternativas de control: biofumigantes, enmiendas y búsqueda de resistencias*" (RTA2014-00063-C04-03) han contribuído a la financiación de los trabajos incluidos en esta Tesis.

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No puedo olvidarme de mis compañeros durante esta travesía, sufridores todos pero llegando (o ya llegados) a buen puerto: Mario González (Mario II), sin tu ayuda no podría haber terminado la Tesis, mil gracias por todo el curro…aprieta que ya queda poco. Paolo, Mª Ángeles, Ángela, Mario I, Joaquín… al personal del Departamento de Ingeniería Forestal, Ramón, Maite, Pilar, Mª Dolores y al de Mejora Genética Vegetal del IAS-CSIC. A los amigos americanos, que tan bien me trataron en USA: Andy, Benja, Jared, Thank you so much! A todos, gracias por hacerme más llevaderos estos años.

Como no, a mi familia, apoyo fundamental en mi vida…pues doctorándome…tela, ¿verdad? No entiendo esto sin vosotros, hasta los niños tienen parte de esta Tesis. Seguiremos consiguiendo nuestros propósitos. Sofía, son ya muchos años y hasta te has manchado las manos inoculando suelo…a la par llegaremos donde nos propongamos.

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Todos y cada uno de vosotros tenéis un pedacito de esta Tesis. Eternamente agradecido.

Córdoba, junio de dos mil diecisiete

TÍTULO DE LA TESIS: Control de la podredumbre radical causada por *Phytophthora cinnamomi* en dehesas mediante biofumigación con *Brassica* spp.

DOCTORANDO/A: Pedro Ríos Castaño

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

Dña. María Esperanza Sánchez Hernández, Profesora Titular del Área de Producción Vegetal del Departamento de Agronomía de la ETSIAM, Universidad de Córdoba; D. Antonio de Haro Bailón, Profesor de Investigación del Consejo Superior de Investigaciones Científicas IAS-CSIC y Dña. María del Perpetuo Socorro Serrano Moral, Investigadora Marie-Curie del Consejo Superior de Investigaciones Científicas IRNAS-CSIC

INFORMAN

Que el trabajo "*Control de la podredumbre radical causada por* Phytophthora cinnamomi *en dehesas mediante biofumigación con* Brassica *spp.*" del que es autor Pedro Ríos Castaño, se ha realizado en el Departamento de Agronomía de la Escuela Técnica Superior de Ingeniería Agronómica y Montes (ETSIAM) perteneciente al Campus de Excelencia Internacional Agroalimentario de Andalucía (ceiA3) en el marco del proyecto de excelencia *"Control de la podredumbre radical de la encina en las dehesas: biofumigación, fertilización y tolerancia natural e inducida"* financiado por la Consejería de Economía, Innovación y Ciencia de la Junta de Andalucía (P10-AGR-6501). El proyecto LIFE+ "*Dehesa ecosystems: development of policies and tools for biodiversity conservation and management*" financiado por la Unión Europea (LIFE11 BIO/ES/000726), y el proyecto "*La Seca de la encina y el alcornoque en la dehesa. Seguimiento temporal de su impacto y alternativas de control: biofumigantes, enmiendas y búsqueda de resistencias*" financiado por el MAPAMA-INIA (RTA2014-00063-C04-03), desarrollados en la Universidad de Córdoba, también han contribuído a financiar los trabajos que han llevado a la elaboración de la Tesis, en colaboración con el Instituto de Agricultura Sostenible (IAS-CSIC) y la Universidad de California en Berkeley (USA). Durante su periodo de formación, el doctorando realizó una estancia de 4 meses en la Universidad de Idaho en

Moscow (USA) bajo la supervisión del doctor M.J. Morra, becado por la Comisión de Másteres y Doctorado de la Universidad de Córdoba. Como resultado de esta colaboración se preparó un artículo científico (*Formulating a* Brassicaceae-*based product effective against* Phytophthora cinnamomi) que se encuentra en fase de revisión para su publicación en la revista *Plant Disease* y que forma parte de la tesis doctoral.

Previamente, en julio de 2013 el doctorando participó en el Curso de Formación (*Training School*) "*Field and laboratory methods for detection of invasive insect pests and pathogens*" en la Universidad de Sarajevo en Bosnia-Herzegovina, financiado por una beca del programa COST de la UE (Action FP1002 PERMIT).

Los resultados que se han ido generando a lo largo de la realización de esta Tesis doctoral se han expuesto y discutido en los foros y congresos científicos nacionales e internacionales que se detallan a continuación:

Comunicaciones presentadas en Congresos Internacionales

1. 15th Congress of the Mediterranean Phytopathological Union. Córdoba, Spain (20-23 June 2017). 1 comunicación.

2. IOBC-WPRS 8th Meeting Integrated Protection in *Quercus* spp. forests. Córdoba, Spain (23-27 October 2016). 1 comunicación.

3. 7th IUFRO Meeting Working Party 7-02-09. Phytophthora in Forest and Natural Ecosystems. Esquel, Argentina (10-14 November 2014). 2 comunicaciones.

4. IOBC-WPRS 7th Meeting Integrated Protection in *Quercus* spp. forests. Avignon, Francia (08-11 October 2013). 1 comunicación.

5. Biomicroworld 2013. V International Conference on Environmental, Industrial and Applied Microbiology. Madrid, Spain (02-04 October 2013). 1 comunicación.

6. 6th IUFRO Meeting Working Party 7-02-09. Phytophthora in Forest and Natural Ecosystems. Córdoba, Spain (09-14 September 2012). 2 comunicaciones.

Comunicaciones presentadas en Congresos y Jornadas Científicas Nacionales

7. 55ª Reunión Científica de la Sociedad Española para el Estudio de los Pastos (SEEP). Lugo-A Coruña (26-29 abril 2016). 1 comunicación.

8. 54ª Reunión Científica de la Sociedad Española para el Estudio de los Pastos (SEEP). Palma de Mallorca (14-17 abril 2015). 1 comunicación.

9. 53ª Reunión Científica de la Sociedad Española para el Estudio de los Pastos (SEEP). Potes, Cantabria (09-12 junio 2014). 1 comunicación.

y han dado lugar a las siguientes publicaciones:

Artículos publicados en revistas científicas indexadas (JCR, Journal Citation Reports)

1. Ríos P, González M, Morra MJ, de Haro A, Sánchez ME (2017) Formulating a Brassicaceae-based product effective against *Phytophthora cinnamomi*. **Plant Disease** (under review). American Phytopathological Society, USA. ISSN 0191-2917.
Índice de impacto JCR 2015: 3.192 Plant Sciences, 1er cuartil.

2. Ríos P, González M, Obregón S, Carbonero MD, Leal JR, Fernández P, de Haro A, Sánchez ME (2017) Biofumigant action of *Brassica* seedmeals against *Phytophthora cinnamomi* in *dehesa* ecosystems. **Phytopathologia Mediterranea** (under review). Mediterranean Phytopathological Union, Italy. ISSN 0031-9465.
Índice de impacto JCR 2015: 1.042 Agronomy, 2º cuartil.

3. Ríos P, Obregón S, González M, de Haro A, Sánchez ME (2016) Screening brassicaceous plants as biofumigants for management of *Phytophthora cinnamomi* oak disease. Forest Pathology 46: 652-659. Wiley-Blackwell, Germany. ISSN 1437-4781. DOI: 10.1111/efp.12287
Índice de impacto JCR 2015: 1.437 Forestry, 2º cuartil.

4. Ríos P, Obregón S, de Haro A, Fernández-Rebollo, P, Serrano MS, Sánchez ME (2016) Effect of *Brassica* biofumigant amendments on different stages of the life cycle of *Phytophthora cinnamomi*. Journal of Phytopathology 164: 582-594. Wiley-Blackwell, Germany. ISSN 0931-1785. DOI: 10.1111/jph.12482
Índice de impacto JCR 2015: 0.945 Plant Sciences, 3er cuartil.

5. Serrano MS, Ríos P, González M, Sánchez ME (2015) Experimental minimum threshold for *Phytophthora cinnamomi* root disease on *Quercus suber*. Phytopathologia Mediterranea 54: 461-464. Mediterranean Phytopathological Union, Italy. ISSN 0031-9465. DOI: 10.14601/Phytopathol_Mediterr-15128
Índice de impacto JCR 2015: 1.042 Agronomy, 2º cuartil.

Artículos publicados en revistas científicas no indexadas y de divulgación científica

6. Serrano MS, Ríos P, González M, Romero MA, Fernández P, Sánchez ME (2017). Integrated control of Phytophthora root rot in oak rangeland ecosystems. IOBC/WPRS Bulletin (in press). International Organization for Biological and Integrated Control of Noxious Animals and Plants, West Palearctic Regional Section (IOBC-WPRS), Darmstadt. ISBN 978-92-9067-282-1

7. Ríos P, Obregón S, de Haro A, Sánchez ME (2014). Screening of potential biofumigant plants against *Quercus* root rot caused by *Phytophthora cinnamomi*. IOBC/WPRS Bulletin 101: 125-132. International Organization for Biological and Integrated Control of Noxious Animals and Plants, West Palearctic Regional Section (IOBC-WPRS), Darmstadt. ISBN 978-92-9067-282-1

8. Serrano MS, Ríos P, De Vita P, Fernández-Rebollo P, Sánchez ME (2013). Selección de morfotipos de encina tolerantes a la podredumbre radical. Vida Rural 362: 22-25. Eumedia, ISSN 1133-8938

Capítulos de libro

1. Ríos P, Obregón S, de Haro A, Sánchez ME (2014). Soil biofumigant treatments for control of the alien pathogen *Phytophthora cinnamomi*. In: Industrial, medical and environmental applications of microorganisms. Current status and trends. Méndez-Vilas A (Ed.). Wageningen Academic Publishers, Wageningen, Netherlands. pp. 243-248. ISBN 978-90-8686-243-6

Artículos publicados en Proceedings

1. Gutiérrez-Hernández O, García LV, De Vita P, Serrano MS, Ramo C, Gutiérrez E, Ríos P, Pérez-Ramos I, Gómez-Aparicio L, Sánchez ME (2015). Influence of multiple stress sources on cork oak (*Quercus suber* L.) seedling susceptibility to *Phytophthora cinnamomi*. Proceedings of the 7th meeting of the International Union of Forest Research Organization (IUFRO) Working Party S07.02.09: Phytophthoras in forests and natural ecosystems. Sutton W, Reeser PW, Hansen EM (Tech coords.). Forest Phytophthoras of the World, Oregon State University. pp. 132-133.

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3. Ríos P, Obregón S, de Haro A, Fernández P, Sánchez ME (2014). Evaluation of biofumigant plants for control of *Quercus* root rot caused by *Phytophthora cinnamomi* in rangelands ecosystems. In: Phytophthoras in Forests and Natural Ecosystems. Proceedings of the 6th

Meeting of the International Union of Forest Research Organizations (IUFRO) Working Party S07-02-09. Forest Phytophthoras of the World, Oregon State University Jung T, Brasier CM, Sánchez ME, Pérez-Sierra A (Eds.), pp. 151

4. Serrano MS, Leal JR, De Vita P, Ríos P, Fernández P, Sánchez ME (2014). Can limestone amendments and pig slurry be considered as control methods for *Quercus ilex* root rot caused by *Phytophthora cinnamomi* in dehesa? Preliminary results from field trials. In: Phytophthoras in Forests and Natural Ecosystems. Proceedings of the 6th Meeting of the International Union of Forest Research Organizations (IUFRO) Working Party S07-02-09. Forest Phytophthoras of the World, Oregon State University Jung T, Brasier CM, Sánchez ME, Pérez-Sierra A (Eds.), pp. 98

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1. Fernández P, Carbonero MD, Leal JR, García AM, Ríos P, Sánchez ME, Obregón S, de Haro A (2016). Biomasa radical incorporada al suelo tras el cultivo de *Brassica carinata* y *Brassica juncea* en la dehesa. Concentración y perfil de glucosinolatos. En: Innovación sostenible en pastos: Hacia una agricultura de respuesta al cambio climático. Actas de la 55ª Reunión Científica de la Sociedad Española para el Estudio de los Pastos. SEEP, A Coruña. pp. 195-200. ISBN: 978-84-608-7722-6

2. Fernández P, Carbonero MD, Leal JR, García AM, Ríos P, Sánchez ME, Obregón S, de Haro A (2015). Producción, concentración y perfil de glucosinolatos de *Brassica carinata* y *Brassica juncea* cultivadas en la dehesa. En: Pastos y Forrajes en el Siglo XXI. Actas de la 54ª Reunión Científica de la S.E.E.P. SEEP, Palma. pp. 193-200. ISBN: 978-84-606-7295-1

3. Fernández P, Carbonero MD, Leal JR, García AM, Ríos P, Sánchez ME, Obregón S, de Haro A (2014). Capacidad de competencia de líneas de *Brassica carinata*, *Brassica juncea* y *Sinapis alba* seleccionadas por su poder biofumigante en cultivos en dehesa. En: Pastos y PAC 2014-2020. Actas de la 53ª Reunión Científica de la Sociedad Española para el Estudio de los Pastos. SEEP, Potes. pp. 169-176. ISBN: 978-84-697-0561-2

El trabajo tiene carácter de investigación y reúne los requisitos necesarios para su exposición y defensa como Tesis Doctoral con mención internacional.

Por todo ello, se autoriza la presentación de la tesis doctoral.

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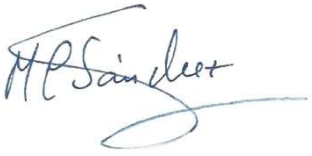

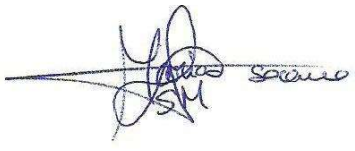

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European/International Mention in the Doctorate Degree

Certificate of stay

I hereby confirm that Mr. / Mrs Pedro Ríos Castaño

has steadily stayed at University of Idaho, Moscow, Idaho, United States

from 10/01/2013 to 01/07/2014 and has successfully developed research in Plant-derived Allelochemicals

that is directly related with his/her PhD thesis in Control of Oak root rot caused by *Phytophthora cinnamomi* in rangelands by biofumigation with Brassica sp.

Signature of the director/responsible of the research group

University of Idaho
Matthew J. Morra
College of | Agricultural and Life Sciences

Matthew J. Morra

Institutional Stamp

Signed in Moscow, 07 of January , 2014

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TESIS DOCTORAL

REFEREE REPORT ON THE PhD THESIS PRESENTED
IN THE UNIVERSITY OF CÓRDOBA (SPAIN)
BY **PEDRO RÍOS CASTAÑO**

TITLE OF THE THESIS:	***Brassicaceae* BIOFUMIGANTS FOR CONTROL OF THE OAK ROOT ROT CAUSED BY *Phytophthora cinnamomi* IN OPEN WOODLANDS (DEHESAS)**

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Rating	Originality	Scientific /technical merit	Planning /methodology
Outstanding			
Excellent			
Very Good	x	x	x
Good			
Sound			
Defficient			

COMMENTS (Please use additional sheets, if necessary):

Very productive dissertation and very useful information on control methods for an important forest pathogen which has been difficult to control

DATE: June 15, 2017

SIGNATURE: Louise-Marie Dandurand

INFORME DOCTORES INTERNACIONALES
TESIS DOCTORAL

REFEREE REPORT ON THE PhD TESIS PRESENTED
IN THE UNIVERSITY OF CÓRDOBA (SPAIN)
BY **PEDRO RÍOS CASTAÑO**

TITLE OF THE THESIS:	***Brassicaceae* BIOFUMIGANTS FOR CONTROL OF THE OAK ROOT ROT CAUSED BY *Phytophthora cinnamomi* IN OPEN WOODLANDS (DEHESAS)**

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Outstanding			
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Very Good	X	X	X
Good			
Sound			
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DATE: 6. June 2017

SIGNATURE: Isabella Børja

Autor/a: **PEDRO RÍOS CASTAÑO**

Título**: Control de la prodredumbre radical causada por *Phytophora cinnamomi* en dehesas mediante biofumigación con *Brassica* spp.**

Director/a/s/es: **María Esperanza Sánchez Hernández, Antonio de Haro Bailón, María Socorro Serrano Moral**

Fecha inscripción: **19/01/2012**

Estudios de doctorado en: **PROGRAMA DE DOCTORADO EN BIOCIENCIAS Y CIENCIAS AGROALIMENTARIAS**

RESUMEN

Los ecosistemas de dehesa (*montados* en Portugal) son uno de los más característicos de la Península Ibérica. Su importancia tanto ecológica como económica ha llevado a la inclusión de este ecosistema entre los protegidos bajo la Directiva HÁBITATS de la Unión Europea (Anexo I de la Directiva del Consejo 92/43/ EEC relativa a la conservación de los hábitats naturales y de la fauna y flora silvestre) y a formar parte de la RED NATURA 2000. Las dehesas son ecosistemas seminaturales creados y mantenidos por el hombre, constituidos por arboleda dispersa, principalmente del género *Quercus,* pastos, matorral, fauna silvestre y ganadería extensiva. La dehesa es ante todo un sistema de producción ecológica en donde la integración de la fauna silvestre, el ganado y la arboleda define el carácter seminatural del ecosistema.

Desde la década de los 90 se viene observando un severo decaimiento de los *Quercus* mediterráneos en las dehesas del suroeste de España y Portugal que está ocasionando una alta mortalidad de encinas y alcornoques. Entre los factores asociados a este decaimiento, la podredumbre radical causada por *Phytophthora cinnamomi* destaca por su gravedad. *Phytophthora cinnamomi* es un patógeno de distribución mundial capaz de infectar a miles de especies huéspedes. Se encuentra en la lista de las 100 peores especies invasoras del mundo, y es especialmente virulento causando la podredumbre radical de encinas y alcornoques que lleva a la muerte del arbolado, que según las condiciones ambientales puede ser un proceso de muerte lenta (decaimiento) o de muerte súbita. En ambos casos, los síntomas observados a nivel aéreo son amarillez y/o marchitez foliar, defoliación y muerte regresiva de brotes y ramas (puntisecado) y a nivel radical, necrosis de las raicillas absorbentes o ausencia de las mismas.

La enfermedad radical es difícil de controlar debido a la longevidad de las esporas de resistencia, su alta capacidad de movimiento a través del agua libre del suelo y a su alto número de huéspedes. En ecosistemas de dehesa, la naturaleza del patosistema *Quercus/ Phytophthora* no permite la utilización de métodos de control legislativo (cuarentenas) o la aplicación masiva de fungicidas, por lo que se debe plantear un sistema de control que integre distintas medidas culturales y biológicas de bajo coste y acordes a la producción ecológica de las dehesas.

En este contexto, la biofumigación se presenta como una técnica candidata a formar parte del manejo integrado de la enfermedad. La biofumigación se basa en la actividad biocida de compuestos volátiles (isotiocianatos, nitrilos, etc.) liberados tras la hidrólisis de los glucosinolatos (GSLs), metabolitos secundarios producidos por especies de la familia *Brassicaceae.* Los GSLs son enzimáticamente hidrolizados en el suelo por la acción de la enzima endógena mirosinasa. Se conocen más de 130 GSLs diferentes, pero el efecto nocivo de éstos contra patógenos, plagas y malas hierbas parece estar relacionado con un perfil concreto de GSLs, o con un GSL específico. Está demostrado que estos productos son tóxicos para un amplio número de patógenos de suelo, incluyendo a hongos, bacterias y nematodos, así como a oomicetos pertenecientes al género *Phytophthora,* pero su efecto sobre *P. cinnamomi* aún es desconocido.

Basándonos en todas estas consideraciones, esta Tesis parte de la hipótesis de que los compuestos volátiles liberados tras la incorporación al suelo de material biofumigante puede minimizar la capacidad de *P. cinnamomi* de infectar raíces de encinas y alcornoques en dehesas.

El primer paso para poder verificar la efectividad de esta estrategia de control ha sido conocer cuál es la densidad de inóculo crítica a partir de la cual se desarrolla la enfermedad radical. En observaciones de campo se han referenciado densidades de inóculo muy variables en dehesas con presencia de *P. cinnamomi* en el sur de España, en una horquilla que varía entre 4 y 2500 Unidades Formadoras de Colonia (CFU)$\times g^{-1}$ de suelo seco. Nuestros experimentos demuestran que infestaciones de suelo a partir de 61 CFU$\times g^{-1}$ dan lugar al desarrollo de los síntomas radicales en *Quercus*, si bien valores tan bajos como 3 CFU$\times g^{-1}$ son suficientes para poder detectar la presencia del patógeno en raíces asintomáticas.

Se evaluó cómo la biofumigación afecta a los distintos estadios vegetativos y reproductivos del ciclo de vida de *P. cinnamomi*. Para ello se ensayaron diferentes

genotipos de tres especies con potencial biofumigante cosechadas en distintos estados fenológicos: *Brassica carinata, B. juncea* y *B. napus*. Se analizó el perfil de GSLs de cada genotipo y su evolución con el desarrollo de la planta, y posteriormente se evaluó el efecto de los volátiles liberados tras la hidrólisis de los tejidos sobre el crecimiento miceliar, la producción de esporangios y zoosporas, la viabilidad de las clamidosporas del patógeno en suelo natural infestado y el desarrollo de la enfermedad *in planta*. De los biofumigantes seleccionados, sólo los genotipos de *B. carinata* y *B. juncea,* ricos en el GSL sinigrina, demostraron potencial para ser usadas contra *P. cinnamomi,* ya que inhibieron su crecimiento miceliar, disminuyeron significativamente la producción de esporangios y disminuyeron la viabilidad de las clamidosporas en el suelo por debajo del umbral mínimo para el desarrollo de la enfermedad en raíces de *Quercus*. Sin embargo, cuando los suelos tratados se testaron sembrando plantas altamente susceptibles al patógeno (*Lupinus luteus*) sólo las plantas que crecieron en suelos biofumigados con *B. carinata* desarrollaron significativamente menos síntomas radicales que aquellas sembradas en suelos no tratados o en suelos biofumigados con el resto de especies.

Se evaluó la efectividad de la harina de semillas de los mismos genotipos debido a su alta concentración en GSLs con respecto a las partes verdes en cualquier estado fenológico. Se comprobó que de nuevo sólo los genotipos ricos en sinigrina resultaron efectivos en la disminución de la viabilidad de las clamidosporas por debajo del umbral crítico para el desarrollo de la enfermedad en *Quercus* y, en este caso, la biofumigación del suelo con estos genotipos dio lugar a que las plantas de *Lupinus* no mostraran síntomas de la enfermedad, difiriendo del elevado nivel de síntomas que se observó en las plantas que crecieron en suelo no biofumigado o biofumigado con harina de semillas de genotipos sin sinigrina. Sin embargo, en ensayos de producción en dehesa de genotipos de *B. carinata* y *B. juncea* ricos en sinigrina se comprobó el escaso rendimiento de estos cultivos, por lo que se concluye que la producción directa de biofumigantes en dehesa no resulta factible y sería necesario producir semilla en otras áreas agrícolas de mayor productividad para ser posteriormente aplicada en suelos de dehesa.

Para confirmar que la efectividad de los biofumigantes testados se debe a su elevado contenido en el GSL sinigrina y conocer si hay otras especies efectivas con distinto perfil de GSLs, se evaluó la actividad biofumigante de 14 especies de plantas

pertenecientes a la familia *Brassicaceae*. En primer lugar, se clasificaron a las especies candidatas en tres grupos en función de su perfil de GSLs: Grupo A, plantas ricas en GSLs aromáticos, Gupo B1, plantas ricas en sinigrina y Grupo B2, plantas ricas en GSLs alifáticos distintos de sinigrina, y posteriormente se evaluó la inhibición del crecimiento micelial de *P. cinnamomi* como indicativo de su capacidad biofumigante, seleccionando especies de distinto grupo para evaluar su efectividad en la disminución de la viabilidad de las clamidosporas en suelo, y el posterior desarrollo de la enfermedad en plantas de *Lupinus*. Los resultados obtenidos confirmaron el papel de la sinigrina como principal GSL con capacidad biofumigante sobre *P. cinnamomi*, ya que únicamente los biofumigantes del Grupo B1 mostraron efecto biocida sobre cultivos *in vitro* de *P. cinnamomi* y sobre las clamidosporas en el suelo, impidiendo el desarrollo de la enfermedad en las plantas que crecieron en los suelos infestados que fueron tratados con estos biofumigantes.

Tras la confirmación de que la capacidad biofumigante contra *P. cinnamomi* reside en el contenido en sinigrina del material empleado, se planteó el desarrollo de un producto *ready-to-use* con una alta concentración en este GSL para su aplicación directa en suelos de dehesa usando como materia prima un residuo industrial y fertilizantes cálcicos de efectividad demostrada contra *P. cinnamomi.* El producto, altamente efectivo *in vitro*, consistió en un extracto enriquecido en GSLs procedente de residuos de *B. juncea* producidos en la industria de obtención de aceites, suplementado con materia verde liofilizada como fuente de mirosinasa y $CaCO_3$ como corrector de pH.

Este trabajo demuestra que la biofumigación es una técnica efectiva que puede ser incorporada en la lucha integrada contra la podredumbre radical de los *Quercus* causada por *P. cinnamomi* en ecosistemas de dehesa. La biofumigación con plantas ricas en sinigrina reduce significativamente la viabilidad de las esporas de resistencia del patógeno en el suelo y minimiza la producción de zoosporas infectivas, disminuyendo significativamente la capacidad de *P. cinnamomi* para infectar raíces y causar enfermedad. Debido a que el método tradicional de enterrado en verde utilizando un cultivo biofumigante producido *in situ* es de difícil aplicación en dehesas por su baja productividad, que también se manifiesta en la producción de semillas (material con mayor contenido en sinigrina), se ha desarrollado un producto efectivo que resulta fácil de obtener, almacenar y usar en dehesas aplicándolo directamente al suelo cuando sea necesario. Además, la fabricación de este producto, usando como materia prima un

residuo de la obtención industrial de aceite, es un respaldo al carácter ecológico que caracteriza la producción en estos ecosistemas.

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1. Ríos P, González M, Morra MJ, de Haro A, Sánchez ME (2017) Formulating a Brassicaceae-based product effective against *Phytophthora cinnamomi*. **Plant Disease** (under review). American Phytopathological Society, USA. ISSN 0191-2917.

Índice de impacto JCR 2015: 3.192 Plant Sciences, 1er cuartil.

2. Ríos P, González M, Obregón S, Carbonero MD, Leal JR, Fernández P, de Haro A, Sánchez ME (2017) Biofumigant action of *Brassica* seedmeals against *Phytophthora cinnamomi* in *dehesa* ecosystems. **Phytopathologia Mediterranea** (under review). Mediterranean Phytopathological Union, Italy. ISSN 0031-9465.

Índice de impacto JCR 2015: 1.042 Agronomy, 2º cuartil.

3. Ríos P, Obregón S, González M, de Haro A, Sánchez ME (2016) Screening brassicaceous plants as biofumigants for management of *Phytophthora cinnamomi* oak disease. **Forest Pathology** 46: 652-659. Wiley-Blackwell, Germany. ISSN 1437-4781. DOI: 10.1111/efp.12287

Índice de impacto JCR 2015: 1.437 Forestry, 2º cuartil.

4. Ríos P, Obregón S, de Haro A, Fernández-Rebollo, P, Serrano MS, Sánchez ME (2016) Effect of *Brassica* biofumigant amendments on different stages of the life cycle of *Phytophthora cinnamomi*. **Journal of Phytopathology** 164: 582-594. Wiley-Blackwell, Germany. ISSN 0931-1785. DOI: 10.1111/jph.12482

Índice de impacto JCR 2015: 0.945 Plant Sciences, 3er cuartil.

5. Serrano MS, Ríos P, González M, Sánchez ME (2015) Experimental minimum threshold for *Phytophthora cinnamomi* root disease on *Quercus suber*. **Phytopathologia Mediterranea** 54: 461-464. Mediterranean Phytopathological Union, Italy. ISSN 0031-9465. DOI: 10.14601/Phytopathol_Mediterr-15128

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Title**: Control de la prodredumbre radical causada por *Phytophora cinnamomi* en dehesas mediante biofumigación con *Brassica* spp.**

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ABSTRACT

Oak rangeland agroforestry systems (*dehesas* in Spain, *montados* in Portugal) are one of the most characteristic ecosystems of the Iberian Peninsula. This is an economically and environmentally valuable ecosystem listed among type habitats protected by the European Union´s Directive on HABITATS (Annex I of Council Directive 92/43/ECC on the Conservation of Natural Habitats and of Wild Fauna and Flora) and taking part of the NATURA 2000 NETWORK. Dehesas are semi-natural ecosystems comprising scattered trees, mainly evergreen oaks belonging to *Quercus* genus, pasture, shrubs, livestock and wild fauna. Dehesa is primarily an ecological production system, where the integration of wild fauna, livestock and trees define the semi-natural character of this ecosystem.

Since the 90´s a severe decline affecting mediterranean *Quercus* has been observed in rangelands of southern Spain and Portugal, leading to a high mortality of holm and cork oak trees. Among decline factors, root rot caused by *Phytophthora cinnamomi* highlights due to its severity. *Phytophthora cinnamomi* has a worldwide distribution, being able to infect thousands of plant hosts. It is included on the IUCN/SSC 100 World´s Worst Invasive Alien Species Catalogue, and it is especially virulent causing root rot in holm and cork oak, leading to the death of trees that, depending on environmental conditions, the disease manifests as a slow death (decline) or a sudden death syndrome. In both situations, symptoms consisted in yellowing and/or crown wilting, defoliation and branch dieback, and at root level, necrosis and loss of feeder roots.

Root rot disease management is difficult due to the longevity of resting spores of *P. cinnamomi* in the soil, due to its rapid and deep dispersal in waterlogged soils, and due

to the large number of hosts. In this rangelands ecosystems, the nature of the *Quercus/Phytophthora* pathosystem does not allow the use of legislative methods for control (quarantines) or the massive application of fungicides, so it should be considered a control system integrating different cultural and biological methods, low costs and suitable for the ecological production of dehesas.

In this context, biofumigation appears as a candidate to become a technique for the integrated disease management. Biofumigation relies on biocide activity of volatile compounds (isothiocyanates, nitriles, etc.) released through the hydrolysis of glucosinolates (GSLs), secondary metabolites produced by *Brassicaceae* species. GSLs are enzymatically hydrolyzed in the soil by the action of the endogenous myrosinase enzyme. More than 130 different GSLs are known, but the noxious action of *Brassicaceae* against pests including pathogens and weeds, seems to be related to their specific GSL profiles, or even one specific GSL. It is a fact that these products inhibit a wide number of soil-borne pathogens, including nematodes, bacteria and fungi, as well as oomycetes belonging to *Phytophthora* genus, although their effect against *P. cinnamomi* remained still unknown.

Based on all these considerations, this Thesis come from the hypothesis that volatile compounds released after the incorporation of biofumigant material to the soil can minimize the ability of *P. cinnamomi* for oak root infection in dehesas ecosystems.

The first step to verify the effectiveness of this control strategy has been to quantify the minimum threshold of inoculum density from which the root disease develops. In field observations, very variable inoculum densities have been reported in dehesas infested by *P. cinnamomi* of southern Spain, ranging from 4 to 2,500 colony forming units (CFU)×g^{-1} of dry soil. Our experiments show that soil infestations over 61 CFU×g^{-1} lead to symptom development in oak roots, whereas asymptomatic root infections occurred from only 3 CFU×g^{-1}.

The effect of biofumigation on the different vegetative and reproductive stages of the pathogen life cycle was evaluated. Different genotypes of three potential biofumigant species, *Brassica carinata, B. juncea* and *B. napus,* harvested at different phenological stages were tested. The GSLs profile of each genotype and its evolution through the plant development were analyzed, and the effect of the volatiles released through the tisular hydrolysis were evaluated on mycelium growth, sporangial and zoospore

production, chlamydospore viability on infested natural soil and disease development *in planta*. Among all biofumigant selected, only both *B. carinata* and *B. juncea* genotypes, with a high content of the GSL sinigrin, showed its potential to be used against *P. cinnamomi*, due to the registered inhibition of mycelium growth, the significant decrease of sporangial production induced, and the decrease of chlamydospore viability on soil below the minimum threshold for *Quercus* root disease development. However, when biofumigated soils were tested by planting highly susceptible hosts (*Lupinus luteus*), only plants growing in soils biofumigated with *B. carinata* developed root sympthoms significantly lower than those planted in non-treated soils or soils biofumigated with other *Brassica* species.

The effect of seedmeals from the same genotypes were evaluated due to its high concentration in GSLs in comparison with green parts at any phenological stage. It was verified that again, only genotypes with a high sinigrin content were effective on the decrease of chlamydospore viability in soil below the minimum threshold for *Quercus* root disease development and, in this case, soil biofumigation with those genotypes leads to *Lupinus* plants did not show disease symptoms, differing from the high symptoms level reached for non-treated soils or soils treated with seedmeals coming from genotypes without sinigrin. However, both *B. carinata* and *B. juncea* genotypes rich in sinigrin yielded very poor productions in dehesa ecosystems, thus it was concluded the unfeasibility of direct production of biofumigants in dehesa and, therefore, it would be needed that seed production will take place in agricultural locations with higher productivity to be later applied in dehesa soils.

To confirm that biofumigant effectiveness is due to its high sinigrin content and know whether other biofumigant species with a different GSLs profile are equally effective, the biofumigant activity of 14 plant species belonging to *Brassicacaceae* family was evaluated. Firstly, the candidate species were classified on three different groups according with their GSLs profile: Group A, plants with high content on aromatic GSLs, Group B1, plants with a high content on sinigrin, and Group B2, plants with high aliphatic GSL content different to sinigrin. Inhibition of *P. cinnamomi* mycelial growth was evaluated as indicative of their biofumigant potential. The next step was to select species belonging to different group to test their effect on the decrease of chlamydospore viability in soil and the later disease development in *Lupinus* plants. Results confirmed the role of sinigrina as main GSL with biofumigant effect against *P.*

cinnamomi, since only plants belonging to Group B1 showed biocide effect on *P. cinnamomi* colonies and on chlamydospore viability in soil, avoiding disease development on *Lupinus* plants growing in infested soils treated with those biofumigants.

After the confirmation that the biofumigant effect on *P. cinnamomi* relies on the sinigrin content of the material used, a *ready-to-use* product with a high concentration on this GSL for its direct application on dehesa soils was formulated. The raw materials used were an industrial waste and calcium fertilizers with demonstrated effectiveness against *P. cinnamomi*. This product, with a high *in vitro* efficacy, consisted on an enriched extract in GSLs coming from *B. juncea* waste produced by oil extraction industry, supplemented with lyophilized green matter as myrosinase source, and $CaCO_3$ as pH corrector.

The current thesis demonstrated that biofumigation is a suitable technique with a high noxious effect against *P. cinnamomi* which can be included in the oak disease integrated management in dehesa woodland ecosystems. Biofumigation with *Brassicaceae* plants rich in sinigrin effectively suppress the viability of *P. cinnamomi* resting spores in the soil, minimizing zoospore production and, consequently, decreasing the ability of *P. cinnamomi* to infect roots and cause oak disease. As the traditional biofumigation strategy using green matter produced in site appears difficult to be applied in dehesa ecosystems due to its low production, also reflected in seed production, an effective product easy to obtain, store and apply has been developped for disease control in dehesa when needed. Even more, the likely commercial manufacture of this kind of products, based on extracts obtained from oil industrial waste, should increase the organic nature of dehesa production and management.

Research articles published on indexed scientific journals (JCR, Journal Citation Reports)

1. Ríos P, González M, Morra MJ, de Haro A, Sánchez ME (2017) Formulating a Brassicaceae-based product effective against *Phytophthora cinnamomi*. **Plant Disease** (under review). American Phytopathological Society, USA. ISSN 0191-2917.

Impact Factor JCR 2015: 3.192 Plant Sciences, 1st quartile.

2. Ríos P, González M, Obregón S, Carbonero MD, Leal JR, Fernández P, de Haro A, Sánchez ME (2017) Biofumigant action of *Brassica* seedmeals against *Phytophthora*

cinnamomi in *dehesa* ecosystems. **Phytopathologia Mediterranea** (under review). Mediterranean Phytopathological Union, Italy. ISSN 0031-9465.

Impact Factor JCR 2015: 1.042 Agronomy, 2nd quartile.

3. Ríos P, Obregón S, González M, de Haro A, Sánchez ME (2016) Screening brassicaceous plants as biofumigants for management of *Phytophthora cinnamomi* oak disease. **Forest Pathology** 46: 652-659. Wiley-Blackwell, Germany. ISSN 1437-4781. DOI: 10.1111/efp.12287

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4. Ríos P, Obregón S, de Haro A, Fernández-Rebollo, P, Serrano MS, Sánchez ME (2016) Effect of *Brassica* biofumigant amendments on different stages of the life cycle of *Phytophthora cinnamomi*. **Journal of Phytopathology** 164: 582-594. Wiley-Blackwell, Germany. ISSN 0931-1785. DOI: 10.1111/jph.12482

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Impact Factor JCR 2015: 1.042 Agronomy, 2nd quartile.

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INTRODUCCIÓN Y OBJETIVOS

CHAPTER 1.
INTRODUCTION AND OBJECTIVES

INTRODUCCIÓN

Los ecosistemas de dehesa (*montados* en Portugal) son uno de los más característicos de la Península Ibérica. Las dehesas son ecosistemas seminaturales creados y mantenidos por el hombre, constituidos por arboleda dispersa, principalmente del género *Quercus,* pastos, matorral, fauna silvestre y ganadería extensiva. En las dehesas españolas, las especies de árboles dominantes son la encina (*Quercus ilex* L. ssp. *ballota* (Desf.) Samp.) y el alcornoque (*Quercus suber* L.), de donde se obtienen los mayores ingresos gracias al corcho y la bellota, un alimento de alto valor nutritivo para el ganado y la fauna silvestre. Su importancia tanto ecológica como económica ha llevado a la inclusión de este ecosistema entre los protegidos bajo la Directiva hábitats de la Unión Europea (Anexo I de la Directiva del Consejo 92/43/ EEC relativa a la conservación de los hábitats naturales y de la fauna y flora silvestre) y a formar parte de la red natura 2000. La dehesa es ante todo un sistema de producción ecológica en donde la integración de la fauna silvestre, el ganado y la arboleda define el carácter seminatural del ecosistema.

La desestabilización de alguno de los elementos que constituyen la dehesa, y que son la base de su sistema de producción, conlleva importantes daños en el ecosistema; lo que supone, además, la no sostenibilidad del mismo y por lo tanto su abandono. Actualmente, este ecosistema se encuentra en peligro debido a distintas amenazas que lo acechan, siendo una de las más importante la pérdida de arbolado debido a la enfermedad radical causada por *Phytophthora cinnamomi.*

Desde la década de los 90 se viene observando un severo decaimiento de los *Quercus* mediterráneos en las dehesas del suroeste de España y Portugal que está ocasionando una alta mortalidad de encinas y alcornoques. Entre los factores asociados a este decaimiento, la podredumbre radical causada por *Phytophthora cinnamomi* destaca por su gravedad. Este oomiceto causa la muerte de las raicillas absorbentes, reduciendo la capacidad del árbol para tomar agua y nutrientes minerales, lo que genera síntomas foliares similares a aquellos debidos a la sequía, siendo capaz de matar al árbol en pocos meses.

Phytophthora cinnamomi es un patógeno de distribución mundial capaz de infectar a miles de especies huéspedes. Se encuentra en la lista de las 100 peores especies invasoras del mundo, y es especialmente virulento causando la podredumbre radical no

sólo de encinas y alcornoques, sino también de especies de alto valor económico como el aguacate o el castaño. El manejo de la podredumbre radical es difícil debido a la longevidad de las esporas de resistencia, su alta capacidad de movimiento a través del agua libre del suelo y a su alto número de huéspedes.

Los métodos de control de la podredumbre radical causada por *P. cinnamomi* deben ser respetuosos con el medio ambiente, debido al carácter seminatural de estos bosques. La lucha contra *P. cinnamomi* en dehesas está basada en la integración de distintas medidas que limiten nuevas infecciones, como son la aplicación de fertilizantes cálcicos al suelo, evitar la siembre de huéspedes herbáceos, evitar las altas cargas ganaderas o mejorar el drenaje del suelo. Todas estas recomendaciones son efectivas, pero ninguna de ellas disminuye la viabilidad de las esporas de resistencia del patógeno presentes en los suelos donde la enfermedad está instalada.

En este contexto, la biofumigación se presenta como una técnica candidata a formar parte del manejo integrado de la enfermedad. La biofumigación se basa en la actividad biocida de compuestos volátiles (isotiocianatos, nitrilos, etc.) liberados tras la hidrólisis de los glucosinolatos (GSLs), metabolitos secundarios producidos por especies de la familia *Brassicaceae.* Los GSLs son enzimáticamente hidrolizados en el suelo por la acción de la enzima endógena mirosinasa. Se conocen más de 130 GSLs diferentes, pero el efecto nocivo de éstos contra patógenos, plagas y malas hierbas parece estar relacionado con un perfil concreto de GSLs, o con un GSL específico. Está demostrado que estos productos son tóxicos para un amplio número de patógenos de suelo, incluyendo a hongos, bacterias y nematodos, así como a oomicetos pertenecientes al género *Phytophthora,* pero su efecto sobre *P. cinnamomi* aún es desconocido.

Basándonos en todas estas consideraciones, esta Tesis parte de la hipótesis de que los compuestos volátiles liberados tras la incorporación al suelo de material biofumigante puede minimizar la capacidad de *P. cinnamomi* de infectar raíces de encinas y alcornoques. La confirmación de la toxicidad de estos volátiles sobre los diferentes estadios vegetativos y reproductivos del patógeno, la identificación del GSL del que provienen, la selección de plantas que lo contienen a las dosis efectivas, y el establecimiento de un método de aplicación en ecosistemas de dehesa, dará lugar a un nuevo, inocuo para el medio ambiente y válido método de control de la enfermedad radical en ecosistemas de dehesa.

OBJETIVOS

El objetivo general de esta Tesis Doctoral es obtener nuevos conocimientos sobre un método de control biológico, la biofumigación, contra la podredumbre radical causada por *Phytophthora cinnamomi* en especies de *Quercus*, que permitan la incorporación de esta estrategia en el control integrado de la enfermedad en ecosistemas de dehesa.

Los objetivos parciales son:

1. Determinar el umbral mínimo de inóculo de *Phytophthora cinnamomi* en el suelo para el desarrollo de la enfermedad radical.
2. Evaluar el efecto biofumigante de material verde de *Brassica* spp. en distintas fases del ciclo de vida del patógeno.
3. Evaluar el efecto biofumigante de harina de semillas de *Brassica* spp. y su producción en ecosistemas de dehesa.
4. Seleccionar especies de Brasicáceas con actividad biofumigante contra *Phytophthora cinnamomi.*
5. Formular un producto biofumigante listo para usar para su aplicación directa en suelos de dehesa infestados por el patógeno.

Los resultados relacionados con el primer objetivo se reflejan en el artículo "*Determination of the minimum threshold of* Phytophthora cinnamomi *in soil for root disease development on Quercus suber*", publicado en ***Phytopathologia Mediterranea*** 54 (3), 461-464 (2015).

Los resultados concernientes al segundo objetivo aparecen en "*Effect of* Brassica *biofumigants amendments on different stages of the life cycle of* Phytophthora cinnamomi*"* publicado en ***Journal of Phytopathology*** 164 (9), 582-594 (2016).

El tercer objetivo se aborda en *"Biofumigant action of* Brassica *seedmeals against* Phytophthora cinnamomi *in dehesa ecosystems",* en revisión en ***Phytopathologia Mediterranea***.

Los resultados correspondientes al cuarto objetivo están publicados en "*Screening brassicaceous plants as biofumigants for management of* Phytophthora cinnamomi *oak disease"*, ***Forest Pathology*** 46, 652-659.

Finalmente, los resultados del quinto objetivo se exponen en *"Formulating a Brassicaceae-based product effective against* Phytophthora cinnamomi*",* en revisión en ***Plant Disease.***

Información sobre las revistas científicas donde se han publicado o se encuentran en revisión los artículos derivados de la Tesis:

Revista: PHYTOPATHOLOGIA MEDITERRANEA

Nombre abreviado: **ISO** Phytopathol. Mediterr. **JCR** Phytopathol Mediterr

ISSN: 0031-9465

Idioma: inglés **País**: Italia

Editorial: Firenze University Press **Dirección**: via Cittadella 7, 50144, Firenze, Italy

Categoría (rango) JCR 2015: Agronomy (37/83), 2º cuartil

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Revista: JOURNAL OF PHYTOPATHOLOGY

Nombre abreviado: **ISO** J. Phytopathol. **JCR** J Phytopathol

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Idioma: multi-idioma **País**: Alemania

Editorial: Wiley-Blackwell **Dirección**: 111 River ST, Hoboken 07030-5774, NJ, Germany

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Revista: FOREST PATHOLOGY

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Revista: PLANT DISEASE

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INTRODUCTION

Oak agroforestry systems (*dehesas* in Spain, *montados* in Portugal) are one of the most characteristic ecosystems at the Iberian Peninsula. Dehesas are semi-natural ecosystems comprising scattered trees, mainly evergreen oaks, and livestock, created and maintained by humans. In Spanish dehesas the dominant tree species are holm oak (*Quercus ilex* L. ssp. *ballota* (Desf.) Samp.) and cork oak (*Quercus suber* L.), providing major incomes from cork and acorn production, a high valuable food resource for livestock and wildlife. These ecosystems are listed among the type habitats protected by the European Union's Directive on Habitats (Annex I of Council Directive 92/ 43/ EEC on the Conservation of natural habitats and of wild fauna and flora) and taking part of the Natura 2000 network. The cohabitation of wild fauna, livestock and trees, defines the semi-natural character of these forests and their organic production.

Destabilization of some of the elements that constitute the dehesa, carries important damages for the ecosystem; which means, moreover, the no-sustainability of the organic production system and, therefore, its abandonment. In recent years, these ecosystems are at risk due to a several threats that are lurking, being one of the most important the loss of trees due to the root disease caused by *Phytophthora cinnamomi*.

Since the 90´s a severe decline affecting Mediterranean *Quercus* species has been detected in rangelands of southern Spain and Portugal, leading to a high mortality of holm and cork oak trees. Among the factors associated with decline, the root rot caused by *P. cinnamomi* highlights due to its severity. This oomycete causes a massive rot of feeder roots, reducing their ability to uptake water and mineral nutrients, leading to foliar symptoms which look like those generated by drought, being able to kill the trees in a few months.

Phytophthora cinnamomi is a serious plant pathogen worldwide, with many woody hosts. It is among the world´s 100 worst invasive alien species. This oomycete is especially virulent causing root rot not only in holm and cork oak, but in high economically valuable species such as avocado and chestnut trees. Root rot disease management is difficult due to the longevity of resting spores of *P. cinnamomi* in the soil, due to its rapid and deep dispersal in waterlogged soils, and due to the large number of hosts.

Control methods against oak root disease caused by *P. cinnamomi* must be environmental friendly due to the semi-natural character of these forests. In dehesa ecosystems the fight against *P. cinnamomi* is based on an integrated management that includes measures to limit new infections, such as application of calcium fertilizers to the soil, avoidance of highly susceptible herbaceous hosts, high livestock densities, or encouraging soil drainage. All these recommendations are quite effective, but none effectively decreases the viability of *P. cinnamomi* resting spores present in the soil of declining oak systems.

In this context, biofumigation appears as candidate. Biofumigation relies on activity of compounds released through the hydrolysis of glucosinolates (GSLs), secondary metabolites produced by *Brassicaceae* species. Once GSLs are enzymatically hydrolyzed by the action of myrosinase, isothiocyanates (ITCs) and other deleterious substances are produced. More than 130 different GSLs are known, but the noxious action of *Brassicaceae* against pests including pathogens and weeds, seems to be related to their specific GSL profiles, or even one specific GSL. It is a fact that these products inhibit a wide number of soil-borne pathogens, including nematodes, bacteria and fungi, as well as oomycetes belonging to *Phytophthora* genus, although their effect against *P. cinnamomi* remained still unknown.

Based on all these considerations, this Thesis come from the hypothesis that volatile compounds released after the incorporation of biofumigant material can minimize the ability of *P. cinnamomi* for oak root infection. The confirmation of the toxicity of these volatiles products on vegetative and reproductive stages of the pathogen, the identification of the GSLs from which they come, the subsequent selection of plants containing them at effective doses, and the establishment of a practical and effective method to apply the biofumigation on dehesa ecosystems, would led to a new, environmentally friendly and suitable control method to the root disease in dehesa ecosystems.

OBJECTIVES

The main objective of this Thesis is to know whether biofumigation is an effective and suitable technique to be incorporated to the integrated disease management of the *Quercus* root rot in dehesa ecosystems.

The partial objectives are:

1. To determine the minimum threshold of *Phytophthora cinnamomi* inoculum required for root disease development.
2. To test the biofumigant effect of green manure from *Brassica* spp. on different stages of the life cycle of the pathogen.
3. To test the biofumigant effect of *Brassica* seed meals and their production in dehesa ecosystems.
4. To select *Brassicaceae* species with biofumigant activity against *Phytophthora cinnamomi.*
5. To formulate a *ready-to-use* product for its direct application on dehesa ecosystems.

Results concerning the first objective are in the research article "*Determination of the minimum threshold of* Phytophthora cinnamomi *in soil for root disease development on Quercus suber*", published in ***Phytopathologia Mediterranea*** 54 (3), 461-464 (2015).

Results related to the second objective are shown in *"Effect of* Brassica *biofumigants amendments on different stages of the life cycle of* Phytophthora cinnamomi*"* published in ***Journal of Phytopathology*** 164 (9), 582-594 (2016).

The third objective has been aborded on *"Biofumigant action of* Brassica *seedmeals against* Phytophthora cinnamomi *in dehesa ecosystems",* under review ***Phytopathologia Mediterranea***.

Results concerning the fourth objective are published on *"Screening brassicaceous plants as biofumigants for management of* Phytophthora cinnamomi *oak disease"* published in ***Forest Pathology*** 46, 652-659.

Results of the research concenrning the fifth objective are in *"Formulating a Brassicaceae-based product effective against* Phytophthora cinnamomi*",* which is under review in ***Plant Disease.***

Information about the journals where the results of this Thesis have been published:

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CAPÍTULO 2.
REVISIÓN BIBLIOGRÁFICA

CHAPTER 2.
LITERATURE REVIEW

LA DEHESA

2.1.Descripción del ecosistema. Situación actual

Los sistemas adehesados son ecosistemas agrosilvopastorales antrópicos, complejos y dinámicos, en su mayoría de bajos insumos y con costes marginales de intensificación complejos y peligrosos, ya que no pueden permitirse el coste medioambiental que supondría. La sostenibilidad ecológica de estos ecosistemas está afectada por numerosos problemas: simplificación de la vegetación, pérdida de diversidad faunística, carga ganadera del sistema superior a su capacidad sustentadora, exceso de artificialidad, laboreo excesivo, desmonte, acumulación de purines, enfermedades y plagas del arbolado, envejecimiento de la masa arbórea, entre otras (Gastó-Cordech et al., 2010).

Al ser la dehesa holística, cualquier aspecto ecológico, económico, cultural, tecnológico y social influye de forma explícita en los equilibrios del ecosistema, afectando a sus mecanismos de autorregulación. Cuando se producen desviaciones, el ecosistema entero sufre desequilibrios complejos.

Uno de los principales componentes de la biogeoestructura de los sistemas adehesados y que tiene una alta repercusión en el ecosistema es el arbolado, donde predominan especies del género *Quercus*, y su estado fitosanitario. A primeros de la década de los 90 del siglo XX se empezó a constatar la existencia de procesos de deterioro y muerte de *Quercus ilex* y *Q. suber* en la cuenca mediterránea, especialmente en el sur de la Península Ibérica (Brasier, 1992). La sintomatología asociada a esos procesos era bastante inespecífica en su apariencia (defoliación, muerte regresiva de brotes y ramos, abundante emisión de brotes adventicios, necrosis del sistema radical, etc.), siendo la muerte del árbol el resultado final tras una larga serie de cambios morfológicos y funcionales (Brasier, 1992; 1996).

Este inusual deterioro fitosanitario de las especies de *Quercus* que ocurría en algunas zonas adehesadas fue considerado al principio bajo el concepto de decaimiento forestal, enfermedad de etiología compleja resultado de la acción de múltiples factores bióticos y abióticos, inespecíficos e intercambiables en el tiempo y en el espacio, que causa un progresivo deterioro y muerte de árboles, principalmente adultos (Manion, 1981; Manion y Lachance, 1992). Algunos aspectos clave ligados a este concepto son:

- La velocidad de deterioro de la masa forestal es lenta y progresiva. En general, transcurren años entre la aparición de los síntomas y la muerte del árbol. Por tanto, la muerte súbita es aparentemente una excepción.
- La mayoría de los árboles afectados son adultos.
- Las causas tienen una etiología compleja causada por factores que se pueden agrupar en tres categorías:
    - Factores predisponentes. Actúan a largo plazo y predisponen a la masa forestal a sufrir los efectos del siguiente grupo de factores, pero por sí mismos no causan síntomas: edad del árbol, predisposición genética, prácticas de manejo, etc.
    - Factores incitantes. Actúan a corto plazo sobre la masa forestal previamente predispuesta y originan los síntomas: aspectos climáticos inusuales (sequías, heladas, etc.) que inciden sobre el árbol y de los que, en ausencia de factores predisponentes, el árbol se recuperaría rápidamente.
    - Factores contribuyentes: Actúan a largo plazo sobre la masa forestal sintomática y provocan su muerte: hongos oportunistas (patógenos de debilidad), plagas de insectos, etc. De nuevo se trata de organismos que no causarían enfermedad o daños si la masa forestal no estuviese previamente deteriorada.

La Tabla 1 centra algunos de estos factores en relación a los sistemas adehesados mediterráneos. Las prácticas ganaderas, selvícolas, culturales, cuestiones económicas de mercado que afectan a la gestión del territorio y de la explotación, los nuevos cambios tecnológicos y sociales pueden ser factores de predisposición que acentúan la susceptibilidad del arbolado a otros factores que sí pueden inducir síntomas de deterioro en un conjunto de especies arbóreas. Como se ha indicado, cuando la masa forestal está sometida a estos factores predisponentes, aspectos climatológicos inusuales o extremos pueden inducir los síntomas del decaimiento. Las alteraciones climatológicas, especialmente en el contexto actual de cambio climático (incremento generalizado de las temperaturas medias anuales, descenso de la precipitación anual con alta variabilidad interanual y estacional, mayores e intensos periodos de sequía y mayor frecuencia de eventos extremos como inundaciones, según muestran diversos modelos y escenarios de simulación en la cuenta mediterránea (Giorgi y Lionello, 2008; García-Ruiz et al.,

2011), pueden inducir síntomas de decaimiento y empeorar el estado general de las coberturas vegetales ya predispuestas, ya que originan desplazamientos y mortalidad en la vegetación existente, además de influir en la susceptibilidad de las especies a la invasión de patógenos y plagas oportunistas. Estos son factores contribuyentes que sobre el arbolado sano no tendrían ninguna incidencia.

Tabla 1. Tipología de factores involucrados en la hipótesis del decaimiento del arbolado presente en los sistemas adehesados

FACTORES DE DECAIMIENTO		
Predisponentes	**Incitantes**	**Contribuyentes**
Prácticas de gestión inadecuadas	Alteraciones climáticas	Fitosanitarios
Sobrepastoreo Poda Descorche Abandono Ausencia de regenerado	Incremento de temperaturas Cambios en la intensidad y distribución de precipitaciones Mayor frecuencia de eventos climáticos extremos	Enfermedades y Plagas (oportunistas)

No obstante, cualquier desviación del estado vegetativo normal de una masa arbórea no debe considerarse intrínsecamente como decaimiento. En la actualidad, en base al trabajo de distintos grupos de investigación que han estudiado cada uno de los posibles factores involucrados y su repercusión en el estado actual de la masa arbolada, el conocimiento adquirido sobre este tema es amplio y ciertos aspectos pueden considerarse contrastados y consolidados.

Al igual que ocurrió con otros problemas fitosanitarios definidos inicialmente como decaimientos, caso de la Tristeza de los cítricos, causada por un virus (Moreno et al., 2008), del decaimiento del peral, causado por un fitoplasma (Seemüller y Schneider., 2004) o del decaimiento de *Quercus robur* y *Q. petraea* en Centroeuropa, provocado por *Phytophthora quercina* (Jung et al., 1999), se sabe que existen enfermedades concretas, resultado de la acción de agentes fitopatógenos concretos (Sánchez et al., 2002; 2006; 2010; Romero et al., 2007), en zonas concretas del conjunto de

formaciones adehesadas de la Península Ibérica que afectan al arbolado y no deben confundirse con situaciones propias de un decaimiento forestal generalizado.

Centrando la atención a Andalucía, en la zona occidental, y especialmente en la provincia de Huelva, la sintomatología observada en los *Quercus* es consecuencia principalmente del agente patógeno *Phytophthora cinnamomi* que causa una enfermedad concreta, la podredumbre radical, donde factores abióticos tales como la alternancia de periodos secos y lluviosos juegan un papel importante en la diseminación de la enfermedad y en la severidad de los síntomas (Sánchez et al., 2002; 2003a; 2010; Caetano et al., 2009; Carrasco et al., 2009; Serrano et al., 2012a; Corcobado et al., 2014). En la Sierra Subbética cordobesa y en las sierras de Málaga y Cádiz, se han observado desecaciones y marchiteces de ramas de *Quercus* asociados con la presencia de lesiones corticales (chancros) consistentes en zonas alargadas de corteza necrosada, visibles más fácilmente cuando se retira la corteza externa. Los chancros de encina y alcornoque son causados por especies del género *Botryosphaeria* (*B. corticola, B. iberica* y *B. dothidea*). Las tres especies anteriores originan chancros, pero únicamente *B. corticola* produce anillamiento y muerte de ramas y la subsecuente marchitez de ramas en árboles no debilitados (Sánchez et al., 2003b). Los chancros del tronco, causados por *B. corticola*, sólo se han estudiado en alcornoques, ya que originan graves pérdidas en calidad y cantidad de corcho. El chancro carbonoso, causado por el ascomiceto xilariáceo *Biscogniaxia mediterranea,* es, sin embargo, un chancro de debilidad que sólo causa necrosis cortical en alcornoques muy debilitados (Jiménez et al., 2005a, 2005b). En lo que respecta a Andalucía oriental, las observaciones realizadas muestran que las masas de encinas y alcornoques sólo sufren el efecto de las sequías, y éstas se recuperaron tras estos periodos secos (Carrasco et al., 2009). En cuanto a las plagas, cabe mencionar los daños producidos por insectos perforadores de ramas y troncos, especialmente por especies de coleópteros de los géneros *Cerambyx* y *Prinobius* (Sánchez-Osorio et al., 2007). Este panorama pone de manifiesto que en Andalucía la mala situación fitosanitaria de las dehesas tiene una explicación contrastada que, en general, no puede relacionarse con un decaimiento generalizado de la masa de encinas y alcornoques a nivel global.

Con todo esto, es importante, para poder aplicar soluciones adecuadas, diferenciar por un lado la incidencia de enfermedades específicas en zonas concretas, que siempre van a

depender de la existencia de condiciones climáticas favorables para la infección y dispersión de los patógenos implicados, y por otro, otras causas de deterioro del arbolado (climáticas, culturales, selvícolas, ecológicas, ganaderas, etc.), que en la mayoría de los casos coexisten en la misma explotación. Dada la multitud de aspectos ecológicos, selvícolas, ganaderos, económicos, culturales, tecnológicos y sociales implicados, resulta esencial integrar todos ellos a la hora de gestionar sistemas físicos y biológicos, como los sistemas adehesados, con el objeto de salvaguardar a largo plazo el ecosistema, la diversidad natural y la productividad. A esto se refiere el concepto de manejo ecosistémico (Christensen et al., 1996). La toma de decisiones con un enfoque ecosistémico permite buscar soluciones integrales para problemas causados por causas múltiples e interdependientes.

2.2.ENFERMEDADES DE LOS *Quercus*

Como se ha mencionado anteriormente, existen agentes únicos que causan deterioro y mortalidad en el arbolado de las dehesas, aunque no se den situaciones de debilitamiento previo. Es por ello que conocer cuáles son las principales enfermedades que pueden afectar a los *Quercus,* los patógenos que las causan, los síntomas que permiten su diagnóstico y las posibles medidas de control son aspectos clave dentro de la gestión de estos ecosistemas.

2.2.1 Enfermedades foliares

2.2.1.1 Repilo

El Repilo es una enfermedad que únicamente afecta a la encina. Está causado por el hongo Deuteromiceto *Fusicladium quercus-ilicis*. Este patógeno se distribuye principalmente por Italia y España, aunque es posible que se encuentre por toda el área mediterránea, coincidiendo con el área de distribución de la encina (Trapero et al., 2000). La enfermedad provoca la caída anticipada de las hojas, por lo que en caso de ataques muy severos puede llegar a defoliar al árbol afectado. Suele presentarse en árboles aislados (Navarro et al., 2008).

El signo más característico del Repilo de la encina es la presencia de manchas de color oscuro de tamaño variable, que frecuentemente aparecen rodeadas por un halo amarillo. Estas manchas corresponden al desarrollo de los conidióforos y conidios bajo la cutícula. Para que se produzca la infección, es necesaria una elevada humedad y una temperatura de 8 a 24ºC (Trapero et al., 2010).

Salvo en ataques muy severos, no es necesario el uso de fungicidas para su control. En el caso de que hubiera que aplicarlos, se recomienda hacerlo antes de los períodos críticos para la infección, a final de otoño y principios de primavera (Trapero et al., 2010).

2.2.1.2 Escobas de bruja

Las Escobas de bruja de la encina es una enfemedad extendida por Italia, Argelia y España, afectando también a *Q. coccifera.* Están causadas por el hongo Ascomiceto *Taphrina kruchii*, que provoca la brotación de las yemas durmientes y el desarrollo de ramas que se ramifican intensamente con entrenudos anormalmente cortos (Torres-Juan 1993). Las hojas de estas ramas presentan enanismo y clorosis y rápidamente se marchitan y caen. Se han descrito epidemias de gran intensidad en encinares de Córdoba y Sevilla (Torres-Juan, 1993).

El principal método de control de las Escobas de bruja es la poda y destrucción de las ramas enfermas, siempre extremando el cuidado a la hora de desinfestar las herramientas antes de cortas nuevas ramas para evitar la propagación de la enfermedad.

2.2.2 Enfermedades corticales (chancros)

Los chancros son síntomas primarios producidos por la necrosis del tejido cortical de troncos y ramas. Como síntomas secundarios se produce amarillez, empardecimiento y marchitez de las hojas en las ramas afectadas, con la posterior defoliación y desecación de las mismas (Sánchez et al., 2003b).

2.2.2.1. Chancros fúngicos

Los chancros fúngicos más frecuentes están causados por hongos Ascomicetos del género Botryosphaeria: B. dothidea (anamorfo: Fussicocum aesculi), B. corticola (anamorfo: Diploida corticola) y B. iberica (anamorfo: Dothiorella iberica) (Alves et al., 2004; Phillips et al., 2005; Romero et al., 2009).

Los tres patógenos causan chancros y muerte regresiva de ramillas y se han asociado al decaimiento de encinas y alcornoques, aunque *B. dothidea* también se ha descrito en *Q. robur* y *Q.* rubra. Tanto *B. dothidea* como *B iberica* son patógenos de debilidad, pero *B. corticola* es un patógeno primario que además causa en alcornoque importantes pérdidas de producción y calidad del corcho, considerándose la enfermedad más común causante de muerte de alcornoques en el noreste de España (Luque et al., 2008).

Los principales métodos de control son de tipo preventivo: evitar heridas en la corteza y eliminación de ramas infectadas (Romero et al., 2009). En el caso del chancro del tronco del alcornoque, se puede llevar a cabo un control químico preventivo mediante la aplicación tras el descorche de benzimidazoles solos o mezclados con derivados cúpricos (Romero et al., 2006; Luque et al., 2008).

2.2.2.2. Chancro carbonoso

El chancro carbonoso está causado por el hongo Ascomiceto *Biscogniauxia mediterranea* (sinónimo: *Hypoxylon mediterraneum*, anamorfo: *Periconiella* sp.). Es un hongo endófito que también se ha asociado al decaimiento de los *Quercus* (Jiménez et al., 2005b). *Biscogniauxia mediterranea* afecta a un gran número de especies del género *Quercus,* siendo la más susceptible *Q. suber.*

El chancro carbonoso aparece principalmente en árboles muy debilitados o muertos (Muñoz y Rupérez, 1987; Jiménez et al., 2005b). El signo más característico de la enfermdad es el estroma carbonoso que se forma en grietas longitudinales de la corteza en los árboles infectados (Jiménez et al., 2005a; 2005b). La zona de la corteza donde se localiza el estroma aparece necrosada, pero el leño subyacente permanece inalterado.

Tradicionalmente para el control del chancro carbonoso se han recomendado medidas de tipo preventivo como podas sanitarias, sellado de heridas y desinfestación de las herramientas de poda (Torres-Juan, 1993), sin embargo, el carácter endófito de *B. mediterranea* hace que estas prácticas no sean suficientes por sí solas, debiéndose adoptar otras medias encaminadas a matener el vigor de los huéspedes y a la disminución del inóculo, destruyendo árboles muertos y leñas con carbón (Torres-Juan, 1993).

2.2.2.3. Chancro bacteriano

El chancro bacteriano o chancro sangrante de la encina está causado por la Enterobacteria *Brenneria quercina*. También afecta a *Q. pirenaica.* El síntoma más caracteristio es la aparición de chancros en la corteza que se extienden hacia la parte interior del troco, de profundidad variable, con necrosis del tejido y abundantes exudaciones (sangrado del tronco). Los exudados se observan principalmente en primavera y otoño, y son de color variable, inicialmente blanco-rojizo-marrón, oscurenciéndose a medida que se van secando hasta alcanzar un marrón oscuro casi

negro. Los *Quercus* que presentan este tipo de chancro muestran como síntomas secundarios pérdida progresiva de vigor, reducción del follaje y caída prematura de las hojas. En brotes, frutos y ramas infectados pueden aparecer abundantes exudados de color pardo, constituidos principalmente por masas de bacterias (Biosca et al., 2003; Melgarejo et al., 2010).

Existe poca información sobre las medidas de control que se pueden llevar a cabo, aunque se pueden extrapolar algunas de las empleadas para combatir otros patógenos que causan enfermedades similares, como *Pseudomonas savastonoi* en olivo (Trapero y Blanco, 2008) o *Erwinia amylovora* en rosáceas silvestres o frutales de pepita (Melgarejo et al., 2010). Estas técnicas consisten en la reducción de la fuente de inóculo mediante la eliminación de los tejidos infectados, y la aplicación de productos derivados del cobre como el caldo bordelés (Trapero y Blanco, 2008).

2.2.3. Enfermedades radicales

2.2.3.1. Colapso tardío en vivero

La enfermedad más importante que afecta a los *Quercus* en viveros forestales es el *damping-off* o colapso tardío causado por especies de *Phytophthora* (*P. cinnamomi, P. cryptogea* y *P. dreschsleri*) y *Cylindrocarpon (C. destructans* y *C. didymum)* prinipalmente (Andicoberry et al., 2001; Sánchez et al., 2002a; 2005).

Los síntomas observados en las plántulas son amarillez y desecación foliar, que comienza por los márgenes y se extiende hacia los nervios centrales, hasta la completa marchitez de las hojas. Estos síntomas se deben a la falta de absorción de agua por la podredumbre de las raicillas absorbentes (Sánchez et al., 2002a; 2005).

Para prevenir estas enfermedades en vivero, especialmente en el caso de *Phytophthora* spp., se debe evitar el encharcamiento de los sustratos, asegurando una buena aireación (Sánchez et al., 2010).

2.3.PODREDUMBRE RADICAL

Como revisa Serrano et al. (2012a) y ya apuntaba Brasier (1996), la principal enfermedad que sufren encinas y alcornoques en el sur de la Península Ibérica es la podredumbre radical causada por el oomiceto *P. cinnamomi*. Este patógeno invasor es la especie principal que se ha asociado consistentemente a la enfermedad, aunque no es la única descrita (Sánchez et al., 2003a; 2005; 2006; Caetano et al., 2009; Corcobado et

al., 2010) ni el único oomiceto, ya que hay estudios que describen la patogenicidad de *Pythium spiculum* en encinas y alcornoques (Romero et al., 2007; Jiménez et al., 2008; Serrano et al., 2012a; De Vita et al., 2013), que está causando daños menos graves y extensos en encinas y alcornoques en las dehesas y montes del sur de la Península Ibérica.

El oomiceto, que no hongo, *P. cinnamomi* está incluido en el catálogo IUCN/SSC de las 100 especies exóticas invasoras más dañinas del mundo (Lowe et al., 2000). Se ha descrito en más de 5000 especies vegetales (Zentmyer, 1980; Grünwald et al., 2011; Jung et al., 2013), entre las que se encuentran las encinas y alcornoques de la Península Ibérica (Sánchez et al., 2002; 2003a; Caetano et al., 2009; Carrasco et al., 2009; Serrano et al., 2012a).

2.3.1 Patogénesis y epidemiología

Este patógeno polífago, con un origen probable en Papúa Nueva Guinea y actualmente presente en gran parte del mundo (Australia, Francia, Italia, México, Nueva Zelanda, Portugal, Rumanía, Eslovaquia, España, Suiza, Reino Unido, Estados Unidos, China y Sudáfrica) (GISD, 2014), es el agente causal de la podredumbre radical en múltiples especies vegetales, principalmente leñosas.

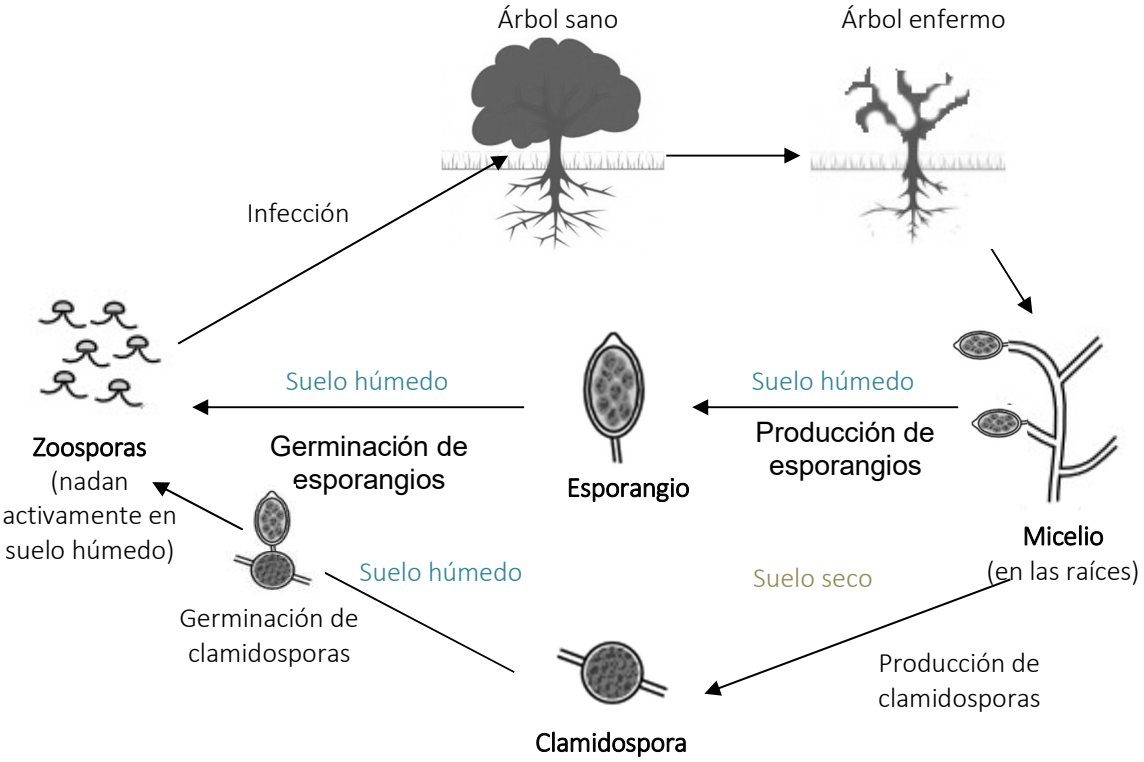
La podredumbre radical es una enfermedad que causa la muerte masiva de raíces absorbentes, reduciendo la capacidad de absorción de agua y nutrientes por parte del árbol. Resulta difícil atribuir el debilitamiento del árbol a la podredumbre radical por los síntomas aéreos, ya que la sintomatología asociada es similar a la del estrés hídrico, consistente en amarillez, marchitez foliar, defoliación, puntisecado, etc. Las condiciones ambientales pueden además contribuir para que un árbol se recupere de los síntomas aéreos, enmascarando a simple vista la posible presencia del patógeno parasitando la raíz. Por último, puede ocurrir que la aparición de síntomas y la muerte de árboles sean rápidas cuando se dan condiciones favorables (muerte súbita).

Phytophthora cinnamomi es un microorganismo heterotálico o autoestéril, es decir, presenta talos o micelios anatómicamente iguales, pero genéticamente distintos, compatibles para la reproducción sexual. Además de en su región de origen, sólo uno de los talos se ha diseminado hacia el resto de su área de distribución actual. No obstante,

se han detectado casos de autofertilidad en plantas infectadas de forma natural (Jayasekera et al., 2007; Crone et al., 2013; Jung et al., 2013).

El ciclo de vida del patógeno se muestra en la Figura 1. Cuando las condiciones ambientales son favorables (hay humedad en el suelo), las masas vegetativas o micelio del oomiceto en el interior de la raíz infectada producen esporangios, que a su vez liberarán zoosporas al suelo. Éstas se mueven tanto de forma activa como pasiva y son atraídas por exudados radicales de las plantas susceptibles. Una vez que entran en contacto con la raíz y la infectan, el patógeno invade la raíz absorbente. El oomiceto se diseminará mediante múltiples ciclos de esporulación desde las raíces infectadas, produciendo más esporangios y zoosporas, hasta que las condiciones ambientales del suelo no sean favorables (baja humedad) o bien la raíz infectada muera. En ese momento, las estructuras de supervivencia que también forman se forman en el micelio (clamidosporas), son liberadas bien al suelo o bien permanecen en fragmentos de raíces no degradadas. Estas estructuras de supervivencia, que pueden tener una viabilidad de varios meses (Jung et al., 2013), germinarán para producir nuevos esporangios y zoosporas infectivas una vez que las condiciones del suelo vuelvan a ser favorables (nuevo periodo húmedo).

Figura 1. Ciclo de vida de *Phytophthora cinnamomi*



Mircetich y Zentmyer (1966) confirmaron que *P. cinnamomi* puede llegar sobrevivir en suelo húmedo después de 6 años en ausencia de huésped, aunque no es un saprofito del suelo. En base a los resultados de Crone et al. (2013) y Jung et al. (2013), se sabe que las clamidosporas son las principales estructuras de supervivencia en condiciones moderadas de sequía entre distintos eventos lluviosos, mientras que las agregaciones hifales e hifas encapsuladas que se forman también en las raíces infectadas, son los propágulos principales de *P. cinnamomi* a largo plazo en condiciones extremas de sequía.

Gómez-Aparicio et al. (2012) muestran en sus modelos que, en condiciones naturales de campo, existe una correlación espacial entre zonas con mayor abundancia de patógeno en suelo y la emergencia y supervivencia del regenerado, aunque no para todas las especies y tipos de bosques. Esto sugiere que debe existir un cierto límite mínimo de inóculo viable en el suelo para que la planta sea infectada. La cuantificación de este umbral es relevante a la hora de tomar decisiones en cuanto al control de la enfermedad, ya que marca un umbral medible a partir del cual existe un riesgo cierto de enfermedad.

Actualmente en la Península Ibérica se han aislado dos poblaciones genéticas (clústeres) diferentes de *P. cinnamomi,* que varían en sus características morfológicas y ratios crecimiento-temperatura (Figura 2). Los patógenos aislados en la Península Ibérica pueden creer en un amplio rango de temperaturas, con un óptimo de crecimiento entre 26 y 30ºC (Sánchez et al., 2002; 2003a; 2006; Caetano et al., 2009). Un hallazgo interesante corresponde a que estos clústeres tienen una distribución geográfica distinta. Un grupo se encuentra localizado en el sur de Portugal y Huelva, y se caracteriza por su amplio rango de temperaturas de crecimiento (mínimo: 1,8ºC y óptimo: 30,1ºC), lo que sugiere una mayor adaptabilidad a futuros cambios climáticos (Caetano et al., 2009). Por otro lado, el clúster aislado mayoritariamente en Extremadura y sur de Andalucía (Cádiz, Sevilla y Córdoba) tiene una temperatura mínima de crecimiento de 5,2ºC y una temperatura óptima en laboratorio de 26,9ºC. La mayor adaptabilidad de la población presente en Portugal y Huelva probablemente haga que este clúster de *P. cinnamomi* sea el que da lugar a una mayor severidad de síntomas en encinas y alcornoques, con mayores posibilidades para extenderse en el territorio (Caetano et al., 2009).

Otro de los factores que influye sobre el patógeno es la humedad del suelo. Se ha observado que *P. cinnamomi* causa síntomas en raíces de *Q. suber* más rápidamente conforme mayor es la humedad en el suelo (González et al., 2014).

Figura 2. Distribución geográfica de los aislados de *P. cinnamomi* en la Península Ibérica. Adaptado de Caetano et al. (2009)

Esto significa que en condiciones de saturación (100% humedad en el suelo) el tiempo necesario para que se observen los síntomas radicales de la enfermedad es inferior frente a otras condiciones de humedad. No obstante, aunque las condiciones de encharcamiento favorezcan el desarrollo rápido de la infección, niveles muy inferiores de humedad (10-30%), también permiten que ocurra la infección y se desarrolle la enfermedad (González et al., 2017b).

2.3.2. Diagnóstico

La sintomatología aérea puede resumirse en función de la rapidez con que se manifiesta, aunque de forma generalizada existe un retraso entre la infección de la raíz y la aparición de síntomas en las copas:

- "Decaimiento lento" (*slow decline*): amarillez, marchitez y caída gradual de hojas, con presencia progresiva de ramas parcial o totalmente defoliadas (puntisecado). Los árboles mueren en un largo período de tiempo (años) desde la aparición de los primeros síntomas en la copa
- "Muerte súbita" (*sudden death*): rápido secado de ramas; las hojas marchitas permanecen un tiempo prendidas y los árboles mueren en un corto período de

tiempo (meses) desde la aparición de los primeros síntomas en la copa. Las hojas muestran un color amarillento o marrón en las primeras etapas de la desecación.

En el decaimiento lento aparecen también exudados gomosos negruzcos (sangraduras) en el tronco (Tuset et al., 1996). Además, los árboles afectados pueden producir, aunque no siempre, brotes adventicios (chupones) a lo largo del tronco y ramas gruesas (Brasier, 1996). Estos son síntomas secundarios originados por la necrosis de las raíces absorbentes, que muestran un color oscuro y se descascarillan fácilmente a consecuencia de la enfermedad. La Figura 3 muestra una guía fotográfica de los principales síntomas.

Dado lo inespecífico de la sintomatología, el diagnóstico de la enfermedad se basa fundamentalmente en el aislamiento e identificación del patógeno a partir de las raicillas infectadas o de la rizosfera.

Figura 3. Sintomatología asociada a la podredumbre radical: (a) Amarillez; (b) defoliación; (c) marchitez generalizada que da lugar al colapso del árbol; (d) exudaciones (sangraduras); (e) corteza muerta; (f) brotes adventicios; (g) puntisecado de ramas

La gran variedad de huéspedes, el periodo a veces largo entre el establecimiento de la infección y la manifestación de síntomas y la longevidad de las estructuras de resistencia del patógeno en el suelo hace que el control de la enfermedad sea complejo.

2.3.3. Control

El gobierno australiano, por ejemplo, ha llevado a cabo de forma experimental distintas actuaciones para la erradicación de este patógeno en sus bosques (AGDE, 2008). Los tratamientos que aplicaron secuencialmente y/o en combinación fueron:

- Destrucción de árboles sanos susceptibles de caer enfermos en las zonas limítrofes a los focos de enfermedad.
- Destrucción de todas las plantas, creando una zona muerta o de barbecho.
- Instalación de barreras físicas y aplicación de riego subterráneo con productos selectivos contra oomicetos (Thiadiazoles y Metalaxil).
- Aplicación aérea de productos selectivos a ultrabajo volumen.
- Inyecciones al suelo, tanto en superficie como en profundidad (± 1 m), con fumigantes químicos (Metam-sodio).

A pesar de lo drástico de las medidas de erradicación adoptadas se comprobó que, a largo plazo, cuando la vegetación volvía a colonizar la zona tratada, volvía a detectase la presencia del patógeno en el suelo. De estas experiencias se concluye que, si bien no es posible la erradicación total del patógeno en las zonas donde ya se ha instalado, se deben aplicar las medidas de control disponibles para prevenir su llegada a zonas libres de la enfermedad y para disminuir su población en zonas ya infectadas, de forma que se consiga mantenerla por debajo del umbral económico de daños. No obstante, este es un aspecto bastante complejo y no abordado hasta la fecha, ya que en el cálculo de ese umbral deben contemplarse multitud de variables ecológicas, económicas, sociales, culturales, paisajísticas, etc., donde muchas de ellas pueden ser intangibles.

El objetivo, por tanto, de las medidas de control a aplicar variarán en función de la presencia o ausencia de la enfermedad en el territorio:

- En zonas libres de enfermedad, las acciones deben enfocarse a evitar la llegada del patógeno y su establecimiento.

- En zonas donde se ha diagnosticado la enfermedad el objetivo debe ser disminuir el riesgo de dispersión del patógeno y/o disminuir la densidad e infectividad del patógeno en el suelo.

La Tabla 2 resume las principales acciones preventivas recomendadas para evitar la dispersión de oomicetos patógenos. La Tabla 3 recoge las principales acciones de control preventivo enfocadas a disminuir el inóculo inicial y/o la tasa de infección de la enfermedad.

Tabla 2. Medidas preventivas para evitar la dispersión *P. cinnamomi* y otros oomicetos que causan podredumbre radical

Ámbito	Zonas con presencia de enfermedad	Zonas libres de enfermedad
Movimiento de suelo y tierra	Evitar el laboreo del suelo: cultivo, control de matorrales y cortafuegos Dejar un residuo herbáceo en el suelo Evitar nuevas construcciones	Evitar acopio de tierra, arena, suelo, procedentes de zonas con presencia de la enfermedad
Flujo de agua superficial y escorrentía	Asegurar un buen drenaje del suelo Evitar cargas ganaderas altas en condiciones de elevada humedad del suelo	Asegurar un buen drenaje del suelo
Tránsito de vehículos, máquinas, personas y animales	Limitar el tránsito de vehículos, máquinas y personas Reducir desplazamientos en periodos en los que el suelo esté seco Limpieza de calzado, aperos, vehículos, etc.	Limpieza de calzado, aperos, vehículos, maquinaria a la entrada a la explotación. Desinfestación de pezuñas de animales si vienen de una explotación infectada

A continuación, se realiza una recopilación de los principales resultados experimentales en relación a las medidas de control de la podredumbre radical.

Tabla 3. Medidas de control para disminuir el inóculo inicial en el suelo y/o la tasa de infección de la enfermedad

Medida	Observación
Fertilización cálcica	Confiere tolerancia a la enfermedad Inhibe la producción (CaO, $CaCO_3$, $CaSO_4$) y germinación de esporangios (CaO, $CaSO_4$, $CaCl_2$)
Fosfonatos	Aplicados a árboles sanos tienen efecto preventivo, mientras aplicados a árboles ya infectados, pero que aún no muestran los síntomas aéreos de la enfermedad radical, tienen efecto curativo
Cultivo / plantación de plantas herbáceas, arbustivos y/o arbóreas	Evitar cultivos de plantas huéspedes del patógeno Utilizar planta forestal libre de patógenos en caso de reforestación
Biofumigación	Enterrado en verde o aplicación de planta/semilla conservada al suelo

2.3.3.1 Medidas de control cultural

En base a resultados de ensayos experimentales en laboratorio, invernadero y en condiciones de campo, se ha demostrado que la aplicación de enmiendas calizas al suelo disminuye significativamente la infectividad de *P. cinnamomi*, dando lugar a una menor tasa de infección radical por la inhibición que induce el ión Ca^{2+} sobre la formación de esporas infectivas (zoosporas) (Serrano et al., 2012b; 2013). Este efecto se consigue con dosis de aplicación que no aumentan el pH del suelo. No obstante, el encalado del suelo no afecta a la viabilidad de las esporas resistentes del patógeno (clamidosporas), que siguen siendo fuente de inóculo (Serrano et al., 2012b; 2013). Es recomendable la fertilización cálcica como medida preventiva de control frente a *P. cinnamomi* cuando se haya confirmado el diagnóstico, aunque no resulte una medida definitiva para impedir nuevas infecciones. Concretamente, tras haberse realizado diversos estudios con distintos productos cálcicos, se ha observado que $CaCO_3$ y $CaSO_4$ reducen la infectividad del inóculo en el suelo, siendo este último compuesto, aplicado en condiciones de campo con dosis de 3550 kg/ha, el que más limita la multiplicación del inóculo infectivo de *P. cinnamomi*, reduciendo significativamente la incidencia de la podredumbre radical (Serrano et al., 2014).

Gómez-Aparicio et al. (2012) indican que la abundancia de patógenos en el suelo, como los oomicetos *P. cinnamomi* y *Pythium* spp., no es aleatoria, sino que responde a una

distribución espacial influída por factores abióticos (textura del suelo) y, particularmente, bióticos (especies arbóreas y arbustivas presentes). Por lo tanto, otro de los aspectos fundamentales a la hora del control es el que hace referencia a las distintas especies hospedantes de *P. cinnamomi*, ya sean sintomáticas o asintomáticas. Distintas investigaciones han concluido que existen cultivos herbáceos susceptibles a este patógeno, ya que son hospedantes del mismo y multiplican el inóculo en el suelo y, por tanto, favorecen la infección de los árboles. Concretamente, se ha demostrado que la tremosilla o altramuz amarillo (*Lupinus luteus*) es un cultivo susceptible sintomático que favorece la multiplicación del patógeno (aumento en la producción de zoosporas y clamidosporas) (Serrano et al., 2010; 2011). Este no es el caso de los cereales (avena y trigo) y la veza (*Vicia sativa*), ya que se ha comprobado que no favorecen la enfermedad, aunque esta leguminosa sea un cultivo hospedante asintomático (Serrano et al., 2012c).

2.3.3.2 Resistencia genética

La información disponible en cuanto a la resistencia de especies arbóreas mediterráneas a *P. cinnamomi* es escasa, si bien sólo la encina y el alcornoque resultan gravemente afectados en condiciones naturales. La resistencia a *P. cinnamomi* de variedades o procedencias de encinas y alcornoques presentes en Andalucía es muy limitada (Tapias et al., 2006; Navarro et al., 2009; Serrano et al., 2012d). Serrano et al. (2012d) subrayan que los cuatro principales morfotipos de encina presentes en Andalucía no muestran diferencias significativas en cuanto a la susceptibilidad de sus raíces al patógeno, que es muy alta en todos los casos. Estos mismos autores indican, no obstante, que dichos morfotipos se pueden clasificar en distintos grupos de susceptibilidad en función del desarrollo de síntomas aéreos: muy susceptibles (microcarpa), susceptibles (expansa) y moderadamente susceptibles (rotundifolia y macrocarpa). Por su parte, resultados descritos por estos mismos autores indican que *Quercus faginea* puede considerarse como una fuente de resistencia a *P. cinnamomi*, ya que el híbrido natural *Q. ilex* subsp. *ballota* × *Q. faginea* muestra niveles de síntomas aéreos y radicales significativamente menores que las encinas de cualquier morfotipo.

2.3.3.3. Control químico

Otra de las medidas de control frente a la podredumbre radical es el uso de fosfonatos. Estos compuestos sistémicos, no fitotóxicos y activos a bajas concentraciones se usan

desde 1977 para el control de enfermedades causadas por oomicetos en especies agrícolas y forestales (McDonald et al., 2001). Actúan principalmente como activadores de resistencia, a través del incremento en la síntesis de fitoalexinas y de la respuesta hipersensible.

Estos productos han demostrado tener eficacia tanto preventiva como curativa ya que, aplicados al árbol sano mediante inyección al tronco, previenen la infección de las raíces por *P. cinnamomi* y aplicados a árboles ya infectados, pero que aún no muestran los síntomas aéreos de la enfermedad radical, evitan su muerte (Navarro et al., 2006; Sánchez et al., 2006). En ensayos de eficacia en plantones de encina y alcornoque se ha demostrado que la materia activa fosetil-aluminio resulta muy efectiva en la prevención de la enfermedad a las dosis recomendadas por los fabricantes para cultivos leñosos, mejorando el efecto preventivo del fosfito potásico (González et al., 2017a). La aplicación de esta última materia activa, que se comercializaba como fertilizante fosfórico a pesar de no aportar fósforo utilizable por la planta, ha sido recientemente prohibida en tanto no se registre como fungicida (Anexo 1 RD 506/2013, www.juntadeandalucia.es/agriculturaypesca/raif/novedades/2014/novedad_140507_02.html). Actualmente se está ensayando en condiciones de campo la eficacia preventiva y curativa del fosetil-Al y de nuevas formulaciones experimentales con efecto inductor de resistencia.

2.3.3.4. Control biológico

El control biológico de una enfermedad se define como la reducción de la densidad o la eficacia del inóculo mediante el uso de uno o más organismos distintos del hombre (Cook y Baker, 1983). Los métodos de control biológico más comunes incluyen el uso de microorganismos antagonistas del patógeno, la solarización y la biofumigación.

Se han descrito ciertos microorganismos antagonistas de *P. cinnamomi in vivo* y en condiciones de invernadero (Weste y Vithange, 1978; Halsall, 1982; Keast et al., 1985; Hardy y Sivasithamparam, 1991). Estos microorganismos son bacterias de los géneros *Mircomonospora, Pseudomonas* y *Streptomyces*. (Turnbull et al., 1992) aunque ninguna de ellas se ha descrito como antagonista de *P. cinnamomi* en Europa.

La solarización consiste en el uso de la energía solar para calentar las primeras capas de suelo mediante el empleo de un plástico y agua. El suelo húmedo se cubre con un

plástico transparente durante los meses de verano por un mínimo de 4-6 semanas, de manera que la temperatura del suelo se incrementa, llegando a evaporar el agua, que se condensa en el plástico favoreciendo a su vez el aumento de la temperatura mediante el efecto invernadero (Mahrer y Katan, 1981). Se ha demostrado que la solarización es efectiva en el control de *P. cinnamomi* (Barbercheck y Von Broembsen, 1986), pero su aplicación en condiciones reales de dehesa conllevaría una alta dificultad.

2.4. BIOFUMIGACIÓN

Angus et al. (1994) definieron biofumigación como la incorporación de tejidos de *Brassica* al suelo para conseguir un efecto supresivo sobre algunos patógenos y plagas del suelo. Es un método de control biológico, ya que trata de reducir la densidad y/o la eficacia del inóculo mediante el uso de otro organismo diferente del hombre, según la definición de Cook y Baker (1983). El interés en la biofumigación es cada vez mayor debido a las crecientes restricciones en el uso de fumigantes químicos y a la tendencia a disminuir el uso de estos productos altamente tóxicos. Actualmente, hay poca información acerca de cómo afecta la biofumigación a las especies de *Phytophthora* (Mazzola y Brown, 2010; Morales-Rodríguez et al., 2012; Pingsheng et al., 2012; Gigot et al., 2013), y en particular a *P. cinnamomi* (Dunne et al., 2003; Morales-Rodríguez et al., 2016). Sin embargo, estudios llevados a cabo sobre otros oomicetos de los géneros *Aphanomyces* y *Pythium* han demostrado que el uso de biofumigantes puede afectar al desarrollo de éstos patógenos, reduciendo así la incidencia de las enfermedades que causan (Lewis y Papavizas, 1971; Manici et al., 2000; Smolinska et al., 1997; Sarwar et al., 1998; Charron y Sams, 1999).

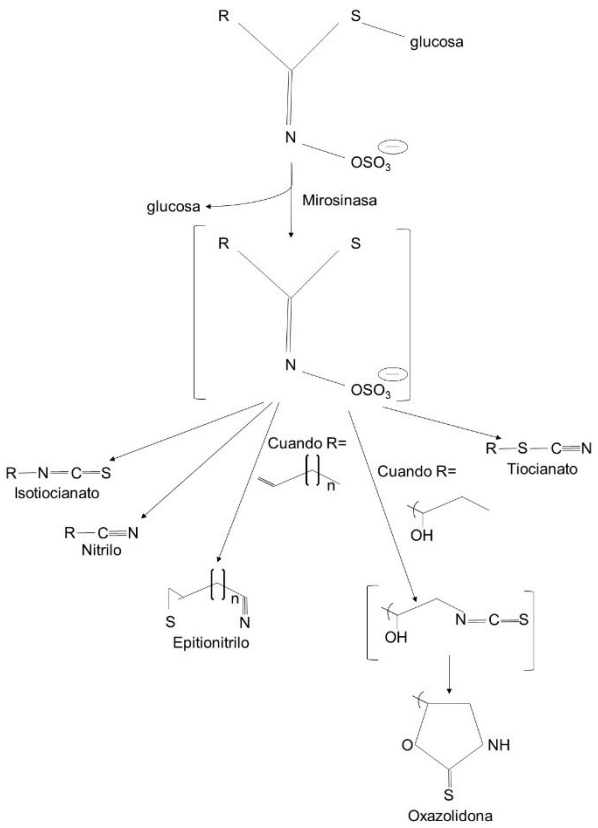
El efecto supresivo de la biofumigación se atribuye a la acción de los compuestos volátiles liberados tras la hidrólisis de los glucosinolatos (GSLs) (Kirkegaard y Sarwar, 1999). Los GSLs son metabolitos secundarios producidos naturalmente en plantas pertenecientes al orden *Capparales*, especialmente en aquellas clasificadas dentro del género *Brassica* (Halkier y Gershenzon, 2006). Se ha demostrado que los productos volátiles formados a partir de la hidrólisis de los GSLs tienen una alta actividad biocida (Brown y Morra, 1997). Los GSLs son hidrolizados en el suelo durante la descomposición de los tejidos de las plantas que los contienen mediante la enzima mirosinasa, una enzima endógena de estas especies vegetales (Sarwar et al., 1998).

Los GSLs tienen una estructura principal bien definida (β-tioglucósidos-N-hidroxisulfatos) con diferentes sustituyentes que pueden ser muy variables (Rosa et al., 1997). La parte aglicónica puede contener cadenas alifáticas, indólicas o aromáticas en función de que su aminoácido precursor sea metionina, triptófano o aminoácidos aromáticos (tirosina o fenilalanina) respectivamente (Giamnoustaris y Mithen, 2006). Es esta naturaleza de la cadena lateral de la que dependen los productos volátiles específicos que se forman tras su hidrólisis (Brown y Morra, 1997) (Figura 4), siendo los isotiocianatos (ITCs) los que presentan una mayor actividad biocida (Brown y Morra, 1997), aunque también se producen otros productos como nitrilos, tiocianatos, epitionitrilos y oxazolidonas (Halkier y Gershenzon, 2006). A pesar de que el papel de los GSLs en el control de patógenos es ampliamente reconocido y referido en la bibliografía, hay autores que afirman que la formación de otros productos solubles en agua, producidos durante la descomposición de los tejidos vegetales y no derivados de los GSLs, contribuyen de manera importante a la mejora del efecto de los biofumigantes (Bending y Lincoln, 1999). En cualquier caso, estos productos no derivados de los glucosinolatos también son compuestos azufrados como disulfuro de carbono (CS_2), disulfuro de dimetilo ($C_2H_6S_2$), sulfuro de dimetilo ($(CH_3)_2S$) o metanotiol (CH_4S), provenientes de la descomposición de los tejidos de brasicáceas (Bending y Lincoln, 1999).

Como se ha indicado, los GSLs son metabolitos secundarios producidos casi exclusivamente en familias del orden *Capparales*: *Tovariaceae*, *Resedaceae*, *Capparaceae*, *Moringaceae* y *Brassicaceae* (Fenwick et al., 1983; Brown y Morra, 1997; Rosa et al., 1997). Son particularmente comunes en la familia *Brassicaceae*, plantas que son ampliamente cultivadas por sus características nutricionales tanto para humanos como para el ganado, y para la producción de aceites de semillas (Fenwick et al., 1983). Esta familia comprende 350 géneros y 2500 especies (Rosa et al., 1997), siendo el género *Brassica* el más estudiado debido al amplio consumo mundial de las plantas pertenecientes a este género con destino a la alimentación humana.

La eficacia biocida de un tratamiento biofumigante parece estar relacionada con su perfil de GSLs o bien con la presencia de un GSL en concreto (Brown y Morra 1997; Sarwar et al., 1998). De este modo, se han descrito efectos contrarios cuando se ha enfrentado a distintos patógenos con un mismo biofumigante.

Figura 4. Mecanismo de acción de la biofumigación (Fuente: Halkier y Gershenzon, 2006)



Así, Smolinska et al. (1997) y Smolinska (2000) demostraron que la biofumigación con *Brassica napus* limita el desarrollo de ciertos hongos como *Sclerotium cepivorum* e incluso de oomicetos como *Aphanomyces euteiches* f. sp. *pisi*, mientras que Mazzola et al. (2001) refieron un aumento de la población de oomicetos del género *Pythium* tras la biofumigación con *B. napus.* En el caso de especies del género *Phytophthora*, también aparecen resultados contrapuestos: Gigot et al. (2013) refirieron una reducción de poblaciones de *P. rubi* tras biofumigar un huerto de frambuesas con *Brassica juncea*, mientras que Mazzola y Brown (2010) afirman que el mismo biofumigante estimula la tasa de infección de *P. cambivora* en condiciones de campo. Es esta selectividad de los GSLs sobre distintos organismos lo que ha llevado a definir modos de acción alternativos de la biofumigación basados en los cambios de las poblaciones microbianas en el suelo. De este modo, Mazzola y Zhao (2010), tras las biofumigación de parcelas de manzanos con *B. juncea* refirieron un aumento de la densidad de *Streptomyces* spp. y una disminución de la densidad de *Rhizoctonia solani* en comparación con suelos sin tratar. En el mismo sentido, Hu et al. (2015) describieron un incremento de poblaciones

de *Firmicutes* spp. y una disminución de las poblaciones de hongos tras la adición de alil-isotiocianatos al suelo en comparación con la aplicación de isotiocianatos puros pertenecientes a otras familias químicas.

El método tradicional para la aplicación de biofumigantes ha sido el uso de la técnica de enterrado en verde, es decir, la integración de un cultivo intercalar, normalmente perteneciente al género *Brassica*, en el sistema rotacional de cultivos. El cultivo de estas plantas tiene efectos beneficiosos durante su crecimiento, como el mantenimiento de una cubierta verde que evita la erosión, la mejora de la estructura del suelo y la puesta a disposición de nutrientes gracias a los bien desarrollados sistemas radicales de las especies de este género. Una vez alcanzada la cantidad de biomasa necesaria, el cultivo se trocea y se incorpora al suelo, manteniendo la humedad del mismo alta y cubriéndolo con plástico para evitar la pérdida de los volátiles (Matthiessen y Kirkegaard, 2006). La efectividad de las enmiedas verdes con cultivos de *Brassica* dependerá de la calidad del cultivo, que viene determinada por la genética del mismo, la especie/variedad utilizada, el momento de siembra/ plantación, la densidad de planta, el tipo y fertilidad de suelo y la climatología durante el período de cultivo (Leonard, 2000). Además, para una liberación eficiente de ITCs durante la incorporación del cultivo es fundamental que exista una correcta rotura celular y la presencia de una adecuada humedad en el suelo (Morra y Kirkegaard, 2002; Morra, 2004). En el caso concreto de las dehesas, hay ciertos factores que limitarían la biofumigación usando la técnica de enterrado en verde, como puede ser la baja producción de biomasa debido a la limitada fertilidad de los suelos, la pedregosidad de los suelos típicos de dehesa, que limitaría su incorporación, y la dificultad para aplicar la humedad necesaria al suelo debido a que el riego no siempre es posible. Como solución a estos problemas, actualmente se comercializan pellets de *Brassica carinata* (BioFence®, Italia) cuyo efecto sobre *P. cinnamomi* ha sido testado *in vitro* (Morales-Rodríguez et al., 2016). Existe poca información acerca de la composición de estos pellets y de sus condiciones de aplicación, pero es una alternativa a tener en cuenta dentro de un programa de lucha integrada contra *P. cinnamomi.*

Aunque los GSLs se encuentran en todos los órganos vegetales de las Brasicáceas, estos están más concentrados en las semillas (Borek y Morra, 2005). La presión en frío de las semillas para la obtención del aceite que contienen genera un subproducto que no sólo conserva una alta concetración de glucosinolatos, sino que también mantiene la

actividad de la enzima mirosinasa de manera que, tras la adicción de agua, genera los productos activos resultantes de su hidrólisis (Brown et al., 1991). Esta técnica evita tener que cultivar *in situ* el biofumigante, pero no asegura las condiciones óptimas para la actividad de la enzima, como un valor de pH adecuado (Iori et al., 1996), ni la cantidad de enzima necesaria para una hidrólisis óptima (Popova et al., 2017).

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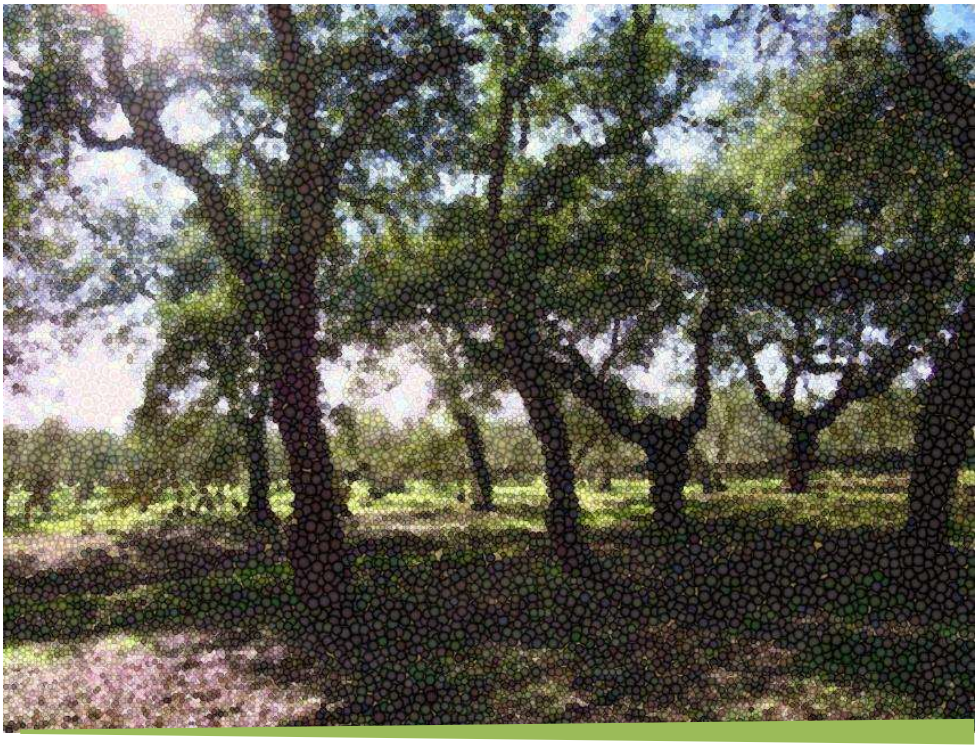
CAPÍTULO 3.

UMBRAL MÍNIMO DE INÓCULO DE *Phytophthora cinnamomi* PARA EL DESARROLLO DE LA ENFERMEDAD RADICAL EN *Quercus suber*

CHAPTER 3.

EXPERIMENTAL MINIMUM THRESHOLD FOR *Phytophthora cinnamomi* ROOT DISEASE ON *Quercus suber*



En este capítulo se recogen los resultados relativos al Objetivo 1 de esta Tesis Doctoral, en el cual se plantea conocer el umbral mínimo de inóculo de *Phytophthora cinnamomi* en suelo necesario para el desarrollo de la enfermedad radical.

Los resultados obtenidos se han recogido en el siguiente artículo publicado en ***Phytopathologia Mediterranea*** 2015, volúmen 54, páginas 461-464 titulado:

"Determination of the minimum threshold of Phytophthora cinnamomi *in soil for root disease development on* Quercus suber".

Autores: María Socorro Serrano, **Pedro Ríos**, Mario González, María Esperanza Sánchez

3.1

Determination of the minimum threshold of *Phytophthora cinnamomi* in soil for root disease development on *Quercus suber*

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RESEARCH PAPERS

Experimental minimum threshold for *Phytophthora cinnamomi* root disease expression on *Quercus suber*

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Summary. *Quercus suber* seedlings were potted in soils infested with increasing concentrations of *Phytophthora cinnamomi* chlamydospores and submitted to weekly flooding for 3 months to favour root infections. Increasing quantities of chlamydospores led to an exponential increase in their ability to germinate. Root symptoms (necrosis and/or absence of feeder roots) were significantly more severe than those recorded in uninfested soil only for plants potted in soils infested with 61 cfu g^{-1} or more. Although generated using potting mix, this minimum threshold represents a tool for checking the potential infectivity of infested soils or to assess the effectiveness of some control methods to reduce soil inoculum. However, a low level of root infection was recorded even at 3 cfu g^{-1}. Therefore, long-term disease risk may be present whenever the pathogen is detectable in oak forest soils.

Key words: chlamydospores, cork oak, infection, inoculum.

Introduction

Quercus is a genus especially threatened by *Phytophthora cinnamomi* in areas of Mediterranean climate, including Spain (Sánchez *et al*., 2002), California (Garbelotto *et al.*, 2006) and Italy (Scanu *et al*., 2013). Highly variable *P. cinnamomi* soil inoculum densities have been reported for diseased oak ecosystems in southern Spain, ranging from 4–49 colony forming units (cfu) g^{-1} dry soil (Romero *et al.*, 2007) to 25-2500 cfu g^{-1} (Gómez-Aparicio *et al*., 2012). Knowledge of the minimum threshold of *P. cinnamomi* inoculum provides an experimental reference point to check the potential infectivity of infested soils or to assess the effectiveness of control methods aimed at reducing soil inoculum (green or mineral amendments, biofumigation). The aim of the research reported here was to determine the threshold of inoculum necessary to cause root disease in cork oak plants under highly favourable experimental conditions for disease development.

Materials and methods

Experiment 1

Two *P. cinnamomi* isolates (PE90 from holm oak, and PA25 from cork oak) were used to obtain inocula at different concentrations. The mother inoculum was prepared following Sánchez *et al*. (2002) and consisted of a water suspension of chlamydospores from both isolates adjusted to 1.5×10^4 chlamydospores mL^{-1} (Romero *et al.*, 2007). Additional inocula of 1500, 150 and 15 chlamydospores mL^{-1} were prepared by successive water dilutions of the original suspension. Four containers, each with 30 L of soil (sand-peat 1:1 vol., 22.5 kg, pH = 6.36) were homogeneously infested with 1 L of each inoculum suspension and a fifth container (control) was prepared by adding 1 L of water (0 concentration). Three 15 mL-soil samples per container (replicates), were plated on NARPH medium as described in Romero *et al.*

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(2007). Individual *Phytophthora cinnamomi* colonies, each derived from at least one viable chlamydospore, were identified and counted. After sampling, each soil was distributed into ten free-draining 3 L capacity plastic pots and 18 month-old *Q. suber* seedlings (replicates) were planted (one per pot) after removing most soil without damaging them. The pots were each placed in a plastic tray without drainage (57 × 41 × 9 cm) and placed in an air-conditioned greenhouse (daily cycle of 25 ± 2°C for 12 h and 10 ± 2°C for 12 h) in a randomized block design. For 2 d a week for the next 3 months the trays were partially filled with tap water, to periodically flood the soil (Serrano *et al.*, 2012). After this time, root rot symptoms were assessed according to the percentage of root necrosis or root absence on a 0–4 scale (0 = 0% necrotic roots, 1 = 10–33%, 2 = 34–66%, 3 = more than 67% necrotic roots, 4 = 100% dead root) (Serrano *et al.*, 2012).

Experiment 2

To narrow the concentration range of inoculum necessary to cause significant root disease, a new mother inoculum suspension was prepared as described above, adjusted to 3×10^3 chlamydospores mL^{-1} and diluted with water to obtain inocula containing 2×10^3, 1.5×10^3, 10^3, 500 or 50 chlamydospores mL^{-1}. Soil mix (above) was infested and processed as described above, and six 18 months-old *Q. suber* seedlings (replicates) were individually potted, incubated, harvested and assessed, as described above.

At the end of both experiments, root segments from plants potted in infested or control soils were plated on NARPH medium for re-isolation of the pathogen.

Data analyses

Inoculum concentration data were transformed to $[(\text{cfu } g^{-1}) + 0.5]^{1/2}$ for ANOVA analysis. A regression curve was performed with data from Experiment 1, to establish the relationship between the amount of chlamydospores added to the soils and the number of viable chlamydospores recovered. Data obtained from root symptom assessments were tested for homocedasticity by the Bartlett's test, and when heterogeneity was detected, angular (Experiment 1) or logarithmic (Experiment 2) transformations were applied to the data. ANOVA was performed for root symptoms and mean values compared by the Tukey's HSD test at $P<0.05$. Statistix 8.0 (Analytical Software) was used for data analyses.

Results and discussion

In Experiment 1, significant (DF = 5, F = 33.67, $P<0.0001$) differences in viable chlamydospores (cfu g^{-1}) were detected depending on the chlamydospore concentration added to the soils, following an exponential relationship: $y = 0.0416\ e^{2.0893x}$ ($R^2 = 0.9393$) (Figure 1). Increasing quantities of chlamydospores in soil led to an exponential increase in their ability to germinate, while for other soilborne pathogens, such as *Fusarium oxysporum* f. sp. *lini*, chlamydospore viability suffers a slight decrease at high initial chlamydospore densities (Couteaudier and Alabouvette, 1990). Only root symptoms recorded in plants growing in soils infested with 1.5×10^4 or 1500 chlamydospores mL^{-1} (256.5 and 54.7 cfu g^{-1}) were significantly (DF = 4, F = 6.29, $P<0.0001$) more severe than those recorded for plants potted in soils infested with the two lowest chlamydospore concentrations and the control soil (Figure 2). Because of the constraints imposed by the pots and by the frequent flooding, all the experimental controls developed low levels of root necrosis; hence such controls need to be included for a correct determination of disease symptoms.

Average numbers of viable chlamydospores obtained from infested soils in Experiment 2 fitted well with the quantities expected according to the relationship presented in in Figure 1. This resulted (on average, and respectively) in 3, 21, 41, 61 and 82 cfu g^{-1} from the initial 50, 500, 10^3, 1.5×10^3, and 2×10^3 chlamydospores mL^{-1} applied. Root symptoms of seedlings potted in soils infested with 61 or 82 cfu g^{-1} were significantly greater (DF = 5, F = 9.48, P=0.0001) than those recorded for plants potted in control soil, while lowest concentrations of viable inoculum did not cause disease (Figure 2). This threshold of 61 cfu g^{-1} was the minimum required to cause cork oak root disease in the highly favourable conditions artificially provided in this experiment. This value is similar to that obtained for *P. capsici*, which requires 41 oospores g^{-1} to produce 50% mortality in pepper plants (Bowers and Mitchell, 1991).

Phytopthora cinnamomi was re-isolated from necrotic roots of plants potted in soil infested with low inoculum concentrations (2.7 cfu g^{-1} in Experiment 1,

or 3, 21 and 41 cfu g^{-1} in Experiment 2), resulting in 14–39% of positive isolations. The pathogen was never recovered from control roots or from soil infested with 0.7 cfu g^{-1} (Experiment 1). These results indicate that infections caused by these low amounts of inoculum lack the ability to progress into significant root mortality. It is possible, however, that in time, these lesions may lead to significant symptoms or new infections. Mitchell (1978) concluded that only 0.6 or 0.9 chlamydospores g^{-1} of *P. citrophtora* or *P. palmivora* were needed to infect, respectively, *Morrenia odorata* or *Carica papaya*, in growth chamber studies, although some of these results pertained to aerial infections. Additionally, these limited infections may allow for persistent survival of the pathogen in a site, as suggested by recrudescence of disease in absence of stringent inoculum eradication (Dunstan *et al*., 2010). Notwithstanding the role played by infections caused by low levels of inoculum, our results suggest that 61 cfu g^{-1} represents a consistent threshold of inoculum capable of inducing significant root infection, at least in the experimental conditions applied in the present study.

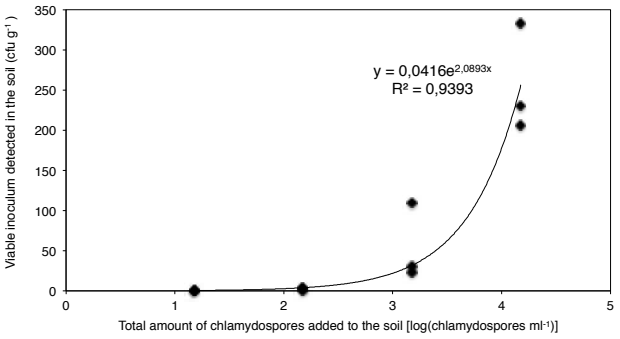

Figure 1. Relationship between numbers of *Phytophthora cinnamomi* chlamydospores added to soil [log (chlamydospores ml^{-1})] and viable chlamydospores detected (cfu g^{-1}). Dots are the obtained values and the line the adjusted exponential curve

Although the minimum threshold determined here can be useful as an experimental reference for checking the potential infectivity of infested soils, or to assess the effectiveness of some control methods to reduce soil inoculum, we are also aware that such a threshold may be different in natural forest soils. It is likely that in natural soils the threshold may be greater than the one determined here, thus our results may be valuable under this precautionary principle. We emphasize that these types of experiments are useful when monitoring disease spread in sites already known to be infested. When assessing risk, a site should be regarded "at risk" whenever the pathogen is detectable in the soil (Dunstan *et al*., 2010).

We conclude that presence/absence of *P. cinnamomi* needs to be accurately monitored at the large geographic scale to identify all cork oak sites at risk and all sites that may be a source of new infestations. Inoculum loads may be calculated to track disease expression and efficacy of disease management approaches. Results from inoculum load studies may indicate which sites may be more at risk and where

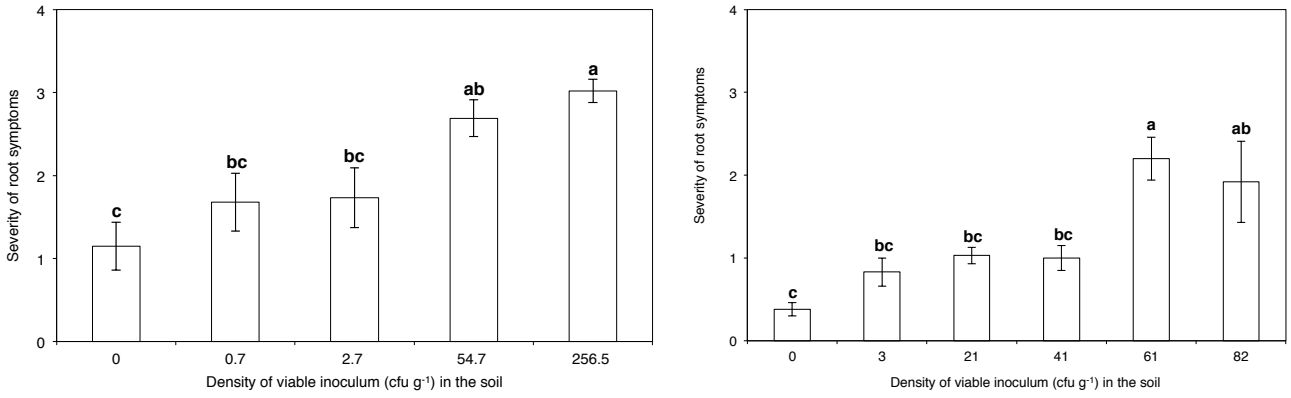

Figure 2. Mean severity of root disease symptoms recorded for cork oaks growing in soils infested with different numbers of *Phytopthora cinnamomi* chlamydospores. Lines are standard errors of ten replicates (Experiment 1, left chart) or six replicates (Experiment 2, right chart). Bars with different letters differ significantly according to Tukey´s HSD test ($P<0.05$).

control measures may be more efficient. This will provide a way to prioritize choices when dealing with the widespread presence of *P. cinnamomi* in oak forest soils in southwestern Spain and southern Portugal (Romero *et al*., 2007), to indicate threats to the survival of oak forests in the region.

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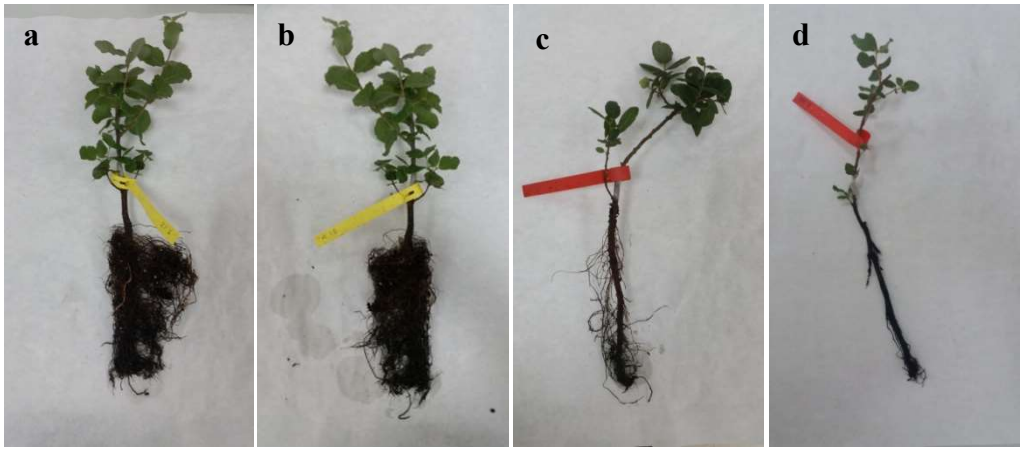
Figura 5: Severidad de síntomas radicales en alcornoques cultivados en suelos infestados con diferentes concentraciones de clamidosporas de *Phytophthora cinnamomi*. (a) suelo testigo no infestado; (b) suelo infestado con 41 CFU×g^{-1} de suelo seco; (c) suelo infestado con 61 CFU×g^{-1} de suelo seco; (d) suelo infestado con 82 CFU×g^{-1} de suelo seco.



CAPÍTULO 4. SELECCIÓN DE ESPECIES BIOFUMIGANTES CON EFECTO TÓXICO PARA *Phytophthora cinnamomi*

CHAPTER 4. SELECTION OF BIOFUMIGANTS SPECIES WITH NOXIOUS EFFECTS ON *Phytophthora cinnamomi*

En este capítulo se recogen los resultados relativos al Objetivo 2 de esta Tesis Doctoral, en el cual se plantea seleccionar especies de crucíferas con poder biofumigante contra *Phytophthora cinnamomi.*

Los resultados obtenidos se han recogido en los siguientes artículos:

Capítulo 4.1, publicado en el ***Journal of Phytopathology*** 2016, volúmen 164, páginas 582-594 titulado:

Effect of Brassica *biofumigants amendments on different stages of the life cycle of* Phytophthora cinnamomi

Autores: **Pedro Ríos**, Sara Obregón, Antonio de Haro, Pilar Fernández-Rebollo, María Socorro Serrano, María Esperanza Sánchez.

Capítulo 4.2, artículo en revisión enviado a ***Phytopathologia Mediterranea*** con el título:

Biofumigant action of Brassica *seedmeals against* Phytophthora cinnamomi *in dehesa ecosystems*

Autores: **Pedro Ríos**, Mario González, Sara Obregón, María Dolores Carbonero, Jose Ramón Leal, Pilar Fernández-Rebollo, Antonio de Haro, María Esperanza Sánchez.

Capítulo 4.3, publicado en ***Forest Pathology*** 2016, volúmen 46, páginas 652-659 titulado:

Screening of Brassicaceous plants as biofumigants for management of Phytophthora cinnamomi *oak disease*

Autores: **Pedro Ríos**¸Sara Obregón, Mario González, Antonio de Haro, María Esperanza Sánchez.

4.1

Effect of *Brassica* biofumigants amendments on different stages of the life cycle of *Phytophthora cinnamomi*

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Journal of Phytopathology 2016, 164, 9, 582-594

Journal of Phytopathology

ORIGINAL ARTICLE

Effect of *Brassica* Biofumigant Amendments on Different Stages of the Life Cycle of *Phytophthora cinnamomi*

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Abstract

The oomycete plant pathogen *Phytophthora cinnamomi* causes a highly destructive root rot that affects numerous hosts. Integrated management strategies are needed to control *P. cinnamomi* in seminatural oak rangelands. We tested how biofumigation affects crucial stages of the pathogen's life cycle *in vitro*, in infested soils under laboratory conditions and *in planta*. Different genotypes of three potential biofumigant plant species (*Brassica carinata, Brassica juncea, Brassica napus*) were collected at different phenological stages, analysed for their glucosinolate contents, and subsequently tested. The most effective genotypes against mycelial growth and sporangial production were further tested on the viability of chlamydospores in artificially infested natural soils and *in planta* on *Lupinus luteus*, a host highly susceptible to *P.cinnamomi*. *Brassica carinata* and *B. juncea* genotypes inhibited mycelial growth, decreased sporangial production, and effectively inhibited the viability of chlamydospores in soil, but only *B. carinata* decreased disease symptoms in plants. Effective genotypes of *Brassica* had high levels of the glucosinolate sinigrin. Biofumigation with Brassica plants rich in sinigrin has potential to be a suitable tool for control of oak root disease caused by *P. cinnamomi* in Spanish oak rangeland ecosystems.

Introduction

Phytophthora cinnamomi Rands is a serious plant pathogen worldwide, with a large number of woody hosts; it is among the world's most invasive species (Lowe et al. 2000). *Phytophthora cinnamomi* causes root rot and is especially virulent on holm oak (*Quercus ilex* L.) and cork oak (*Q. suber* L) in rangeland ecosystems, and on chestnut (*Castanea sativa* Mill.) and avocado (*Persea americana* Mill.) in southern Europe. Zoospores of *P. cinnamomi* are generated in sporangia produced by the germination of resistant spores present in the soil. The biflagellated zoospores move through water in the soil and infect susceptible host feeder roots. Once roots are infected, mycelium develops inside plant tissues and, under conducive conditions the pathogen enters asexual sporulation by generating new sporangia on the root surface (Hardham 2005).

Root rot disease management is difficult due to the longevity of resting spores of *P. cinnamomi* in the soil (both sexually produced oospores and asexually produced chlamydospores), due to its rapid and deep dispersal in waterlogged soils, and due to the large number of hosts. At present, control of *P. cinnamomi* in oak rangelands is based on a combination of techniques that decrease incidence of *Phytophthora*, but do not affect the viability of *P. cinnamomi* resting spores. These include limestone amendments (Serrano et al. 2012a), phosphite trunk injections, selection of oak morphotypes tolerant to the disease (Serrano et al.

2012b), limiting soil movement and not planting herbaceous species susceptible to *P. cinnamomi* (Serrano et al. 2010).

Soil borne pathogens are commonly treated with chemical fumigants in agricultural situations, but would be detrimental to many species in seminatural ecosystems. Biofumigation use natural products to control pests, pathogens and weeds based on the biocidal action of volatile compounds released through the hydrolysis of glucosinolates (GSLs) (Kirkegaard et al. 1993), secondary metabolites produced in plants belonging to the order Capparales and especially those in the genus *Brassica* (Halkier and Gershenzon 2006). GSLs have a well-defined main structure (β-thioglucoside-N-hydroxysulfates) with variable substituents. The aglycone part may contain aliphatic, indole, or aromatic side chains depending on whether the amino acid precursor was methionine, tryptophan or an aromatic amino acid (tyrosine or phenylalanine), respectively (Giamnoustaris and Mithen 2006). GSLs are not bioactive until they have been enzymatically hydrolyzed to various volatile breakdown products (isothiocyanates (ITCs), nitriles, thiocyanates, epithionitriles, oxazolidine-2-thiones, and epithionitriles) by the endogenous plant enzyme myrosinase (thioglucoside glucohydrolase, E.C.3.2.1.147) (Halkier and Gershenzon 2006). Pure ITCs are active against soilborne pathogens (Smith and Kirkegaard 2002). Some studies have evaluated the antifungal activity of different *Brassica* tissues against different soilborne pathogens (Lazzeri and Manici 2000), but little is known about their activity on *Phytopththora* species (Dunne et al. 2003a,b).

Biofumigation can be applied as an isolated measure or as part of an integrated control program, being major issues to reach a high release of biologically active products derived from GLSs hydrolysis, the method applied for incorporation of the biofumigant material to the soil, and soil moisture at incorporation time (Morra and Kirkegaard 2002). Combined biofumigation and other techniques such as soil solarization, enhance its pesticide effectiveness (Keinath 1996).

Brassica spp. green manure crops are the traditional technology for providing benefits as disease suppression in different farming systems (Matthiessen and Kirkegaard 2006), but there are also references about their role as trap crops (Muller 1999; Thorup-Kristensen et al. 2003; Schlathoelter 2004). There is a trend to use *Brassica*-derived GSL-rich material such as *Brassica* pellets, oil or seed meal amendments to achieve pesticidal effects (Lazzeri et al. 2004; Matthiessen and Kirkegaard 2006), turning industrial wastes into useable byproducts.

The genus *Brassica* comprises 41 species and three of them, *Brassica juncea* L. Czern & Coss (Indian mustard, genome AABB), *Brassica napus* L. (rapeseed, genome AACC), and *Brassica carinata* A. Braun (Ethiopian mustard, genome BBCC) are allotetraploids, genetically related, and well adapted to the rain-fed Mediterranean region. These three *Brassica* species have several desirable agronomic characteristics, including high biomass and seed yield, well-developed root systems, and resistance to drought, diseases and pests (Lazzeri et al. 2013). These characteristics make them potential biofumigant candidates in the semiarid climate of oak rangelands in southern Spain.

The aims of this work were to: i) test the allotetraploid *Brassica* genotypes for their biofumigant activity against *P. cinnamomi*, ii) assess which growth stages of the *Brassica* species are the most inhibitory or biocidal to the pathogen, and iii) explore any associations between inhibition of *P. cinnamomi* and *Brassica* genotype GSLs profiles.

Material and methods

Plant material

Brassica spp. were cultivated in an experimental field located at the Institute of Sustainable Agriculture (IAS) in Córdoba, Spain (37.8°N, 4.8°W). The climate is Mediterranean, with an average annual rainfall of 650 mm. The soil is sandy-loam, classified as a Typic Xerofluvent.

Two genotypes from each of three *Brassica* species (*B. napus*, *B. carinata* and *B. juncea*) were selected on the basis of the seed GSL content. The two genotypes of *B. napus*: Bn-Lewis (double low variety = low erucic acid and low glucosinolate content in seed) and Bn-Salamander (single low variety = low erucic acid content in seed) were provided by Dr. Delourme (INRA, Rennes, France). Genotypes of *B. carinata* (Bc-IASC1 and Bc-IAS119) and *B. juncea* (Bj-Tezla and Bj-552) were obtained from the Plant Breeding Group at IAS (Córdoba, Spain) after several cycles of genetic selection for agronomic performance and seed GSL composition under Mediterranean climate conditions (Font et al. 2006): Bc-IASC1 and Bj-Tezla were selected as low GLS content varieties, while Bc-IAS119 and Bj-552 represented high GLS content varieties.

Plants were grown in the 2011/2012 growing season. For each selected genotype, the plot unit consisted of three rows 6 m long, with 0.8 m between

rows. The above-ground parts (leaves, stems, flowers, seeds) of five plants from each genotype were harvested at three growth stages: end of stem elongation, full flowering, and maturity, corresponding to the BBCH (Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie) scale codes 49, 65 and 79 respectively (Lancashire et al. 1991). Immediately after harvesting, plants were washed with abundant tap water and superficially disinfected by soaking them in aqueous 10% sodium hypochlorite for 10 s. After surface drying, plants were weighed to obtain their fresh weights, frozen (24 h at −20°C) and lyophilized for 1 week using a freeze-drier (Telstar model Cryodos-50, Terrasa, Spain). After lyophilization, plant material was weighed to obtain the dry weight, and ground in coffee grinders for subsequent use.

Phytophthora isolate

All experiments were conducted using *P. cinnamomi* isolate PE90, isolated from *Quercus ilex* ssp. *ballota*. Isolate PE90 was from the western population in the south of the Iberian Peninsula (Caetano et al. 2009). Previously, differences in virulence of *P. cinnamomi* isolates to some hosts have been reported, but no significant differences among *P. cinnamomi* isolates were found when infecting holm or cork oaks (Robin et al. 1998).

Glucosinolate analysis

GSL composition for each *Brassica* species, genotype and phenological growth stage, and samples of the original seeds, was determined by High Performance Liquid Chromatography (HPLC). For each sample, 100 mg dry weight of lyophilized ground *Brassica* material was placed in 2.5 ml of 70% aqueous methanol and heated at 75°C for 15 min (to deactivate the myrosinase); 200 μl of 10 mM Glucotropaolin (Benzylglucosinolate) was used as the internal standard. The samples (2 ml) were centrifuged (5 min at 5000 g) and 1 ml of the combined GSL extracts was pipetted onto the top of an ion-exchange column containing 1 ml Sephadex DEAE-A25 (formate form). GSL desulphation was achieved by the addition of 75 μl of purified Sulphatase (E.C. 3010601, type H-1 from *Helix pomatia*) (Sigma, St. Louis, MO, USA) solution. Desulphated GSLs were eluted with 2.5 ml (0.50 × 5) Mili-Q (Merck Millipore, Darmstadt, Germany) ultra-pure water and analysed with a Model 600 HPLC instrument (Waters) equipped with a Model 486 UV tunable absorbance detector (Waters) at a wavelength of 229 nm. Separation was by a Lichrospher 100 RP-18 in Lichrocart 125-4 column, with 5 μm particle size (Merck Millipore). HPLC solvents and gradients agreed with the ISO protocol (ISO Norm, 1992). The HPLC chromatogram was compared to the desulpho-GSL profiles of three certified reference materials recommended by the EU (CRMs 366, 190 and 367) (Whatelet et al. 1991). The amount of each GSL present in the sample was calculated by the mean of the internal standard and expressed as μmol/g of dry weight. The total GSL content was computed as the sum of all the individual GSLs present in the sample. Data were corrected for UV response factors for different types of GSLs (ISO Norm, 1992).

Mycelial growth

Agar plugs (6 mm diameter) were cut from the edges of *P. cinnamomi* colonies actively growing in carrot-agar (CA 20%) (25°C, 3 days of incubation in the dark) and transferred to the centre of Petri dishes (90 mm diameter) containing fresh CA. These dishes, without lids, were immediately inverted over plastic beakers (internal upper diameter 90 mm; height 120 mm; 0.4 l volume) containing different amounts of lyophilized plant material. Beakers were hermetically sealed with Parafilm® to avoid the loss of volatiles. Four different amounts were tested: the equivalent of 5, 10 and 20 g of fresh plant material, and a 0 g control.

The minimum dose, 5 g, was fixed according to the average yield of *Brassica* genotypes in a two-years field experiment (2010–2011 and 2011–2012) carried out in a semiarid oak rangeland (7206 kg/ha, Fernández-Rebollo et al. 2015; P. Fernández-Rebollo, unpublished data), and extrapolating this amount to the area of a Petri dish (63.62 cm^2). The required weight of lyophilized plant material corresponding to the doses of fresh material was individually calculated for each *Brassica* genotype and phenological stage, with the amount of water lost during the lyophilization process taken into account. The equivalent of 5, 10 and 20 g of fresh matter were prepared by adding the appropriate volume of deionized water to the dry, ground plant material.

One beaker was prepared for each genotype, phenological stage, and dose tested in a complete randomized experimental design. The experiment was repeated four times. The radial growth of colonies was measured daily until the colonies of the controls had completely covered the surface of the media in the dishes (4 days). Dishes were removed from the beakers, covered with a new sterile Petri dish lid, and incubated for 1 week at 25°C in the dark to evaluate colony growth postexposure to the volatiles.

Sporangial and zoospore production

Isolate PE90 was grown on pea agar (PA, 2%; Trione 1974) for 4 days at 25°C in the dark. Agar plugs (6 mm diameter) taken from the edge of actively growing colonies were placed in the centre of empty Petri dishes (6 cm diameter). Sterilized mineral salt solution (MSS, Chen and Zentmeyer 1970) was poured into each dish until the liquid reached the plug edge without covering it. These dishes were incubated at 25°C in the light for 48 h, at which point there were floating mycelium at the edge of the agar plugs, but no sporangia. Empty Petri dishes (6 cm diameter) were filled with the appropriate volume of deionized water and lyophilized material corresponding to 5 g of fresh matter for each *Brassica* genotype at full flowering or mature growth stages. A lidless 6 cm diameter Petri dish containing the *P. cinnamomi* culture and a 6 cm dish containing each biofumigant were placed together in 15 cm diameter Petri dishes. The 15 cm dishes were immediately covered and sealed with Parafilm® and incubated at 25°C in the light for 9 h, when the control cultures of *P. cinnamomi* achieved maximum sporangial production (Serrano et al. 2012a). Mature sporangia generated from the floating mycelium were counted under an inverted microscope (Eclipse Ti, Nikon Instruments, Tokyo, Japan; ×400). One dish of each biofumigant genotype and phenological stage was prepared, and one control dish was included in a complete randomized experimental design. The experiment was repeated four times.

To test zoospore release, fresh 6 cm Petri dishes were prepared and incubated as described above until maximum sporangial production (57 h), and were placed in 15 cm Petri dishes as described above. Dishes were subject to a 30 min cold-shock (4°C) and returned to 25°C for 2 h in order to stimulate zoospore release. One dish was prepared for each biofumigant genotype and phenological stage tested, and included a control. The experiment was a complete randomized design, and was repeated four times. The average number of zoospores produced per ml was quantified by counting zoospores in three aliquots (1 ml) of homogenized MSS solution from each dish using a Neubauer counting chamber (0.1 μl).

Chlamydospore viability in soil

To test the effect of biofumigants against resting spores of *P. cinnamomi*, 30 kg of natural soil free of the pathogen was taken from an asymptomatic oak rangeland located in the north of Córdoba province (southern Spain). The soil was typical of oak rangeland ecosystems in Córdoba: acidic with low fertility. In the autumn of 2010 absence of *P. cinnamomi* was confirmed in six soil samples (400 g per sample) from the rhizosphere of six holm oak trees. The soil was air dried at room temperature and sieved (2 mm pore diameter). Ten grams of homogenized dry soil was suspended in 100 ml of sterilized water agar (0.2%) and shaken. One-millilitre aliquots were taken from the soil-water-agar mix, and plated on Petri dishes containing 20 ml of NARPH medium, using a sterile glass spreader to distribute the material over the agar surface. This dilution was previously shown to produce a countable number of colonies from soil samples of declining oaks (Romero et al. 2007). For each soil sample, a total of 20 Petri dishes were prepared. Dishes were incubated at 24°C in the dark for 24 h, when the agar surface of each dish was washed with sterile water, removing the soil-water-agar mix. Dishes were re-incubated at 24°C in darkness for another 48 h. No colonies of *P. cinnamomi* were obtained. The same sampling was repeated in the autumn of 2011 and 2012. Colonies of *P. cinnamomi* were not observed to grow from any sample.

The soil (30 kg) for the experiments was oven-dried at 28°C and sieved (2 mm pore size). Inoculum for soil infestation was a water suspension of chlamydospores prepared as follows: isolate PE90 of *P. cinnamomi* was plated in Petri dishes (90 mm diameter) containing 20 ml of carrot broth (20%) and incubated at 25°C in the dark. After 4 weeks of incubation, the liquid medium was filtered aseptically and the mycelium washed with sterile deionized water. Washed mycelium was suspended in sterile water at a rate of three Petri dishes per 100 ml and placed in a blender for 3 min to break up the mycelial aggregates and obtain free chlamydospores. Aliquots were taken from the homogenized suspension, and chlamydospores were counted in a Neubauer counting chamber (0.1 μl) (Romero et al. 2007). Chlamydospore concentration was adjusted to 1.5×10^4/ml; the inoculum was carefully homogenized with the soil to obtain a final concentration of 650 chlamydospores per g of dry soil. Lyophilized material (equivalent to 1, 5 or 10 g of fresh material) of one genotype of each *B. napus, B. carinata* and *B. juncea* at full flowering was placed at the bottom of 250 ml plastic beakers; beakers of biofumigant-free controls were included. Infested soil (225 ml) was poured into the beakers; each beaker was immediately covered, hand shaken, and mixed before adding water. The final soil moisture was 10% (w/w). All beakers were incubated in a growth chamber in the dark, with temperature

adjusted to 25°C (12 h) and 16°C (12 h), during 0 (starting point), 1, 4 and 8 days.

One beaker was prepared for each biofumigant genotype, dose (including one biofumigant-free control) and incubation time tested. The experiment was a complete randomized design, and was repeated four times. In addition, soil pH was determined before and after each biofumigant treatment in a 1:2.5 soil—water suspension using a pH-meter (Crison GLP21, Crison Instruments, Barcelona, Spain).

A second set of experiments was performed with open beakers. As described above, one beaker was prepared for each biofumigant genotype, dose (including one biofumigant-free control) and incubation time tested. The experiment was a complete randomized design, and was repeated four times.

For both experiments (closed or open containers), after each incubation period soils were air dried for 4 days at room temperature. Ten grams of homogenized dry soil was suspended in 100 ml of sterilized water-agar (Roko industries, Asturias, Spain) (0.2%), shaken and analysed following Romero et al. (2007): 1 ml aliquots were taken from each soil-water-agar mix, and plated onto *Phytophthora* selective NARPH medium, using a sterile glass spreader to distribute the material evenly over the agar surface. For each soil sample, a total of 20 Petri dishes were prepared. Dishes were incubated at 24°C for 24 h in the dark, and the agar surface of each dish was washed with sterile water to remove the soil-water-agar mix. Dishes were incubated again at 24°C for a further 48 h in the dark, and the colonies identified as *P. cinnamomi* based on morphology were counted. Inoculum densities were expressed as colony forming units per g of dry soil (cfu/g).

Disease symptoms *in planta*

After sampling, the soil used in the chlamydospore viability experiments was mixed by biofumigant genotype and dose, homogenized and distributed into small plastic pots (75 ml). Seeds of *Lupinus luteus*, a species highly susceptible to *P. cinnamomi* (Serrano et al. 2010), were germinated in a moist container until the radicle was approximately 3 cm long. Seedlings were planted individually in pots. The experimental unit was a pot with one *Lupinus* seedling. A total of 40 pots were prepared for each biofumigant genotype, including the biofumigant-free control soil in a complete randomized experimental design with 40 replicates (pots). All pots were incubated in a growth chamber and received constant irrigation, keeping the soil moisture close to 100%. Day/night growth conditions were 12 h/12 h at 25°C/16°C with a constant humidity of 70%. After 2 months, symptoms of *P. cinnamomi* were evaluated according to the percentage of root necrosis on a 0–4 scale, being 0 = 0% necrotic root; 1 = 1–33%; 2 = 34–66%; 3 = more than 67%; 4 = dead root (Serrano et al. 2010). Ten plants were chosen at random from each biofumigant treatment and the controls, and six root segments per plant were plated on NARPH medium for re-isolation of the pathogen.

Data analysis

Data (number of sporangia, number of zoospores per ml and cfu/g + 0.5) were square-root transformed prior to analysis to ensure a continuous distribution. All the data were tested for homocedasticity using Bartlett's test prior to performing ANOVA. For all ANOVA, the repeated experiments (replicates) were combined in a single analysis.

Different ANOVA models were applied, depending on the experiment. Firstly, a four-way ANOVA was used to analyse the inhibition of mycelial growth, considering the biofumigant genotype, phenological stage, dose and replicate as independent variables. Secondly, a three-way ANOVA was used to analyse the sporangial and zoospore production data, with biofumigant genotype, phenological stage and replicate as independent variables. Chlamydospore viability in the soil was also investigated using a four-way ANOVA as described for mycelial growth, but with main effects of biofumigant genotype, dose, incubation time and replicate. Finally, to assess the effect of biofumigation on root symptom development, a two-way ANOVA was used with biofumigant and replicate as main effects.

F- and P-values were inspected for model suitability and significance of main effects and double interactions between variables. When main effects and interactions were significant at $P < 0.05$, data were grouped by that main effects and the mean values obtained compared by the Tukey's HSD test ($P < 0.05$). Statistix software 8.0 (Analytical Software, Tallahassee, FL, USA) was used for all the statistical analyses.

Results

GSL profiles in seeds

Significant amounts of 10 GSLs were identified and quantified in the seed of selected genotypes of *B. napus*, *B. carinata* and *B. juncea*: seven aliphatic, two indolic and one aromatic (Table 1). The GSL content

Table 1 Trivial name, chemical class, systematic name and abbreviation of the glucosinolates identified in seeds of *Brassica napus*, *Brassica carinata* and *Brassica juncea* genotypes

Chemical class	Trivial name	Systematic name	Abbreviation
Aliphatic	Progoitrin	R-2-Hydroxy-3-butenyl glucosinolate	PRO
	Epiprogoitrin	S-2-Hydroxy-3-butenyl glucosinolate	E-PRO
	Sinigrin	2-Propenyl glucosinolate	SIN
	Gluconapoleiferin	2-Hydroxy-4-pentenyl glucosinolate	GNL
	Glucoalyssin	5-Methylsulphinylpentyl glucosinolate	GAL
	Gluconapin	3-Butenyl glucosinolate	GNA
	Glucobrassicanapin	4-Pentenyl glucosinolate	GBN
Indolic	4-Hydroxyglucobrassicin	4-Hydroxy-3-indolymethyl glucosinolate	4-OHGBS
	Glucobrassicin	3-Indolymethyl glucosinolate	GBS
Aromatic	Gluconasturtin	2-Phenylethyl glucosinolate	GST

Table 2 Glucosinolate profiles and concentrations (μmol/g dry weight) in seeds of the selected *Brassica napus*, *Brassica carinata* and *Brassica juncea* genotypes

Species	Genotype	Total GSLs	PRO[a]	E-PRO[a]	SIN[a]	GNL[a]	GAL[a]	GNA[a]	4-OHGBS[a]	GBN[a]	GBS[a]	GST[a]	Others
B. napus	Bn-Lewis	15.94	7.29	0.10	0.00	0.21	0.00	3.39	3.29	0.93	0.12	0.37	0.25
B. napus	Bn-Salamander	87.84	59.46	1.43	0.00	6.84	0.74	7.23	3.98	5.11	0.07	1.80	1.18
B. carinata	Bc-IASC1	86.01	1.47	0.00	79.36	0.00	0.00	0.66	2.86	0.00	0.45	0.46	0.75
B. carinata	Bc-IAS119	103.55	3.68	0.00	90.70	0.00	0.14	2.67	5.33	0.00	0.29	0.41	0.33
B. juncea	Bj-Tezla	87.33	0.53	0.00	79.16	0.00	0.00	4.16	2.14	0.00	0.05	0.53	0.86
B. juncea	Bj-552	105.10	0.00	0.00	99.88	0.00	0.03	0.65	3.36	0.07	0.01	0.17	0.95

[a]Abbreviation of GSL trivial name. See Table 1.

of Bn-Lewis and Bn-Salamander seeds confirmed them as double low and single low (high GSL) varieties respectively (Table 2). The content of all the aliphatic GSLs (especially progoitrin) was lower in Bn-Lewis compared with Bn-Salamander, with no major differences between the two cultivars in the content of indolic or aromatic GSLs. All genotypes of *B. carinata* and *B. juncea* had high GSL contents in seed, with aliphatic GSLs accounting for >90% of the total; sinigrin was the most abundant GSL.

GSL profiles in green parts at different phenological stages

Aliphatic GSLs were found in the above-ground, vegetative parts of all three *Brassica* species (Table 3). The double low variety Bn-Lewis had a very low GSL content at all three phenological stages analysed, with a small increase as the plant approached maximum maturity. Despite the high GSL content in seeds, vegetative parts of Bn-Salamander had low GSLs, similar to that of Bn-Lewis, with no change in GSL content during its maturation. Progoitrin, gluconapoleiferin and glucobrassicanapin were the predominant aliphatic GSLs in both genotypes of *B. napus* (Table 3).

The total GSL content of Bc-IASC1 at the end of stem elongation was twice the GSL content of Bn-Salamander at the same stage (26.57 and 10.59 μmol/g dry matter respectively, Table 3), despite similar GSL contents in seeds (86.01 and 87.84 μmol/g dry matter respectively, Table 2). There was a decrease in GSL content in the vegetative parts of both genotypes of *B. carinata* (Bc-IASC1 and Bc-IAS119) from the stem elongation stage to full flowering, and continuing into maturity. The GSL content of *B. carinata* was similar to *B. napus* at maturity (Table 3). The progression of GSL content of *B. juncea* genotypes during their growth was similar to *B. carinata* genotypes, with a decrease in GSL content from stem elongation to maturity (Table 3). Sinigrin was the most abundant GSL in the vegetative parts of *B. carinata* and *B. juncea* genotypes, representing more than 90% of the total GSL content, regardless of the phenological stage analysed.

Effect on mycelial growth of *P. cinnamomi*

Cultures of *P. cinnamomi* exposed to *B. carinata* or *B. juncea* genotypes did not show any growth at any dose or phenological stage tested, in contrast with

Table 3 Glucosinolate profiles and concentrations (μmol/g dry weight) in the vegetative above-ground parts of *Brassica napus*, *Brassica carinata* and *Brassica juncea* genotypes at different phenological stages

Species	Genotype	Total GSLs	PRO[a]	SIN[a]	GNL[a]	GNA[a]	GBN[a]	Others
Stem elongation (code 49 of the BBCH scale)[b]								
B. napus	Bn-Lewis	3.00	1.13	0.00	0.01	0.63	0.22	1.01
B. napus	Bn-Salamander	10.59	3.85	0.00	2.12	0.68	3.54	0.40
B. carinata	Bc-IASC1	26.57	0.10	25.29	0.00	0.14	0.03	1.01
B. carinata	Bc-IAS119	40.74	0.32	39.11	0.00	0.47	0.00	0.84
B. juncea	Bj-Tezla	53.99	0.24	52.78	0.00	0.44	0.00	0.53
B. juncea	Bj-552	27.71	0.28	26.53	0.00	0.53	0.02	0.35
Flowering stage (code 65 of the BBCH scale)[b]								
B. napus	Bn-Lewis	5.09	2.15	0.00	0.33	0.65	1.04	0.92
B. napus	Bn-Salamander	8.59	4.10	0.00	1.72	0.55	1.46	0.76
B. carinata	Bc-IASC1	9.31	0.32	8.28	0.00	0.00	0.00	0.71
B. carinata	Bc-IAS119	16.07	0.37	15.33	0.00	0.00	0.00	0.37
B. juncea	Bj-Tezla	25.79	0.32	25.01	0.00	0.00	0.00	0.46
B. juncea	Bj-552	27.00	0.28	26.56	0.00	0.00	0.00	0.16
Maturity stage (code 79 of the BBCH scale)[b]								
B. napus	Bn-Lewis	6.84	2.66	0.00	0.37	0.80	2.18	0.83
B. napus	Bn-Salamander	11.49	7.99	0.00	1.51	0.75	1.02	0.22
B. carinata	Bc-IASC1	8.17	0.18	7.84	0.00	0.00	0.00	0.15
B. carinata	Bc-IAS119	13.61	0.45	12.97	0.00	0.00	0.00	0.19
B. juncea	Bj-Tezla	17.90	0.16	17.47	0.00	0.00	0.00	0.27
B. juncea	Bj-552	13.34	0.16	12.13	0.00	0.80	0.00	0.25

[a]Abbreviation of GSL trivial name. See Table 1.

[b]Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie scale (Lancashire et al. 1991).

colonies exposed to *B. napus* (Table 4). ANOVA showed significant differences in mycelial growth depending on biofumigant species ($F = 2506.6$; $P < 0.0001$), growth stage ($F = 52.2$; $P < 0.0001$), dose ($F = 6.6$; $P = 0.0037$) and the interaction between growth stage and dose ($F = 26.7$; $P < 0.0001$). Significance was not obtained depending on the replicate neither on the rest of double interactions considered.

When cultures of *P. cinnamomi* were re-incubated in the absence of volatiles from the *B. carinata* and *B. juncea* genotypes, no growth was observed regardless of *Brassica* growth stage or dose. In contrast, colonies exposed to volatiles from both genotypes of *B. napus* were able to continue growing after removal from exposure to the volatiles, and fully colonized the plates after 1 week of incubation at 25°C.

Effect on sporangial and zoospore production

Experiments on inhibition of sporangial production and germination were conducted with *Brassica* material from the flowering and maturity stages at the minimum effective dose noted above for mycelial growth: the equivalent of 5 g of fresh material. No stem extension stage material was used due to low crop yield (P. Fernández-Rebollo, unpublished data) and its low dry matter content (average water content at stem extension = 91.9%; flowering = 83.1%; maturity = 68.4%), although the three stages tested proved equally effective inhibiting mycelial growth.

ANOVA showed significant differences in the number of sporangia produced depending on biofumigant genotype ($F = 84.3$; $P < 0.0001$), growth stage ($F = 10.3$; $P = 0.005$) and the interaction between both variables ($F = 9.5$; $P = 0.0001$). Significance was not obtained depending on the replicate neither on the rest of double interactions considered.

Brassica carinata and *B. juncea* genotypes significantly inhibited sporangial production compared to controls at both growth stages tested. *Brassica napus* Bn-Lewis did not significantly decrease sporangial yield. Flowering stage material from Bn-Salamander weakly inhibited sporangial production of *P. cinnamomi* although at maturity even stimulated sporangial production (Table 5).

There were significant differences in the number of zoospores per ml produced depending on the biofumigant species ($F = 18.2$; $P < 0.0001$), growth stage ($F = 14.1$; $P = 0.002$) and the interaction between both variables ($F = 8.6$; $P = 0.0002$), but no significance was neither obtained depending on the replicate or on the rest of double interactions considered.

Table 4 Maximum radial growth (mm) and standard error of *Phytophthora cinnamomi* colonies exposed for 72 h to volatiles released by different *Brassica* biofumigants. For each row, values with different letters differ significantly according to Tukey's HSD test at $P < 0.05$

Genotype	Growth stage	Dose (g)			
		0 (control)	5	10	20
Brassica napus					
Bn-Lewis	Stem elongation stage (code 49 BBCH scale)[a]	26.7 ± 0.8	25.6 ± 1.2	25.1 ± 1.5	22.2 ± 2.6
Bn-Salamander		26.7 ± 0.9	25.0 ± 0.3	25.3 ± 0.4	24.7 ± 0.3
Bn-Lewis	Flowering stage (code 65 BBCH scale)[a]	30.4 ± 2.1	30.5 ± 2.5	30.4 ± 2.7	29.7 ± 2.1
Bn-Salamander		29.7 ± 2.6 a	27.5 ± 2.8 a	25.6 ± 2.1 ab	22.4 ± 1.7 b
Bn-Lewis	Maturity stage (code 79 BBCH scale) [a]	34.7 ± 0.9 a	33.6 ± 0.6 ab	32.4 ± 0.5 ab	31.1 ± 0.4 b
Bn-Salamander		34.6 ± 0.3 a	31.4 ± 0.5 ab	28.4 ± 1.2 b	21.7 ± 2.0 c
Brassica carinata					
Bc-IASC1	Stem elongation stage (code 49 BBCH scale)[a]	26.9 ± 0.5 a	0.0 b	0.0 b	0.0 b
Bc-IAS119		26.1 ± 0.3 a	0.0 b	0.0 b	0.0 b
Bc-IASC1	Flowering stage (code 65 BBCH scale)[a]	29.7 ± 2.6 a	0.0 b	0.0 b	0.0 b
Bc-IAS119		30.1 ± 2.3 a	0.0 b	0.0 b	0.0 b
Bc-IASC1	Maturity stage (code 79 BBCH scale) [a]	32.9 ± 0.6 a	0.0 b	0.0 b	0.0 b
Bc-IAS119		33.8 ± 0.5 a	0.0 b	0.0 b	0.0 b
Brassica juncea					
Bj-Tezla	Stem elongation stage (code 49 BBCH scale)[a]	26.5 ± 1.1 a	0.0 b	0.0 b	0.0 b
Bj-552		25.7 ± 0.3 a	0.0 b	0.0 b	0.0 b
Bj-Tezla	Flowering stage (code 65 BBCH scale)[a]	32.0 ± 2.7 a	0.0 b	0.0 b	0.0 b
Bj-552		32.6 ± 2.4 a	0.0 b	0.0 b	0.0 b
Bj-Tezla	Maturity stage (code 79 BBCH scale) [a]	33.5 ± 1.6 a	0.0 b	0.0 b	0.0 b
Bj-552		34.4 ± 0.4 a	0.0 b	0.0 b	0.0 b

[a]Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie scale (Lancashire et al. 1991).

Table 5 Average number and standard error of sporangia and zoospores per Petri plate produced by *Phytophthora cinnamomi* cultures exposed to volatiles released by the *Brassica* biofumigants tested. Values with different letters in each column differ significantly according to Tukey's HSD test at $P < 0.05$

Species	Genotype	Number of sporangia		Zoospore production (millions/ml)	
		Flowering stage (code 65)[a]	Maturity stage (code 79)[a]	Flowering stage (code 65)[a]	Maturity stage (code 79)[a]
Control	-	53.0 ± 4.9 a	47.5 ± 2.9 b	15.3 ± 1.2 bc	15.3 ± 1.2 bc
B. napus	Bn-Lewis	51.5 ± 5.2 a	30.2 ± 3.2 b	12.6 ± 0.4 c	13.6 ± 0.3 c
	Bn-Salamander	25.7 ± 1.5 b	80.2 ± 6.9 a	18.0 ± 2.4 ab	14.9 ± 0.6 c
B. carinata	Bc-IASC1	3.5 ± 2.2 c	6.2 ± 2.3 c	20.2 ± 0.8 ab	18.9 ± 0.6 a
	Bc-IAS119	4.7 ± 2.7 c	10.5 ± 3.3 c	15.1 ± 0.2 bc	13.3 ± 0.2 c
B. juncea	Bj-Tezla	5.0 ± 2.6 c	4.0 ± 1.3 c	21.6 ± 0.8 a	14.3 ± 0.9 c
	Bj-552	4.0 ± 2.1 c	4.5 ± 1.3 c	16.0 ± 0.4 bc	17.8 ± 0.5 ab

[a]Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie scale (Lancashire et al. 1991).

Any biofumigant species significantly decreased zoospore production at any growth stage in comparison with the controls (Table 5) and even some stimulation was recorded with the Bj-Tezla genotype at the flowering stage.

Effect on chlamydospore viability in soil

Material from the flowering stage of genotypes Bc-IAS119 and Bj-552 were tested for the chlamydospore recovery from soil experiments due to their high GSL content, effectiveness against mycelial growth and sporangial production, and lack of stimulation of zoospore release at any stage. Material from Bn-Salamander at the flowering stage was used as a negative control. Values of soil pH before the biofumigant treatment ranged between 5.90 and 5.94, with no change after the addition of 1 g of biofumigant matter. Soils in which 5 g and 10 g of biofumigant were added had pH values of 5.96–5.99 and 6.30–6.35

respectively, regardless of the plant species and genotype tested.

In closed container experiments, ANOVA showed significant differences in chlamydospore viability depending on biofumigant genotype ($F = 14.3$; $P = 0.0004$), dose ($F = 68.1$; $P < 0.0001$), exposure time ($F = 31.1$; $P < 0.0001$), and the double interactions between genotype and exposure time ($F = 4.9$; $P = 0.0107$) and dose and exposure time ($F = 5.3$; $P = 0.0002$). No significance was obtained depending on replicate neither on the rest of double interactions considered.

Viable chlamydospore numbers decreased significantly after 1 day in soils treated with Bj-552 at all doses tested in comparison with the untreated soils and these decreases persisted at 4 and 8 days of exposure (Table 6). However, Bc-IAS119 did not cause a significant decrease on chlamydospore viability until at least 4 days of incubation, regardless of dose (Table 6). Bn-Salamander-treated soil effects were indistinguishable from the control soil (Table 6).

When soil experiments were performed in open containers, ANOVA showed significant differences in chlamydospore viability depending on biofumigant genotype ($F = 26.36$; $P < 0.0001$), dose ($F = 4.05$; $P = 0.0229$), exposure time ($F = 882.03$; $P < 0.0001$), and the double interaction between biofumigant and exposure time ($F = 19.91$; $P < 0.0001$), but no significance was obtained depending on replicate neither on the rest of double interactions considered.

Significant differences in chlamydospore viability were observed between the control soils and soils treated with Bc-IAS119 and Bj-552 after 8 days of incubation, with no significant differences among doses. Chlamydospore viability from Bn-Salamander-treated soil was always indistinguishable from the control soil (Table 6).

Effect on disease symptoms *in planta*

Two months after seedlings of *L. luteus* were planted in infested and biofumigated soil, all plants showed foliar symptoms (yellowing and wilting) and root symptoms (necrosis). Significant differences in root necrosis were found among biofumigant genotype applied to the soil ($F = 4.3$; $P < 0.0001$) (Table 7), but not among replicates, nor for the double interaction between variables. Only soil treated with 10 g of Bc-IAS119 significantly decreased root necrosis in comparison with control plants. Interestingly, Bn-Salamander-treated soil increased root necrosis at the maximum dose tested.

Phytophthora cinnamomi was always reisolated from necrotic root segments from all treatments.

Discussion

Above-ground, vegetative material of each *Brassica* species had similar GSL profiles to those observed in their respective seed, but the total GSL content was always lower in vegetative material for each genotype tested, as Halkier and Gershenzon (2006) have previously reported. Important differences in GSL patterns between *B. napus* and *B. carinata* or *B. juncea* were detected, independently of the genotype tested. All *Brassica* species tested maintained similar GSL profiles in vegetative material during their development and maturation, with progoitrin being dominant in *B. napus* and sinigrin in *B. carinata* and *B. juncea*. The lack of sinigrin in the two *B. napus* (genome AACC)

Table 6 Average number and standard error of viable *Phytophthora cinnamomi* chlamydospores (cfu/g of dry soil) after soil exposure to volatiles released by different *Brassica* spp. biofumigants at the flowering stage. Values with different letters in each column differ significantly according to Tukey's HSD test at $P < 0.05$

			Exposure time (days) in closed containers			Exposure time (days) in open containers		
Brassica species	Genotype	Dose (g)	1	4	8	1	4	8
-	Control	-	108.7 ± 10.1 a	177.0 ± 11.7 a	88.2 ± 9.6 a	27.7 ± 1.5	37.2 ± 3.2 bc	103.2 ± 7.7 a
Brassica napus	Bn-Salamander	1	107.8 ± 11.3 a	192.9 ± 2.5 a	81.9 ± 3.4 a	29.3 ± 2.8	36.0 ± 4.7 bc	89.7 ± 6.3 a
B. carinata	Bc-IAS119	1	105.2 ± 7.6 a	52.7 ± 8.8 b	41.2 ± 3.4 b	26.7 ± 2.7	31.5 ± 4.1 c	73.5 ± 2.2 b
Brassica juncea	Bj-552	1	46.5 ± 9.2 b	75.2 ± 11.2 b	34.2 ± 5.2 b	25.0 ± 1.1	30.0 ± 2.5 c	65.7 ± 4.8 b
B. napus	Bn-Salamander	5	92.8 ± 9.5 a	167.7 ± 6.7 a	83.7 ± 4.4 a	31.2 ± 3.0	35.4 ± 1.8 bc	95.3 ± 4.5 a
B. carinata	Bc-IAS119	5	83.5 ± 4.3 ab	65.2 ± 14.1 b	42.7 ± 7.5 b	23.7 ± 1.5	43.7 ± 2.8 ab	62.2 ± 7.2 b
B. juncea	Bj-552	5	49.0 ± 7.9 b	46.7 ± 5.3 b	40.0 ± 6.4 b	28.7 ± 2.1	37.5 ± 4.1 bc	62.5 ± 4.3 b
B. napus	Bn-Salamander	10	110.0 ± 2.1 a	198.0 ± 12.4 a	89.1 ± 5.1 a	34.2 ± 2.4	42.5 ± 4.5 ab	91.8 ± 3.8 a
B. carinata	Bc-IAS119	10	72.5 ± 3.1 ab	65.5 ± 9.9 b	49.0 ± 6.4 b	29.9 ± 2.9	47.0 ± 3.1 a	64.5 ± 2.1 b
B. juncea	Bj-552	10	49.0 ± 6.6 b	54.2 ± 13.4 b	28.7 ± 3.4 b	28.2 ± 1.8	37.5 ± 5.5 bc	69.0 ± 4.0 b

Table 7 Average values and standard error of root necrosis recorded on *L. luteus* growing in soil infested with *Phytophthora cinnamomi* chlamydospores (650 cfu/g dry soil) and biofumigated. Values with different letters differ significantly according to Tukey's HSD test at $P < 0.05$

Brassica species	Genotype	Dose (g)	Root symptoms
-	Control	-	3.07 ± 0.08 b
Brassica napus	Bn-Salamander	1	3.08 ± 0.08 b
Brassica carinata	Bc-IAS119	1	3.05 ± 0.08 b
Brassica juncea	Bj-552	1	3.07 ± 0.09 b
B. napus	Bn-Salamander	5	3.14 ± 0.08 b
B. carinata	Bc-IAS119	5	2.92 ± 0.09 ab
B. juncea	Bj-552	5	3.11 ± 0.09 b
B. napus	Bn-Salamander	10	3.57 ± 0.11 c
B. carinata	Bc-IAS119	10	2.80 ± 0.08 a
B. juncea	Bj-552	10	3.15 ± 0.08 b

genotypes and the high content of sinigrin in the two genotypes of *B. carinata* and *B. juncea* support the general idea that genes for the synthesis of sinigrin are located in genome B, present in both the allotetraploids *B. carinata* and *B. juncea* (Rosa 1999). Although there were no changes in the GSL pattern of the vegetative material of the *B. napus*, *B. carinata* and *B. juncea* genotypes throughout their growth, GSLs quantities varied. While *B. juncea* and *B. carinata* had the highest GSL content during stem extension and the lowest at maturity, *B. napus* did not differ markedly during development. This behaviour has been reported by some other authors, (Bellostas et al. 2007) and is also confirmed for *Brassica* spp. cultivated in southern Spain.

The biocidal action of biofumigant *Brassica* spp. against pests, pathogens and weeds seems to be related to their specific GSL profile, or even related to only one specific type of GSL. The relationship between inhibition of soilborne pathogens and sinigrin content in biofumigant plants has been reported before (Olivier et al. 1999), but little information about the mechanisms of activity is available. Our work suggests that 2-propenyl glucosinolate (sinigrin) is involved in the biofumigant action of *B. juncea* and *B. carinata* against *P. cinnamomi*. However, further research with other plants rich in sinigrin is needed to confirm this hypothesis.

The mycelial growth of *P. cinnamomi* was inhibited even at the lowest dose tested for both genotypes of *B. juncea* and *B. carinata* at every phenological stage screened, but there was no effect of *B. napus*. Dunne et al. (2003a), reported a 100% of inhibition of radial colony growth for one isolate of *P. cinnamomi* from Australia exposed to *B. juncea* at flowering (reporting <10 μmol/g dry matter of sinigrin), resulting in the death of the pathogen. *Brassica carinata* and *B. juncea* genotypes tested in this study also exhibited similar biocidal action, with a sinigrin concentration similar to or even lower (7.84 μmol/g dry matter in Bc-IASC1) compared to that tested by Dunne et al. (2003a). However, the ability of *P. cinnamomi* to infect oak roots does not depend on saprophytic growth, but the ability of chlamydospores to germinate and produce infective zoospores through sporangial production. Although in general, the biofumigants tested did not inhibit zoospore release once the sporangia were formed and even some stimulant effect was occasionally obtained, we demonstrated that volatiles released by the hydrolysis of *B. juncea* and *B. carinata* GSLs from vegetative material at flowering or maturity caused significant inhibition of sporangial production, which could potentially prevent secondary infections. Dunne et al. (2003b) also reported a decrease in *P. cinnamomi* sporangial production using *B. juncea* but showed no effect on zoospore release. There are some references regarding the inhibition of *B. napus* against several soilborne oomycete and fungal pathogens, such as *Aphanomyces euteiches* f. sp. *pisi* or *Sclerotium cepivorum* (Smolinska et al. 1996; Smolinska 2000) but this does not appear to be the case for the oomycete *P. cinnamomi*. In general, *B. napus* genotypes tested had no effect on the different stages of the *P. cinnamomi* life cycle that were tested, but it is remarkable that there was a significant increase in sporangial production in response to volatiles from mature Bn-Salamander plants (approx. 2×). There are reports of the ability of *B. napus* to increase the infectious potential of some soil pathogens belonging to the genus *Pythium* (Mazzola et al. 2001), which is closely related to *Phytophthora*. Based on these observations, it seems reasonable to discourage the use of *B. napus* as a biofumigant against *P. cinnamomi*, as some genotypes might increase the infectivity of the pathogen in the soil.

Several researchers have evaluated biofumigation of other *Phytophthora* species. For example, Gigot et al. (2013) tested different Brassica seed meals as biofumigants in raspberry orchards and found that *B. juncea* reduced populations of *P. rubi* in the soil. In contrast, Mazzola and Brown (2010) reported that seed meal of *B. juncea* stimulated the infection rate of *P. cambivora* under field conditions. Our results demonstrate the effectiveness of *B. juncea* Bj-552 in reducing *P. cinnamomi* infectivity in soil experiments, as previously observed by Dunne et al. (2003b), and occurred from the first day of soil incubation in closed containers. After a longer exposure time to volatiles, *B. carinata*

Bc-IAS119 was an equally efficacious biofumigant in soil. In addition, there was apparently no relationship between the effectiveness of the biofumigants and the amount of plant tissue applied to the soil, as similar results were obtained at every dose tested, suggesting that the maximum effect on reduction of chlamydospore viability was already obtained at the minimum dose tested. Moreover, both biofumigants were effective in open containers after an extended exposure time, suggesting the potential usefulness of biofumigation in the field with or without covering the soil. It has been demonstrated that the effectiveness exhibited by both biofumigants was not based on inhibition of zoospore release (no effect was observed on the zoospore stage of *P. cinnamomi*), but mainly on inhibition of chlamydospore viability and sporangial production. Flowering stage Bn-Salamander had no effect on *P. cinnamomi* in soil, despite the ability of this genotype to stimulate zoospore release at maturity.

Differences between results obtained with closed or open containers can be related to several factors. Variations in moisture content has been suggested by Omirou et al. (2013) as a factor affecting dissipation of pure broccoli-released GSLs in soils under different moisture contents, with faster hydrolysis under high moisture levels. In closed containers, soil moisture is maintained throughout the whole experiment; likely giving rise to a faster hydrolysis of GSLs. Moisture content could also influence the soil population of specialized microorganisms able to degrade GSLs (Gimsing et al. 2006). Despite the faster and greater decrease in chlamydospore viability in covered soil, the direct application of biofumigant matter rich in sinigrin without covering the soil appears to be a suitable and practical choice for field application in rangelands ecosystems.

Results obtained in plant experiments were not as definitive as those in the soil experiments; only *B. carinata* Bc-IAS119 at a dose 10 g was able to significantly reduce root symptoms in comparison with seedings in control soil. This was possibly due to the high quantity of spores used to infest the soil (650 chlamydospores/g of soil) and the extreme susceptibility of the host plant used (Serrano et al. 2010). Although high sinigrin-content biofumigants are able to drastically decrease the number of viable chlamydospores in soil, the reduced number of resting spores after soil biofumigation could still remain above the minimum threshold for *L. luteus* to become severely infected. It is unknown how many *P. cinnamomi* chlamydospores are needed to produce root disease on *L. luteus*. Bowers and Mitchell (1991) reported that 41 oospores/g of *Phytophthora capsici* are needed to produce a 50% mortality in pepper plants, and similar quantities were found to cause necrosis on rootlets of *Q. suber* (Martin et al. 2014). These quantities are similar to those present in our soils after successful biofumigation.

Soils inhabited by *P. cinnamomi* that infects oak roots in the rangelands of southern Spain are characterized by low pH (5.4–6.1), low fertility; low organic matter and mineral nutrient content (Parras-Alcántara et al. 2014). The application of green biofumigants rich in sinigrin, in addition to a direct control of the pathogen, could potentially improve the soil by increasing its pH, enhancing its organic matter content and increasing its microbiota populations, including potential pathogen antagonists (Mazzola and Zhao 2010). Several reports describe alternative modes of action of biofumigants based on induced changes in soil microbial populations (Mazzola et al. 2007; Hu et al. 2015). The selectivity of different GSLs to different pathogens is likely dependent on soil microbial populations; Mazzola and Zhao (2010) reported that after biofumigation with *B. juncea* seed meal in an apple orchard, the amended soil had significantly higher densities of *Streptomyces* spp. and decreased density of *Rhizoctonia solani* compared to untreated controls. Hu et al. (2015) described a significant increase of Firmicutes bacterial populations and a decrease in soil fungal populations when pure allyl-ITCs were added to the soil, in comparison with pure ITCs from other chemical families.

Phytophthora cinnamomi is a polycyclic pathogen (Hardham 2005). A reduction of primary inoculum in soil (chlamydospores), together with a significant reduction of secondary inoculum (zoospores) is needed to reduce epidemics. This work highlights the potential of selected genotypes of *Brassica* biofumigants to significantly decrease chlamydospore viability in soil and indirectly inhibit zoospore release through inhibition of sporangial production, even when no direct effect on zoospore production from already formed sporangia was recorded. This work represents the first step in understanding how biofumigation might be used in oak rangelands affected by root disease. Short term research goals are to select more appropriated biofumigant species and genotypes on the basis of their high sinigrin content and test their performance in seminatural oak rangelands ecosystems.

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4.2

Biofumigant action of *Brassica* seedmeals against *Phytophthora cinnamomi* in dehesa ecosystems

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Biofumigant action of *Brassica* seedmeals against *Phytophthora cinnamomi* in *dehesa* ecosystems

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Summary. The oomycete plant pathogen *Phytophthora cinnamomi* causes a highly destructive root rot that seriously affects oaks in semi-natural woodlands (*dehesa*). Biofumigation with *Brassica* spp. is a promising tool for disease management. The effectiveness of ground seeds from *B. carinata* and *B. juncea* to inhibit the mycelial growth and significantly decrease chlamydospore viability of *P. cinnamomi* in soil was established, in contrast with the inability shown by *B. napus* seedmeals. A significant

reduction of root necrosis in *Lupinus* plants was also achieved when soils were treated with *B. carinata* or *B. juncea* seedmeals. Glucosinolate content analyses indicate that seedmeal effectiveness was related with a high content in sinigrin (2-propenyl glucosinolate). Biofumigation with seedmeals rich in sinigrin should be considered as an effective measure to be incorporated in the integrated control of the oak disease caused by *P. cinnamomi* in *dehesa* ecosystems, but as seed production levels in *dehesa* appear so low, it should be best practiced on agricultural lands.

Key words: *Brassica carinata*, *Brassica juncea*, *Brassica napus*, sinigrin.

Introduction

There is a social refusal to the use of chemical fumigants to control soil-borne plant pathogens, mainly due to environmental and health constraints (Alabouvette, 2006). Looking for environmentally friendly fumigation methods capable of controlling or at least limiting the spread of such pathogens is a continuous search. The root disease caused by *Phytophthora cinnamomi* Rands is killing thousands of oak trees and other woody species around the world (Brasier, 1996; Hardham, 2005), being especially virulent on holm and cork oaks in the south of the Iberian Peninsula (Sánchez *et al*., 2006). In this geographical area these evergreen oaks grow in agroforestry systems called *dehesa*. As semi-natural production systems, chemical fumigation to control *P. cinnamomi* is not recommended, and biofumigation appears to be a promising alternative. Biofumigation is based on the release of isothiocyanates (ITCs), toxic products resulting from the hydrolysis of glucosinolates (GSLs) (Kirkegaard *et al*., 1993), secondary metabolites produced in plants belonging to the order Capparales (Halkier and Gershenzon, 2006), after their incorporation to the soil as fresh material or seedmeals (Brown and Morra, 1997). There are more than 130 different GSLs

commonly found in *Brassica* spp. (Agerbirk and Olsen, 2012) with different noxious activity on a given target pathogen. GSL-containing plants have been demonstrated to reduce weeds (Rice *et al*., 2007; Fourie *et al*., 2015), pests (Elberson *et al*., 1997) and soil-borne pathogens (Bomford *et al*., 2009; Mazzola *et al*., 2015). The biocide action against *P. cinnamomi* of green tissues of some *Brassica* biofumigants was recently reported (Morales-Rodríguez *et al*., 2016; Ríos *et al*., 2016b), but did not explore the potential of their seeds as biofumigant material.

The mechanical incorporation of cover crops into the soil is the most common way of carrying out biofumigation. Soil moisture (rainfall or irrigation) is needed at incorporation time to ensure the hydrolysis of GSLs (Morra and Kirkegaard, 2002), as well as efficient plant tissue breakdown. However, the cold crushing of seeds for oil removal in the oil and biodiesel industry results in a seedmeal product which contains high concentrations of GSLs and preserves myrosinase activity (Morra, 2004). As with cover crops, the use of seedmeal as biofumigant requires wet soil, but the disruption of plant tissue has been maximized, and thereby the GSL hydrolysis occur faster. Another advantage of using seedmeals is that their application can be done at any time and simultaneously with other products, allowing a better adjustment to soil moisture content or pathogen life cycle, thus increasing the efficiency of disease control (Mazzola *et al*., 2015).

The chance of biofumigant crops in *dehesa* ecosystems and their compatibility with the seminatural character of this agroforestry system makes biofumigation a promising tool for control of *P. cinnamomi* root rot affecting oak trees. However, there are factors which may constrain biofumigation in *dehesa*: low soil fertility may limit biomass and seed production; the stoniness of some areas may hamper an effective incorporation of fresh biomass into the soil; and, because irrigation is not feasible, the high

unpredictability of rainfall within a year may slow down the hydrolysis of GSLs once the green matter is incorporated. Collected seeds, once ground, may be used as seedmeal amendment when the soil is naturally wet, overlapping with the more active period of *Phytophthora* pathogens, or prior to aforestation actions. The adoption of biofumigation by farmers will depend on the ability of seedmeals to control *P. cinnamomi*, the dose needed and the potential seed yield in *dehesa* systems.

The aim of this work was to test the effectiveness of ground seeds from three *Brassica* species, *B. napus, B. carinata* and *B. juncea* to be used against *P. cinnamomi* oak disease and explore the possibility producing such seeds in Spanish *dehesa* systems.

Materials and methods

Seedmeal material

Two genotypes of three *Brassica* species were tested: *B. napus* L., genotypes Bn-Lewis and Bn-Salamander, provided by Dr. Delourme (INRA, Rennes, France); *B. carinata* A.Braun genotypes Bc-IASC1 and Bc-IAS119, and *B. juncea* (L.)Czern. genotypes Bj-552 and Bj-Tezla, obtained by the Plant Breeding Group at the Institute of Sustainable Agriculture (IAS) in Córdoba (Spain) after several cycles of genetic selection for agronomic performance and seed GSL composition (Font *et al*., 2006). All plants were cultivated under Mediterranean climate conditions in an experimental field located at the Institute of Sustainable Agriculture in Córdoba, Spain (37.8º N, 4.8º W). At the end of their life cycle, seeds were collected, washed, lyophilized and ground to a fine powder in a blender Janke and Kunkel, Model A10 mill (IKA Labortechnik Staufen, Germany). GSL composition for each species and genotype was determined by High Performance Liquid Chromatography (HPLC) according with Font *et al*. (2005), as fully described in Ríos *et al*. (2016a). The GSL content of Bn-Lewis and Bn-

Salamander seeds conformed with double low and single low (high GSL) varieties respectively (Ríos *et al*., 2016a). The total GSL content (especially progoitrin) was 15.94 μmol g^{-1} dry weight in Bn-Lewis and 87.84 μmol g^{-1} in Bn-Salamander seeds. All genotypes of *B. carinata* and *B. juncea* had high GSL contents in seeds (86.01 to 105.10 μmol g^{-1}), with sinigrin (2-propenyl glucosinolate) accounting for > 90% of the total (Ríos *et al*., 2016a).

Oomycete material and *in vitro* experiments

Biological tests were carried out with *P. cinnamomi* strain PE90, isolated from *Quercus ilex* spp. *ballota* and previously characterized as belonging to the most aggressive population in the south of the Iberian Peninsula (Caetano *et al*., 2009). Agar plugs (6 mm diameter) taken from the edge of *P. cinnamomi* colonies actively growing in carrot-agar medium (CA) (25ºC, 4 days of incubation in the dark) were cut and transferred to the centre of Petri dishes (9 cm diameter) containing fresh CA medium and immediately turned over and placed as lid of plastic beakers (9 cm internal upper diameter; 120 mm height; 0.4 l in volume) containing each lyophilized seed material plus 10 ml of deionized water. Four doses were tested: 0 (control), 0.2, 0.5 and 1.0 g of seed powder. Beakers were hermetically sealed with Parafilm® to avoid loss of the volatiles released. Four beakers (replicates) were prepared for each biofumigant and dose tested in a completely randomized experimental design; subsequently the experiment was repeated four times. The radial growth of colonies was measured daily until controls had completely covered the surface of the substrate in the dishes. Data of maximum radial growth were recorded after 3 days of incubation.

Dishes were removed from the beakers, covered with a fresh sterile Petri dish lid, and incubated for 1 week at 25º C in the dark to evaluate colony growth post-exposure to the volatiles

Data of maximum radial growth were checked for normality and homoscedasticity using the Shapiro-Wilk and Levene's tests respectively, and a two-way ANOVA was performed with biofumigant genotype and doses as independent variables. When significance was obtained for $p < 0.05$, mean values were compared by Tukey´s HSD test (Statistix software 9.0).

Soil experiments

One genotype of each *B. napus*, *B. carinata* or *B. juncea* was selected to be tested against the viability of resting spores (chlamydospores) of *P. cinnamomi* in the soil. Natural soil was taken from an asymptomatic *dehesa* located in the north of Córdoba province (southern Spain). The soil was typical of *dehesa* ecosystems in Córdoba: acidic with low fertility (Parras-Alcántara *et al*., 2014). The absence of the pathogen in 10 soil samples was assessed following the method reported by Romero *et al*. (2007) and no pathogen colonies were recorded in any sample. The soil was air dried, sieved (2 mm pore size), and artificially infested with a water suspension of *P. cinnamomi* chlamydospores prepared as described in Romero *et al*. (2007): isolate PE90 of *P. cinnamomi* was plated in Petri dishes (9 cm diameter) containing 20 ml of carrot broth (20%) and incubated at 25ºC in the dark. After 4 weeks of incubation, the liquid medium was filtered aseptically and the mycelium washed with sterile deionized water. Washed mycelium was suspended in sterile water at a rate of three Petri dishes per 100 ml and placed in a blender (Oster^{TM} Pulse-matic 16, London) for 3 min at maximum speed (liquefy) to break up the mycelial aggregates and obtain free chlamydospores.

149 Aliquots were taken from the homogenized suspension, and chlamydospores counted in
150 a Neubauer counting chamber (0.1 µl). Concentration was adjusted to 1.5×10^4
151 chlamydospores ml^{-1}. The inoculum was carefully mixed with the soil to obtain a final
152 concentration of 650 chlamydospores per g of dry soil. Lyophilized seed meal material
153 (0.1, 0.5, or 1.0 g) was then placed at the bottom of 250 ml plastic beakers; beakers of
154 biofumigant-free controls were included. Infested soil (225 ml; 292.5 g) was poured
155 into the beakers; each beaker was immediately covered, hand shaken, and mixed before
156 adding water to a final moisture content of 10% (w/w). All beakers were incubated in a
157 growth chamber in the dark, with the temperature adjusted to 25ºC (12h) and 16ºC (12h).
158 Incubation times were 0 (starting point), 1, 4 and 8 days. Four replicate beakers were
159 prepared for each biofumigant species, dose and incubation time tested in a complete
160 randomized design. After each incubation period, soils were air dried for 4 days at room
161 temperature. Ten grams of homogenized dry soil per beaker was suspended in 100 ml
162 0.2% sterilized water-agar (Roko Industries, Asturias, Spain), shaken and analysed
163 following Romero *et al*. (2007): 1 ml aliquots were taken from each soil-water-agar mix,
164 and plated onto *Phytophthora* selective NARPH medium (Hüberli *et al*., 2000), using a
165 sterile glass spreader to distribute the material over the agar surface. For each soil
166 sample, a total of 20 Petri dishes were prepared. Dishes were incubated at 24ºC for 24 h
167 in the dark before washing the agar surface of each dish with sterile water to remove the
168 soil-water-agar mix. Dishes were incubated again at 24ºC for an additional 48 h period
169 in the dark. Colonies identified as *P. cinnamomi* based on hyphal morphology (presence
170 of rounded hyphal swellings in clusters together with chlamydospores) observed under
171 the inverted microscope were counted (Romero *et al.*, 2007). Inoculum densities were
172 expressed as colony forming units per g of dry soil (CFU g^{-1}). To fit continuity, data
173 were transformed to $[(\text{CFU } g^{-1}) + 0.5]^{1/2}$, checked for normality and homoscedasticity

by using the Shapiro-Wilk and Levene's tests respectively, and a three-way ANOVA was performed considering biofumigant, dose and incubation period as independent variables. When significance was obtained at $p < 0.05$, mean values were compared by the Tukey´s HSD test (Statistix software 9.0).

Plant experiments

After sampling for chlamydospore viability, soils biofumigated at different doses of the same seedmeal were mixed, as well as the unfumigated control soils. Homogenised soils (three soils treated with the three biofumigant species plus the untreated control soil) were distributed in 40 plastic pots per biofumigant (replicates, 75 ml of soil per pot).

Seeds of *Lupinus luteus* L., a species highly susceptible to *P. cinnamomi* (Serrano *et al*., 2010), were germinated in damp chambers and when the radicles were approximately 3 cm long, seedlings were planted individually in the pots, making a total of 40 seedlings (replicates) per biofumigated or control soil. All pots were incubated at 25ºC (day) /16ºC (night) with constant irrigation, keeping the soil moisture close to 100%. After 1 month, symptoms of *P. cinnamomi* root disease were evaluated based on the percentage of root necrosis on a 0-4 scale, being 0 = 0% necrotic root; 1 = 1-33%; 2 = 34-66%; 3 = more than 67%; 4 = dead root (Serrano *et al*., 2010). Root symptom data were tested for normality and homoscedasticity using the Shapiro-Wilk and Levene's tests respectively, and a one-way ANOVA performed with biofumigant as independent variable for $p < 0.05$, and mean values compared using the Tukey´s HSD test. Ten plants were chosen at random from each biofumigant treatment and control, and six root segments per plant were plated on NARPH medium for re-isolation of the pathogen.

199 **Seed production**

200 To evaluate seed yield of the effective *Brassica* species as well as to test the best rate of
201 sowing for high seed production, an experiment was conducted during 2012-2013 and
202 2013-2014 in a *dehesa* system located at Hinojosa del Duque, Spain (38º 29'50''N; 5º
203 6'56''O; 543 m asl). The region has a Mediterranean-type climate, characterized by a
204 moderate rainfall with hot summers and cool winters. Weather data during the two
205 growing seasons are in Figure 1. The soil at the experimental plots was: sandy, pH 5.8,
206 organic matter content 2.24 %, cation exchange capacity (CEC) 84.7 meq kg^{-1},
207 exchangeable base cations K^+ 3.2 meq kg^{-1}, Ca^{2+} 29.7 meq kg^{-1}, Mg^{2+} 30.1 meq kg^{-1} and
208 Na^+ 2.3 meq kg^{-1} and available P (Olsen) 7.5 mg kg^{-1}. The experiment was a randomized
209 factorial design, with the two genotypes of both *B. carinata* and *B. juncea* species,
210 previously characterized as noxious to *P. cinnamomi*, as main factors subjected to two
211 types of sowing procedure and replicated three times. Types of sowing tested were (i)
212 line sowing with a dose of 150 seeds m^{-2}, 33 cm line apart and 50 seeds m^{-1} (around 6
213 kg ha^{-1}); (ii) broadcast sowing with a dose of 300 seeds m^{-2} (around 12 kg ha^{-1}). The
214 elemental plots had a dimension of 3×2 m with 2 m between adjacent plots.
215 In both years, the seeds were sown in mid-November. Seedbeds were prepared with a
216 chisel plow to a depth of approximately 150 mm (two passes in cross directions) and
217 before sowing, organic fertilizer (9N:18P:16K:17.5S) was applied as a basal application
218 at a rate of 570 kg ha^{-1}. The seeds were distributed by hand and covered by means of a
219 shallow cultivation action to ensure a good contact between seed and soil. Once the
220 crops reached the complete maturity, all the plants of each plot were harvested by
221 cutting the above-ground material. For determination of seed yielding, siliquas were
222 unshelled and seeds collected, cleaned and weighed using a digital scale. A two-way
223 ANOVA was performed to compare, in each growing season, seed yield (kg per ha)

among species/genotype and type of sowing. Data were tested for normality and homoscedasticity using the Shapiro-Wilk and Levene's tests, respectively. Tukey´s HSD test was applied for post-hoc comparisons ($\alpha = 0.05$).

Results

Inhibition of mycelial growth

Average values ± SE of maximum radial growth reached by *P. cinnamomi* colonies exposed to volatiles from biofumigants tested are in Table 1. ANOVA showed significant differences among doses (DF = 3, F = 1169.23, $p < 0.0001$), genotype (DF = 5, F = 898.66, $p < 0.0001$) and the interaction dose×genotype (DF = 15, F = 106.70, $p < 0.0001$). At every dose tested, both genotypes of *B. carinata* and *B. juncea* significantly reduced the mycelial growth of *P. cinnamomi* when compared with dose 0. Bc-IAS-C1, Bc-IAS-119 and Bj-552 lead to a total inhibition of *P. cinnamomi* mycelial growth even at the lowest dose tested. In contrast, colonies exposed to both genotypes of *B. napus* did not differ from control colony growth even at the highest dose tested.

When Petri dishes were re-incubated after exposure to the volatiles, those previously exposed to *B. carinata* or *B. juncea* genotypes remain unable to grow. In contrast, all cultures exposed to volatiles released by *B. napus* genotypes resumed growth and filled the plates after incubation at 25ºC.

Inhibition of chlamydospore viability

Bn-Salamander was the genotype of *B. napus* selected to test ability to inhibit chlamydospore viability in the soil, as it was the genotype with the highest value of GSL content. Genotypes Bc-IAS-119 and Bj-552 were selected since they reached a

100% of mycelial growth inhibition at the lowest dose tested and they had the highest GSL content of *B. carinata* and *B. juncea* respectively (Ríos *et al*., 2016b).

Results obtained are in Table 2. ANOVA showed significant differences in chlamydospore viability in soil depending on biofumigant seedmeal ($DF = 2$, $F = 289.55$, $p < 0.0001$), but no differences were detected depending on dose ($DF = 2$, $F = 0.06$, $p = 0.9386$) nor time of exposure to volatiles ($DF = 2$, $F = 0.71$, $p = 0.4958$).

All soils treated with Bn-Salamander did not differ in *P. cinnamomi* chlamydospore viability from infested and unfumigated control soils, in contrast with Bc-IAS119 and Bj-552 genotypes, which significantly decreased chlamydospore viability from the first day of exposure. No significant differences were observed between Bc-IAS119 and Bj-552 genotypes.

Effect on disease symptoms development

After 1 month of incubation, *Lupinus* seedlings growing in control soils (infested and no biofumigated) developed foliar and root symptoms typical of the root rot caused by *P. cinnamomi*. Foliar symptoms consisted of yellowing, foliar wilting and defoliation, and root symptoms consisted of root necrosis. Table 3 shows mean values recorded for root rot symptoms. ANOVA revealed significant differences in root symptoms depending on the biofumigant applied to the soil ($DF = 3$, $F = 43.87$, $p < 0.0001$). As expected, root symptoms did not differ for *Lupinus* plants growing in soils treated with Bn-Salamander or growing in untreated control soil. However, roots symptoms were significantly lower when plants grew in soil biofumigated with Bc-IAS119 or Bj-552 in comparison with plants growing in control soils, being Bj-552 the more effective seedmeal decreasing root necrosis induced by *P. cinnamomi* infections.

Phytophthora cinnamomi was always re-isolated from necrotic root segments from plants growing in every infested soil.

Seed production

Temperatures and rainfall recorded during the field experiments are in Figure 1. In the first growing season, annual rainfall recorded was 610 mm, while it only reached 401 mm in the second season. November 2012 was rainy, especially after sowing (129 mm), and followed by a cold winter (-4ºC absolute minimum temperature in January and February). In contrast, November 2013 was dry after an October with 107 mm of precipitation and followed by a warm winter (absolute minimum temperatures never fell below -2°C). Seed yields of *Brassica* spp. are in Table 4. ANOVA showed that seed yields were affected by the interaction of species/genotype × type of sowing in both years ($F = 4.19$, $p = 0.02$ in 2012-2013; and $F = 8.32$, $p = 0.001$ in 2013-2014).

In the first growing season, seeds were harvested on June 17th. Seed production was very low for *B. juncea* genotypes and null for *B. carinata* genotypes. Seed production of Bj-Tezla was significantly higher than Bj-552, although it was below the seed dose sown in most of the plots. Broadcast sowing significantly increased seed yield of Bj-Tezla.

In the second growing season, the harvest date was June 6th for both genotypes of *B. juncea*. Seeds of *B. carinata* genotypes were collected on June 9th for line sowing and June 30th for broadcast sowing respectively. Global seed production increased in this second growing season, with significant differences among genotypes. Bc-IAS-119 achieved the lowest seed production level in both types of sowing. The level of seed production of *B. juncea* genotypes was higher than *B. carinata* ones, although no significant differences were found between them in line sowing or between Bj-552 and

298 Bc-IAS-C1 in broadcast sowing. In contrast with 2012-2013 results, seed yield was
299 significantly higher with line sowing in comparison with broadcast sowing (Table 3).
300

301 ## Discussion

302 *Brassica* species contain GSLs in all their organs, although their concentration is
303 especially high in seeds (Halkier and Gershenzon, 2006). However, plant breeding
304 programs have tended to drastically reduce GSL levels to make them edible to livestock
305 (Tripathi and Mishra, 2007). Ríos *et al*. (2016a; 2016b) showed that sinigrin (2-
306 propenyl glucosinolate) present in green parts of some *Brassicaceae* species had a high
307 biofumigant potential against the oomycete *P. cinnamomi*. Green matter from plants
308 rich in sinigrin significantly reduced the ability of *P. cinnamomi* resting spores to
309 germinate in the soil (Ríos *et al*. 2016a; 2016b), although their potential to decrease
310 disease symptoms in highly susceptible hosts (*L. luteus*) growing in biofumigated soils
311 appeared quite low. Additionally, the effectiveness of commercial pellets based on *B.*
312 *carinata* dry green matter has been recently reported to reduce *P. cinnamomi* inoculum
313 density in experimental conditions, and thus to protect seedlings of *Quercus cerris* from
314 root infections (Morales-Rodríguez *et al*., 2016), but this is a host species with a low
315 susceptibility to the pathogen. In this work, we demonstrated the ability of *Brassica*
316 seedmeals rich in sinigrin to effectively reduce *P. cinnamomi* chlamydospore viability
317 in the soil and to protect *L. luteus* against the root necrosis caused by the pathogen. As
318 Ríos *et al*. (2016a) reported, inoculum load remaining in the soil after biofumigation
319 with green manure of *B. juncea* Bj-552 (28-69 CFU g^{-1}) or *B. carinata* Bc-IAS-119 (23-
320 83 CFU g^{-1}) probably exceed the minimum threshold for *L. luteus* root rot. This
321 infective inoculum load remains unknown for *L. luteus*, but information exists for the
322 pathosystem *Quercus-P. cinnamomi*. Serrano *et al*. (2015) reported that a minimum of

61 viable chlamydospores g^{-1} soil was needed to cause root disease on *Q. suber*, a highly susceptible host (Robin *et al*., 2001) under experimental conditions. Inoculum loads in the soil after biofumigation with *Brassica* seedmeals were much lower than those reached with green matter: 2-13 CFU g^{-1} for Bj-552 or 5-20 CFU g^{-1} for Bc-IAS-119, even adding doses 10 times lower to the soil.

This effectiveness of seedmeals in comparison with green matter appears directly related to their sinigrin concentration: four times higher in seeds for Bj-552 and six times for Bc-IAS-119 (Ríos *et al*., 2016a). As previously reported (Ríos *et al*., 2016b), sinigrin seems to be directly linked to the biofumigant action of Brassicaceae plants against *P. cinnamomi*. Consequently, *B. napus* Bn-Salamander seedmeal, rich in GSLs but with no sinigrin content, was ineffective at controlling *P. cinnamomi* in soil and *in planta*, as previously reported by Ríos *et al*. (2016b) for green manure of Brassicaceae rich in other GSLs apart from sinigrin.

In general, seed yields in *dehesa* were low comparing with production reported from other agricultural areas around the world. Meena *et al*. (2013) explored *B. juncea* seed yield from multiple locations in India and reported values between 1200-1520 kg ha^{-1} and similar levels were found by Singh *et al*. (2014), more than 10 times the production reached in our study. Pan *et al*. (2012) reported a seed production of 797-2572 kg ha^{-1} for *B. carinata* in Canada. Field trials and agronomic studies on *B. juncea* and *B. carinata* conducted at Córdoba resulted in seed yield ranging from 2560 to 2567 Kg ha^{-1} for *B. juncea*, and 2688 to 3152 ha^{-1} for *B. carinata* (Fereres *et al*. 1983). Recent trials in rain-fed but fertile soils of southern Spain reached seed yields around 1200 Kg ha^{-1} for *B. carinata* (Junta de Andalucía, 2008).

In this study, seed production was especially low in the first growing season, likely due to the lack of plant establishment, leading to a null seed yield for *B. carinata*. This could

348 be associated with the weather conditions just after sowing, in which a high rainfall was
349 recorded, these crops being highly sensitive to waterlogging (López Bellido, 2003). In
350 addition, the minimum temperatures fell sharply during January and February when
351 plants had not yet reached the rosette stage, and they do not tolerate temperatures below
352 -2 or -3ºC (López Bellido, 2003). In fact, frost damage was visible in leaves of both *B.*
353 *carinata* genotypes and *B. juncea* Bj-552. Increasing the sowing dose from 150 to 300
354 seeds m^{-2}, seed yield decreased, as reported by Pan *et al*. (2012), who found a decrease
355 in *B. carinata* seed production when the sowing dose was increased from 200 to 400
356 seed m^{-2}. A higher sowing dose could be feasible when GSL-containing plants will be
357 used as cover crops to be incorporated into the soil and resulting in an intense plant
358 tissue breakdown (Björkman *et al*., 2015) but for seed production, doses of 6 kg ha^{-1} are
359 recommended (López Bellido, 2003). According with our results, a minimum seed
360 production of around 500 kg ha^{-1} would be needed to biofumigate 1 ha (0.03 g of
361 seedmeal per 100 g of soil), a yield which is likely beyond the potential seed production
362 in dehesa. Therefore, it would be more effective to allocate *Brassica* crops for seed
363 production to more fertile soils.
364 As conclusion, our results suggest that the direct application of seedmeal of *B. juncea* or
365 *B. carinata* varieties rich in sinigrin to infested soils should be considered as an
366 effective measure to be incorporated in the integrated control of oak disease in *dehesa*
367 ecosystems, since root disease symptoms appeared significantly reduced after soil
368 treatment. This effectiveness against *Phytophthora* disease was not achieved by green
369 matter, even at maturity stage (Ríos *et al*., 2016a). However, as seed production levels
370 in *dehesa* systems appear so low, it should be best practiced on agricultural lands.
371 Nevertheless, seedmeal biofumigation can slow disease spread although not eradicate

372 inoculum. Consequently, root disease may still be present wherever the pathogen was
373 detectable in *dehesa* soils.
374

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382

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Table 1. Maximum radial growth and standard error of *P. cinnamomi* cultures on CA medium after 3 days of exposure to volatiles released by different *Brassica* spp. biofumigant seedmeals. Values with different letters differ significantly according with Tukey´s HSD test ($p < 0.05$).

***Brassica* spp.**	**Genotype**	**Dose (g)**							
		0.0 (control)		**0.2**		**0.5**		**1.0**	
B. napus	Bn-Lewis	28.8 ± 0.6	a	28.3 ± 0.8	a	27.8 ± 1.0	a	28.3 ± 0.6	a a
	Bn-Salamander	29.8 ± 0.3	a	27.0 ± 0.4	a	27.8 ± 0.5	a	27.5 ± 0.3	a a
B. carinata	Bc-IAS-C1	29.5 ± 0.5	a	0.0 ± 0.0	c	0.0 ± 0.0	c	0.0 ± 0.0	c c
	Bc-IAS-119	29.8 ± 0.6	a	0.0 ± 0.0	c	0.0 ± 0.0	c	0.0 ± 0.0	c c
B. juncea	Bj-Tezla	29.3 ± 0.8	a	9.3 ± 3.1	b	0.0 ± 0.0	c	0.0 ± 0.0	c c
	Bj-552	28.8 ± 0.3	a	0.0 ± 0.0	c	0.0 ± 0.0	c	0.0 ± 0.0	c c

Table 2. Average number and standard error of viable *P. cinnamomi* chlamydospores (CFU g^{-1} of dry soil) after soil exposure to volatiles released by different *Brassica* biofumigants seeds at different doses and time of exposure. Values with different letters in each column differ significantly according with Tukey´s HSD test ($p < 0.05$).

***Brassica* spp.**	**Genotype**	**Dose (g)**	**Exposure time (days)**					
			1		**4**		**8**	
Control	-	0.0	104.7 ± 14.2	a	126.5 ± 13.0	a	106.6 ± 5.8	a
		0.1	100.7 ± 8.1	a	94.7 ± 17.0	a	90.1 ± 3.2	a
B. napus	Bn-Salamander	0.5	103.1 ± 4.2	a	94.7 ± 16.5	a	99.5 ± 10.1	a
		1.0	102.1 ± 4.5	a	88.1 ± 6.3	a	86.9 ± 31.5	a
		0.1	11.8 ± 1.9	b	7.9 ± 2.1	b	7.7 ± 0.5	b
B. carinata	Bc- IAS-119	0.5	13.2 ± 1.8	b	20.5 ± 4.8	b	6.4 ± 1.0	b
		1.0	14.6 ± 2.8	b	15.8 ± 5.4	b	5.0 ± 1.6	b
		0.1	12.1 ± 1.9	b	8.0 ± 1.4	b	8.5 ± 2.3	b
B. juncea	Bj-552	0.5	13.4 ± 2.3	b	5.7 ± 1.5	b	6.0 ± 1.4	b
		1.0	7.2 ± 1.8	b	20.7 ± 5.9	b	2.9 ± 0.7	b

511 **Table 3.** Seed yield (kg ha^{-1}) and standard error of two genotypes of *Brassica juncea* and *Brassica carinata* according with type of sowing
512 (line/broadcast with double seed dose) during the growing seasons 2012/2013 and 2013/2014 at Hinojosa del Duque (Spain). Values with
513 different letters within growing season differed significantly according with Tukey´s HSD test ($p < 0.05$).

514

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***Brassica* spp.**	**Genotype**	**2012/2013**		**2013/2014**	
		Line sowing	**Broadcast sowing**	**Line sowing**	**Broadcast sowing**
B. carinata	Bc-IAS-C1	0	0	98.93 ± 6.39 a	24.73 ± 1.66 c
	Bc-IAS-119	0	0	16.97 ± 1.49 c	5.27 ± 0.55 d
B. juncea	Bj-Tezla	12.71 ± 0.84 b	15.87 ± 0.45 a	118.21 ± 4.88 a	60.36 ± 11.35 b
	Bj-552	3.47 ± 0.66 c	4.79 ± 0.12 c	106.99 ± 6.26 a	31.68 ± 4.91 c

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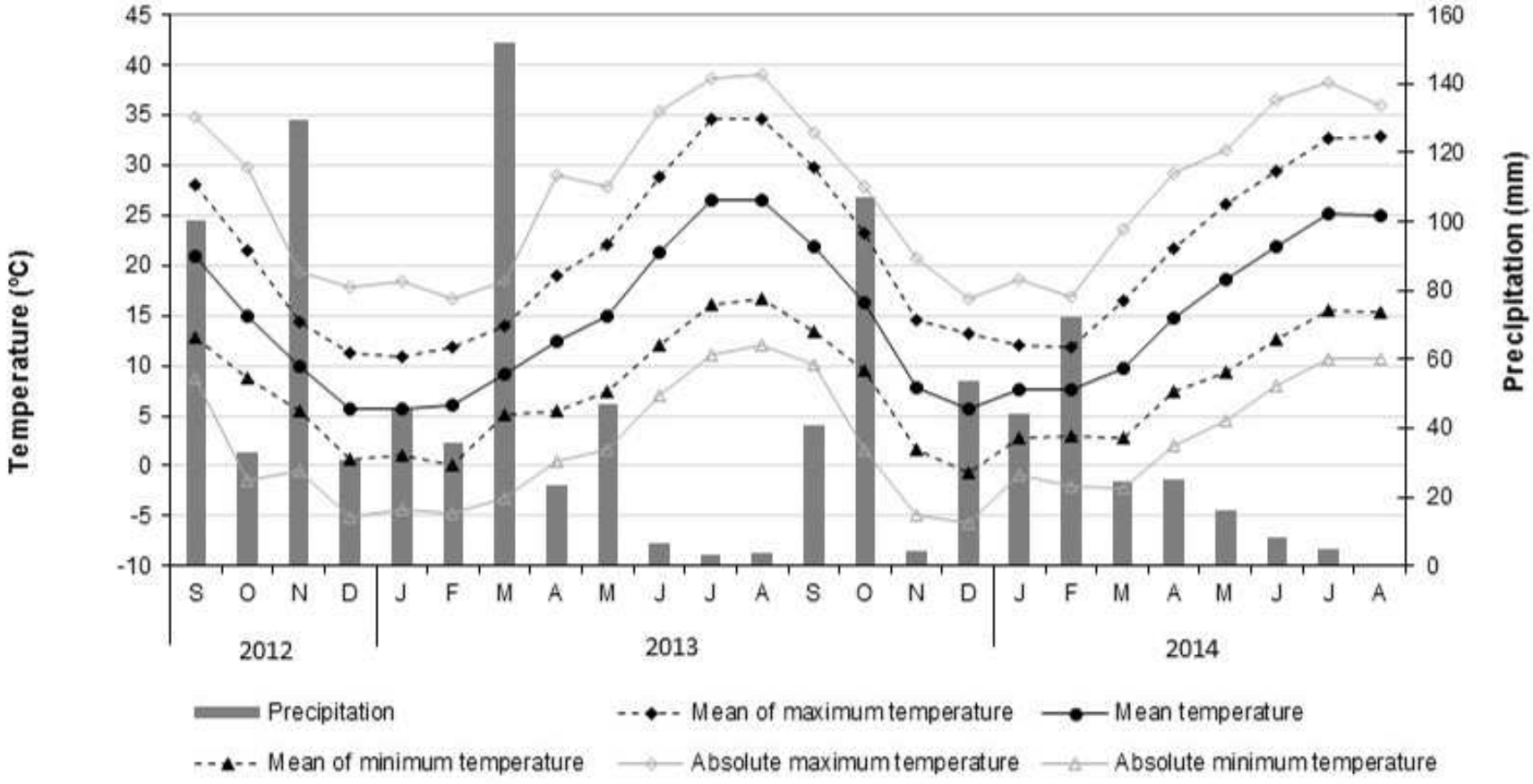
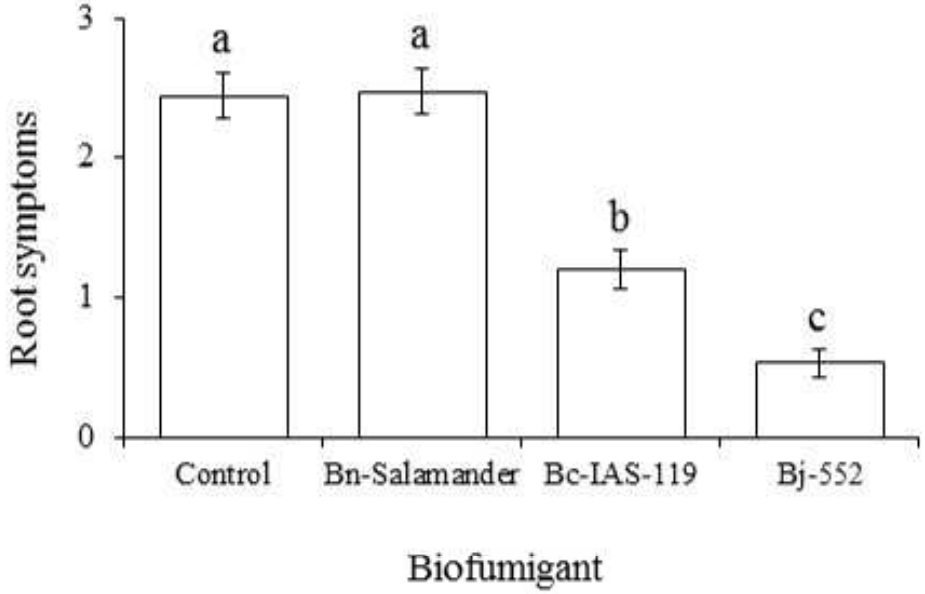
520

521 **Figure 1.** Temperature and precipitation data along the growing seasons 2012-2013 and 2013-2014. Data from Agencia Estatal de Meteorología,
522 Hinojosa del Duque station, Spain.
523
524 **Figure 2.** Average values and standard error of root symptoms showed by *L. luteus* growing in soils infested with *P. cinnamomi* chlamydospores
525 and biofumigated. Values with different letters differ significantly according with the Tukey´s HSD test ($p < 0.05$).


Temperature (°C)
Precipitation (mm)
45
40
35
30
25
20
15
10
5
0
-5
-10
160
140
120
100
80
60
40
20
0
S O N D J F M A M J J A S O N D J F M A M J J A
2012
2013
2014
Precipitation
Mean of maximum temperature
Mean temperature
Mean of minimum temperature
Absolute maximum temperature
Absolute minimum temperature


Root symptoms
3
2
1
0
a
a
b
c
Control
Bn-Salamander
Bc-IAS-119
Bj-552
Biofumigant

4.3

Screening of brassicaceous plants as biofumigants for management of *Phytophthora cinnamomi* oak disease

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ORIGINAL ARTICLE

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Screening brassicaceous plants as biofumigants for management of *Phytophthora cinnamomi* oak disease

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Summary

Brassicaceous plants rich in glucosinolates have been used as biofumigants for the management of soilborne pathogens. Efficacy of *Brassica* plant tissue has mainly been attributed to toxic isothiocyanates released upon the hydrolysis of glucosinolates. Management of *Phytophthora cinnamomi*, the causal agent of oak root rot in rangeland ecosystems using biofumigation, is promising, but requires further validation. The biofumigation activity of 14 brassicaceous plants was evaluated under experimental conditions. All evaluated plants rich in sinigrin suppressed (100%) the mycelial growth of *P. cinnamomi*, while plants rich in aromatic or other aliphatic glucosinolates had little or no suppressive effect. Simulating soil amendment in field conditions, the effects on natural soil artificially infested with *P. cinnamomi* chlamydospores were examined with *Brassica juncea*, *Eruca vesicaria* and *Lepidium sativum*, three species with different glucosinolate profiles. Only *B. juncea* decreased the viability of chlamydospores significantly in comparison with untreated soil only 1 day after biofumigation, whereas *E. vesicaria* needed 8 days to reach significance and *L. sativum* had no effect at all. Despite the decreases in soil inoculum, biofumigation with *B. juncea* did not prevent the root infections in a highly susceptible host (*Lupinus luteus*). However, biofumigation with plants rich in sinigrin, such as *B. juncea*, decreased *P. cinnamomi* soil inoculum under the experimental minimum threshold for oak disease expression. Although biofumigation should be considered as an effective measure to be incorporated in integrated control of the oak disease, biofumigation by itself would not be effective enough for the substantial suppression of *P. cinnamomi* inoculum.

1 | INTRODUCTION

Many woody species are threatened by *Phytophthora cinnamomi* Rands., one of the world's 100 worst invasive alien species (Lowe, Browne, & Boudjelas, 2000). This oomycete is causing high mortality levels in oak rangeland forests ('dehesas') in southern Spain and Portugal (Brasier, 1996; Sánchez, Caetano, Ferraz, & Trapero, 2002; Sánchez, Caetano, Romero, Navarro, & Trapero, 2006). Dehesas are semi-natural ecosystems comprising scattered trees, mainly evergreen oaks, and livestock, created and maintained by humans (Scarascia-Mugnozza, Oswald, Piussi, & Radoglou, 2000). In Spanish dehesas, the dominant tree species are holm oak (*Quercus ilex* L. ssp. *ballota* [Desf.] Samp.) and cork oak (*Quercus suber* L.), providing major incomes from cork and acorn production, a high valuable food resource for livestock and wildlife. These ecosystems are listed among the type habitats protected by the European Union's Directive on Habitats (Annex I of Council Directive 92/43/EEC on the Conservation of natural habitats and of wild fauna and flora). The cohabitation of wild fauna, livestock and trees, defines the semi-natural character of these forests and their organic production. Control methods against oak root disease caused by *P. cinnamomi* must be environmental friendly. In dehesa ecosystems, the fight against

P. cinnamomi is based on an integrated management that includes measures to limit new infections, such as the application of calcium fertilizers to the soil (Serrano, De Vita, Fernández-Rebollo, & Sánchez, 2012), avoidance of highly susceptible herbaceous hosts (Serrano, Fernández-Rebollo, De Vita, & Sánchez, 2012), high livestock densities or encouraging soil drainage (Fernández, Carbonero, Sánchez, & Trapero, 2008). All these recommendations are quite effective, but none effectively decreases the viability of *P. cinnamomi* resting spores present in the soil of declining oak systems (Serrano, De Vita et al., 2012). In this context, biofumigation appears a potential tool for use in oak rangeland ecosystems for disease management. Biofumigation is based on the toxicity of volatiles released by plants in the order Capparales, which contains 15 families, including *Brassicaceae* (cruciferous or brassicaceous plants; Halkier & Gershenzon, 2006). These volatiles arise during the lysis of secondary metabolites (glucosinolates, GSLs) by the endogenous plant enzyme myrosinase (thioglucoside glucohydrolase, E.C.3.2.1.147; Halkier & Gershenzon, 2006), yielding toxic products such as isothiocyanates (ITCs), nitriles, thiocyanates, oxazolidine-2-thiones and epithionitriles, noxious to weeds and soilborne pests and pathogens (Angus, Gardner, Kirkegaard, & Desmarchelier, 1994; Mattner et al., 2008; Sarwar, Kirkegaard, Wong, & Desmarchelier, 1998). The main objective of this work was to test the biocidal action against *P. cinnamomi* of brassicaceous plant species with different GSL profiles which are well adapted to grow in the Mediterranean climate, in order to select potential biofumigants for declining oak rangelands. To achieve these goals, (1) GSL profiles of 14 brassicaceous plants were assessed and (2) the suppression of *P. cinnamomi* mycelial growth by the release of volatile compounds was tested for each plant species; three species with different GSL profiles were selected to assess (3) the effect on the viability of *P. cinnamomi* chlamydospores in simulated soil amendments in field conditions and (4) the effect of selected biofumigants on the development of root infection symptoms *in planta*.

2 | MATERIALS AND METHODS

Fourteen plant species in the *Brassicaceae* were selected based on their performance to grow in rangeland ecosystems: *Brassica carinata* Braun, *Brassica juncea* (L.) Czern., *Brassica rapa* L., *Brassica nigra* Koch, *Sinapis flexuosa* Poir., *Sinapis alba* L., *Sinapis arvensis* L., *Eruca sativa* Mill., *Eruca vesicaria* (L.) Cav., *Diplotaxis virgata* (Cav.) DC., *Lepidium sativum* L., *Armoracia rusticana* G. Gaertn., B. Mey. & Scherb., *Cardaria draba* (L.) Desv. and *Erucastrum virgatum* C. Presl. Most of these plant species (i.e. *D. virgata*, *Sinapis* spp., *Eruca* spp. and *B. nigra*) are native to the Mediterranean Basin, and wild and cultivated forms can be found in the fields. Other plant species such as *L. sativum*, *C. draba* or *A. rusticana* are naturalized with a long history of cultivation by Mediterranean civilizations (Gómez-Campo, 1999). *Brassica juncea* and *B. carinata* (natives to Middle East and Ethiopia, respectively) were recently introduced as new oilseed crops because they are well adapted to the Mediterranean climate, showing agronomic characteristics (high biomass, high seed yield and resistance to drought, diseases and pests) as desirable as the native or naturalized plant species (De Haro et al., 1998).

Plants were cultivated in an experimental orchard at the Institute of Sustainable Agriculture (IAS, Córdoba, Spain; 37.8° N, 4.8° W) in the 2011/2012 growing season. The above-ground parts (leaves, stems and flowers) of 10 plants from each species were harvested at full flowering growth stage, corresponding to code 65 of the BBCH (Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie) scale (Lancashire et al., 1991). Immediately after harvesting, the plants were washed with abundant tap water and superficially disinfested by soaking them in aqueous 10% sodium hypochlorite for 10 s. The plants were superficially air-dried and weighed to obtain fresh weights, frozen (24 hr at −20°C) and freeze-dried (Virtis Wizard 2.0, Genesis Lyophilizer). After lyophilization, the dry material was again weighed to obtain dry weights and ground to a fine powder in a Janke and Kunkel, Model A10 mill (IKA-Labortechnik) for later use.

2.1 | Glucosinolate analysis

GSL composition for each plant species was determined by high-performance liquid chromatography (HPLC) according to Font, Del Río-Celestino, Cartea, and de Haro-Bailón (2005). For each sample, 100 mg dry weight lyophilized ground material was submitted to a two-step GSL extraction carried out in a water bath at 75°C to inactivate myrosinase. In the first step, the sample was heated for 15 min in 2.5 ml 70% aqueous methanol with 200 µl 10 mM of the internal standard glucotropaeolin (benzyl-glucosinolate) for *Brassica* and *Armoracia* species, and sinigrin (2-propenyl glucosinolate) as internal standard for the other plant species analysed. A second extraction was applied after centrifugation (5 min at 5000 ***g***) using 2 ml 70% aqueous methanol. One millilitre of the combined GSL extracts was pipetted onto the top of an ion-exchange column containing 1 ml Sephadex DEAE-A25 in the formate form. GSL desulphation was carried out by the addition of 75 µl of purified sulphatase (E.C. 3010601, type H-1 from *Helix pomatia*; Sigma) solution. Desulphated GSLs were eluted with 2.5 ml (0.50 × 5) Milli-Q (Millipore) ultrapure water and analysed with a Model 600 HPLC instrument (Waters) equipped with a Model 486 UV tunable absorbance detector (Waters) at a wavelength of 229 nm. Separation was carried out on a LiChrospher 100 RP-18 in Lichrocart 125-4 (125-mm length, 4-mm-inner diameter) column, 5 µm particle size (Merck). HPLC solvents and gradients followed the ISO protocol (ISO Norm, 1992). The HPLC chromatogram was compared with the desulpho-GSL profiles of three certified reference materials recommended by EU (CRMs 366, 190 and 367; Whatelet, Wagstaffe, & Boenke, 1991) and with those of pure reference glucosinolates (PHYTOPLAN Diehm and Neuberger GmbH). The amount of each individual GSL present in the sample was calculated using the mean of the internal standard and expressed as µmol g^{-1} dry weight. Total GSL content was computed as the sum of all individual GSLs present in the sample. Data were corrected for

UV response factors for different types of GSLs (ISO Norm, 1992).

2.2 | Inhibition of mycelial growth

The experiments were conducted with *P. cinnamomi* PE90, isolated from *Q. ilex* ssp. *ballota* (Sánchez, Sánchez, Navarro, Fernández, & Trapero, 2003) and belonging to the western population of the pathogen in the south of the Iberian Peninsula (Caetano, Ávila, Sánchez, Trapero, & Coelho, 2009). Previously, differences in virulence of *P. cinnamomi* isolates to some hosts have been reported, but no significant differences among *P. cinnamomi* isolates were found when infecting holm or cork oaks (Caetano et al., 2009; Robin, Deprez-Loustau, Capron, & Delatour, 1998; Sánchez et al., 2002).

For testing the effectiveness of biofumigants against mycelial growth, agar plugs (6 mm diameter) were cut from the edges of *P. cinnamomi* PE90 colonies actively growing on carrot agar (CA 20%) and transferred to the centre of Petri dishes (90 mm diameter) containing fresh CA. These dishes, without lids, were immediately inverted over plastic beakers (internal upper diameter 90 mm; height 120 mm; 0.4 L volume) containing 0 g (control), 2 or 5 g of rehydrated plant material and hermetically sealed with Parafilm® and scotch tape to avoid the loss of volatiles before incubation at 25°C in the dark. The required weight of lyophilized plant material and deionized water corresponding to the doses of fresh material selected was individually calculated for each species, taking into account the amount of water lost during lyophilization. Four beakers were prepared for each biofumigant and dose tested in a complete randomized experimental design; subsequently, the experiment was repeated four times. The radial growth of colonies was measured daily until controls had completely covered the surface of the substrate in the dishes. Data of maximum radial growth were recorded after 3 days of incubation, and when no radial growth was observed, data (0.0 mm) were not included in the statistical analysis. Data were checked for normality and homoscedasticity using the Shapiro–Wilk and Levene's tests, respectively, and a three-way ANOVA was performed with biofumigant species, doses tested and repetitions as independent variables. As significance was not observed for repeated experiments at $p < .05$, data were combined in a single analysis, leading to four values (replicates) per treatment. When significance was obtained at $p < .05$, mean values were compared by Tukey's HSD test for $p < .05$ (Statistix software 9.0).

Dishes were removed from the beakers, covered with a fresh sterile Petri dish lid and incubated for 1 week at 25°C in the dark to evaluate the colony growth post-exposure to the volatiles.

2.3 | Chlamydospore viability in soil

Three plant species, each belonging to one of the three groups detected based on GSL profiles, were chosen to test their effects on the viability of *P. cinnamomi* resting spores in soil. Natural soil free of the pathogen was taken from an asymptomatic oak rangeland located in the north of Córdoba Province (southern Spain). The soil was typical of oak rangeland ecosystems in Córdoba: acidic with low fertility (Parras-Alcántara et al., 2014). The absence of the pathogen in 10 samples of natural soil was assessed following the method reported by Romero et al. (2007) and no pathogen colonies were recorded in any sample. The soil was air-dried, sieved (2 mm pore size) and artificially infested with a water suspension of *P. cinnamomi* chlamydospores prepared as described in Romero et al. (2007): isolate PE90 of *P. cinnamomi* was plated in Petri dishes (90 mm diameter) containing 20 ml of carrot broth (20%) and incubated at 25°C in the dark. After 4 weeks of incubation, the liquid medium was filtered aseptically and the mycelium washed with sterile deionized water. Washed mycelium was suspended in sterile water at a rate of three Petri dishes per 100 ml and placed in a blender (Oster™ Pulse-matic 16, London) for 3 min at the maximum speed (liquefy) to break up the mycelial aggregates and obtain free chlamydospores. Aliquots were taken from the homogenized suspension and chlamydospores counted in a Neubauer counting chamber (0.1 µl). Chlamydospore concentration was adjusted to 1.5×10^4 chlamydospores ml^{-1}. The inoculum was carefully mixed with the dry and sieved natural soil to obtain a final concentration of 650 chlamydospores per g of dry soil. For each selected biofumigant, lyophilized material equivalent to 2 g of fresh material was placed at the bottom of 250-ml plastic beakers; beakers of biofumigant-free controls were included. Infested soil (225 ml; 292.5 g) was poured into the beakers; each beaker was immediately covered, hand-shaken and mixed before adding water to a final moisture content of 10% (w/w). All beakers were incubated in a growth chamber in the dark, with the temperature adjusted to 25°C (12 h) and 16°C (12 h). Incubation times were 0 (starting point), 1, 4 and 8 days. Four replicate beakers were prepared for each biofumigant species and incubation time tested in a complete randomized design. After each incubation period, soils were air-dried for 4 days at room temperature. Ten grams of homogenized dry soil per beaker was suspended in 100 ml 0.2% sterilized water agar (Roko Industries, Asturias, Spain), shaken and analysed following Romero et al. (2007): 1 ml aliquots were taken from each soil–water–agar mix and plated onto *Phytophthora*-selective NARPH medium, using a sterile glass spreader to distribute the material over the agar surface. For each soil sample, a total of 20 Petri dishes were prepared. The dishes were incubated at 24°C for 24 h in the dark before washing the agar surface of each dish with sterile water to remove the soil–water–agar mix. The dishes were incubated again at 24°C for an additional 48-h period in the dark. Colonies identified as *P. cinnamomi* based on hyphal morphology (presence of rounded hyphal swellings in clusters together with chlamydospores) observed under the inverted microscope were counted (Romero et al., 2007). Inoculum densities were expressed as colony-forming units per g dry soil (CFU g^{-1}). To fit continuity, data were transformed to $([\text{CFU } g^{-1}] + 0.5)^{1/2}$, checked for normality and homoscedasticity by using the Shapiro–Wilk and Levene's tests, respectively, and a three-way ANOVA was performed

TABLE 1 Glucosinolate (GSL) profiles and concentrations (μmol g^{-1} dw) in the above-ground parts of brassicaceous biofumigants

Group	Biofumigant species	Total GSLs	Aliphatic							Aromatic		Indolic		
			PRO	SIN	GRA	GNA	GSAT	GBN	GER	GTL	SBN	4-OMGBS	NGBS	Others
A	*Sinapis arvensis*	11.53	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	7.67	1.49	2.17	0.20
	Sinapis flexuosa	28.66	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	27.98	0.00	0.00	0.68
	Sinapis alba	29.96	0.00	0.00	0.00	0.00	0.00	0.00	0.00	11.99	17.51	0.00	0.00	0.46
	Lepidium sativum	35.53	0.00	0.00	0.00	0.00	0.00	0.00	0.00	35.22	0.00	0.00	0.00	0.31
B1	*Armoracia rusticana*	49.91	0.00	49.58	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.33
	Brassica carinata	15.06	0.00	14.19	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.87
	Brassica juncea	25.79	0.00	25.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.78
	Brassica nigra	19.30	0.00	17.79	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.51
B2	*Cardaria draba*	50.86	0.00	0.00	34.40	0.00	0.00	0.00	6.30	0.00	8.44	0.00	0.00	1.72
	Eruca vesicaria	34.14	0.00	0.00	19.62	0.00	12.62	0.00	1.62	0.00	0.00	0.00	0.00	0.28
	Eruca sativa	41.55	0.00	0.00	22.29	0.00	12.02	0.00	6.96	0.00	0.00	0.00	0.00	0.28
	Erucastrum virgatum	56.71	0.00	0.00	28.68	0.00	16.65	0.00	11.10	0.00	0.00	0.00	0.00	0.28
	Diplotaxis virgata	36.44	2.06	0.00	0.00	31.74	0.00	1.80	0.00	0.00	0.00	0.00	0.00	0.84
	Brassica rapa	32.81	8.11	0.00	0.00	20.89	0.00	1.87	0.00	0.00	0.00	0.00	0.72	1.22

A, high aromatic GSL content; B1, high sinigrin content; B2, high aliphatic GSL content different from sinigrin; PRO, progoitrin; SIN, sinigrin; GRA, glucoraphanin; GNA, gluconapin; GSAT, glucosativin; GBN, glucobrassicanapin; GER, glucoerucin; GTL, glucotropaeolin; SBN, sinalbin; 4-OMGBS, 4-methoxyglucobrassicin; NGBS, neoglucobrassicin.

considering biofumigant species, incubation periods and replicates as independent variables. When significance was obtained at $p < .05$, mean values were compared using Tukey's HSD test for $p < .05$ (Statistix software 9.0).

2.4 | Plant experiments

After sampling for chlamydospore viability, soil biofumigated with each plant species was mixed (including the not biofumigated control soils), homogenized and treated soil distributed in 40 plastic pots (replicates, 75 ml of soil per pot). Seeds of *Lupinus luteus* L., a species highly susceptible to *P. cinnamomi* (Serrano et al., 2010), were germinated in damp chambers. When the radicles were approximately 3 cm long, 40 seedlings per biofumigated or control soil were planted individually in the pots. All the pots were incubated at 25°C (day)/16°C (night) with a constant irrigation, keeping the soil moisture close to 100%. After 2 months, symptoms of *P. cinnamomi* root disease were evaluated based on the percentage of root necrosis on a 0–4 scale: 0 = 0% necrotic root; 1 = 1–33%; 2 = 34–66%; 3 = more than 67%; and 4 = dead root (Serrano et al., 2010). Root symptom data were tested for normality and homoscedasticity using the Shapiro–Wilk and Levene's tests, respectively, and a two-way ANOVA performed with biofumigant and replicate as independent variables. When significance was obtained at $p < .05$, mean values were compared using Tukey's HSD test at $p < .05$. Ten plants were chosen at random from each biofumigant treatment and the controls, and six root segments per plant were plated on NARPH medium for reisolation of the pathogen.

3 | RESULTS

3.1 | GLS profiles

GSL profiles of the 14 potential biofumigant plant species tested are given in Table 1. Based on the chemical nature of their main GSLs, the plants were divided into two groups: plants with high aromatic GSL content (group A) and those with a high content of aliphatic GSLs (group B). In turn, group B was subdivided into two subgroups, B1, including plants rich in sinigrin, and B2, plants rich in aliphatic GSLs other than sinigrin.

3.2 | Inhibition of mycelial growth

Inhibition of mycelial growth for each biofumigant tested is shown in Table 2. There were significant differences in *P. cinnamomi* mycelial growth depending on the biofumigant tested (ANOVA, $F = 349.8$, $p < .0001$), dose ($F = 43.5$, $p < .0001$) and the interaction between both variables ($F = 5.9$, $p < .0001$). Compared with the control cultures (dose 0), all biofumigants in the B1 group, three *Brassica* species (*B. carinata*, *B. juncea* and *B. nigra*) and *A. rusticana*, achieved 100% inhibition, even at the lowest dose tested (2 g). In all these cases, after the exposure to the biofumigant, *P. cinnamomi* did not grow again, showing a fungicidal action of these biofumigants against the pathogen. Other biofumigants in group A, such as *S. alba* and *L. sativum*, and in group B2, such as *C. draba*, *E. vesicaria*, *E. sativa* or *E. virgatum*, produced a significant mycelial growth inhibition in comparison with controls. However, after exposure to these A and B2 biofumigants,

TABLE 2 Maximum radial growth (mm ± *SE*) of *Phytophthora cinnamomi* colonies exposed to volatiles released by biofumigants

		Dose (g)		
GLS group	Biofumigant species	0 (control)	2	5
A	*Sinapis arvensis*	31.0 ± 0.6	29.0 ± 1.3	29.2 ± 1.8
	Sinapis flexuosa	28.6 ± 0.2 a	25.6 ± 1.0 a	19.6 ± 2.2 b
	Sinapis alba	30.2 ± 0.8 a	2.1 ± 0.9 b	1.2 ± 0.7 b
	Lepidium sativum	30.5 ± 0.8 a	11.0 ± 2.6 b	8.6 ± 1.8 b
B1	*Armoracia rusticana*	28.6 ± 1.0	0.0	0.0
	Brassica carinata	29.5 ± 0.7	0.0	0.0
	Brassica juncea	27.2 ± 0.2	0.0	0.0
	Brassica nigra	28.6 ± 0.9	0.0	0.0
B2	*Cardaria draba*	28.6 ± 0.8 a	19.4 ± 2.2 b	16.1 ± 2.2 b
	Eruca vesicaria	27.6 ± 0.8 a	15.9 ± 3.0 b	10.1 ± 1.5 b
	Eruca sativa	29.1 ± 1.1 a	4.2 ± 0.8 b	5.7 ± 2.7 b
	Erucastrum virgatum	28.7 ± 1.1 a	1.5 ± 0.3 b	1.7 ± 0.5 b
	Diplotaxis virgata	29.0 ± 0.8	26.0 ± 0.6	22.7 ± 0.7
	Brassica rapa	29.3 ± 0.3 a	23.7 ± 2.0 a	16.5 ± 2.4 b

Values obtained after 3 days of exposure.
For each row, values with different letters differ significantly according to Tukey's HSD test ($p < .05$).
A, high aromatic GSL content; B1, high sinigrin content; B2, high aliphatic GSL content different from sinigrin.

P. cinnamomi colonies were able to resume the growth until culture dishes were fully colonized, demonstrating a fungistatic action against *P. cinnamomi*. The other plant species tested did not produce a significant decrease in mycelial growth, or did so only at the highest dose tested. Based on these results, the biofumigants selected for soil experiments were *L. sativum* as the representative of group A (near 100% of the aromatic GSL glucotropaeolin, Table 1), *B. juncea* as the representative of group B1 (near 100% of the aliphatic GSL sinigrin, Table 1) and *E. vesicaria* of group B2 (two main aliphatic GSLs, glucoraphanin and glucosativin, and no sinigrin content). Apart from these considerations, these three particular species were selected on the basis of good agronomic characteristics and high biomass production when cultivated in oak rangeland systems (A. de Haro, unpublished data).

3.3 | Chlamydospore viability in soil

The viability of *P. cinnamomi* chlamydospores in soils exposed to the biofumigants tested is presented in Table 3. ANOVA showed significant differences depending on biofumigant species ($F = 69.3$; $p < .0001$) and exposure time ($F = 4.13$; $p = .0335$). No significance was observed between replicates or in the cases of other double interactions considered.

Viable chlamydospore numbers decreased significantly after 1 day in soils treated with *B. juncea* in comparison with the untreated soils; this decrease continued to 4 and 8 days of exposure (Table 3). *Eruca vesicaria* resulted in a significant decrease in chlamydospore viability until 8 days of incubation, whereas *L. sativum* did not differ from control soils at any of the times tested.

Two months after *L. luteus* plants were potted in infested, biofumigated soils and in non-biofumigated soil (control plants), foliar wilting and root rot were assessed. Significant differences in root symptoms were found between treatments ($F = 4.18$; $p = .0073$), but none of the biofumigants tested induced a significant decrease in root symptoms in comparison with control soils (Table 4). *Phytophthora cinnamomi* was always reisolated from necrotic root segments from every treatment to which infested soils were exposed.

4 | DISCUSSION

Several studies dealing with the *in vitro* and *in vivo* effectiveness of biofumigants against soilborne pathogens obtained different or even contradictory conclusions when the same biofumigant was tested against different pathogens. For example, *Brassica napus* appeared an effective biofumigant against *Sclerotinia sclerotiorum*, decreasing potato mortality by 39.2% (Ojaghian, Cui, Li, & Zhang, 2012), inhibiting oospore germination of *Aphanomyces euteiches* f. sp. *pisi* (Smolinska, Morra, Knudsen, & Brown, 1997) or decreasing apple root infections caused by *Rhizoctonia* sp. (Mazzola, Granatstein, Elfving, & Mullinix, 2001). In contrast, *B. napus* was ineffective for controlling *Pythium* sp. in apple orchards, even increasing the population of this pathogen (Mazzola, Reardon, & Brown, 2012). Generally speaking, the inhibitory effect of a particular biofumigant appears to be specific to certain pathogens (Mazzola et al., 2001; Ojaghian et al., 2012; Smolinska et al., 1997), and this behaviour seems to depend on the GSL profile (Brown & Morra, 1997; Sarwar et al., 1998). In this work, we found that brassicaceous plants with a

TABLE 3 Number of viable *Phytophthora cinnamomi* chlamydospores (CFU g^{-1} dry soil ± *SE*) after exposure of infested soil to volatiles released by biofumigants

		Exposure time (days)		
GLS group	**Biofumigant species**	**1**	**4**	**8**
	Control	84.2 ± 6.5 a	91.2 ± 3.2 a	114.2 ± 5.8 a
A	*Lepidium sativum*	85.0 ± 4.1 a	95.0 ± 5.8 a	100.7 ± 1.2 a
B1	*Brassica juncea*	36.0 ± 10.8 b	38.8 ± 5.8 b	44.3 ± 6.8 c
B2	*Eruca vesicaria*	75.7 ± 3.7 a	72.7 ± 2.2 a	71.0 ± 1.5 b

For each column, the values with different letters differ significantly according to Tukey's HSD test ($p < .05$).
A, high aromatic GSL content; B1, high sinigrin content; B2, high aliphatic GSL content different from sinigrin.

TABLE 4 Average values (±*SE*) of root symptoms on *Lupinus luteus* growing in soils infested with *Phytophthora cinnamomi* (650 chlamydospores g^{-1}) and biofumigated (0 = asymptomatic root, 4 = dead root)

GLS group	Biofumigant species	Root symptoms
	Control	$3.25 \pm 0.41^{a,b}$
A	*Lepidium sativum*	3.40 ± 0.31^{a}
B1	*Brassica juncea*	3.07 ± 0.09^{b}
B2	*Eruca vesicaria*	$3.28 \pm 0.38^{a,b}$

Values with different letters differ significantly according to Tukey's HSD test ($p < .05$).
A, high aromatic GSL content; B1, high sinigrin content; B2, high aliphatic GSL content different from sinigrin.

high content of the aliphatic GSL sinigrin are effective biofumigants against *P. cinnamomi*. These B1 group plants (*A. rusticana*, *B. carinata*, *B. juncea*, *B. nigra*) totally inhibited *P. cinnamomi* mycelial growth, demonstrating their biocidal action, as previously reported by Dunne, Dell, and Hardy (2003) when *P. cinnamomi* colonies were exposed to *B. juncea*. Other potential biofumigants without high sinigrin content, such as *S. flexuosa*, *S. alba* and *L. sativum* (group A, rich in aromatic GSLs) or *B. rapa*, *C. draba*, *E. vesicaria*, *E. sativa* and *E. virgatum* (group B1, rich in aliphatic GSLs different from sinigrin), were able to significantly inhibit the mycelial growth, but only a fungistatic effect was obtained.

In soil experiments, *B. juncea*, chosen as representative of group B1, reduced the viability of *P. cinnamomi* chlamydospores by 58% on average, with no notable variation between the incubation times tested (1–8 days). In contrast, *L. sativum* (group A) and *E. vesicaria* (group B2) gave low levels of inhibition, only significant for *E. vesicaria* after 8 days of incubation (37% inhibition). The different behaviour observed between *B. juncea* and *E. vesicaria* could be based on the different molecular weights of the ITCs released from the hydrolysis of the different aliphatic GSLs (Aires et al., 2011), which determine the volatility and consequently could influence their abilities to reach *P. cinnamomi* chlamydospores in the soil. When sinigrin (2-propenyl glucosinolate) is broken down by myrosinase activity, a highly volatile product of low molecular weight, allyl-ITC, is released. In contrast, sativin-ITC, the product of the hydrolysis of glucosativin (4-mercaptobutyl glucosinolate; Aires et al., 2011), released when *E. vesicaria* is added to soil, has a higher molecular weight. Aromatic GSLs from *L. sativum* were not effective at all in the soil, although the inhibition of mycelial growth occurred *in vitro*. The low volatility of the aromatic ITCs, in comparison with aliphatic ones, resulted in a slow rate of increase in ITC concentration in the headspace, as Sarwar et al. (1998) demonstrated.

In planta experiments showed that the biofumigated soils were unable to decrease root necrosis caused by *P. cinnamomi* on *L. luteus*, a highly susceptible host (Serrano et al., 2010). Recently, Morales-Rodríguez, Vettraino, and Vannini (2016) reported the effectiveness of commercial pellets produced from *B. carinata* to reduce *P. cinnamomi* inoculum density in experimental conditions and thus to protect seedlings of a moderately susceptible host (*Q. cerris*) from root infections. Even considering that soil biofumigated with *B. juncea* led to root necrosis values significantly lower than those obtained for plants growing in inoculated soils not exposed to biofumigation, these values would not be an effective disease control in this highly susceptible herbaceous host. Inoculum load remaining in the soil after *B. juncea* biofumigation probably exceeds the minimum threshold for *L. luteus* root rot. This infective inoculum load is unknown for *L. luteus*, but information exists for the pathosystem *Quercus*–*P. cinnamomi* in oak rangelands. Serrano, Ríos, González, and Sánchez (2015) reported that a minimum of 61 viable chlamydospores g^{-1} soil were needed to cause root disease on *Q. suber*, a susceptible host (Robin, Capron, & Desprez-Loustau, 2001), under experimental conditions. Bowers and Mitchell (1991) reported 41 oospores g^{-1} of *Phytophthora capsici* as the minimum density required to cause 50% mortality in pepper plants. Chlamydospore densities in soils biofumigated with *B. juncea* were below these minimum thresholds, and thus, *B. juncea* biofumigation could be effective in decreasing the destruction of a susceptible host such as *Q. suber*.

On the other hand, Mitchell (1978) concluded that only 0.6 or 0.9 chlamydospores g^{-1} of *P. citrophthora* or *P. palmivora* were required to infect *Morrenia odorata* or *Carica papaya*, respectively, in growth chamber studies, although some of these results refer to aerial infections. Equally, Serrano et al. (2015) obtained *Q. suber* root infections at low inoculum densities (2.7 CFU g^{-1}), even when progress to a significant oak root mortality was not achieved. Considering that a significant decrease in inoculum mediated by soil biofumigation resulted in a limited level of root infections and an inability to develop the significant oak root mortality in the short term, it may allow for persistent

survival of the pathogen in a site. This possibility is suggested by resurgence of disease in the long term in the absence of stringent inoculum eradication (Dunstan et al., 2010).

In conclusion, biofumigation with plants rich in sinigrin, such as *B. juncea*, is effective in significantly decreasing *P. cinnamomi* inoculum load under experimental conditions. Although biofumigation should be considered an effective measure to be incorporated in the integrated control of oak disease in the dehesa, we are also aware that this measure can slow the disease spread. However, it would not eradicate the inoculum. Consequently, root disease may still be present whenever the pathogen is detectable in the soils from oak forests.

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Figura 6.1: Efecto de los volátiles liberados por materia verde de *Brassica* spp. sobre el crecimiento micelial de *P. cinnamomi*. (a) contenedores dispuestos para la exposición de los cultivos a los volátiles; (b) resultados tras 4 días de exposición de las colonias de *P. cinnamomi* a los volátiles. Cada columna corresponde a un genotipo biofumigante: de izquierda a derecha *B. napus* Bn-Lewis; *B. napus* Bn-Salamander; *B. juncea* Bj-Tezla; *B. juncea* Bj-552; *B. carinata* Bc-IAS119; *B. carinata* Bc-IASC1. Cada fila corresponde a una dosis, de arriba a abajo: testigo 0 g; 5 g; 10 g; 20 g.

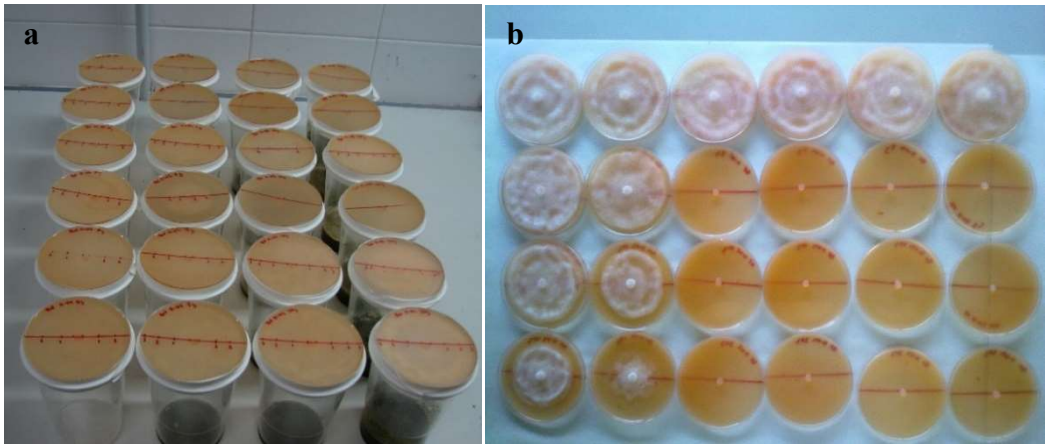


Figura 6.2: Efecto de los volátiles liberados por materia verde de *Brassica* spp. sobre la producción de esporangios y zoosporas de *P. cinnamomi*. Placas dispuestas para la exposición de los cultivos de *P. cinnamomi* en solución salina MSS a los volátiles liberados por el biofumigante. En el centro, placa testigo sin material biofumigante.

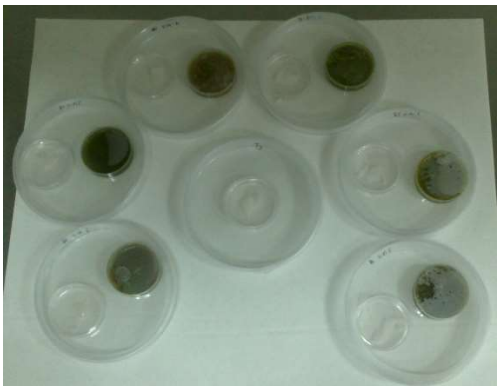


Figura 6.3: Efecto de los volátiles liberados por materia verde de *Brassica* spp. sobre la viabilidad de las clamidosporas de *P. cinnamomi* en suelo natural infestado. (a) infestación del suelo con la suspensión acuosa de clamidosporas (1.5×10^4 clamidosporas×ml^{-1}); (b) adición del material biofumigante a los contenedores; (c) adición del suelo infestado; (d) incubación de los contenedores cerrados a 16-25ºC (12h/12h) protegidos de la luz; (e) extensión de muestras de suelo biofumigado en placas de Petri con el medio selectivo para *Phytophthora* spp. NARPH.

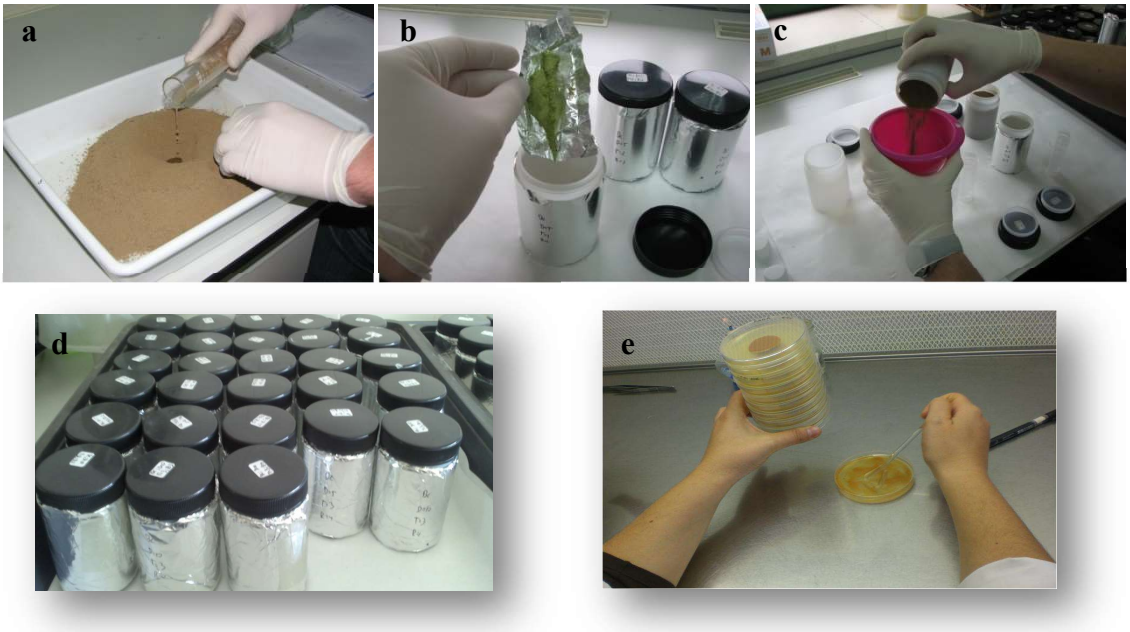


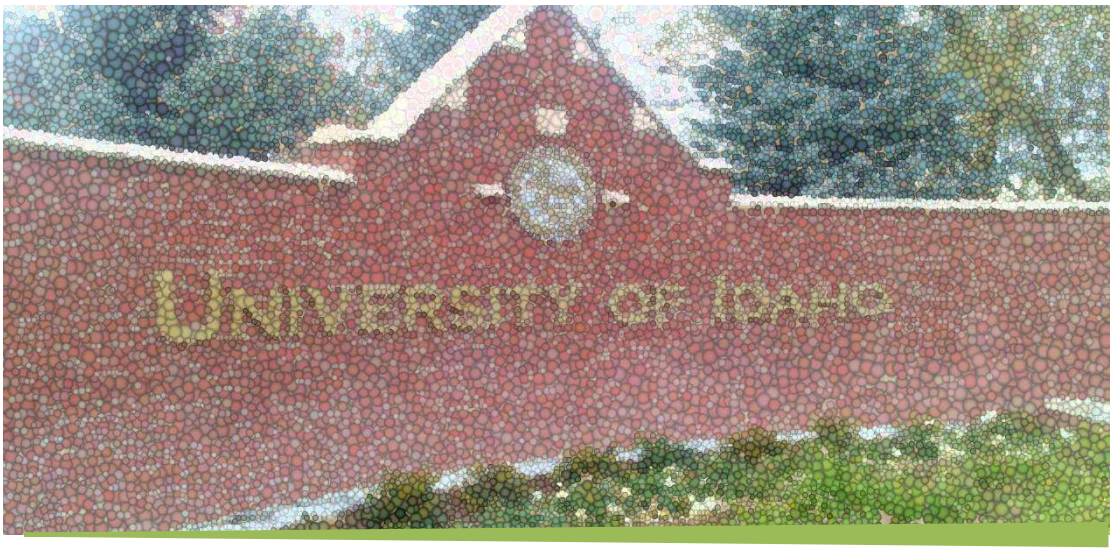
Figura 6.4: Severidad de síntomas radicales en plantas de *Lupinus luteus* cultivadas en (a) suelo infestado con 650 clamidosporas×g^{-1} de suelo sin biofumigar (testigo); (b) suelo infestado y biofumigado con harina de semillas de *Brassica juncea;* (c) suelo infestado y biofumigado con harina de semillas de *B. carinata*; (d) suelo infestado y biofumigado con harina de semillas de *B. napus*.

CAPÍTULO 5.

FORMULACIÓN DE UN PRODUCTO BIOFUMIGANTE EFECTIVO CONTRA *Phytophthora cinnamomi*

CHAPTER 5.

FORMULATING A BRASSICACEAE-BASED PRODUCT EFFECTIVE AGAINST *Phytophthora cinnamomi*



En este capítulo se recogen los resultados relativos al Objetivo 5 de esta Tesis Doctoral, en el cual se plantea formular un producto biofumigante listo para usar para su aplicación directa en suelos de dehesa infestados por el patógeno.

Los resultados obtenidos se han recogido en el siguiente artículo enviado a **Plant Disease** (under review):

"Formulating a Brassicaceae-*based product effective against* Phytophthora cinnamomi*"*

Autores: **Pedro Ríos**, Mario González, Matthew J. Morra; Antonio de Haro; María Esperanza Sánchez

5.1

Formulating a *Brassicaceae*-based product effective against *Phytophthora cinnamomi*

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plant disease

Formulating a Brassicaceae-based product effective against Phytophthora cinnamomi

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Keywords:	biofumigation, sinigrin, seed meal extract, root rot

Formulating a *Brassicaceae*-based product effective against *Phytophthora cinnamomi*

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Abstract

Biofumigation using crop plowdowns is an effective environmentally friendly method to control root rot caused by *Phytophthora cinnamomi*, but growth of *Brassica* crops is not always possible in soils where this pathogen is present. Our objective was to develop a formulated *Brassicaceae*-based product that can be applied to control Phytophthora oak disease, thus avoiding the need to grow a biofumigant crop. *Brassica juncea* extracts obtained from seed meal containing the glucosinolate sinigrin were combined with different enzyme sources in the form of dried green plant tissues to release the volatile inhibitory

compound 2-propenyl isothiocyanate. *Brassica juncea*, *Brassica nigra*, *Armoracia rusticana, Sinapis flexuosa*, and *Eruca vesicaria* green tissues all were effective myrosinase sources and when combined with *B. juncea* extract, inhibited mycelial growth. A *Brassicaceae*-based product derived from an oil waste seed meal was identified as an effective treatment that can be used as an alternative to a biofumigant crop plowdown to potentially control Phytophthora oak disease.

Keywords: biofumigation, sinigrin, seed meal extract, root rot

Introduction

The oomycete *Phytophthora cinnamomi* Rands. is included on the IUCN/SSC 100 World´s Worst Invasive Alien Species catalogue (Lowe et al. 2000), being described as a pathogen of more than 3000 plant species (Grünwald et al. 2011; Jung et al. 2013). Root rot caused by *P. cinnamomi* is threatening woodlands called “dehesa”, economically and environmentally valuable ecosystems listed among type habitats protected by the European Union´s Directive on Habitats (Annex I of Council Directive 92/43/ECC on the Conservation of Natural Habitats and of Wild Fauna and Flora). Dehesas are seminatural woodland ecosystems constituted by scattered trees, mainly evergreen oaks, and livestock, as generated anthropogenically by the clearing of Mediterranean forests for new pasture zones (Scarascia-Mugnozza et al. 2000). It is estimated that *P. cinnamomi* is currently killing thousands of oak trees every year (Romero et al. 2007).

Several methods have been investigated to control pathogen spread, from chemical soil treatments such as metalaxyl (Coffey et al. 1984) or endotherapy with potassium phosphonate (Fernández-Escobar et al. 1999; Solla et al. 2009)

to organic treatments such as soil calcium amendments (Serrano et al. 2012). None of these treatments effectively reduce chlamydospore populations in soil, the primary inoculum source for the pathogen. As an alternative, Ríos et al. (2016a; 2016b) demonstrated that biofumigation effectively reduced the primary and secondary inocula of *P. cinnamomi*. Biofumigation relies on activity of compounds released through the hydrolysis of glucosinolates (GSLs), secondary metabolites produced by *Brassicaceae* species. Once GSLs are enzymatically hydrolyzed by the action of myrosinase (thioglucoside glucohydrolase, E.C.3.2.1.147), isothiocyanates (ITCs) and other deleterious substances are produced (Halkier and Gershenzon 2006). More than 130 different GSLs are known (Agerbirk and Olsen 2012), but the noxious action of *Brassicaceae* against pests including pathogens and weeds seems to be related to their specific GSL profiles, or even one specific GSL. Ríos et al. (2016a; 2016b) reported the biocidal action of biofumigants against *P. cinnamomi* as directly related to content of the glucosinolate sinigrin that produces a volatile ITC. Additionally, the suppression of mycelial growth induced by volatiles released by the biofumigant can be used as an indicator of the ability of such products to significantly decrease the viability of *P. cinnamomi* chlamydospores in soil.

The use of biofumigant crops relies on tissue incorporation at a specific growth stage of the crop that may not correspond to an optimal time required for pathogen control. The aim of this work was to identify a *Brassicaceae*-based product that can be easily deployed during the period most appropriate for controlling Phytophthora oak disease in seminatural dehesa ecosystems, thus avoiding dependency on optimal growth stage of the biofumigant crop. Because we previously observed that the greatest biofumigant action was obtained when

effective *Brassicaceae* species were applied to soil as seed meals as compared to green tissues (Ríos et al. unpublished), our focus here was on using Brassicaceae seed meal extracts.

Materials and Methods

Mustard seed meal and extract preparation. Seed meal from *B. juncea* Pacific Gold, a variety well adapted to climate of the Northwestern United States, was used. Seeds were cold pressed to remove approximately 90% of the oil (Peterson et al. 1983). Mustard seed meal was extracted as described by Popova et al. (2017) with 73% (v/v) ethanol at a seed meal to liquid ratio of 1:20 (v/v) using an end-to-end shaker at room temperature for 2 h. Residual solids were separated by filtering, filtrates were concentrated by evaporation, and the concentrate was freeze-dried to obtain a powder (mustard extract). The extract did not contain any residual myrosinase activity.

***Brassicaceae* green tissues.** Different, well characterized *Brassicaceae* plants were used as sources of myrosinase for the extract: *Brassica juncea* (BJ-Tezla, Ríos et al. 2016a), *Sinapis flexuosa*, *Lepidium sativum*, *Armoracia rusticana*, *Brassica nigra*, *Eruca vesicaria,* and *Diplotaxis virgata* (Ríos et al. 2016b). Plants were cultivated in an experimental orchard at the Institute of Sustainable Agriculture (IAS, Córdoba, Spain; 37.8° N, 4.8° W) under a Mediterranean climate. The above-ground parts of five plants per biofumigant species were harvested at full flowering, corresponding to code 65 of the BBCH (Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie) scale (Lancashire et al. 1991). Immediately after harvesting, the plants were washed with abundant tap water and lightly disinfected by soaking them in aqueous 10% sodium hypochlorite for 10 s. After evaporation of water on external plant

surfaces, the plants were weighed to obtain their fresh weights, frozen (24 h at -20°C), and lyophilized (Virtis Wizard 2.0, Genesis Lyophilizer). After lyophilization, dry material was weighed, ground to a fine powder in a Janke and Kunkel, Model A10 mill (IKA-Labortechnik), and stored for later use.

GSL analysis. GSL profiles and concentrations (µmol g^{-1} dry weight) in *B. juncea* BJ-Tezla were analyzed as described in Ríos et al. (2016a). GSL profiles and concentrations in the above-ground parts of the other brassicaceous biofumigants can be found in Ríos et al. (2016b). GSL concentrations in the mustard extract were quantified using an Agilent 1200 Series HPLC system with a diode array detection system after separation on an Agilent XDB C18 (1.8 µm, 4.6×50 mm) column (Agilent, Santa Clara, CA, USA) as previously reported (Popova and Morra 2014).

Phytophthora isolate. In all biological tests, a well characterized isolate of *P. cinnamomi* obtained from oak roots was used (PE90, Caetano et al. 2009). The isolate was stored under mineral oil at the oomycete collection of the Agronomy Department, University of Córdoba, Spain.

Biofumigant products. Biofumigant products tested were a mix of the mustard extract as a source of sinigrin and different doses of brassicaceous green powder material as myrosinase sources. The doses tested were chosen based on their respective sinigrin concentrations relative to previous results of effectiveness against *P. cinnamomi* (Ríos et al. 2016a; 2016b). This dose was 0.16 g of the mustard extract containing 46.4 µmol sinigrin. For testing, the biofumigant products (mustard extract + brassicaceous green powder) were rehydrated (10 ml distilled water per gram of dry product) and pH was measured

(Crison, GLP21). When pH was below 6.5, $CaCO_3$ (98.5% Panreac) was added to adjust to pH 6.5, the optimum pH for myrosinase activity (Iori et al. 1996).

Inhibition of *P. cinnamomi* mycelial growth. In the first experiment, Bj-Tezla green powder by itself was tested to obtain the minimum quantity unable to inhibit mycelial growth of *P. cinnamomi* following the methodology detailed in Ríos et al. (2016a). Agar plugs (6 mm diameter) were taken from the edges of PE90 colonies actively growing in carrot-agar medium (CA 2%) after 3 days of incubation at 25°C in the dark. Plugs were positioned in the center of Petri dishes (90 mm diameter) containing fresh CA and immediately placed face down as lids of plastic beakers (internal upper diameter 90 mm; 120 mm high; 0.4 L volume) containing the material under test. Doses tested were: 0.00 (control), 0.01, 0.05, 0.10, 0.12, and 0.17 g of green dry material. Previously, Ríos et al. (2016a) found that 2 g of rehydrated Bj-Tezla green material exhibited 100% mycelial growth inhibition (Ríos et al. 2016a). After addition of 10 ml of distilled water per beaker, all beakers were immediately covered with the Petri dishes and hermetically sealed with Parafilm to avoid loss of released volatiles. Four replicates were used to test each dose including the zero dose control. Mustard extract at the chosen dose (0.16 g) was also tested alone for mycelial growth inhibition. Beakers were incubated at 25°C in the dark, and radial growth of the colonies was measured after 6 days of incubation when control colonies covered the entire surface of the agar medium. Inhibition of mycelial growth was calculated as a percentage based on radial growth recorded for the control colonies. When mycelial growth inhibition reached 100%, dishes were removed from the beakers, covered with a new sterile Petri dish lid, and incubated for 1 week at 25°C in the dark to check the ability of the colonies to grow after exposure to volatiles released by the biofumigant.

The second experiment tested the ability of the mustard extract (0.16 g) to inhibit *P. cinnamomi* mycelial growth after addition of Bj-Tezla green powder at doses causing 0% inhibition in the previous experiment: 0.01 to 0.12 g. Biofumigant products also contained $CaCO_3$ to adjust pH. Methodology was as described above including control beakers without any biofumigant product. Dishes showing 100% mycelial growth inhibition were removed from the beakers, covered with a new sterile Petri dish lid, and incubated for 1 week at 25°C in the dark to evaluate colony growth post-exposure to the volatiles.

Finally, following the same methodology a third experiment was performed to test different sources of myrosinase activity. *Sinapis flexuosa* and *Lepidium sativum* rich in aromatic GSLs (Group A, Ríos et al. 2016b), *Armoracia rusticana* and *Brassica nigra* rich in the aliphatic GSL sinigrin (Group B1, Ríos et al. 2016b), and *Eruca vesicaria* and *Diplotaxis virgata* rich in aliphatic GSLs different from sinigrin (Group B2, Ríos et al. 2016b) were tested at a dose of 0.12 g of lyophilized green powder plus 0.16 g of mustard extract. All biofumigants contained similar quantities of their main group of GSLs relative to *B. juncea* Bj-Tezla with the exception of *A. rusticana*, which was tested at a 50% dose because it contained twice as much sinigrin as Bj-Tezla. Resultant biofumigant products also contained $CaCO_3$ to adjust pH to 6.5. When 100% inhibition of *P. cinnamomi* mycelial growth was not reached, a double dose of green powder was additionally tested. When 100% inhibition was achieved, dishes were removed from the beakers and incubated as already described to evaluate subsequent colony growth.

Results

Table 1 shows the results obtained in the first experiment. From 0.01 to 0.12 g of *B. juncea* Bj-Tezla green powder did not inhibit *P. cinnamomi* mycelial growth. A similar result was obtained with 0.16 g of *B. juncea* Pacific Gold mustard extract.

In the second experiment when *B. juncea* Bj-Tezla green powder was tested together with *B. juncea* Pacific Gold mustard extract, total inhibition of mycelial growth was recorded with 0.10 and 0.12 g of green powder (Table 2). However when colony growth post-exposure to the volatiles was evaluated, only colonies previously exposed to the product containing 0.12 g of green powder were unable to grow.

When other sources of myrosinase activity were tested at doses similar to *B. juncea* Bj-Tezla, species rich in sinigrin (Group B1) caused total inhibition of *P. cinnamomi* mycelial growth (Table 3). *Sinapis flexuosa* (Group A) and *E. vesicaria* (Group B2), species rich in GSLs different from sinigrin, exhibited the same inhibition as sinigrin-containing Group B1 plants, leading to total inhibition of *P. cinnamomi* mycelial growth. However, *L. sativum* (Group A) and *D. virgata* (Group B2) induced some degree of growth inhibition (68% and 28%, respectively) only when the dose was doubled. In contrast to all other species tested, colonies were able to continue growing post-exposure to volatiles.

Discussion

The effectiveness of *Brassica* green manures against different soil pathogens depends on quality of the *Brassica* crop, which is determined by genetics of the *Brassica* species/variety used, timing of planting, sowing density, soil nutrition, soil type, and day length (Leonard 2000). In addition, efficient release of ITC during tissue incorporation requires extensive cell disruption and the presence

of adequate moisture (Morra and Kirkegaard 2002; Morra 2004). In dealing with dehesa woodlands, there are factors that may constrain biofumigation: low soil fertility may limit biomass production; stoniness of some areas may hamper effective incorporation of fresh biomass into soil; and because irrigation is not feasible, the high unpredictability of rainfall within a year may slow GSL hydrolysis once green matter is incorporated.

To avoid dependence on *in situ* cropping, our work indicates that easy to obtain and use biofumigant products provide biocidal action against *P. cinnamomi*. The proposed product is based on a seed meal extract rich in sinigrin that is generated from oil industry waste and low quantities of lyophilized green material. Both components could be cropped in locations more fertile than dehesa woodlands. The mustard extract is not active until a source of myrosinase activity (lyophilized green material) and water are added at an optimal pH of 6.5 (Iori et al. 1996).

Lyophilized green matter from *Brassicaceae* plants rich in sinigrin (*B. juncea*, *B. nigra* or *A. rusticana*) at a 2-g dose effectively inhibited *P. cinnamomi* mycelial growth and decreased chlamydospore density when added to infested soil (Ríos et al. 2016b). Doses of green powders tested in this work (0.12 g for *Brassica* spp. and 0.06 g for *A. rusticana*) were not effective by themselves at inhibiting mycelial growth. However, *B. juncea*, *B. nigra*, and *A. rusticana* green powders contained adequate amounts of myrosinase activity for releasing 2-propenyl ITC from the mustard extract and endogenous sinigrin contained within the tissues. *Brassica juncea* Bj-Tezla contains 25 µmol (Ríos et al. 2016a), *A. rusticana* 50 µmol and *B. nigra* 18 µmol sinigrin per gram of green powder (Ríos et al. 2016b) that was likely hydrolyzed along with mustard extract sinigrin. Similarly, *E.*

vesicaria is also a viable myrosinase source, but this green powder contains the glucosinolates glucoraphanin and glucosativin that produce volatile methylated isothiocyanates potentially active against *P. cinnamomi* (Ríos et al. 2016b). *Sinapis flexuosa* likewise is a good myrosinase source to hydrolyze sinigrin in *B. juncea* mustard extract as demonstrated by total growth inhibition. Unlike the previously mentioned species, endogenous aromatic glucosinolate hydrolysis products most likely did not contribute to observed inhibition because previous investigations showed no mycelial growth effects at a dose of 2 g (Ríos et al. 2016b). These results suggest a lack of substrate specificity for myrosinase contained in various species. In contrast, *L. sativum* (endogenous aromatic glucosinolate substrate) and *D. virgata* (endogenous aliphatic glucosinolate substrate) were poor myrosinase sources for releasing 2-propenyl ITC from *B. juncea* mustard extract either because the contained myrosinases are substrate specific or low in concentration. These same species, which have previously been shown ineffective against *P. cinnamomi* by themselves (Ríos et al. 2016b), induced a fungistatic effect only when green powder amounts were doubled.

The use of $CaCO_3$, a common calcium fertilizer, as a pH buffer may contribute to biofumigant effectiveness. $CaCO_3$ has proven activity against *P. cinnamomi* sporangial production in soil (Serrano et al. 2012), reducing zoospore production (secondary inoculum). However, Ca^{2+} does not eliminate resistant spores (chlamydospores, primary inoculum) in soil to the same extent as does biofumigation with sinigrin-rich plants (Ríos et al. 2016a; 2016b).

The resulting dry product (extract - lyophilized matter - $CaCO_3$) can be directly used in dehesa woodlands as an amendment when soil is naturally wet, overlapping with the more active period of *Phytophthora* pathogens or prior to

afforestation actions, thus increasing the efficiency of disease control (Mazzola et al. 2015).

In conclusion, this research documents the potential viability of a ready-to-use biofumigant product that combines the biocidal action of *B. juncea* amendments enhanced through the increase in sinigrin concentration and the suppressive action of calcium fertilizers. We identified plant tissues of specific species that can be used as myrosinase sources to release sufficient amounts of 2-propenyl ITC for *P. cinnamomi* inhibition. This product can be applied at an appropriate time for *P. cinnamomi* control without suffering restrictions that limit the use of *in situ Brassica* crops in traditional biofumigation strategies. Additionally, this product increases the value of what is now often considered an industrial waste.

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Table 1. Mycelial radial growth and mycelial growth inhibition of *P. cinnamomi* cultures on CA medium after 6 days of exposure to volatiles released by different biofumigants at pH 6.5

Biofumigant product	Dose (g)	Mycelial radial growth (mm)	Mycelial growth inhibition (%)
Brassica juncea Bj-Tezla' green powder	0.00	40.0	-
	0.01	40.0	0
	0.05	40.0	0
	0.10	40.0	0
	0.12	40.0	0
	0.17	00.0	100
Brassica juncea Pacific Gold' mustard extract	0.16	40.0	0

Table 2. Mycelial radial growth and mycelial growth inhibition of *P. cinnamomi* cultures on CA medium after 6 days of exposure to volatiles released by different biofumigant products at pH 6.5

Biofumigant product		Mycelial radial growth (mm)	Mycelial growth inhibition (%)	Further mycelial growth
Brassica juncea Bj-Tezla' green powder (g)	*Brassica juncea* Pacific Gold' mustard extract (g)			
0.00	0.00	40.0	-	-
0.01	0.16	40.0	0	-
0.05	0.16	36.0	10	Yes
0.10	0.16	00.0	100	Yes
0.12	0.16	00.0	100	No

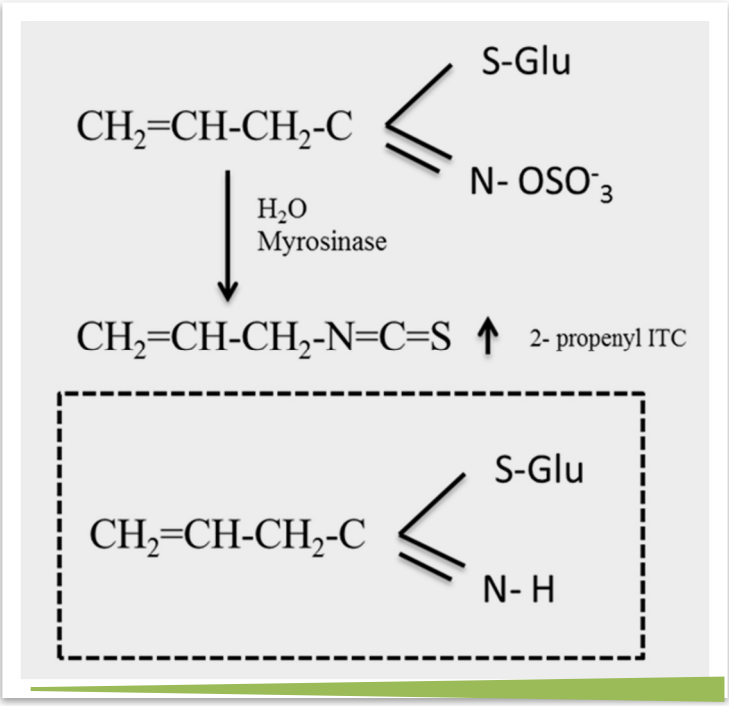
Table 3. Mycelial radial growth and mycelial growth inhibition of *P. cinnamomi* cultures on CA medium after 6 days exposed to volatiles released by different biofumigant products at pH 6.5. Group A: biofumigants rich in aromatic GSLs, Group B1: biofumigants rich in GSL sinigrin, Group B2: biofumigants rich in aliphatic GSLs different from sinigrin (Ríos et al. 2016b).

Group	Biofumigant product			Mycelial radial growth (mm)	Mycelial growth inhibition (%)	Further mycelial growth
	Brassicaceae species	Green powder (g)	*Brassica juncea* Pacific Gold' mustard extract (g)			
-	-	-	0.00	40.0	-	-
A	*Sinapis flexuosa*	0.12	0.16	00.0	100	No
A	*Lepidium sativum*	0.12	0.16	40.0	0	-
A	*Lepidium sativum*	0.24	0.16	12.8	68	Yes
B1	*Armoracia rusticana*	0.06	0.16	00.0	100	No
B1	*Brassica nigra*	0.12	0.16	00.0	100	No
B2	*Eruca vesicaria*	0.12	0.16	00.0	100	No
B2	*Diplotaxis virgata*	0.12	0.16	40.0	0	-
B2	*Diplotaxis virgata*	0.24	0.16	29.0	28	Yes

Figura 7: Extracción del concentrado de sinigrina a partir de (a) torta de semilla desgrasada de *Brassica juncea* var. Pacific Gold, la cual se somete a (b) un proceso de maceración en etanol y prensado en frío que da lugar a (c) un extracto acuoso del que se obtiene (d) el extracto sólido tras un proceso de secado.

CAPÍTULO 6.
DISCUSIÓN

CHAPTER 6.
DISCUSSION



Dentro del género *Phytophthora* se encuentran numerosos agentes fitopatógenos que han causado y están causando serios problemas tanto en cultivos agrícolas como en ecosistemas naturales (Zentmyer, 1980; Erwin y Ribeiro, 1996; Jung et al., 2000). En el caso concreto de los ecosistemas de dehesa de la Península Ibérica, se ha demostrado que *Phytophthora cinnamomi* es el principal agente asociado a la muerte de especies del género *Quercus* (Brasier, 1996; Sánchez et al., 2002; 2006), base fundamental sobre el que se asienta la sostenibilidad tanto ecológica como económica de este ecosistema (San Miguel, 1994; Costa et al., 2006). *Phytophthora cinnamomi* causa podredumbre radical, impidiendo que los árboles infectados puedan abastercese de agua y nutrientes, lo que lleva a su muerte (Erwin y Ribeiro, 1996; Sánchez et al., 2002).

El control de la enfermedad mediante métodos químicos es una opción interesante pero aún por precisar: a pesar de que se ha demostrado que la aplicación de fosfito potásico mediante inyección al tronco resulta efectiva en la prevención de la enfermedad radical causada por *P. cinnamomi* a las dosis recomendadas (Hardy et al., 2001; Sánchez et al., 2006), la reciente prohibición de su comercialización como fertilizante impide su uso en tanto no se registre como fungicida (Anexo 1 RD 506/2013, www.juntadeandalucia.es/agriculturaypesca/raif/novedades/2014/novedad_140507_02.html). Otras materias activas efectivas contra especies del género *Phytophthora* como el metalaxil (Cho, 1981; Coffey y Bower, 1984; Pegg et al., 1987; Swart y Denman, 2000) no se adecuaría al sistema de producción ecológica propio de la dehesa, ya que este tipo de fungicida sistémico debería aplicarse al suelo, lo que entraría en contradicción con el aprovechamiento ganadero extensivo y la presencia de fauna silvestre típica de la dehesa. Otros fosfonatos registrados como fungicidas, como el fosetil-aluminio, han demostrado una elevada eficacia preventiva y curativa frente a la podredumbre radical en encinas y alcornoques infectados en condiciones controladas, pero aún no está demostrada su eficacia en condiciones de campo si se aplican mediante inyección al tronco (González et al., 2017).

La búsqueda de resistencia genética contra este patógeno es otra alternativa, pero la lenta tasa de crecimiento de las especies de *Quercus*, la compleja genética de estas especies (Coelho et al., 2006) y la posibilidad de una adaptación del patógeno (León, 2013) hacen que no se contemple esta medida a corto/medio plazo.

Actualmente los métodos de control de efectividad demostrada y aplicación real se engloban en un sistema de manejo integrado de la enfermedad que incluye medidas

preventivas destinadas a limitar nuevas infecciones, como evitar la siembra de tremosilla (*Lupinus luteus*), cultivo forrajero altamente susceptible a la infección radical (Serrano et al., 2010) que favorece la multiplicación del patógeno en las raíces infectadas y favorece el aumento de densidad de inóculo en el suelo mediante el aporte de clamidosporas y la estimulación de la germinación de esporangios (Serrano et al., 2011; 2012b). Por otro lado, la aplicación al suelo de enmiendas calizas inhibe la producción y germinación de esporangios a partir de las clamidosporas de *P. cinnamomi*, disminuyendo la tasa de infección y reduciendo la sintomatología radical en encinas (Serrano et al., 2012a). Serrano et al. (2014) recomiendan el uso de $CaSO_4$ a dosis de 3.500 $kg \times ha^{-1}$. Además, está demostrado que una buena nutrición cálcica confiere tolerancia a la podredumbre radical, disminuyendo significativamente el desarrollo de síntomas radicales en plantones de encina con concentraciones de Ca^{2+} superiores a lo habitual (Serrano et al., 2013). Todas estas medidas de control cultural resultan efectivas minimizando el impacto de la enfermedad en las dehesas, pero ninguna de ellas disminuye de manera significativa la viabilidad de las esporas de resistencia de *P. cinnamomi* en el suelo.

El objetivo de este trabajo ha sido evaluar la biofumigación como una técnica de control biológico aplicable en ecosistemas de dehesa que podría añadirse a las anteriormente mencionadas, especialmente si resulta efectiva contra las estructuras de resistencia del patógeno.

El primer paso para poder establecer la efectividad de este tipo de técnicas de control es conocer la densidad de inóculo de *P. cinnamomi* en el suelo a partir de la cual se desencadena la enfermedad radical. Existen referencias sobre densidades de inóculo en suelos de dehesa con presencia de la enfermedad radical causada por *P. cinnamomi*, que varían en una amplia horquilla de entre 4 y 2.500 Unidades Formadoras de Colonia por gramo de suelo seco ($CFU \times g^{-1}$) (Romero et al., 2007; Gómez-Aparicio et al., 2012). Nuestros resultados demuestran que infestaciones de suelo a partir de 61 $CFU \times g^{-1}$ dan lugar al desarrollo de los síntomas radicales en *Quercus,* si bien valores tan bajos como 3 $CFU \times g^{-1}$ son suficientes para poder detectar la presencia del patógeno en las raíces asintomáticas. Se han referenciado umbrales similares para otras especies del género, como *P. capsici*, que produce una mortalidad del 50% en plantas de pimiento con densidades de inóculo de 41 $CFU \times g^{-1}$ (Bowers y Mitchell, 1991).

El umbral mínimo de inóculo para el desarrollo de la enfermedad radical en *Quercus* fue determinado en experimentos llevados a cabo bajo condiciones controladas, pero marca una referencia a la hora de conocer el potencial de los suelos de dehesa infestados para inducir la enfermedad y poder evaluar la biofumigación como efectiva si es capaz de disminuir la densidad de inóculo en el suelo por debajo de este umbral de referencia.

No obstante, hay que señalar que la infección asintomática de la raíz de *Quercus* tiene lugar con densidades tan bajas como 3 $CFU \times g^{-1}$, lo que implica que, a largo plazo, la enfermedad podrá desarrollarse en cualquier suelo con presencia de *P. cinnamomi*, como han comprobado en Australia tras la adopción de medidas muy drásticas que perseguían la erradicación del patógeno (AGDE, 2008). Estas medidas incluyeron la destrucción de todas las plantas, creando una zona muerta o de barbecho en los focos de enfermedad y zonas limítrofes, la instalación de barreras físicas, la aplicación de fungicidas selectivos (thiadiazoles y metalaxil) a ultrabajo volumen de forma aérea y por riego subterráneo, así como el tratamiento posterior del suelo, tanto en superficie como en profundidad (± 1 m), con fumigantes químicos (metam-sodio). A pesar de lo drástico de las medidas de erradicación adoptadas, se comprobó como a largo plazo, cuando la vegetación volvía a colonizar la zona tratada, volvía a detectase la presencia del patógeno en el suelo (Dunstan et al., 2010).

Una vez conocido el umbral a partir del cual se validaría la técnica, se evaluó cómo la biofumigación afecta a los distintos estadios vegetativos y reproductivos del ciclo de vida de *P. cinnamomi*. Para ello se ensayaron dos genotipos diferentes de tres especies con portencial biofumigante, *Brassica carinata, B. juncea* y *B. napus*, cosechadas en distintos estados fenológicos. Existen referencias bibliográficas que relacionan el efecto inhibitorio de una especie biofumigante con un glucosinolato (GSL) específico de su perfil (Smolinska et al., 1997; Mazzola et al., 2001; Ojaghian et al., 2012), por lo que conocer la composición de GSLs de cada una de los genotipos seleccionados es un factor clave. A este respecto, se comprobó que las tres especies mantenían un perfil similar de GSLs durante su desarrollo y maduración, siendo la sinigrina (2-propenil glucosinolato) el GSL predominante en los genotipos de *B. carinata* y de *B. juncea*, y la progoitrina (2(R)-2-hidroxi-3-butenil glucosinolato) en los genotipos de *B. napus*. De los biofumigantes seleccionados, sólo los genotipos de *B. carinata* y *B. juncea,* ricos en sinigrina, demostraron su potencial para ser usadas contra *P. cinnamomi*, ya que inhibieron su crecimiento micelial, disminuyeron significativamente la producción de

esporangios, y disminuyeron la viabilidad de las clamidosporas en el suelo por debajo del umbral mínimo para el desarrollo de la enfermedad en *Quercus.* Olivier et al. (1999) referenciaron el efecto inhibitorio del GSL sinigrina sobre patógenos del suelo. Sin embargo, la información disponible sobre la efectividad de las especies de *Brassica* ensayadas en la inhibición de distintas fases del ciclo de vida de *Phytophthora* spp. resulta muy contradictoria: Mazzola y Brown (2010) afirman que la biofumigación con *B. juncea* aumentó la tasa de infección en *P. cambivora*, mientras que Gigot et al. (2013), usando el mismo biofumigante, redujeron la población de *P. rubi.* En nuestros experimentos, *B. napus* no sólo no inhibió a *P. cinnamomi* en ningún estadio de su ciclo de vida, sino que incluso puede llegar a estimular la tasa de infección debido al aumento del número de esporangios que produce, lo cual es acorde con los resultados obtenidos por Mazzola et al. (2001) al estudiar el efecto de *B. napus* sobre patógenos pertenecientes al género *Pythium*. Sin embargo, existen referencias sobre la capacidad inhibitoria de *B. napus* sobre otros oomicetos como *Aphanomyces euteiches* (Smolinska et al., 1997; Smolinska, 2000).

A pesar de los buenos resultados obtenidos en la reducción de la viabilidad de las clamidosporas en el suelo mediante biofumigación con partes verdes de *B. carinata* y *B. juncea*, ricas en sinigrina, los resultados obtenidos en los ensayos *in planta*, realizados con *L. luteus*, una especie altamente susceptible a la infección (Serrano et al., 2010), no resultaron completamente satisfactorios. Sólo las plantas que crecieron en suelos biofumigados con *B. carinata* desarrollaron un nivel de síntomas radicales significativamente menor que las plantas que crecieron en suelos no tratados o en suelos tratados con el resto de biofumigantes.

Las especies pertenecientes al género *Brassica* contienen GSLs en todos sus órganos, pero es en las semillas donde su concentración es más alta (Halkier y Gershenzon, 2006). Por ello, se evaluó la efectividad de la harina de semillas de los mismos genotipos de las especies biofumigantes anteriormente ensayadas. Se comprobó que al igual que en los ensayos con material verde, sólo los genotipos ricos en sinigrina resultaron efectivos en la disminución de la viabilidad de las clamidosporas por debajo de umbral crítico para el desarrollo de la enfermedad en *Quercus* y, en este caso, la biofumigación del suelo con harina de semillas de estos genotipos ricos en sinigrina dio lugar a que las plantas de *L. luteus* no mostraran síntomas radicales de la enfermedad, difiriendo del elevado nivel de síntomas que se observó en las plantas que crecieron en

suelo no biofumigado o biofumigado con harina de semillas de genotipos sin sinigrina (*B. napus*).

En ensayos de producción en dehesa de genotipos de *B. carinata* y *B. juncea* ricos en sinigrina, se comprobó el escaso rendimiento de estos cultivos. A lo largo de dos campañas, la máxima producción de semillas se obtuvo con un genotipo de *B. juncea* que alcanzó algo más de 100 kg×ha^{-1}, lejos de los 500 kg×ha^{-1} requeridos para igualar las dosis aplicadas en los ensayos en condiciones controladas, y de los alrededor de 3.000 kg×ha^{-1} obtenidos en ensayos agronómicos realizados en la provincia de Córdoba con *B. carinata* y *B. juncea* (Fereres et al., 1983). Estos datos implican que la producción directa de biofumigantes efectivos contra *P. cinnamomi* en dehesa no resulta factible, y sería necesario producir semillas en otras áreas agrícolas de mayor productividad para ser cosechadas y posteriormente aplicadas en suelos de dehesa.

Para confirmar que la efectividad de los biofumigantes testados se debe a su elevado contenido en sinigrina y conocer si hay otras plantas que con distinto perfil de GSLs resulten igualmente efectivas, se evaluó la actividad biofumigante de material verde perteneciente a 14 especies de plantas de la familia *Brassicaceae.* En primer lugar se clasificaron las especies candidatas en tres grupos en función de su perfil de GSLs: Grupo A, plantas ricas en GSLs aromáticos; Grupo B1, plantas ricas en sinigrina; y Grupo B2, plantas ricas en GSLs alifáticos distintos de sinigrina. Posteriormente se evaluó la inhibición del crecimiento micelial de *P. cinnamomi* como indicativo de su capacidad biofumigante, seleccionando especies de distinto grupo para evaluar su efectividad en la disminución de la viabilidad de las clamidosporas en el suelo y el posterior desarrollo de la enfermedad en plantas de *Lupinus.* Los resultados de estos experimentos confirmaron que las especies biofumigantes pertenecientes al Grupo B1 no sólo inhibieron el crecimiento micelial de las colonias, sino que demostraron acción biocida sobre las mismas, mientras que sólo algunas especies pertenecientes a los grupos A y B2 inhibieron significativamente el crecimiento micelial, pero sólo mostraron acción fungistática.

La capacidad biocida de la sinigrina se confirmó en los ensayos en suelo y en planta, ya que la viabilidad de las clamidosporas disminuyó significativamente y por debajo del umbral mínimo sólo en los suelos tratados con biofumigantes pertenecientes al grupo B1 y además, las plantas de *Lupinus* que crecieron en suelos infestados y tratados con estos biofumigantes mostraron síntomas radicales significativamente más bajos que las

plantas que crecieron en suelos infestados y no biofumigados. Estos resultados confirman lo señalado por Brown y Morra (1997) y Sarwar et al. (1998), que relacionaban el efecto inhibitorio de los biofumigantes con su perfil de GSLs. Esta capacidad de inhibición podría explicarse por diferencias en la volatilidad de los productos derivados de la hidrólisis de los distintos GSLs, debido a su vez a diferencias en su peso molecular, tal y como afirmaron Aires et al. (2011), aunque parece estar más relacionada con la selectividad de dichos volátiles a nivel de especie fitopatógena (Smolinska et al., 1997; Mazzola et al., 2001; Ojaghian et al., 2012).

Tras la confirmación de que la capacidad biofumigante contra *P. cinnamomi* reside en el contenido en sinigrina del material vegetal empleado, se planteó el desarrollo de un producto *ready-to-use* con una alta concentración de este GSL para su aplicación directa en suelos de dehesa usando como materia prima un residuo industrial y fertilizantes cálcicos de efectividad demostrada contra *P. cinnamomi* (Serrano et al., 2012a). El producto, altamente efectivo *in vitro*, tiene como materia básica principal los residuos generados en la industria de obtención de aceites de semillas de *B. juncea*. Una vez obtenido el contenido graso de las semillas, el subproducto generado se sometió a un nuevo proceso de extracción y concentración de los GSLs que persisten en el residuo, sinigrina casi en su totalidad. Para darle estabilidad al extracto y que no se degrade, se inactiva a la enzima mirosinasa, por lo que para su posterior utilización se añadió materia verde liofilizada como fuente de actividad mirosinasa. Finalmente se corrigió el pH de la mezcla con carbonato cálcico ($CaCO_3$), consiguiéndose así un doble beneficio: por un lado, se optimiza la hidrólisis de la sinigrina al aumentar el pH del producto hasta el óptimo para la actividad de la enzima (pH= 6.5, Iori et al., 1996), y por otro se mejora la efectividad del producto, ya que el $CaCO_3$ posee una eficacia demostrada en la inhibición de la producción de esporangios de *P. cinnamomi* en el suelo, reduciendo de esta manera la producción de zoosporas infectivas (Serrano et al., 2012a; 2014). Este producto puede aplicarse en el momento más apropiado para el control de *P. cinnamomi*, evitándose los problemas que conlleva la técnica tradicional de enterrado en verde, y más teniendo en cuenta la baja productividad de los biofumigantes activos frente a *P. cinnamomi* cuando se cultivan en las condiciones edáficas y climáticas más características de la dehesa. Por otro lado, se pone en valor un residuo industrial, lo que se adapta perfectamente a la producción ecológica propia de la dehesa.

A modo de conclusión general, este trabajo investigador demuestra que la biofumigación es una técnica efectiva que puede ser incorporada en la lucha integrada contra la podredumbre radical de los *Quercus* causada por *P. cinnamomi* en ecosistemas de dehesa. La biofumigación con plantas ricas en sinigrina reduce significativamente la viabilidad de las esporas de resistencia del patógeno en el suelo, minimizando así la producción de zoosporas infectivas, lo que lleva a una disminución significativa de la capacidad de *P. cinnamomi* para infectar raíces y causar enfermedad.

Debido a que el método tradicional de enterrado en verde utilizando un cultivo biofumigante producido *in situ* es de difícil aplicación en dehesas por la baja productividad que muestran los cultivos efectivos contra *P. cinnamomi*, y que también se manifiesta en la producción de semillas (material con mayor contenido en sinigrina), se ha desarrollado un producto efectivo que resulta fácil de obtener, almacenar y usar en dehesas aplicándolo directamente al suelo cuando sea necesario. Además, la fabricación de este producto, usando como materia prima un residuo de la obtención industrial de aceite, es un respaldo al carácter ecológico que caracteriza la producción de estos ecosistemas.

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CAPÍTULO 7. CONCLUSIONES

CHAPTER 7. CONCLUSIONS

CONCLUSIONES

1. El umbral mínimo de inóculo de *Phytopththora cinnamomi* requerido para el desarrollo de la enfermedad radical en alcornoques bajo condiciones altamente favorables es de 61 UFC g-1 de suelo seco, mientras que las infecciones asintomáticas ocurren a partir de solo 3 UFC g-1 de suelo seco (Serrano et al., 2015. Phytopathologia Mediterranea 54, 461-464).
2. Las plantas pertenecientes a la familia *Brassicaceae* con alto contenido en el glucosinolato alifático sinigrina (2-propenil glucosinolato) son biofumigantes efectivas contra *P. cinnamomi* (Ríos et al. 2016. Journal of Phytopathology 164, 582-594; Ríos et al. 2016. Forest Pathology 46, 652-659).
3. La biofumigación con materia verde con alto contenido en sinigrina, disminuye significativamente la viabilidad de las clamidosporas (fuente de inóculo primario) en el suelo por debajo del umbral mínimo para el desarrollo de la enfermedad radical (Ríos et al. 2016. Journal of Phytopathology 164, 582-594; Ríos et al. 2016. Forest Pathology 46, 652-659; Ríos et al. Phytopathologia Mediterranea, under review)
4. La biofumigación con materia verde con alto contenido en sinigrina disminuye significativamente la producción de esporangios, fuente de inóculo secundario (zoosporas) (Ríos et al. 2016. Forest Pathology 46, 652-659).
5. El uso de especies que no tienen sinigrina en su perfil de glucosinolatos no sólo no tienen eficacia como biofumigantes, sino que además pueden incrementar la infectividad del patógeno en el suelo, como ha sido observado en el caso de *Brassica napus* (Ríos et al. 2016. Journal of Phytopathology 164, 582-594; Ríos et al. 2016. Forest Pathology 46, 652-659).

6. La biofumigación con harina de semillas de *Brassica* sp. con alto contenido en sinigrina no sólo reduce la viabilidad de las clamidosporas en el suelo, sino que además reduce significativamente la severidad de síntomas radicales en una especie altamente susceptible como el *Lupinus luteus* (Ríos et al 2016. Phytopathologia Mediterranea, under review)
7. La baja producción de especies biofumigantes en condiciones de dehesa desaconseja la estrategia tradicional de enterrado en verde (Ríos et al 2016. Phytopathologia Mediterranea, under review).
8. La formulación *ready-to-use* desarrollada en esta Tesis, combina la acción biocidida de los extractos de harinas de semilla con alta concentración en sinigrina, y la conocida acción supresiva de los fertilizantes cálcicos. Además, el producto puede ser fácilmente aplicado en ecosistemas de dehesas en el momento más adecuado (Ríos et al 2017. Plant Disease, under review).

El presente estudio demuestra que la biofumigación es una técnica efectiva para su integración en sistema de control integrado de la podredumbre radical causada por *P. cinnamomi*. La biofumigación con plantas que tienen un alto contenido de sinigrina reduce significativamente la viabilidad de las clamidosporas, minimizando la esporulación y, por consiguiente, disminuyendo la capacidad del patógeno de infectar raíces, consiguiendo de este modo una menor incidencia de la enfermedad radical. Como el método tradicional de aplicación de la biofumigación enterrando la biomasa producida *in situ* no es recomendable en condiciones de dehesas, se ha formulado un producto efectivo y fácil de aplicar para el control de la enfermedad. Este producto tiene como valor añadido el hecho de que su formulación, que tiene como materia prima un extracto obtenido de un residuo de la industria de la

producción de aceites, refuerza el carácter ecológico de las producciones y el manejo de los ecosistemas de dehesa.

CONCLUSIONS

1. The minimum threshold of *Phytophthora cinnamomi* required to cause cork oak root disease under highly favourable conditions is 61 CFU g^{-1} dry soil, whereas asymptomatic root infections occurred from only 3 CFU g^{-1} (Serrano et al., 2015. Phytopathologia Mediterranea 54, 461-464).
2. Brassicaceous plants with a high content of the aliphatic glucosinolate sinigrin (2-propenyl glucosinolate) are effective biofumigants against *P. cinnamomi.* (Ríos et al. 2016. Journal of Phytopathology 164, 582-594; Ríos et al. 2016. Forest Pathology 46, 652-659).
3. Biofumigation with high sinigrin-content green material significantly decrease chlamydospore (primary inoculum) viability in soil under the minimum threshold for oak root disease development (Ríos et al. 2016. Journal of Phytopathology 164, 582-594; Ríos et al. 2016. Forest Pathology 46, 652-659; Ríos et al 2017. Phytopathologia Mediterranea, under review).
4. Biofumigation with high sinigrin-content green material significantly decrease *P. cinnamomi* sporangial production, source of secondary inoculum (zoospores) (Ríos et al. 2016. Forest Pathology 46, 652-659).
5. The use of species with no sinigrin in their GSL profile did not show efficiency as biofumigants against *P. cinnamomi*, and even more, they might increase the infectivity of the pathogen into the soil, as observed for *Brassica napus* green material (Ríos et al. 2016. Journal of Phytopathology 164, 582-594; Ríos et al. 2016. Forest Pathology 46, 652-659).
6. Biofumigation with seedmeals from *Brassica* spp. with a high content in sinigrin not only decreases chlamydospore viability in soil, but also significantly reduces

root symptoms in a highly susceptible species such as *Lupinus luteus* (Ríos et al 2017. Phytopathologia Mediterranea, under review).

7. Low production of effective biofumigant species recorded in dehesa systems discourage their culture on site for a traditional (green) biofumigation strategy (Ríos et al 2017. Phytopathologia Mediterranea, under review).
8. The *ready-to-use* formulations developed on this Thesis, combines the biocidal action of seedmeal extracts rich in sinigrin and the already known suppressive action of calcium fertilizers, and can be easily applied in dehesa ecosystems at any time (Ríos et al 2017. Plant Disease, under review).

The current study demonstrated that biofumigation is a suitable technique with a high noxious effect against *P. cinnamomi*. Biofumigation with *Brassicaceae* plants rich in sinigrin effectively suppress the viability of resting spores, minimizing secondary sporulation and, consequently, decreasing the ability of *P. cinnamomi* to infect roots and diminishing the incidence of the disease caused by this pathogen. As the traditional biofumigation strategy using green matter produced in site appears difficult to be applied in dehesa ecosystems, an effective and easy to apply product has been developped for disease control. Even more, the likely commercial manufacture of this kind of products, based on extracts obtained from oil industrial waste, should increase the organic nature of dehesa production and management.